Programa de Doctorado en Biomedicina y Biotecnología

Light and drought: bioprospecting the microbiota of highly-irradiated and dry environments

MEMORIA PRESENTADA POR ESTHER MOLINA MENOR, CANDIDATA AL GRADO DE DOCTORA POR LA UNIVERSITAT DE VALÈNCIA

CODIRECTORES: DR. MANUEL PORCAR MIRALLES, DR. ALBERT QUINTANA ROMERO Y DR. JULI PERETÓ MAGRANER

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“The theatre is so endlessly fascinating because it's so accidental. It's so much like life”

Arthur Miller

El **Dr. MANUEL PORCAR MIRALLES**, Investigador Indefinido Doctor de la Universitat de València (Instituto de Biología Integrativa de Sistemas, I2SysBio, UV-CSIC), el **Dr. ALBERT QUINTANA ROMERO**, Profesor Agregat del Departament de Biologia Cel·lular, Fisiologia i Immunologia de la Universitat Autònoma de Barcelona (Institut de Neurociències, INc, UAB), el **Dr. JULI PERETÓ MAGRANER**, Catedrático de la Universitat de València, y la **Dra. MARÍA LUISA GIL HERRERO**, Catedrática de la Universitat de València:

AUTORIZAN la presentación de la memoria titulada '*Light and drought: bioprospecting the microbiota of highly-irradiated and dry environments*' y **CERTIFICAN** que los resultados que incluye fueron obtenidos bajo la codirección de los doctores **MANUEL PORCAR MIRALLES**, **ALBERT QUINTANA ROMERO** y **JULI PERETÓ MAGRANER** en el Instituto de Biología Integrativa de Sistemas por **ESTHER MOLINA MENOR**, y que reúne los requisitos necesarios para ser defendida como tesis doctoral y optar al grado de Doctora por la Universitat de València.

Y para que conste, firman el siguiente certificado en Paterna a 19 de junio de 2023.

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Agradecimientos

Decía Arthur Miller, dramaturgo, que *el teatro es tan infinitamente fascinante porque es muy accidental, como la vida*. Pese a los guiones, encima de un escenario son muchas las cosas que se escapan al control de los actores. El teatro es improvisación, resolución de problemas, creatividad, arte, imaginación y, ante todo, el trabajo de un equipo. El teatro es el resultado de la fascinación por todo aquello que accidentalmente termina sucediendo en el escenario. Igual que la vida. Igual que la ciencia. Imagino que esa fascinación fue la que ya de pequeña, como cuenta mi madre, me hizo querer ser *envestigadora*. Durante estos años son muchas las personas que me han acompañado en este duro y fascinante camino. No puedo ahora hacer otra cosa que agradecer a todas ellas el tener hoy este trabajo en las manos.

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Table of contents

Introduction

1. Microbial diversity

Our planet's biodiversity is dominated by microorganisms. It has been estimated that the Earth hosts from 10^{11} to 10^{12} microbial species, what could be translated into 10^{30} prokaryotic cells living in the planet. However, the vast majority are still unknown and/or have never been cultivated in laboratories (Locey and Lennon, 2016). Although the largest microbial diversity is concentrated in the so-called 'big five' habitats (deep oceanic subsurface, deep continental subsurface, soil, upper oceanic sediment and oceans), prokaryotes inhabit all the possible ecological niches and colonize even the most remote, isolated and often extreme environments (Flemming and Wuertz, 2019; Rampelotto, 2013; Shu *et al*., 2022). It is in these environments, harsh and extreme from an anthropological point of view, from where many innovative and revolutionary biotechnological tools and products have been developed.

1.1. Measures of diversity and Next Generation Sequencing (NGS)

Estimating the number of living organisms on Earth has historically been of interest for ecologists. This has extended to the microbial world in the last decades (Pedrós-Alió and Manrubia, 2016). The microbial diversity is in the spotlight of many studies since the development of metagenomic techniques, that allow large-scale studies on the structure, function and evolution of microbiomes (Haegeman *et al*., 2013). The development and widespread use of Next Generation Sequencing techniques (NGS), which include whole-genome shotgun metagenomic sequencing, RNA sequencing (metatranscriptomics) and high-throughput 16S rRNA gene sequencing (metataxonomy), has made available valuable information about taxa, genes and metabolic properties of microbial species and communities that was previously unknown (Wensel *et al*., 2022).

However, evaluating and estimating diversity is a challenging task and subject of intense debate. From the taxonomic point of view, the most common measures of diversity are based on the estimation of either the number of microbial species or their relative abundances in specific environments. The main parameters used are α-diversity (richness or number of observed taxa in a sample) and β-diversity (variability in microbial communities among samples) (Walters and Martiny, 2020). These estimations, though, present some difficulties. First, the definition of species within the prokaryotic world is controversial. Thus, Operational Taxonomic Units (OTUs) or, more recently, Amplicon Sequence Variants (ASVs) are used instead in metataxonomic studies (Callahan *et al*., 2017; Pedrós-Alió, 2006). Second, the difficulties in measuring rare species or highly diverse communities leads to make assumptions on abundance distribution of microbial species (Haegueman *et al*., 2013).

Microbial diversity varies among ecosystems and is influenced by abiotic factors, such as pH, desiccation, temperature or salinity, not only from the taxonomic but also from the metabolic and functional point of view (Ruhl *et al*., 2022). Under extreme conditions, microbial diversity tends to decrease and so does functional diversity (Fierer and Jackson, 2006; Maestre *et al*., 2015; Rousk *et al*., 2010; Ruhl *et al*., 2018; Sharp *et al*., 2014; Yang *et al*., 2016). However, in terms of specific functions related to survival under those conditions, an enrichment in genes associated to resistance to osmotic stress or dormancy, as well as reduced abundance of genes involving species relationship (such as antibiotic resistance or virulence factors) has been reported (Song *et al*., 2019). Interestingly, in a thawed permafrost, Yuan *et al*. (2018) described decreasing functional diversity and increasing community diversity in moderate-to-extensively thawed areas.

Microbial genomes and metabolic characteristics can help to better understand microbial diversity. This functional diversity is often ignored, but is crucial for understanding how microbial communities are shaped and how they behave under different conditions (Johnson and Pomati, 2020; Li *et al*., 2015;

Miller *et al*., 2009). In fact, functional diversity may give a more valuable and reliable information about the processes that take place in a specific environment and its properties than taxonomic profiles (Fontana *et al*., 2018; Hillebrand and Matthiessen, 2009).

Despite the extensive use of high-throughput 16S rRNA gene sequencing (or metataxonomy) as a cost-reduced NGS strategy, it has some limitations. Metataxonomy has limited resolution and gives a general overview of the taxonomic bacterial profiles no further than at the genus level as a consequence of the error associated to the technologies used. Moreover, the need for previous acid nucleic amplification leads to biases towards specific taxonomic groups and difficulties in estimating real abundances, and it gives little information about functional profiles (Johnson *et al*., 2019; Winand *et al*., 2019).

Metagenomics, instead, allows a more precise identification: there are no amplification biases, it also considers functional information, and has more power to detect specific less abundant taxa. However, it requires higher investment, higher DNA concentrations, databases are not that complete, it is difficult to assembly genomes of less abundant taxa and host DNA may interfere with microbiota detection (Breitwieser and Salzberg, 2019; Durazzi *et al*., 2021; Wensel *et al*., 2022). Nevertheless, the choice of one technique or the other would depend on the objectives and the biological question to answer.

NGS has dramatically changed the way microbiology and microbial biotechnology are understood. In clinical microbiology, it can easily assist and guide diagnosis and treatment of infectious diseases. In microbial ecology, it has unveiled a great previously-unknown microbial diversity; whereas in bioprospecting, it can efficiently improve biomolecule mining (Gaston *et al*., 2022; Gu *et al*., 2019; Maansson *et al*., 2016; Yu *et al*., 2020).

1.2. Culturability and microbial dark matter

If the Earth is home for 10^{11}-10^{12} microbial species, only 10^{4} have been cultivated in laboratories (Locey and Lennon, 2016). The evidence that the count of viable cells retrieved in cultures was far from the count under the microscope was named ‘the great plate count anomaly’, which suggested that many cells were reluctant to be cultured (Staley and Konopma, 1985). Although microbes were discovered in the 17th century thanks to the invention of microscopes, it was not until 19th century that the first laboratory cultures were performed.

Since then, the discovery of new microorganisms has strongly depended on their isolation in pure culture. However, culturing methods have systematically biased the culture of microorganisms towards a few easy-to-grow taxa (Overmann *et al*., 2017). Thus, the development of NGS allowed the identification of a large number of taxa that had never been isolated in laboratories. This uncultured diversity of species, summed to the genetic information encoded in their genomes, the context-dependent biomes and the ecological and evolutionary dynamics of the populations, is known as the microbial dark matter (Zha *et al*., 2022).

NGS, specifically metagenomics, have shed light not only in the discovery of new taxa, but also in understanding the behavior of those taxa within microbial communities. Current microbiology focuses on the isolation of those uncultured microorganisms as the underexplored microbial dark matter may encode an also underexplored metabolic diversity useful for biotechnological applications (Overmann *et al*., 2017; Rinke *et al*., 2023).

Even though NGS gives useful information about the existing species and genetic diversity, bioprospecting and the development of new biotechnological tools based on microorganisms still relies on their isolation under laboratory conditions. In fact, the complete and detailed characterization of the physiological traits of bacterial species is only possible through culturing (Vartoukian *et al*., 2010). Although not all the microbial diversity can be, to date, cultured, molecular techniques often fail in detecting rare taxa that are accessible through culturing techniques (Pedrós-Alió, 2006). Cultures are, thus, still indispensable for microbiology.

That said, the functional information obtained through NGS is useful to guide and assist the design of isolation protocols and culture media. Moreover, *in situ* sequencing technologies such as Nanopore sequencing can be used to detect hot spots in microbial diversity in bioprospecting expeditions (Latorre-Pérez *et al*., 2021). It is therefore important the combination of both culture-dependent and culture-independent experimental approaches to retrieve as much information as possible from the studied environments and select the proper conditions for the isolation of new or targeted microorganisms (Satari *et al*., 2020; Tidjani Alou *et al*., 2020; Vidal-Verdú *et al*., 2022 a) (Figure 1).

Great efforts are, hence, being made in order to develop innovative culturing techniques that allow us to isolate the yet uncultured diversity and to exploit the biotechnological potential of their metabolisms (Figure 1).

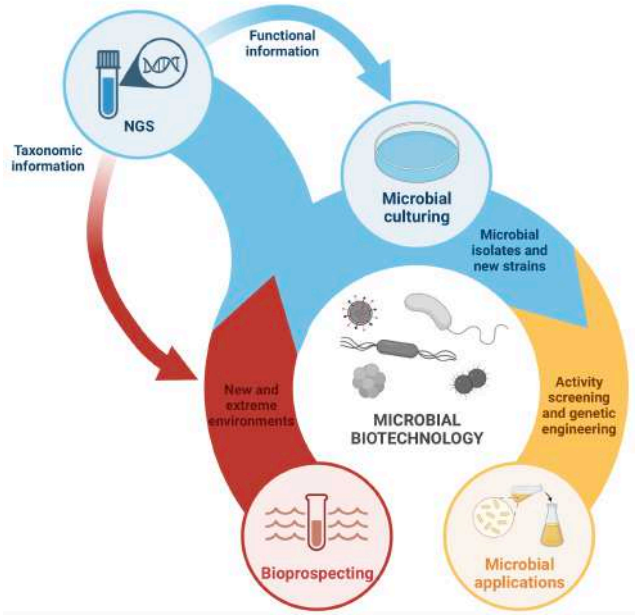

Figure 1. Bioprospecting for the development of new microbial applications. Created with BioRender.com.

1.3. Challenges in culturomics and new culturing strategies

As stated by Pedrós-Alió (2007), any microorganism would be susceptible to be cultured provided that the conditions are suitable for their growth. However, culturing microorganisms is still challenging given the complexity of natural microbial communities and the differences between the laboratory conditions and their natural habitats. Traditional culture media offer a static and homogeneous environment for species to grow individually, in contrast to the natural environment in which bacteria and other organisms live, which is diverse, changing and heterogeneous (Overmann *et al*., 2017).

The reasons for the unculturability of most taxa are varied but can be classified into six categories, as reviewed by Lewis *et al*. (2021): identification of substrates and growth conditions (carbon and nitrogen sources, vitamins, cofactors, inorganic compounds, etc.), resuscitation of dormancy, symbiotic interdependencies, physical contact or spatial proximity, physicochemical environmental conditions (temperature, pH, salinity, redox conditions, etc.), and low abundance and competition.

Of those, the identification of proper substrates and the optimal physicochemical environmental conditions can be addressed through simple strategies such as the combination and the design of new media (Figure 2E). For example, Kaboré *et al*. (2019) described an innovative culture media for marine bacteria that included sponge skeleton given the natural relationship that *Gemmata* spp. stablish with marine sponges in their habitat. On their part, Vartoukian *et al*. (2016) described the importance of siderophore supplementation and helper strains in the isolation of previously-uncultured bacteria from the oral microbiota. Moreover, widely used laboratory culture media often contain high nutrient concentrations that inhibit the growth of oligotrophic bacteria in favor of copiotrophic strains, but marine environments, for example, are in many cases oligotrophic habitats (Yin *et al*., 2013). In this case, the use of filtered and autoclaved seawater has been successful for isolating oligotrophic marine bacteria, and the use of diluted conventional media increase the diversity of microbial cultured communities (Figure 2D) (Cho *et al*., 2004; Sun *et al*., 2019).

Other improvements in culturing methods mimic the environmental conditions in which bacteria naturally live through more sophisticated strategies, such as the so-called ichip (Nichols *et al*., 2010). Moreover, the isolation in pure culture of bacteria that live in consortia is not always possible, as they may need to grow close to each other in order to develop visible colonies. Thus, co-cultures are being established (D'Onofrio *et al*., 2010).

- Ichip

Isolation chip or ichip is a device consisting on small diffusion chambers that is inoculated and incubated *in situ* in the sampling point (Figure 2A). Thus, microbial cells are grown in exactly their natural environmental conditions. This device has been used to culture previously uncultured bacteria, to isolate toxic compound-degrading bacteria or in de discovery of novel antibiotics (Lodhi *et al*., 2018; Nichols *et al*., 2010; Polrot *et al*., 2022; Sherpa *et al*., 2015).

- Dilution-to-extinction

This method is based on the use of low-nutrient media and the dilution of cell samples to a small and known number of them, with the intention of growing individual cells in multiple-well plates (Figure 2B). It has been very useful in the isolation of novel and oligotrophic marine bacteria, but has also allowed the characterization of bacteria producing antitumor compounds (Benítez *et al*., 2021; Buchholz *et al*., 2021; Castro *et al*., 2017; Derrien *et al*., 2004; Song *et al*., 2009).

- Co-cultures

Co-culture techniques are based on the growth of bacteria in a way that different species can interact, as they do in their natural habitats (Figure 2C) (Goers *et al*., 2014). The reason for which co-culture is interesting is not only to study how different species relate and communicate, but also to improve and

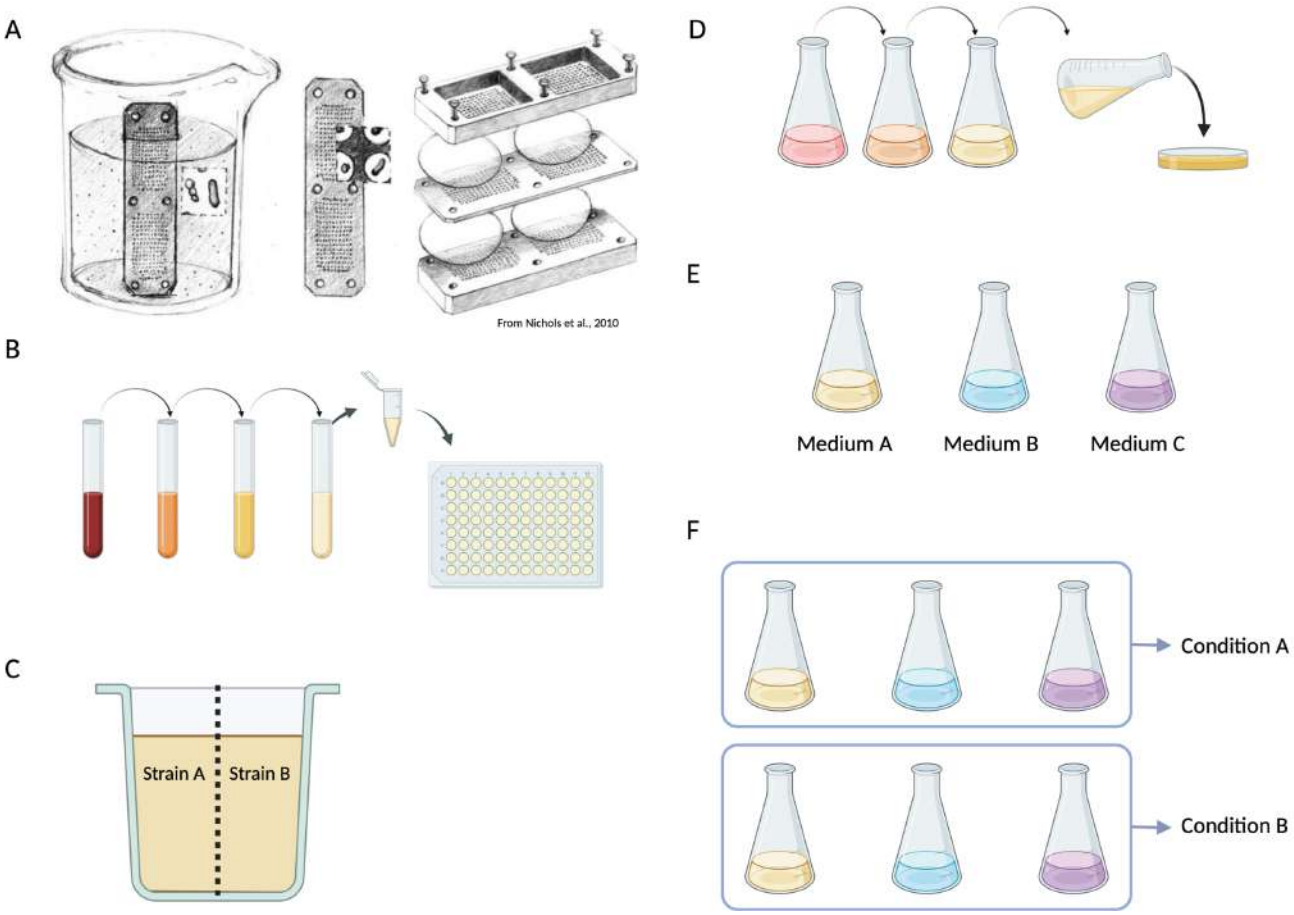

Figure 2. New microbial culturing strategies. (A) ichip (Nichols et al., 2010). (B) Dilution-to-extinction. (C) Co-culture. (D) Medium dilution. (E) Media combination. (F) Combination of media and incubation conditions. Created with BioRender.com.

enhance their grown under laboratory conditions, as some of them need microbial consortia to grow. Moreover, co-cultures are also interesting in the search for novel antimicrobial compounds and secondary metabolites, as many biosynthetic routes are silent under traditional laboratory growth conditions (Arora *et al*., 2020; Bertrand *et al*., 2014; Lodhi *et al*., 2018).

Finally, as the microorganisms retrieved in pure culture may differ depending on the culturing techniques employed, considering the application in parallel of different culture-dependent strategies or growth conditions is a feasible way to easily increase the culturable microbial diversity (Figure 2F) (Köpke, 2005; Vartoukian *et al*., 2010).

2. Bioprospecting highly irradiated and dry environments

Bioprospecting is the search for microorganisms, or their parts (i.e., enzymes), to develop biotechnological tools with biomedical, pharmaceutical, agricultural or industrial applications (Ayilara *et al*., 2022; Saeed et a., 2022; Stirk *et al*., 2022; Tanner *et al*., 2017). Highly irradiated and dry environments, either natural (e.g., deserts) or artificial (e.g., solar panels) possess specific characteristics that make them interesting from the biotechnological perspective, as the microorganisms living on them develop mechanisms to cope with high doses of radiation (particularly, UV radiation) and desiccation.

Radiation, specifically sun radiation, includes infrared, visible light and ultraviolet (UV), which can be further divided into UV-A, UV-B and UV-C. The most damaging UV light, UV-C, is filtered by the stratospheric ozone layer of the atmosphere. Thus, UV sun radiation reaching the Earth's surface is mainly UV-A and some UV-B (Hargreaves *et al*., 2007). However, it is enough to have an impact on living organisms and to shape the microbial biocenosis living on exposed areas (Dorado-Morales *et al*., 2016).

Desiccation, on the other side, is an intrinsic characteristic of drylands and refers to the lack of water. Drylands, which represent the largest biome on the Earth's surface, include desert environments, which are characterized by scarce rainfall and can be further classified into hot (Sahara, Mojave, Atacama or Tabernas Desert, among others) and cold deserts (such as Antarctica). In the context of climate change, drylands are expected to increase in the following years. Thus, understanding how microorganisms live and adapt to these environments is of great relevance in order to face the challenges of this global threat. In fact, the use of microorganisms that have been exposed to drought stress, among others, has already been explored as a possibility to improve forest response and increase resilience to climate change (Ahmed *et al*., 2022; Allsup *et al*., 2023; Makhalanyane *et al*., 2015; Maestre *et al*., 2015).

2.1. Polar niches

Although polar environments in Arctic and Antarctic poles share some climatic features and register the lowest temperatures in the globe, the environmental conditions in both locations are different. Antarctica is extremely dry and is considered a cold desert, whereas in the Arctic some areas register stormy and wet winters. However, polar microbial surfaces are exposed to high doses of solar UV radiation given the reduction in ozone in the stratospheric atmosphere layer that happen in light-dark cycles every six months (Cowan *et al*., 2014; Dahms *et al*., 2011; Di Giuseppe *et al*., 2012).

Despite the variety of abiotic stressors to which living organisms are subjected to in polar regions (e.g., freezing, low temperatures and low nutrient availability), these environments still harbor suitable niches for microorganisms to grow (Cowan *et al*., 2014; Varin *et al*., 2012). Specifically, soil niches such as microbial mats or permafrost, revealed higher microbial diversities than expected, also with prevalence of highly-resistant taxa such as *Deinococcota* (former *Deinococcus-Thermus*), with respect to less hostile environments (Cowan *et al*., 2014). Moreover, the study of different Antarctic plant-associated microbial niches revealed microbiomes mainly composed of *Actinomycetota*, *Pseudomonadota* and *Bacillota* (former *Actinobacteria*, *Proteobacteria* and *Firmicutes*, respectively) (Zhang *et al*., 2020).

However, in the context of climate change, the microbial communities living in these areas are expected to be altered. Specifically, ice thawing permafrost and melting glaciers will increase moisture and wet previously dried niches, and increased solar radiation has already been reported (Cowan *et al*., 2014; Turner and Overland, 2009).

The biotechnological potential of microorganisms from Polar regions lies in the isolation and characterization of cold-adapted enzymes, such as esterases, lipases, DNases and proteases, but also in the synthesis of pigments as a response to abiotic stressors (e.g., UV-radiation) (De Santi *et al*., 2016; Silva *et al*., 2021). Moreover, polar bacteria have been reported to bioremediate toxic compounds, such as perchlorate (Acevedo-Barrios *et al*., 2022).

2.2. Hot deserts

Although deserts are traditionally considered lifeless habitats, they host a wide microbial diversity. In fact, the upper part of soil in drylands such as arid deserts hosts a specific and well-adapted microbial biocenosis dominated by extremophilic bacteria (Belov *et al*., 2018; Perera *et al*., 2018).

The microbial communities inhabiting hot deserts are mainly composed of microalgae, cyanobacteria and bacteria within the phyla *Actinomycetota* and *Pseudomonadota*, followed by *Bacillota*, similarly to those described above for polar soil habitats, but there are also representatives of the highly-resistant *Deinococcota* (Belov *et al*., 2018; Perera *et al*., 2018; Zhang *et al*., 2020). These microorganisms are able to cope with high UV radiation, high temperatures and low water and nutrient availability, among other stresses (Azua-Bustos *et al*., 2012; Saul-Tcherkas *et al*., 2013). This microbial diversity, which has huge biotechnological potential, is also threatened by the increasing aridity derived from climate change (Azua-Bustos and González-Silva, 2014; Maestre *et al*., 2015).

For example, patented strains of *Acidithiobacillus* bacteria isolated from the Atacama Desert are useful in bioleaching (metal extraction driven by microorganisms), which is of high interest for biomining operations (Ohata *et al*., 2013; Sugio *et al*., 2009). Other strains are useful in bioremediation (e.g., arsenic bioremediation in drinking water), which is more efficient than conventional chemical and physical remediation methods (Campos *et al*., 2009). Moreover, for biomedicine, the abundance of antibiotic-producing bacteria is especially relevant (Astakala *et al*., 2022; Azua-Bustos and González-Silva, 2014; Goodfellow *et al*., 2018; Rateb *et al*., 2011; Schulz *et al*., 2011).

2.3. Biocrusts

Biological soil crusts (i.e., biocrusts) are worldwide-distributed associations of soil particles and microorganisms, among which there can be found bacteria, algae, archaea and bryophytes, characteristic of low-water and scarce vascular plant cover ecosystems. They are found in dry-to-hyper-arid regions, but also in cold environments such as polar regions, in which freezing limits water availability (Weber *et al*., 2022).

Biocrusts are relevant players in drylands. They influence the characteristics on the surrounding soils and thus the microbial communities being established. Moreover, the mosses in biocrusts participate in alleviating the effects of climate change and increased aridity on the microbial communities (Delgado-Vaquerizo *et al*., 2018). The biotechnological interest of microorganisms in biocrusts lies on their role in land restoration and fight desertification and other climate change consequences (Chilton *et al*., 2022; Maestre *et al*., 2017). Moreover, the synthesis of biotechnologically relevant secondary metabolites, such as the sunscreen scytonemin, and exopolysaccharides (EPS) has also been reported for microorganisms isolated from biocrusts (Couradeau *et al*., 2016; Xue *et al*., 2021).

2.4. Solar panels

The solar panels' microbiome throughout the planet has been studied in the last decade. Solar panels have proven a desert-like environment colonized by bacteria adapted to tolerate stresses that are characteristic to deserts (irradiation, temperature

fluctuations, low nutrient availability and desiccation) (Dorado-Morales *et al*., 2016). Moreover, a study on the colonization process of solar panels suggested that airborne transport of desert dust may be the origin of the solar panel microbiome. This would be supported by the evidence that some of the most abundant taxa (*Deinococcus* and *Hymenobacter*), are also desert airborne bacterial markers (Meola *et al*., 2015; Tanner *et al*., 2020).

The microbial biocenosis living on solar panels worldwide is dominated by *Deinococcus*, *Sphingomonas* and *Hymenobacter*. These taxa are well known for their robustness against abiotic stressors. However, their dominance in the solar panel microbiome comes with time, as the environmental conditions that bacteria experience shape the microbiota from generalist taxa, initially, to a more specialized and adapted community. This pattern is supported by the high similarities found among solar panels distributed in different latitudes (Dorado-Morales *et al*., 2016; Lim *et al*., 2019; Liu *et al*., 2022; Moura et. al., 2021; Park *et al*., 2022; Porcar *et al*., 2018; Tanner *et al*., 2018; Tanner *et al*., 2020).

Bacteria living on solar panels are interesting from the pharmaceutical and biotechnological points of view given their ability to synthetize antioxidant compounds, such as carotenoids. These carotenoids have demonstrated their antioxidant power and protection against oxidative stress in a *Caenorhabditis elegans* model (Tanner *et al*., 2019). Moreover, robust bacteria such as *Deinococcus* are being explored as chassis for industrial purposes, as the processes to which they are subjected to are often harsh, and *Sphingomonas* are also useful in bioremediation (Gerber *et al*., 2015; White *et al*., 1996).

3. Industrial potential of extremophiles

The extremobiosphere encompasses all biomes subjected to harsh environmental conditions. These conditions vary from extreme desiccation (e.g., hyper-arid deserts), high pressure (e.g., deep-sea sediments), extreme temperatures (e.g., permafrost soils or hot deserts), low nutrient availability (e.g., polar mats), high irradiation and salinity, etc. (Sayed *et al*., 2020).

Extremophilic environments have proven a rich source of biotechnologically relevant microorganisms (Table 1). Rare, unusual and extreme environments host microbes adapted to live under such conditions. These microbes have developed mechanisms to survive and cope with the resulting stresses. The mechanisms and the products derived from them hold great promise from the ecological, agricultural, industrial, pharmaceutical and biomedical point of view (Dumorné *et al*., 2017; Kochhar *et al*., 2022).

3.1. Extremozymes

Extremozymes, which are the enzymes isolated from extremophilic organisms, have industrial applications given their natural robustness (e.g., thermostability and salt tolerance). Extremozymes are highly valuable since most of industrial processes take place under harsh conditions of temperature, salinity or pH (Dumorné *et al*., 2017; Sarmiento *et al*., 2015).

Table 1. industrial potential of extremophilic microorganisms and the products derived from them.

STRESS RESISTANCE	PRODUCTS/USES	EXAMPLES	APPLICATION	REFERENCE
Radioresistance	Pigments	Carotenoids	Antioxidants, anticancer molecules, antimicrobial	Tian and Hua, 2010; Nawaz *et al.* , 2020
	Antibiotics	Chaxamycin D	Antibacterial	Rateb *et al.* , 2011
		Mutactimycin AP	Antibacterial	Astakala *et al.* , 2022
Desiccation	Polymers	Exopolysaccharydes (EPS)	Antiinfamatory, antimicrobial, antibiofilm	Angelin and Kavitha, 2020
High temperature	Antibiotic	Abenquines	Antibacterial and antifungal	Schulz *et al.* , 2011
	Enzymes	Celullases and xylanases	Paper industry, environmental contamination	Unsworth *et al.* , 2007; Sunna *et al* ., 2003
		Lipases and proteases	Detergents	Dumorné *et al* ., 2017
		taq polymerase	Molecular biology (PCR)	Bell, 1989
Low temperature	Antibiotic	Microcin B	Antibacterial	Lee *et al* ., 2017
	Enzymes	Lipases and proteases	Detergents	Zhang *et al.* , 2011
	Secondary metabolites	Biocontrol agents	Biopesticides	Torracchi *et al* ., 2020
	Pigments	Violacein	Sunscreen, antimicrobial, antioxidant	Silva *et al* ., 2021
High salinity	Biosurfactants		Biofilm inhibition in implants and catheters	Kochhar *et al* ., 2022
	Bioplastics	PHAs	Drug delivery	Coker *et al.* , 2016
	Antibiotics	Halocins	Antibacterial	Coker *et al.* , 2016

One of the best known extremozymes is the *taq* polymerase used in molecular biology for the Polymerase Chain Reaction (PCR). This polymerase was first isolated from the thermophilic bacterium *Thermus aquaticus* that lives in hot springs (Chien *et al*., 1976). Moreover, other thermophilic enzymes such as cellulases, amylases, proteases or lipases, as well as many other polymer-degrading enzymes have also been characterized (van der Burg., 2003). Specifically, detergents possess thermophilic and psychrophilic enzymes with protease, lipase, cellulase and amylase activities (Sarmiento *et al*., 2015; van der Burg., 2003). Moreover, low-temperature active enzymes are also of interest for the food, cosmetic and pharmaceutical industries (van der Burg, 2003).

Some extremozymes are active in high salt concentration environments or extreme pH. Halophilic enzymes are of high interest for synthetic processes in low water activity and in the presence of organic solvents (Klibanov, 2001; Raddadi *et al*., 2013), whereas acidophilic and alkalophilic ones are of interest for the food industry, biofuel and ethanol production, detergents and biomining (Dumorné *et al*., 2007; Golyshina and Timmis, 2005; van der Burg, 2003).

Biodiscovery of new extremophilic microorganisms and their extremozymes is an opportunity for industry to change towards a more sustainable production system. Specifically, the use of bacteria able to grow fast in versatile conditions (broad range of temperatures, use of alternative carbon sources, high salt or metal tolerance and reduced use of water) may avoid other microorganisms' contamination, which is one of the most important challenges for bioprocesses. This would, thus, simplify production by reducing the costs of keeping sterility, allowing continuous processes and reducing energy consumption. Moreover, the use of polyextremophilic organisms is preferable over chemical synthesis. For example, the biosynthesis of nanoparticles by metal-resistant bacteria is less costly, more efficient and ecological that the alternative chemical process (Atalah *et al*., 2022; Chen and Jiang, 2018).

Apart from extremozymes, other non-enzymatic molecules can be isolated from extremophilic organisms. For example, some psychrophilic bacteria protect plants against pathogens through the synthesis of secondary metabolites and are used in biopesticides; some can synthesize molecules that inhibit biofilm formation, a major concern in food industry (Núñez-Montero and Barrientos, 2018; Torracchi *et al*., 2020). Finally, others produce highly valued compounds in cosmetics and biomedicine, such as pigments or therapeutic molecules.

3.2. Carotenoids and other pigments

Carotenoids are a group of compounds within the isoprenoids characterized by their red-to-yellow pigmentation. They are responsible for the color of many fruits and vegetables, but they are also synthesized by bacteria and algae (Paniagua-Michel *et al*., 2012). Both phototrophic and non-phototrophic microorganisms synthetize these pigments as they confer protection against reactive oxygen species (ROS) and the resulting oxidative stress. However, humans and most animals must acquire them from the environment, mainly through the diet (Tian and Hua, 2010; Toews *et al*., 2017).

Oxidative stress is a natural condition consequence of an imbalance between pro-oxidative molecules and antioxidative molecules. Although pro-oxidative molecules are subproducts of the cellular metabolism, it has been demonstrated that prolonged oxidative stress plays an important role in the development and progression of different pathologic states, among which cancer, neurodegenerative and cardiovascular diseases outstand (Batty *et al*., 2022; Chen and Zhong, 2014; Gorrini *et al*., 2013).

Oxidative stress is generated as a response to different environmental stressors to which organisms, including microorganisms, are subjected. This is the reason why extremophilic microorganisms are considered a promising source of antioxidant molecules. For example, the phylum *Deinococcota*, which has demonstrated high tolerance and resistance to radiation, oxidation, desiccation and high temperatures, synthetizes unique carotenoids

such as deinoxanthin or thermozeaxanthin. *Actinomycetota*, a major soil and desert inhabitant, hosts a wide diversity of colorful bacteria due to the synthesis of varied carotenoids. Moreover, microbial strains from thermophilic environments, from natural radioactive sites and from Antarctica have also been studied for their carotenogenesis (Asker *et al*., 2007; Lutnaes *et al*., 2004; Mandelli *et al*., 2012; Sandmann., 2021; Silva *et al*., 2021; Tian and Hua, 2021; Ron *et al*., 2018).

The interest of carotenoids and other pigments goes beyond their antioxidant effects. Their use in therapy is also related to their antimicrobial effect (antibiotic, antifungal and antiviral), and to their cytotoxic and antiproliferative effect with direct impact on anticancer activity (Nawaz *et al*., 2020; Tapia *et al*., 2021). Moreover, some of them may be used as biodegradable sunscreens (Gabani and Singh, 2013). Carotenoids are, thus, highly valuable molecules for the pharmaceutical industry that can be obtained from microbial sources. Their therapeutic effects are mediated by their ability to scavenge ROS molecules and have been reported in aging processes, cardiovascular diseases, Alzheimer's disease and cancer (Balendra and Singh., 2021; Leoncini *et al*., 2015; Przybylska and Tokarczyk, 2022; Sztretye *et al*., 2019).

3.3. Therapeutic molecules

Extremophilic organisms are a source of varied therapeutic molecules, apart from the above-described pigments. Among them, antimicrobials (antibiotics and antifungals) are of major interest. Antibiotics are a diverse group of clinically-relevant molecules that have largely been isolated from microorganisms within *Actinomycetota*, a major inhabitant of desert environments (Azua-Bustos and González-Silva, 2014; Durand *et al*., 2019; Hui *et al*., 2021; Zhao *et al*., 2018). Specifically, chaxamycins A-D, Mutactimycin AP and abenquines A-D, among others, have been isolated from microorganisms of the Atacama Desert (Astakala *et al*., 2022; Rateb *et al*., 2011; Schulz *et al*., 2011). Moreover, halocins and diketopiperazines have been isolated from halophilic microorganisms, whereas psychrophilic bacteria from Antarctica have also proved a good source of antimicrobials against a wide range of human pathogenic bacteria (Coker, 2016; Núñez-Montero and Barrientos, 2018).

Beyond antimicrobials, substances with antitumor, anticancer or antiproliferative effect have also been isolated and characterized from extremophilic microorganisms. Radioresistant microorganisms develop enzymatic and non-enzymatic mechanisms to repair radiation-damaged DNA. As DNA damage can further cause cancer, possible applications of these molecules in cancer therapeutics may be explored (Gabani and Singh, 2013). In particular, the carotenoid deinoxanthin has been studied for its apoptotic effect in cancer cells, and berkelic acid, synthetized by bacteria living in a metal-contaminated lake has shown activity against ovarian cancer cell lines (Choi *et al*., 2014; Stierle *et al*., 2006). Moreover, microorganisms exposed to high radiation synthetize Mycosporine-like aminoacids (MAAs), which have antioxidant properties, but also antiproliferative activity and sunscreen effect (Gabani and Singh, 2013; Nayak *et al*., 2021).

Halophilic bacteria are also a source of biosurfactants and polyhydroxyalcanoates (PHAs), with special interest for the pharmaceutical industry. On one hand, biosurfactants are amphiphilic molecules that decrease surface tension between phases and act as emulsifiers, and have proven useful in combating cell adhesion and biofilm formation of pathogenic bacteria in implants and catheters (Kochhar *et al*., 2022). On the other hand, PHAs are non-cytotoxic, biocompatible and biodegradable bioplastics that are good alternatives to petroleum-derived plastics for drug delivery. Moreover, gas-filled recombinant vesicles from these microbes are also promising in this area (Coker, 2016; Kochhar *et al*., 2022).

4. Challenges we tackled

The work described in this thesis aims at characterizing the microbial profiles associated to different natural and artificial surfaces subjected to irradiation, through a holistic strategy including both culture-independent and culture-dependent

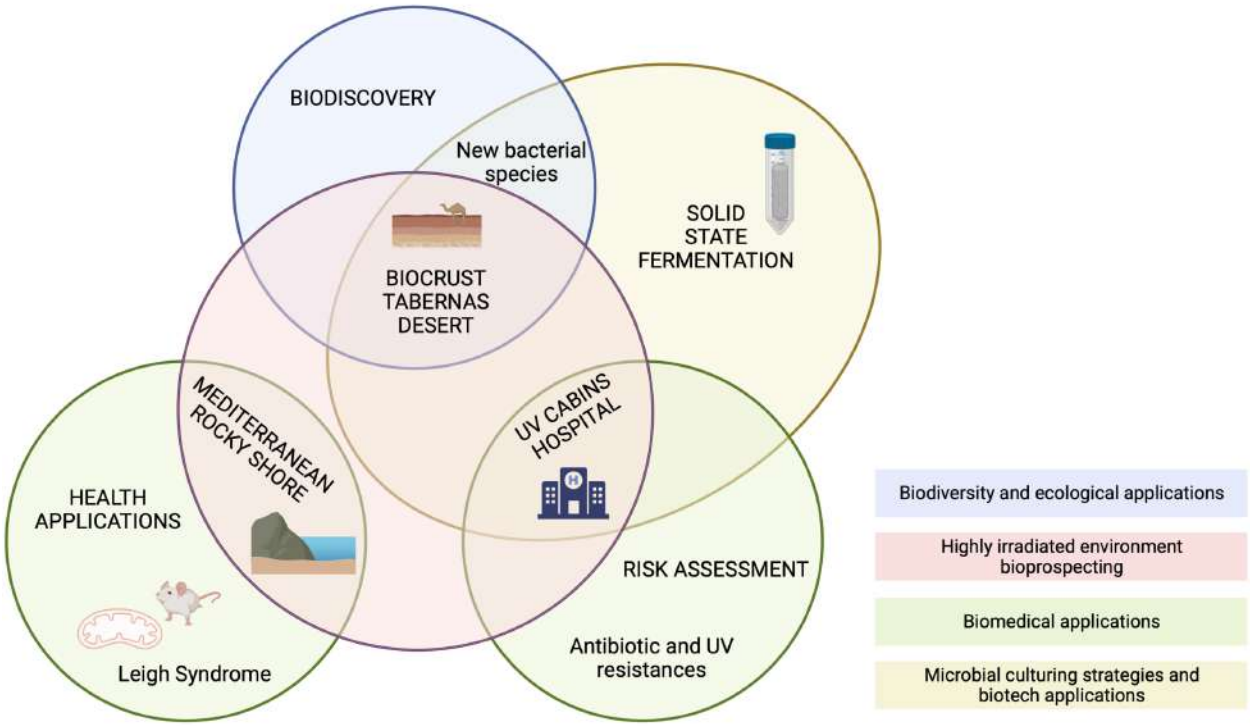

Figure 3. Challenges tackled in the thesis. Created with BioRender.com

techniques. Specifically, the microbial communities from the supralittoral zone of the Mediterranean coast in three different locations (Vinaròs, Cullera and Dénia, Spain) and from biocrusts from the Tabernas Desert (Almería, Spain) have been studied. Moreover, the microorganisms living on the surfaces of UV cabins in a hospital facility have also been explored.

The biotechnological applications and biomedical implications of these microorganisms has been investigated. Specifically, the use of microorganisms isolated from the supralittoral coast in therapeutics, as well as the potential resistances to antibiotics and UV light in a hospital have been assessed. From the ecological point of view, we have emphasized improving the recovery of the maximum microbial diversity and the description of novel taxa through the combination of simple culturing strategies. Furthermore, use of alternative culture configurations through solid state fermentation has been studied, with implications in industrial biosynthetic processes, particularly for extremophilic and/or drought-adapted microorganisms (Figure 3).

Objectives

This thesis aims at describing microbial communities living in highly-irradiated and dry environments, from both ecological and biotechnological perspectives. For this purpose, the following specific objectives were set:

- Studying the microbial communities from natural highly-irradiated environments (Chapters 1 and 2).
- Studying the microbial communities from artificial highly-irradiated environments (Chapter 3).
- Describing new microbial species isolated from extreme environments (Chapter 2).
- Assesing the potential of combining culturing strategies in isolating highly diverse microbial taxa (Chapters 1, 2 and 3).
- Exploring combined and innovative microbial culturing strategies and their implications in industrial biosynthetic processes (Chapter 4).
- Assessing the therapeutic potential of environmental microorganisms (Chapter 4).

Chapter 1: Microbial communities of the Mediterranean rocky shore

Publication I: Molina-Menor, E., Tanner, K., Vidal-Verdú, À., Peretó, J., and Porcar M. (2019). Microbial communities of the Mediterranean rocky shore: ecology and biotechnological potential of the sea-land transition. *Microbial Biotechnology*, *12,* 1359-1370.

Publication I

Microbial communities of the Mediterranean rocky shore: ecology and biotechnological potential of the sea-land transition

Esther Molina-Menor[1†], Kristie Tanner[1,2†], Àngela Vidal-Verdú[1], Juli Peretó[1,2,3] and Manuel Porcar[1,2*†]

1. Institute for Integrative Systems Biology I2SysBio, Universitat de València-CSIC, Paterna 46980, Spain.
2. Darwin Bioprospecting Excellence S.L. Parc Científic Universitat de València, Paterna 46980, Spain.
3. Departament de Bioquímica i Biologia Molecular, Universitat de València, Burjassot 46100, Spain.

†These authors contributed equally to this work.

Summary

Microbial communities from harsh environments hold great promise as sources of biotechnologically relevant strains and compounds. In the present work, we have characterized the microorganisms from the supralittoral and splash zone in three different rocky locations of the Western Mediterranean coast, a tough environment characterized by high levels of irradiation and large temperature and salinity fluctuations. We have retrieved a complete view of the ecology and functional aspects of these communities and assessed the biotechnological potential of the cultivable microorganisms. All three locations displayed very similar taxonomic profiles, with the genus *Rubrobacter* and the families *Xenococcaceae*, *Flammeovirgaceae*, *Phyllobacteriaceae*, *Rhodobacteraceae* and *Trueperaceae* being the most abundant taxa; and *Ascomycota* and halotolerant archaea as members of the eukaryotic and archaeal community respectively. In parallel, the culture-dependent approach yielded a 100-isolates collection, out of which 12 displayed high antioxidant activities, as evidenced by two *in vitro* (hydrogen peroxide and DPPH) and confirmed *in vivo* with *Caenorhabditis elegans* assays, in which two isolates, CR22 and CR24, resulted in extended survival rates of the nematodes. This work is the first complete characterization of the Mediterranean splash-zone coastal microbiome, and our results indicate that this microbial niche is home of an extremophilic community that holds biotechnological potential.

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Introduction

The interphase between marine and land environments is an ecologically complex habitat in which selection pressures from both environments can co-occur. Some of those pressures are high salinity, dehydration, wind and sun exposition, extreme temperature oscillations and mechanical stress associated with seawater splash, often with sand or pebbles, with strong abrasive effects. The aquatic to land transition has been reported to be linked to a narrow gradient in species distribution in function of the distance to the water line, as for example in cyanobacteria in an English lake (Pentecost, 2014). Regarding marine environments, the microbial ecology of rocky shores has previously been analysed (Chan *et al*., 2003; Langenheder and Ragnarsson, 2007; Pinedo *et al*., 2007; Brandes *et al*. 2015), including its links with oil spills and biodegradation (Alonso-Gutiérrez *et al*., 2009). However, and in contrast with the well-studied microbial ecology of the intertidal zone (for a review, see Mitra *et al*., 2014), a holistic study on the microbial ecology of the marine supralittoral Mediterranean rocky shore has not been addressed previously.

Harsh, extremophilic environments can be sources of biotechnologically relevant bacteria and therefore hold great promise for the biotechnological industry (Raddadi *et al*., 2015). For example, extremophilic microorganisms can yield enzymes such as lipases and esterases that can be used under a wide range of conditions and may have relevant applications in the food, detergent and biofuel industries (Fuciños *et al*., 2012). There are many other examples of biotechnologically relevant microorganisms from extreme environments, including the well-known case of *Thermus aquaticus*, which produces the widely used Taq polymerase; or the hyperthermophilic biofuel-producing archaea that live in deep-sea hydrothermal vents (Chien *et al*., 1976; Nishimura and Sako, 2009).

The present study focuses on the microorganisms that inhabit the rocky areas of the supralittoral zone (the area just above the tide line that is subjected regularly to splash but is not permanently underwater) of the Mediterranean coast. Surface-associated microbial communities that are sun-exposed are often rich in microorganisms that produce pigments, including carotenoids (Dorado-Morales *et al*., 2015; Kumar *et al*., 2015; Shindo and Misawa, 2014; Tanner *et al*., 2017). These pigments play a key role in radiation tolerance (Tian and Hua, 2010; Klindworth *et al*., 2013; Sandmann, 2015; Tanner *et al*., 2018), and they are valuable for the food, pharmacological and cosmetic industries as colourants, antioxidants and protectors against solar radiation respectively (Sandmann, 2015). Therefore, we hypothesized that rough conditions of the supratidal zone may be associated with the presence of biotechnologically relevant microbial taxa. From this hypothesis, we have, in the present work, compared three different supralittoral coastal locations of the Mediterranean West coast and combined culturing techniques and high throughput sequencing data (16S rRNA amplicon and metagenomic sequencing) in order to shed light on the taxonomic composition of these communities, and to explore the biotechnological potential of the culturable strains.

Results

High-throughput 16S rRNA analysis

High-throughput 16S rRNA sequencing of the samples revealed that, based on the comparison of the richness value (number of different species; Figure I.1A) and the diversity (Shannon index; Figure I.1B), the alpha diversity was not significantly different among the locations. Moreover, the shape of the rarefaction curve at OTU level (Operational Taxonomic Unit) showed that the sequences covered the majority of taxa present in the samples (Figure I.S1).

However, the composition of the bacterial communities varied depending on the location, as represented in the Principal Coordinates Analysis (PCoA; Figure I.2A). Samples from Dénia showed the highest intragroup homogeneity, whereas samples from Vinaròs and Cullera displayed higher differences between replicates. Nevertheless, samples from all

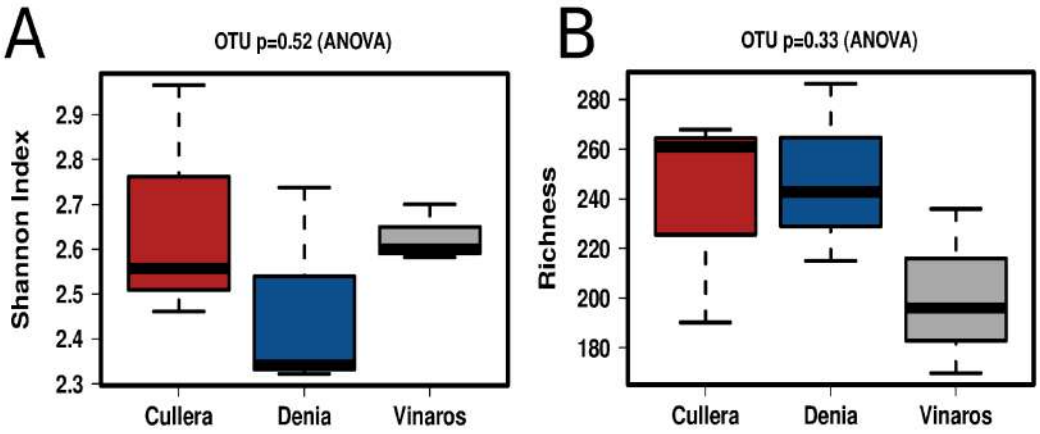

Figure I.1. Box plots showing the values of alpha diversity indexes in the sampled locations on the Mediterranean rocky-shore. (A) Observed richness at OTU level (number of OTUs). (B) Shannon index of diversity.

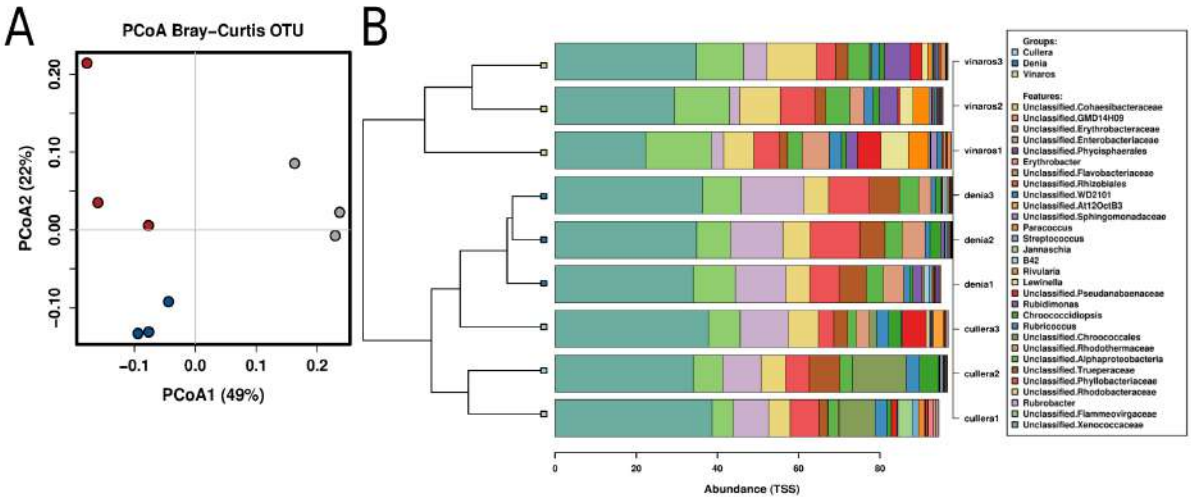

Figure I.2. (A) Principal coordinates analysis (PCoA) based on Bray-Curtis distances between OTUs in bacterial communities of three different locations. (B) Clustered-Barchart showing the top 30 most abundant genera in terms of relative abundance.

three locations could be distinguished in the plot. The variability explained by both axes is high enough to conclude that the microbial communities among the three locations are different. Moreover, the representation of the relative abundances (TSS) of the top 30 most abundant genera showed that the microbial composition was generally similar along the locations (Figure I.2B), although some taxa such as the genus *Rubrobacter* in Vinaròs or the genus *Rubricoccus* in Dénia allowed the differentiation of specific regions (Table I.1). Eleven out of the 30 most abundant genus were significantly different at least in one location. A list of the 30 more significantly different genus is shown in (Table I.S1). The original data have been deposited with the NCBI SRA accession number PRJNA556782.

Shotgun metagenomic analysis

The three locations exhibited similar taxonomic profiles according to the metagenomics analysis. The most abundant bacterial phyla were the same ones observed with high-throughput 16S rRNA sequencing, with *Cyanobacteria* being the most abundant in all three locations. Moreover, other taxa, such as the families *Rhodobacteraceae*, *Flammeovirgaceae*,

Table I.1. Top 30 most abundant genera and P-values for the One-Way ANOVA statistical analysis of their distributions among the three sampled locations.

TAXA	P labelA	P (Tukeys) Dénia-Cullera	P (Tukeys) Vinaròs-Cullera	P (Tukeys) Vinaròs-Dénia
Rubrobacter	0.0011*	0.083	0.0096*	0.00091*
Rubricoccus	0.0018*	0.0014*	0.057	0.026*
Unclassified.Flammeovirgaceae	0.006*	0.22	0.0051*	0.04*
Rubidimonas	0.0075*	0.58	0.0078*	0.024*
Unclassified.Erythrobacteraceae	0.0085*	0.011*	0.018*	0.9
Unclassified.Alphaproteobacteria	0.019*	0.052	0.02*	0.71
Unclassified.Cohaesibacteraceae	0.021*	0.021*	0.062	0.66
Rivularia	0.026*	1	0.04*	0.038*
Unclassified.Rhodobacteraceae	0.037*	1	0.058	0.052
Unclassified.WD2101	0.039*	1	0.06	0.054
Unclassified.Chroococcales	0.045*	0.063	0.068	1
Lewinella	0.052	1	0.073	0.075
Unclassified.Trueperaceae	0.066	0.29	0.45	0.056
Unclassified.Phyllobacteriaceae	0.091	0.094	0.87	0.18
Unclassified.Xenococcaceae	0.1	0.85	0.1	0.21
Unclassified.GMD14H09	0.12	1	0.17	0.16
Unclassified.Sphingomonadaceae	0.13	0.88	0.24	0.13
Unclassified.Flavobacteriaceae	0.13	1	0.16	0.17
B42	0.18	0.19	0.95	0.28
Unclassified.Rhodothermaceae	0.24	0.23	0.47	0.82
Unclassified.Rhizobiales	0.25	0.72	0.55	0.22
Chroococcidiopsis	0.28	0.4	0.3	0.97
Erythrobacter	0.28	0.29	0.42	0.95
Unclassified.Phycisphaerales	0.32	0.29	0.66	0.73
Unclassified.Pseudanabaenaceae	0.34	0.51	0.93	0.33
Jannaschia	0.39	0.44	0.47	1
Paracoccus	0.44	0.78	0.41	0.78
Unclassified.At12OctB3	0.46	0.49	0.55	0.99
Streptococcus	0.54	0.91	0.52	0.76
Unclassified.Enterobacteriaceae	0.77	0.91	0.75	0.95

Trueperaceae and the genus *Rubrobacter*, belonging to the phyla *Proteobacteria*, *Bacteroidetes*, *Deinococcus-Thermus* and *Actinobacteria* respectively, were also detected (Figures I.S2A, I.S3A and I.S4A). Metagenomic sequencing allowed the identification of abundant taxa in the *Cyanobacteria* phylum, including the genera *Staniera*, *Pleurocapsa*, *Myxosarcina* and *Xenococcus*, in contrast to the high-throughput 16S rRNA, which mainly showed unclassified *Xennococcaceae* taxa.

Archaeal and eukaryotic communities proved very diverse, with a high number of salt-adapted microorganisms in the former and a large fraction of *Ascomycota* in the latter (Figures I.S2, I.S3, I.S4B and C). Salt-adapted archaea included members of *Halococcus*, *Halobacteriaceae* (*Haladaptatus* and *Halalkalicoccus*), *Haloarculaceae*, *Haloferaceae*, *Halorubraceae* and *Natrialbaceae* families, as well as methanogenic archaea (members of the *Methanosarcinaceae* family; Figures I.S2B, I.S3B and I.S4B). Among the diversity of *Ascomycota*, the most abundant taxa were *Glonium stellatum*, *Cenococcum geophilum, Coniosporium apollinis* and *Lepidopterella palustris* (Figures I.S2C, I.S3C and I.S4C).

The functional analysis of the samples revealed a high representation of enzymes related to oxidative stress, being peroxiredoxin (EC 1.11.1.15) and peroxidase (EC 1.11.1.7) the most abundant activities, and displaying the highest values in Cullera and Vinaròs respectively. Thioredoxin-related enzymatic activities (EC 1.8.4.8; EC 1.8.1.9; EC 1.8.4.10) were homogeneously represented in all three samples, as well as superoxide dismutase (EC 1.15.1.1). Other enzymes such as glutathione transferase (EC 2.5.1.18) or glutathione peroxidase (EC 1.11.1.9) varied among locations, with the former being more represented in Vinaròs and Cullera than in Dénia, and the latter being more abundant in Vinaròs. Among the genes related to carotenoid biosynthetic routes, the abscisic acid 80-hydroxylase (EC 1.14.14.137) was particularly represented in Cullera, whereas sphingolipid-related genes such as glucosyl ceramidase proved to be

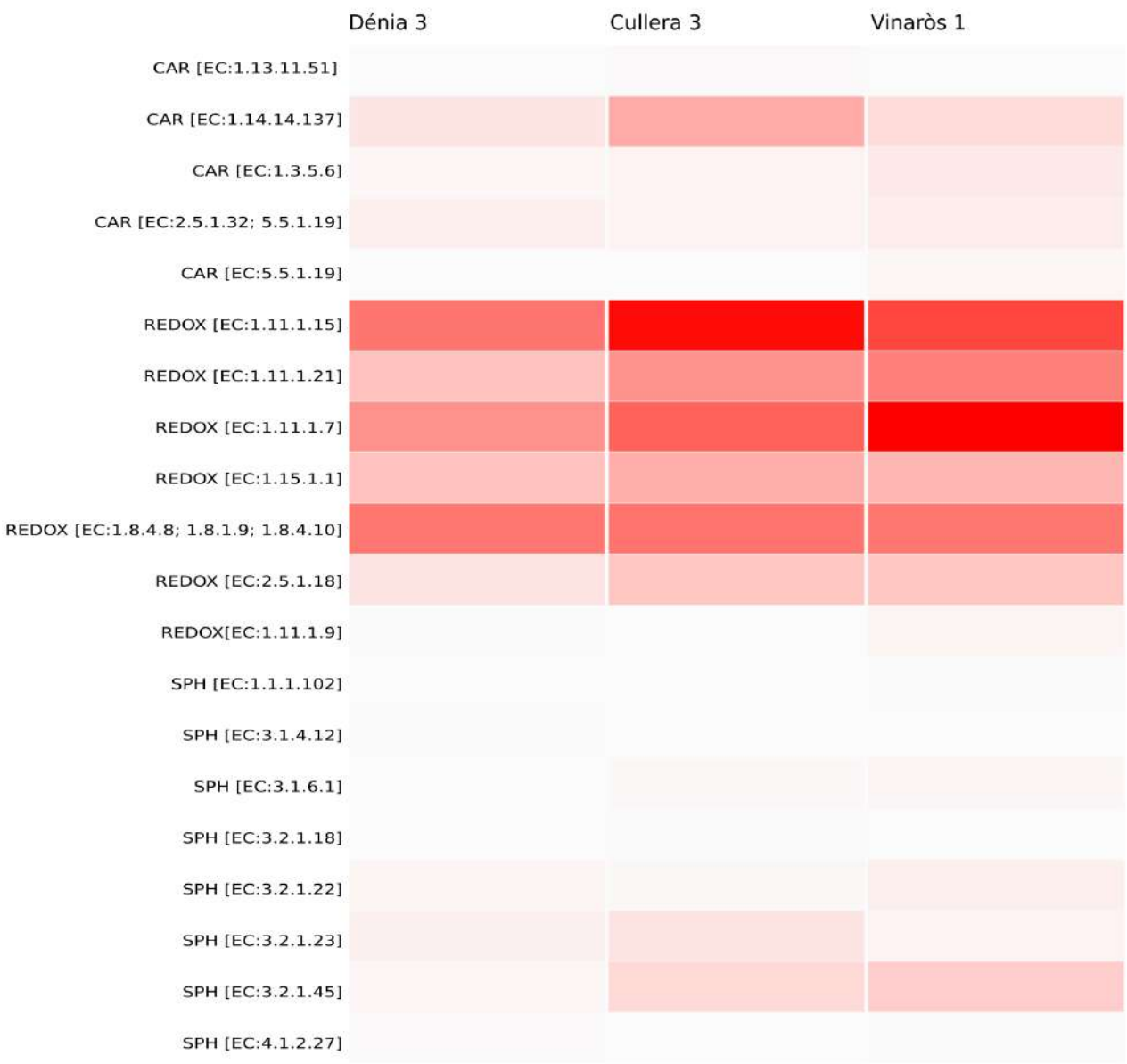

Figure I.3. Heatmap representing the functional analysis carried out through metagenomics sequencing. Enzymes related to carotenoid biosynthesis (CAR), oxidative stress (REDOX) and sphingolipid biosynthesis (SPH) are shown in the Y-axis.

frequent in Vinaròs (EC 3.2.1.45; Figure I.3). The original data have been deposited with the NCBI SRA accession number PRJNA556786.

Strain collection and identification

Culturing the samples on LB and Marine Agar yielded a large diversity of colonies in terms of colour, shape and morphology. A total of 100 strains were isolated and named with a code, after the location (C: Cullera, D: Dénia, V: Vinaròs) and the origin (M: Marine water, R: Rock surface). In our conditions, there was no significant fungal growth in any of the samples. The colonies observed on Marine Agar displayed the widest range of colours (wine-red, red, pink and orange, among others) in comparison with the ones observed on LB media, which were mostly yellowish and cream-coloured. Due to the known relation between the presence of pigments and antioxidant power, the main criterion for colony selection was the colour (Pawar *et al*., 2015).

A collection of the 100 selected isolates in pure culture was established. A total of 34 isolates were initially identified through colony PCR and 16S rRNA Sanger sequencing. Although an initial step of

incubation at 100°C was added to the PCR protocol of the isolates whose amplification had failed, some remained non-identified and therefore their total DNA was extracted to repeat the PCR. Finally, 56 of the isolates remained unidentified. Among theidentified isolates, there were many *Bacillus* spp. (*B. oleronius*, *B. licheniformis*, *B. marisflavi*, *B. salsus* and *B. altitudinis*) and *Halobacillus* spp. (*H. trueperi* and *H. faecis*) as well as other species such as *Micrococcus antarcticus*, *Micrococcus luteus*, *Staphylococcus pasteuri*, *Vibrio tubiashii* and *Virgibacillus halodenitrificans* (Table I.S2).

Antioxidant activity

In order to select and establish a collection of isolates with antioxidant properties, a high-throughput screening of the 100 isolates was performed by growing them on solid media containing H_2O_2. *Planomicrobium glaciei* and *E. coli* JM109 were used as positive and negative controls respectively. Strain JM109, with no known reports of antioxidant effect, exhibited a weak growth on the first (OD_{600} 1) and, sometimes, second dilution (OD_{600} 10^{-1}). This led us to the criterion to consider positive antioxidant producers those strains able to grow on H_2O_2-containing plates at least up to threefold dilutions (OD_{600} 10^{-2}). A total of 12 isolates were thus selected (Table I.2) based on their ability to grow on 1 mM H_2O_2 plates as described above.

DPPH-based assays are widely used to detect and quantify the antioxidant power of plants or bacterial extracts. These assays are based on the decrease of DPPH absorbance at 517 nm in presence of antioxidant factors. The oxidative stress-resistant isolates selected from the H_2O_2 assay (shown in Table I.2) were further tested using this method. CR17, CR21 and CR57 could not be tested due to poor growth in liquid culture, which made it impossible to obtain a concentrated extract, prepared as described in Experimental Procedures. 16S rRNA sequences were compared using NCBI BLAST tool. Isolates CR10-VR2 and CR22-CR28 were 100% identical in their 16S rRNA sequence, and therefore only one of them was selected for further assays (CR10 and CR22 respectively).

The test resulted in a general decrease in absorbance in all the samples, suggesting that the extracts were able to scavenge the DPPH. The isolates that proved more effective as antioxidants were CR22, CR24 and CR28, with values of scavenged DPPH over 30% (Figure I.4A). DR12 displayed low DPPH scavenging values maybe due to failure of the pigment extraction. Surprisingly, the control samples *P. glaciei* and JM109 did not display the expected effect. A set of three strains that had previously shown a protective effect against oxidative stress in a *Caenorhabditis elegans* model and a set of three *E. coli* strains (JM109, HB101 and DH5a) were also tested (Figure I.4D).

The two strains with the best results in the *in vitro* assays (CR22 and CR24) were selected for further *in vivo* antioxidant assays in the model organism *C. elegans*, where both proved able to display an important antioxidant activity (Figure I.4B).

Table I.2. List of selected isolates, percentage of identity with the closest type strain, sequence similarity and results obtained in the H_2O_2 assay.

SAMPLE	CLOSEST TYPE STRAIN	%	H_2O_2 ASSAY (dilution at which the isolate remains viable)
CR10	*Micrococcus luteus* (CP001628)	99.77	3
CR17	*Virgibacillus halodenitrificans* (AY543169)	99.58	7
CR21	Non-identified	-	4
CR22	*Virgibacillus halodenitrificans* (AY543169)	99.37	4
CR24	*Halobacillus trueperi* (AJ310349)	98.31	6
CR28	*Virgibacillus halodenitrificans* (AY543169)	100	6
CR37	*Bacillus marisflavi* (LGUE01000011)	100	4
CV44	Non-identified	-	3
CV67	*Bacillus oleronius* (X82492)	97.32	4
DM10	Non-identified	-	3
DR12	Non-identified	-	3
VR1	*Bacillus altitudinis* (ASJC011000029)	100	6
VR2	*Micrococcus luteus* (CP001628)	99.35	3
Positive control	*P. glaciei*		8
Negative control	*E. coli* (JM109)		1

Nematodes subjected to oxidative stress after being treated with isolates CR22 and CR24 displayed survival rates higher than the untreated worms and similar to those observed in the worms treated with vitamin C (survival rates of around 55%–65%).

Discussion

We report here, for the first time, and by using culture-dependent and independent (NGS) techniques, the microbiomes of the rocky-coastal surface of the supralittoral zone in three regions on the Mediterranean western coast. The three sampled sites, covering a coast line of about 260 km, displayed remarkably similar taxonomic profiles in terms of richness and microbial diversity, but still could perfectly be differentiated thanks to the significant difference in abundances of specific taxa, which suggest that the microbial composition of the Mediterranean supratidal zone, at least in eastern Spain, is stable but not identical within rocky locations. The studied communities were particularly dominated by bacterial strains previously described as thermophilic, halotolerant or radioresistant, such as the species within the genus *Rubrobacter* (Jurado *et al*., 2012), and pigmented isolates, as is the case of species within the *Flameovirgaceae* family, like *Tunicatimonas pelagia* and *Porifericola rhodea* (Yoon *et al*., 2011, 2012).

Truepera radiovictrix, characterized by an optimum growth temperature of 50°C and an extreme resistance to ionizing radiation, was first isolated from a hot spring in a geothermal area close to the Azores (Albuquerque *et al*., 2005; Ivanova *et al*., 2011). Moreover, the *Truepera* genus has been previously found in Lake Lucero Playa (New Mexico, USA), a particularly hostile environment as the lake dries periodically (Sirisena *et al*., 2018). This is, to the best of our knowledge, the first report of sea-inhabiting *Truepera* in a non-thermal environment, and it is tempting to hypothesize that the genus *Truepera* might have a similar ecological niche (radiation- and desiccation- resistance) than *Deinococcus*, but in saline environments, as a consequence of both its radiation resistance and halotolerance (Albuquerque *et al*., 2005).

Shotgun metagenomic analysis confirmed the similarity between the communities of the three sampled locations, as discussed above from the high-throughput 16S rRNA results, particularly at higher taxonomic (i.e., family) levels. Nevertheless, the results at lower taxonomic levels varied considerably among sequencing techniques. One of the largest differences at the species level was observed within the Cyanobacterial group. In particular, high-throughput 16S rRNA revealed a large abundance of *Xennococcaceae*, whereas shotgun metagenomic sequencing revealed a more diverse population including members of *Pleurocapsa*, *Myxosarcina*, *Stanieria* and *Xenococcus*, as previously reported for marine environments (Burns *et al*., 2004; Alex *et al*., 2012; Yu *et al*., 2015; Brito *et al*., 2017).

The eukaryotic fraction of the samples was mainly composed of Ascomycota, such as *Glonium stellatum*. The genus *Glonium* includes saprophytic *Dothideomycetes* that produce darkly pigmented apothecia, which could contribute to the dark colour of the sampled rocks (Spatafora *et al*., 2012). Other species detected in the samples included as follows: *Cenococcum geophilum*, an ectomycorrhizal fungus previously described in coastal forest soils (Matsuda *et al*., 2015) and previously demonstrated to grow at up to 100 mM of NaCl (Obase *et al*., 2010); *Coniosporium apollinis*, a rock-inhabiting fungi previously isolated from the Mediterranean basin (Sterflinger *et al*., 1997); and *Lepidopterella palustris*, typically a freshwater fungus (Shearer *et al*., 2009), with this being, to the best of our knowledge, the first description of this species in a salt water habitat.

Taken together, the results obtained from both high-throughput 16S rRNA and metagenomic sequencing suggest that the sampled communities are composed of a diverse array of fungi (mainly belonging to the phylum *Ascomycota*), cyanobacteria (mainly *S. cyanosphaera* and *Pleurocapsa* spp., but also *Myxosarcina* spp. and *Xenococcus* spp.) and salt-adapted archaea, which remain rather stable among the three different sampled locations. From the functional point of view, metagenomics sequencing showed abundance of enzymes involved in oxidative

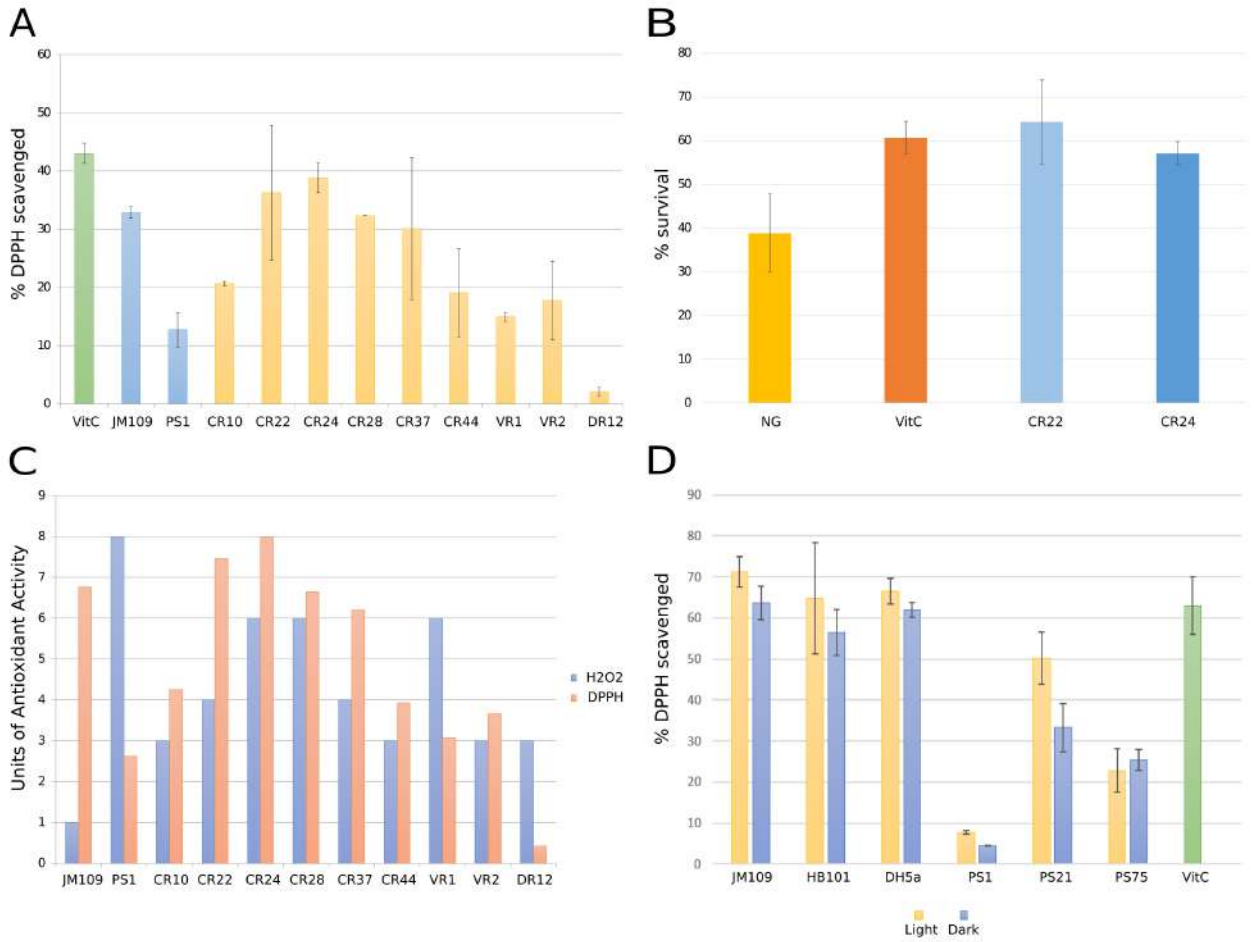

Figure I.4. (A) Antioxidant activity as measured through DPPH assay as described in EP. Absorbance was measured at 517 nm after 30 min of incubation with DPPH 50 µM. DPPH scavenged (%) is represented in Y-axis. VitC, vitamin C (0.5 µg/mL solution). (B) Antioxidant activity *in vivo* (using the model organism *C. elegans*). Y-axis indicates percentage of surviving worms after 5 h of incubation under oxidative stress (H_2O_2). Worms were treated with either a control diet (NG), a diet supplemented with the known antioxidant vitamin C as a positive control (VitC), or a diet supplemented with the selected strains CR22 and CR24. (C) Comparative analysis of the results obtained with H_2O_2 and DPPH assays. Values in Y-axis are normalized with respect to the highest value obtained in both assays. (D) DPPH assay with positive and negative controls. Absorbance was measured at 517 nm after 30 min of incubation with DPPH 50 µM. DPPH scavenged percentage is represented in Y-axis. VitC, Vitamin C, 0.5 µg/mL solution. Light and dark conditions are represented.

stress, mainly peroxidase, peroxiredoxin and thioredoxin, but also catalase and glutathione transferase. In contrast with this, enzymes involved in carotenoid or sphingolipid biosynthesis, which also play a role in the protection against oxidative stress, were less abundant and varied among locations, being abscisic acid 80-hydroxylase (EC 1.14.14.137) in Cullera and glucosylceramidase (EC 3.2.1.45) in Vinaròs the ones with the highest values.

From the collection of cultured microorganisms, a total of 12 isolates were selected for their high antioxidant activity as measured by the oxidative stress assay performed with H_2O_2. Of those, *M. luteus* has been reported to encode genes related to resistance and tolerance to oxidative stress (superoxide dismutase and NADP reductase; Lafi *et al*., 2017). The DPPH assay was performed to dismiss false positives through the H_2O_2 assay. In general, the results correlated well with the ones previously observed in the H_2O_2 assay. It is important to note that, although DR12 displayed low scavenging in the DPPH assay, the extraction of pigments from this isolate was sub-optimal, since the pellet remained pink-coloured after the extraction process. Surprisingly, the control samples *P. glaciei* and JM109

did not display the expected effect in terms of antioxidant activity. On one hand, *P. glaciei* was expected to be one of the most antioxidant isolates, as its antioxidant activity was demonstrated in previous *in vivo* assays in *C. elegans* (Tanner *et al*., 2019) and in the H_2O_2 assay. Nevertheless, it was the worst strain in terms of DPPH scavenging. On the other hand, *E. coli* JM109, with no previous reports on antioxidant activity, resulted in high DPPH scavenging. This raise concerns on the suitability of DPPH-methods in bioprospecting for the determination of antioxidant activity and highlights the importance of using several alternative methods as the best option to have a proxy of the *in vivo* antioxidant effects. Nevertheless, the *in vivo* antioxidant assay performed in *C. elegans* allowed to confirm the antioxidant activity detected in the DPPH and H_2O_2 tests. Specifically, CR22 and CR24 displayed an antioxidant activity similar to the one observed in Vitamin C (Figure I.4B).

In general, though, the correlation between both methods was good, as the isolates with higher survival in the presence of H_2O_2 also displayed higher DPPH-scavenging ability (Figure I.4C). Nevertheless, there were some isolates that displayed different results depending on the method, in particular VR1 and CR37. Differences in VR1 could be the result of catalase activity, which may have enhanced its growth on the H_2O_2-supplemented plates. On the contrary, differences between both methods for CR37 could be caused by a deficient growth in solid medium. Once again, these results highlight the limitation of using a single screening technique for the selection of microbial strains with antioxidant activities. A collection of both positive and negative controls (in terms of theoretical antioxidant activity) were tested using both assays (H_2O_2 and DPPH). PS1, PS21 and PS75 (*P. glaciei* 423, 97.38% ID; *Rhodobacter maris* JA276, 98.89% ID; and *Bacillus megaterium* NBRC 15308, 100% ID respectively) were the three control strains selected, all of them recovered from solar panels and previously tested in *C. elegans* for *in vivo* protection against oxidative stress (Tanner *et al*., 2019). Three different strains of *E. coli* were chosen as negative controls (JM109, BH101, DH5a). For the DPPH assay, the isolates were grown under both light and dark conditions, in order to determine whether the light had a negative impact on the production of pigments or other antioxidant factors, as it is known that many pigments, particularly carotenoids, are prone to photodegradation (Boon *et al*., 2010). For the *E. coli* strains, no significant differences were observed between growth in dark and light conditions, whereas PS21 proved very sensitive to light (Figure I.4D). Moreover, the scavenging effect of the JM109 strain was also observed in the other two *E. coli* strains, confirming that the extracts obtained from *E. coli* contain compounds that are indeed able to react with DPPH. Even though *R. maris* and *B. megaterium* displayed better antioxidant properties than *P. glaciei*, which was again comparable to the negative control of methanol, they yielded lower DPPH-based activity than *E. coli* strains.

The biotechnological potential of extremophiles is well known, and saline environments are no exception to this rule (de Lourdes Moreno *et al*., 2013). However, and in contrast with the well-studied intertidal zone (Mitra *et al*., 2014), the supralittoral zone has been poorly studied to date. Interestingly, this zone experiences much higher selection pressures than the intertidal zone since while the intertidal zone is basically a marine environment which is only transiently and partially exposed to land conditions, the supralittoral zone forces organisms to adapt to a sea/land intermediate habitat where both marine and land stresses are present.

This work is the first holistic (using culture-dependent, culture-independent and biological activity assays) approach studying the microbial ecology and biotechnological potential, in terms of antioxidant properties, of the supralittoral zone of the Mediterranean rocky shore. Our results suggest that the western coastline of the Mediterranean Sea harbours a stable microbial community that is conserved among different locations, with cyanobacteria as the majoritarian bacterial taxon, followed by members of the *Flameovirgaceae* family and members of the *Rubrobacter* genus, as well as

eukaryotic and archaeal members, such *Ascomycota* and halotolerant archaea. Furthermore, *in vitro* and *in vivo* assays demonstrate that this environment is a potential source of microorganisms with antioxidant activities that could hold potential for a wide range of applications in the food, cosmetic or pharmacological industries.

Experimental procedures

Sampling

Samples were collected from three different locations on the Mediterranean Western coast, in Eastern Spain: Vinaròs (Castelló), Cullera (València) and Dénia (Alacant). Three samples of dark-stained rock, at least two metres apart from each other and thus considered as biological replicates, were collected from the supralittoral (splash) zone of each location by scraping the surface with a sterile blade. Samples of the adjacent marine water were also taken, and both types of samples (scrapped rock and sea water samples) were separately stored in Falcon tubes in 15% glycerol, transported to the laboratory on ice and then stored at -20°C until required.

High-throughput rRNA and metagenomic sequencing

Total DNA was isolated from the samples with the PowerSoil DNA Isolation kit (MO BIO laboratories, Carlsbad, CA, USA) following the manufacturer's instructions. The quantity and quality of the isolated DNA was assessed using a Nanodrop-100 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and purified DNA samples were sequenced by Life Sequencing SL (València, Spain). On one hand, the hypervariable V3- V4 regions of the 16S rRNA gene was amplified as described by Klindworth *et al*. (2013) and sequenced on the high-throughput NextSeq 500 (Illumina) platform. Greengenes database was used for the taxonomic analysis. The statistical analysis was carried out with Calypso web tool (version 8.84; http://cgenome.net). The statistical comparison of the relative abundances between locations at the genus level was calculated through One-Way Anova test (Tables I.1 and I.S1). Richness and Shannon index box plots, PCoA, relative abundances clustering and rarefaction curve were also constructed with Calypso.

On the other hand, shotgun metagenomic sequencing was performed on the NextSeq500 Illumina platform, with paired-end sequences and reads of 150 base pairs. The obtained sequences were filtered by using 'Bbtools' version 37.28 (https://jgi.doe.gov/data-and-tools/bbtools/) in order to avoid ends holding quality values under the Q20 standards. Lectures coming from human contamination were also dismissed by mapping them against the reference human genome (GRCh37d5) version 0.7.15. Assembly was carried out with 'SPAdes' (Bankevich *et al*., 2012) version 3.9. ORFs prediction was carried out by 'MegaGeneMArk' (Zhu *et al*., 2010) version 3.38 and rRNA prediction, by 'RNAmmer' (Lagesen *et al*., 2007) version 1.2. Functional annotation of the predicted CDS was carried out with BLAST2go (Conesa *et al*., 2005) version 4.1.9. The Clustergrammer on-line software (Fernández *et al*., 2017) was used for the functional analysis heatmap construction, by using a correlation type distance and average linkage.

Isolation and identification of bacterial strains

Three different growth media were used for this study: Lysogenic Broth (LB, composition in g/L: 10 tryptone, 10 NaCl, 5.0 yeast extract, 15 agar); Reasoner's 2A agar (R2A, composition in g/L: peptone 0.5, casaminoacids 0.5, yeast extract 0.5, dextrose 0.5, soluble starch 0.5, K_2HPO_4 0.3, $MgSO_4$ 0.05, sodium pyruvate 0.3, 15 agar); and Marine Agar (composition in g/L: peptone 5.0, yeast extract 1.0, ferric citrate 0.1, NaCl 19.45, $MgCl_2$ 5.9, Na_2SO_4 3.24, $CaCl_2$ 1.8, KCl 0.55, $NaHCO_3$ 0.16, KBr 0.08, $SrCl_2$ 0.034, H_3BO_3 0.022, Na_4O_4Si 0.004, NaF 0.024, NH_4NO_3 0.0016, Na_2HPO_4 0.008, 15 agar). The scraped rock samples were homogenized in the Falcon tube by vigorously mixing with a vortex, and serial dilutions were cultured on the different media and incubated at room temperature for 7 days. Marine water samples were also cultured in the same conditions. After 1 week of incubation, individual colonies were selected based on colony pigmentation and isolated by independent re-streaking on fresh medium. Pure cultures were then cryo-preserved at -80°C in 20% glycerol (vol:vol) until required.

Colony PCR and, when needed, DNA extracts of each of the isolated strains, were used for taxonomic identification through 16S rRNA gene sequencing using universal primers 28F (5'-GAG TTT GAT CNT GGC TCA G-3') and 519R (5'-GTN TTA CNG CGG CKG CTG-3'). Colony PCR was performed with an initial step of incubation at 95°C for 5 min to lyse cells followed by PCR amplification (30 cycles of 30 s at 95°C, 30 s at 54°C, 30 s at 72°C, followed by 10 min at 72°C). The DNA extraction was done following the Latorre *et al*. (1986) protocol. Amplifications were verified by electrophoresis in a 0.8% agarose gel and then amplicons were precipitated overnight in isopropanol 1:1 (vol:vol) and potassium acetate 1:10 (vol:vol; 3 M, pH 5). DNA pellets were washed with 70% ethanol and resuspended in 30 µl Milli-Q water. BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) was used to tag amplicons, which were sequenced with the Sanger method by the Sequencing Service (SCSIE) of the University of València (Spain). All sequences were manually edited with Pregap4 (Staden Package, 2002) to eliminate low-quality base calls, and final sequences were compared by EzBioCloud 16S tool (https://sourceforge.net/projects/staden/).

Antioxidant activity

Hydrogen peroxide assay. The collection of isolates was initially screened for antioxidant activity by applying oxidative stress to the isolated colonies through the addition of hydrogen peroxide (H_2O_2) to the growth medium. In order to do so, isolates were grown on solid media for 4 days or until reaching enough biomass. Then, the optical density at 600 nm (OD_{600}) was measured, adjusted to a value of 1, and serial dilutions prepared up to seven times fold. Two microlitres of each dilution were placed on a LB or Marine Agar place, to which 1 mM H_2O_2 had been previously added. The plates were incubated at room temperature and in the dark to avoid degradation of the H_2O_2, and results were recorded after two, four and six days. Two strains were used as controls for the assay: PS1 (*Planomicrobium glaciei* 423, 97.38% ID) and *Escherichia coli* JM109 as a positive and negative control for antioxidant activity respectively. *Planomicrobium glaciei* is a pigmented microorganism whose antioxidant activity has previously been reported *in vivo* using a *Caenorhabditis elegans* model (Tanner *et al*., 2019).

DPPH assay. Since the H_2O_2 assay can result in false-positive results due to catalase activity, a second assay using 2,2-diphenyl-1-picrylhydrazyl (DPPH) was performed to dismiss false positives in the H_2O_2 assay and to confirm the antioxidant activity of the selected strains (the ones with the best antioxidant activity according to the previous assay). Pigments were extracted from the isolates based on the protocols described by Brand-Williams *et al*. (1995), von Gadow *et al*. (1997) and Su *et al*. (2015), with the modifications suggested by Sharma and Bhat (2009). Briefly, the isolates were grown overnight in liquid LB medium and OD_{600} was measured and normalized at a value of 1.2. Cells were then harvested by centrifugation at 11,300 g for 3.5 min, and the pellets resuspended in 500 µL of methanol, vigorously vortexed and sonicated for 5 min (Ultrasonic bath XUBA1, Grant Instruments, Royston, UK). The supernatant was collected after centrifugation at 11,300 g for 3 min and kept in the dark until the assay was performed. The extraction was repeated as described until a colourless pellet was obtained.

For the DPPH assay, 600 µl of the extract in methanol were mixed with 400 µl of DPPH solution (50 µM in methanol) and incubated for 30 min in the dark. The negative control sample consisted of DPPH mixed with methanol. Absorbance was measured at 517 nm (Ultrospec 200 UV/V Visible Spectrophotometer, Pharmacia Biotech, Piscataway Township, NJ, USA). A standard curve with a control antioxidant, ascorbic acid (vitamin C) was performed at 10, 5, 1, 0.5, 0.1, 0.05 and 0.01 µg/mL concentrations in methanol. The detection threshold was established at 0.5 µg/mL of vitamin C, as lower concentrations of vitamin C did not change DPPH absorbance (data not shown). DPPH scavenging ability was quantified by measuring the decrease in the absorbance of this compound at 517 nm, and the percentage of scavenged DPPH was calculated using the following formula:

$$\%DPPH = \left(1 - \frac{Abs\ 517\ nm\ Extract}{Abs\ 517\ nm\ Control}\right) \times 100$$

In vivo *oxidative stress assays with* C. elegans

Wild-type *C. elegans* strain N2 (Bristol, UK) was routinely propagated at 20°C on Nematode Growth Medium (NGM) plates supplemented with *E. coli* strain OP50 as the regular food source.

Nematodes were synchronized by isolating eggs from gravid adults at 20°C. Synchronization was performed on NGM plates with different treatments: *E. coli* OP50 was supplied as a negative control; *E. coli* OP50 plus vitamin C (vitC) at 10 µg/mL as a positive control; and, finally, *E. coli* OP50 plus one of the selected isolates was used in order to test the effect of administrating the selected strains. Duplicates were performed for every condition. Bacterial strains were grown overnight in liquid LB medium at 28°C and 11,300 g. Then, OD_{600} was adjusted to 30 and 50 µl of the bacterial suspension were added to the plates.

The synchronized worms were incubated for 3 days on the previously described plates, until reaching young adult stage. Then, young adult worms were selected for each treatment (n = 50) and incubated at 20°C on the corresponding treatment, until reaching 5-day adult stage. The selected worms were then transferred to plates containing basal medium supplemented with 2 mM H_2O_2 and incubated for 5 h at 20°C. After incubation, survival rates for each condition (negative control, positive control and bacteria-fed worms) were recorded by manually counting the number of living versus dead worms.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

MP conceived the work. MP, KT and EMM collected the samples. EMM, KT and AVV performed the culture-based characterization, and KT carried out the bioinformatic analysis. All authors (MP, KT, AVV, EMM and JP) analysed the results, wrote and approved the manuscript.

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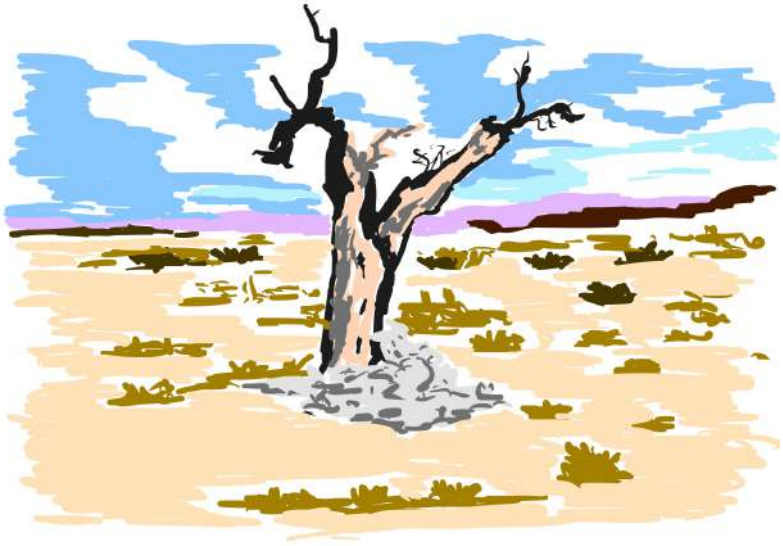
Supporting information

Suporting information may be found in Appendix B.

Chapter 2: The Tabernas Desert microbial communities



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Publication II

High culturable microbial diversity from a European desert: the Tabernas Desert

Esther Molina-Menor[1], Helena Gimeno-Valero[2], Javier Pascual[2], Juli Peretó[1,2,3] and Manuel Porcar[1,2*]

1. Institute for Integrative Systems Biology I2SysBio (University of València-CSIC), Paterna, Spain.
2. Darwin Bioprospecting Excellence S.L. Parc Científic Universitat de València, Paterna, Spain.
3. Departament de Bioquímica i Biologia Molecular, Universitat de València, Burjassot, Spain.

Summary

One of the most diverse ecological niches for microbial bioprospecting is soil, including that of drylands. Drylands are one of the most abundant biomes on Earth, but extreme cases, such as deserts, are considered very rare in Europe. The so-called Tabernas Desert is one of the few examples of a desert area in continental Europe, and although some microbial studies have been performed on this region, a comprehensive strategy to maximize the isolation of environmental bacteria has not been conducted to date. We report here a culturomics approach to study the bacterial diversity of this dryland by using a simple strategy consisting of combining different media, using serial dilutions of the nutrients, and using extended incubation times. With this strategy, we were able to set a large (254 strains) collection of bacteria, the majority of which (93%) were identified through 16S ribosomal RNA (rRNA) gene amplification and sequencing. A significant fraction of the collection consisted of *Actinobacteria* and *Proteobacteria*, as well as *Firmicutes* strains. Among the 254 isolates, 37 different genera were represented, and a high number of possible new taxa were identified (31%), of which, three new *Kineococcus* species. Moreover, 5 out of the 13 genera represented by one isolate were also possible new species. Specifically, the sequences of 80 isolates held a percentage of identity below the 98.7% threshold considered for potentially new species. These strains belonged to 20 genera. Our results reveal a clear link between medium dilution and isolation of new species, highlight the unexploited bacterial biodiversity of the Tabernas Desert, and evidence the potential of simple strategies to yield surprisingly large numbers of diverse, previously unreported, bacterial strains and species.

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Introduction

Only a small fraction of the microbial diversity of our planet can be cultured under traditional microbiological tools, media, and strategies (Overmann *et al*., 2017; Steen *et al*., 2019). The use of ribosomal RNA (rRNA) genes and other genomic sequences as key markers in microbial ecology led to the discovery of a previously unknown microbial diversity (Rappé and Giovannoni, 2003). Traditional culturing techniques have allowed the isolation of rapidly-growing, easy-to-culture microbial taxa, thus relegating the vast majority of the microbial world to the imprecise group of "unculturable" organisms, which has been addressed as the "microbial dark matter" (Bernard *et al*., 2018; Bowman, 2018).

The generally accepted "1% culturability paradigm," which refers to the fact that around 99% of environmental microorganisms are in fact unculturable, was recently revised by Martiny (2019). The evidence based on the study of different environments no longer supports some of the interpretations. However, even though this "1%" is probably no longer acceptable, as discussed by Steen *et al*. (2019), microbiologists are still far from culturing most of the existing bacterial diversity.

One of the reasons behind the obstacles in culturing environmental bacteria is the difficulty in mimicking the particular environmental conditions that certain microbes require for growth, as standard laboratory conditions may differ from the natural habitats (Stewart, 2012). Small variations in a wide variety of parameters make almost impossible to cover the immense number of different micro-environments that can be found in nature. Moreover, not only abiotic factors but also the interactions between species and metabolic cooperation are critical for the development of specific taxa that may be reluctant to grow under laboratory conditions (Vartoukian *et al*., 2010).

Multi-omic data have broadened our knowledge on previously unknown microbial communities and taxa (Vilanova and Porcar, 2016). Specifically, metataxonomy, which refers to the high-throughput characterization of the microbiota emphasizing on the taxonomic status and relationships between taxa (Marchesi and Ravel, 2015), has shed light on the actual composition of microbial communities, whereas metagenomics has partially unveiled some key ecological interactions, with no need for cultivating microorganisms (Lok, 2015).

Metagenomic data have been used in order to design tailormade culture media for specific samples (Gutleben *et al*., 2020), but the inference of metabolic features and the functional analysis depends on the availability of complete annotated genes or genomes in public databases, what leads to failure in function assignment due to mislead annotations in genomes (Choi *et al*., 2016). All possible novel metabolic pathways are as diverse as the microorganisms that feature them, highlighting the countless potential higher diversity of bacterial applications that can derive from the uncultured microbial world (Overmann *et al*., 2017).

Sophisticated culture-dependent techniques have already been developed, such as the so-called ichip device, which is based on the cultivation of microorganisms by incubating the chips *in situ*, overcoming the problem of mimicking the environmental conditions (Berdi *et al*., 2017), or reverse genomics, which uses genomic data in order to obtain targeted antibodies to capture and sort specific cell types (Cross *et al*., 2019). Moreover, culturing gut microbes through techniques, such as "dilution to extinction" (Gross *et al*., 2015), in complete anaerobic flow work (Browne *et al*., 2016) and the selection of micro colonies in combination with multiple incubation conditions (Lagier *et al*., 2016), among other efforts in cultivating "uncultured" bacteria, have resulted in a considerable increase in the number of identified species (Martiny, 2019). Moreover, increasing the incubation times and diluting media have also proven to be useful for this purpose (Gutleben *et al*., 2020).

Soils are known to harbor a wide bacterial diversity with an immense potential in biotechnology and biomedicine. *Actinobacteria* are the major inhabitants of soil environments and one of the most ancient

groups of bacteria (Battistuzzi *et al*., 2004), which play an important role from the ecological point of view in maintenance processes (Lewin *et al*., 2016). They are well-known because of their ability to produce a wide range of bioactive secondary metabolites with immense potential and applications, in particular due to their role in the synthesis of antibiotics (Zhao *et al*., 2018), but also enzymes, such as cellulases, which can be used in the industrial-scale breakdown of cellulosic plant biomass into simple sugars that can then be converted into biofuels (Lewin *et al*., 2016).

Among the different terrestrial ecosystems and soil environments, drylands have been discovered as a source of biotechnologically-relevant bacterial strains (Azua-Bustos and González-Silva, 2014; Mohammadipanah and Wink, 2016). Extreme environments, such as deserts, have been found to host a wide diversity of microbial taxa adapted to live under such harsh conditions of temperature, desiccation, and radiation, among which low water and nutrient availability are the main limiting factors for organisms (Saul-Tcherkas *et al*., 2013). These factors shape the local biocenosis and act as a selection pressure toward interesting mechanisms to overcome the hurdles of living in an extreme ecological niche. Both culture-dependent and -independent studies have been performed in the Atacama, the Sahara, or the Gibson deserts (Lester *et al*., 2007; Azua-Bustos *et al*., 2014; Belov *et al*., 2018). However, European arid lands have been poorly studied from both points of view.

The Tabernas Desert, located in the province of Almeria (southeastern Spain), is a particular, not very much studied region to date in terms of bacterial culturability. It is formed by marls, sandstone, and scarce vegetation. The annual rainfall is below 250 mm, its climate is considered hot semiarid to desert climate, depending on the altitude, and it has interesting, well-developed biocrusts.

Biological soil crusts are multi-organism consortia worldwide—spread in dryland landscapes, being estimated that around 12% of the total terrestrial surface is covered by biocrust (Rodríguez-Caballero *et al*., 2018). Specifically, Miralles *et al*. (2020) recently reported how biocrusts influenced the microbial communities in the surrounding soil, which also depend on multiple environmental factors, such as pH, temperature, or salinity, and other biotic interactions. Moreover, Maestre *et al*. (2013) have studied in detail the effect of climate change on the microbial biocenosis associated with biocrust in different areas, including Sorbas, which is in the vicinities of the Tabernas Desert. The studies revealed that changes in biocrusts play a major role in further modelling the composition of microorganisms.

In the present work, we have characterized the microbial communities of the Tabernas Desert by using improved culturomics techniques that have allowed the identification of an unprecedented rich diversity of bacterial strains from this unique European dryland. We hypothesized that by combining culture laboratory techniques and increasing incubation times, a higher diversity of bacterial taxa would be isolated. The results obtained confirm that simple strategies in culturomics can lead to a better understanding of the culturable fraction of microbial communities, including the isolation of new and potentially new microbial taxa.

Materials and Methods

Sample Collection

Biocrust samples were obtained in September 2018 at the Tabernas Desert, in the vicinity of the Natural Park. Sampling was carried out by taking in Falcon tubes the upper 1–2 cm of biocrust-covered surfaces in three different locations (geolocation 1: 37.0083240, -2.4532390; geolocation 2: 37.0215300, -2.4323020; geolocation 8: 37.0226290, -2.4295450), and a total of eight samples were collected of different biocrusts in terms of color and appearance (Figure II.1), which were further used for culturing assays. Sample 1.1.1 corresponded to a black stained biocrust; sample 1.2.1 was from a white-pink biocrust; samples 1.3.1, 2.1.1, and 8.1.1 were all white biocrust with similar appearance; sample 1.4.1 was a yellowish biocrust; sample 2.5.1 was a white smooth biocrust; and sample 8.3.1 was a dark yellow biocrust (Table II.1).

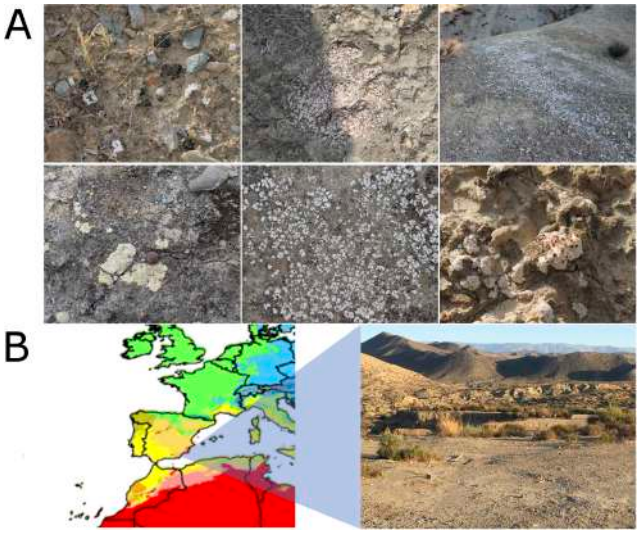


Figure II.1 (A) Biocrust sampling sites in the Tabernas Desert. Six different biocrusts are shown. (B) Climate map zoom of Spain in the Köppen-Geiger climate classification map (1980-2016) from Beck et al. (2018). The different climate areas are represented in colors. Warm-arid regions, corresponding to desert environments, are represented in red. Picture of the sampling area in the vicinity of the natural Park of the Tabernas Desert (Almería, southeastern Spain).

Table II.1. List of biocrust samples.

	SAMPLE	APPEARANCE
Geolocation 1	1.1.1	Black
	1.2.1	White-pink
	1.3.1	White
	1.4.1	Pale yellow
Geolocation 2	2.1.1	White
	2.5.1	White smooth
Geolocation 8	8.1.1	White
	8.3.1	Dark yellow

Isolation of Bacterial Strains

The samples were homogenized by mixing 1 g of biocrust with 1 ml of sterile phosphate buffer saline (PBS) 1X. Then, serial dilutions of the suspensions were prepared, and 50 µl of the 10^{-3}–10^{-5} samples was spread on Petri dishes containing either Tryptic Soy Agar (TSA) medium (composition in g/L: 15.0 tryptone, 5.0 soya peptone, 5.0 sodium chloride, 15.0 agar) or Reasoner's 2A (R2A) medium (composition in g/L: 1 peptone, 0.5 yeast extract, 0.5 dextrose, 0.5 soluble starch, 0.3 dipotassium phosphate, 0.05 magnesium phosphate, 0.3 sodium pyruvate, 15.0 agar) at concentrations 1X (standard concentration), 0.1X, and 0.01X (10 and 100 times diluted, respectively). TSA is a rich media used for general purposes, whereas R2A is a nutrient-poor media that favours the isolation of slow-growing and oligotrophic bacteria. Media were sterilized by autoclaving at 121 °C for 20 min. Agar was autoclaved separately to the nutrient solution of each medium and added just before pouring the media into the plates. After 2 weeks of incubation at room temperature (23 °C), individual colonies were selected based on their color and morphology and isolated by independent re-

streaking on fresh media in order to obtain them in pure culture. New colonies were also selected a month of incubation, in order to identify, small, slow-growing microorganisms. A total of 254 isolates were cryopreserved as a glycerol stock (20% glycerol in PBS, vol:vol) at -80 °C until required.

Colony Identification Through 16S rRNA Sequencing

A loopful of 3 ml of microbial biomass from grown plates was suspended in 100 ml of Milli-Q sterile water and then boiled for 10 min before the PCR in order to ensure cellular lysis. Colony PCR was used for taxonomic identification through 16S rRNA gene sequencing by using the universal primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') (Edwards *et al.*, 1989) and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Stackebrandt and Liesack, 1993). PCR was carried out with a step of incubation at 94 °C for 5 min, then 24 cycles of denaturation at 94 °C for 15 s, annealing at 48 °C for 15 s, elongation at 72 °C for 5 min, and a final elongation step at 72 °C for 5 min.

Amplifications were visualized by electrophoresis in a 1.2% agarose gel stained with GelRed nucleic acid gel stain (Biotium, CA, United States) (100 V for 30 min). Amplicons were precipitated overnight in isopropanol 1:1 (vol:vol) and potassium acetate 1:10 (vol:vol) (3 M, pH 5) at -20 °C. DNA was pelleted by centrifugation at 12,000 rpm for 10 min, then washed with ethanol 70%, and resuspended in the required 15 ml of Milli-Q water. BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, United States) was used to tag amplicons, which were sequenced with

the Sanger method by the Sequencing Service (Servei Central de Suport a la Investigació Experimental SCSIE) of the University of València (Spain). All sequences were manually edited with Trev (Bonfield and Staden, 2002) to eliminate low-quality base calls, and final sequences were compared by EzBioCloud 16S identification BLAST tool to nucleotide databases. Primers 341R (5'-CTGCTGCCTCCCGTAGG-3') (Muyzer *et al.*, 1996) and 1055F (5'-ATGGCTGTCGTCAGCT-3') (Harms *et al.*, 2003) were used for whole 16S rRNA sequencing in order to fully identify the isolates holding an identity lower than 98.7% with the closest type strain by sequencing partially the 16S rRNA gene. The MEGA7 tool was used to assemble the whole 16S rRNA gene sequence. The quality of the chromatograms was checked with the Sequence Scanner software. The quality value (QV) threshold was 20. In the case of competing peaks, the highest peak in both forward and reverse sequences after the assembly was chosen. The sequences were manually revised afterward and have been deposited under the GenBank/EMBL/DDBJ accession numbers MT749781–MT750013 and MN069869–MN069868.

In order to analyze the closest environmental clone or isolates of our strains, an extensive blast was carried out against the NCBI Nucleotide collection (nr/nt) databases optimized for highly similar sequences.

Results

Isolation of Bacterial Strains

Culturing different biocrust samples from the Tabernas Desert yielded a large number of different colonies in terms of color, shape, and morphology (Figure II.2). A total of 254 strains were isolated in pure culture, which were named as T or R depending on the medium from which they had been isolated (TSA or R2A, respectively) and numbered consecutively. There was no significant fungal growth, and most of the bacterial colonies displayed bright colors, being the most abundant those of red, pink, orange, and yellow-pigmented bacteria. There were also dark stained isolates, and some others changed from orange to purple with time. The morphology of the isolates was also diverse, being particularly

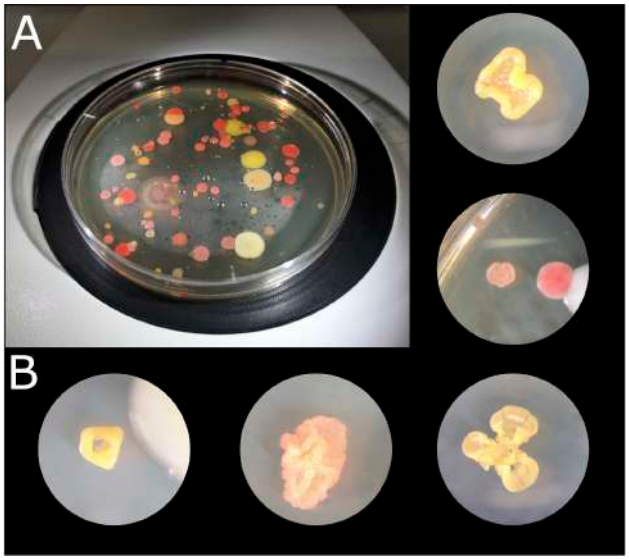


Figure II.2. Microbial colonies obtained after culturing biocrust samples at 23 °C for 2 weeks. (A) TSA 1X plate of sample 1.4.1. (B) Microbial colonies under the binocular loupe.

curious those growing in 3D structures and cell clumps (Figure II.2B). Isolates T1 to T159 were isolated from TSA plates after 2 weeks of incubation, of which 131 were obtained in pure culture and cryopreserved for further use; isolates R1 to R115 were selected from R2A plates, of which only 76 were obtained in pure culture. It was not possible to obtain all the selected colonies in pure culture due to crossed contamination or growth failure after several re-streaking steps. After a month of incubation of the plates, small colonies were also selected, and 47 of them were obtained in pure culture, being 20 of them originally from TSA and 27 from R2A plates.

Colony Identification Through 16S rRNA Sequencing

From the 254-strains collection, a total of 236 isolates (93%) were identified, belonging to 37 different genera, and only 18 isolates remained not identified due to either a lack of amplification in PCR or failure in sequencing. A total of 62 isolates were identified as *Arthrobacter* spp., which was the most abundant genus representing 24.8% of the collection. The second most represented genus was *Mycolicibacterium*, holding a 7.09% of abundance, similar to the abundances of *Microbacterium* and *Roseomonas*. In contrast, the less abundant genera were *Agrococcus*, *Amycolatopsis*, *Azospirillum*,

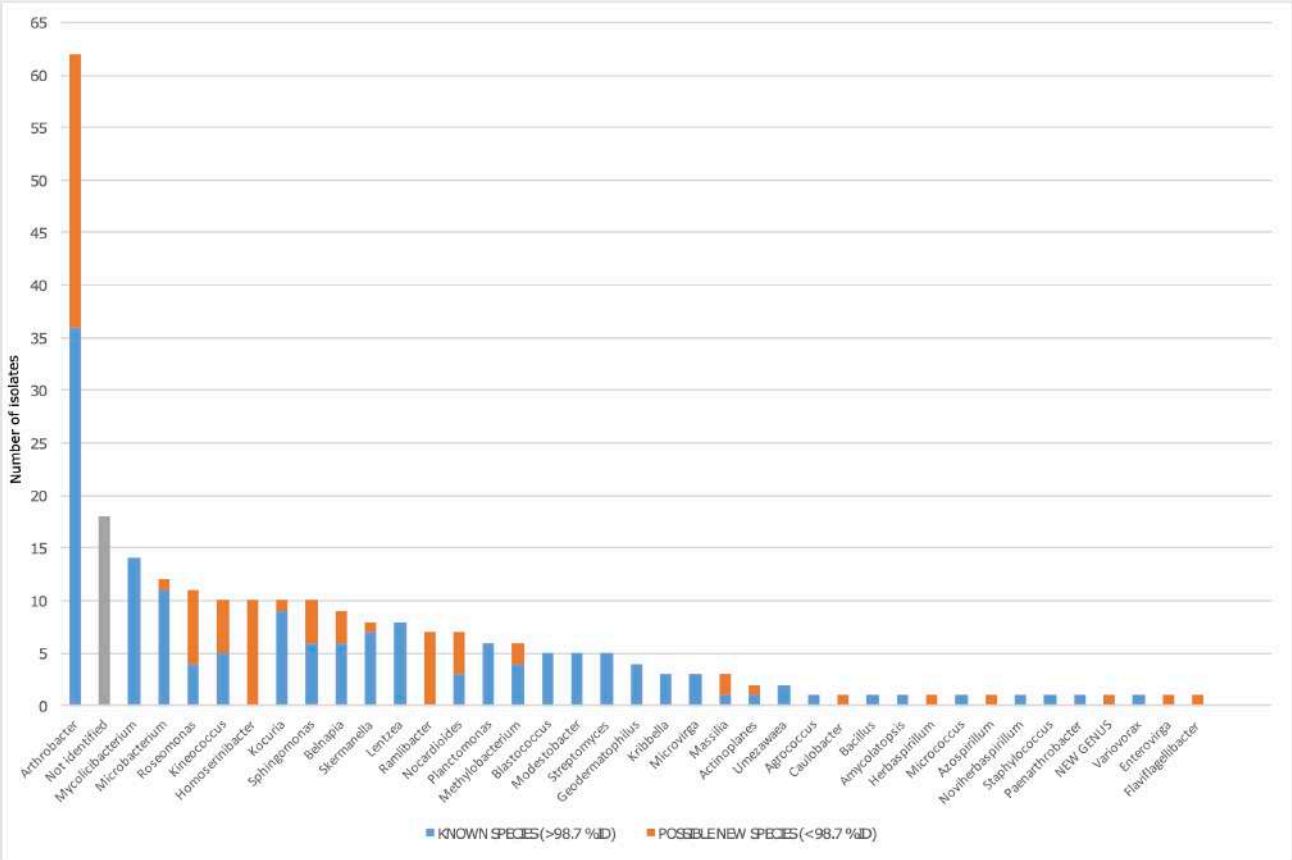


Figure II.3. Bacterial diversity among the 254 isolates from the Tabernas Desert collection. The blue bar represents the number of fully identified isolates in each genus (>98.7% of identity against 16S rRNA databases). The orange bar represents the number of possible new species in each genus (as defined by <98.7% of identity against 16S rRNA databases). Not identified isolates are shown in gray.

Bacillus, *Caulobacter*, *Enterovirga*, *Flaviflagellibacter*, *Herbaspirillum*, *Micrococcus*, *Noviherbaspirillum*, *Paenarthrobacter*, *Staphylococcus*, and *Variovorax*, being represented only by one isolate each (Figure II.3).

A total of 80 isolates in the collection showed a percentage of identity lower than 98.7%, suggesting that they could represent new species within that genus (Chun *et al*., 2018). Out of the 80 possible new species, 26 isolates were identified as *Arthrobacter* spp., whereas the rest were distributed among half of the genera detected. Interestingly, between the genera that had at least one possible new species, five of them were represented by just one isolate (*Caulobacter*, *Herbaspirillum*, *Azospirillum*, *Enterovirga*, and *Flaviflagellibacter*) (Figure II.3). For example, we isolated five *Kineococcus* strains as being possible new species, and three were further identified and described. The three new species have recently been published with valid names *Kineococcus vitellinus* sp. nov., *Kineococcus indalonis* sp. nov., and *Kineococcus siccus* sp. nov. (Molina-Menor *et al*., 2020). Moreover, the isolates identified as *Homoserinibacter* and *Ramlibacter*, which were 10 and 7, respectively, were all potential new species, and one isolate in the collection showed an ID value lower than 95%, which is the threshold value for new genus description (Chun *et al*., 2018). This possible new genus, represented by the isolate named as T16, would be closely related to *Roseomonas*, within the *Acetobacteraceae* family. Other genera identified in the collection were *Actinoplanes*, *Belnapia*, *Blastococcus*, *Geodermatophilus*, *Kocuria*, *Kribbella*, *Lentzea*, *Massilia*, *Methylobacterium*, *Microvirga*, *Modestobacter*, *Nocardioides*, *Planctomonas*, *Skermanella*, *Sphingomonas*, *Streptomyces*, and *Umezawaea* (Figure II.3).

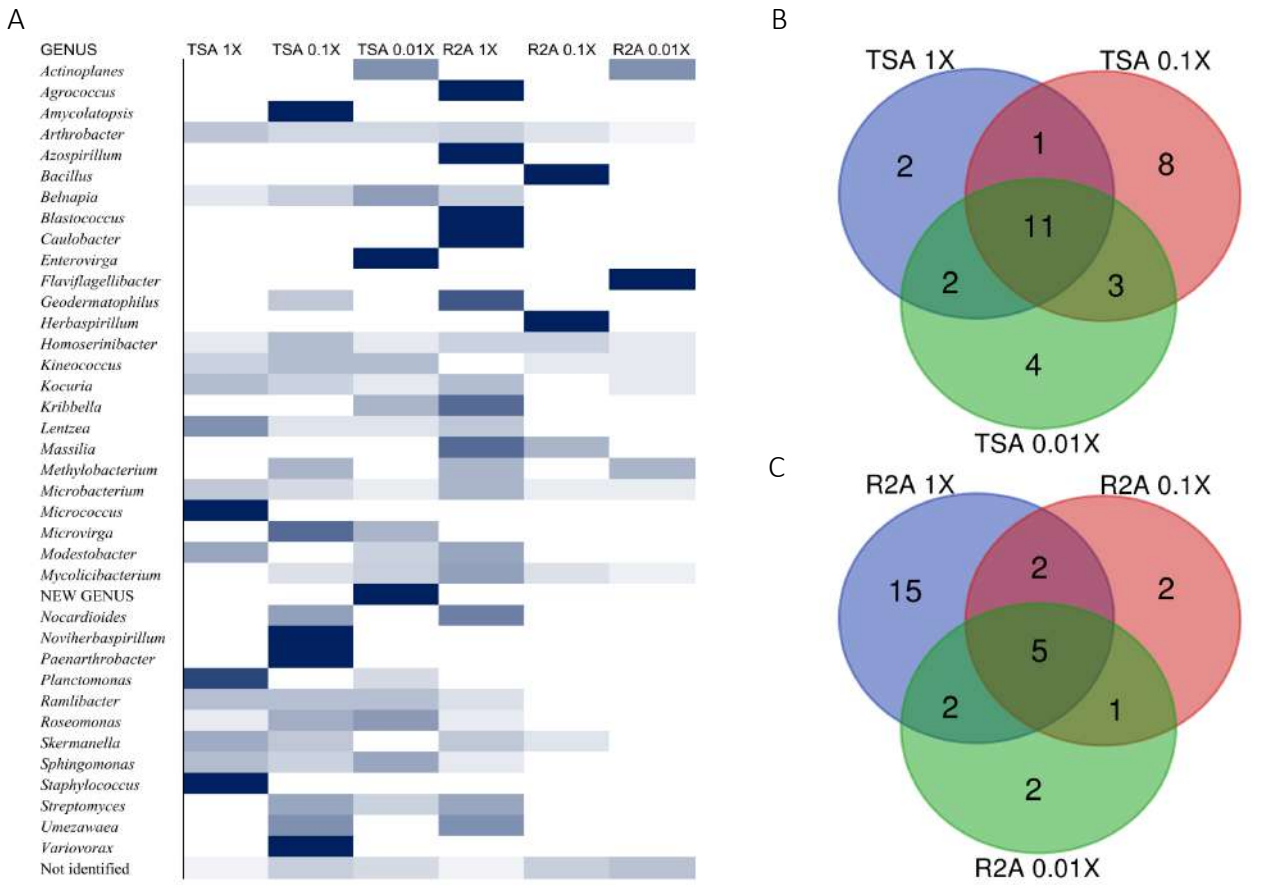


Figure II.4. Taxonomic diversity as a function of the culture media and dilutions used. (A) Heatmap of relative abundances pf the different genera, possible new genera and non-identified isolates among media (TSA at 1, 0.1 and 0.01X; R2A at 1, 0.1 and 0.01X). The genera are listed in alphabetical order. Possible new genera and non-identified isolates are included. (B) Venn diagram of shared and exclusive taxa at the genus level in TSA media at three different concentrations (1, 0.1 and 0.01X). Possible new genera and non-identified groups are included. (C) Venn diagram of shared and exclusive taxa at the genus level in R2A media at three different concentrations (1, 0.1 and 0.01X). Possible new genera and non-identified groups are included.

As for the isolation conditions, it was possible to analyze the bacterial diversity according to the media from which the genera had been isolated, as well as the incubation time required for their isolation. Almost 60% of the genera were isolated from at least two different media (TSA or R2A at three concentrations), whereas 15 genera were isolated just from one of the tested conditions (Supplementary Table II.1). The quantitative distribution of genera among the different isolation media is shown in Figure II.4A. In the case of *Arthrobacter* and *Microbacterium*, isolates were identified in all six media but, most frequently, in the most concentrated ones. Others, such as *Actinoplanes*, were isolated from the most diluted concentration on TSA and R2A, and the possible new genus was isolated from TSA 0.01X. Non-identified strains were also isolated from all six media, although the higher abundance was detected in the less concentrated ones, especially on R2A 0.01X. Interestingly, the five *Blastococcus* isolates grew on R2A 1X (Figure II.4A).

The co-isolation of genera in the different nutrient conditions was compared through Venn diagram representations, which are shown in Figures II.4B,C, listed in Tables II.2, II.3, and in Figure II.5. In the case of TSA media, 11 different genera were detected in the three concentrations assayed. The concentration that allowed the isolation of more exclusive taxa was the intermediate 0.1X, with eight unique taxa, in contrast with two on TSA 1X and four on TSA 0.01X (Figure II.4B). On the contrary, for R2A media, the concentrated R2A 1X seemed the best media for

Table II.2. List of coincident genera in the TSA media as it is compared in the Venn diagram in Figure II.4B.

MEDIA	N.	GENERA
TSA 1X; TSA 0.1X; TSA 0.01X	11	*Lentzea, Belnapia, Kocuria, Arthrobacter, Ramlibacter, Homoserinibacter, Sphingomonas, Roseomonas, Kineococcus, Microbacterium,* Not identified
TSA 1X; TSA 0.1X	1	*Skermanella*
TSA 0.1X; TSA 0.01X	3	*Streptomyces, Mycolicibacterium, Microvirga*
TSA 1X; TSA 0.01X	2	*Planctomonas, Modestobacter*
TSA 1X	2	*Micrococcus, Staphylococcus*
TSA 0.1X	8	*Methylobacterium, Geodermatophilus, Nocardioides, Variovorax, Umezawaea, Amycolatopsis, Paenarthrobacter, Noviherbaspirillum*
TSA 0.01X	4	New genus, *Enterovirga, Actinoplanes, Kribbella*

isolating different genera, being only five of them common for the three concentrations. Only two exclusive taxa were isolated from 0.1 and 0.01 R2A (Figure II.4C). In general, thus, bacterial genera tended to be shared by the TSA media, regardless of the dilution. In contrast, genera isolated from R2A tended to concentrate in the non-diluted medium.

The comparison, regardless of the dilution factor, of TSA and R2A media in terms of genera diversity revealed that one fourth of the groups (as non-identified isolates and the possible new genus were included) had been exclusively isolated in TSA, whereas around 20% were unique taxa for R2A (Figure II.5). This revealed a clear preference of some genera to grow under specific environments, as almost half of them were only isolated from one of the media used. Interestingly, all the genera that were isolated exclusively in TSA were represented by one isolate, except for *Planctomonas*, which was represented by six (five from TSA 1X and one from TSA 0.01X). In the case of the unique R2A taxa, apart from the five *Blastococcus* and three *Massilia* strains, the rest were also represented by one isolate.

Moreover, the distribution of the possible new species and the non-identified isolates was different among the media dilution used (Figure II.6). The ones that gave the higher fraction of potential new taxa were the lowest nutrient concentrations (media TSA 0.1 and 0.01X and all the R2A combinations). Specifically, the best results for new taxa isolation were obtained in R2A 0.1X, whereas the highest fraction of non-identified ones was obtained in R2A 0.01X. Interestingly, the sum of both groups of isolates (potential new species and unidentified ones) comprised a similar percentage in these two media, being almost 65%, which is approximately a threefold increase with respect to the TSA 1X group.

Regarding the differences in diversity depending on the incubation time after which each bacterial strain was isolated, the comparison of the groups, at the genus level, isolated after 2 weeks and after 1 month of incubation revealed that most of the taxa had been already detected in the first selection (Figure II.7A). However, four genera were exclusively isolated after 1 month, which were *Caulobacter*, *Geodermatophilus*, *Azospirillum*, and *Blastococcus* (Figure II.7B). Moreover, there was no significant difference in isolating potentially new taxa, as the percentage of isolates showing an identity percentage below 98.7 with the closest blast was similar between both selection times.

Finally, the differences in diversity observed depending on the biocrust sample and sampling site were analyzed (Supplementary Tables II.2–4). The geolocation that yielded the highest number of colonies was geolocation 1, with as much as 60% of the total isolates. On the contrary, geolocations 2 and 3 yielded a total of 51 isolates each. In terms of number of different genera identified, the most diverse sample was 1.4.1, from which 23 genera were isolated in pure culture. Although sample 1.4.1 was also the one that yielded the highest number of colonies, 30% of them corresponded to *Arthrobacter* spp.

Arthrobacter spp., which was the most abundant genus, was isolated from seven out of the eight samples, with different abundances. Particularly, in sample 2.1.1, half of the isolates belonged to this

Table II.3. List of coincident genera in the R2A media as it is compared in the Venn diagram in Figure II.4C.

MEDIA	N.	GENERA
R2A 1X; R2A 0.1X; R2A 0.01X	5	*Mycolicibacterium, Arthrobacter, Homoserinibacter, Microbacterium, N* ot identified
R2A 1X; R2A 0.1X	2	*Skermanella, Massilia*
R2A 0.1X; R2A 0.01X	1	*Kineococcus*
R2A 1X; R2A 0.01X	2	*Kocuria, Methylobacterium*
R2A 1X	15	*Lentzea, Agrococcus, Umezawaea, Nocardioides, Geodermatophilus, Blastococcus, Modestobacter, Curvibacter, Sphingomonas, Caulobacter, Roseomonas, Azospirillum, Belnapia, Streptomyces, Kribbella*
R2A 0.1X	2	*Bacillus, Herbaspirillum*
R2A 0.01X	2	*Actinoplanes , Flaviflagellibacter*

genus. Interestingly, 8 of the 10 *Homoserinibacter* spp. were from sample 1.4.1, but none had been isolated from geolocations 2 and 3, and the *Kineococcus* genus was also unique for the four samples of geolocation 1.

Ecological Novelty

Many of our strains were not phylogenetically related with any previously cultured strain. Specifically, 10.6% of the isolates shared less than 98.7% of 16S rRNA gene sequence similarity against any isolate previously studied, and 5.5% of the strains shared less than 98.7% regarding any environmental clone. Moreover, 24.8% of our isolates showed similarities with, to date, uncultured microorganisms (Supplementary Table II.5).

Up to 40.5% of the closest neighbors of our strains were environmental clones or isolates associated with soil environments, being 4.7% of them specifically from deserts around the globe, such as the Mojave Desert (Southwestern United States) and the Badain Jaran Desert (China), among others. Others (8.66%) were closely linked to clones or isolates inhabiting extreme environments, such as oil or heavy metal-contaminated soils, or cold environments, such as glaciers and permafrost samples. Furthermore, 8.3% of our strains were related to environmental clones or isolates found in aquatic habitats, mainly rainwater but also lakes, fish ponds, and freshwater sediments. Interestingly, 8.7% of the closest relatives corresponded to plant-associated clones, either associated with the rhizosphere, phyllosphere, or endophytic bacteria, and a small fraction representing 4.7% of the collection was similar to animal-associated bacteria, such as the gut of insects or corals.

Discussion

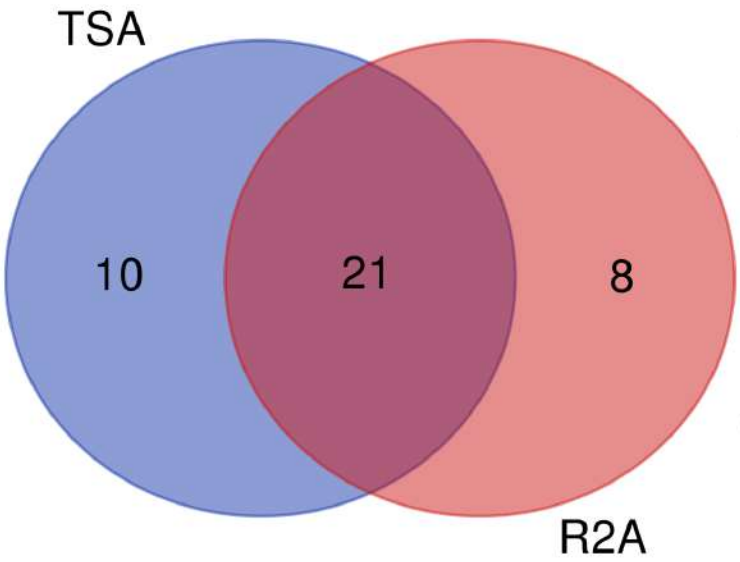
The Tabernas Desert Soils Harbor a Wide Bacterial Diversity

Although drylands and cold and hot deserts are a source of biotechnologically-relevant bacterial strains (Azua-Bustos and González-Silva, 2014; Mohammadipanah and Wink, 2016), the unique ecosystem of the Tabernas Desert remained poorly unexplored from the culturomics point of view. In the present study, we established a large collection consisting of, at least, 254 bacterial strains from 37 genera, in addition to one possible new genus, which belonged to three phyla.

The distribution of phyla in the collection resembles the microbial communities studied in other desert environments, such as the Atacama, the Gibson, or the Sahara Desert, with *Actinobacteria* as the most abundant phylum, followed by *Proteobacteria* and, to less extent, *Firmicutes*, among the culturable fraction (Belov *et al*., 2018; Schulze-Makuch *et al*., 2018). These taxa are also the ones previously detected in airborne sand particles from the Sahara Desert (Meola *et al*., 2015). Moreover, 19 different families were identified in the present work within those phyla: ten *Actinobacteria* families, five α-*Proteobacteria*, two β-*Proteobacteria*, and two *Firmicutes*. *Actinobacteria*, as reported by many authors, are one of the most common inhabitants in soil, but also particularly abundant in what is considered the extremobiosphere (Bull, 2011; Mohammadipanah and Wink, 2016).

The role of *Actinobacteria* in the synthesis of bioactive microbial metabolites with high pharmacological and commercial interests, such as antibiotics, is well-known. In particular, *Microbacterium* and *Lentzea* isolates, as well as other represented genus to lesser extent, such as *Streptomyces*, *Amycolatopsis*, and *Actinoplanes*, may be playing a role in the modulation of communities through competitors' growth inhibition (Tao *et al*., 2016; Zhao *et al*., 2018). Nithya *et al*. (2015) reported the isolation of 16 actinobacterial strains from the desert in Arabia Saudi that exhibited antimicrobial potential, and Hoshino *et al*. (2018) described for the first time a new type of macrolactams named as umezawamides as they are synthetized by the genus *Umezawaea*. Moreover, Wichner *et al*. (2017) reported the isolation of six new metabolites that had ever been traced before from microbial sources by a novel strain of *Lentzea* sp. from the Atacama Desert, which confirms the potential that these bacteria hold in the synthesis of interesting secondary metabolites.

A



B

TSA	*Enterovirga*	*Planctomonas*
	Variovorax	*Microvirga*
	Noviherbaspirillum	*Paenarthrobacter*
	New genus	*Staphylococcus*
	Micrococcus	*Amycolatopsis*
Shared	*Lentzea*	*Methylobacterium*
	Nocardioides	*Kineococcus*
	Kocuria	*Geodermatophilus*
	Arthrobacter	*Mycolicibacterium*
	Ramlibacter	*Umezawaea*
	Homoserinibacter	*Actinoplanes*
	Sphingomonas	*Modestobacter*
	Not identified	*Skermanella*
	Roseomonas	*Microbacterium*
	Belnapia	*Kribbella*
	Streptomyces	
R2A	*Caulobacter*	*Flaviflagellibacter*
	Herbaspirillum	*Azospirillum*
	Massilia	*Agrococcus*
	Bacillus	*Blastococcus*

Figure II.5. Taxonomic diversity as a function of the culture media and dilutions used. (A) Venn diagram comparing the genera isolated in TSA medium and R2A medium, considering all the three dilutions used for microbial growth (1, 0.1 and 0.01X). Possible new genera and non-identified groups are included. (B) List of genera represented in the Venn diagram.

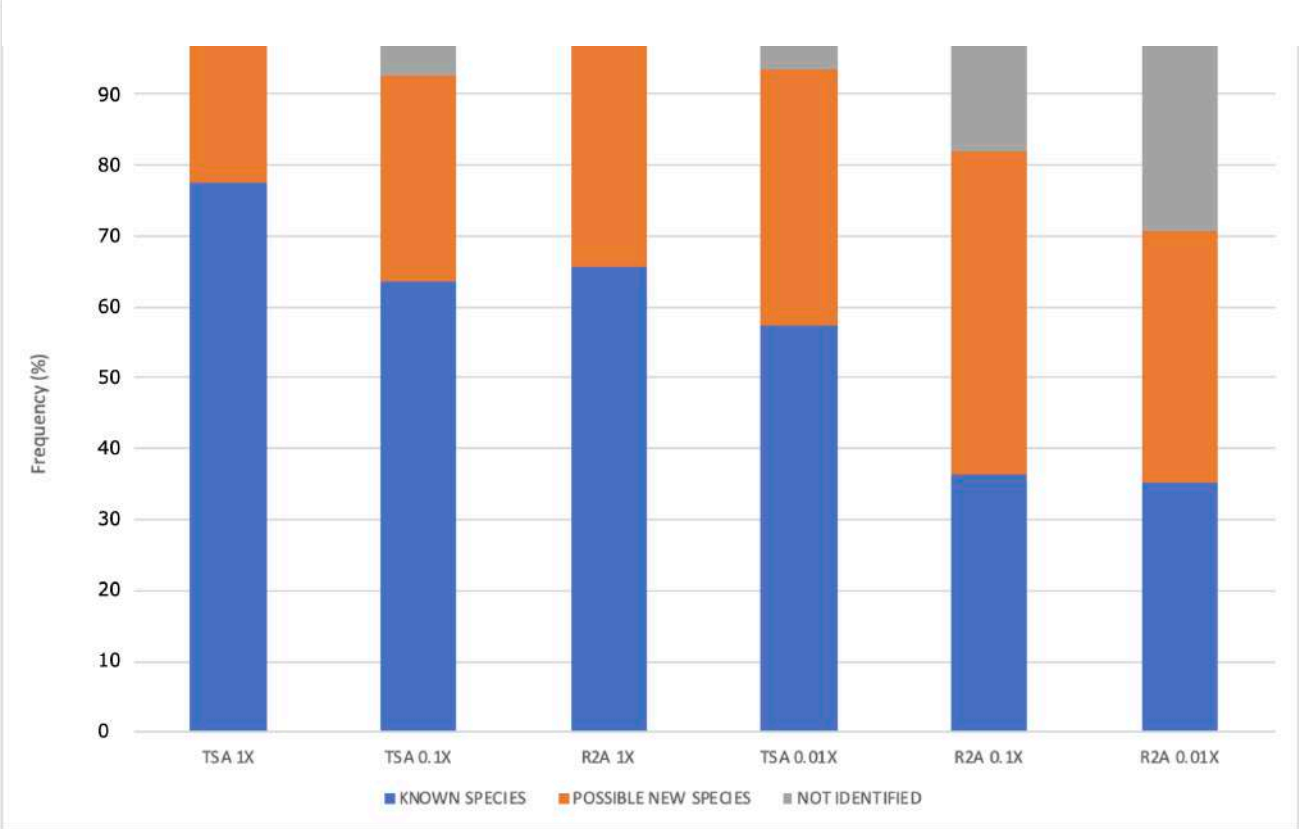
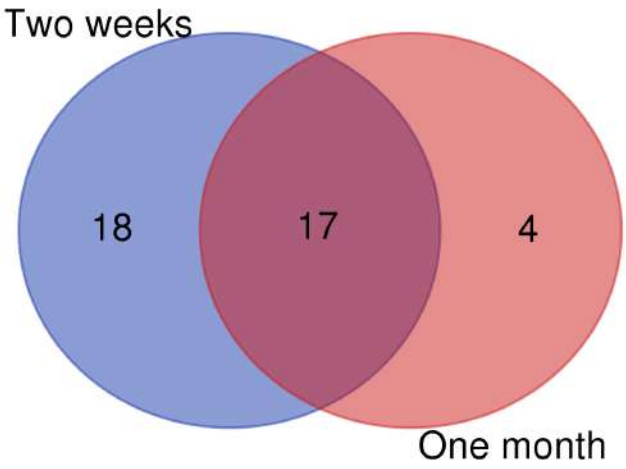


Figure II.6. Frequency (%) of possible new species, known species, and non-identified isolates as function of the culture media and the dilution used. The media are ordered according to the concentration of protein hydrolysate. Two different media, TSA and R2A, were used at three different concentrations: 1, 0.1 and 0.01X.

Other groups within the *Actinobacteria* clade have been described as important complex organic matter-degrading bacteria, such as *Actinomycetales*, which are proficient degraders of polysaccharide of plant,

A



B

Two weeks	*Lentzea*	*Microvirga*
	Flaviflagellibacter	*Actinoplanes*
	Variovorax	*Paenarthrobacter*
	Enterovirga	*Skermanella*
	Noviherbaspirillum	*Staphylococcus*
	New genus	*Herbaspirillum*
	Micrococcus	*Massilia*
	Agrococcus	*Bacillus*
	Umezawaea	*Amycolatopsis*
Shared	*Nocardioides*	*Belnapia*
	Kocuria	*Streptomyces*
	Arthrobacter	*Methylobacterium*
	Ramlibacter	*Kineococcus*
	Homoserinibacter	*Mycolicibacterium*
	Sphingomonas	*Modestobacter*
	Not identified	*Microbacterium*
	Roseomonas	*Kribbella*
	Planctomonas	
One month	*Caulobacter*	*Azospirillum*
	Geodermatophilus	*Blastococcus*

C

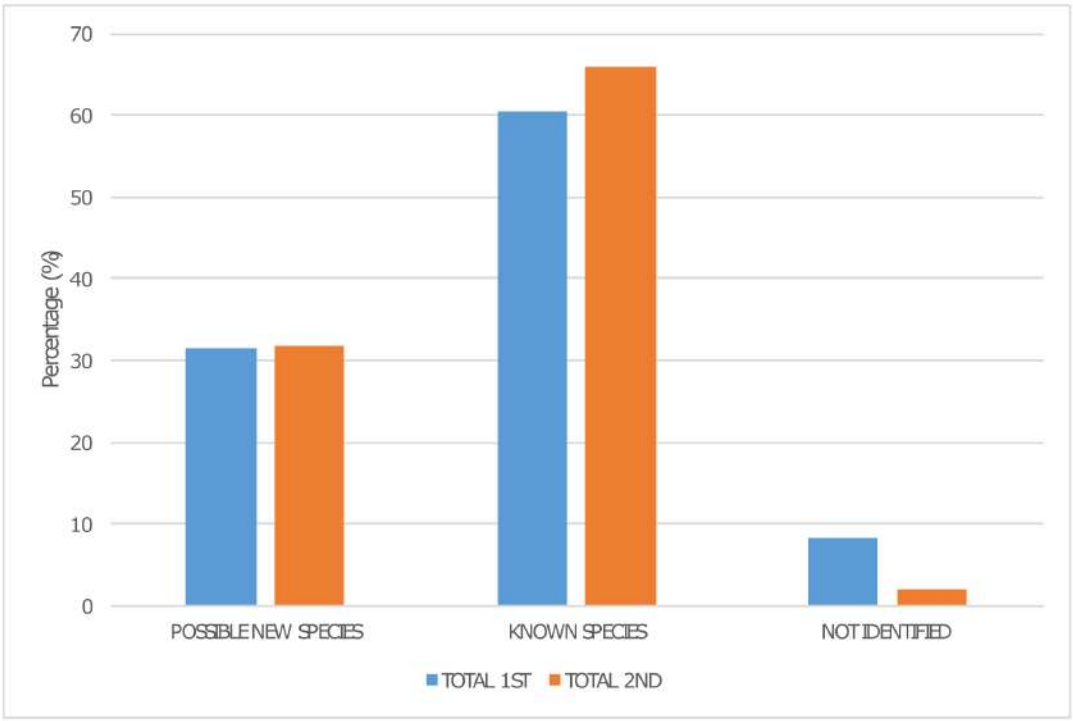


Figure II.7. Taxonomic diversity as a function of isolation date. (A) Venn diagram comparing the genera isolated after 2 weeks and a month of incubation. New genus and non-identified groups are included. (B) List of genera represented in the Venn diagram. (C) Bar plot representing the percentage of possible new taxa, known taxa, and non-identified isolates at the genus level after 2 weeks (1st selection) and a month of incubation (2nd selection).

animal, or fungal origin in soils. This polysaccharide degradation has a crucial impact in the carbon sources reuse cycles (Yeager *et al.*, 2017). Glucosidases, glycoside hydrolases, carbohydrate esterases, and other related enzymatic activities are abundant in the analyzed genomes of the three new *Kineococcus* strains described from Tabernas indeed (Molina-Menor *et al.*, 2020).

Actinobacterial species can play also an important role in bioremediation. The ability to degrade recalcitrant compounds and other environmental contaminants, such as heavy metals, has been reported for species within the *Actinomycetales* clade, what would have an application in the bioconversion of wastes into high-value products (Masuda *et al.*, 2012; Inoue *et al.*, 2016; Alvarez *et al.*, 2017; Herrero *et al.*, 2018). *Kocuria* and *Microbacterium* species isolated from

rhizosphere have been used for the bioremediation of pesticides, such as lindane (Abhilash *et al*., 2011), and *Microbacterium* spp. have also been found to potentially degrade organophosphorus pesticides and polycyclic aromatic hydrocarbons (PAHs) (Panneerselvan *et al*., 2018). Moreover, some of the *Roseomonas* species have been first isolated from oil-contaminated soils (Chaudhary and Kim, 2017; Subhash and Lee, 2018), and *Arthrobacter* species have demonstrated their potential to degrade polycyclic aromatic compounds (Gil *et al*., 2000; Muangchinda *et al*., 2017), which is interesting as they are one of the main inhabitants of soil environments (Mongodin *et al*., 2006).

In fact, *Arthrobacter* is the most abundant taxa in the collection established from the Tabernas Desert, with as many as 62 strains assigned, 26 of which being possible new species. *Arthrobacter* spp. do not only play a major role in the maintenance of natural processes in soils, such as the degradation of organic matter (Yeager *et al*., 2017) or the recycling of different carbon sources (Adams *et al*., 2011; Anderson *et al*., 2012; Berlemont and Martiny, 2013), but also in their recovery after fires (Fernández-González *et al*., 2017) and in the rhizosphere as common plant growth-promoting bacteria, preventing other bacteria and nematodes infections (Topalovic *et al*., 2020). They are common inhabitants of extreme environments, such as warm or cold arid deserts, and the robustness of this genus makes it interesting from the biotechnological point of view as it can be a source of bioactive compounds, such as the highly valued carotenoids (Fong *et al*., 2001). This, summed up to the diversity that has been found in the collection, opens up possible new biotechnological applications in several areas for the isolates within this genus.

The samples from the Tabernas Desert display a wide range of colors, probably due to the presence of these carotenoids, which may be playing a major role in their natural protection against oxidative stress and other consequences of sun exposure. The relationship between the synthesis of carotenoids, among other pigments, and the resistance to UV radiation in highly irradiated environments has been well described (Villareal *et al*., 2016; Peyrat *et al*., 2019). Not only *Arthrobacter* but also the genus *Microbacterium*, the second most abundant one with 14 representatives, is also known for its ability to synthetize carotenoids. Han *et al*. (2016) and Reis-Mansur *et al*. (2019) reported the UV resistance of two carotenoid-producing *Microbacterium* spp., both isolated from Antarctica. Furthermore, the genus *Methylobacterium*, represented by six isolates in the collection, has been described to synthetize pink carotenoids, which presumably act as antioxidants against reactive oxygen species (ROS) generated by UVA and UVB stresses (Sundin *et al*., 2002; Gao and Garcia-Pichel, 2011). Moreover, Yoshida *et al*. (2017) described an avobenzone-like compound isolated from several strains within the genus *Methylobacterium* with UVA-absorption and photostability activities, whose similarity to avobenzone supports its potential use as a commercial sunscreen ingredient.

The resistance to a wide range of stressors, particularly the resistance to gamma or UV radiation, has been largely described for the genera identified in the collection. Species within the genera *Roseomonas* (Kim *et al*., 2018), *Kocuria* (Gholami *et al*., 2015; Mehrabadi *et al*., 2016), or *Geodermatophilus* (Montero-Calasanz *et al*., 2013; Hezbri *et al*., 2016), among others, have been described as highly resistant to radiation. Moreover, *Sphingomonas* species, which represent almost 4% of the collection, have been reported to be one of the main inhabitants of solar panels. These artificial devices, exposed to the maximum sun radiation and distributed worldwide, are colonized by a stable and diverse microbial community dominated by highly resistant bacteria (Dorado-Morales *et al*., 2016). Interestingly, Yu *et al*. (2015) reported for the first time the resistance to radiation for bacteria that had been isolated from the Taklamakan Desert belonging to the genera *Nocardioides* and *Microvirga*, among others. These two genera are represented by seven and three isolates in the collection, respectively.

As expected according to the origin of the samples, most of the taxa have been previously described as soil inhabitants, with representatives having been isolated from desert environments indeed. Not only *Arthrobacter* and *Microbacterium*, as mentioned above, but also many other species identified come from similar ecological niches. *Roseomonas* and *Kineococcus* species (Yokota *et al*., 1993; Lee, 2009; Liu *et al*., 2009; Nie *et al*., 2012b; Ramaprasad *et al*., 2015; Chaudhary and Kim, 2017; Kim *et al*., 2017), the only species in the genus *Homoserinibacter* (Kim *et al*., 2014) or *Lentzea terrea*, *Lentzea soli*, and *Lentzea jiangxiensis*, among other representatives of the genus *Lentzea*, are examples of taxa that have been described as members of soil microbial communities (Li *et al*., 2012, 2018a,b). Moreover, other genera, such as *Skermanella*, *Belnapia*, or *Blastococcus*, which are comprised by few members, have also representatives that come from soil or desert ecosystems (Reddy *et al*., 2006; Zhang *et al*., 2015; Castro *et al*., 2018; Yang *et al*., 2019).

Although culture-independent approaches have led to the discovery of large unknown microbial diversity, these techniques can only predict to a certain extent the biotechnological potential of the identified microorganisms. The isolation of culturable, novel bacterial or fungal taxa under laboratory conditions is thus a crucial step for the development of innovative and new whole cell-based applications. For example, desert-inhabiting extremophilic bacteria have demonstrated to be useful in improving plant toleration to drought (Marasco *et al*., 2012; Rolli *et al*., 2015). Moreover, Sibanda *et al*. (2017) reported a culture-based screening to identify strains able to degrade some of the compounds present in carwash effluents, such as PAHs, heavy metals, and other pollutants.

Extremophilic bacteria have proven to be an excellent source of highly resistant proteins for several industries, such as in biofuel production, biorefinery, bioremediation, or the revalorization of agricultural waste products, such as lignocellulosic material (Zhu *et al*., 2020). However, many of these applications rely on genetic engineering techniques and synthetic biology and represent only a fraction of their possibilities.

Simple Culture-Based Strategies Rescue Significant Fractions of Previously Unknown Bacterial Diversity

The Tabernas Desert is particularly diverse in novel bacteria. Many of the closest relatives of our strains are environmental clones that inhabit a wide range of environments, mainly terrestrial ecosystems, including extreme environments, such as hot and cold deserts. These results highlight the importance of our collection of microorganisms from an ecological point of view (Supplementary Table II.5) since we have been able to access many bacteria never before studied, including through molecular tools.

Our methodology and results have, thus, important implications in terms of defining the best strategies for the discovery of new genetic variants of the soil bacteriome. We found a clearly different pattern in the two media we analyzed. Indeed, the composition in terms of nutrients and concentration is considerably different between TSA and R2A media. TSA is a rich media, which differs from the natural environments in terms of nutrient availability as these compounds are not usually abundant, whereas R2A is a poor media with limited amount of nutrients and carbon sources. TSA media seem to be equally good for yielding different isolates, according to the high number of shared taxa (Figure II.4B), whereas for R2A, it is clear that 1X combination, which is already a diluted media, is a suitable concentration for microbial growth because further dilutions yielded a very low number of colonies after 1 month (Figure II.4C). However, the highest number of non-identified isolates was isolated from R2A 0.01X, with as much as five isolates that represented 27.8% of them (Figure II.4A).

Arthrobacter, *Homoserinibacter*, and *Microbacterium* were identified in all of the tested media, whereas *Actinoplanes* or *Microvirga*, among others, was only found in diluted media (Figure II.4A and Supplementary Table II.1). The ability of *Microvirga* species to grow on different media has been previously reported. Species within this genus are able

to grow both in concentrated media, such as TSA, and in diluted ones, such as R2A (Dahal and Kim, 2017), but others, such as *Microvirga*, are unable to grow in nutrient abundance (Huq, 2018). *Nocardioides* spp., *Geodermatophilus* spp., and *Umezawaea* spp. have been identified in TSA 0.1X and R2A 1X, in which the nutrients are at the same order of magnitude, suggesting a more specific requirement, even though there are reports of the isolation and growth of species within these genera in a broader number of media (Nie *et al*., 2012a; Montero-Calasanz *et al*., 2013; Hezbri *et al*., 2016).

In contrast with this, the genera *Kocuria*, *Kineococcus*, and *Mycolicibacterium* were isolated from five out of the six media used. In the case of *Kineococcus* spp., there are previous reports on their isolation from a wide diversity of media and their ability to grow on them too, including diluted TSA 0.1X (Liu *et al*., 2009), the rich starch-casein agar medium (Duangmal *et al*., 2008; Li *et al*., 2015), or pure TSA (Xu *et al*., 2017), which also happens for *Mycolicibacterium* species, as reported by Nouioui *et al*. (2019). Moreover, Kaewkla and Franco (2013) reported a similar strategy of isolating novel culturable strains from trees by extending incubation times and using diluted media, which resulted in the isolation of a wide diversity of genera and new species, including several *Nocardioides*, *Kribbella*, and *Amycolatopsis* isolates, which have grown preferably in intermediate to low concentrations in the case of the samples from the Tabernas Desert.

Of the 39 groups, including non-identified isolates and the possible new genus, 15 were identified in just one media, which represents almost 40% of the diversity at the genus level. This confirms that the diversity observed is highly dependent on the use of a combination of culture media, which supports the idea that combining media and diluting them increase the chances of finding different and new taxa.

It is interesting to note that the potentially new genus we identified, which is closely related to the *Roseomonas* clade according to the 16S rRNA sequencing analysis, was isolated from the most diluted TSA media. Moreover, the strain R60, isolated from the lower concentration of R2A, was initially identified as another possible new genus and is now classified within the genus *Flaviflagellibacter*, as it has been recently published by Dong *et al*. (2019), after the beginning of this work. Thus, isolate R60 is now a possible new species in this novel genus, which includes only one species, *Flaviflagellibacter deserti*, that was first isolated from R2A media plates that had been incubated for 10 days, although it showed the ability to grow also in more concentrated media, such as TSA and LB, suggesting a flexible behavior in terms of nutrient availability for this genus.

The fact that around 25% of the taxa at the genus level were exclusively isolated in TSA and 20% in R2A suggests that even applying dilution factors to the media and increasing the incubation times, culturing samples in a selection of media is worth it in order to obtain a higher diversity (Figure II.5), and that the selection of the proper media is critical for it. Moreover, only 3 out of the 18 exclusive groups, *Planctomonas* in TSA and *Massilia* and *Blastococcus* in R2A, are represented by more than one isolate, which suggests that the less abundant taxa in culture collections are the ones with more specific requirements for growth. Interestingly, the only species described within *Planctomonas* was first isolated after 4 weeks of incubation (Liu *et al*., 2019).

The differences observed in the distribution of potential new species depending on the isolation media suggest that low nutrient concentrations increase the chances of identifying new taxa (Figure II.6), which is in accordance with the results observed in terms of diversity at the genus level (Figures II.4B,C). The percentage of possible new species increases, whereas the concentration of nutrients decreases. The concentrations in media TSA 0.1X and R2A 1X as well as and the concentrations in TSA 0.01X and R2A 0.1X are in the same order of magnitude. Interestingly, the most diluted media, R2A 0.01X, was not the best one in terms of possible new taxa, but yet exhibited the highest fraction of non-identified isolates, which can at least partially correspond to new species (Figure II.6). The results obtained support the idea that traditional, concentrated media have

favored the isolation of well-known, specific groups of fast-growing taxa. Almost 80% of the isolated strains in TSA 1X have been already described, whereas the fraction of unknown isolates (possible new species and non-identified ones) comprises more than 60% of the total in highly diluted media (R2A 0.1 and 0.01X) (Figure II.6).

Finally, the moment in which the different genera were isolated reveals that most of them could already be detected after 2 weeks of growth (Figure II.7A). However, four genera were exclusively isolated after a month, confirming that extending incubation times is important in order to obtain a higher microbial diversity, especially for isolating slow-growing and oligotrophic bacteria. One of these genera was *Blastococcus*, which is in accordance with the long incubation times reported by Yang *et al*. (2019) for the isolation of *Blastococcus deserti* in diluted media.

Surprisingly, the fraction of possible new species was similar after 15 days and 1 month of growth, but the percentage of nonidentified isolates was higher in the first sampling (Figure II.7C). Song *et al*. (2016) studied the effect of nutrient availability and incubation times in the development of soil microbial communities, revealing that the highest bacterial diversity is found in intermediate nutrient concentrations, as copiotrophic bacteria rapidly grow in concentrated media, whereas only resistant oligotrophic strains develop in highly diluted ones, which is in accordance with the results described above. Moreover, they observed that the peak in diversity is reached at the same time regardless of the nutrient concentration, which may explain the constant fractions of known and unknown diversity discussed at two sampling times.

Taken together, our results reveal that simple culturomics approaches, such as using combinations of media, long incubation times, and extreme dilutions of the nutrients, can be useful to yield a large diverse set of culturable microbial strains if applied to biodiverse natural samples, since we were able to build a diverse collection of bacterial strains from a few samples using these simple techniques. Our findings, combined with similar efforts, can help to fill the gap between the high numbers of bacterial genetic variants identified in culture-independent next generation sequencing (NGS) studies and the–up to date–still very low fraction of culturable microorganisms. This has, well beyond microbial ecology, important biotechnological implications.

Data availability statement

The datasets generated for this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, MT749781–MT750013 and MN069867–MN069869.

Author contributions

MP conceived the work. EM-M, HG-V, and MP carried out the sampling. EM-M and HG-V performed all the experiments. EM-M, HG-V, JuP, JaP, and MP analyzed the results, wrote, and approved the manuscript. All authors contributed to the article and approved the submitted version.

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Supplementary Material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.583120/full#supplementary-material and in Appendix B.

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Conflict of interest

MP and JuP are founders of Darwin Bioprospecting Excellence S.L. HG-V is an employee of Darwin Bioprospecting Excellence S.L.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publication III

Kineococcus vitellinus sp.nov., *Kineococcus indalonis* sp. nov. and *Kineococcus siccus* sp. nov., isolated nearby the Tabernas Desert (Almería, Spain)

Esther Molina-Menor[1], Helena Gimeno-Valero[2], Javier Pascual[2], Juli Peretó[1,2,3] and Manuel Porcar[1,2*]

1. Institute for Integrative Systems Biology I2SysBio (Universitat de València -CSIC), Calle del Catedràtic Agustin Escardino Benlloch 9, 46980 Paterna, Spain.
2. Darwin Bioprospecting Excellence S.L. Parc Científic Universitat de València, Calle del Catedràtic Agustin Escardino Benlloch 9, 46980 Paterna, Spain.
3. Departament de Bioquímica i Biologia Molecular, Universitat de València, Calle del Dr. Moliner 50, 46100 Burjassot, Spain.

Summary

Three novel Gram-positive, aerobic, chemoheterotrophic, motile, non-endospore-forming, orange-pigmented bacteria designated strains T13^T, T90^T and R8^T were isolated from the Tabernas Desert biocrust (Almería, Spain). Cells of the three strains were coccus-shaped and occurred singly, in pairs or clusters. The three strains were oxidase-negative and catalase-positive, and showed a mesophilic, neutrophilic and non-halophilic metabolism. Based on the 16S rRNA gene sequences, the closest neighbours of strains T13^T, T90^T and R8^T were *Kineococcus aurantiacus* IFO 15268^T, *Kineococcus gypseus* YIM 121300^T and *Kineococcus radiotolerans* SRS 30216^T (98.5%, 97.1% and 97.9% gene sequence similarity, respectively). The genomes were sequenced, and have been deposited in the GenBank/EMBL/DDBJ databases under the accession numbers JAAALL000000000, JAAALM000000000 and JAAALN000000000, respectively, for strains T13^T, T90^T and R8^T. The average nucleotide identity (ANIb) and digital DNA-DNA hybridization (dDDH) values confirmed their adscription to three new species within the genus *Kineococcus*. The genomic G + C content of strains T13^T, T90^T and R8^T ranged from 75.1% to 76.3%. The predominant fatty acid of all three strains was anteiso-$C_{15:0}$. According to a polyphasic study, strains T13^T, T90^T and R8^T are representatives of three new species in the genus *Kineococcus*, for which names *Kineococcus vitellinus* sp. nov. (type strain T13^T = CECT 9936^T = DSM 110024^T), *Kineococcus indalonis* sp. nov. (type strain T90^T = CECT 9938^T = DSM 110026^T) and *Kineococcus siccus* sp. nov. (type strain R8^T = CECT 9937^T = DSM 110025^T) are proposed.

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Introduction

The genus *Kineococcus*, belonging to the family *Kineosporiaceae* [1], was first proposed by Yokota *et al*. in 1993 [2]. At the time of writing, the genus *Kineococcus* is comprised of 11 species with valid published names (http://lpsn.dsmz.de/) which were isolated from soil [2–5], saline environments [6–8], plants [9,10] and radioactive work areas [11]. This genus includes species with the ability to grow in a wide range of temperatures [5] and high salt concentrations [8,9]. Their resistance to multiple stresses and extreme conditions has also been reported by several authors, with *Kineococcus radiotolerans* standing out among them because of its high resistance to radiation [11].

The genus *Kineococcus* unites Gram-stain-positive, aerobic, catalase-positive and oxidase-negative microorganisms. Colonies are cream to orange in colour, and cells are motile and occur singly, in pairs or in clusters. The major cellular fatty acid is $C_{15:0}$ anteiso, whereas diphosphatidylglycerol and phosphatidylglycerol are the major polar lipids. The genomic DNA G + C content of the *Kineococcus* species ranges from 73.9% to 74.2% [12].

During a study on the culturable microbial diversity of biocrust in dry environments exposed to high solar radiation, three different strains, T13^T, T90^T and R8^T, were isolated. The objective of the study was to characterize in detail the microbial communities from the Tabernas Desert (Almería, Spain), with particular emphasis in the isolation of extremophilic bacteria. The bacterial strains isolated from similar environmental niches, such as solar panels or the intertidal coastal zone, are able to tolerate UV radiation, and could thus be potentially useful in the synthesis of antioxidant compounds [13,14]. Among them, pigments such as carotenoids are particularly valuable for the biotechnological and pharmaceutical industries. In this paper, we describe the isolation and polyphasic characterization of these bacteria, and propose them as new species of the genus *Kineococcus*, for which the names *Kineococcus vitellinus* sp. nov., *Kineococcus indalonis* sp. nov. and *Kineococcus siccus* sp. nov. are proposed.

Materials and Methods

Isolation of Strains and Culture Conditions

Biocrust samples were collected in the vicinity of the Tabernas Desert (37.002404 N, 2.450655 W) in September 2018. One gram of each biocrust sample was resuspended in one millilitre of phosphate-buffered saline (PBS) pH 7.4. The suspensions were vigorously shaken, spread on 1X, 0.1X and 0.01X trypticase soy agar (TSA; in g/L: 15 tryptone, 5 NaCl, 5 soya peptone), and Reasoner's 2A agar (R2A; in g/L: 1 peptone, 0.5 yeast extract, 0.5 dextrose, 0.5 soluble starch, 0.3 dipotassium phosphate, 0.05 magnesium sulphate heptahydrate, 0.3 sodium pyruvate) media and incubated at 23 °C for one week. T13^T and T90^T strains were isolated from 0.1X and 0.01X TSA plates, respectively, whereas R8^T was isolated from a 0.1X R2A plate. All three strains were purified and cultivated by re-streaking on fresh media. For cryopreservation at -80 °C, cell suspensions in trypticase soy broth (TSB) were supplemented with 15% (v/v) glycerol. To determine the taxonomic status of strains T13^T, T90^T and R8^T, six reference type strains were used in this study: *Kineococcus radiotolerans* DSM 14245^T, *Kineococcus aurantiacus* DSM 7487^T, *Kineococcus gypseus* DSM 27627^T and *Kineococcus aureolus* DSM 102158^T from the DSMZ—German Collection of Microorganisms and Cell Cultures (Leibniz Institute, Braunschweig, Germany), and *Kineococcus gynurae* NBRC 103943^T and *Kineococcus mangrovi* NBRC 110933^T from the NITE Biological Resource Center (Tokyo, Japan). The selection of reference strains was based on the comparison of 16S rRNA gene sequences of the three isolates against the EzBioCloud database (http://www.ezbiocloud.net/) as detailed below. The comparative analysis conditions were selected according to the available literature on the species within this genus. Unless otherwise specified, all nine strains were grown on TSA media at 30 °C.

DNA Extraction and Sequencing

Extraction of genomic DNA was carried out using the DNeasy PowerSoil kit (QUIAGEN, Hilden, Germany) according to the manufacturer's instructions. A PCR of

the whole 16S rRNA gene was performed with universal primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') [15] and 1492R (5'-GGTTACCTTGTTACGACTT-3') [16] (94 °C for 5 min, 24 cycles of denaturation at 94 °C for 15 s, annealing at 48 °C for 15 s and elongation at 72 °C for 5 min, and a final elongation step at 72 °C for 5 min). The 16S rRNA gene sequence length of the strains T13^T, T90^T and R8^T were 1415 bp (accession number MN069869), 1425 bp (MN069867) and 1404 bp (MN069868), respectively. The draft genome of strains T13^T, T90^T and R8^T was sequenced using a MiSeq sequencer (Illumina, San Diego, CA, USA), and the Nextera XT Prep Kit protocol was used for library preparation.

Phylogenetic Analysis

We identified the closest relatives of the three strains T13^T, T90^T and R8^T by comparing their 16S rRNA gene sequences against the EzBioCloud database update 2020.05.13 (http://www.ezbiocloud.net/). Maximum-likelihood (ML) (Figure III.1) [17] and neighbour-joining trees (NJ) (Figure III.2) [18] were inferred with the software MEGA X v.10.1.7 (https://www.megasoftware.net/). The Tamura–NeiG+I evolutionary model was used for the ML tree, whereas the Kimura two-parameter model was used for the NJ tree. The reliability of the branch patterns was assessed using bootstrap analysis based on 500 replicates for the ML tree and on 1000 replicates for the NJ tree [19]. *Acidimicrobium ferrooxidans* DSM 10331^T was used as an outgroup for the phylogenetic analyses.

Genomic Analysis

The FastQC tool [20] was used to assess the quality of the sequence reads. Genome assembly of paired reads was performed using SPAdes 3.12.0 [21]. The draft genomes were annotated using the RAST tool kit (RASTk) [22] integrated in PATRIC v.3.5.41 (https://www.patricbrc.org). The circular genomic maps were also obtained from PATRIC v.3.5.41. The whole-genome shotgun projects of strains T13^T, T90^T and R8^T have been deposited in GenBank/EMBL/DDBJ under accession numbers JAAALL000000000,

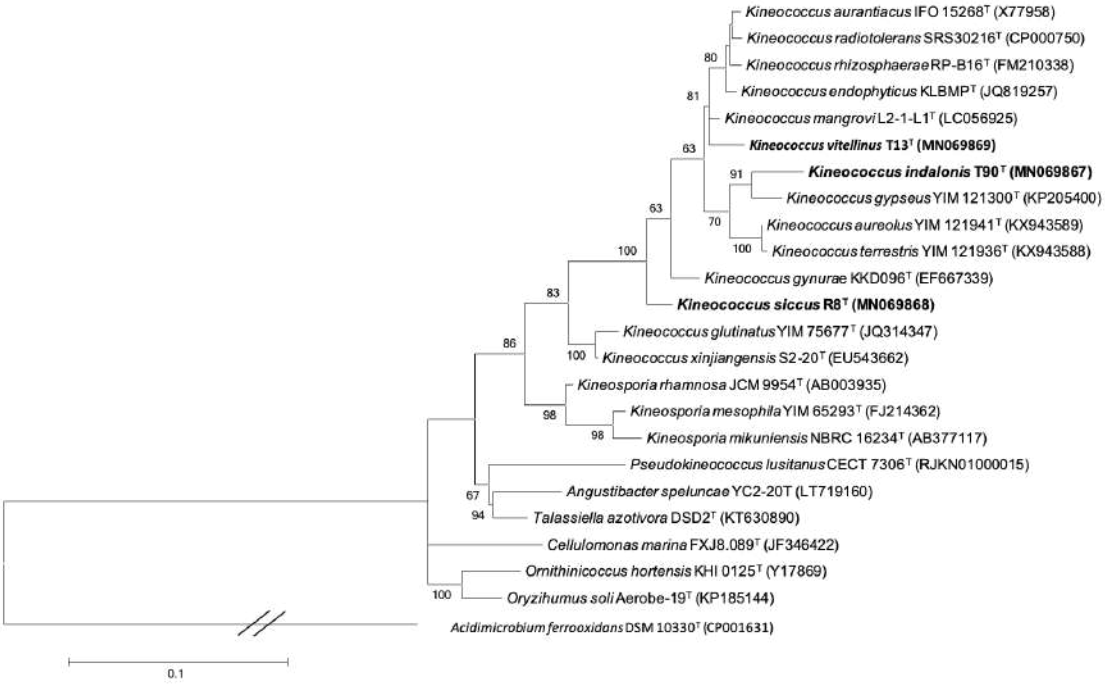

Figure III.1. Maximum likelihood phylogenetic tree showing the relationships between strains T13^T, T90^T and R8^T and other members of the family *Kineosporiaceae* based on 16S rRNA sequences. The optimal evolutionary model of nucleotide substitution applied was Tamura-Nei G+I. Numbers at branch points refer to bootstrap percentages based on 500 replicates (values under 50% are not indicated). *Acidimicrobium ferrooxidans* DSM 10331^T (CP001631) was used as an outgrop. Bar 0.1 fixed nucleotide substitutions per site.

JAAALM000000000 and JAAALN000000000, respectively. The completeness and contamination of the genomes was analysed with the bioinformatic tool CheckM v.1.0.6 [23].

Pairwise average nucleotide identity values (ANIb) [24] were calculated between strains T13^T, T90^T and R8^T and their closest type strains whose genomes were publicly available, by using the JSpeciesWS online tool [25]. Additionally, digital DNA-DNA hybridization (dDDH) pairwise values were also obtained using the Genome-to-Genome Distance Calculator 2.1 (GGDC) tool [26]. As recommended for incompletely sequenced genomes formula 2 was used for calculating the digital DDH values [26].

Morphological and Biochemical Characteristics

The phenotypic characteristics of the bacterial cultures were determined after one week of growth at 30 °C on TSA medium following the procedures outlined previously by other authors [27,28]. Cell morphology was observed under the microscope with crystal violet glass stain. Motility was analysed on wet mounts. The presence of flagella was determined with the staining method described by Heimbrook *et al*. [29].

Growth under microaerophilic conditions was tested by incubating the plates in a candle jar. The ability to grow in anaerobiosis was determined with the BD GasPakTM EZ pouch system (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Catalase activity was determined by using 30% (v/v) H_2O_2, recording bubble production as a positive result. Oxidase activity was determined with Oxidase Test Stick for microbiology (PanReac AppliChem, Barcelona, Spain). Gram type was determined by using KOH 3% (w/v), with cell lysis as a positive result for Gram-negative

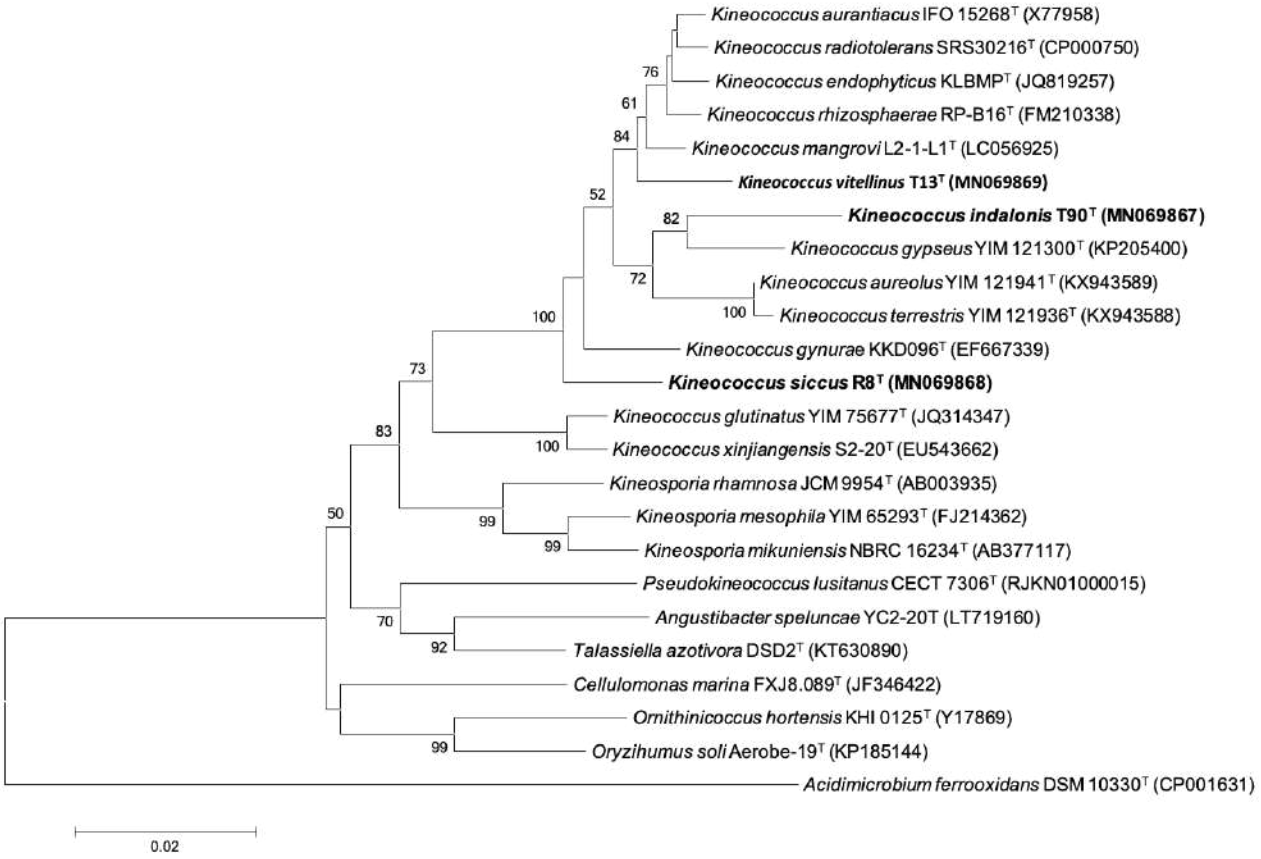

Figure III.2. Neighbour-joining phylogenetic tree showing the relationships between strains T13^T, T90^T and R8^T and other members of the family *Kineosporiaceae* based on 16S rRNA sequences. The optimal evolutionary model of nucleotide substitution applied was Kimura two-parameter model (K2P). Numbers at branch points refer to bootstrap percentages based on 1000 replicates (values under 50% are not indicated). *Acidimicrobium ferrooxidans* DSM 10331^T (CP001631) was used as an outgrop. Bar 0.02 fixed nucleotide substitutions per site.

bacteria. Growth at different temperatures (4, 10, 15, 25, 30, 37, 40 and 45 °C) and at various final NaCl concentrations (0.0–4.0% at intervals of 0.5%, and 4.0–10.0% at intervals of 1.0%) was examined by cultivating the isolates on TSA medium. The ability to grow at different pH values (4.0–11.0 at intervals of 1.0 pH unit) was examined in TSB using the MES (pH 4–6), HEPES (pH 7–8) and CHES (pH 9–11) buffers at 10 mM. Carbon source assimilation and enzymatic activities were determined using the API 20NE and API ZYM system strips (bioMérieux, Marcy-l'Étoile, France) according to manufacturer's instructions, as well as BIOLOG GENIII MicroPlates (BIOLOG Inc., Hayward, CA, USA).

Chemotaxonomic Analysis

For fatty acid analysis, T13^T, T90^T and R8^T and their closest relatives were grown on TSA at 26 °C for 72 h. Analysis of cellular fatty acids was carried out using the Sherlock Microbial Identification System (version 6.1, MIDI, Inc., Newark, DE, USA) [30]. Fatty acids were analysed on an Agilent 6859 gas chromatography system and using the MIDI method TSBA6 [31], according to the manufacturer's instructions.

Results and Discussion

Phylogenetic Analysis

The almost-complete 16S rRNA gene sequences showed that the three strains were phylogenetically related to representatives of the genus *Kineococcus*. According to the EzBioCloud database tool (http://www.ezbiocloud.net/), the closest type strains of T13^T, T90^T and R8^T are *K. aurantiacus* IFO 15268^T (98.5% 16S rRNA gene sequence similarity), *K. gypseus* YIM 121300^T (97.1%) and *K. radiotolerans* SRS 30216^T (97.9%), respectively. Since these 16S rRNA gene sequence similarities are lower than 98.7%, we have evidence that suggests that the strains T13^T, T90^T and R8^T may belong to new species [32,33]. According to these results, *K. aurantiacus* DSM 7487^T and *K. radiotolerans* DSM 14245^T were selected as comparative reference strains for T13^T; *K. gypseus* DSM 27627^T, *K. aureolus* DSM 102158^T and *K. mangrovi* NBRC 110933^T for T90^T; and *K. radiotolerans* DSM 14245^T, *K. aurantiacus* DSM 7487^T and *K. gynurae* NBRC 103943^T for R8^T.

The three isolates are classified into the genus *Kineococcus* in both 16S-rRNA-based phylogenetic trees constructed by ML and NJ. Specifically, T13^T showed an external position in the cluster formed by *K. aurantiacus* IFO 15268^T, *K. mangrovi* L2-1-L1^T, *K. endophyticus* KLBMP^T, *K. rhizosphaerae* RP-B16^T and *K. radiotolerans* SRS30216^T (Figures III.1 and III.2). Strain T90^T formed a monophyletic group with *K. gypseus* YIM 121300^T (Figures III.1 and III.2) while strain R8^T appeared as an external taxon within the genus *Kineococcus* (Figures III.1 and III.2). The phylogenetic inference of the three strains based on the 16S rRNA was supported by high bootstrap values.

The ANI and digital DDH values between strains T13^T vs. *K. radiotolerans* SRS 30216^T and R8^T vs. *K. radiotolerans* SRS 30216^T were 80.6–24.20% and 77.40–41.73%, respectively. As the values were higher than the threshold established to circumscribe prokaryotic species, namely 95% for ANI values [34] and 70% for dDDH [26], both genome-related indexes [35] confirmed the adscription of strains T13^T and R8^T to hitherto unknown species. On the other hand, the closest relative of T90^T is *K. gypseus* YIM 121300^T. It must be noted that the genome of *K. gypseus* YIM 121300^T (97.1%) was not publicly available at the time of writing the manuscript. However, since the 16S rRNA gene sequence similarity between T90^T and its closest relative K. *gypseus* YIM 121300^T is <98.7%, it is not necessary to calculate any overall genome relatedness index (OGRI) to propose T90^T as an independent genomospecies [32].

Genomic Characteristics

The circular map highlighting the main genomic features of the three strains is shown in Figures III.S2–III.S4. The draft genome of strains T13^T, T90^T and R8^T consisted of 705, 1067 and 502 contigs, yielding a total length of 4,857,076, 4,498,067 and 4,581,425 bp, respectively. The genomic G + C content of strains T13^T, T90^T and R8^T was 75.4%, 76.3% and 75.1%, respectively. This genomic G + C content is in accordance with other *Kineococcus* species, and

further confirms their adscription to the *Kineococcus* genus [2,7,8]. A total of 5039, 4690 and 4633 coding sequences (CDSs) were predicted for strains T13^T, T90^T and R8^T, respectively, of which 1894, 2906 and 2832 were proteins with functional assignments. In the case of tRNA genes, a total of 45 were predicted for strains T13^T and R8^T, and 46 were predicted for strain T90^T. Regarding rRNA genes, six of them were identified in T13^T; whereas five genes were found for T90^T and R8^T. The genome completeness values of strains T13^T, T90^T and R8^T were 98.9%, 98.1% and 99.2%, respectively; and the levels of contamination were 1.1%, 1.7% and 0%, respectively. Therefore, the draft genomes showed high enough quality for further analysis [32].

By analysing the draft genome of strains T13^T, T90^T and R8^T, their ability to synthesise diphosphatidylglycerol and phosphatidylglycerol was predicted based on the presence of genes coding for cardiolipin synthase A/B (EC:2.7.8.-) and ribosomal-protein-serine acetyltransferase (EC 2.3.1.-). However, the synthesis of phosphatidylinositol (EC 2.7.8.11), a major polar lipid of several *Kineococcus* species, could not be predicted in any of the three genomes [2,8,11]. Furthermore, the synthesis of meso-diaminopimelic acid in the three strains was predicted based on the presence of the enzyme diaminopimelate epimerase (EC 5.1.1.7), and the synthesis of menaquinones was predicted based on the identification of the enzyme demethylmenaquinone methyltransferase (EC 2.1.1.163). Therefore, the chemotaxonomic profile of the three new strains is in accordance with other *Kineococcus* species, corroborating their inclusion into the genus *Kineococcus* [2,7,8].

Phenotypic Characterization

Strains T13^T, T90^T and R8^T proved to be aerobic, Gram-positive, motile and coccus-shaped (1 µm in diameter). Like other members of the genus *Kineococcus*, cells of the three strains occur singly, in pairs or in clusters (Figure III.S1). Cell motility was confirmed by the presence of flagella. Colonies were orange-coloured, with irregular margins and a rough surface. T13^T colonies were paler than other *Kineococcus* strains. T90^T colonies changed in colour to dark orange-greenish after incubation at low temperatures (below 15 °C). The colonies displayed a diameter of around 1–2 mm after 3–4 days of growth, although T90^T colonies displayed a larger diameter.

All three strains were able to grow between 10 and 30 °C (optimum 25–30 °C). Furthermore, strain R8^T was able to grow at 4 °C, while strains T13^T and T90^T could grow up to 37 and 40 °C, respectively (Table III.1). Strains T90^T and R8^T could grow at NaCl concentrations up to 4%, while T13^T could grow at concentrations up to 5% (optimum for the three strains, 0% NaCl). *K. radiotolerans* DSM 14245^T, *K. aurantiacus* DSM 7487^T, *K. gypseus* DSM 27627^T and *K. gynurae* NBRC 103943^T showed a salinity tolerance range similar to our strains, while K. *aureolus* DSM 102158^T and *K. mangrovi* NBRC 110933^T were able to grow at concentrations up to 8% and 9% NaCl, respectively (Table III.1). Even though the three strains were found to be neutrophilic (optimum growth at pH 7), strains T13^T and T90^T were able to grow at alkaline values (up to pH 9 and 10, respectively), whereas R8^T could grow weakly at pH 5. Other *Kineococcus* species such as *K. aureolus* DSM 102158^T, *K. aurantiacus* DSM 7487^T, *K. gypseus* DSM 27627^T, *K. mangrovi* NBRC 110933^T and *K. gynurae* NBRC 103943^T were able to tolerate alkaline pH (Table III.1). All three strains were able to grow under microaerophilic conditions, but no growth was observed for any of them in anaerobiosis.

Strains T13^T, T90^T and R8^T, like their closest relatives, showed a positive response for esculin hydrolysis, β-galactosidase, esterase (C4), esterase lipase (C8), leucine arylamidase, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and β-glucosidase. On the other hand, strains T13^T, T90^T and R8^T and their closest neighbours showed a negative response for nitrate reduction, indole production, glucose fermentation, arginine dihydrolysis and N-acetyl-β-glucosaminidase. Strains R8^T and *K. gynurae* NBRC 103943^T showed urease activity, while strain T90^T, *K. gynurae* NBRC 103943^T, *K. aureolus* DSM 102158^T and *K. gypseus* DSM 27627^T were able to hydrolyse gelatine (Table III.1). In API 20

Table III.1. Differential characteristics between strains T13^T, T90^T, R8^T and related type strains of the genus *Kineococcus*. Strains: 1, T13^T; 2, T90^T; 3, R8^T; 4, *Kineococcus radiotolerans* DSM 14245^T; 5, *Kineococcus aureolus* DSM 102158^T; 6, *Kineococcus aurantiacus* DSM 7487^T; 7, *Kineococcus gypseus* DSM 27627^T; 8, *Kineococcus mangrovi* NBRC 110933^T; 9, *Kineococcus gynurae* NBRC 103943^T. Data in the present study were obtained from experiments carried out under identical conditions. All strains were positive for the following characteristics: Gram reaction, catalase activity, esculin hydrolysis, β-galactosidase, esterase (C4), esterase lipase (C8), leucine arylamidase, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and β-glucosidase. All strains were negative for the following characteristics: oxidase, nitrate reduction, indole formation, glucose fermentation, arginine dihydrolysis, capric acid assimilation and N-acetyl- β-glucosaminidase. +, positive; -, negative; W, weak reaction.

CHARACTERISTIC	1	2	3	4	5	6	7	8	9
Isolation source	Biocrust	Biocrust	Biocrust	Radioactive work area [11]	Saline sediment [8]	Soil [1]	Saline sediment [6]	Mangrove sediment [7]	Medicinal plant [9]
Growth at/in:									
Temperature range (°C)	10-37	10-40	4-30	4-37	10-45	04-40	15-37	10-30	4-30
pH range	6-9	6-10	5-8	6-8	7-11	5-9	5-10	5-9	5-10
NaCl tolerance (%, w/v)	0-5	0-4	0-4	0-4	0-8	0-4	0-4	0-9	0-6
Carbon source utilization (API 20NE)									
D-glucose	W	-	+	+	+	+	+	+	+
L-arabinose	-	-	+	+	+	+	+	+	+
D-mannose	-	-	+	+	+	+	+	+	+
D-mannitol	-	-	+	+	+	+	W	+	+
N-acetyl-glucosamine	-	-	+	W	-	+	-	W	-
D-maltose	-	-	+	W	+	+	W	+	+
Potassium gluconate	-	-	W	+	+	W	-	+	W
Adipic acid	-	-	W	W	W	-	-	-	-
Malic acid	-	-	-	W	+	-	W	+	W
Trisodium citrate	-	-	-	-	W	-	-	-	W
Phenylacetic acid	-	-	-	-	W	-	-	-	-
Enzymatic activity (API 20NE)									
Urease	-	-	+	-	-	-	-	-	+
Gelatinase	-	+	-	-	+	-	+	-	+
Enzymatic activity (API ZYM)									
Alkaline phosphatase	-	+	+	+	-	+	-	+	+
Lipase (C14)	-	-	-	+	-	-	-	-	-
Valine arylamidase	+	+	+	+	+	+	+	-	-
Cystine arylamidase	-	-	+	+	-	+	+	-	-
Trypsin	-	-	-	+	-	-	-	-	-
α-galactosidase	+	+	+	+	+	+	-	+	+
β-glucuronidase	-	-	-	+	-	-	-	-	-
α-glucosidase	+	+	-	+	-	-	+	+	+
α-manosidase	+	+	-	-	+	+	-	-	+
α-fucosidase	+	-	+	-	+	-	-	-	-

NE strips, strain R8^T proved able to use all the saccharides tested (Table III.1). On the contrary, strain T13^T was only able to grow weakly with glucose, while T90^T could not grow in any of the carbon sources tested. Furthermore, BIOLOG assays revealed that strains T13^T, T90^T and R8^T were able to oxidize 31, 62 and 26 out of the 71 tested carbon sources, respectively, and these were mainly organic acids and amino acids (Table III.S1). This suggests that strain T90^T displays a more polytrophic metabolism than strains T13^T and R8^T.

The major fatty acids for strains T13^T, T90^T and R8^T were anteiso-$C_{15:0}$ (96.1%, 73.9% and 94.0%, respectively). Similarly, their closest relatives showed high amounts of anteiso-$C_{15:0}$ (Table III.2), corroborating the inclusion of the three strains in the genus *Kineococcus* [2,8,11]. The presence of anteiso-$C_{15:0}$ fatty acid in the cell membranes has been associated with resistance to low temperatures [36,37]. In fact, this fatty acid has been found in the psychrophilic members of the genus *Flavobacterium* [38] isolated from a cold desert environment. Moreover, anteiso-$C_{15:0}$ fatty acid can play an

TABLE III.2. Cellular fatty acid composition of strains T13^T, T90^T and R8^T and their closest relatives. * Summed features represent groups of fatty acids that cannot be separated with the chromatographic system. Sum in feature 2 corresponds to $C_{13:0}$ 3-OH/iso-$C_{15:1}$ I/iso-$C_{15:1}$ H. Sum in feature 3 corresponds to iso-$C_{16:1}$ I/$C_{14:0}$ 3-OH/$C_{12:0}$ alde?. Sum in feature 5 corresponds to iso-$C_{17:1}$ I/anteiso-$C_{17:1}$ B.; Strains: 1, T13^T; 2, T90^T; 3, R8^T; 4, *Kineococcus radiotolerans* DSM 14245^T; 5, *Kineococcus aureolus* DSM 102158^T; 6, *Kineococcus aurantiacus* DSM 7487^T; 7, *Kineococcus gypseus* DSM 27627^T; 8, *Kineococcus mangrovi* NBRC 110933^T; 9, *Kineococcus gynurae* NBRC 103943^T. Data for reference strains were obtained in the present study. Values are shown as percentages. – means not detected; tr, <1.0% trace.

FATTY ACIDS	1	2	3	4	5	6	7	8	9
Saturated									
$C_{14:0}$	tr	1.2	1.37	-	1.8	1.3	2.1	3.9	1.6
iso-$C_{14:0}$	-	1.7	1.34	-	2.0		1.8	7.1	2.9
$C_{15:0}$	-	tr	-	-	1.7	1.0	2.6	-	2.2
iso-$C_{15:0}$	tr	1.3	-	-	tr	-	-	-	-
anteiso-$C_{15:0}$	96.1	73.9	94.0	97.9	78.8	75.9	73.0	84.4	74.2
$C_{16:0}$	-	tr	tr	-	2.0	1.8	2.2	tr	1.8
$C_{18:0}$	-	-	tr	-	tr	1.5	1.2	-	-
Unsaturated									
AT 12-13 $C_{13:1}$	-	-	-	-	-	3.4	-	-	3.4
anteiso-$C_{15:1}$ A	2.6	16.9	-	tr	6.7	-	12.3	-	-
Hydroxylated									
$C_{14:0}$ 2-OH	-	-	-	-	-	5.4	-	tr	4.0
$C_{17:0}$ 2-OH	-	-	-	-	-	-	-	2.8	-
$C_{17:0}$ 3-OH	-	3.4	tr	1.2	3	2.4	3.5	-	1.3
Summed*									
Sum in feature 2	-	-	-	-	-	2.7	-	-	2.8
Sum in feature 3	-	-	-	-	-	2.7	-	-	2.4
Sum in feature 5	-	-	-	-	-	2.0	-	-	-

important role in biofilm formation [39,40], which is in accordance with the biofilms and cell clumps observed for the three *Kineococcus* strains under liquid growth conditions.

Unlike their closest relatives, strains T90^T and *K. gypseus* DSM 27627^T also showed high amounts of anteiso-$C_{15:1}$ (16.9%) (Table III.2). Furthermore, the presence or absence of other minor fatty acids allowed us to differentiate the new strains from their closest neighbours (Table III.2).

Conclusions

During a study on the microbial communities associated with biocrust in sun-exposed and dry surfaces, the three strains T13^T, T90^T and R8^T were isolated in pure culture. All three strains, which were phylogenetically related to the genus *Kineococcus*, were characterized using a polyphasic approach. According to the phenotypic, genomic and phylogenetic analyses, strains T13^T, T90^T and R8^T should be considered as new species within the *Kineococcus* genus, for which the names *Kineococcus vitellinus* sp. nov., *Kineococcus indalonis* sp. nov. and *Kineococcus siccus* sp. nov., respectively, are proposed.

Description of Kineococcus vitellinus *sp. nov.*

Kineococcus vitellinus (vi.tel.li'nus. N.L. masc. adj. Vitellinus Egg-Yolk-Coloured)

Cells are Gram-positive, motile, non-endospore-forming, catalase-positive, oxidase-negative cocci (1 µm in diameter). Cells occur singly, in pairs or in clusters. Colonies are 1–1.5 mm in diameter, circular, rough and pale orange. Temperature range for growth is 10–37 °C, with an optimum at 25–30 °C. Growth occurs at pH 6–9 (optimum pH 7.0) and tolerates up

to 5% NaCl (w/v), with optimum at 0% NaCl (w/v). Esculin hydrolysis, β-galactosidase, esterase (C4), esterase lipase (C8), leucine arylamidase, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-glucosidase, valine arylamidase, α-galactosidase, α-glucosidase, α-mannosidase and α-fucosidase activities are detected. Nitrate reduction, indole production, glucose fermentation, arginine dihydrolysis, urease, gelatinase, alkaline phosphatase, lipase (C14), cystine arylamidase, trypsin, β-glucuronidase and N-acetyl-β-glucosaminidase are not detected. Using API 20NE test kit, this species is weakly positive for the assimilation of glucose and negative for arabinose, mannitol, N-acetyl-glucosamine, maltose, potassium gluconate, malic acid, mannose, capric acid, adipic acid, trisodium citrate and phenylacetic acid. Using BIOLOG GENIII MicroPlates, this species is positive for the utilization of D-raffinose, α-D-glucose, D-sorbitol, pectin, Tween 40, dextrin, α-D-lactose, D-mannose, D-mannitol, D-maltose, D-melibiose, D-fructose, L-alanine, D-trehalose, β-methyl-D-glucoside, D-galactose, myo-inositol, D-cellobiose, D-salicin, glycerol, gentiobiose, N-acetyl-D-glucosamine, glucuronamide, acetoacetic acid, sucrose, D-fructose-6PO_4, D-turanose, L-rhamnose, L-pyroglutamic acid, stachyose and L-serine; and negative for the utilization of gelatine, p-hydroxy-phenylacetic acid, glycyl-L-proline, D-galacturonic acid, methyl pyruvate, γ-amino-butyric acid, D-arabitol, L-galactonic acid lactone, D-lactic acid methyl ester, α-hydroxy-butyric acid, L-arginine, D-gluconic acid, L-lactic acid, β-hydroxy-D,L-butyric acid, 3-methyl-D-glucoside, L-aspartic acid, D-glucuronic acid, citric acid, α-keto-butyric acid, D-fucose, D-glucose-6-PO_4, L-glutamic acid, α-keto-glutaric acid, N-acetyl-β-F-mannosamine, L-fucose, L-histidine, mucic acid, D-malic acid, propionic acid, N-acetyl-D-galactosamine, D-aspartic acid, quinic acid, L-malic acid, acetic acid, N-acetyl neuraminic acid, inosine, D-serine, D-saccharic acid, bromo-succinic acid and formic acid. The major fatty acid is anteiso-$C_{15:0}$. The type strain T13^T (CECT 9936^T = DSM 110024^T) was isolated nearby the Tabernas Desert in Almería (Spain), from biocrust samples. The G + C content of the type strain is 75.4%. The GenBank/EMBL/DDBJ accession number for the 16S rRNA sequence isMN069869, and the genome accession number is JAAALL000000000.

Description of Kineococcus indalonis *sp. nov.*

Kineococcus indalonis (in.da.lo'nis. N.L. gen. n. indalonis of Indalo, Which Is a Prehistoric Symbol Found in Rock Paintings in Almería (Spain), Referring to the Place Where the Microorganism Was Isolated).

Cells are Gram-positive, motile, non-endospore-forming, catalase-positive, oxidase-negative cocci (1 μm in diameter). Cells occur singly, in pairs or in clusters. Colonies are small (1 mm in diameter), circular with irregular margins, rough and pale orange, but below 20 °C the colour changes from orange to dark greenish. Temperature range for growth is 10–40 °C, with an optimum at 25–30 °C. No growth is observed below 10 or above 40 °C. Growth occurs at pH 6–10 (optimum, 6–9) and tolerates up to 4% NaCl (w/v), with optimum at 0% (w/v). Esculin hydrolysis, β-galactosidase, gelatinase, esterase (C4), esterase lipase (C8), leucine arylamidase, α-chymotrypsin, acid phosphatase, alkaline phosphatase, valine arylamidase, α-galactosidase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, α-mannosidase and β-glucosidase activities are detected. Nitrate reduction, indole production, glucose fermentation, arginine dihydrolysis, urease, lipase (C14), cystine arylamidase, trypsin, β-glucuronidase, α-fucosidase and N-acetyl-β-glucosaminidase are not detected. Using API 20NE test kit, this species is negative for the assimilation of glucose, arabinose, mannitol, N-acetyl-glucosamine, maltose, potassium gluconate, malic acid, mannose, capric acid, adipic acid, trisodium citrate and phenylacetic acid. Using BIOLOG GENIII MicroPlates, this species is positive for the utilization of D-raffinose, α-D-glucose, D-sorbitol, gelatine, pectin, Tween 40, dextrin, α-D-lactose, D-mannose, D-mannitol, glycyl-L-proline, D-galacturonic acid, γ-amino-butyric acid, D-maltose, D-melibiose, D-fructose, D-arabitol, L-alanine, L-galactonic acid lactone, D-lactic acid methyl ester, d-trehalose, β-methyl-D-glucoside, D-galactose, myo-inositol, L-arginine, D-gluconic acid, β-hydroxy-D,L-butyric acid, D-cellobiose, D-salicin, 3-methyl-D-glucoside,

glycerol, L-aspartic acid, D-glucuronic acid, citric acid, α-keto-butyric acid, gentiobiose, N-acetyl-D-glucosamine, D-fucose, D-glucose-6-PO_4, L-glutamic acid, glucuronamide, α-keto-glutaric acid, acetoacetic acid, sucrose, N-acetyl-β-D-mannose, L-fucose, D-fructose-6PO_4, L-histidine, mucic acid, D-malic acid, propionic acid, D-turanose, N-acetyl-D-galactosamine, L-rhamnose, D-aspartic acid, L-pyroglutamic acid, L-malic acid, acetic acid, stachyose, D-serine, L-serine and bromo-succinic acid; and negative for the utilization of p-hydroxy-phenylacetic acid, methyl pyruvate, α-hydroxy-butyric acid, L-lactic acid, quinic acid, N-acetyl neuraminic acid, inosine, D-saccharic acid and formic acid. The major fatty acids for strain T90^T are anteiso-$C_{15:0}$ and anteiso-$C_{15:1}$ A. The type strain T90^T (CECT 9938^T = DSM 110026^T) was first isolated nearby the Tabernas Desert in Almería (Spain), from biocrust samples. The G + C content of the type strain is 76.3%. The GenBank/EMBL/DDBJ accession number for the 16S rRNA sequence is MN069867, and the genome accession number is JAAALM000000000.

Description of Kineococcus siccus *sp. nov.*

Kineococcus siccus (*siccus*. L. masc. adj. *Siccus*, Dry)

Cells are Gram-positive, motile, non-endospore-forming, catalase-positive, oxidase-negative cocci (1 µm in diameter). Cells occur singly, in pairs or in clusters. Colonies are orange, circular, with irregular margins and variable size (1–2 mm diameter). Temperature range for growth is 4–30 °C with an optimum at 25–30 °C. No growth is observed at 40 °C. Growth occurs at pH 5–8 (optimum 6–7) and tolerates up to 4% NaCl (w/v), with optimum at 0% (w/v). Esculin hydrolysis, β-galactosidase, urease, esterase (C4), esterase lipase (C8), leucine arylamidase, α-chymotrypsin, acid phosphatase, alkaline phosphatase, valine arylamidase, cystine arylamidase, α-galactosidase, naphthol-AS-BI-phosphohydrolase, α-fucosidase and β-glucosidase activities are detected. Nitrate reduction, indole production, glucose fermentation, arginine dihydrolysis, gelatinase, lipase (C14), trypsin, α-glucosidase, β-glucuronidase, α-mannosidase and N-acetyl-β-glucosaminidase are not detected. Using API 20NE test kit, this species is positive for the assimilation of glucose, arabinose, mannose, mannitol, N-acetyl-glucosamine and maltose; weak for the assimilation potassium gluconate and adipic acid and negative for the assimilation of malic acid, capric acid, trisodium citrate and phenylacetic acid. Using BIOLOG GENIII MicroPlates, this species is positive for the utilization of pectin, Tween 40, dextrin, D-fructose, D-arabitol, L-alanine, D-galactose, D-gluconic acid, L-lactic acid, D-cellobiose, 3-methyl-D-glucoside, glycerol, D-glucuronic acid, gentiobiose, glucuronamide, acetoacetic acid, L-fucose, D-fructose-6-PO_4, mucic acid, D-malic acid, D-turanose, D-aspartic acid, quinic acid, L-malic acid, acetic acid and D-serine; and negative for the utilization of D-raffinose, α-D-glucose, D-sorbitol, gelatine, p-hydroxy-phenylacetic acid, α-D-lactose, D-mannose, D-mannitol, glycyl-L-proline, D-galacturonic acid, methyl pyruvate, γ-amino-butyric acid, D-maltose, D-melibiose, L-galactonic acid lactone, D-lactic acid methyl ester, α-hydroxy-butyric acid, D-trehalose, β-methyl-D-glucoside, myo-inositol, L-arginine, β-hydroxy-D,L-butyric acid, D-salicin, L-aspartic acid, citric acid, α-keto-butyric acid, N-acetyl-D-glucosamine, D-fucose, D-glucose-6-PO_4, L-glutamic acid, α-keto-glutaric acid, sucrose, N-acetyl-β-D-mannose, L-histidine, propionic acid, N-acetyl-D-galactosamine, L-rhamnose, L-pyroglutamic acid, stachyose, N-acetyl neuraminic acid, inosine, L-serine, D-saccharic acid, bromo-succinic acid and formic acid. The major fatty acid is anteiso-$C_{15:0}$. The type strain R8^T (CECT 9937^T = DSM 110025^T) was first isolated nearby the Tabernas Desert in Almería (Spain), from biocrust samples. The G + C content of the type strain is 75.1%. The GenBank/EMBL/DDBJ accession number for the 16S rRNA sequence is MN069868, and the genome accession number is JAAALN000000000.

Supplementary Materials

The following are available online at http://www.mdpi.com/2076-2607/8/10/1547/s1 and in Appendix B.

Author Contributions

M.P. conceived the work. M.P., E.M.-M. and H.G.-V. carried out the sampling. E.M.-M. and H.G.-V. performed the identification. E.M.-M. carried out the phenotypic characterization. J.P. (Javier Pascual) supervised the bioinformatic and phylogenetic analysis. E.M.-M., H.G.-V., J.P. (Javier Pascual), J.P. (Juli Peretó) and M.P. analysed the results, wrote and approved the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Publication IV

Belnapia mucosa sp. nov. and *Belnapia arida* sp. nov., isolated from desert biocrust

Esther Molina-Menor[1], Àngela Vidal-Verdú[1], Leila Satari[1], Javier Pascual[2], Juli Peretó[1,2,3] and Manuel Porcar[1,2*]

1. Institute for Integrative Systems Biology I2SysBio (Universitat de València-CSIC), Calle del Catedràtic Agustin Escardino Benlloch 9, 46980 Paterna, Spain.
2. Darwin Bioprospecting Excellence S.L. Parc Científic Universitat de València, Calle del Catedràtic Agustin Escardino Benlloch 9, 46980 Paterna, Spain.
3. Departament de Bioquímica i Biologia Molecular, Universitat de València, Calle del Dr. Moliner 50, 46100 Burjassot, Spain.

Summary

Two novel Gram-staining-negative, aerobic, cocci-shaped, non-motile, non-spore forming, pink pigmented bacteria designated strains T6^T and T18^T, were isolated from a biocrust (biological soil crust) sample from the vicinity of the Tabernas Desert (Spain). Both strains were catalase-positive and oxidase-negative, and grew under mesophilic, neutrophilic and non-halophilic conditions. According to the 16S rRNA gene sequences, strains T6^T and T18^T showed similarities with *Belnapia rosea* CGMCC 1.10758^T and *Belnapia moabensis* CP2C^T (98.11 and 98.55% gene sequence similarity, respectively). The DNA G+C content was 69.80 and 68.96% for strains T6^T and T18^T, respectively; the average nucleotide identity by blast (ANIb) and digital DNA–DNA hybridization (dDDH) values confirmed their adscription to two novel species within the genus *Belnapia*. The predominant fatty acids were summed feature 8 ($C_{18:1}$ω7c/$C_{18:1}$ω6c), $C_{16:0}$, $C_{18:1}$ 2-OH and summed feature 3 ($C_{16:1}$ω7c/$C_{16:1}$ω6c. According to the results of the polyphasic study, strains T6^T and T18^T represent two novel species in the genus *Belnapia* (which currently includes only three species), for which names *Belnapia mucosa* sp. nov. (type strain T6^T = CECT 30228^T = DSM 112073^T) and *Belnapia arida* sp. nov. (type strain T18^T = CECT 30229^T = DSM 112074^T) are proposed, respectively.

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The genus *Belnapia* was first described by Reddy *et al*. [1] and it is, at the time of writing, comprised of three species, which were all isolated from soil samples: *Belnapia moabensis* [1], *Belnapia rosea* [2] and *Belnapia soli* [3]. In this study we describe the polyphasic characterization of two strains, namely $T6^T$ and $T18^T$, which were isolated from biocrust (biological soil crust) samples from south-eastern Spain during a study on the microbial diversity of European arid regions.

Strains $T6^T$ and $T18^T$ were isolated in the vicinity of the Tabernas Desert (Almería, Spain) during a study on the culturable microbial diversity in European drylands [4]. The Tabernas Desert is considered the only arid desert in Europe. Biocrust samples were obtained from near the Tabernas Desert Parc (37.002404° N, 2.450655° W) and homogenized in phosphate buffered saline (PBS; NaCl 8.0 g/L, KCl 0.2 g/L, Na_2HPO_4 1.44 g/L, KH_2PO_4 0.24 g/L) pH 7.4 (1 g in 1 ml). The suspensions were then spread on 1, 0.1 and 0.01X trypticase soy agar (TSA; 15 g/L tryptone, 5 g/L NaCl, 5 g/L soya peptone), and Reasoner's 2A Agar (R2A; 1 g/L peptone, 0.5 g/L yeast extract, 0.5 g/L dextrose, 0.5 g/L soluble starch, 0.3 g/L dipotassium phosphate, 0.05 g/L magnesium sulphate heptahydrate, 0.3 g/L sodium pyruvate). Agar was autoclaved separately and added before plating at a final concentration of 15 g/L. The plates were incubated at 23 °C for 1 week. Strain $T6^T$ was isolated from 0.1X TSA plates, whereas $T18^T$ was isolated from a 0.01X TSA plate. The isolation of the strains was carried out by re-streaking on fresh media until a pure culture was obtained. Cell suspensions in TSA and R2A were cryopreserved at −80 °C with 15% glycerol (v/v). Their taxonomic status was determined by a polyphasic approach. On the basis of the results from phylogenetic, phenotypic and chemotaxonomic analysis, it is concluded that strains $T6^T$ and $T18^T$ are related to members of the genus *Belnapia* and representatives of two novel species. In the present work, the reference strains *B. rosea* DSM 23312^T, *B. moabensis* DSM 16746T and *B. soli* DSM 28067^T, from the DSMZ (German Collection of Microorganisms and Cell Cultures, Leibniz Institute, Braunschweig, Germany), and strains $T6^T$ and $T18^T$ were all grown in parallel on R2A media at 30 °C, unless otherwise specified.

The phenotypic characteristics of $T6^T$ and $T18^T$ were analysed after 1 week of growth at 30 °C. A Gram staining test was carried out with KOH 3 % (w/v), recording viscosity as a negative result. Oxidase activity was tested by using the commercial Oxidase Test Stick for microbiology (PanReac AppliChem). Catalase activity was tested with hydrogen peroxide 30% (v/v), recording bubble formation as a positive result. Cell morphology was observed under an optical microscope with crystal violet glass stain. Growth at different temperatures (4, 10, 15, 20, 23, 30, 37, 40, 42 and 45 °C) and NaCl concentrations (0.0–4.0% at intervals of 0.5%) was checked on R2A. Growth at different pH values (4.0–10.0 at intervals of 1.0 pH unit) was examined by growing the strains in liquid R2A using the buffers MES (pH 4–6), HEPES (pH 7–8) and CHES (pH 9–10) at a final concentration of 10 mM. Growth under microaerophilic and anaerobic conditions was tested by incubating the plates in a candle jar and with the BD GasPak EZ pouch system (Becton, Dickinson), respectively. Carbon source assimilation and enzymatic activities were checked using the API 20NE and API ZYM system strips (bioMérieux) according to manufacturer's instructions. BIOLOG GENIII MicroPlates (BIOLOG) were also used to determine carbon source assimilation.

Strains $T6^T$ and $T18^T$ and the reference strains *B. rosea* DSM 23312^T, *B. moabensis* DSM 16746^T and *B. soli* DSM 28067^T were grown on R2A medium at 30 °C for 72 h for analysis of cellular fatty acids. The analysis was carried out following the protocol recommended by MIDI Microbial Identification System (version 6.1, MIDI, Inc, Newark, DE, USA) [5]. The fatty acids content was analysed on a 6850 gas chromatography system (Agilent) using the TSBA6 method [6].

Genomic DNA extraction was carried out using the DNeasy Power Soil kit (Qiagen), according to the manufacturer's instructions, but incubating at 65 °C after the addition of C1. Whole 16S rRNA gene PCR

was carried out with universal primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) [7] and 1492R (5′-GGTTACCTTGTTACGACTT-3′) [8] following procedures described previously [4]. Phylogenetic trees based on the 16S rRNA gene sequences were reconstructed using the maximum-likelihood (ML) [9] and neighbour-joining (NJ) [10] methods with the software mega X v.10.1.7. The TamuraNei G+I evolutionary model and the Kimura two-parameter model were used for the ML and NJ trees, respectively. The reliability of the branch patterns was assessed using bootstrap analysis based on 500 and 1000 replicates, respectively, for the ML and the NJ trees [11].

The draft genome of strains T6^T and T18^T were sequenced with the NovaSeq 6000 system (Illumina; 2x150 bp paired-end sequencing). The genomic DNA was randomly fragmented by sonication, then DNA fragments were end polished, A-tailed and ligated with the full-length adapters for Illumina sequencing. Further PCR amplification was carried out with P5 and indexed P7 oligonucleotides, and PCR products for the final construction of the libraries were purified with an AMPure XP system. Libraries were then checked for size distribution by Agilent 2100 Bioanalyzer (Agilent Technologies), and quantified by real-time PCR. The FastQC tool (v0.11.5) [12] was utilized to assess the quality of the sequence reads. There were 14 375 848 and 12 879 132 paired-end reads for strains T6^T and T18^T, respectively before filtering. After quality filtering, there were considered to be 13 106 155 and 11 792 897 paired-end sequences the genomes of strains T6^T and T18^T, respectively. Genome assembly of paired reads was performed using the '--isolate' mode in SPAdes (3.14.1) [13]. Assembly statistics were calculated with QUAST (v.5.0.2) [14] and the completeness and contamination levels were evaluated with CheckM (v.1.1.3) [15]. The draft genomes were annotated using the RAST tool kit (RAStk) [16] integrated in PATRIC v.3.6.8. The draft genomes were analysed with the TYGS tool [17] in order to identify the most closely related type strains to T6^T and T18^T with publicly available genomes and to calculate digital DNA–DNA hybridization (dDDH) indexes. JSpecies [18] was used for calculating the average nucleotide identities according to blast (ANIb) between genome pairs. UBCG (v.3.0) [19] was used for reconstructing the phylogenomic tree among the selected strains based on a multiple alignment of a set of 92 housekeeping genes. We selected the alignment method codon and inferred the phylogenetic relationships with FastTree. The reliability of the branch patterns was assessed using bootstrap analysis based on 100 replicates.

Table IV.1. Differential phenotypic characteristics between strains T6^T, T18^T and the other members of the genus *Belnapia*. Strains: 1, T6^T; 2, T18^T; 3, *Belnapia moabensis* DSM 16746^T; 4, *Belnapia rosea* DSM 23312^T; 5, *Belnapia soli* DSM 28067^T. Data for reference strains were obtained in the present study. +, Positive; –, negative; w, weak reaction. All strains are positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase and napthol-AS-BI-phosphohydrolase. All strains are negative for lipase (C14), valine arylamidase, cystine arylamidase, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, fermentation of glucose, arginine dihydrolysis, aesculin hydrolysis and the assimilation of d-mannitol, N-acetyl-glucosamine, maltose, capric acid, trisodium citrate and phenylacetic acid.

Characteristic	**1**	**2**	**3**	**4**	**5**
Isolation source	Biocrust	Biocrust	Soil crust	Forest soil	Grass soil
Growth at/in					
Temperature range (°C)	4-42	4-40	4-40	4-40	4-40
pH range	5-9	5-9	5-9	5-9	5-9
NaCl tolerance (%, w/v)	0-1.5	0-1.5	0-2	0-1.5	0-3
Carbon source utilization (API 20NE)					
D-glucose	-	-	-	-	w
L-arabinose	-	-	w	w	w
D-mannose	-	-	-	w	-
Potassium gluconate	w	-	-	w	-
Adipic acid	w	-	-	+	+
Malic acid	w	-	-	-	w
Enzymatic activity (API 20NE)					
Nitrate reduction	-	-	-	+	-
Indole production	-	-	-	+	-
Urease	-	w	+	w	W
Gelatin	-	-	+	-	-
Enzymatic activity (API ZYM)					
Trypsin	-	-	+	-	-

Strains T6^T and T18^T were aerobic, Gram-staining-negative, non-motile and coccus-shaped (0.8–1.0 µm in diameter). The cells of both strains occurred singly, as in other members of the genus Belnapia. Colonies were pink, irregular and mucous. T6^T colonies were paler than those of the rest of the members of the genus *Belnapia*. After 3–5 days of growth at 30 °C, the colonies of both strains displayed a diameter of around 3–4 mm.

Both strains were able to grow at between 4 and 40 °C (optimum at 30 °C). Moreover, T6^T was able to

grow at up to 42 °C. Both strains showed tolerance to up to 1.5% (w/v) of NaCl (optimum 0–1%). *B. moabensis* DSM 16746^T and *B. rosea* DSM 23312^T showed similar NaCl tolerances, in contrast with *B. soli* DSM 28067^T, which was able to grow at concentrations of up to 3%. All five strains were able to grow at between pH 5 and 9, with an optimum at 6–7 (Table IV.1).

Strains T6^T and T18^T showed, like the other members of the genus Belnapia, a positive response for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase and napthol-AS-BI- phosphohydrolase. In contrast, T6^T and T18^T and their relatives of the genus Belnapia showed a negative response for lipase (C14), valine arylamidase, cystine arylamidase, α-chymotripsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl- β- glucosaminidase, α-manosidase, α-fucosidase, fermentation of glucose, arginine dihydrolysis and aesculin hydrolysis. *B. moabensis* DSM 16746^T showed positive response for trypsin and gelatin, while *B. rosea* DSM 23312^T showed positive response for reduction of nitrate and indole production. T6^T was negative for urease, in contrast to the other four strains, which were positive or weakly positive for this activity. In API 20 NE strips, T18^T was not able to assimilate any of the saccharides tested, whereas T6^T could grow weakly with potassium gluconate, adipic acid and malic acid (Table IV.1). Furthermore, the results of the BIOLOG GENIII able to oxidize five and four out of the 71 carbon sources, respectively (Table IV.S1, available in the online version of this article). In contrast, the reference strains *B. moabensis* DSM 16746^T, *B. rosea* DSM 23312^T and *B. soli* DSM 28067^T were able to oxidize 41, 25 and 16 carbon sources, respectively. This indicates that the reference strains present a

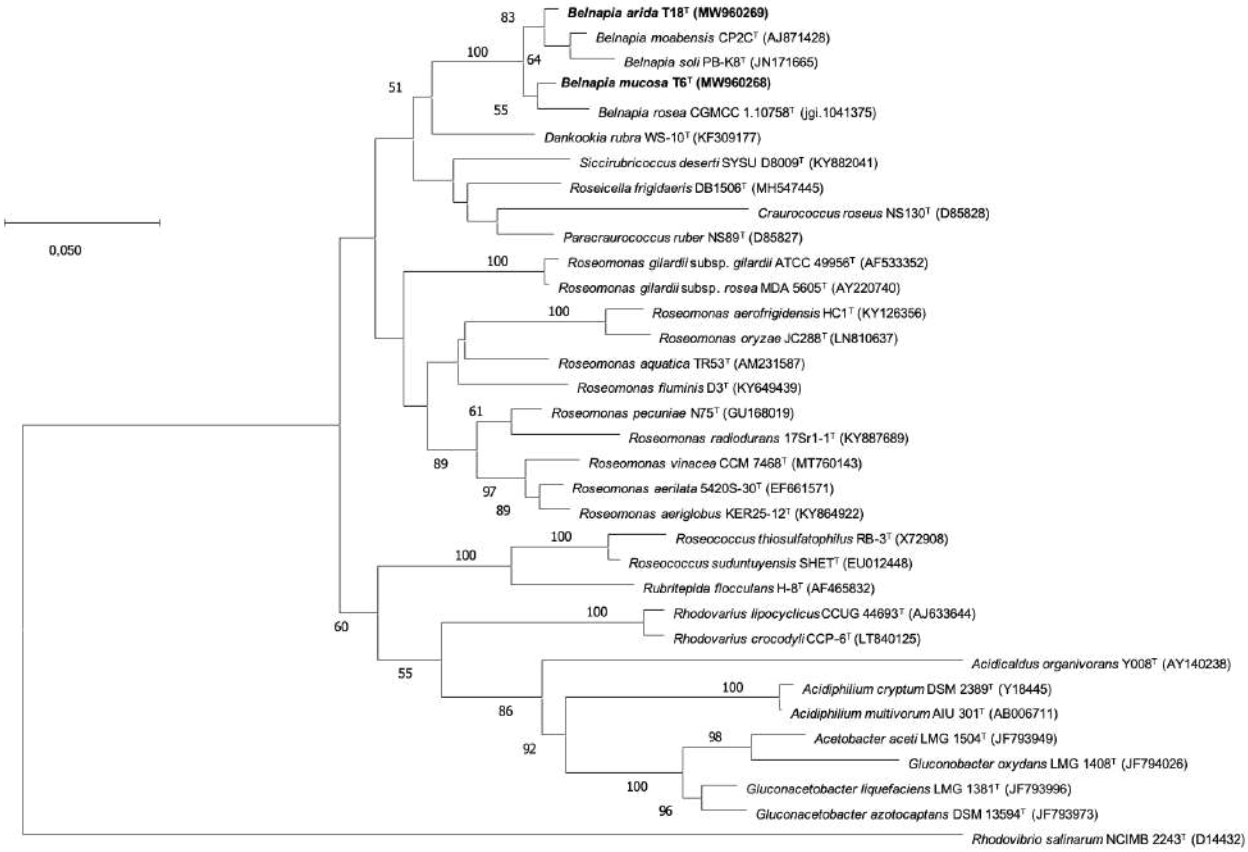

Figure IV.1. Maximum likelikood phylogenetic tree showing the relationships between strains T6^T, T18^T and other members of the family *Acetobacteraceae* based on 16S rRNA gene sequences. The optimal evolutionary model of nucleotide substitution applied was Tamura-Nei G+I. Numbers at branch points refer to bootstrap percentages based on 500 replicates (values under 50% are not indicated). *Rhodovibrio salinarum* NCIMB 2243^T (D14432) was used as an outgroup. Bar, 0.05 fixed nucleotide substitutions per site.

more polytrophic metabolism than strains T6^T and T18^T.

Almost complete 16S rRNA gene sequences were obtained. The 16S rRNA gene sequence lengths of strains T6^T and T18^T are 1392 (accession number MW583035) and 1383 bp (MW583036), respectively. According to the EzBioCloud database tool, the most closely related type strains of T6^T were *B. rosea* CGMCC 1.10758^T (98.11%), *B. moabensis* CP2C^T (97.38%) and *B. soli* PB-K8T (96.80%); whereas the closest relatives of T18^T were *B. moabensis* CP2C^T (98.55%), *B. soli* PB-K8^T (97.54%) and *B. rosea* CGMCC 1.10758^T (97.40%). The type strains *B. rosea* DSM 23312^T, *B. moabensis* DSM 16746^T and *B. soli* DSM 28067^T were, thus, selected as comparative reference strains, which were obtained from the DSMZ-German Collection of Microorganisms and Cell Cultures (Leibniz Institute, Braunschweig, Germany).

The phylogenetic positions of strains T6^T and T18^T within the genus *Belnapia* were confirmed by both 16S-rRNA-based ML and NJ phylogenetic tress (Figure IV.1 and IV.S1). T6^T grouped with *B. rosea* CGMCC 1.10758^T, whereas T18^T showed an external position in the cluster formed by *Belnapia moabensis* CP2C^T and *Belnapia soli* PB-K8^T in both trees. This phylogenetic inference was supported by high bootstrap values.

The draft genomes of strains T6^T and T18^T consisted of 220 and 355 contigs, respectively, which constituted a total length of 6449681 and 6937094 bp, respectively. The N50 values were 328 210 and 194 160 for T6^T and T18^T respectively. The genomic DNA G+C contents were 69.80 and 68.96% for T6^T and T18^T respectively, which is in accordance with the values previously described for the rest of the species within the genus *Belnapia* and further confirms their adscription to this genus [1–3]. A total of 6369 and 7450 coding sequences (CDSs) were predicted for strains T6^T and T18^T, of which 3380 and 3480, respectively, corresponded to proteins with functional assignment. Regarding the prediction of tRNA and rRNAs, a total of 49 and 47 tRNAs, and 3 and 2 rRNAs were predicted for strains T6^T and T18^T, respectively. The 16S rRNA gene sequences of strains T6^T and T18^T were also extracted from the genome, which were 1496 and 1494 bp long, respectively (accession numbers MW960268 and MW960269, respectively). According to the EzBioCloud database tool, the most closely related type strains to T6^T were *B. rosea* CGMCC 1.10758^T (98.16%), *B. moabensis* CP2C^T (97.45%) and *B. soli* PB-K8^T (96.80%); whereas

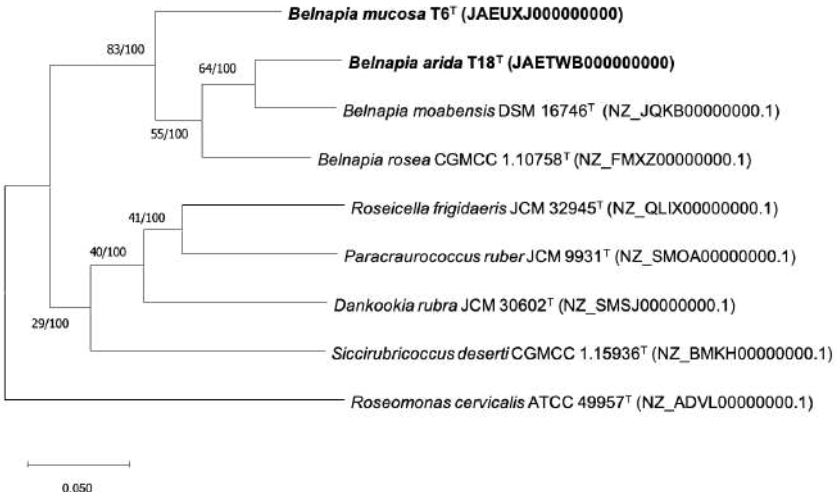

Figure IV.2. Phylogenomic tree showing the relationships between strains T6^T, T18^T and other members of the family *Acetobacteraceae* based on a multiple alignment of a set of 92 gene sequences using the UBCG (v.3.0) [19] pipeline. Bootstrap analysis was carried out using 100 replicates. Gene support indices (maximum value; 92 genes) and percentage bootstrap values (maximum value; 100%) are given at the branch points. *Roseomonas cervicalis* ATCC 49957^T was used as an outgroup. Bar 0.050 substitutions per position.

the most closely related type strains to T18^T were *B. moabensis* CP2C^T (98.58%), *B. soli* PB-K8^T (97.54%) and *B. rosea* CGMCC 1.10758^T (97.45%). The completeness values of genomes were 100 and 99.5% for strains T6^T and T18^T, respectively; and the levels of contamination were 0.5 and 2.49%, respectively. The draft genomes showed, thus, sufficient quality for further analysis.

In order to obtain a more accurate phylogenetic inference of novel strains, a phylogenomic tree based on nucleotide sequences was reconstructed (Figure IV.2). The phylogenomic tree corroborated that the two strains represent members of the genus *Belnapia*. Strain T18^T was most closely related to *B. moabensis* DSM 16746^T, while T6^T showed an external position to the rest of the members of the genus *Belnapia*. The type strain of *B. soli* was not included in this analysis because its genome was not publicly available at the time of writing.

The ANIb and digital DDH values between strains T6^T and T18^T and other related species were calculated (Table IV.S2). The ANIb and dDDH values of strain T6^T vs. *Belnapia rosea* CGMCC 110758^T were 83.26 and 29 %, respectively; the ANIb and dDDH values of strain T18^T vs. *Belnapia moabensis* DSM 16746^T were 88.47 and 40.5 %, respectively. Moreover, both genome indexes were calculated between strains T6^T and T18^T, which were 82.96 and 28.5 % for ANIb and dDDH, respectively. As the values were below the thresholds established to circumscribe prokaryotic species, namely 95 % for ANI values [20] and 70 % for dDDH [21], both genome indexes confirmed the classification of strains T6^T and T18^T as representing novel species [22].

The analysis of the genome of strains T6^T and T18^T allowed the prediction of their ability to synthesize phosphatidylglycerol, phosphatidylcholine and diphosphatidylglycerol on the basis of the presence of genes coding for phosphatidylglycerol phosphatase (EC 3.1.3.27), phosphatidylethanolamine N-methyltransferase (EC 2.1.1.17) and cardiolipin synthase (EC 2.7.8.-) respectively. This polar lipids' profile is in agreement with the polar lipid analyses results available for other species of the genus *Belnapia* [1–3]. Furthermore, their ability to synthesize phosphatidylethanolamine was predicted on the basis of the presence of the genes coding for phosphatidylserine synthase (EC 2.7.8.8) and phosphatidylserine decarboxylase (EC 4.1.1.65), which had been previously described for *B. soli* [3].

Table IV.2. Cellular fatty acid composition (percentages) of strains T6^T, T18^T and the members of the genus *Belnapia*. Strains: 1, T6^T; 2, T18^T; 3, *Belnapia moabensis* DSM 16746^T; 4, *Belnapia rosea* DSM 23312^T; 5, *Belnapia soli* DSM 28067^T. TR, trace (<1.0%); -, not detected.

	1	2	3	4	5
Saturated					
$C_{9:0}$	tr	tr	-	tr	tr
$C_{10:0}$	-	tr	-	tr	tr
$C_{14:0}$	1.9	tr	-	tr	1.2
$C_{16:0}$	15.7	12.9	13.2	11.3	16.8
$C_{18:0}$	3.6	1.8	1.9	1.2	2.6
$C_{19:0}$ cyclo *w 8c*	-	-	-	1.8	2.3
Unsaturated					
$C_{16:1}$ *w 5c*	-	tr	-	1.0	tr
$C_{18:1}$ *w 5c*	-	-	-	tr	-
$C_{18:1}$ *w 9c*	5.4	3.5	1.7	1.2	2.4
Hydroxylated					
$C_{12:0}$ 2-OH	6.4	3.9	2.0	1.6	2.3
$C_{16:0}$ 3-OH	-	tr	-	tr	1.4
$C_{18:1}$ 2-OH	12.2	10.0	8.2	9.0	10.7
$C_{18:0}$ 3-OH	1.4	-	-	-	tr
Summed Features*					
Sum in Feature 2	1.1	tr	tr	tr	1.3
Sum in Feature 3	10.3	12.2	10.2	19.2	16.5
Sum in Feature 8	41.4	51.5	62.0	50.6	40.4

The major fatty acid for strains T6^T and T18^T was summed feature 8 ($C_{18:1}$ω7c/$C_{18:1}$ω6c) (41.4 and 51.5%, respectively). However, there were also high amounts of $C_{16:0}$ (15.7 and 12.9%, respectively for T6^T and T18^T), $C_{18:1}$ 2-OH (12.2 and 10.0%, respectively) and summed feature 3 ($C_{16:1}$ω7c/$C_{16:1}$ω6c) (10.3 and 12.2%, respectively) (Table IV.2). This is in accordance with the profiles obtained for the members of the genus *Belnapia*, which also showed high amounts of summed feature 8, summed feature 3, $C_{18:1}$ 2-OH and $C_{16:0}$, thus confirming the inclusion of both strains within the genus *Belnapia*.

The results of the phenotypic, chemotaxonomic, genomic and phylogenetic analyses confirm that strains T6^T and T18^T should be considered as each representing a novel species within the genus *Belnapia*, for which the names *Belnapia mucosa* sp.

nov. and *Belnapia arida* sp. nov., respectively, are proposed.

Description of *Belnapia mucosa* sp. nov.

Belnapia mucosa (mu. co'sa. L. fem. adj. *mucosa*, mucous, slimy).

Colonies are circular, smooth, mucous, convex and pale-pink. Cells are Gram-reaction-negative, coccoid (0.8–1.0 μm) and non-motile. Growth occurs at 4–42 °C (optimum at 30 °C) and pH 5–9 (optimum 6–7), and it can tolerate up to 1.5 % (w/v) NaCl (optimum 0–1%). This species grows under aerobic and microaerophilic conditions, no growth is observed under anaerobic conditions. Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities are detected. Lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, nitrate reduction, indole production, glucose fermentation, arginine dihydrolysis, urease, aesculin hydrolysis and gelatinase are not detected. According to API 20 NE test kits, this species is weakly positive for the assimilation of potassium gluconate, adipic acid and malic acid and negative for the assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, maltose, capric acid, trisodium citrate and phenylacetic acid. According to BIOLOG GENIII MicroPlates, this species is positive for the utilization of D-galactonic acid lactone, β-hydroxy-D,L-butyric acid, D-glucuronic acid, glucuronamide and acetoacetic acid; and negative for raffinose, α-D-glucose, Tween 40, dextrin, lactose, D-mannose, D-mannitol, glycyl-L-proline, D-galacturonic acid, methyl pyruvate, γ-aminobutyric acid, maltose, melibiose, D-fructose, D-arabitol, L-alanine, D-lactic acid methyl ester, α-hydroxybutyric acid, trehalose, methyl β-D-glucoside, D-galactose, myo-inositol, L-arginine, D-gluconic acid, L-lactic acid, cellobiose, D-salicin, 3-methyl-D-glucoside, glycerol, L-aspartic acid, citric acid, α-ketobutyric acid, gentiobiose, N-acetyl-D-glucosamine, D-fucose, D-glucose-6-phosphate, L-glutamic acid, α-ketoglutaric acid, sucrose, N-acetyl-β-D-mannosamine, L-fucose, D-fructose-6-phosphate, L-histidine, mucic acid, D-malic acid, propionic acid, turanose, N-acetyl-D-galactosamine, L-rhamnose, D-aspartic acid, L-pyroglutamic acid, quinic acid, L-malic acid, acetic acid, stachyose, N-acetyl neuraminic acid, inosine, D-serine, L-serine, D-saccharic acid, bromo-succinic acid and formic acid. The major fatty acids are summed feature 8 ($C_{18:1}$ω7c/$C_{18:1}$ω6c), $C_{16:0}$, $C_{18:1}$ 2-OH and summed feature 3 ($C_{16:1}$ω7c/$C_{16:1}$ω6c).

The type strain T6^T (CECT 30228^T = DSM 112073^T) was first isolated from the Tabernas Desert in Almería (Spain) from a biocrust sample. The DNA G+C content of the type strain is 69.80%. The DDBJ/ENA/GenBank accession number for the 16S rRNA gene sequence is MW960268 and the genome accession number is JAEUXJ000000000.

Description of *Belnapia arida* sp. nov.

Belnapia arida (a' ri. da. L. fem. adj. *arida*, dry, referring to the isolation of the strain from an arid soil)

Colonies are circular, smooth, mucous, convex and pink. Cells are Gram-reaction-negative, coccoid-shaped (0.8–1.0 μm) and non-motile. Growth occurs at 4–40 °C (optimum at 30 °C) and pH 5–9 (optimum 6–7), and it can tolerate up to 1.5% (w/v) NaCl (optimum 0–1%). This species grows under aerobic and microaerophilic conditions, no growth is observed under anaerobic conditions. Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and urease activities are detected. Lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, nitrate reduction, indole production, glucose fermentation, arginine dihydrolysis, aesculin hydrolysis and gelatinase are not detected. According to API 20 NE test kits, this species is negative for the assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid. According to BIOLOG GENIII

MicroPlates, this species is positive for the utilization of D-gluconic acid, β-hydroxy-D,L-butyric acid, L-pyroglutamic acid and L-malic acid; and negative for the utilization of raffinose, α-D-glucose, D-sorbitol, gelatin, pectin, p-hydroxyphenylacetic acid, Tween 40, dextrin, lactose, D-mannose, D-mannitol, glycyl-L-proline, D-galacturonic acid, methyl pyruvate, γ-aminobutyric acid, maltose, melibiose, D-fructose, D-arabitol, L-alanine, L-galactonic acid lactone, D-lactic acid methyl ester, α-hydroxybutyric acid, trehalose, methyl β-D-glucoside, D-galactose, myo-inositol, L-arginine, L-lactic acid, cellobiose, D-salicin, 3-methyl-D-glucoside, glycerol, L-aspartic acid, D-glucuronic acid, citric acid, α-ketobutyric acid, gentiobiose, N-acetyl-D-glucosamine, D-fucose, D-glucose-6-phosphate, L-glutamic acid, glucuronamide, α-ketoglutaric acid, acetoacetic acid, sucrose, N-acetyl-β-D-mannosamine, L-fucose, D-fructose- 6-phosphate, L-histidine, mucic acid, D-malic acid, propionic acid, turanose, N-acetyl-D-galactosamine, L-rhamnose, D-aspartic acid, quinic acid, acetic acid, stachyose, N-acetyl neuraminic acid, inosine, D-serine, L-serine, D-saccharic acid, bromosuccinic acid and formic acid. The major fatty acids are summed feature 8 ($C_{18:1}$ω7c/$C_{18:1}$ω6c), $C_{16:0}$, summed feature 3 ($C_{16:1}$ω7c/$C_{16:1}$ω6c) and $C_{18:1}$ 2-OH.

The type strain T18^T (CECT 30229^T = DSM 112074^T) was first isolated from the Tabernas Desert in Almería (Spain) from a biocrust sample. The DNA G+C content of the type strain is 68.96%. The DDBJ/ENA/GenBank accession number for the 16S rRNA gene sequence is MW960269 and the genome accession number is JAETWB000000000.

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Author contributions

E.M.M., sampling, experimental procedures, bioinformatic analysis, writing and approving the manuscript. À.V.V., experimental procedures, bioinformatic analysis, writing and approving the manuscript. L.S., experimental procedures, bioinformatic analysis, writing and approving the manuscript. A.C., experimental procedures, bioinformatic analysis, writing and approving the manuscript. J. Pascual, supervising experimental work, bioinformatic analysis, writing and approving the manuscript. J. Peretó, writing and approving the manuscript. M.P., conceived the work, sampling, writing and approving the manuscript.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Supplementary material

Additional information may be found in Appendix B.

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Chapter 3: The microbial communities associated to UV cabins in the Hospital General de València

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Publication V

Microbial communities on UV cabins in the dermatology service of a Spanish hospital: ecology and resistance to UV light and antibiotics

Esther Molina-Menor[1], Nicolás Carlotto[1], Àngela Vidal-Verdú[1], Amparo Pérez-Ferriols[2], Gemma Pérez-Pastor[2] and Manuel Porcar[1,2*]

1. Institute for Integrative Systems Biology I2SysBio, Universitat de València-CSIC, Spain.
2. Servicio de Dermatología, Consorcio Hospital General de València, Spain.
3. Darwin Bioprospecting Excellence S.L. Parc Científic Universitat de València, Spain.

Summary

Microorganisms colonize all possible ecological habitats, including those subjected to harsh stressors such as UV radiation. Hospitals, in particular the UV cabins used in phototherapy units, constitute an environment in which microbes are intermittently subjected to UV irradiation. This selective pressure, in addition to the frequent use of antibiotics by patients, may represent a threat in the context of the increasing problem of antimicrobial resistance. In this work, a collection of microorganisms has been established in order to study the microbiota associated to the inner and outer surfaces of UV cabins and to assess their resistance to UV light and the antibiotics frequently used in the Dermatology Service of a Spanish hospital. Our results show that UV cabins harbor a relatively diverse biocenosis dominated by typically UV-resistant microorganisms commonly found in sun-irradiated environments, such as *Kocuria*, *Micrococcus* or *Deinococcus* spp., but also clinically relevant taxa, such as *Staphylococcus* or *Pseudomonas* spp. The UV-radiation assays revealed that, although some isolates displayed some resistance, UV is not a major factor shaping the biocenosis living on the cabins, since a similar pool of resistant microorganisms was identified on the external surface of the cabins. Interestingly, some *Staphylococcus* spp. displayed resistance to one or more antibiotics, although the hospital reported no cases of antibiotic-resistance infections of the patients using the cabins. Finally, no association between UV and antibiotic resistances was found.

This work is under review in Scientific Reports since April 2023.

Introduction

Microorganisms (not only bacteria and archaea, but also eukaryotes) can resist both ultraviolet radiation (UV) and high doses of ionizing radiation [1]. UV-resistant organisms are widely distributed in many ecosystems, but they are particularly frequent on sun-irradiated environments such as building surfaces, deserts or solar panels [2-4].

Radiation-resistant bacteria belong to different taxonomic groups. Although some clades are known by their natural resistance to radiation and high temperatures, such as the *Deinococcota* phylum (former *Deinococcus-Thermus*), radiation-resistant bacteria are also represented in *Pseudomonadota*, *Bacillota*, *Actinomycetota* or *Bacteroidota*, previously *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes*, respectively [5]. In addition to their general non-pathogenic condition, they can even contribute to other organisms, such as plants, fighting pathogenic microorganisms [6].

Pathogenic bacteria, in particular those causing health problems to humans and other animals, show different sensitivities to UV radiation [7]. In fact, irradiation is used as an antimicrobial strategy in different laboratory and hospital devices such as UV sterilization lamps and microbiology cabins [8]. Besides, UV light and other light-based strategies have been also proposed as potential strategies to treat microbial infections in patients [9, 10]. However, UV sterilization has some limits as its efficiency depends on factors such as microbial species and state of cultures, or the nature of the surfaces, among others [7, 11].

UV light covers the spectrum wavelength between 100 and 400 nm. It can be further subdivided into three regions: UVA (315-400 nm), UVB (280-315 nm) and UVC (100-280 nm) [12]. Each UV light range has a different effect on living organisms, being UVC the most energetic, and thus dangerous, radiation. However, solar radiation that reaches the Earth's surface is UVB and UVA, as UVC is absorbed by the atmosphere [13]. Although UVB is also mainly filtered, the small fraction that gets to the surface causes different deleterious effects on the organisms, such as skin tanning and sunburns in animals. UVB light is directly absorbed by DNA molecules causing mutations, which is associated with the development of several types of skin cancer. Moreover, it is major responsible of killing airborne bacteria subjected to sunlight [14]. In contrast, UVA light is the most penetrating one and represents 95 % of the UV light that reaches our planet's surface. Its penetrating power has an impact on photoaging, but it can also contribute to DNA damage by interacting with already existing photoproducts [13, 15-16].

The damaging effects of UV light is both direct (changes in biomolecules) and indirect, via the increase in reactive oxygen species (ROS). It includes changes in DNA, such as the formation of pyrimidine dimers, but also structural changes in proteins, lipids, and physiological stress that leads to loss of cell viability [16-18]. Interestingly, and beyond the antiseptic effect of UV treatment described above, both UVB and UVA, at controlled doses, can be used for therapeutic purposes to treat cutaneous affections.

Phototherapy is the controlled use of light of different wavelengths to treat health problems, mostly skin disorders [19]. It is commonly used in newborns developing jaundice, to treat the accumulation of bilirubin [20], but also in the treatment of psoriasis, chronic eczema, mycosis fungoides or vitiligo, among other diseases [21-22].

Antimicrobial resistance, AMR, has risen as one of the main threats for global health. Specifically, the World Health Organization (WHO) estimates that by 2050, around 10 million people will die from infections with no available treatments [23]. Although the selection of resistances is a natural phenomenon resulting from the imposed selective pressure of using antibiotics, their abuse and misuse, among other factors, has accelerated their spread [24-25]. Specifically, hospitals and intensive care units (ICUs) represent environments in which the risk of acquiring nosocomial multidrug resistant infections increases [26]. The systematic use of antibiotics in hospital environments, in addition to the abundance of more

susceptible patients, favors the spread of AMR among microorganisms [27].

The studies regarding AMR and radiation mainly focus on the effect of IR on the degradation of both antibiotic products and the inactivation of gene synthesis [28], or the treatment of infections with specific IR [29]. Although there is some evidence that the use of radiation may favor the selection of antibiotic resistant bacteria, there are no previous reports on their study in phototherapy services [30].

In the present work, we aimed at studying the microbial diversity in a previously unreported niche: therapeutic UV light hospital cabins, and to explore whether there is a link between the origin of the samples (taken either inside or outside the cabins) and UV resistance. Additionally, we have explored the co-occurrence of UV resistance and antibiotic resistance. Such co-occurrence may be of special interest in order to inform UV-based antimicrobial and disinfection policies in hospital facilities [26, 31].

Results

Ecology and microbial diversity

Culturable microbial collection and identification

The establishment of a microbial collection resulted in the isolation of 169 strains. A total of 164 isolates were identified, of which 155 corresponded to bacterial species. The identification through 16S rRNA gene or ITS sequence sequencing revealed that the isolates belonged to 44 different genera, among which the genera *Staphylococcus* (29 isolates), *Kocuria* (17 isolates), *Micrococcus* (17 isolates) and *Pseudomonas* (11 isolates) were the most abundant ones. In contrast, genera *Erwinia*, *Fredinandcohnia*, *Lysinibacillus*, *Mixta*, *Moraxella*, *Peribacillus*, *Pseudoxanthomonas*, *Rhodococcus*, *Robertmurraya*, *Roseomonas*, *Pantoea*, *Psychrobacillus*, *Domibacillus*, *Kosakonia*, *Ustilago*, *Cryptococcus*, *Cystobasidium* and *Rhodotorula* were just represented by one isolate each (Figures V.1A and V.1B).

Taking into consideration the isolation source, 81 isolates were originating from the inner surface of the cabins, being *Staphylococcus* (17 isolates) the most abundant genera. In contrast, 88 microbial isolates came from the outer surface of the cabins, among which *Staphylococcus* (12 isolates), *Pseudomonas* (11 isolates) and *Kocuria* (10 isolates) were the most abundant ones. Moreover, 14 genera were exclusively isolated from each location (inside and outside), whereas 16 were common for both sampling sites (Figure V.2). However, it has to be stressed that most of the "exclusive" taxa were represented by just one isolate, with the exception of *Pseudomonas* spp. When comparing the cabins, the four most abundant genera (*Staphylococcus*, *Kocuria*, *Micrococcus* and *Pseudomonas*), as well as *Bacillus*, were isolated from all of them and cabin two was the one with the highest number of exclusive taxa (11) regardless of the isolation source (Figure V.S1C). However, the exclusivity inside and outside was similar (Figures V.S1A and V.S1B).

Next Generation Sequencing: high-throughput 16S rRNA gene sequencing

Three different α-diversity indexes were calculated: richness, Shannon index and Simpson index. Richness refers to the total number of amplicon sequence variants (ASVs, or clones), Shannon measures the number of different taxa and their abundances, whereas Simpson quantifies how the sequences are distributed among ASV. That is, the number of bacteria per ASV. Although the α-diversity (ASV level) was higher in the samples from the outside than from the inside of the cabins, the Wilcoxon test did not find significant differences given the low number of replicates from each cabin and location. Similarly, the Shannon and Simpson indexes revealed higher diversity values for the outside of cabins two, three and four, but again, these results were not significantly different (Figure V.3A, Figures V.S3A and V.S3B).

The representation of the β-diversity in a principal component analysis (PCoA) showed that the outer surfaces of the cabins displayed higher similarities than the inner surfaces among cabins. Moreover, samples from both the inside and the outside of cabins one and two were similar in terms of microbial composition but plotted separately in the PCoA

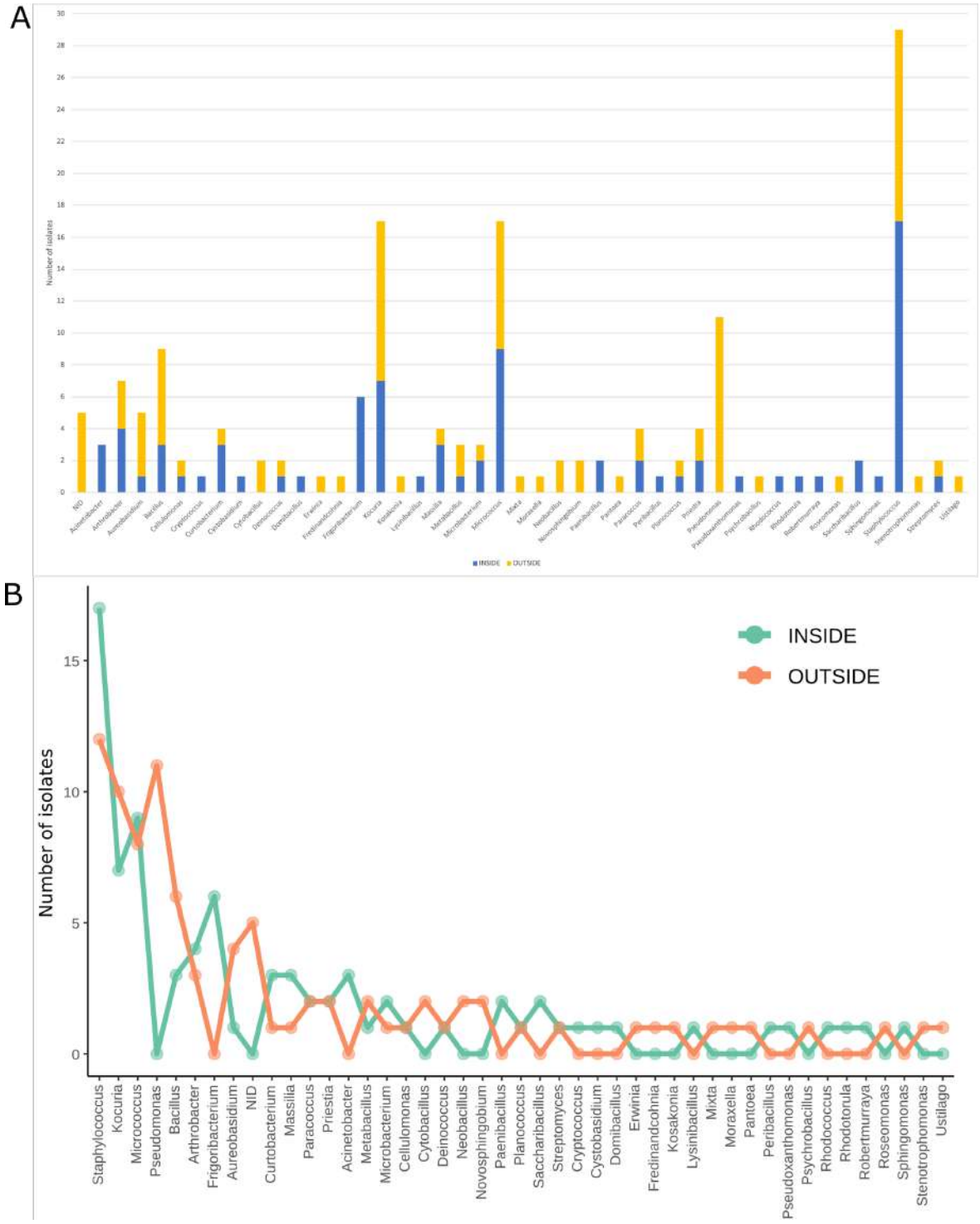

Figure V.1. Culturable microbial diversity. (A) Histogram showing the number of strains isolated from inside (blue) and outside (yellow) the cabins. The genera are listed in alphabetical order. Non-identified isolates (NID) are also included. (B) Lineplot representing the number of strains isolated from inside (green) and outside (orange) from the most abundant to the less abundant.

(Figure V.3B). The PERMANOVA test confirmed that the microbiomes were significantly different both between cabins and sample locations.

At the phylum level (updated according to the new nomenclature for prokaryotic phyla [32]), all the samples displayed similar bacterial profiles, with *Pseudomonadota*, *Actinomycetota* and *Bacillota* as the predominant taxa. Moreover, *Cyanobacteria* and *Bacteroidota* were also abundant, although not in the case of sample C4D1 (inside of cabin four) (Figure V.4A and Figure V.S4A), in which *Bacillota* was the predominant phylum. However, none of the phyla

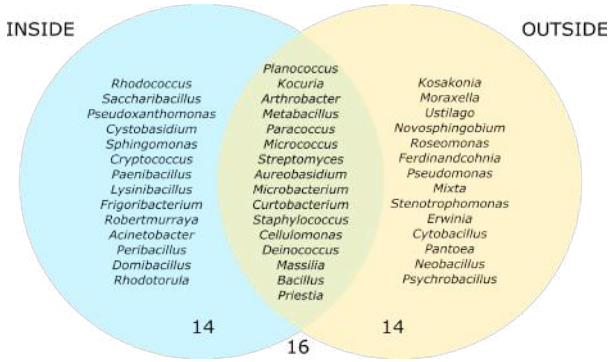

Figure V.2. Venn diagram showing the exclusive and shared cultured genera isolated from inside and outside the cabins.

was statistically more abundant in any of the cabins or locations (inside/outside) according to the DESeq2 test.

At the genus level, the most similar cabins in terms of bacterial composition were cabin one and two. The genera *Staphylococcus*, *Rubellimicrobium* and *Paracoccus* were especially abundant there. Moreover, *Pseudomonas*, *Kocuria*, *Sphingomonas* and *Corynebacterium* were among the most abundant ones in the majority of the samples. Samples C1D3 (inside of cabin one), C3D1 (inside of cabin three) and C4D1 (inside of cabin four) were the most different ones (Figure V.3B). In C1D3 there was a significant higher abundance of an unknown genus within the order *Enterobacterales*. In C3D1 there was higher abundance of *Pseudomonas* and an uncultured genus within the order *Cyanobateriales*. In C4D1 *Halomonas* was especially overrepresented, whereas *Melittangium* was characteristic for samples of cabin two (Figure V.4B and Figure V.S4B). Furthermore, some genera were significantly more abundant outside the cabins, such as *Oligella*, *Serratia*, *Cobetia* and *Carnobacterium*, whereas only *Providencia* was more abundant inside them according to a DESeq2 test.

Given the previous experience in irradiated environments, the knowledge on the natural skin microbiota, and the abundances found in the previous experiments, a selection of relevant taxa was analyzed in order to determine whether there were differences in their distribution inside and outside. Moreover, the abundances of the top ten abundant genera were also plotted. That is, in alphabetical order, the genera *Corynebacterium*, *Deinococcus*, *Hymenobacter*, *Kocuria*, *Micrococcus*, *Paracoccus*, *Pseudomonas*, *Rubellimicrobium*, *Sphingomonas* and *Staphylococcus* (Figure V.5 and Figure V.S5). None of the studied genera revealed any significant difference on the distribution among locations, except for *Staphylococcus* (Figure V.5C). Although *Deinococcus* was more abundant on the inner surfaces, these results were not significant according to the Wilcoxon test (Figure V.S5A). This tendency was also observed in the case of *Sphingomonas* (Figure V.S5D).

Finally, in order to identify the most frequent species within *Staphylococcus*, successive BLAST were performed with the most abundant ASV. Among the 61 ASV identified as *Staphylococcus*, *S. epidermitis* and *S. aureus* were between the ten more abundant

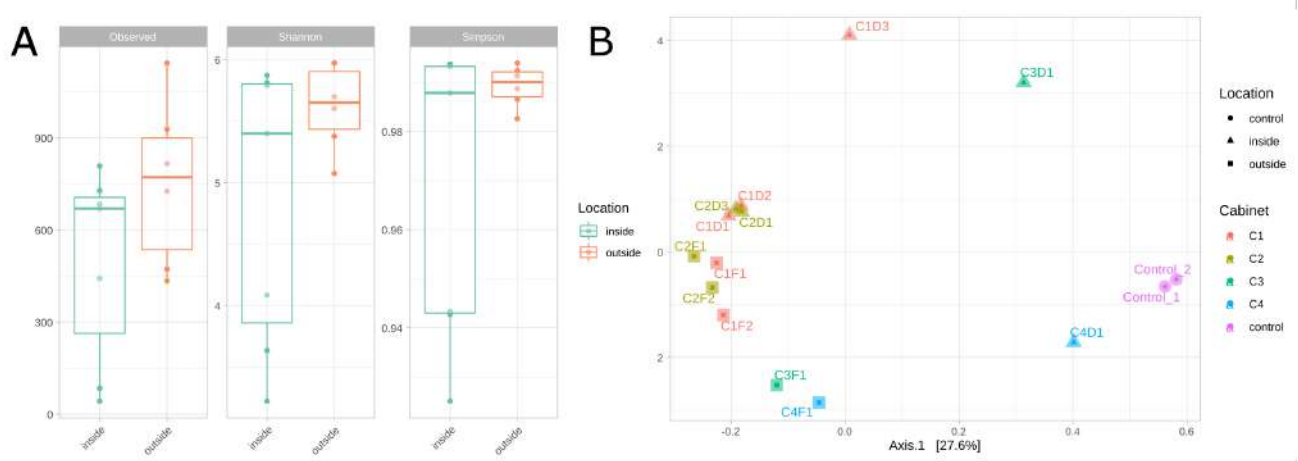

Figure V.3. Microbial diversity in the cabins. (A) α-diversity at the ASV level (clones) observed through Wilcoxon test and measured by Shannon and Simpson indexes. (B) PCoA showing the β-diversity of the samples from four different cabins both from inner and outer surfaces. Control samples corresponding to the DNA extraction kits used are included.

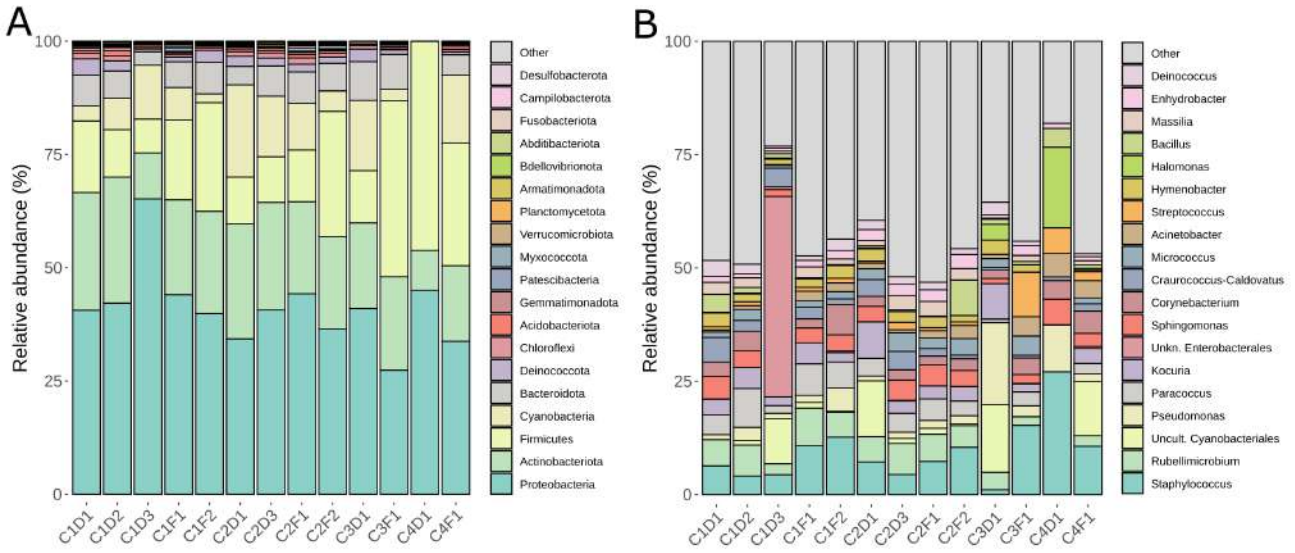

Figure V.4. Relative abundances (%) of bacteria in the sampled cabins as deduced by high-throughput 16S rRNA gene sequencing. (A) Relative abundances at the phylum level. (B) Relative abundances at the genus level.

species, along with *S. caprae*, *S. capitis*, *S. cohnii*, and *S. haemolyticus*.

Biological activity assays

UV-radiation resistance assay

The resistance to UV irradiation of the isolates from the subset of the microbial collection (Table V.S2) was assessed by quantifying the colony forming units (CFU) after treatments with 15 s and 30 s of exposure to UV. The survival rates were calculated by dividing the number of CFUs after irradiation between the CFUs observed in a non-exposed control replicate. Survival rates values close to 1 represented a high resistance to UV whereas those close to 0 represented a low resistance to UV (Figure V.6A).

From the strains that were exclusively isolated inside the cabins, *Kocuria polaris* was the only one not showing a significant decrease of survival after 15 s of UV exposure. However, 30 s of exposure resulted in a sharp and significant decrease, with a survival rate close to 0. In contrast, *Acinetobacter variabilis* and *Microbacterium esteraromaticum* showed a linear decrease in the survival rate, with values after 15 s and 30 s close to 0.5 and 0 respectively, whereas, the survival rates of *Frigoribacterium faeni*, *Lysinibacterium halotolerans*, *Paracoccus panacisoli* and *S. epidermitis*, showed a marked reduction to almost 0 after 15 s exposure to UV, revealing a high susceptibility to UV exposure.

From the strains that were exclusively isolated outside the cabins, there were two of them showing no significant decrease of survival rate after 15 s exposure to UV: *Arthrobacter agilis* and *Deinococcus ficus*. Although 30 s exposure to UV led to a significant decrease in the survival rate, these two isolates showed a difference from *K. polaris* as the values were not close to 0, suggesting a mid-resistance to the treatment. This was especially evident for *D. ficus* with a survival rate at 30 s higher than 0.75. The rest of the group formed by *Kocuria palustris*, *Priestia aryabhattai*, *Pseudomonas stutzeri*, *S. haemolyticus* and *Stenotrophomonas rhizophila* showed survival rates significantly different to the control and with values close to 0 even with 15 s of UV exposure.

Regarding the species that were isolated both inside and outside the cabins, all of them showed a similar output comparing the lineplot of the inside isolate to the outside isolate, revealing similar resistance patterns. The only exception was *Micrococcus luteus*, showing the outer strain a higher resistance to 15 s of exposure. Interestingly, the isolates of *Arthrobacter bussei* showed a similar output to the outer isolates *A agilis* and *D. ficus*, with survival rate values between 1 and 0.5 after 15 s and 30 s of UV exposure. Finally, *Curtobacterium flaccumfaciens* and *Kocuria arsenatis*

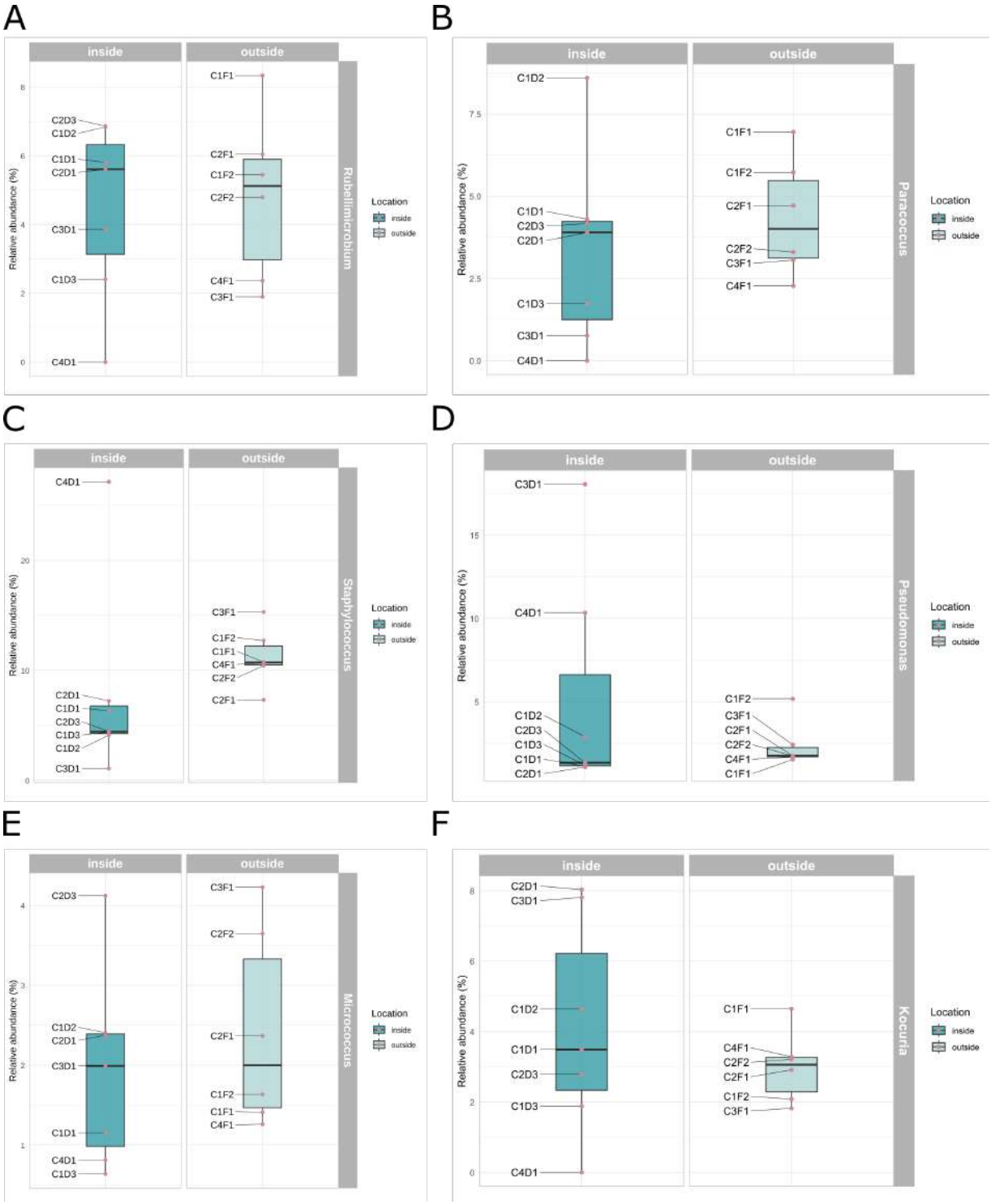

Figure V.5. Relative abundances (%) at the genus level of specific taxa based on their abundance and relevance for the study. (A) Abundance of *Rubellimicrobium*. (B) Abundance of *Paracoccus*. (C) Abundance of *Staphylococcus*. (D) Abundance of *Pseudomonas*. (E) Abundance of *Micrococcus*. (F) Abundance of *Kocuria*.

also showed a considerable resistance after 15 s exposure to UV, whereas *Bacillus altitudinis*, *S. cohnii* and *S. hominis* showed a strong decrease in the resistance to UV treatment already at 15 s for both inside and outside isolates.

To further test if the UV irradiation from the cabins could shape the surface-associated microbiome leading to an enrichment of UV resistant species in the inside of the cabins, we compared in figures V.6B and C the survival rates after 15 s and 30 s UV exposure, respectively, for all the isolates tested in figure V.6A.

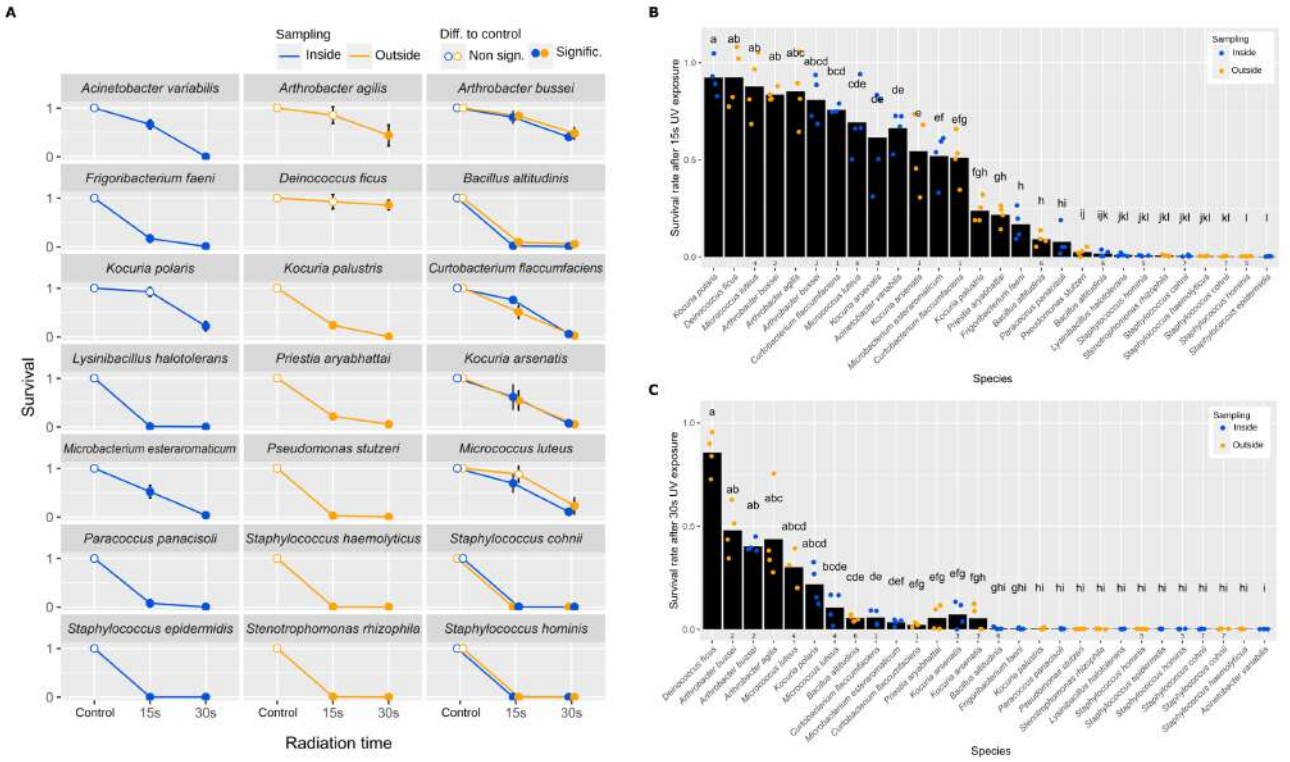

Figure V.6. Survival rate of bacterial isolates after UV-irradiation treatment. (A) Lineplots showing the survival rate of cell suspensions for each of the selected isolates. Results for isolates of the same species that were taken from the inside and the outside of the UV cabins are plotted in the same facets to facilitate the comparison. The mean is depicted with a circle and standard deviation is depicted with vertical black lines. t-tests for the difference of the mean to a theoretical survival rate value of 1 were performed for 15 s and 30 s groups. Significance is stated as filled circles for $p<0.05$. (B-C) Survival rate of all the strains for treatments with 15 s (B) or 30 s (C) exposure to UV irradiation. Kruskal Wallis test was performed to assess the differences among all the survival rates. Each dot represents a replicate, while the black columns represent the average. Isolates with the same letter above the column are not statistically different ($p\geq0.05$). Isolates taken both outside and inside samples are underlined. Results for all the panels were obtained from 4 replicates for each isolate.

However, there was no clear evidence that supported this hypothesis. Although *K. polaris* inside showed the highest survival rate after 15 s, this was not significantly different to the next four species (*D. ficus*, *M. luteus*, *A. bussei* and *A. agilis*), which were isolated outside the cabins (Figure V.6B). Moreover, a similar pattern was observed at 30 s of exposure, in which the significance group for the highest survival rates was formed by four isolates coming from the outer surfaces and two coming from the inner ones (Figure V.6C). At the strain level, *M. luteus* and *B. altitudinis* isolated from outside displayed higher survival rates than the inside isolates, whereas *C. flaccumfaciens* behaved contrarily after 15 s UV exposure. In the case of the 30 s treatment, only *B. altitudinis* behaved differently between treatments, being more resistant the outer strain.

Antibiotic resistance assay

To study a possible occurrence of AMR in isolates from the microbiomes of the UV cabins, we tested the resistance to antibiotics of the isolates from the subset of the microbial collection (Table V.S2). Quality control strains were included in all the experiments and gave the expected MIC results (in µg/ml): *E. coli* for amoxiclavulanic acid (AMC; 0.5 – 2), doxycycline (DXT; 0.5 – 2) and gentamicin (GEN; 0.25 – 1); and *S. aureus* for mupirocin (MUP; 0.06 – 0.25), azithromycin (AZM; 0.5 – 2) and clindamycin (CD; 0.06 – 0.25). The classification of the isolates as resistant (R) or sensitive (S) to the tested antibiotics was determined according to clinical breakpoints established by EUCAST (EUCAST Clinical Breakpoint Tables v.12.0), which classifies microorganisms into susceptible at standard doses (S), susceptible increased exposure (I) or resistant (R). However, there were not available data for some species or antibiotics given the environmental origin of the tested isolates. In those

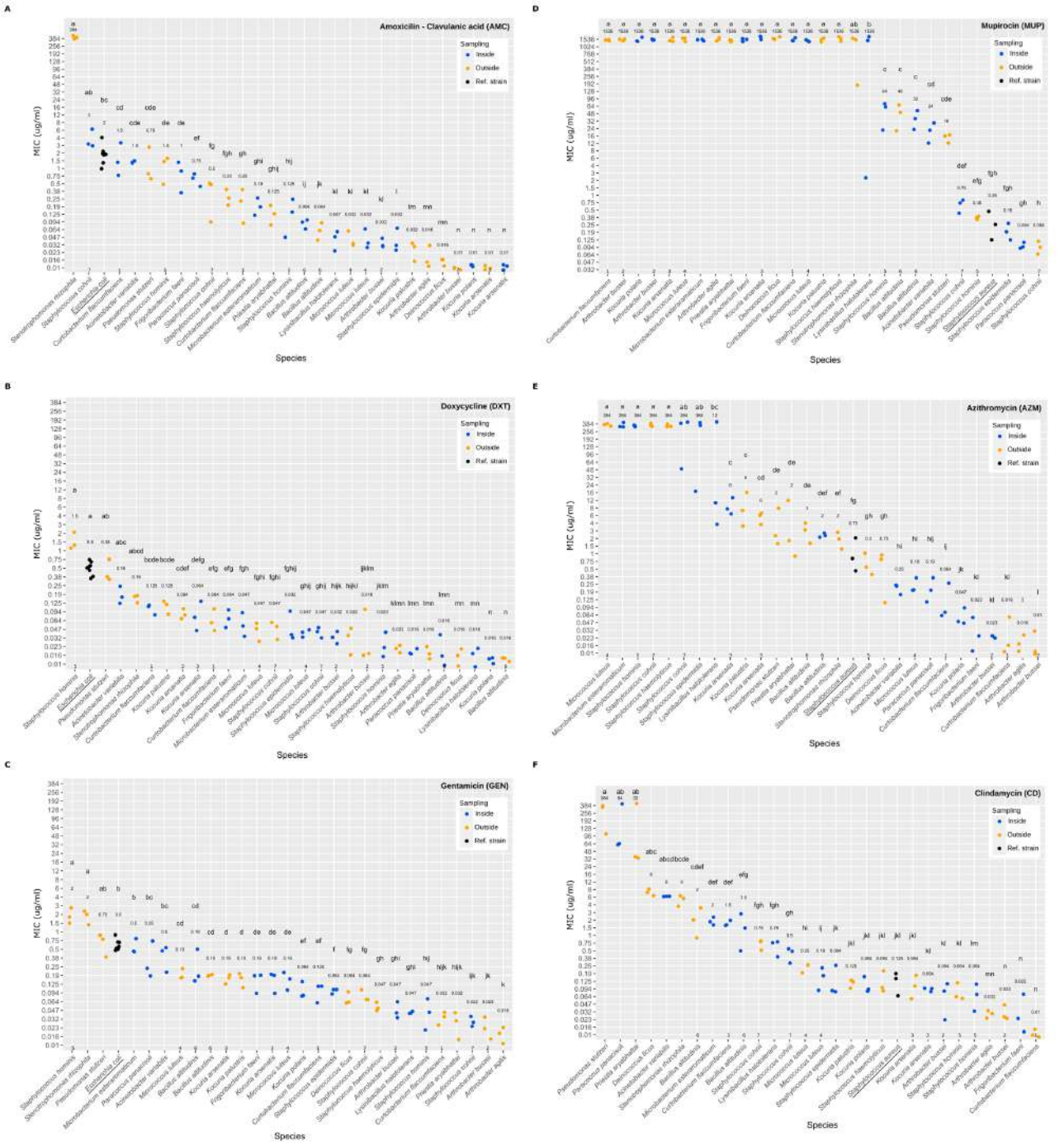

Figure V.7. Minimum Inhibitory Concentration (MIC) test strips (MTSs) displaying the resistance of bacterial isolates to antibiotics commonly used in the Dermatology Service of the HGUV. (A) Amoxicillin clavulanic acid (AMC). (B) Doxycycline (DXT). (C) Gentamicin (GEN). (D) Mupirocin (MUP). (E) Azithromycin (AZM). (F) Clindamycin (CD). Kruskal Wallis test was performed to assess for differences among MIC values. Each dot represents a replicate, while the number over the dots states the median value for each isolate. Isolates with the same letter above the column are not statistically different from each other ($p \geq 0.05$). Isolates taken both from outside and inside the cabins are underlined. Reference strains are depicted with black dots. Expected MIC range for reference strains in µg/ml: AMC 2 – 8; MUP 0.06 – 0.25; CD 0.06 – 0.25; AZM 0.5 – 2; GEN 0.25 – 1; DXT 0.5 – 2. Results for MTSs were obtained from 3 replicates for each isolate.

cases, only the MIC values are commented and further discussed.

The six antibiotics revealed two different patterns. On the one hand, the tested strains showed variable susceptibilities to AMC, DXT, GEN, and CD (Figures V.7A, B, C, F). In contrast, there was a clear cluster of resistant strains to MUP and AZM (Figures V.7D and E).

In the case of AMC, the majority of the tested isolates were sensitive (S or I) according to the EUCAST criteria, with the exception of *S. rhizophila* which was resistant (R, Figure V.7A). In regard to DXT, *S. hominis* appeared as I, with a MIC value between 1 and 2 µg/ml, whereas the rest of the tested strains gave MIC values below 1 (Figure V.7B). Similarly, most of the selected strains were S to GEN, with non-related species threshold established at 0.5 µg/ml, and 4 µg/ml for *Pseudomonas* spp. and *Acinetobacter* spp. However, in this case, *S. hominis* and *S. rhizophila* were R (Figure V.7C).

In the case of MUP and AZM, there was a significant cluster of R strains (Figures V.7D and E, respectively), with interest on *Staphylococcus* spp. in both cases: *S. haemolyticus* for MUP (R threshold at 256 µg/ml) and *S. hominis*, *S. epidermitis*, *S. cohnii* and *S. haemolyticus* for AZM (R threshold at 2 µg/ml). Moreover, both *S. cohnii* isolates displayed MIC values above the breakpoint established for *Staphylococcus* spp. for CD (Figure V.7F).

As for the strains with no registry of their resistances, their MIC values were diverse. AMC median MIC values ranged from 3 to 0.01, with the highest value at 6 µg/ml. DXT values ranged from 1.5 to 0.016, with the highest point on 4 µg/ml. GEN values varied from 2 to 0.016, being 3 µg/ml the top value. MUP values were from 1536, the top score, to 0.064 µg/ml. AZM values ranged from 384 (highest value) to 0.01 µg/ml, and CD values were from 384 (highest score) to 0.01 µg/ml.

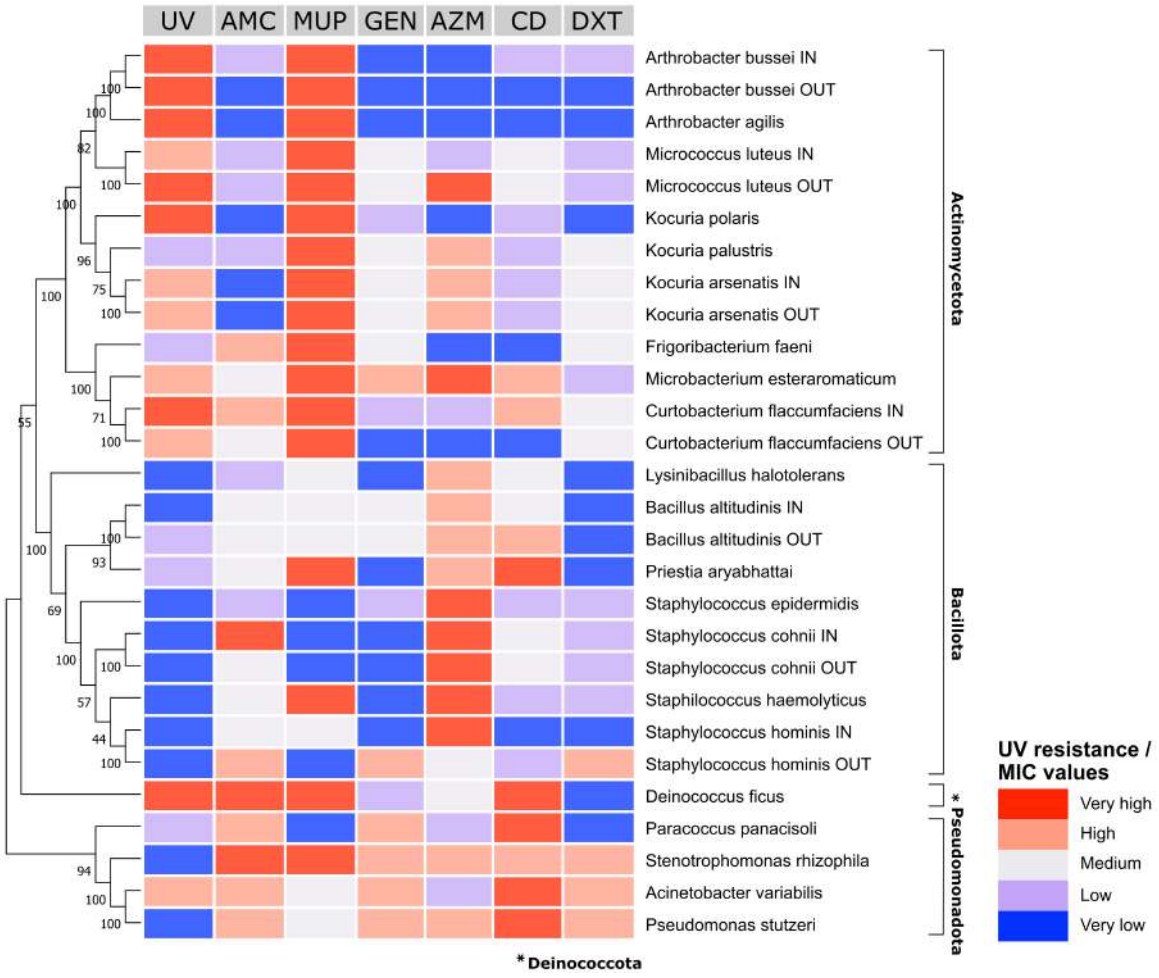

Figure V.8. Heatmap assessing correlating the phylogenetic distance of bacterial isolates and the results of biological tests (15 s of UV irradiation and antibiotic MIC tests). UV-resistance is divided in to five categories based on non-overlapping significance groups from figure V.6. MIC values are divided into five categories based on non-overlapping significance groups from figure V.7. Figures and significance groups [very high - high - medium - low - very low]: UV Fig V.6B [ab - e - XX - h - kl], AMC Fig V.7A [a - de - ghij - l - n], DXT Fig V.7B [ab - XX - c - XX - fh], GEN Fig V.7C [a - e - hi - k - mn], MUP Fig V.7D [ab - cde - g - i - kl], AZM Fig V.7E [ab - d - XX - f - hk], CD Fig V.7F [ab - e - XX - h - kl]. XX depicts non-used classification level.

Phylogenetic interpretation

From the phylogenetic perspective, there was found out a clear tendency on *Actinomycetota* to resist UV. In contrast, species within the phylum *Bacillota* appeared as the most sensitive to this treatment. Regarding antibiotics, as stated above, there was not a significant enrichment on resistant isolates. However, MIC values were diverse. Specifically, there is interest on the resistances displayed by *staphylococci* to DXT, GEN, MUP, AZM and CD. Although the displayed values may appear as high, the available literature on clinical specimens did not catalogue most of them as resistant.

Discussion

In the present work, we analyzed the microbiomes associated to the inner and external surfaces of UV cabins used in the Dermatology Service of the Hospital General de València to treat skin pathologies. First, we wanted to shed light on whether the UV irradiation shaped the microbial communities of the cabins. Second, we wanted to explore the possible correlation between UV light-resistance and resistance to antibiotics commonly used to treat skin pathologies. For this, we used a double strategy based on culture-dependent (culturomics, colony identification and biological activity tests) and independent techniques (high-throughput 16S rRNA gene sequencing).

From the culturomics point of view, the microbial profiles we found are moderately diverse and include both environmental and human associated microbial taxa (Figure V.1A). From the most abundant taxa to the least, the high abundances of *Staphylococcus*, and to a lesser extent of *Micrococcus* and *Bacillus*, are not surprising since these genera are naturally present on the human skin [33-34]. Moreover, *Kocuria* and *Pseudomonas* have also been associated with skin disorders, such as psoriasis, and some *Arthrobacter* species are opportunistic human pathogens, such as *A. creatinolyticus* or *A. woluwensis*, or have been isolated from human clinical specimens [35-38].

However, both *Kocuria* and *Arthrobacter* species inhabit soils, being, thus, common environmental species [2], and the large genus *Bacillus* not only includes pathogenic species such as *B. cereus* or *B. anthracis*, but is also a typical environmental species in different natural habitats [39-40]. Some of these genera are also known by their tolerance to biotic and abiotic stressors. Specifically, *Micrococcus* spp. and *Kocuria* spp. have been isolated from polar environments and reported to be resistant to radiation [41-44], whereas *Arthrobacter* spp. are present in hot deserts [2, 45-46]. Moreover, *Bacillus* spp. are well known by their tolerance to stress given their ability to form resistance spores [47].

From the clinical point of view, we found relevant to analyze the presence of some health-threatening genera, such as *Acinetobacter* or *Pseudomonas*. Both genera have been described to have an innate adaptation ability, including the acquisition of antibiotic resistances [48-50]. Among them, *A. baumanii* and *P. aeruginosa* strains fall into the ESKAPE group of multi-drug resistant bacteria [51-52]. Moreover, other present genera such as *Rhodococcus*, *Roseomonas* or *Stenotrophomonas* host species that cause infection to immunocompromised patients, and *Cryptococcus* spp. have been reported to cause opportunistic infections [52-56].

Apart from the above-described ones, there is also a less abundant representation of some environmental-associated taxa, many of which have been isolated from varied environments such as *Deinococcus*, *Domibacillus*, *Pantoea* or *Sphingomonas* [57-60]. Others have been linked to isolated cases of fungaemia, bacteremia or sepsis, such as *Aureobasidium*, *Kosakonia*, *Lysinibacillus* or *Massilia* [61-64]. However, *Massilia* is also a common soil-inhabitant [65-66]. Regarding fungi, the ones we isolated in pure culture belonged to the genera *Aureobasidium* (five isolates), *Ustilago*, *Cystobasidium*, *Rhodotorula* and *Cryptococcus* (the last four represented by just one isolate). Despite the low number of fungal isolates selected, the fact that the yeast genera *Aureobasidium*, *Rhodotorula* and *Cryptococcus* had previously been reported to inhabit different hospital facilities is in accordance with our results [67].

The comparison of the isolation surface (inside or outside the cabins) revealed similar microbiomes. However, the existence of a cluster of exclusive genera in each location reveals some differences from the culturable point of view. The dominance of *Staphylococcus* in both isolation sources is in accordance with its widely known role in skin pathology [68]. In contrast, *Pseudomonas*, a sensitive genus, is only detected outside [69]. Curiously, all six isolates identified as *Frigoribacterium* spp. were isolated from the inner surfaces of the cabins (Figure V.1A). This genus was first described as a psychrophilic genus isolated from dust in a cattle barn in Finland [70].

As revealed by high-throughput 16S rRNA gene sequencing, the all the cabins and locations display similar taxonomic profiles in terms of α-diversity. However, in terms β-diversity some differences are observed at the genus level (Figures V.3A and B). The fact that cabin one (working in UVA) and cabin two (working in UVB) are the most similar ones suggests that UV does not have a significant effect in shaping the microbial biocenosis in our studies (Figure V.3B).

At the phylum level, the predominant phyla found (*Pseudomonadota*, *Actinomycetota* and *Bacillota*) are in accordance with the already described profiles found in hospitals by other authors. Moreover, the comparison of the microbial profiles found in the samples at the genus level with those of hospitals is also in accordance with previous studies, particularly due to the presence of *Staphylococcus*, *Pseudomonas*, *Acinetobacter* and *Streptococcus* (Figures V.4A and B) [27, 71]. Interestingly, the presence of the genera *Rubellimicrobium*, *Deinococcus*, *Bacillus*, *Hymenobacter* and *Sphingomonas* is in line with the already described microbial communities living on solar panels [3]. This may suggest that the studied microbiomes are a combination of both highly-irradiated surfaces, such as solar panels, and hospital environments. Although, at the genus level, there are differences between both cabins and locations (Figure V.4B), the most relevant genera according to their environmental or clinical interest do not show significant differences in their distribution, with the exception of *Staphylococcus* (Figure V.5C).

Finally, the genera *Rubellimicrobium*, *Paracoccus* and *Corynebacterium* are among the most abundant genera through NGS whereas they are completely absent in the strain collection (Figures V.4B and V.1A, respectively). Biases in culturing techniques are well-known and our results support the importance of combining both culture-dependent and culture-independent techniques in microbial ecology.

We tested the hypothesis that the resistance to UV irradiation of the species isolated inside the cabins would be higher than that of the species isolated outside the cabins. However, our results did not support this statement (Figure V.6). All the strains isolated exclusively from the inner surfaces showed a significant decrease in the survival rate after 15 s or 30 s of UV irradiation, with the only exception being *K. polaris* at 15 s of UV irradiation treatment. Similarly, in the group of isolates obtained exclusively from the outer surfaces there were only two strains whose survival did not decrease significantly after 15 s of UV exposure: *A. agilis* and *D. ficus*. Moreover, in the comparison of species isolated from both the inner and outer surfaces of the cabins, no relevant differences were found out. There was only one outside *M. luteus* isolate displaying higher resistance that the inner strain (Figure V.6). This suggests that, in this case, UV exposure is no causing an adaptive response for bacteria (Figure V.6A).

According to this experiment, the most resistant strains were *A. bussei, A. agilis, K. polaris, D. ficus* and *M. luteus* (Figures V.6B and C). Some *Kocuria* strains have been reported as highly resistant to different types of radiation, as well as to synthesize carotenoids and encode genes related to oxidative stress [41, 44, 72-74]. Moreover, *Deinococcus* spp. have been extensively studied for its high resistance to radiation, which is a result of a combination of mechanisms such as robust DNA repair systems regulatory proteins, enzymatic and non-enzymatic antioxidant strategies [74-75]. On its part, the genus *Arthrobacter* hosts several multi-resistant species to different abiotic stressors [76-77]. Specifically, *A. agilis* has been

reported to produce the C50 carotenoid bacterioruberin [78]. Finally, *Micrococcus* spp. have DNA repair mechanisms fundamental on their resistance to UV light [79-80], and *M. luteus* strains have been reported to resist high doses of gamma radiation [42]. These taxa are, thus, highly resistant to radiation and other stresses, and naturally inhabit soil and desert-like environments [2-3].

Even though for most of the strains the survival rate after UV treatment was significantly reduced compared to the non-irradiated control, many of them showed mid-viability after 15 s of treatment. This is the case of *A. variabilis* and *M. esteraromaticum*, isolated from the inner surfaces of the cabins, and *A. bussei, C. flaccumfaciens* and *K. arsenatis*, isolated both from the inside and the outside. Some *Acinetobacter* species have demonstrated to be able to cope with oxidative stress [81-82]. Moreover, the genus *Microbacterium* has extensive background on UV resistance and carotenoid synthesis as well [83-85]. In contrast, less has been described about *Curtobacterium* spp., but still there are reports on their tolerance to UV [86].

The reasons for the absence of an enrichment on resistant species inside the cabins may be varied (Figures V.6A and B). On the one hand, both surfaces are accessible to patients, which may be in contact constantly with both surfaces. This would explain also the high similarities found in terms of diversity. Moreover, the stress to which they are subjected (short pulses of UV light) may be less intense than the stress tested under laboratory conditions. From this perspective, the species tested may not represent a threaten.

As stated in the introduction, hospitals and sanitary environments increase the population of multidrug resistant pathogens. Considering that the surface of UV-cabins is constantly in contact with patients with skin pathologies, who may be in complementary treatments with antibiotics, and that the use of radiation may favor the selection of antibiotic resistant bacteria [30], we hypothesized that the isolates taken from the inside and the outside of these cabins may present an altered susceptibility to antibiotics. In this regard, we assessed the antibiotic resistance of the isolates from Table V.S2 to six antibiotics widely used in dermatology: AMC, DXT, GEN, MUP, AZM and CD (Figure V.7). The classification of the strains as sensitive or resistant has been done according to the European Committee on Antimicrobial Susceptibility (EUCAST) as expressed in the instructions of the MTS.

The presence of some resistant strains is confirmed, with special interest on the genus *Staphylococcus*. In this regard, there were found isolates resistant to DXT and GEN (*S. hominis* isolated from outside), MUP (*S. haemolyticus*), AZM (both *S. cohnii* isolates, *S. haemolyticus* and *S. hominis* from inside) and CD (both *S. cohnii*). The resistance to MUP is remarkable, as this antibiotic is specifically used in the treatment of topic dermal infections by Gram-positive cocci, which also explains the resistance values displayed by most of the tested strains (which are not Gram-positive cocci). Moreover, the resistance of *S. haemolyticus* to MUP has been already reported and is mediated by the gene *mupA* [87], and the rest of the mentioned staphylococci have reports on their multi-resistances [88-90]. There were some differences between strains isolated from different surfaces belonging to the same species, such as the case of *S. hominis*: the inside isolate is resistant to AZM whereas the outside one is resistant to DXT and GEN. As for *S. rhizophila*, our strain showed resistance to AMC, and there are reports on their multi-resistance to many antibiotics [91]. Regarding the strains for which a breakpoint has not been established, no susceptibilities can be assigned.

Taken together, the results obtained from both resistance assays (UV and antibiotics) reveal that most of microorganisms are not resistant to the antibiotics tested. Those that are, which are the staphylococci, do not show UV-resistance. The rest of the isolates we tested lack clinical interest as they are not common human pathogens. Therefore, the microbial community of the cabins is mainly composed of antibiotic-sensitive microorganisms which display a diverse sensitivity to UV light, and a few potential pathogenic microorganisms that are sensitive to UV

light (and that should thus be eliminated easily with UV-based sterilization devices). Moreover, the lack of reports of infections associated with the cabins supports a lack of substantial threat in their microbial content. However, the combination of the presence in the cabins of some microbial pathogens and the presence of antibiotic resistant genes poses an obvious potential problem linked to horizontal gene transfer (Figure V.8).

Materials and Methods

Sample collection

Dust samples from the inner and the outer surfaces of four UVA and UVB cabins were taken in June 2021, in the Dermatology Service of the Hospital General de València, Spain. The cabins sampled were: 1) PUVA 700 Waldmann (Villingen-Schwenningen, Germany), 2) UV7001K UVA/UVB Waldmann (Villingen-Schwenningen, Germany), 3) and 4) UV7002 UVA/UVB Waldmann (Villingen-Schwenningen, Germany). Samples (in duplicate or triplicate) were obtained by scrubbing the surface with a sterile swab (FLOQSwabs™ hDNA Free, Copan Flock Technologies SRL, Brescia, Italy) and immediately stored in sterile tubes with 500 µL of Phosphate Buffer Saline (PBS) 1X until processed in the laboratory.

Isolation of microbial strains

Samples were thoroughly shaken with vortex. As most samples were very clear, suggesting a low microbial load, 20 µL of the direct suspensions were spread on Petri dishes in duplicate, on five different culture media: TSA (composition in g/L: 15.0 tryptone, 5.0 soya peptone, 5.0 sodium chloride, 15.0 agar), Nutrient Agar (composition in g/L: peptone 5.0, meat extract 3.0, agar 15.0), Columbia Blood (catalogue number: CM0331B, ThermoFisher Scientific Inc., Massachusetts, USA), R2A (composition in g/L: 1 peptone, 0.5 yeast extract, 0.5 dextrose, 0.5 soluble starch, 0.3 dipotassium phosphate, 0.05 magnesium phosphate, 0.3 sodium pyruvate, 15.0 agar) and Yeast Mold (composition in g/L: malt extract 3.0, yeast extract 3.0, dextrose/glucose 10.0, peptone soybean 5.0, agar 15.0). Samples were incubated at 25 and 37 °C for one week. Colonies were then selected according to morphological traits, such as color or shape, and isolated independently by re-streaking on fresh media. When pure cultures were obtained, strains were cryopreserved as glycerol stocks (12 % glycerol in their isolation media) at -80 °C until required.

Colony identification (16S rRNA/ITS gene sequencing)

Microbial biomass from grown plates of pure cultures was suspended in 100 µL of Milli-Q sterile water. Cells were lysed by heat shock in two cycles of boiling-freezing steps. PCR was carried out for the taxonomic identification through 16S rRNA gene sequencing. Colony PCR and amplicon precipitation were carried out following the procedures previously described by Molina-Menor *et al*. (2021) [2]. Sequencing was performed with Sanger by Eurofins Genomics (Ebersberg, Germany). Trev tool (Staden Package, 2002) was used to manually edit 16S rRNA sequences in order to eliminate low-quality base calls. Sequences where then compared by EzBioCloud 16S rRNA BLAST tool to nucleotide databases. The sequences have been deposited under the GenBank/EMBL/DDBJ accession numbers OQ221901-OQ222055 (bacterial sequences) and OQ208835-OQ208843 (fungal sequences). Redundancy of the isolates was checked among the ones with the same identification that had been isolated from the same sample and media by Blast to Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The identifications are listed in Table V.S1.

High-throughput 16S rRNA gene sequencing (metataxonomics)

Total DNA extraction was carried out with the DNease PowerSoil kit (MO BIO laboratories, Carlsbad, CA, USA). In order to consider potential microbial contamination of the reagents used, two negative controls consisting of pure water, instead of samples, and processed with the same kit were included. Given that very low DNA concentrations were obtained, the amplification of the 16S rRNA gene through PCR was carried out following the protocol described by Molina-Menor *et al*. (2021) [2]. For those samples failing to be amplified with 16S rRNA gene primers, a

PCR with ITS region primers ITS3 and ITS4 [92] was carried out under the following conditions: initial step at 95 °C for 5 min, followed by 30 cycles of 30 s at 94 °C for denaturation, 30 s at 53 °C for annealing and 30 s at 72 °C for elongation, and a final step of elongation of 5 min at 72 °C. Samples with a clear amplification band in the electrophoresis gel were selected for sequencing, ensuring the representation of all four cabins and sampling points (inside and outside). The samples selected consisted of: three samples from inside and two from outside cabin one, two samples from inside and two from outside cabin two, one sample from inside and one sample from outside cabin three, and one sample from inside and one sample from outside cabin four. Both sequencing (Illumina) and the bioinformatic analysis were carried out by Darwin Bioprospecting Excellence SL (Paterna, Spain). Rarefaction curves were plotted to check the sequencing depth (Figure V.S2).

UV-radiation resistance assay

Based on the microbial identifications, a subset of isolates was selected from the collection to further perform UV irradiation and antibiotic resistance assays (from now on, sub-collection). Isolates from the most represented taxa were selected considering to have (1) a wide diversity of genera tested for each surface and (2) species that were isolated both from the inside and outside of the cabins (Table V.S2).

Liquid cell cultures of selected isolates were serially diluted in PBS to an OD_{600} of $10^{-4}/10^{-5}$ in order to obtain isolated colonies after inoculation on R2A agar plates. Aliquots of 100 µl of the cultures were plated in triplicate. Two replicates were then exposed to UVC light for 15 s or 30 s, while the other replicate was the non-irradiated control. UVC irradiation was performed with a VILBER LOURMAT UV lamp emitting 354 nm light with an intensity of 340 µW/cm^2 at 15 cm of distance. Plates were incubated at 30 °C and colonies were counted 24 to 72 h post inoculation depending on each strain. Number of colonies in the irradiated plates were compared to the number of colonies in the control non-irradiated plates.

Antibiotic resistance assay

Minimum Inhibitory Concentration (MIC) tests were carried out with the commercial MIC Test Strip (MTS) by Liofilchem SRL (Roseto degli Abruzzi (TE), Italy). Amoxicillin*-clavulanic acid (2/1) (ref: 920240, 0.016-256 mg/L), azithromycin (ref: 920300, 0.016-256 mg/L), clindamycin (ref: 920720, 0.016-256 mg/L), doxycycline (ref: 921560, 0.016-256 mg/L), gentamicin (ref: 920090, 0.016-256 mg/L) and mupirocin (ref: 920380, 0.064-1024 mg/L) were selected among the antibiotics that are commonly used in the Dermatology Service of the Hospital General de València (AMC: amoxicillin-clavulanic acid; AZM: azithromycin; CD: clindamycin; DXT: doxycycline; GEN: gentamycin; MUP: mupirocin). Growth on Mueller Hinton-agar media was tested prior to the assay in order to ensure that all the strains were able to grow on it. The experiment was carried out following the manufacturer's instructions, using OD_{600} instead of McFarland turbidity standards to assess cell concentration. For this, PBS cell-resuspensions for each strain were diluted to OD_{600} 0.1, 0.5 and 1, and 100 µl were plated on Mueller Hinton agar plates. We selected the dilution for inoculation for each strain based on confluent cell-growth after 24, 48 and 72 h incubation at 30 °C. MIC was registered 24 h, 48 h and 72 h for each sample. The quality check strains were *Staphylococcus aureus* (ATCC 29213 – WDCM 00131) for AZM, MUP and CD; and *Escherichia coli (*ATCC 25922 – WDCM 00013) for DXT, AMC and GEN. The strains were purchased from DSMZ (German Collection of Microorganisms and Cell Cultures, Leibniz Institute, Braunschweig, Germany). The MICs for control strains showed minor changes throughout the three days that lasted each replicate, but they were always inside the expected range as referred by the manufacturer. The results were interpreted following the manufacturer's instructions, according to the European Committee on Antimicrobial Susceptibility testing (EUCAST) (EUCAST Clinical Breakpoint Tables v.12.0).

Phylogenetic tree

The 16S rRNA gene sequences of the type strains of the sub-collection (Table V.S2) were retrieved from EzBioCloud (www.ezbiocloud.net). Phylogenetic

analysis was carried out with MEGA11 software (v.11.0.13). Sequences were aligned with MUSCLE algorithm and the phylogenetic tree was constructed using the Neighbour Joining method [93]. The branch pattern reliability was checked with bootstrap analysis based on 100 replicates with nucleotide p-distance substitution model including transitions and transversion.

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Author contributions

E.M.M., A.P.F, G.P.P and M.P. conceived the work. E.M.M., N.C. and À.V.V. carried out the experimental procedures. E.M.M., N.C, À.V.V, A.P.F, G.P. and M.P. analyzed the results, wrote and approved the manuscript. All the authors agree to publish the manuscript.

Data availability statement

The 16S rRNA gene sequences have been deposited under the GenBank/EMBL/DDBJ accession numbers OQ221901-OQ222055 (bacterial sequences) and OQ208835-OQ208843 (fungal sequences).

Additional information

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Supplementary material

Supplementary material may be found in Appendix B.

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Chapter 4: Applications of light and drought-adapted microorganisms

Publication VI: Molina-Menor, E., Vidal-Verdú, À., Gomis-Olcina, C., Peretó, J. and Porcar, M. (2023). A 3D printed plastic frame deeply impacts yeast cell growth. *bioRxiv,* 2023.06.16.545257.

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Publication VI

A 3D printed plastic frame deeply impacts yeast cell growth

Esther Molina-Menor[1], Àngela Vidal-Verdú[1], Carlos Gomis-Olcina[1], Juli Peretó [1,2,3] and Manuel Porcar[1,3*]

1. Institute for Integrative Systems Biology I2SysBio, Universitat de València-CSIC, Paterna 46980, Spain.
2. Departament de Bioquimica i Biologia Molecular, Universitat de València, Burjassot 46100, Spain.
3. Darwin Bioprospecting Excellence S.L. Parc Científic Universitat de València, Paterna 46980, Spain.

Summary

Solid State Fermentation (SSF) processes have been explored for yeast growth and protein and metabolites production. However, most of these processes lack standardization. In this work, we present a polylactic acid (PLA) 3D printed matrix that dramatically enhances yeast growth when embedded in liquid media compared to equivalent static cultures, and changes yeast expression patterns at the proteome level. Moreover, differences in sugar assimilation and ethanol production, as the main product of alcoholic fermentation, are observed. Our results suggest that these matrixes may be useful for a vast range of biotechnological applications based on yeast fermentation.

This work was uploaded to biorXiv and is under review in

Frontiers in Bioengineering and Biotechnology since June 2023

https://www.biorxiv.org/content/10.1101/2023.06.16.545257v1

Introduction

Microorganism-culturing techniques have drastically evolved in the last years. Specifically, since the development of high-throughput DNA sequencing technologies, several groups have worked in the improvement of culturing conditions in order to increase the number of microbial species grown in labs and to improve their growth (Overmann *et al*., 2017).

Industrial microorganisms are mainly grown in planktonic, continuous (chemostat) or batch cultures (Carsanba *et al*., 2021; Jiao *et al*., 2022). In particular, yeast industrial production can be approached through different bioreactor configurations, but all of them usually in liquid or submerged fermentation (SmF). On one hand, batch culturing is the simplest and cheapest strategy, not only from the economic point of view but also considering the workload. In batch culturing, the culture is prepared once, simplifying sterilization and stock preparation. However, the main disadvantage is growth inhibition due to the accumulation of metabolic products, some of which are toxic (Carsanba *et al*., 2021). On the other hand, continuous cultures avoid this limitation by uninterrupted feeding and removing of products. It allows monitoring and comparing in real time different parameters, but it is not always a feasible strategy as some products may require the culture to reach stationary phase to be synthesized (Carsanba *et al*., 2021). In between there can be found fed-batch culturing, the most widely used strategy, in which the effect of growth inhibition is lower than in conventional batch (Carsanba *et al*., 2021).

Mimicking the environmental conditions that microbes find in their natural habitats is one of the aims of culturomics, not only for cultivating more species but also to improve their growth in terms of production performance, and also in terms of energy and economic savings. For example, some authors studied the effects on cell immobilization over the conventional batch mode in the synthesis of specific compounds (citronellol) and found a lower subproduct production (Esmaeili *et al*., 2012), improving, thus, the recovering of the product. For their part, Raghavendran *et al*. (2020) developed a system to reduce the cost of aeration of batch yeast cultures by using a fluidic oscillator that generated microbubbles. Moreover, some authors have worked on the growth of bacteria on solid surfaces in order to enable biofilm formation, as some bacterial species require this for optimal growth (Gich *et al*., 2012).

Solid-State Fermentation (SSF) has been explored and partially developed as a culture technique that improves oxygen transfer, avoids foaming in cultures and mimics the natural growth conditions of some microorganisms, such as filamentous fungi, yeast or biofilm-forming bacteria (Hölker and Lenz, 2005; Lima-Pérez *et al*., 2019). This microbial-growth alternative is based on the fermentation of microorganisms on an organic solid moist surface, such as agricultural waste, or inert solid surfaces like polyurethane foam (PUF) to which nutrients are added. Although there are some studies on the promising role of SSF in the biosynthesis of specific compounds and the advantages that it holds over SmF, at industrial scale, the growth of microorganisms to produce either biomass or secondary metabolites is still mainly SmF (López-Pérez and Viniegra-González, 2016; Singhania *et al*., 2009; Valdés-Velasco *et al*., 2022).

In the line of this, here we report an attempt to standardize yeast growth on solid matrixes with an unprecedented approach. Based on a preliminary test on sand, cotton and sponges, we describe a culturing strategy consisting on geometrical 3D solid tough polylactic acid (PLA) matrixes embedded in liquid media for sourdough yeast *Saccharomyces cerevisiae* production and analyze how culture conditions modulate their growth and their proteomes.

Materials and Methods

3D-matrixes design

3D-printed cylinders were designed by Marker Station 3D (València, Spain). The design consisted in a cylindric spiral with regular holes (1X scale: 23.7 x 24.4 x 94.8 mm). The designs were printed in black tough polylactic acid (PLA) in an Ultimaker S3 3D printer (Figure VI.1).

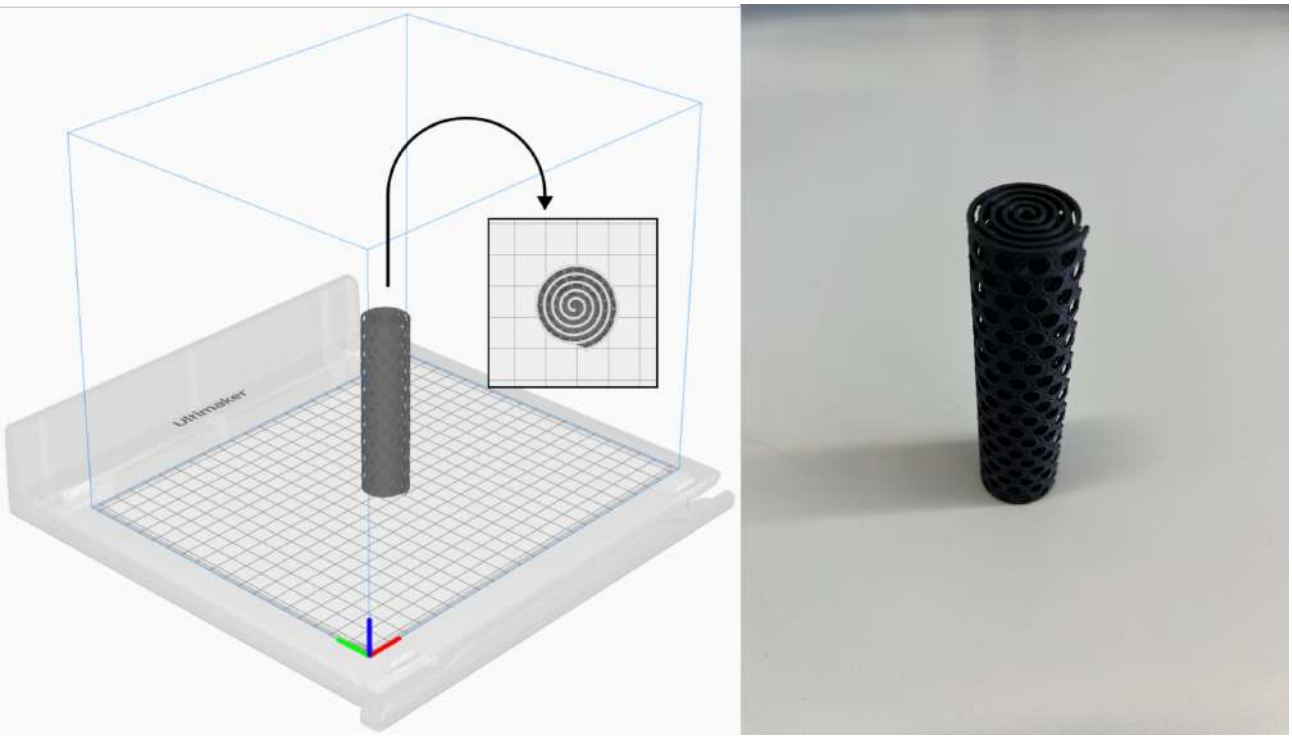

Figure VI.1. 3D printed PLA matrix design.

SSF experiment

Yeast precultures were grown overnight in YPD medium (in g/L: 10 yeast extract, 20 peptone, 20 dextrose) at 30 °C. Cultures were adjusted to a final optical density of 600 nm (OD_{600}) of 0.1 in YPD. SSF matrixes were submerged in 1/10 (v:v) bleach in order to disinfect them for 20-30 min. Then, they were thoroughly washed in sterile distilled water in three cleaning steps. The SSF matrixes were placed in 50 mL tubes, and 25 mL of the adjusted yeast culture was poured into them. The growth controls consisted of 25 mL of culture in 100 mL flasks for agitated/shaking conditions (SK) (180 rpm) and 25 mL of culture in 50 mL tubes for static conditions (ST). The experiments were carried out in triplicate (biological replicates). A tube without yeast was used for sterility control. All tubes were incubated overnight at 30 °C and centrifuged in 50 mL tubes for 5 min at 4500 rpm. After discarding the supernatant, cells were resuspended in sterile PBS for cleaning. A centrifuging step was repeated under the same conditions. After discarding the supernatant, cells were finally resuspended in sterile PBS at a final volume of 25 mL. OD_{600} was, then, measured. One-way ANOVA followed by Tukey's multiple comparisons test was performed using GraphPad Prism version 9.0.0 for macOS, GraphPad Software, San Diego, California USA, www.graphpad.com.

Metabolite measurement

D-glucose and ethanol content were measured in overnight culture supernatants after centrifuging the cultures for 10 min at 5000 rpm in a Y15 Automated Analyzer (BioSystems, Barcelona, Spain). One-way ANOVA followed by Tukey's multiple comparisons test was performed using GraphPad Prism version 9.0.0 for macOS, GraphPad Software, San Diego, California USA, www.graphpad.com.

Proteomics

One experiment (in triplicate) was carried out for proteome analysis. After collecting and cleaning the cells, pellets were directly frozen and stored at -80°C until required. Proteomic analysis was carried out by the Central Service for Experimental Research (SCSIE, Universitat de València). Proteins were separated by 1D SDS PAGE electrophoresis and identified through LC-MSMS. Differential proteomic analysis was carried out through SWATH. Proteomic analysis was done by Darwin Bioprospecting Excellence SL (Paterna, València).

Results

SSF experiment

Preliminary assays of SSF approaches using solid substrates such as sand, cotton and sponges revealed enhanced yeast growth (data not shown) compared to traditional SmF cultures. Therefore, an experiment with standardized 3D matrixes was designed. Growing yeast in alternative culture approaches (liquid shaken cultures-SK, liquid static cultures-ST and 3D-SSF cultures) resulted in significantly different cell yields measured by OD (Figure VI.2A) (p-values<0.05). Specifically, the highest biomass was obtained through conventional SK cultures, followed by 3D-SSF and ST culturing. Although 3D-SSF culturing did not result in similar cell-yields compared to SK, it significantly enhanced growth compared to the equivalent submerged culture without agitation (ST).

In order to determine whether the observed increased biomass may result from increased oxygen through embedded bubbles inside 3D matrixes, an experiment was carried out in which tubes with 3D matrixes were subjected agitation prior to incubation or hit in order to remove air bubbles. However, no significant differences were found among these three conditions and the rest of the controls displayed values accordingly to the previous experiments (p-values>0.05) (Figure VI.2B).

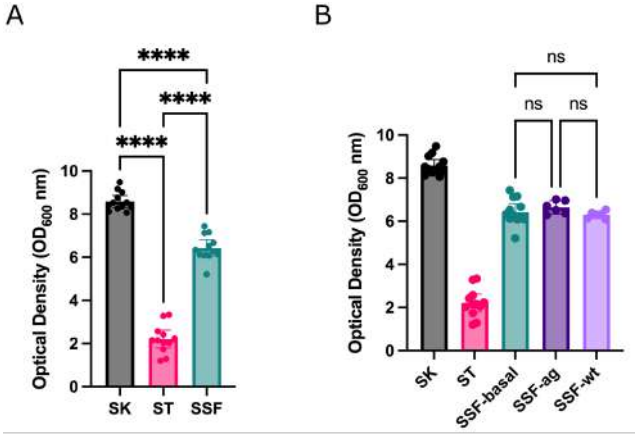

Figure VI.2. Yeast growth (OD_{600} nm) inside 3D matrixes. Shaking and static controls are included. Confidence interval of twelve replicates (biological replicates) is represented in the error bar. (A) SSF experiment. (B) SSF experiment in different configurations: with the PLA matrix and static conditions (3D basal), agitated to increase bubbles or hit to remove any air bubble (without bubbles; wt). Shaking and static controls are included.

Proteomics

Proteomic analysis was carried out in order to determine whether the observed enhanced growth in 3D matrixes was accompanied by differences in expression at the proteome level. The three conditions (agitated/shaking control (A), static control (E) and 3D-SSF matrix (N)) were analyzed in triplicate. Significant differences were found at the protein profile level as revealed in the PCA, in which there can be observed a clear separation of samples among conditions, as well as grouping of the biological replicates (Figure VI.3). Moreover, the number of differentially expressed proteins in the comparison of 3D-SSF with SK and ST controls was higher in the former than in the latter, suggesting that in terms of protein expression 3D-SSF culturing resembled more ST than SK fermentation.

With the purpose of determining the differences at the proteome level underlying this phenomenon, an analysis of differential expression of all the identified proteins was carried out. The proteins that were significantly over or under-represented in N-SSF samples with respect to A-shaking and E-static were identified prior to perform an enrichment analysis with STRING (https://string-db.org/), which is based on a protein-protein interaction database. This analysis allowed the identification of the metabolic pathways (KEGG pathways, https://www.kegg.jp/) that outstood in each of the groups with respect to the others. We hypothesized that metabolic routes involved in processes such as alcoholic fermentation and oxidative stress may display different expression patterns among conditions. Thus, a specific research and analysis on genes within these routes were performed (Figure VI.3B,C).

The analysis of proteins in SK and ST conditions compared to 3D-SSF revealed some patterns and significantly different expression levels. Regarding alcoholic fermentation proteins, the detected pyruvate decarboxylases (PDC1 and PDC5) were significantly more expressed in SK, while there were

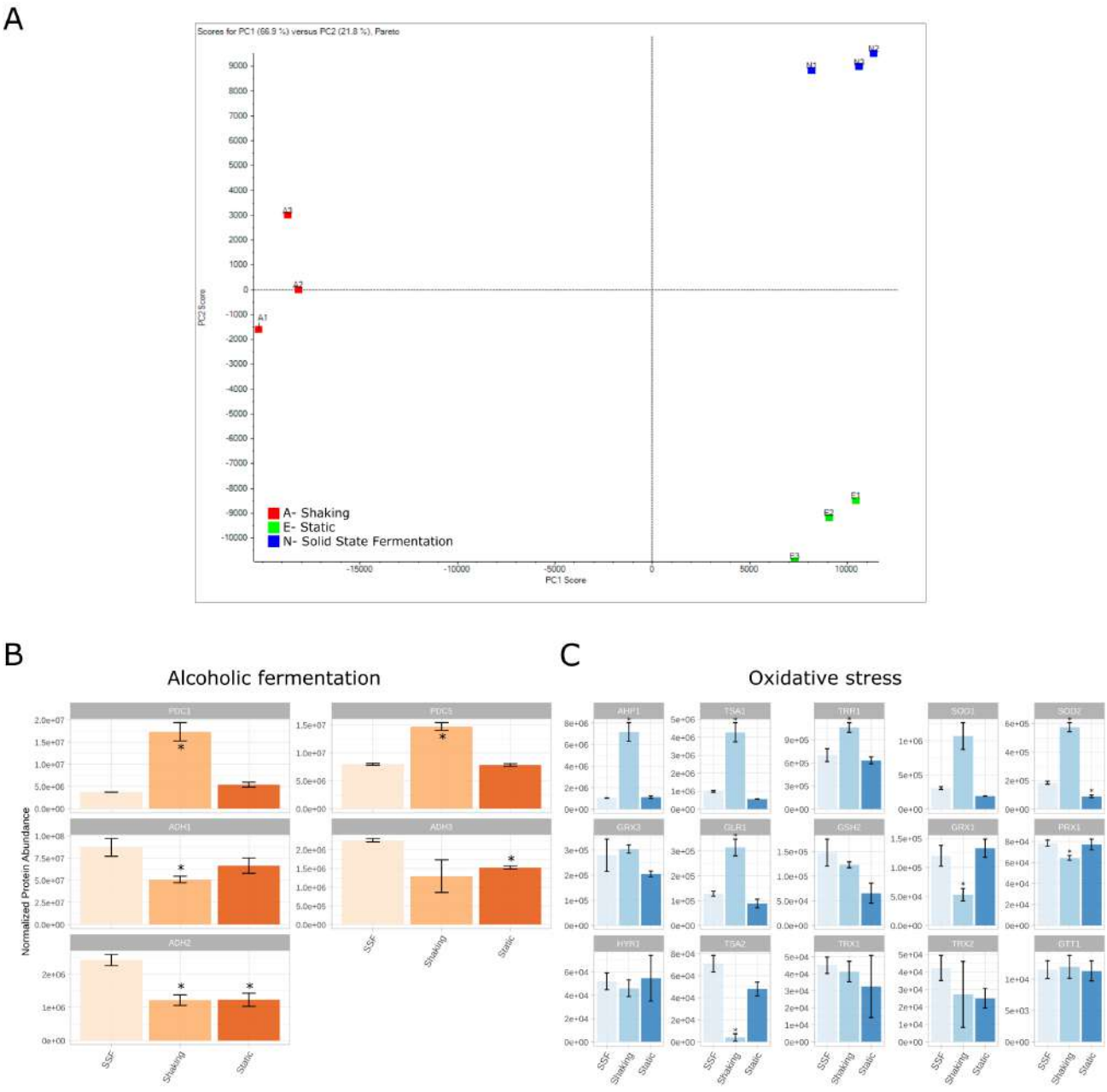

Figure VI.3. Proteomic analysis. (A) PCA showing the differential proteomic analysis (SWATH) of yeast cultures grown in the three conditions tested: agitated/shaking control (A), static control (E) and 3D matrixes (N). (B) Alcoholic fermentation protein expression levels. (C) Oxidative stress protein expression levels. * represents differential expression compared to SSF conditions.

no differences for ST. PDC6 was not detected. In contrast, alcohol dehydrogenases (ADH1, ADH2 and ADH3) were in general terms overexpressed in 3D-SSF cultures with respect to SK conditions (Figure VI.3B).

Regarding proteins involved in oxidative stress, superoxide dismutases (SOD1 and SOD2), thioredoxin peroxidases (TSA1 and AHP1), glutathione reductase (GLR1) and thioredoxin reductase (TRR1) were overexpressed in SK cultures, in contrast to thioredoxin peroxidases TSA2 and PRX1, which were underexpressed in SK cultures (Figure VI.3C). SOD2 was also underexpressed in ST cultures, whereas the rest of the studied proteins (glutathione peroxidase (HYR1), glutathione transferase (GTT1), glutaredoxins (GRX1 and GRX3), glutathione synthase (GSH2) and thioredoxins (TRX1 and TRX2)) did not reveal clear differences among conditions.

Metabolite measurement

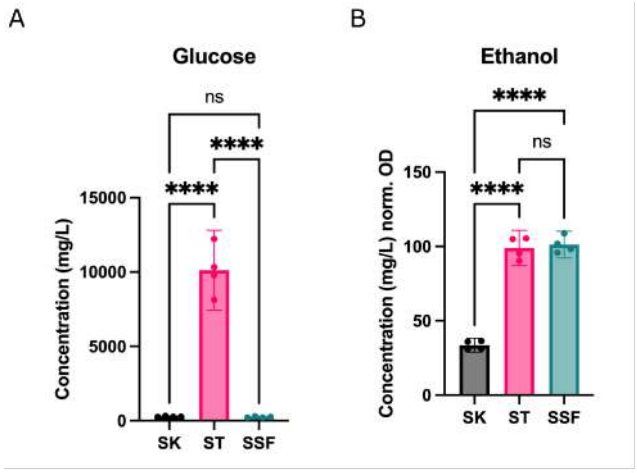

Figure VI.4. Metabolite profiles of cultures. (A) Sugar content (D-glucose) after fermentation (mg/L). (B) Ethanol production during fermentation (mg/L) normalized by cell density (OD_{600} nm).

Given the observed differences found at biomass yield and protein expression levels, we decided to explore the impact of fermentation in the cultures. Thus, D-glucose and ethanol contents were measured in the media after yeast growth and compared to the initial content in YPD medium (Figure VI.4).

YPD media was prepared at 20 g/L of D-glucose (quantified 19.32 g/L after autoclaving, +- 0.03 g/L). D-glucose content was reduced to half in ST cultures (10.12 g/L), indicating poor sugar assimilation, whereas in both SK and SSF remaining sugar content was very low (280 and 245 mg/L, respectively) (Figure VI.4A). However, ethanol yields (normalized by the OD_{600} nm values achieved by each culture) were very similar in both ST and SSF cultures, and higher with respect to SK conditions (Figure VI.4B).

Discussion

In this work we aimed at assessing whether the use of alternative culturing configurations based on standardized SSF inert matrixes may have an impact on yeast cell growth, not only in terms of the achieved cell densities but also in terms of protein expression patterns and metabolism. In the light of the results obtained, we conclude that solid, inert matrixes such as plastic cylinder results in a dramatic increase in yeast biomass production, and that different culturing setups result in different cell yields and also different protein profiles, which may be interesting from the biotechnological point of view and industrial biotechnology.

First, comparing yeast culturing under agitated and non-agitated (static) liquid cultures revealed, as expected, lower cell densities for static cultures. Microbial liquid cultures of aerobic organisms are usually agitated in order to favor the access to oxygen and nutrients, as oxygen poorly diffuses into water (Somerville and Proctor, 2013). Thus, it is expected that cultures incubated under shaking conditions achieve higher biomass yield. However, our results revealed that SSF matrixes embedded in liquid media and incubated under static conditions were able to enhance yeast growth with respect to the equivalent control culture in static (Figure VI.2).

We hypothesized, then, that 3D matrixes may trap oxygen molecules in bubbles that enhanced somehow growth. In fact, the use of inert media such as PUF increases the ratio area/volume favoring air spread and oxygen transfer in SSF (López-Pérez and Viniegra-González, 2016). Moreover, some authors have studied the effect of recipients in gas permeability and their impact on fermentation processes, such as the case of kimchi in onggi vessels, facilitating CO_2 dispersion (Kim and Hu, 2023). However, no differences have been found when trying to, manually, change this condition (Figure VI.2B).

We explored how proteomes were affected by different incubation formats. The analysis of differential expression revealed clearly different protein profiles, and SSF cultures resemble more those of ST rather than SK ones, according to the distribution on the PCA and the number of differentially expressed proteins (Figure VI.3). As we hypothesized that both ST and SSF conditions (thus static ones) may favor alcoholic fermentation over respiratory metabolism, proteins involved in that pathway were specifically analyzed. In this regard, the overexpression pattern found for ADH1, ADH2 and ADH3 under SSF conditions, as well as to some extent under ST conditions, is in line with this hypothesis indicating enhanced fermentation in those cultures. Moreover, the profiles obtained for pyruvate

decarboxylases (PDC1 and PDC5) are also coherent with their role in the cytosolic synthesis of acetyl-CoA required for lysine and lipid biosynthesis, essential processes for cell growth (Flikweert *et al*., 1996; Pronk *et al*., 1996).

Moreover, as a consequence of aerobic metabolism, Reactive Oxygen Species (ROS) and Oxidative Stress (OS) are generated. The analysis of the main proteins involved in the protection against OS and detoxification of ROS (see Herrero *et al*. 2008 for a review), revealed differences among culturing conditions. Specifically, the main defenses against OS associated to the operation of Krebs cycle (SOD1, SOD2, TRR1 and AHP1) were overexpressed under shaking conditions with respect to SSF. This is expected as respiratory metabolism is favored over fermentative pathways in the presence of oxygen. Moreover, the expression patterns found for TSA1 and TSA2 peroxiredoxins are also consistent with the literature. Both peroxiredoxins are homologue proteins that can complement one to the other, but TSA1 basal expression levels are significantly higher than those of TSA2 (Wong *et al*., 2002). In the case of SK conditions, TSA1 is enough to maintain its function, but in SSF and ST a complementation between TSA1 and TSA2 is observed (Figure VI.3C).

We also assessed sugar consumption and ethanol production (Figure VI.4), as D-glucose consumption is a common parameter measured to estimate the efficiency of SSF processes. For example, Christen *et al*. (1993) evidenced enhanced growth and total D-glucose consumption of *Candida utilis* grown on sugarcane. We expected higher use of sugars in agitated cultures given the higher OD observed, as well as higher ethanol biosynthesis in both static and SSF. D-glucose was almost completely used by yeast in shaking and SSF cultures (more than 99%), but around 50 % remained in static tubes (Figure VI.4A). However, ethanol yields normalized by OD in static and SSF were equivalent (Figure VI.4B). As this is unexpected, we hypothesize that under conditions of sugar depletion yeast may have started to consume ethanol, which could be supported by ADH2 overexpression, as it converts ethanol to acetaldehyde once preferable carbon sources are scarce and metabolism switches towards assimilation of nonfermentable carbon sources (Figure VI.3B).

As reviewed by López-Pérez and Viniegra-González (2016), SSF culturing strategies have been used for the production of proteins and metabolites by different yeast species, such as *Pichia pastoris*, *Kluyveromyces marxianus*, *K. lactis*, *S. cerevisiae* or some *Candida* spp., among others. The products obtained include proteins such as lipases and lytic enzymes, but also ethanol and aromatic compounds. Valdés-Velasco *et al*. (2022) described differences in the biosynthesis of lipopeptide biosurfactant in *Bacillus* strains being cultured under SmF or SSF, revealing that the use of PUF as an inert support for bacteria yielded higher productivity. Moreover, as reviewed by Wang *et al*. (2019) and described by Lahiri *et al*. (2021), incubating bacteria in static or in agitated conditions influences the biosynthesis of cellulose in terms of yield and form (either reticular films on the surface in static or sphere-like particles under shaking conditions) and offers different applications. Moreover, industrial biosynthetic processes may also consider the combination of different fermentation configurations, as Piecha *et al*. (2022) evaluated the production of polyhydroxyalcanoates (PHAs) and concluded that the combination of SSF and SmF would increase the productivity of the process. Altogether, alternative culturing strategies have an impact on the biosynthetic abilities of microorganisms and can be exploited for biotechnological benefit.

Although SmF has advantages for industrial processes, SSF holds potential for sustainable industrial production processes (Carsanba *et al*., 2021; Doriya *et al*., 2016). SSF has demonstrated to allow production with lower water requirement, which is of special interest in yeast as they naturally grow in low water availability environments, but also for bacteria that could adapt to these conditions (Couto and Sanromán, 2006; López-Perez and Viniegra-González, 2015). Moreover, SSF is an opportunity for recycling and revaluation of agricultural waste (Chilakamarry *et al*., 2022). That said, and to the best of our knowledge,

inert, plastic matrixes have not yet been massively implemented to boost yeast biomass production.

Our studies serve as a starting point in the development of 3D structures that may change culture dynamics in terms of achieved biomass and metabolic fluxes for yeast-led industrial processes. Our findings arise some questions regarding the processes involved in the observed differences at biomass and proteome levels, and the metabolic fluxes. Further research is needed in order to elucidate the mechanisms underlying these results. In this regard, not only the development of matrixes with different geometric patterns or materials (e.g., autoclavable materials) would be of interest, but also the exploration of the role of other parameters that allow a deeper comprehension of the process are worthwhile studies. For example, sugar and ethanol quantification at different incubation times would give information about D-glucose consumption rates by each culture, ethanol synthesis rates and, in case of need, ethanol assimilation. Moreover, CO_2 measurements may inform about the fermentation process. Also, investigating proteins related to functions such as quorum sensing or biofilm formation may shed some light on other processes that affect microbial growth.

Finally, many authors have outlined the limitations of SSF in large scale production processes (Chilakamarry *et al*., 2022; Piecha *et al*., 2022). Taken together, the results we show in the present article may be a first step towards a new view of SSF-like fermentation in yeast.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

MP conceived the work. EMM, ÀVV and CGO performed the experiments. All the authors (MP, EMM, ÀVV, CGO and JP) analyzed the results, wrote and approved the manuscript.

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Publication VII

Maltodextrin administration ameliorates brain pathology in a mouse model of mitochondrial disease

Adán Domínguez-Martínez[1], **Esther Molina-Menor[2]**, Marcos Blanco-Ramos[1], Andrea Urpi[1], Juli Peretó[2,3,4], Manuel Porcar[2,4] and Albert Quintana[1,5*].

1. Institut de Neurociències, Universitat Autònoma de Barcelona, Campus de Bellaterra.
2. Institute for Integrative Systems Biology I2SysBio, Universitat de València-CSIC.
3. Departament de Bioquimica i Biologia Molecular, Facultat de Ciències Biològiques, Universitat de València.
4. Darwin Bioprospecting Excellence S.L. Parc Científic Universitat de València.
5. Departament de Biologia Cel·lular, Fisiologia i Immunologia, Universitat Autònoma de Barcelona.

Summary

Mitochondrial dysfunction lead to a wide group of progressive and fatal pathologies known as mitochondrial diseases (MD). One of the most common pediatric representation of MD is Leigh Syndrome, affecting 1/40.000 births. LS is characterized by neurodegeneration in specific brain areas, such as brainstem and basal ganglia, and by respiratory and motor alterations. However, the results obtained from clinical trials based on antioxidant therapies are controversial. Thus, the development novel antioxidant strategy is required to improve the efficacy of current palliative treatments. In this regard, Ndufs4KO mouse model is a suitable model to test new drugs in the field of MD and LS. Therefore, we set to assess the therapeutic potential of oral administration of *Micrococcus luteus*, a high-antioxidant content microorganism. Incidentally, we identified that while *M. luteus* administration did not possess any beneficial actions, the cryopreservant maltodextrin (MDX), included in the preparation, ameliorated the phenotype of Ndufs4KO mice. Our results show that MDX treatment at a concentration of 30 g/L increased lifespan and reduced microglial reaction compared to vehicle-treated Ndufs4KO mice. However, no improvement in locomotion nor respiratory function was observed in MDX-treated mice compared to vehicle-treated Ndufs4KO mice. Metataxonomic characterization of intestinal microbiome identified differential profiles in Ndufs4KO mice at the genus level. Furthermore, MDX treatment increased the variability of the abundance of *Akkermansia* sp. Thus, this work paves the way for further studies to confirm the therapeutic potential of MDX in mitochondrial disease.

This work was uploaded to bioRxiv on June 2023.

Introduction

Mitochondria are organelles that play a central role in homeostasis and survival as they provide the energy required for proper cell function (Friedman & Nunnari, 2014). Mutations that impair normal mitochondrial function have a huge impact on organs with high energy requirement, such as the brain or muscles, and lead to a wide group of progressive and often fatal pathologies known as primary mitochondrial diseases (MD) (Vafai & Mootha, 2012; Ng & Turnbull, 2016).

MD have a prevalence of 1/5000 newborns, being Leigh Syndrome (LS) the most common pediatric presentation (1/40000 births). LS onset is typically described between 3 and 12 months of age, although onset in prenatal and adult life has also been reported, and is commonly caused by mitochondrial complex I deficiency (almost 50 % patients) (Gerards et al. 2016). However, over 75 mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) mutations have been described to cause LS (Lake et al., 2016). Despite the genetic and clinical heterogeneity, LS patients commonly present symmetrical brain lesions, mainly in the brainstem and basal ganglia, together with failure to thrive, persisting vomiting, hypotonia, motor deficits and respiratory failure (Arii & Tanabe, 2000; Rahman, 2012; Sofou *et al*., 2014).

Unfortunately, the severity of the disease, the variability in its progression and the paucity of good animal models difficult and limit our understanding of the pathophysiology of LS. Moreover, current treatment for LS and other MD are limited and palliative, thus urging the need to develop novel drugs to cope with the symptomatology (Lake *et al*., 2016). In this regard, the characterization of a mouse model lacking the *Ndufs4* gene (Ndufs4KO), essential for the mitochondrial complex I of the respiratory chain, represented an important step forward in the study of the disease (Kruse *et al*., 2008; Quintana *et al*., 2010).

Ndufs4KO mice faithfully recapitulate human LS condition, being, thus, suitable for testing new drugs and therapeutic approaches (Bolea *et al*., 2019). These animals show increased oxidative stress (OS) and reactive oxygen species (ROS) levels in affected brain areas, leading to neurodegeneration and glial activation (Angelova & Abramov, 2018; Liu *et al*., 2015; Quintana *et al*., 2010). OS causes lipid peroxidation and neuron membrane alteration, DNA damage (especially mitochondrial DNA damage), protein oxidation and subsequent alteration of signaling pathways (Cobb & Cole, 2015; Grimm & Eckert, 2017; Lavie, 2015). Moreover, these high OS and ROS levels seem to be responsible for the symptomatology of MD and LS given the observed susceptibility of the nervous system to OS damage (Ng & Turnbull, 2016; Vafai & Mootha, 2012). Furthermore, the model also shows motor alterations, respiratory deficits, epilepsy and premature death and has revealed the prominent role of the dorsal brainstem in disease progression and lethality (Bolea *et al*., 2019; Quintana *et al*., 2012). Given the role of OS and ROS in MD, antioxidants have proven a good palliative treatment to ameliorate the clinical progression of LS in animal models in preclinical assays (Liu *et al*., 2015). Even though the role of antioxidant treatments in clinical trials remains unclear due to failure of several approaches in improving clinical progression (Parkinson *et al*., 2013), it has been described that administration of the antioxidant EPI-743 ameliorated symptomatology of LS pediatric patients (Martinelli *et al*., 2012). For this reason, novel antioxidant-based strategies are still of interest in the field of LS treatment (Enns, 2014).

In the last decades, the role of microbiota in health and disease has been in the spotlight of many scientists. The microbiota can be defined as the microbial population inhabiting a specific environment at a specific time, and particularly gut microbiota has received interest since differences between healthy and ill patients of a group of diseases have been found. It has been suggested that microbes inhabiting the intestinal tract of animals, including humans, may contribute to the global health state of the individual and, therefore, to the appearance and progression of certain pathologies (e.g., neurologic, respiratory, autoimmune and metabolic disorders).

However, whether changes in microbiota are cause or result of these altered states remains a question to be answered in most cases (Fan & Pedersen, 2021; Lynch & Pedersen, 2016).

Specifically, microorganisms inhabiting the intestinal tract can modulate OS levels and the antioxidant capacity of the host in the central nervous system. Thus, their contribution to the development of several neurodegenerative disorders is being increasingly recognized (Dumitrescu *et al*., 2018). In the era of pre, pro and postbiotics (live biotherapeutic microorganisms), an approach that deeply studies the effect of potentially new probiotic microorganisms or specific substances on the microbiota, and further in the general health state of the individuals is of interest (Cunningham *et al*., 2021; Soheili *et al*., 2022).

In this regard, carotenoids are pigments present in many phototrophic and non-phototrophic organisms (including vegetables, algae and bacteria) with widely-known antioxidant properties (Milani *et al*., 2017). In fact, microbial communities from extremophilic and highly irradiated environments (e.g., solar panels, the supralittoral area of the coast or deserts) are enriched in carotenoid-synthetizing microorganisms with biotechnological potential in biomedicine given their antioxidant capacity (Asker *et al*., 2007; Molina-Menor *et al*., 2019; Tanner *et al*., 2019; Tapia *et al*., 2021; Tian & Hua, 2010, Molina-Menor *et al*, 2020). These antioxidant properties are of interest since ROS molecules are generated not only during aerobic metabolism, but also during pathological processes in degenerative diseases and under abiotic stress conditions (Apel & Hirt, 2004; Stahl & Sies, 2005).

We hypothesized that antioxidant microorganisms and/or microbial extracts may have a therapeutic effect on MD. Therefore, we set to develop a complete strategy to characterize the effect of carotenoid-rich microbial formulations, containing freeze-dried microorganisms and maltodextrin (MDX), on the phenotype of Ndufs4KO. Our results indicate that while no significant effect of microorganisms per se was observed, the administration of maltodextrin leads to an amelioration of clinical and histological signs in Ndufs4KO, which may be mediated by an effect on microbiota profiles.

Materials and Methods

Animal model

Ndufs4$^{\Delta/\Delta}$ (Ndufs4KO) mouse generated by Dr. Palmiter lab (Kruse *et al*., 2008) and characterized by Quintana *et al*. (2010) were used in this study. All mice were on a C57BL6/6J background after backcrossing for at least 10 generations. Deletion of the exon 2 in Ndufs4 gene was confirmed by PCR. Female and male mice of the same age were used and maintained on a rodent diet (5053, Picolab) and water available *ad libitum*, in an animal facility at 22 °C with a 12 h light-dark cycle. All procedures were approved by the animal care committees at the Universitat Autònoma de Barcelona and Generalitat de Catalunya.

Physiological and behavioral parameters

Body weight and gait/postural alterations were measured every day for each mouse. Mice were euthanized after losing 20 % of their maximum weight.

Maltodextrin dose-response study

Low-DE maltodextrin (MDX) (Mane Iberica S.A) was diluted in drinking water at different concentrations (1.5, 5, 15 and 30 g/L), and provided to the animals *ad libitum*. Water was used as vehicle (VEH, no treatment). MDX beverage was replaced twice a week to avoid drink contamination by fungi. MDX treatment started after weaning at P21. Given the severe locomotion alterations of Ndufs4KO mice, MDX beverage was poured over chao pellets (one per animal) and placed at the same location in the cage daily.

Behavioral assays

Behavioral assays were performed at early-stage (P30-35), mid-stage (P40-45) and late-state (P50-55). Phenotypic markers (weight, hydration and survival) were also registered and analyzed as stated below:

	Behavioral tests			Phenotypic markers		
	Rotarod	Plethys.	Open Field	Weight	Hydration	Ataxia Curling Twisting
Age (days)	P30 P40 P50	P32 P42 P52	P35 P45 P55	Daily	Daily	Daily

Rotarod test

A rotarod device was used to assess motor coordination and global physical condition of animals as described (Bolea *et al*., 2019). Mice were placed on a gradually accelerating rotating drum (from 4 rpm to 50 rpm) for a maximum of 3 min. Each trial ended when the mouse fell off the rod. The assay consisted in 3 trials, separated by a 5 min rest period.

Open Field

Mice were placed in the center of an open-field arena (650 x 365 x 400 mm) and allowed to move freely for 45 min in order to assess locomotor activity as described (Bolea *et al*., 2019). Travelled distance and velocity average were analyzed in two periods of time: the first 5 min, and the rest 40 min. The time spent inside and outside the center of the cage was also analyzed. EthoVision tracking software (Noldus) was used for analysis.

Whole-body plethysmography

Ventilatory function was assessed through whole-body plethysmography under unrestrained conditions as described (Prada-Dacasa et al., 2020). Mice were placed in an airtight chamber that allowed free movement and spontaneous breathing. The system was calibrated to 1 mL. Mice were monitored for 1 h: 45 min of adaptation and 15 min of measurement. Respiratory frequency (RF; breaths/min) and tidal volume normalized per body weight (TV; µL/g) were measured.

Tissue preparation and immunofluorescence

Brains were dissected after euthanasia, fixed overnight in 4 % paraformaldehyde (PFA) in PBS (pH 7.4) and then cryopreserved in a PBS solution with 30 % sucrose (w/v) as described (Quintana et al, 2010). Mouse brains were sectioned at 30 µm in a cryostat and stored in PBS solution for further use. Brain sections containing the VN were obtained from bregma -5.6 to bregma -6.96. For immunofluorescence, brain sections were rinsed in 0.2 % Triton X-100 in PBS (PBST). An incubation step of 60 min at room temperature (RT) was done with a solution of 10 % Normal Donkey Serum (NDS) in PBST to block non-specific binding. The sections were then incubated overnight with primary antibodies αGFAP Ch (ref PA1-10004) 1:2000 and αIba1 Rb (ref 019-19741) 1:2000 at 4 °C. Brain sections were further washed in PBST and incubated for 1 h at RT with secondary antibodies αCh 488 (green) 1:500 and αRb 594 (red) 1:500 in 1% NDS-PBST, and finally washed with PBS and rinsed in water before mounting onto glass slides for microscopic analysis.

High-throughput 16S rRNA gene sequencing (metataxonomy)

DNA extractions were carried out with the Stool DNA Isolation Kit Dx from NORGEN Biotek (Ontario, Canada) following the manufacturer's instructions. Gut samples in PBS 1X were first centrifuged for 12 min at 12000 rpm at room temperature. The pellets were then resuspended in 400 µL of PBS 1X, which were further used for the DNA extraction. The conserved regions V3 and V4 of the bacterial 16S rRNA gene were amplified with primers 5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG 3′ and 5′- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C -3', respectively. Libraries were constructed following the Illumina's standard protocol and Illumina MiSeq platform (2x300 pb) was used for amplicon sequencing. Bioinformatic analysis was carried out by Darwin Bioprospecting Excellence S.L.

Statistics

GraphPad v8.0 software was used for statistical analysis. The tests were chosen according to the experimental design, as stated in figure legends.

Statistical significance is also stated in the legend. Data are shown as the mean±SEM.

Results

Maltodextrin administration extends survival in Ndufs4KO mice

To assess the potential role of a microorganism-based antioxidant administration we delivered an extract of *Micrococcus luteus*, a microorganism we had previously identified to possess high antioxidant properties (Molina-Menor *et al*., 2019), to a cohort of Ndufs4KO mice. Similarly, to LS patients (Sofou *et al*., 2014) Ndufs4KO mice present a severe phenotype including death at early stage (around week 7) (Quintana *et al*., 2012). Administration of *M. luteus* mixed in the drinking water (5 g/L) led to a significant extension of the lifespan of Nduf4KO compared to our historical record for Ndufs4KO mice (median survival of 69 vs 43 days, respectively (Figure VII.1A). However, similar effects were obtained when a cohort of Ndufs4KO mice receiving 5g/L maltodextrin, used as a cryopreservant in the preparation of the *M. luteus* compared to a vehicle (water)-treated Ndufs4KO mice (Figure VII.1B).

To determine if the lifespan survival of Ndufs4KO mice is dependent on MDX dosage, a dose-response study was designed. To that end, we administered increasing concentrations of MDX (1.5, 5, 15 and 30 g/L) or vehicle (water). Subsequently a log-rank test was performed to analyze the effect of different doses

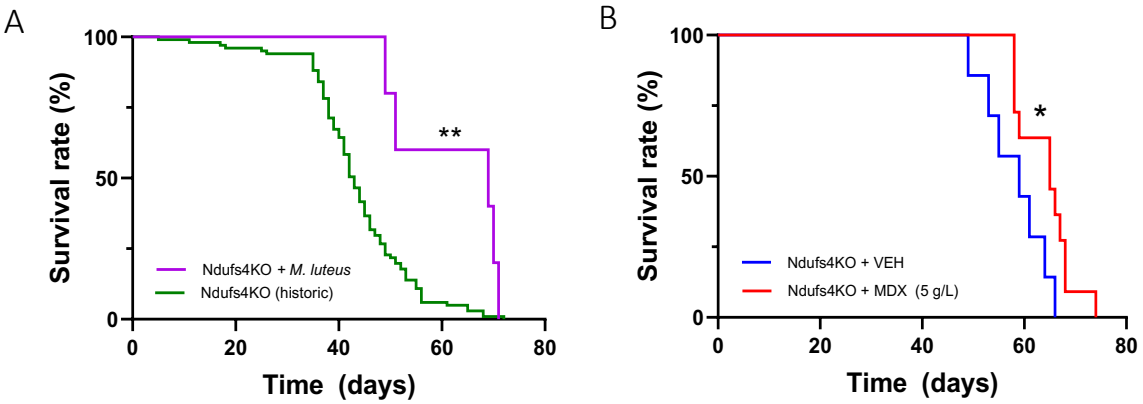

Figure VII.1. Maltodextrin administration extends lifespan in Ndufs4KO mice. (A) Survival curve of Ndufs4KO mice receiving a 5 g/L solution of *Micrococcus luteus* and maltodextrin (n=5) in the drinking water vs. a comparative historical survival of the Ndufs4KO colony (n=101). **p<0.01 vs. Ndufs4KO historical record, Long-rank test. (B) Survival curve of Ndufs4KO mice receiving a 5 g/L solution of maltodextrin in the drinking water (MDX, n=11) vs. mice receiving regular drinking water (VEH, n=7). *p<0.05 vs. VEH, Long-rank test.

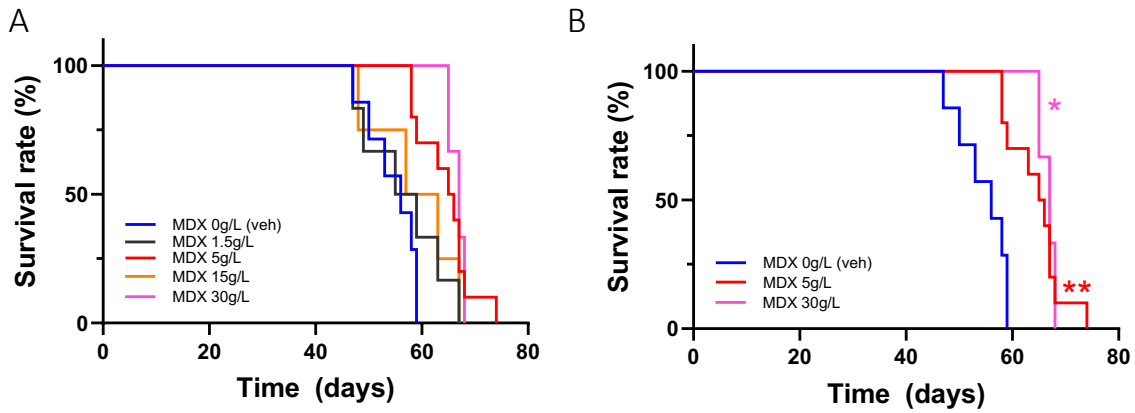

Figure VII.2. Effect of increasing maltodextrin concentrations on Ndufs4KO survival. (A) Combined survival curves of 0-30 g/L MDX doses. (0 g/L -veh- n=7, 1.5 g/L n=6, 5 g/L n=10, 15 g/L n=4, 30 g/L n=3) of MDX. Doses of 5 g/L and 30 g/L MDX in the drinking water led to an extension of the lifespan in Ndufs4KO mice compared to vehicle (water)-treated mice. (B) Survival curves of statistically significant MDX concentrations. * p<0.05, **p<0.01, Log-rank test vs vehicle.

of MDX on the survival of Ndufs4KO mice. Our results showed that MDX significantly increased survival in a compared to the vehicle-treated group, obtaining a significant lifespan extension in Ndufs4KO mice treated with 30 g/L and 5 g/L of MDX (Figure VII.2). Median survivals in the MDX 5 g/L and 30 g/L groups were 66 and 67 days, respectively, significantly higher than in vehicle-treated group, which showed a median rate of survival of 55 days. Noteworthy, no effect was observed at an intermediate dose of 15 g/L, even though an increase in median survival was found (56 vs. 60, veh vs 15 g/L, respectively).

Maltodextrin administration does not improve clinical signs in Ndufs4KO mice

Ndufs4KO mice develop severe motor and breathing deficits as the pathology progresses (Quintana *et al.*, 2010). Therefore, we set to assess whether the beneficial effects of MDX treatment (5 g/L or 30 g/L) in lifespan could also contribute to reduced Ndufs4KO symptomatology.

Ndufs4KO mice show reduced spontaneous locomotion and mobility (Chen *et al.*, 2017). To assess the effect of MDX treatment on spontaneous locomotion in this mouse model animals treated with different doses of this compound were subjected to the open field (OF) test for 45 minutes, at early (P35), mid (P45) and late (P55) stage of the disease. During the first 5 minutes, corresponding to novelty-induced locomotion, Ndufs4KO mice moved significantly less than WT animals, regardless of the treatment, being already evident in MDX-treated mice at P35. However, no differences were observed between VEH- or MDX-treated Ndufs4KO mice (Figure VII.3A).

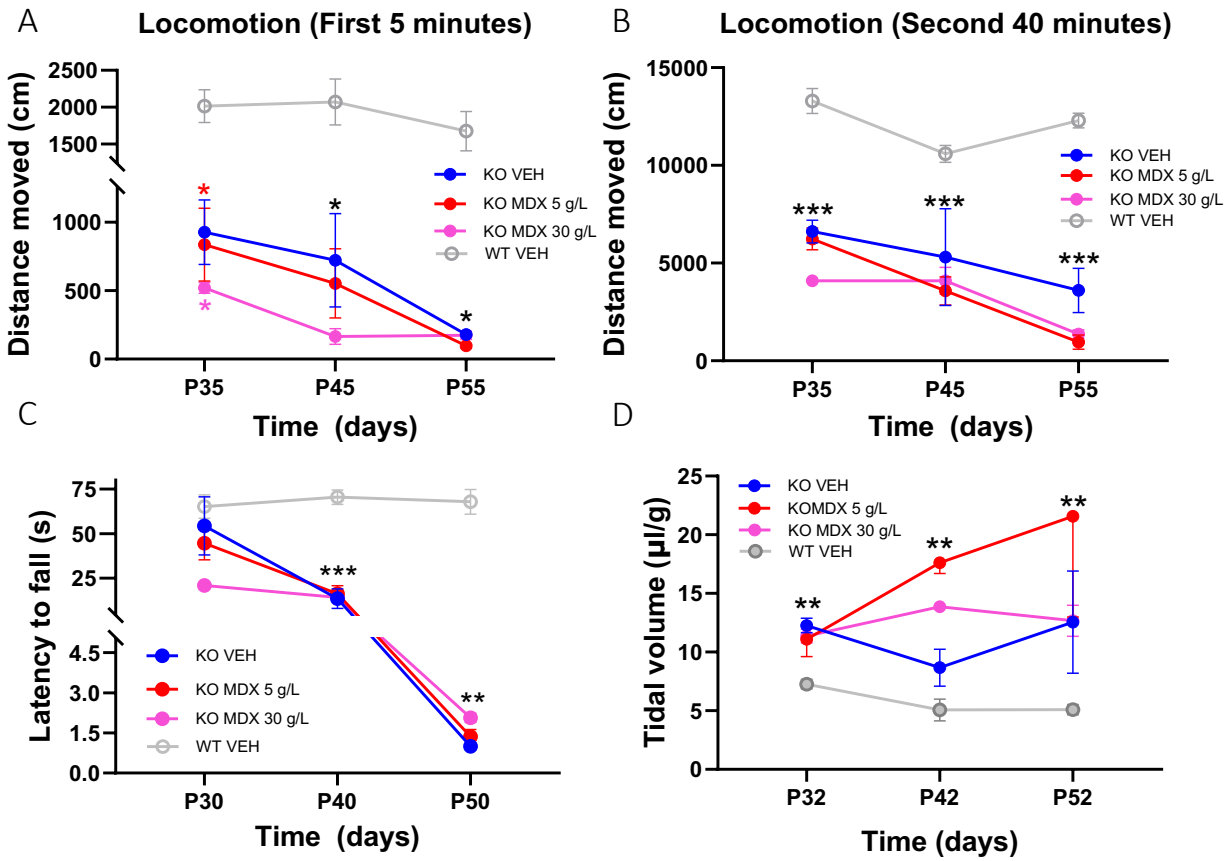

Figure VII.3. MDX administration does not correct motor or respiratory deficits in Ndufs4KO mice. (A-B) Novelty-induced minute locomotion in WT or Ndufs4KO mice treated with either water (VEH) or 5 g/L or 30 g/L MDX in the drinking water during the first 5 minutes (A) or subsequent 40 minutes (B) in the open field test. (C) Rotarod performance (latency to fall) in WT or Ndufs4KO mice treated with either water (VEH) or 5 g/L or 30 g/L MDX in the drinking water. (D) Respiratory function (tidal volume) in WT or Ndufs4KO mice treated with either water (VEH) or 5 g/L or 30 g/L MDX in the drinking water * p<0.05, **p<0.01, ***p<0.001, mixed-model analysis. Black asterisks denote overall effect KO vs WT. Colored asterisks denote difference of the indicated group vs WT mice. n=3-6 per group.

In a similar fashion, analysis of the subsequent 40 minutes revealed significant differences between WT and Ndufs4KO mice, with no observable effect of MDX treatments in the performance of Ndufs4KO mice (Figure VII.3B).

Motor coordination was evaluated by the rotarod test at different stages of the disease. Animals lacking *Ndufs4* present impaired performance on the rotarod test after P40 (Figure VII.3C), indicating that motor coordination is affected in these animals, as described (Kruse *et al.*, 2008; Sofou *et al.*, 2014). No improvement was observed in animals treated with different doses of MDX when compared to vehicle-group, indicating that MDX has no effect on gross motor coordination in the Ndufs4KO mice in any stage of the disease (Figure VII.3C).

Ndufs4KO mice show respiratory alterations that have been linked to mortality (Quintana *et al.*, 2012) and human patients (Lake *et al.*, 2016). Specific respiratory parameters such as Tidal Volume (TV) are altered in the Ndufs4KO mice, accompanied with other breathing deficits such as hypo- and hyperventilation, gasping and apnea (Bolea *et al.*, 2019). To assess the effect of MDX treatment on the respiratory function of Ndufs4KO mice, we analyzed TV by unrestrained whole-body plethysmography in awake mice at early, mid, and late (P52) stages of the disease (Figure VII.3D), as described (Prada-Dacasa *et al.*, 2020). In agreement with this, analysis of TV revealed significant differences in Ndufs4KO mice compared with WT mice. However, no significant differences were observed between VEH- and MDX-treated Ndufs4KO (Figure VII.3D). Thus, our results suggest that MDX treatment does not affect the development of clinical signs in Ndufs4KO mice.

Maltodextrin administration reduces glial reactivity in Ndufs4KO mice

LS is characterized by symmetrical brain neuroinflammation followed by neurodegeneration in some brain nuclei, such us brainstem and basal ganglia in humans (Arii & Tanabe, 2000). In this regard, the Ndusf4KO mice the most affected area is the vestibular nucleus (VN) (Quintana *et al.*, 2010).

To define the effect of MDX treatment on the overall neuroinflammatory phenotype in Ndufs4KO mice, immunofluorescence analysis for glial reactivity markers was performed in the VN of vehicle- and MDX-treated and MDX-treated (30 g/L) WT and Ndufs4KO mice (Figure VII.4). As expected, our results showed marked induction of astroglial marker glial

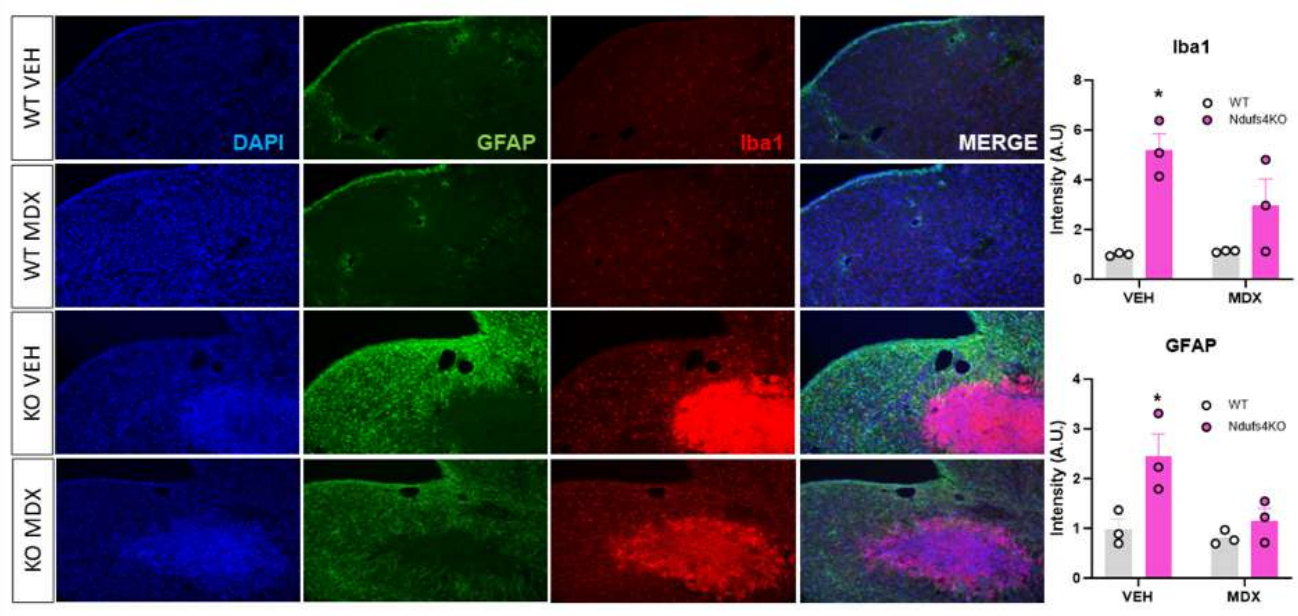

Figure VII.4. MDX administration reduces glial reactivity in the brainstem of Ndufs4KO mice. Immunofluorescence and quantification of the microglial marker Iba 1 (red), astroglial marker GFAP (green) in the vestibular nucleus region of VEH- and MDX-treated WT and Ndufs4KO mice. Nuclear counterstain (DAPI, blue) is also shown. * p<0.05 vs. WT VEH, two-way ANOVA Tukey's multiple comparisons. n=3 per group.

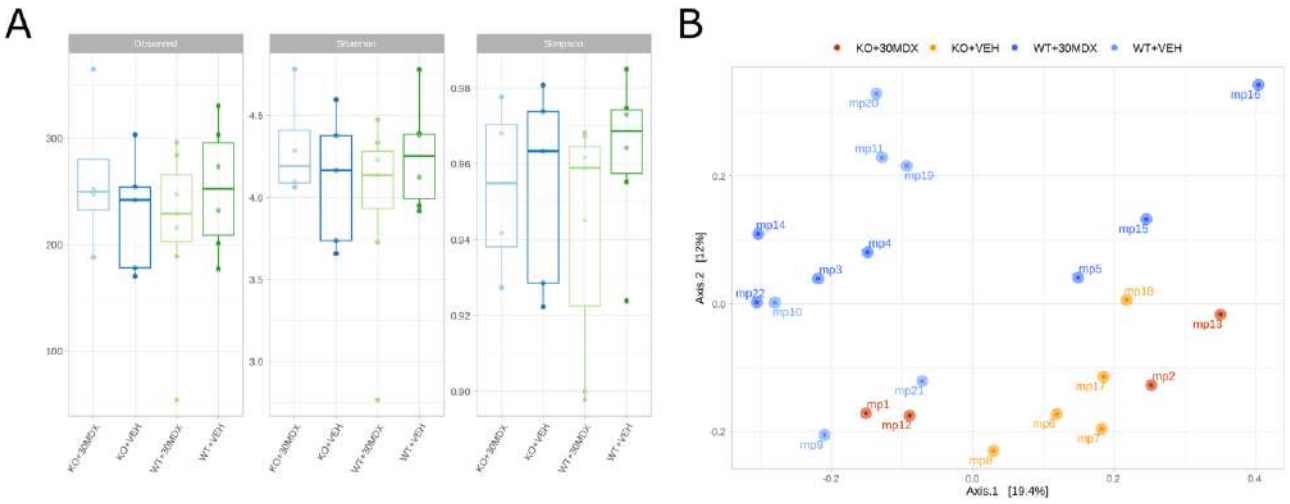

Figure VII.5. α- and β-diversity in caecal samples from VEH- and MDX-treated WT and Ndufs4KO mice. (A) α-diversity results at the ASV (Amplicon Sequence Variant) level. (B) β-diversity at the ASV level. n= 4-7per group.

fibrillary acidic protein (GFAP) and the microglial reactivity marker ionized calcium-binding adapter molecule 1 (Iba1) in the VN of VEH-treated Ndufs4KO mice compared to WT mice. However, this increase in glial reactivity was not present in MDX-treated Ndufs4KO mice (Figure VII.4), suggesting a decreased inflammatory tone in Ndufs4KO mice after MDX treatment.

Altered intestinal microbiome profile in Ndufs4KO mice

We hypothesized that maltodextrin may be acting through regulation of intestinal microbiota in Ndufs4KO mice in a prebiotic manner. Thus, given the role that prebiotics play in modulating the host microbiota, high-throughput 16S rRNA gene sequencing of samples from the intestinal tract (caecum) was carried out in VEH- and MDX-treated (30 g/L) WT and Ndufs4KO mice. Metataxonomic analyses revealed a high variability between replicates within the same group, being thus very similar the resulting bacterial profiles among them. In terms of alpha-diversity (species richness), no significant differences were found (Figure VII.5A). In contrast, beta-diversity revealed a phenotype-dependent gut bacterial composition, but not a treatment dependent one (Figure VII.5B).

No significant differences were observed at the phylum level among Ndufs4KO and WT mice (Figure VII.6). However, the comparison at the genus level (Figure VII.7A-B) revealed that the abundance of the genus *Akkermansia* was higher in Ndufs4KO mice (VEH) compared to WT (VEH) mice (Figure VII.7B). Furthermore, the abundance variability of *Akkermansia* was increased by MDX treatment, independent of the genotype (Figure VII.7B).

Independent of the treatment, group analysis between Ndufs4KO and WT mice was able to identify differences in several genera. In particular, Ndufs4KO mice presented increased abundance of *Bilophila* and *Ruminococcus.* On the other hand, reduced

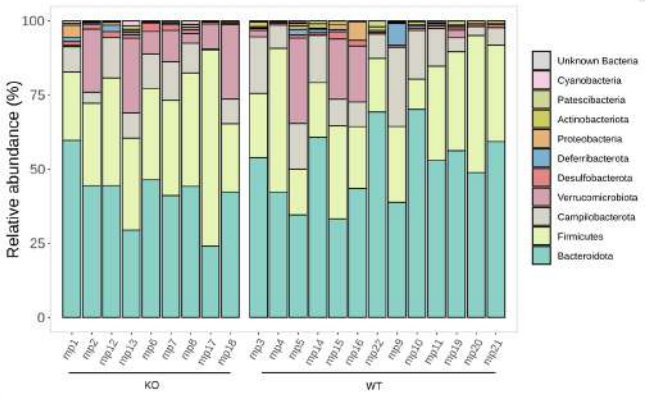

Figure VII.6. Bacterial profiles at the phylum level in caecal samples from VEH- and MDX-treated WT and Ndufs4KO mice. Similar profiles were obtained in all groups. n= 9-13 per group.

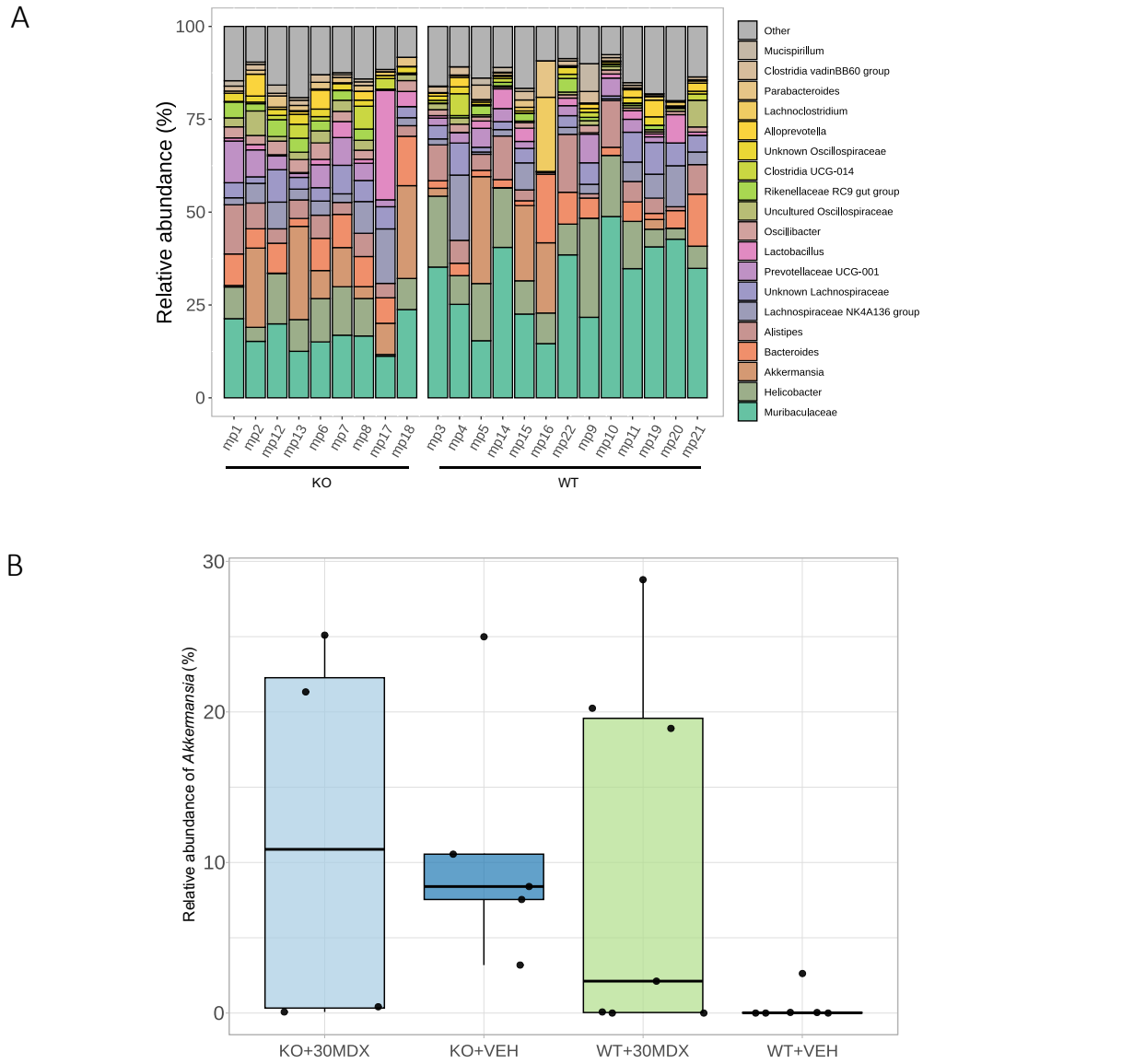

Figure VII.7. Bacterial profiles at the genus level in caecal samples from VEH- and MDX-treated WT and Ndufs4KO mice. (A) Global bacterial profiles at the genus level. (B) Relative abundances of *Akkermansia.* n= 4-7per group.

abundance of *Muribaculaceae* was found in caeca from Ndufs4KO mice (Figure VII.8).

Discussion

There is an urgent need to identify novel treatments for LS. Among the different treatment options, antioxidants have been shown to possess some value in animal models of Leigh Syndrome (de Haas *et al*., 2017; Liu *et al*., 2015). However, antioxidant approaches have had limited success in clinical settings. To that end, we hypothesized that high antioxidant loads may overcome this limitation, in line with previous studies (Liu *et al*., 2015). To that end, we set to use a high-antioxidant content microorganism, *Micrococcus luteus* (Molina-Menor *et al*., 2019), as a potential therapy for the Ndufs4KO (Quintana *et al*., 2010), a well-validated model of Leigh Syndrome. While we could not identify any beneficial effect attributed to *M. luteus per se*, here we show that, in a serendipitous discovery, the administration of maltodextrin (MDX), a cryopreservant used in the preparation of bacterial solutions, was sufficient to improve the phenotype of the Ndufs4KO mice.

MDX has many applications, mainly in food industry and as therapeutic approach in some pathologies

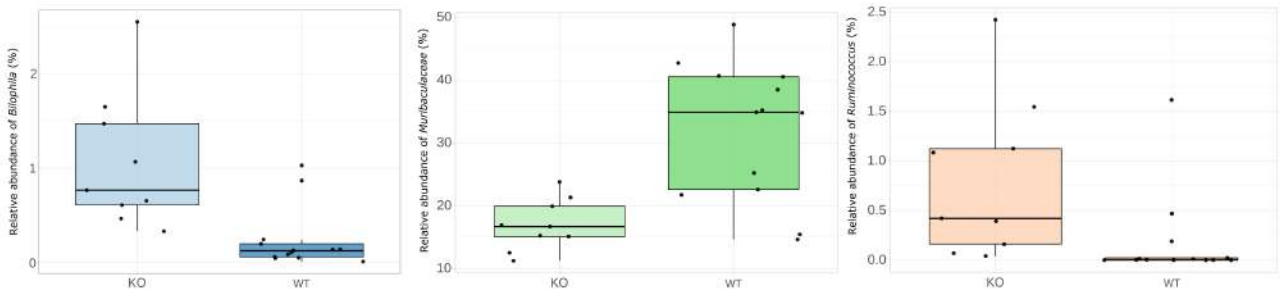

Figure VII.8. Relative abundance of *Bilophila* (left), *Muribaculaceae* (middle), and *Ruminococcus* (right) by phenotype. n= 4-7per group.

related to nutrition and dehydration, where it has been demonstrated that is safe for human consumption (Hofman *et al*., 2016). However, the use of complex sugars for the treatment of LS or other MD had yet to be explored. Our results show a clear lifespan extension in Ndufs4KO mice treated with either 5 or 30 g/L of MDX, which was the highest dose test. Surprisingly, no effect on lifespan was observed with a dose of 15 g/L MDX. While this may be due to individual differences and/or statistical power, it could also underscore that the effect of MDX is not completely dependent on concentration and that may require the interaction with other factors. However, based on present results, we anticipate that the maximum dose-response effect on survival has not been reached. Therefore, further studies increasing the concentration of MDX will be necessary to define the dose leading to the maximal effect. In this regard, MDX at 30 g/L has a modest effect on the lifespan of Ndufs4KO mice when compared to another therapies for MD that have been tested in the same mouse model. Hypoxia (Ferrari *et a*l., 2017), hyperoxia (Jain *et al*., 2019), rapamycin (Johnson *et al*., 2013) and tetracyclines (Perry *et al*., 2021) treatment result in higher survival rates when compared to our results with MDX at 30 g/L in Ndufs4KO mice. However, we cannot exclude that higher doses of MDX may lead to similar effects on the lifespan of these animals.

The beneficial effects of MDX could be related to the capacity of MDX to keep Ndufs4KO mice hydrated for longer periods of time, and to the glucose from MDX molecule that provides the necessary energy for physiological processes to these animals. Here, it is important to point that at late-stage of the disease, Ndufs4KO animals show severe locomotion alterations that hinder their capacity to drink and feed (Quintana *et al*., 2010).

Despite the encouraging results in lifespan extension in the MDX-treated Ndufs4KO mice, no clear effects were observed on the motor and respiratory alterations present in these animals. Analysis of locomotion in the open field test or motor coordination by rotarod test in the MDX-treated Ndufs4KO did not show any significant difference when compared to vehicle-treated group. However, the rotarod test evaluates gross coordination rather than fine motor coordination (Shiotsuki *et al*., 2010). For this reason, it remains to be determined whether MDX treatment affects other tests such as foot-print test and horizontal bar test, instead of rotarod which may be too demanding to appreciate differences in this severe phenotype.

Respiratory alterations are well described in Ndufs4KO mice (Quintana *et al*., 2012) and have been associated to increased lethality in both the Ndufs4KO model (Quintana *et al*., 2012) and LS patients (Arii & Tanabe, 2000). However, whole-body plethysmography analyzing TV, did not reveal significant differences between the MDX and vehicle-treated Ndufs4KO mice. In this regard, we and others have recently identified that spontaneous seizures are a driving cause of the lethal phenotype of the Ndufs4KO (Bolea *et al*., 2019; Bornstein *et al*., 2022) independent to motor and respiratory alterations. Thus, future studies to uncover whether MDX

treatment ameliorates the epileptogenic phenotype are warranted.

Despite the lack of physiological and behavior deficits, MDX treatment is able to reduce the neuroinflammatory profile of Ndufs4KO mice in an affected area such as the VN. In this regard, MDX treatment at a concentration of 30 g/L reduces astrocyte and microglia-containing lesions in the VN of Ndufs4KO mice when compared to vehicle-treated Ndufs4KO mice. In this regard, increased glial reactivity has been associated with poor outcome and death in Ndufs4KO mice (Quintana *et al*., 2012). In line with this, interventions leading to reduced inflammation (Johnson *et al*., 2013, Liu *et al*., 2015, Perry *et al*., 2021) or targeting immune cells (Aguilar *et al*., 2022; Stokes *et al*., 2022) have led to increased lifespan in the Ndufs4KO mice. Thus, it is likely that the reduction of glial activity is playing a role in the beneficial effects of MDX treatment.

However, it is still unclear how MDX may exert its beneficial effects. It has been described that MDX possesses antioxidant capabilities (Hofman *et al*., 2016). However, it is likely that this effect may be locally observed in the gut, rather than the brain. Hence, given the known and growing relationship between microbiota, brain and inflammation (Rutsch *et al*., 2020), we hypothesized that MDX may be affecting microbiota in the intestines of Ndufs4KO mice in a prebiotic manner. In this regard, even though interindividual variability has limited the ability to identify differences at the phyla level, our metataxonomic analyses have identified several genera altered in the intestines of Ndufs4KO mice compared to WT. Among them, we have detected an increase in the relative abundance of *Bilophila* and *Ruminococcus,* coincident with reports in neurodegenerative diseases such as Parkinson's Disease (Li *et al*., 2023). Along the same lines, *Muribaculaceae* has been suggested to negatively correlate with inflammation (Shang *et al*., 2021). Thus, the alterations observed in intestinal microbiota in Ndufs4KO mice seem to point to an enhanced inflammatory status in these mice, which may contribute to pathology progression, in line with recent reports (Bitto et al., 2023). As mentioned above, no robust effects were found in bacterial profiling after MDX administration. However, *Akkermansia* was significantly increased in Ndufs4KO VEH mice compared to WT VEH controls. The role of *Akkermansia* in physiology and pathology is gaining traction, having been described both positive and negative roles in inflammation and pathological progression in different paradigms (Wang *et al*., 2022; Zhang *et al*., 2019). MDX treatment, both in Ndufs4KO and WT mice, was associated with an increase in the variability of *Akkermansia* abundance, which could underlie some of the beneficial effects of MDX. In this regard, further in-depth studies to identify possible correlations between microbiome profiles, *Akkermansia* abundance, MDX treatment, and lifespan extension can help identify the underlying factors driving the beneficial effects of MDX administration in Ndusf4KO.

In conclusion, our results pave the way for the development of MDX as a potential novel treatment for Leigh Syndrome, and identify alterations in the intestinal microbiota in Ndus4KO that may be involved in the progression of the disease.

Author contributions

ADM, EMM, MBR and AU carried out the experiments, and generated and analyzed data. JP, MP and AQ planned, designed, and supervised the experiments and obtained resources. ADM, EMM and AQ wrote the manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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General results and discussion

This thesis has focused on the study of highly irradiated environments, their microbial structures and biomedical implications. The microbiome of natural sun-exposed environments or artificial UV-exposed surfaces has been assessed along this thesis. Previous work on solar panels had revealed that a highly-diverse biocenosis lived on the surface of these worldwide distributed man-made structures (Dorado-Morales *et al*., 2016; Tanner *et al*., 2020). This biocenosis shared some features with those of desert environments, which outstand as a possible source of microbes through airborne particles. Moreover, other sun-exposed environments such as outdoor, wasted chewing gum also displayed similarities given the presence of highly-resistant taxa such as *Deinococcus* and *Sphingomonas* (Satari *et al*., 2020). In this work we aimed at studying natural and artificial UV-exposed surfaces with the goal of isolating biotechnologically-relevant culturable microorganisms. Moreover, we have explored strategies to increase the microbial diversity cultured in laboratories through simple and non-expensive experimental designs.

1. Combining media: a key for increasing diversity

The use of a combination of culturing methods and media has been systematically applied in bioprospecting and in microbial ecology. For example, Gong *et al*. (2017) used four media with alternative carbon sources in the screening for lignocellulolytic activities (cellulolytic, xylanolytic and lignolytic), and discussed how the use of each of them favored the isolation of bacteria with specific lytic activities. Similarly, Moote *et al*. (2021) concluded that combining media, as well as combining culture strategies, was critical for increasing the diversity of cultured microorganisms (including the isolation of new taxa) in the case of fastidious bacteria from the gastrointestinal tract of animals. Moreover, Kothe *et al*. (2021) outlined not only the potential of media combination, but also the importance of the parallel study of the samples through culture-independent techniques based on genomic and metagenomic sequencing.

Here, we have systematically used a combination of culture media and culture incubation conditions in

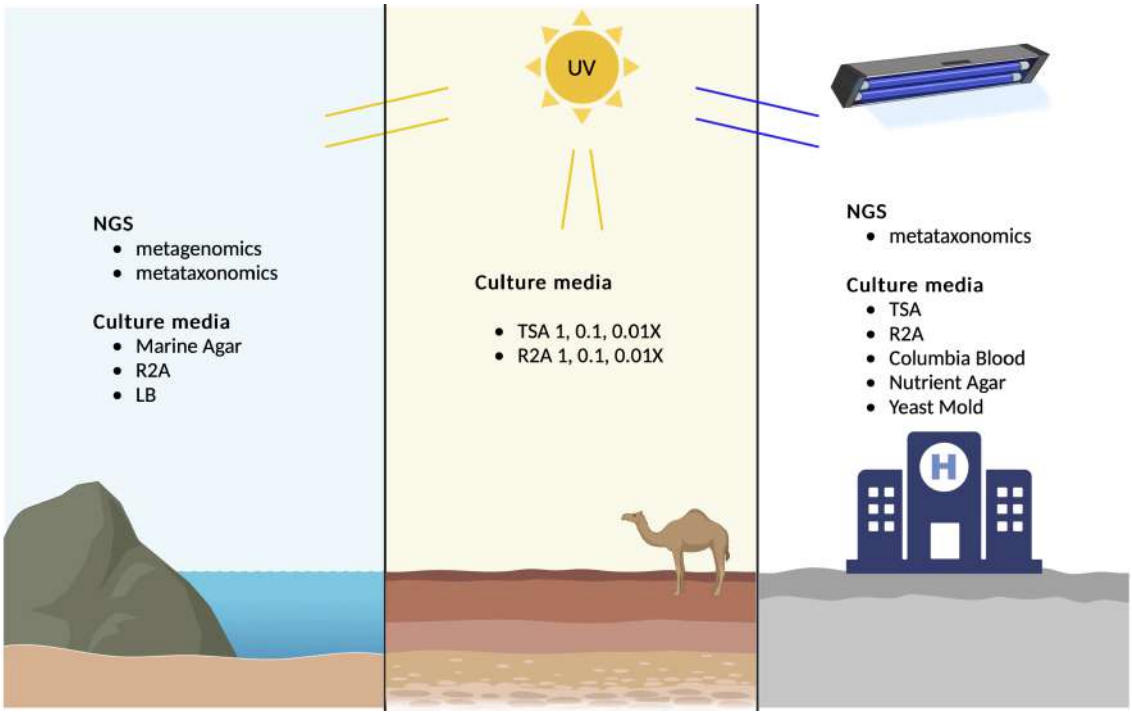

Figure 4. Sampled highly-irradiated environments and the culture-dependent and independent strategies followed. From left to right: the supralittoral zone of the coast (Publication I), desert biocrust (Publications II-IV) and UV lamps in a hospital facility (Publication V). Created with BioRender.com

order to maximize the microbial diversity retrieved in pure culture. With this, not only a high number of different taxa, but also potentially new microorganisms and biotechnologically-relevant strains have been isolated. First, in the isolation of microorganisms from different points of the rocks in the Mediterranean coast (Marine Agar, R2A and LB) (Publication I). Then, in the isolation of biocrust-associated microorganisms from the Tabernas Desert (Almería, Spain) (TSA and R2A at 1, 0.1 and 0.01X concentrations) (Publications II-IV). Finally, in the isolation of microbes from UV cabins in a dermatology service of a hospital (TSA, R2A, Columbia Blood, Nutrient Agar and Yeast Mold) (Publication V) (Figure 4).

Specifically, the use of low-nutrient media, such as R2A or Nutrient Agar, as well as dilutions of TSA, favored the isolation of a higher genera diversity. In the case of the samples from the Tabernas Desert, TSA 0.1X and R2A 0.01X led to the isolation of the highest number of exclusive taxa when compared to the rest of dilutions of each of them (Figures II.4B and C). Even though the differences were not that marked for the samples from the UV cabins of the hospital, R2A, Nutrient Agar and Yeast Mold media together allowed the isolation of 16 exclusive taxa (Figure 5A). Therefore, the combination of media influenced the resulting diversity found in culture. Besides, all five new species isolated from Tabernas (*Belnapia mucosa*, *Belnapia arida*, *Kineococcus vitellinus*, *Kineococcus indalonis* and *Kineococcus siccus*) were so from diluted TSA and R2A plates (Publications III and IV).

In addition to media combination, biocrust samples were incubated for a long time and colonies were picked at two incubation times, whereas samples from the UV cabins were incubated at two different temperatures. In both cases, the combination of media with these strategies led to the isolation of a wide diversity of strains belonging to 37 and 44 different genera, respectively. Although the highest impact on diversity was due to media combination, still there were four taxa exclusively isolated after one month of incubation of the samples from Tabernas, and nine genera from the hospital samples exclusively isolated at 37 °C (including the *Deinococcus* spp. isolates) (Figure II.7A and Figure 5B). Thus, again, our results indicated that these two simple strategies help to increase diversity in microbial collections.

Regarding the diversity found in the culturable fraction of microorganisms from the Mediterranean rocky-shore, it is important to outline that more than

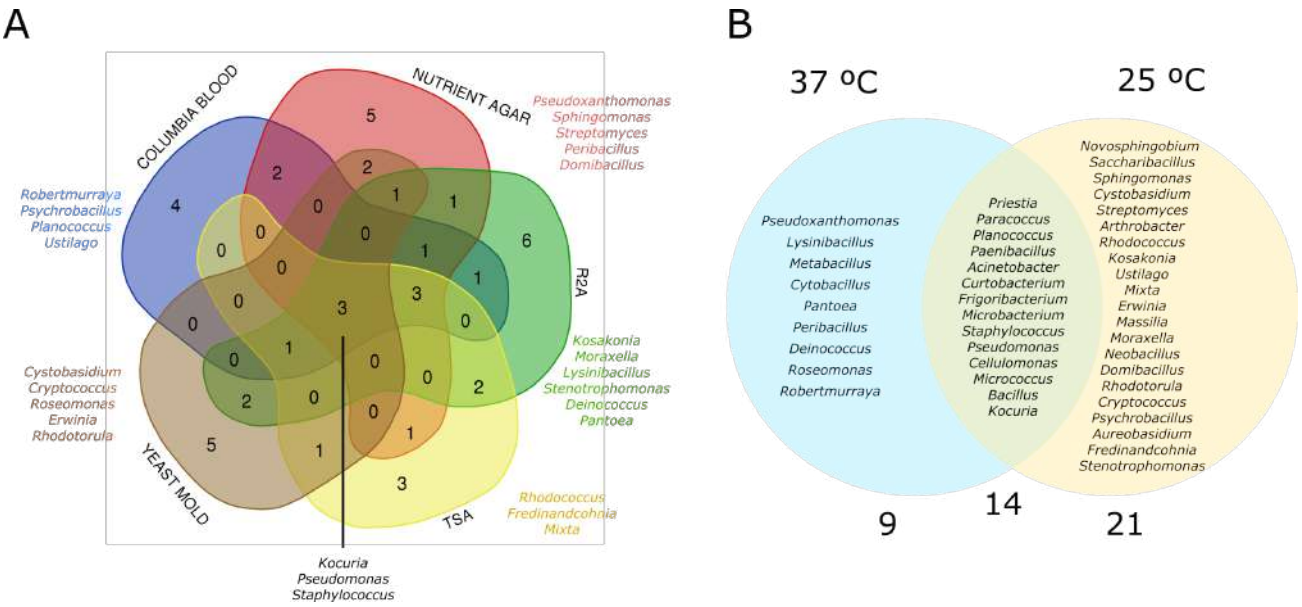

Figure 5. Microbial diversity isolated from the UV cabins of the Hospital General de València (Publication V). (A) Venn diagram showing the shared and exclusive taxa between the culture media used. (B) Venn diagram showing the shared and exclusive taxa between incubation at 25 and at 37 °C.

half of the isolates could not be identified. The identification protocol followed in the laboratory was optimized later for the Tabernas Desert samples. Thus, the low diversity found (most of the isolates belonged to the genera *Bacillus* and *Halobacillus*) may be an artifact as a consequence of the low performance of colony PCR, but a relatively large proportion of almost identical microorganisms cannot be ignored either (Table I.S2).

In addition to the potential of media combination in the isolation of a wide range of microorganisms, the design of new culturing strategies also allows the cultivation of specific microorganisms with different outcomes. This is the case of the use of 3D matrixes for Solid State Fermentation (SSF) (Figure VI.1), which resulted in the obtention of different growth and protein expression patterns in microorganisms, particularly in yeast (Publication VI). Our matrixes dramatically fostered cell-yields compared to the equivalent cultures under static conditions and appeared to favour alcoholic fermentation, although further research is needed in order to elucidate the impact of the matrixes in other processes (e.g., quorum sensing or biofilm formation) (Figure VI.2 and Figure VI.3B).

2. Culture-independent and culture-dependent approaches: differences and similarities in highly irradiated environments

The use of a combination of culture-dependent and culture-independent techniques allows a deeper comprehension of the microbial complexity and diversity in the studied environments. In this regard, our bioprospecting expeditions usually have combined both strategies in order to explore together the biotechnological potential of the samples and the taxonomical and ecological characteristics of the microbial communities (Satari *et al*., 2020; Tanner *et al*., 2018; Vidal-Verdú *et al*., 2022 a; Vidal-Verdú *et al*., 2022 b). In this thesis, we have studied from both perspectives the diversity inhabiting the Mediterranean supralittoral zone and the UV cabins of a hospital.

Through metataxonomy and metagenomic analysis, it was possible to determine that, at the phylum level, the communities studied in all three highly-irradiated environments were dominated by *Pseudomonadota*, *Actinomycetota* and *Bacteroidota*. *Cyanobacteria* was the most abundant taxa in the Mediterranean coast samples, but those of biocrust and the hospital shared also high abundances of this genus. *Acidobacteriota* and *Bacillota*, instead, appeared as representatives of the hospital UV cabins and biocrust samples, respectively (Latorre-Pérez *et al*., 2021; Publications I and V).

Regarding the abundances of highly-resistant taxa, it is relevant to outline that *Deinococcota* members were present in all the cases. Particularly, there were *Truepera* spp. in biocrust samples and in samples from the rocky-shore, and *Deinococcus* spp. in the samples from the UV cabins (Latorre-Pérez *et al*., 2021; Publications I and V). Interestingly, the genus *Truepera* has been described as slightly halophilic (which is in accordance with its presence in marine environments and the desert), whereas *Deinococcus* display varied responses to growth in moderate salt concentrations and is also found on solar panels (Albuquerque *et al*., 2005; Brooks and Murray, 1981; Dorado-Morales *et al*., 2016; Porcar *et al*., 2018; Tanner *et al*., 2020).

At the genus level, some similarities could also be observed. *Rubrobacter* was among the most abundant genera in samples from the rocky-shore and biocrust (Publication I; Latorre-Pérez *et al*., 2021). Biocrust and UV cabin samples were also abundant in *Rubellimicrobium*, *Sphingomonas* and *Hymenobacter*, similarly to what is found on solar panels (Publication V; Dorado-Morales *et al*., 2016; Porcar *et al*., 2018). *Paracoccus*, instead, was present in both the rocky-shore and the UV cabins (Publications I and V). The presence of all of them agrees with the already described microbial communities inhabiting deserts (including saline environments within deserts) (Belov *et al*., 2018).

In the case of the samples from the Mediterranean coast, though, most of the detected taxa remained unclassified and some discrepancies between

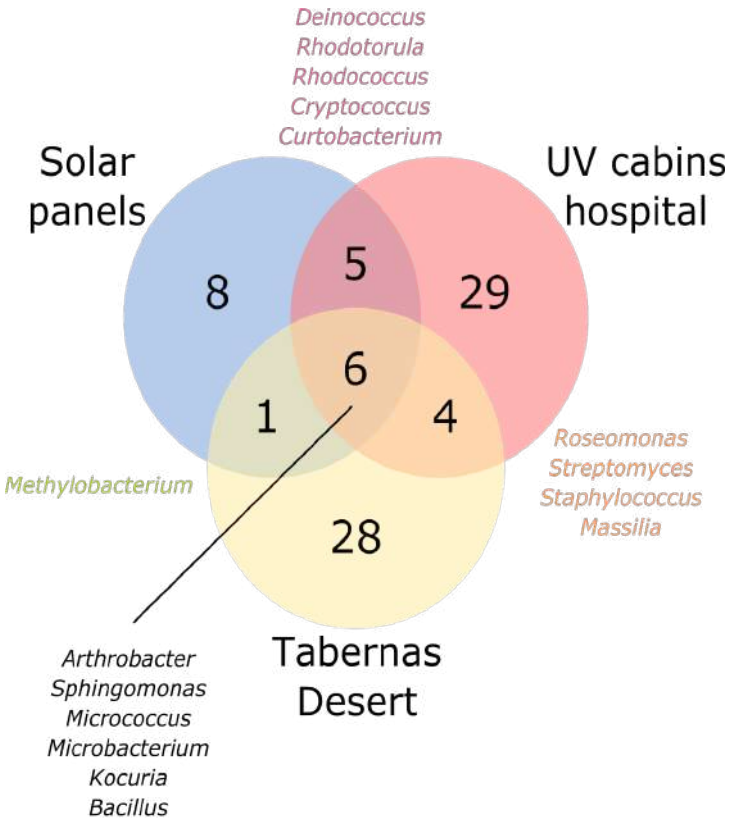

Figure 6. Venn diagram showing the shared and exclusive taxa in the microbial collections established from solar panels samples (data from Dorado-Morales et al. (2016), Porcar et al. (2018) and Tanner et al. (2018)), Tabernas Desert and hospital UV cabins (Publications II and V).

metagenomic and metataxonomic data with respect to the classification of cyanobacterial genera were observed (Publication I).

In terms of culturable microorganisms, the collection of the Mediterranean rocky-shore did not show similarities to the microbial collections established from samples of the Tabernas desert and the UV cabins of the hospital, despite the presence of some *Micrococcus* spp. Particularly, this collection was mainly composed of *Bacillus* and *Halobacillus* species, which is in accordance with its marine or saline origin (Table I.S2) (Liu *et al*., 2017; Mondol *et al*., 2013).

In contrast, both the collection of microorganisms isolated from Tabernas and from the hospital shared features with those already described for solar panels by Dorado-Morales *et al*. (2016), Porcar *et al*. (2018) and Tanner *et al*. (2018) (Figure 6). Specifically, *Arthrobacter*, *Sphingomonas*, *Micrococcus*, *Microbacterium*, *Kocuria* and *Bacillus* spp. were all isolated from these three highly UV-exposed environments. Moreover, the Tabernas and the hospital collections shared also isolates belonging to *Roseomonas*, *Streptomyces*, *Staphylococcus* and *Massilia*, whereas the collections of the hospital and solar panels shared *Deinococcus*, *Rhodotorula*, *Rhodococcus*, *Cryptococcus* and *Curtobacterium*. Therefore, these environments share a biocenosis rich in UV-resistant microorganisms (Rasuk *et al*., 2017; Srinivasan *et al*., 2015).

The combination of culture-independent and culture-dependent techniques allowed a more accurate characterization of the microbial communities. First, it confirmed that the low diversity found in the culturable fraction of microorganisms from the Mediterranean rocky shore was not reliable and was influenced by culturing methods and PCR performance. For example, no cyanobacteria were cultured while they were the most abundant taxa in metagenomics, which may very likely related to the culture media used (Publication I). Then, in the case of Tabernas and the UV cabins revealed that taxa that had not been cultivated, such as *Hymenobacter* or

Rubellimicrobium, characteristic of solar panels, were also inhabiting these environments (Latorre-Pérez *et al*., 2021; Publication V). Moreover, Latorre-Pérez *et al*. (2021) established a parallel microbial collection from biocrust samples of the Tabernas desert. They found a similar diversity to the one described in Publication II and analyzed the differences between culturing and sequencing data. Their comparison revealed that, although it had been possible to identify most of the culturable microorganisms through 16S rRNA gene sequencing, some of the target abundant highly-resistant taxa identified though metataxonomy had not been cultivated in the laboratory. Similarly, *Rubellimicrobium*, *Paracoccus* and *Corynebacterium* were abundant in the UV cabins and only detected through sequencing techniques (Publication V).

As stated in the Introduction, both strategies have limitations in analyzing microbial diversity, but it is by combining them that it is possible to determine in a more reliable way the composition of microbial communities.

3. Biotechnological and biomedical potential of extremophilic microorganisms from highly irradiated and low-water environments

The biotechnological potential of extremophiles has been deeply studied given their applications in industrial, biomedical and environmental biotechnologies, among others (Dumorné *et al*., 2017; Raddadi *et al*., 2015). In the present work, we have explored the potential and the resistance of microorganisms subjected to two main abiotic stressors (UV radiation and desiccation) but also others such as antibiotics (Publications I, V and VII).

The collections of microorganisms established from the sampled sites (i.e., the supratidal zone of the Mediterranean coast, biocrust from the Tabernas Desert and UV cabins of a valencian hospital) revealed a wide diversity of biotechnologically-relevant taxa (Publications I, II and V). Specifically, *Arthrobacter*, *Sphingomonas*, *Micrococcus*, *Microbacterium*, *Kocuria, Bacillus, Roseomonas*, *Streptomyces*, *Staphylococcus* and *Massilia* species were present in both the Tabernas Desert and the Hospital General collection. Moreover, *Bacillus* marine isolates were also part of the Mediterranean rocky-shore collection. The uses of microorganisms within all these genera vary from promoting plant growth (Plant Growth Promoting Bacteria, PGPB), serving as biosynthetic cell factories for industrial purposes (e.g., secondary metabolite production and compound degradation) or producing pigments with antioxidant, antitumor and antimicrobial effect (Table 2). Remarkably, the Scandinavian company Promar AS (Norway) applied for the patent of the use of bacterial pigments that prevented the effects of radiation. The pigment sarcinaxanthin, isolated from a strain of *M. luteus* of Trondheim Fjord (Norway), and related carotenoids were proposed to be used as sunscreens in pharmaceutical and cosmetic compositions (Goksøyr, Patent Application Pub. No. US 2013/0078203 A1, 2013). Of all the described uses, the antioxidant effect of pigment compounds was of interest in this work.

Recent definitions on antioxidants refer to them as substances that prevent or delay undesired oxidation reactions, which may be caused by ROS or RNS (Reactive Nitrogen Species) (Apak, 2019; Halliwell and Gutteridge, 2015). In this regard, the antioxidant potential of strains isolated from the Mediterranean supratidal zone was assessed through both *in vitro* and *in vivo* assays in a *C. elegans* model.

Our comparisons, though, revealed some problems in translation and discrepancies between *in vitro* methods. As stated by Apak (2019) and Apak *et al*. (2013), not only there is a lack of standardization in antioxidant measurement protocols but also the results of two assays may not be identical for the same sample given the thermodynamic and kinetic particularities of each reagent (e.g., DPPH). Thus, measures of antioxidant activity or antioxidant power are controversial. As reported in Publication I, a combination of methods (i.e., H_2O_2 and DPPH assays) may be more informative in order to have an overview of the antioxidant potential of the sample.

Moreover, one of the main disadvantages of *in vitro* testing is failure in reproducing the conditions given in a living organism (*in vivo* assays). In the case of the

Table 2. Biotechnological applications of the genera isolated in the microbial collections of this thesis.

GENUS	LOCATION				USES	REFERENCES
	MED. R-S	TAB. D	HOS. G. V	SOL. PAN		
Arthrobacter		X	X	X	Antioxidant and antitumor pigment production (carotenoid), plant growth promotion and stress management, biosurfactant production and bioremediation of crude oil in soils	Afra et al., 2017; Ali et al., 2020; Bhat et al., 2022; Flegler et al., 2021; Hajfarajollah et al., 2018; Krishnan et al., 2016; Sun et al., 2022; Wietz et al., 2012; Yu et al., 2022
Sphingomonas		X	X	X	Antioxidant pigment production (carotenoid nostoxanthin), plant growth promotion and increased drought tolerance, bioremediation of crude oil in soils and bisphenol A	Asaf et al., 2017; Asker et al., 2007; Kikukawa et al., 2021; Oshiman et al., 2007; Wang et al., 2022 a
Micrococcus	X	X	X	X	Antioxidant pigment production (e.g., carotenoid sarcinaxanthin), plant growth promotion and stress management, bioremediation of crude oil in soils and copper	Ali et al., 2020; Anwar et al., 1977; Bhat et al., 2022; Mafi et al., 2021; Netzer et al., 2010; Ungers et al., 1968
Microbacterium		X	X	X	Antioxidant pigment production (carotenoid), antifungal production and bioremediation of crude oil in soils	Ali et al., 2020; Avramov et al., 2016; Patel et al., 2022; Xie et al., 2021
Kocuria		X	X	X	Antibiotic synthesis and biodiesel production from cooking oil	Najjar et al., 2021; O'Mahony et al., 2001
Bacillus	X	X	X	X	Cell factory and molecule biosynthesis (biotechnological chassis), enzymes production, plant growth promotion and biosurfactant production	Bhat et al., 2022; Contesini et al., 2018; Hajfarajollah et al., 2018; Liu et al., 2019; Schwechheimer et al., 2016
Streptomyces		X	X		Antimicrobial and antioxidant compound production, biodegradation of synthetic polymers, biotechnological chassis for protein expression and secondary metabolites production	Hamed et al., 2018; Hwang et al., 2014; Rammali et al., 2022; Rodríguez-Fonseca et al., 2021
Staphylococcus		X	X		Food industry starter and biotechnological chassis for protein expression	Löfblom et al., 2017
Massilia		X	X		Bioremediation of phenanthrene, antibiotic and anticancer pigment production (i.e., violacein)	Lou et al., 2016; Myeong et al., 2016
Deinococcus			X	X	Robust biotechnological chassis, antioxidant and anticancer pigment production (i.e., deinoxanthin)	Choi et al., 2014; Gerber et al., 2015
Rhodotorula			X	X	Biosynthesis of oleochemicals, carotenoids, enzymes and lipids	Mussagy et al., 2022; Zhao et al., 2021
Rhodococcus			X	X	Biosynthesis of valuable compounds (e.g., biosurfactants, carotenoid pigments, PHAs and antimicrobial substances), biodegradation of organic compounds and pollutants	Cappelletti et al., 2020; Kim et al., 2018
Curtobacterium			X	X	Plant growth promotion and pathogen protection	Bulgari et al., 2014
Methylobacterium		X		X	Pigment production (carotenoid), bioremediation of herbicides and heavy metals and plant pathogen inhibition	Dourado et al., 2015

DPPH assay, the evaluation of the antioxidant power of pigmented molecules was assessed in an organic environment (i.e., methanol), which differs from the physiological conditions given in an organism (in this case, *C. elegans*) (Publication I). Also, Xie and Schaich (2014) outlined some limitations to DPPH-based antioxidant measures in complex samples, such as the bacterial extracts studied, as different molecules may have different behavior in reacting against DPPH radicals. Out results indeed confirm that strains that have proven to confer protection against oxidative stress in a *C. elegans* model give false negative results in a DPPH-based assay.

Having all of this in mind, we decided to explore the biomedical potential application of some of the isolated strains in a model of a human mitochondrial disease (MD) (Publication VII). Previous evidence on antioxidants ameliorating the symptomatology of Leigh Syndrome (LS) supported our hypothesis that bacteria synthetizing antioxidant compounds, such as carotenoids, may have a similar impact (Enns, 2014; Martinelli *et al*., 2012). In the light of these previous reports, a collaboration between our group in the Universitat de València (UV) and the group of Mitochondrial Neuropathology in the Universitat Autònoma de Barcelona (UAB) was set. Our collaboration aimed at studying whether environmental carotenoid-synthetizing bacterial strains isolated from extremophilic environments and synthetizing carotenoids may improve the phenotype of the disease in the Ndufs4KO mice model.

Although probiotics have traditionally been food-related or commensal microorganisms, in the last decade the number of probiotic strains being isolated

or discovered from other sources is growing. Moreover, the use of metagenomic strategies to compare, for example, microbiomes of patients with and without specific conditions (e.g., allergic and non-allergic) allows the identification of differences in microbiota and target taxa to be proposed as probiotics (Papadimitriou *et al*., 2015).

In this regard, we decided to assess the potential of oral administration of a microbial preparation based on strain CR10 identified as *M. luteus*, with the hope that its antioxidant capacity could have beneficial effects on the diseased mice (Publication I). It was not the case, but serendipity led us to identify that the cryopreservant (maltodextrin, MDX) was responsible of the observed effects, which were mainly a reduction in neuroinflammation and increased lifespan (Figure VII.1, Figure VII.2 and Figure VII.4). Also, our preliminary assays and gut microbiota analysis revealed that, although there were no highly differentiated intestinal microbial profiles, the genus *Akkermansia* may be favored by MDX (Figure VII.7). This is of interest because the genus *Akkermansia* has recently been associated with changes in inflammation and proposed as a potential probiotic (Wang *et al*., 2022 b; Zhang *et al*., 2019). Ironically, after discarding the effect of microorganisms in microbial preparations on the observed phenotype, a possible 'microbial connection' mediated by MDX arises as a potential key factor in LS. This could hopefully one day be partially addressed through the modification of the gut microbiota, as MDX may be doing.

The hypothesis that MDX has an effect on the gut microbiota, which in turns modulates the response to the disease is very tempting. The use of pre- and probiotics in other neurodegenerative disorders, such as Parkinson's or Alzheimer's disease, has already been reported. As gut microbes modulate neurotransmitter and immune responses in the host, alterations in microbiota may also contribute to developing these disorders. Thus, both probiotics and prebiotics are effective in ameliorating the symptoms and preventing diseases progression (Abraham *et al.*, 2019; Tamtaji *et al*., 2019). Moreover, other increasing public health problems such as obesity are also being addressed through microbial supplementation (Green *et al*., 2020). For example, strains of *Limosilactobacillus* have been explored given the antioxidant protection that they confer in the gut as probiotics. However, research is needed in this field in order to elucidate the real antioxidant potential of each strain, the doses and treatment times required to obtain specific effects and to find reliable evidence of their effectivity in clinical trials (Paulino do Nascimento *et al*., 2022).

Finally, the potential of yeast as cell factories in biotechnology and food industries is well reported (Karim *et al*., 2020; Wagner and Alper, 2016). Given the urgent need for developing more sustainable production systems, the use of microorganisms that somehow increase their biosynthetic abilities in less water or with lower energy requirements is of great interest. In this regard, SSF strategies hold advantages over SmF, such as lower production costs (e.g., low equipment, low price of substrates, minimum sterility requirements) and higher yields (Doriya *et al*., 2016; Hölker and Lenz, 2005). But above all, SSF strategies change the metabolic fluxes and biosynthetic abilities of microorganisms, which may constitute indeed an opportunity to explore new biotechnological uses of microorganisms (Publication VI; Hölker and Lenz, 2005).

Our work on SSF aimed at studying whether solid matrixes may influence yeast growth and their impact on biotechnology. Specifically, the main goal was to propose a standardized method for SSF as the lack of reproducibility and standardization in these fermentation processes has been reported time and again (Hölker and Lenz, 2005; Singhania *et al*., 2009). Although we did not try to scale up the results, we found that the use of standard matrixes results in consistent and reproducible results (Publication VI).

Further work on this area may attempt to elucidate the impact of matrixes on routes that may be of interest for industrial product biosynthesis, to characterize metabolic fluxes and modify them, as well as to translate the results into larger fermentation volumes, as it has already been reported

that some specific secondary metabolites are solely synthetized during growth on solid substrates (Hölker and Lenz, 2005)

4. Concluding remarks and future prospects

Along this thesis we have outlined the biotechnological potential of environmental microbial strains isolated from extreme environments. However, the development of biotechnological tools based on microorganisms needs further work on the deep characterization of their genotypic and phenotypic characteristics. Moreover, the use of microorganisms and their products requires a detailed comprehension of their biosynthetic abilities, with special attention to their behavior in large-scale production processes. In this regard, not only a full characterization of microorganisms but also scaling-up experiments would be needed to assess their industrial potential (Linke *et al*., 2023).

Therefore, further research on the potential of the identified taxa would be required in order to define real uses of the isolates within microbial collections. For example, compound biosynthesis (e.g., carotenoids) or stress tolerance (e.g., drought resistance and pollutant bioremediation) are relevant work areas in current microbial biotechnology (de Lorenzo, 2022; Revuelta *et al*., 2016). Biotechnological applications need experiments that go beyond defining the possible uses of each strain and testing their abilities under laboratory conditions (e.g., antioxidant activity measures or survival to desiccation). For that reason, experiments that allow the translation of results into real-life applications would be required and constitute an underexplored work field in this thesis (e.g., *in vivo* testing of antioxidant compounds in cell-lines or experimental models, enhancing product biosynthesis in low-water activity environments through genetic engineering, scale-up experiments and mid- and large-batch fermentation trials) (Dassprakash *et al*., 2012; López *et al*., 2021).

Regarding the assessment of the biomedical potential of potentially antioxidant microbial species, this work has just outlined the possible use of extremophilic bacteria in therapeutics given their ability to synthetize antioxidant compounds and preliminarily explored their effect. As reviewed by Gulcin (2020), understanding the antioxidant mechanisms and reaction kinetics of specific molecules is important in the screening of antioxidants. Thus, further research on identifying which molecules and mechanisms are responsible of the observed effect and the conditions under which the strains, or their products, are effective is urgent. Moreover, deeply investigating the biosynthetic routes and the most favorable conditions for their production in cell factories is crucial for their real application.

With respect to the studies on LS, our hypothesis that microbial strains from extreme niches may be used in therapeutics for mitochondrial diseases has not been confirmed to date. However, our findings on the possible role of MDX modulating the gut microbiota and inflammation suggest a possible role of this sugar as a prebiotic, whichever are the underlying mechanisms. Therefore, there is still an open field of research to unveil the function of this compound in the animal model, as well as to study a possible microbial approach based on *Akkermansia* spp. Nevertheless, other antioxidant synthetizing microorganisms may be further investigated in this area.

Additionally, the known biosynthetic potential of yeast cell-factories may be exploited by testing new culturing techniques, such as the previously described 3D-SSF matrixes. Not only scaling-up experiments, but also genetic engineering and metabolic flux modeling would be worthwhile research areas in the synthesis of biotechnologically-valuable compounds. For example, some authors have reported the deletion of *ADH2* gene, among others, to increase ethanol yield in *S. cerevisiae* fermentation, which is overexpressed under SSF conditions (Yang *et al*., 2022; Publication VI). Moreover, alternative materials (e.g., autoclavable plastics that reduce workload) and geometric designs may also be explored.

Although the identification and characterization of microbial taxa is just a first step in the biotechnological pipeline, it is still a relevant task in

microbial ecology and taxonomy, as the approximation to the microbial dark matter for the development of new microbial biotechnological tools may only be addressed through the discovery of new microorganisms. Indeed, new and unconventional microbes hold huge biotechnological potential through genetic engineering for industrial applications (Blombach *et al*., 2022; Vartoukian *et al*., 2010).

Ultimately, and as last concluding remarks, highly-irradiated and dry environments host both known and yet unknown microorganisms with enough potential to be explored for creative and innovative biotechnological applications. The unveiled microbial dark matter of our planet will be, certainly, a source of new and efficient developments for the biomedical and environmental sectors, which is dramatically needed in the present situation of climatic and environmental crisis.

Conclusions

In the present thesis, the ecology and biotechnological potential of natural and artificial highly irradiated and dry environments has been explored. The general conclusions drawn from this work are listed below:

- The combination of culturing strategies (media combination, extended incubation times and different incubation temperatures) results in obtaining highly diverse microbial collections, which has a profound impact on their biotechnological potential.
- Different locations along the Mediterranean rocky shore host a stable biocenosis rich in biotechnologically relevant microbial strains with biomedical potential.
- Biocrust of the Tabernas Desert host diverse microbial communities in which a significant fraction of isolates could represent new bacterial species. Among them, three *Kineococcus* and two *Belnapia* species have been described and fully characterized.
- The microbial communities associated to UV cabins in a hospital facility is composed of both typically environmental microorganisms adapted to highly-irradiated environments (e.g., solar panels) and hospitals, and the analysis of biological activities reveals no significant threats in the selection of UV and antibiotic resistant microorganisms.
- The application of NGS in different extreme environments, such as the supralittoral zone and UV phototherapy cabins, has allowed the comparison of microbiomes subjected to irradiation and desiccation, and reveals differences and similarities with other sun-exposed environments, specifically regarding the presence of highly-resistant microorganisms such as *Truepera* spp. or *Deinococcus* spp., among others.
- Yeast display different growth and protein expression patterns when they are cultivated under SSF conditions through the use of solid 3D-printed matrixes, compared to traditional SmF culturing, which has implications on biosynthetic processes driven by yeast cells and opens research to enhancing the production of metabolites and/or genetic engineering. The use of these solid matrixes allows high flexibility regarding the assessment of alternative designs.
- The administration of microbial preparations resulted in the serendipitous discovery of the beneficial effects of MDX in ameliorating the symptomatology of LS, which are increased lifespan and reduced neuroinflammation. Moreover, MDX may play a role in shaping the gut microbiota of treated mice, as evidenced by increased variability in the abundance of *Akkermansia* spp.

Resumen en castellano

Introducción

1. Diversidad microbiana

La biodiversidad de nuestro planeta está dominada por microorganismos. Se ha estimado que la Tierra alberga de 10^{11} a 10^{12} especies microbianas, lo que podría traducirse en un total de 10^{30} células procariotas viviendo en nuestro planeta. Sin embargo, la inmensa mayoría de ellas son todavía desconocidas y/o no se han cultivado nunca en el laboratorio (Locey y Lennon, 2016). A pesar de que la mayor parte de la diversidad microbiana se concentra en los llamados 'cinco grandes' hábitats (el subsuelo profundo oceánico, el subsuelo profundo continental, el suelo, los sedimentos oceánicos y los océanos), los procariotas habitan todos los nichos ecológicos posibles y colonizan incluso los ambientes más remotos, aislados y, en ocasiones, extremos (Flemming y Wuertz, 2019; Rampelotto, 2013; Shu *et al*., 2022). Es precisamente en esos ambientes, adversos y extremos desde el punto de vista antropológico, de donde se han desarrollado muchas herramientas y productos biotecnológicos innovadores y revolucionarios.

1.1 Diversidad microbiana

Estimar el número de organismos vivos en la Tierra ha sido históricamente uno de los intereses de los ecólogos. Esto se ha extendido al mundo microbiano en las últimas décadas (Pedrós-Alió y Manrubia, 2016). La diversidad microbiana está en el punto de mira de muchos estudios desde el desarrollo de las técnicas metagenómicas, que permiten estudios a gran escala acerca de la estructura, función y evolución de los microbiomas (Haegeman *et al*., 2013). El desarrollo y uso extendido de las técnicas de secuenciación de nueva generación (Next Generation Sequencing, NGS), que incluyen la secuenciación metagenómica de genomas completos, la secuenciación del ARN (metatranscriptómica) y la secuenciación masiva del gen 16S del ARN ribosómico (metataxonomía), ha hecho disponible información valiosa sobre taxones, genes y propiedades metabólicas de las especies y comunidades microbianas que era anteriormente desconocida (Wensel *et al*., 2022).

Sin embargo, evaluar y estimar la diversidad es todavía una tarea difícil y sujeta a gran debate. Desde el punto de vista taxonómico, las medidas más habituales de diversidad se basan en la estimación del número de especies microbianas o de sus abundancias relativas en ambientes específicos. Los principales parámetros utilizados son α-diversidad (riqueza o número de taxones observados en una muestra) y β-diversidad (variabilidad en las comunidades microbianas entre muestras) (Walters y Martiny, 2020). Estas estimaciones, no obstante, no son fáciles. En primer lugar, la definición de especie en el mundo procariota es controvertida. Por eso, en su lugar, en estudios de metataxonomía se utilizan los conceptos de unidad taxonómica operativa (Operational Taxonomic Unit, OTU) o, más recientemente, variantes de secuencia de amplicones (Amplicon Sequence Variants, ASVs) (Callahan *et al*., 2017; Pedrós-Alió, 2006). En segundo lugar, se hacen asunciones sobre las abundancias de las especies microbianas debido a las dificultades al medir especies raras o las comunidades altamente diversas llevan a (Haegeman *et al*., 2013).

La diversidad microbiana varía entre ecosistemas y está influenciada por factores abióticos, como el pH, la desecación, la temperatura o la salinidad, no solo desde el punto de vista taxonómico sino también desde el punto de vista metabólico y funcional (Ruhl *et al*., 2022). Bajo condiciones extremas, la diversidad microbiana tiende a decrecer, y también lo hace la diversidad funcional (Fierer y Jackson, 2006; Maestre *et al*., 2015; Rousk *et al*., 2010; Ruhl *et al*., 2018; Sharp *et al*., 2014; Yang *et al*., 2016). Sin embargo, en términos de funciones específicas relacionadas con la supervivencia bajo esas condiciones, se ha encontrado un enriquecimiento en genes asociados a la resistencia a estrés osmótico o estado de latencia, así como una reducción en genes implicados en las relaciones entre especies (como resistencias a antibióticos o factores de virulencia) (Song *et al*., 2019). Curiosamente, en un *permafrost* en deshielo,

Yuan *et al*. (2018) describieron una decreciente diversidad funcional y una creciente diversidad en la comunidad en áreas moderada y ampliamente desheladas.

Los genomas microbianos y las características metabólicas pueden ayudar a entender mejor la diversidad microbiana. Esta diversidad funcional se ignora con frecuencia, pero es crucial para entender cómo se modelan las comunidades microbianas y cómo se comportan bajo diferentes condiciones (Johnson y Pomati, 2020; Li *et al*., 2015; Miller *et al*., 2009). De hecho, la diversidad funcional podría dar una información más valiosa y fiable que los perfiles taxonómicos acerca de los procesos que tienen lugar en un ambiente específico y sus propiedades (Fontana *et al*., 2018; Hillebrand y Matthiessen, 2009).

La secuenciación masiva del gen 16S del ARN ribosómico (o metataxonomía) presenta algunas limitaciones a pesar de su uso extendido como estrategia NGS de bajo coste. La metataxonomía tiene resolución limitada y da una descripción general de los perfiles taxonómicos bacterianos no más allá del nivel de género, a consecuencia del error asociado a las tecnologías utilizadas. La necesidad de amplificación previa de ácidos nucleicos produce sesgos que favorecen ciertos grupos taxonómicos y dificulta la estimación de las abundancias reales, y además aporta poca información sobre los perfiles funcionales (Johnson *et al*., 2019; Winand *et al*., 2019).

La metagenómica, sin embargo, permite una identificación más precisa: no hay sesgos de amplificación, considera también la información funcional, y tiene mayor poder de detección de taxones específicos menos abundantes. Sin embargo, requiere una mayor inversión, mayores concentraciones de ADN, las bases de datos no están tan completas, es difícil ensamblar genomas de taxones menos abundantes y el ADN del huésped puede interferir con la detección de la microbiota de interés en el estudio (Breitwieser y Salzberg, 2019; Durazzi *et al*., 2021; Wensel *et al*., 2022). No obstante, la elección de una técnica u otra dependerá de los objetivos y la pregunta biológica a contestar.

La NGS ha cambiado drásticamente la manera en la que se entienden la microbiología y la biotecnología. En microbiología clínica, puede fácilmente asistir y guiar el diagnóstico y tratamiento de enfermedades infecciosas. En ecología microbiana, ha revelado una gran diversidad microbiana hasta ahora desconocida; mientras que en bioprospección puede mejorar el minado de biomoléculas de manera eficiente (Gaston *et al*., 2022; Gu *et al*., 2019; Maansson *et al*., 2016; Yu *et al*., 2020).

1.2 Cultivabilidad y la materia oscura microbiana

Si la Tierra alberga 10^{11}-10^{12} especies microbianas, solo 10^{4} han podido ser cultivadas (Locey y Lennon, 2016). La evidencia de que el conteo de células viables recuperadas en cultivos estaba lejos de los conteos realizados bajo el microscopio se denominó 'la gran anomalía del recuento en placa', que sugería que muchas células se resistían a ser cultivadas (Staley y Konopma, 1985). Aunque los microorganismos se descubrieron en el siglo XVII gracias a la invención de los microscopios, los primeros cultivos no se llevaron a cabo hasta el siglo XIX.

Desde entonces, el descubrimiento de nuevos microorganismos ha dependido fuertemente de su aislamiento en cultivo puro. Sin embargo, las técnicas de cultivo han sesgado sistemáticamente el cultivo de microorganismos hacia unos cuantos taxones fáciles de crecer (Overmann *et al*., 2017). Por ello, el desarrollo de la NGS permitió la identificación de un gran número de taxones que nunca habían sido aislados en laboratorios. Esta diversidad de especies incultivables, sumado a la información genética codificada en sus genomas, los biomas dependientes de contexto, y las dinámicas ecológicas y evolutivas de las poblaciones, es lo que se conoce como la materia oscura microbiana (Zha *et al*., 2022).

La NGS, especialmente la metagenómica, ha arrojado luz no solo en el descubrimiento de nuevos taxones, sino también en entender el comportamiento de esos taxones dentro de las comunidades microbianas. La microbiología actual se centra en el aislamiento de esos microorganismos incultivables dado que la infraexplorada materia oscura microbiana podría

codificar una también infraexplorada diversidad metabólica útil para aplicaciones biotecnológicas (Overmann *et al*., 2017; Rinke *et al*., 2023).

A pesar de que la NGS da información útil sobre las especies existentes y la diversidad genética, la bioprospección y el desarrollo de nuevas herramientas biotecnológicas basadas en microorganismos todavía depende de su aislamiento en condiciones de laboratorio. De hecho, la caracterización completa y detallada de las características fisiológicas de las especies bacterianas solo es posible a través del cultivo (Vartoukian *et al*., 2010). Aunque no toda la diversidad microbiana puede ser, hasta ahora, cultivada, las técnicas moleculares a menudo fallan en detectar taxones raros que sí son accesibles a través de técnicas de cultivo (Pedrós-Alió, 2006). Los cultivos son, por tanto, indispensables todavía para la microbiología.

No obstante, la información funcional obtenida a través de la NGS es útil para guiar y asistir el diseño de protocolos de aislamiento y medios de cultivo. En este sentido, las tecnologías de secuenciación *in situ* como la secuenciación Nanopore puede usarse para detectar puntos calientes de diversidad microbiana en expediciones de bioprospección (Latorre-Pérez *et al*., 2021). Es, por tanto, importante la combinación tanto de estrategias experimentales dependientes de cultivo como independientes de cultivo para recoger tanta información como sea posible de los ambientes estudiados, y seleccionar así las condiciones adecuadas para el aislamiento de microorganismos nuevos o diana (Satari *et al*., 2020; Tidjani Alou *et al*., 2020; Vidal-Verdú *et al*., 2022 a) (Figura 1).

Se están haciendo, por tanto, grandes esfuerzos para desarrollar técnicas innovadoras de cultivo que nos permitan aislar la diversidad todavía no cultivada y explotar el potencial biotecnológico de sus metabolismos (Figura 1).

1.3 Retos en culturómica y nuevas estrategias de cultivo

Tal y como dijo Pedrós-Alió (2007), cualquier microorganismo sería susceptible de ser cultivado si se dieran las condiciones adecuadas para su crecimiento. Sin embargo, cultivar microorganismos es todavía difícil dada la complejidad de las comunidades microbianas naturales y las diferencias entre las condiciones de laboratorio y sus hábitats naturales. Los medios de cultivo tradicionales ofrecen un ambiente estático y homogéneo para el crecimiento de las especies individualmente, a diferencia del ambiente natural en el que viven las bacterias y otros organismos, que es diverso, cambiante y heterogéneo (Overmann *et al*., 2017).

Los motivos para la actual imposibilidad de cultivo de la mayoría de los taxones son diversos, pero pueden clasificarse en seis categorías, tal y como revisa Lewis *et al*., (2021): identificación de los sustratos y condiciones de crecimiento (fuentes de carbono y nitrógeno, vitaminas, cofactores, compuestos inorgánicos, etc.), salida de estado de latencia, interdependencias simbióticas, contacto físico o proximidad espacial, condiciones ambientales fisicoquímicas (temperatura, pH, salinidad, condiciones redox, etc.), y baja abundancia y competencia.

De ellas, la identificación de los sustratos adecuados y de las condiciones ambientales fisicoquímicas óptimas puede abordarse a través de estrategias simples como la combinación y el diseño de nuevos medios de cultivo (Figura 2E). Por ejemplo, Kaboré *et al*. (2019) describieron un innovador medio de cultivo para bacterias marinas que incluía esqueleto de esponjas dada la relación simbiótica que las bacterias del género *Gemmata* establecen con las esponjas marinas en su hábitat. Por su parte, Vartoukian *et al*. (2016) describieron la importancia de la suplementación con sideróforos y especies ayudantes (*helper*) en el aislamiento de bacterias antes incultivadas de la microbiota oral. Además, los medios de cultivo de laboratorio más ampliamente utilizados a menudo contienen elevadas concentraciones de nutrientes que inhiben el crecimiento de bacterias oligótrofas, en favor de las cepas copiotrofas, pero los ambientes marinos, por ejemplo, son en muchos casos ambientes oligótrofos (Yin *et al*., 2013). En este caso, el uso de agua de mar filtrada y autoclavada ha resultado exitoso para el aislamiento de bacterias

marinas oligótrofas, y el uso de medios convencionales diluidos aumenta la diversidad de las comunidades microbianas cultivadas (Figura 2D) (Cho *et al*., 2004; Sun *et al*., 2019).

Otras mejoras en los métodos de cultivo imitan las condiciones ambientales en las que las bacterias viven de manera natural mediante estrategias más sofisticadas, como el llamado ichip (Nichols *et al*., 2010). Además, el aislamiento en cultivo puro de las bacterias que viven en consorcios no es siempre posible, dado que pueden necesitar crecer cerca unas de otras para desarrollar colonias visibles. Por ello, se están estableciendo co-cultivos (D'Onofrio *et al*., 2010).

- Ichip

El chip de aislamiento o ichip es un dispositivo que consiste en un conjunto de pequeñas cámaras de difusión que son inoculadas y cultivadas *in situ* en el punto de muestreo (Figura 2A). De este modo, las células microbianas crecen exactamente en sus condiciones ambientales naturales. Este dispositivo se ha utilizado para cultivar bacterias antes incultivables, para aislar bacterias degradadoras de compuestos tóxicos o para descubrir nuevos antibióticos (Lodhi *et al*., 2018; Nichols *et al*., 2010; Polrot *et al*., 2022; Sherpa *et al*., 2015).

- Dilución a extinción

Este método se basa en el uso de medios de cultivo de baja concentración de nutrientes y en la dilución de las muestras de células hasta conseguir un número pequeño y conocido de ellas, con el objetivo de crecer células individuales en placas multipocillo (Figura 2B). Ha resultado muy útil en el aislamiento de nuevas bacterias marinas oligótrofas, pero también ha permitido la caracterización de bacterias que producen compuestos antitumorales (Benítez *et al*., 2021; Buchholz *et al*., 2021; Castro *et al*., 2017; Derrien *et al*., 2004; Song *et al*., 2009).

- Co-cultivos

Las técnicas de co-cultivo se basan en el crecimiento de bacterias de manera que diferentes especies pueden interaccionar, de la misma forma que lo hacen en sus hábitats naturales (Figura 2C) (Goers *et al*., 2014). La razón por la que los co-cultivos son interesantes no es solo el estudio de cómo diferentes especies se relacionan y comunican, sino también la mejora y favorecimiento de su crecimiento bajo condiciones de laboratorio, ya que algunas de ellas necesitan los consorcios microbianos para crecer. Además, los co-cultivos son también interesantes en la búsqueda de nuevos compuestos antimicrobianos y metabolitos secundarios, pues muchas rutas biosintéticas están silenciadas en condiciones tradicionales de cultivo en laboratorio (Arora *et al*., 2020; Bertrand *et al*., 2014; Lodhi *et al*., 2018).

Finalmente, dado que los microorganismos aislados en cultivo puro pueden diferir dependiendo de las técnicas de cultivo empleadas, considerar la aplicación en paralelo de diferentes estrategias o condiciones de cultivo es una manera factible de aumentar fácilmente la diversidad microbiana cultivable (Figure 2F) (Köpke, 2005; Vartoukian *et al*., 2010).

2. Bioprospección de ambientes altamente irradiados y secos

La bioprospección es la búsqueda de microorganismos, o de sus partes (i.e., enzimas), para el desarrollo de herramientas biotecnológicas con aplicaciones biomédicas, farmacéuticas, agrícolas o industriales (Ayilara *et al*., 2022; Saeed et a., 2022; Stirk *et al*., 2022; Tanner *et al*., 2017). Los ambientes altamente irradiados y secos, bien naturales (e.g., desiertos) o bien artificiales (e.g., placas solares), poseen características específicas que los hacen interesantes desde la perspectiva biotecnológica, pues los microorganismos que viven en ellos desarrollan mecanismos para lidiar con altas dosis de radiación (en particular, radiación UV) y desecación.

La radiación, en concreto la radiación solar, incluye infrarrojo, luz visible y luz ultravioleta (UV), que puede dividirse a su vez en UV-A, UV-B y UV-C. La luz UV más dañina, UV-C, se filtra en la capa de ozono estratosférica de la atmósfera. Por ello, la radiación solar UV que llega a la superficie de la Tierra es mayoritariamente UV-A y algo de UV-B (Hargreaves *et*

al., 2007). No obstante, es suficiente para tener un impacto en los organismos vivos y para modelar la biocenosis microbiana que vive en áreas expuestas (Dorado-Morales *et al*., 2016).

La desecación, por otra parte, es una característica intrínseca de las tierras áridas y hace referencia a la falta de agua. Las tierras áridas, que representan el bioma más extenso de la superficie terrestre, incluyen ambientes desérticos, que se caracterizan por las precipitaciones escasas y pueden ser a su vez clasificados en desiertos cálidos (los desiertos del Sáhara, Mojave, Atacama o Tabernas, entre otros) y desiertos fríos (como la Antártida). En el contexto del cambio climático, se espera que aumenten las tierras áridas en los próximos años. Por ello, entender cómo los microorganismos viven y se adaptan a estos ambientes es de gran relevancia para afrontar los retos de esta amenaza global. De hecho, el uso de microorganismos que han estado expuestos a estrés por sequía, entre otros, ya se ha explorado como una posibilidad para mejorar la respuesta e incrementar la resiliencia de los bosques al cambio climático (Ahmed *et al*., 2022; Allsup *et al*., 2023; Makhalanyane *et al*., 2015; Maestre *et al*., 2015).

2.1 Nichos polares

Aunque los ambientes polares en los polos ártico y antártico comparten algunas características climáticas y registran las temperaturas más bajas del globo, las condiciones ambientales en ambas localizaciones son diferentes. La Antártida es extremadamente seca y se considera un desierto frío, mientras que en el Ártico algunas áreas registran tormentas e inviernos húmedos. No obstante, las superficies microbianas polares están expuestas a elevadas dosis de radiación solar UV dada la reducción de ozono en la estratosfera, que se da en ciclos de luz-oscuridad de seis meses (Cowan *et al*., 2014; Dahms *et al*., 2011; Di Giuseppe *et al*., 2012).

Pese a la variedad de estreses abióticos a los que los organismos vivos están sometidos en las regiones polares (e.g., congelación, temperaturas bajas y baja disponibilidad de nutrientes), estos ambientes todavía albergan nichos adecuados para el crecimiento de microorganismos (Cowan *et al*., 2014; Varin *et al*., 2012). En especial, los nichos terrestres como los tapetes microbianos o el *permafrost*, revelaron diversidades microbianas más elevadas de lo esperado, también con prevalencia de taxones altamente resistentes como *Deinococcota* (anteriormente *Deinococcus-Thermus*), en comparación con ambientes menos hostiles (Cowan *et al*., 2014). Además, el estudio de diferentes nichos microbianos asociados a plantas en la Antártida reveló microbiomas principalmente compuestos por *Actinomycetota*, *Pseudomonadota* y *Bacillota* (antes *Actinobacteria*, *Proteobacteria* y *Firmicutes*, respectivamente) (Zhang *et al*., 2020).

Sin embargo, en el contexto del cambio climático, se espera que se alteren las comunidades microbianas que viven en estas áreas. Especialmente, el *permafrost* y los glaciares en deshielo aumentarán la humedad y humedecerán nichos antes secos, y ya se han registrado incrementos en la radiación solar (Cowan *et al*., 2014; Turner y Overland, 2009).

El potencial biotecnológico de los microorganismos de regiones polares reside en el aislamiento y la caracterización de enzimas adaptadas al frío, como esterasas, lipasas, ADNasas y proteasas, pero también en la síntesis de pigmentos como respuesta a estreses abióticos (e.g., radiación UV) (De Santi *et al*., 2016; Silva *et al*., 2021). Además, las bacterias polares han demostrado biorremediar compuestos tóxicos, como el perclorato (Acevedo-Barrios *et al*., 2022).

2.2 Desiertos

Aunque los desiertos son tradicionalmente considerados hábitats sin vida, albergan una amplia diversidad microbiana. De hecho, la superficie del suelo en tierras secas como los desiertos áridos alberga una biocenosis microbiana específica y bien adaptada dominada por bacterias extremófilas (Belov *et al*., 2018; Perera *et al*., 2018).

Las comunidades microbianas que habitan los desiertos cálidos están principalmente compuestas por microalgas, cianobacterias y bacterias de los filos *Actinomycetota* y *Pseudomonadota*, seguidos de *Bacillota*, de manera similar a aquellos descritos

anteriormente para los suelos polares, pero también hay representantes del filo altamente resistente *Deinococcota* (Belov *et al*., 2018; Perera *et al*., 2018; Zhang *et al*., 2020). Estos microorganismos son capaces de lidiar con elevada radiación UV, altas temperaturas y baja disponibilidad de agua y nutrientes, entre otros estreses (Azua-Bustos *et al*., 2012; Saul-Tcherkas *et al*., 2013). Esta diversidad microbiana, que tiene gran potencial biotecnológico, está también amenazada por la creciente aridez derivada del cambio climático (Azua-Bustos y González-Silva, 2014; Maestre *et al*., 2015).

Por ejemplo, cepas patentadas de *Acidithiobacillus* aisladas del desierto de Atacama son útiles en biolixiviación (extracción de metales por microorganismos), que es de interés para las operaciones de biominado (Ohata *et al*., 2013; Sugio *et al*., 2009). Otras cepas son útiles en biorremediación (e.g., biorremediación de arsénico en agua potable), que es más eficiente que el proceso químico convencional y los métodos físicos de remediación (Campos *et al*., 2009). Además, para biomedicina, la abundancia de bacterias productoras de antibióticos es especialmente relevante (Astakala *et al*., 2022; Azua-Bustos y González-Silva, 2014; Goodfellow *et al*., 2018; Rateb *et al*., 2011; Schulz *et al*., 2011).

2.3 *Biocrusts*

Las costras biológicas del suelo (i.e., *biocrusts*) son asociaciones de partículas del suelo y microorganismos, entre los cuales podemos encontrar bacterias, algas, arqueas y briófitos, distribuidas por todo el planeta, características de los ecosistemas donde escasea el agua y hay escasa vegetación vascular. Se encuentran en regiones secas a híper áridas, pero también en ambientes fríos como las regiones polares, en las que la congelación limita la disponibilidad de agua (Weber *et al*., 2022).

Los *biocrusts* son importantes en las tierras áridas. Influyen en las características de los suelos de alrededor y, por tanto, en las comunidades microbianas que se establecen. Además, los musgos en los *biocrust* participan en aliviar los efectos del cambio climático y la creciente aridez en las comunidades microbianas (Delgado-Vaquerizo *et al*., 2018). El interés biotecnológico de los microorganismos en *biocrusts* recae en su papel en la restauración de suelos y en la lucha contra la desertización y otras consecuencias del cambio climático (Chilton *et al*., 2022; Maestre *et al*., 2017). Además, se han descrito microorganismos aislados de *biocrust* que sintetizan metabolitos secundarios biotecnológicamente relevantes, como el protector solar escitonemina, y exopolisacáridos (EPS) (Couradeau *et al*., 2016; Xue *et al*., 2021).

2.4 Placas solares

El microbioma de las placas solares alrededor del planeta ha sido estudiado en la última década. Las placas solares han demostrado ser un ambiente similar al de los desiertos colonizado por bacterias adaptadas a tolerar estreses característicos de los mismos (irradiación, fluctuaciones de temperatura, baja disponibilidad de nutrientes y desecación) (Dorado-Morales *et al*., 2016). Además, un estudio sobre el proceso de colonización de placas solares sugirió que las partículas de polvo en suspensión transportadas por el aire podrían ser el origen del microbioma de las placas solares. Esto estaría apoyado por la evidencia de que algunos de los taxones más abundantes (*Deinococcus* e *Hymenobacter*), son también marcadores bacterianos aerotransportados del desierto (Meola *et al*., 2015; Tanner *et al*., 2020).

La biocenosis microbiana que vive en las placas solares de todo el mundo está dominada por *Deinococcus*, *Sphingomonas* e *Hymenobacter*. Estos taxones son bien conocidos por su robustez frente a estreses abióticos. Sin embargo, su dominancia en el microbioma de las placas solares llega con el tiempo, ya que las condiciones ambientales que experimentan modelan la microbiota desde taxones generalistas, inicialmente, hacia una comunidad más especializada y adaptada. Este patrón está apoyado por las grandes similitudes encontradas entre placas solares distribuidas en diferentes latitudes. (Dorado-Morales *et al*., 2016; Lim *et al*., 2019; Liu *et al*., 2022; Moura

et. al., 2021; Park *et al*., 2022; Porcar *et al*., 2018; Tanner *et al*., 2018; Tanner *et al*., 2020).

Las bacterias que viven en las placas solares son interesantes desde el punto de vista farmacéutico y biotecnológico dada su habilidad para sintetizar compuestos antioxidantes, como los carotenoides. Estos carotenoides han demostrado su poder antioxidante y protección frente al estrés oxidativo en un modelo de *Caenorhabditis elegans* (Tanner *et al*., 2019). Además, bacterias robustas como *Deinococcus* se estudian como chasis para fines industriales, dado que los procesos a los que se ven sometidos son habitualmente duros, y bacterias del género *Sphingomonas* son útiles en biorremediación (Gerber *et al*., 2015; White *et al*., 1996).

3. Potencial industrial de los extremófilos

La extremobiosfera comprende todos los biomas sujetos a condiciones ambientales severas. Estas condiciones varían desde desecación extrema (e.g., desiertos hiperáridos), altas presiones (e.g., sedimentos profundos en el mar), temperaturas extremas (e.g., suelos de *permafrost* o desiertos cálidos), baja disponibilidad de nutrientes (e.g., tapetes polares), elevada irradiación y salinidad, etc. (Sayed *et al*., 2020).

Los ambientes extremófilos han demostrado ser ricos en microorganismos biotecnológicamente relevantes (Tabla 1). Los ambientes raros, inusuales y extremos albergan microbios adaptados a vivir bajo esas condiciones. Estos microorganismos han desarrollado mecanismos para sobrevivir y lidiar con los estreses resultantes. Los mecanismos y los productos derivados de ellos tienen gran potencial desde los puntos de vista ecológico, agrícola, industrial, farmacéutico y biomédico (Dumorné *et al*., 2017; Kochhat *et al*., 2022).

3.1 Extremozimas

Las extremozimas, que son las enzimas aisladas de organismos extremófilos, tienen aplicaciones industriales dada su natural robustez (e.g., termoestabilidad y tolerancia a sal). Las extremozimas son muy valiosas porque la mayoría de procesos industriales tienen lugar en condiciones hostiles de temperatura, salinidad o pH (Dumorné *et al*., 2017; Sarmiento *et al*., 2015).

Una de las extremozimas más conocidas es la *taq* polimerasa utilizada en biología molecular para la Reacción en Cadena de la Polimerasa (PCR). Esta polimerasa se aisló por primera vez de la bacteria termófila *Thermus aquaticus* que vive en fuentes termales (Chien *et al*., 1976). Además, se han caracterizado otras enzimas termófilas como celulasas, amilasas, proteasas o lipasas, así como otras enzimas degradadoras de polímeros (van der Burg., 2003). Por ejemplo, los detergentes contienen enzimas termófilas y psicrófilas con actividades proteasa, lipasa, celulasa y amilasa (Sarmiento *et al*., 2015; van der Burg., 2003). Del mismo modo, las enzimas activas a bajas temperaturas son también de interés para las industrias alimentaria, cosmética y farmacéutica (van der Burg., 2003).

Algunas extremozimas son activas en ambientes de elevada concentración de sales o de pH extremo. Las enzimas halófilas son de interés para procesos sintéticos en baja actividad de agua y en presencia de solventes orgánicos (Klivanov, 2001; Raddadi *et al*., 2023), mientras que las acidófilas y alcalófilas son de interés para la industria alimentaria, la producción de biofuel y etanol, los detergentes y el biominado (Dumorné *et al*., 2007; Golyshina y Timmis, 2005; van der Burg, 2003).

El biodescubrimiento de nuevos microorganismos extremófilos y sus extremozimas es una oportunidad para la industria de cambiar hacia un sistema de producción más sostenible. En concreto, el uso de bacterias que pueden crecer rápido en condiciones versátiles (amplio rango de temperaturas, uso de fuentes de carbono alternativas, tolerancia de grandes cantidades de sal o metales, y uso reducido de agua) puede prevenir la contaminación de otros microorganismos, que es uno de los retos más grandes de los bioprocesos. Esto podría, por tanto, simplificar la producción mediante la reducción de los costes de mantenimiento de la esterilidad, permitiendo los procesos en continuo y reduciendo el consumo energético. Además, el uso de organismos poliextremófilos es preferible frente a la síntesis

química. Por ejemplo, la biosíntesis de nanopartículas por bacterias resistentes a metales es menos costosa, más eficiente y ecológica que el proceso químico alternativo (Atalah *et al*., 2022; Chen y Jiang, 2018).

Además de las extremozimas, otras moléculas no enzimáticas se pueden aislar de organismos extremófilos. Por ejemplo, hay bacterias psicrófilas que protegen a las plantas de patógenos mediante la síntesis de metabolitos secundarios y se utilizan como biopesticidas; otras pueden sintetizar moléculas que inhiben la formación de biofilms, una de las mayores preocupaciones de la industria alimentaria (Núñez-Montero y Barrientos, 2018; Torracchi *et al*., 2020). Finalmente, otras producen compuestos de alto valor en cosmética y en biomedicina, como pigmentos o moléculas terapéuticas.

3.2 Carotenoides y otros pigmentos

Los carotenoides son un grupo de compuestos dentro de los isoprenoides caracterizados por su pigmentación rojo-amarillenta. Son responsables del color de muchas frutas y vegetales, pero también los sintetizan las bacterias y algas (Paniagua-Michel *et al*., 2012). Tanto microorganismos fotótrofos como no fotótrofos sintetizan estos pigmentos ya que confieren protección frente a las especies reactivas de oxígeno (ROS) y el estrés oxidativo resultante. Sin embargo, los humanos y la mayoría de animales necesitan adquirirlos del ambiente, principalmente a través de la dieta (Tian y Hua, 2010; Toews *et al*., 2017).

El estrés oxidativo es una condición natural que se da a consecuencia de un desequilibrio entre moléculas pro-oxidantes y antioxidantes. Aunque las moléculas pro-oxidantes son subproductos del metabolismo celular, se ha demostrado que el estrés oxidativo prolongado juega un papel importante en el desarrollo y progresión de diferentes estados patológicos, entre los cuales destacan el cáncer, las enfermedades neurodegenerativas y cardiovasculares (Batty *et al*., 2022; Chen y Zhong, 2014; Gorrini *et al*., 2013).

El estrés oxidativo se genera en respuesta a diferentes estreses ambientales a los que los organismos, incluyendo a los microorganismos, están sometidos. Este es el motivo por el cual los organismos extremófilos se consideran una fuente prometedora de moléculas antioxidantes. Por ejemplo, el filo *Deinococcota*, que ha demostrado elevada tolerancia y resistencia a radiación, oxidación, desecación y elevadas temperaturas, sintetiza carotenoides únicos como la deinoxantina y la termozeaxantina. El filo *Actinomycetota*, habitante mayoritario del suelo y el desierto, incluye una gran diversidad de bacterias coloreadas debido a la síntesis de diferentes carotenoides. Además, las cepas microbianas de ambientes termófilos, de sitios naturalmente radiactivos y de la Antártida también han sido estudiadas por su capacidad de sintetizar carotenoides (Asker *et al*., 2007; Lutnaes *et al*., 2004; Mandelli *et al*., 2012; Sandmann., 2021; Silva *et al*., 2021; Tian y Hua, 2021; Ron *et al*., 2018).

El interés de los carotenoides y otros pigmentos va más allá de sus efectos antioxidantes. Su uso en terapia está también relacionado con su efecto antimicrobiano (antibiótico, antifúngico y antiviral), y su efecto citotóxico y antiproliferativo con impacto directo en actividad anticancerígena (Nawaz *et al*., 2020; Tapia *et al*., 2021). Además, algunos de ellos podrían ser utilizados como protectores solares biodegradables (Gabani y Singh, 2013). Los carotenoides son, por tanto, moléculas altamente valiosas para la industria farmacéutica que pueden obtenerse de fuentes microbianas. Sus efectos terapéuticos están mediados por su capacidad de neutralizar moléculas ROS y se han descrito en procesos de envejecimiento, enfermedades cardiovasculares, Alzheimer y cáncer (Balendra y Singh., 2021; Leoncini *et al*., 2015; Przybylska y Tokarczyk, 2022; Sztretye *et al*., 2019).

3.3 Moléculas terapéuticas

Los organismos extremófilos son fuente de una gran variedad de moléculas terapéuticas, a parte de los pigmentos descritos anteriormente. Entre ellos, los antimicrobianos (antibióticos y antifúngicos) son de gran interés. Los antibióticos son un grupo diverso de moléculas clínicamente relevantes que han sido en gran medida aisladas de microorganismos del grupo

Actinomycetota, uno de los principales habitantes de los ambientes desérticos (Azua-Bustos y González-Silva, 2014; Durand *et al*., 2019; Hui *et al*., 2021; Zhao *et al*., 2018). En especial, las chaxamicinas A-D, la mutactimicina AP y las abenquinas A-D, entre otras, han sido aisladas de microorganismos del desierto de Atacama (Astakala *et al*., 2022; Rateb *et al*., 2011; Schulz *et al*., 2011). Además, las halocinas y diketopiperazinas se han aislado de microorganismos halófilos, mientras que las bacterias psicrófilas de la Antártida también han demostrado ser una buena fuente de antimicrobianos contra un amplio rango de bacterias patógenas humanas (Coker, 2016; Núñez-Montero y Barrientos, 2018).

Más allá de los antimicrobianos, sustancias con efecto antitumoral, anticancerígeno o antiproliferativo también se han aislado y caracterizado de microorganismos extremófilos. Los microorganismos radiorresistentes desarrollan mecanismos enzimáticos y no enzimáticos para reparar el ADN dañado por la radiación. Dado que el daño en el ADN puede causar cáncer, podrían explorarse las posibles aplicaciones de estas moléculas en terapias contra el cáncer (Gabani y Singh, 2013). En concreto, el carotenoide deinoxantina se ha estudiado por su efecto apoptótico en células cancerígenas, y el ácido berkelico, sintetizado por bacterias que viven en un lago contaminado por metales, tiene actividad contra líneas celulares de cáncer de ovario (Choi *et al*., 2014; Stierle *et al*., 2006). Además, los microorganismos expuestos a elevada radiación sintetizan aminoácidos tipo micosporina (MAAs), que tienen propiedades antioxidantes, pero también actividad antiproliferativa y efecto protector solar (Gabani y Singh, 2013; Nayak *et al*., 2021).

Las bacterias halófilas son también fuente de biosurfactantes y polihidroxialcanoatos (PHAs), con especial interés para la industria farmacéutica. Por un lado, los biosurfactantes son moléculas anfifílicas que reducen la tensión superficial entre fases y actúan como emulsionantes, y han demostrado ser útiles en combatir la adhesión celular y la formación de biopelículas de bacterias patógenas en implantes y catéteres (Kochhar *et al*., 2022). Por otro lado, los PHAs son bioplásticos no citotóxicos, biocompatibles y biodegradables que son buenas alternativas a los plásticos derivados del petróleo para la administración de fármacos. Además, las vesículas recombinantes llenas de gas de estos microbios son también prometedoras en esta área (Coker, 2016; Kochhar *et al*., 2022).

4. Retos abordados

El trabajo descrito en esta tesis pretende caracterizar los perfiles microbianos asociados a diferentes superficies naturales y artificiales sometidas a irradiación, a través de una estrategia holística que incluye tanto técnicas independientes de cultivo como técnicas dependientes de cultivo. En concreto, se han estudiado las comunidades microbianas de la zona supralitoral de la costa mediterránea en tres puntos (Vinaròs, Cullera y Dénia, España) y del biocrust del desierto de Tabernas (Almería, España). Además, también se han explorado los microorganismos que viven en la superficie de las cabinas UV de un hospital.

Se han investigado las aplicaciones biotecnológicas y las implicaciones biomédicas de estos microorganismos. Concretamente, se ha abordado el uso de microorganismos aislados de la zona supralitoral de la costa en terapia, así como las potenciales resistencias a antibióticos y luz UV en un hospital. Desde el punto de vista ecológico, hemos puesto énfasis en mejorar la recuperación de la máxima diversidad microbiana y la descripción de nuevos taxones a través de la combinación de estrategias simples de cultivo. Además, el uso de configuraciones alternativas de cultivo como la fermentación en fase sólida se ha estudiado, con implicaciones en los procesos biosintéticos industriales, especialmente para microorganismos extremófilos y/o adaptados a la falta de agua (Figura 3).

Resultados generales y discusión

Esta tesis se ha centrado en el estudio de ambientes altamente irradiados, sus estructuras microbianas e implicaciones biomédicas. El microbioma de ambientes naturales expuestos al sol o artificiales

expuestos a luz ultravioleta (UV) se ha evaluado a lo lardo de esta tesis. Trabajos previos en placas solares revelaron que en la superficie de estas estructuras artificiales distribuidas por todo el planeta hay una diversa biocenosis (Dorado-Morales *et al*., 2016; Tanner *et al*., 2020). Esta biocenosis comparte características con ambientes desérticos, que parecen ser la posible fuente de microorganismos a través de partículas transportadas por el aire. Además, otros ambientes expuestos al sol, como los chicles desechados al aire libre, también presentan similitudes dada la presencia de taxones altamente resistentes como *Deinococcus* y *Sphingomonas* (Satari *et al*., 2020). En este trabajo teníamos como objetivo estudiar superficies naturales y artificiales expuestas a UV con interés en aislar microorganismos cultivables biotecnológicamente relevantes. Además, hemos explorado estrategias para aumentar la diversidad cultivada en laboratorios a través de diseños experimentales simples y de bajo coste.

1. Combinar medios: la clave para aumentar la diversidad

El uso de una combinación de medios de cultivo se ha aplicado de manera sistemática en bioprospeción y ecología microbiana. Por ejemplo, Gong *et al*. (2017) utilizaron cuatro medios con fuentes alternativas de carbono en el cribado de actividades lignocelulolíticas (celulolítica, xilanolítica y lignolítica), y discutieron cómo el uso de cada una de ellas favorecía el aislamiento de bacterias con actividades líticas específicas. De manera similar, Moote *et al*. (2021) concluyeron que la combinación de medios, así como la combinación de estrategias de cultivo, era crítica para aumentar la diversidad de microorganismos (incluyendo el aislamiento de nuevos taxones) en el caso de bacterias fastidiosas del tracto gastrointestinal de animales. Además, Kothe *et al*. (2021) destacaron no solo el potencial de la combinación de medios, sino también la importancia del estudio en paralelo de las muestras mediante técnicas independientes de cultivo basadas en la secuenciación genómica y metagenómica.

Aquí hemos utilizado una combinación de medios de cultivo y condiciones de incubación de manera sistemática para maximizar la diversidad microbiana aislada en cultivo puro. Con esto, se han aislado no solo un número elevado de taxones diferentes, sino también microorganismos potencialmente nuevos y cepas biotecnológicamente relevantes. En primer lugar, en el aislamiento de microorganismos en puntos diferentes de las rocas de la costa mediterránea (agar marino, R2A y TSA) (Publicación I). Después, en el aislamiento de microorganismos asociados a *biocrust* del desierto de Tabernas (Almería, España) (TSA y R2A, a concentraciones 1X, 0.1X y 0.01X) (Publicaciones II-IV). Finalmente, en el aislamiento de microbios de las cabinas UV del servicio de dermatología de un hospital (TSA, R2A, *Columbia Blood*, *Nutrient Agar* y *Yeast Mold*) (Publicación V) (Figura4).

En concreto, el uso de medios de bajo contenido en nutrientes, como R2A o *Nutrient Agar*, así como diluciones de TSA, favoreció el aislamiento de una mayor diversidad de géneros. En el caso de las muestras del desierto de Tabernas, los medios TSA 0.1X y R2A 0.01X permitieron aislar el mayor número de taxones exclusivos en comparación con el resto de diluciones de cada uno de ellos (Figuras II.4B y C). Aunque las diferencias no fueron tan notorias para las muestras de las cabinas UV del hospital, los medios R2A, Agar nutritivo y Yeast Mold juntos permitieron aislar 16 taxones exclusivos (Figura 5A). Por tanto, la combinación de ellos influyó en la diversidad resultante en cultivo. A parte, las cinco nuevas especies aisladas de Tabernas (*Belnapia mucosa*, *Belnapia arida*, *Kineococcus vitellinus*, *Kineococcus indalonis* y *Kineococcus siccus*) se aislaron de placas diluidas de TSA y R2A (Publicaciones III y IV).

Además de la combinación de medios, las colonias de las muestras de *biocrust* se incubaron durante un tiempo largo y las colonias se seleccionaron a dos tiempos de incubación, mientras que las muestras de las cabinas UV se incubaron a dos temperaturas diferentes. En ambos casos, la combinación de medios con estas estrategias permitió aislar una gran diversidad de cepas pertenecientes a 37 y 44 géneros diferentes, respectivamente. Aunque la combinación de medios fue lo que tuvo mayor impacto en la

diversidad, cuatro géneros se aislaron de manera exclusiva después de un mes de incubación de las muestras de Tabernas, y nueve géneros de las muestras del hospital se aislaron exclusivamente a 37 °C (incluyendo los aislados de *Deinococcus* spp.) (Figura II.7A y Figura 5B). Por tanto, de nuevo, nuestros resultados indicaron que estas dos estrategias simples ayudan a aumentar la diversidad de las colecciones microbianas.

Respecto a la diversidad encontrada en la fracción cultivable de microorganismos de la zona rocosa de la costa mediterránea, es importante destacar que más de la mitad de los aislados no pudo identificarse. El protocolo de identificación seguido en el laboratorio se optimizó más tarde para las muestras del desierto de Tabernas. Por tanto, la baja diversidad encontrada (la mayoría de los aislados pertenecían a los géneros *Bacillus* y *Halobacillus*) podría ser un artefacto del bajo rendimiento de la PCR, pero no se puede ignorar tampoco una proporción relativamente grande de microorganismos casi idénticos (Tabla I.S2).

Además del potencial de la combinación de medios en el aislamiento de un amplio rango de microorganismos, el diseño de nuevas estrategias de cultivo también permite el cultivo de microorganismos específicos con resultados diferentes. Es el caso del uso de las matrices 3D de SSF (Figura VI.1), que resultó en la obtención de patrones diferentes de crecimiento y expresión de proteínas en microorganismos, particularmente en levaduras (Publicación VI). Nuestras matrices incrementaron notablemente los rendimientos celulares comparado con los cultivos equivalentes en condiciones estáticas y parecieron favorecer la fermentación alcohólica, aunque se necesita más investigación para averiguar el impacto de las matrices en otros procesos (e.g., *quorum sensing* o formación de biopelículas) (Figura VI.2 y Figura VI.3B).

2. Estrategias independientes y dependientes de cultivo: diferencias y similitudes en ambientes altamente irradiados

El uso de una combinación de técnicas dependientes e independientes de cultivo permite conocer en mayor profundidad la complejidad y diversidad microbiana en los ambientes estudiados. A este respecto, nuestras expediciones de bioprospección habitualmente combinan ambas estrategias para explorar de manera conjunta el potencial biotecnológico de las muestras y las características taxonómicas y ecológicas de las comunidades microbianas (Satari *et al*., 2020; Tanner *et al*., 2018; Vidal-Verdú *et al*., 2022 a; Vidal-Verdú *et al*., 2022 b). En esta tesis, hemos estudiado desde ambas perspectivas la diversidad que habita en la zona supralitoral mediterránea y las cabinas UV de un hospital. En el caso de las muestras de *biocrust* del desierto de Tabernas, los datos metagenómicos discutidos provienen de Latorre-Pérez *et al*. (2021).

A través del análisis metataxonómico y metagenómico, fue posible determinar que, a nivel de filo, las comunidades estudiadas en los tres ambientes altamente irradiados estaban dominados por *Pseudomonadota*, *Actinomycetota* y *Bacteroidota*. *Cyanobacteria* fue el taxón más abundante en las muestras de la costa mediterránea, pero las de *biocrust* y el hospital también compartían grandes abundancias de este género. *Acidobacteriota* y *Bacillota*, sin embargo, aparecieron como representativas de las cabinas UV del hospital y de *biocrust*, respectivamente (Latorre-Pérez *et al*., 2021; Publicaciones I y V).

En cuanto a las abundancias de taxones altamente resistentes, es relevante destacar que había miembros de *Deinococcota* en todos los casos. En particular, se encontraron *Truepera* spp. en las muestras de *biocrust* y de la costa rocosa, y *Deinococcus* spp. en las muestras de las cabinas UV (Latorre-Pérez *et al*., 2021; Publicaciones I y V). Curiosamente, el género *Truepera* se ha descrito como ligeramente halófilo (lo cual está de acuerdo con su presencia en ambientes marinos y el desierto), mientras que *Deinococcus* muestra respuestas variadas en concentraciones moderadas de sal y se encuentra también en placas solares (Albuquerque *et al*., 2005; Brooks y Murray, 1981; Dorado-Morales *et al*., 2016; Porcar *et al*., 2018; Tanner *et al*., 2020).

A nivel de género, también se observaron algunas similitudes. *Rubrobacter* estaba entre los géneros más abundantes en las muestras de la costa rocosa y el *biocrust* (Publicación I; Latorre-Pérez *et al*., 2021). Las muestras de *biocrust* y las cabinas UV eran abundantes en *Rubellimicrobium*, *Sphingomonas* y *Hymenobacter*, de manera similar a lo encontrado en placas solares (Publicación V; Dorado-Morales *et al*., 2016; Porcar *et al*., 2018). *Paracoccus*, sin embargo, estaba presente tanto en la costa rocosa como en las cabinas UV (Publicaciones I y V). La presencia de todos ellos concuerda con las comunidades microbianas ya descritas en los desiertos (incluidos los ambientes salinos dentro de los desiertos) (Belov *et al*., 2018).

En el caso de las muestras de la costa mediterránea, no obstante, la mayoría de taxones quedaron sin clasificar y se encontraron discrepancias entre los datos de metagenómica y metataxonomía en cuanto a la clasificación de los géneros de cianobacterias (Publicación I).

En términos de microorganismos cultivables, la colección del litoral rocoso mediterráneo no presentó similitudes con las colecciones establecidas a partir de las muestras del desierto de Tabernas y de las cabinas UV del hospital, a excepción de la presencia de algunas especies de *Micrococcus*. En concreto, esta colección estaba principalmente compuesta de especies de *Bacillus* y *Halobacillus*, lo cual está de acuerdo con su origen marino o salino (Tabla I.S2) (Liu *et al*., 2017; Mondol *et al*., 2013).

En contraposición, tanto la colección de microrganismos aislados de Tabernas como la de los del hospital compartían aspectos con las ya descritas en placas solares por Dorado-Morales *et al*. (2016), Porcar *et al*. (2018) y Tanner *et al*. (2018) (Figura 6). Especialmente, *Arthrobacter*, *Sphingomonas*, *Micrococcus*, *Microbacterium*, *Kocuria* y *Bacillus* spp. se aislaron todos de estos tres ambientes altamente expuestos a UV. Además, las colecciones de Tabernas y del hospital también compartían aislados pertenecientes a *Roseomonas*, *Streptomyces*, *Staphylococcus* y *Massilia*, mientras que las del hospital y las placas solares compartían *Deinococcus*, *Rhodotorula*, *Rhodococcus*, *Cryptococcus* y *Curtobacterium*. Por lo tanto, estos ambientes comparten una biocenosis rica en microorganismos resistentes a UV (Rasuk *et al*., 2017; Srinivasan *et al*., 2015).

La combinación de técnicas independientes y dependientes de cultivo permitió una caracterización más precisa de las comunidades microbianas. En primer lugar, confirmó que la baja diversidad encontrada en la fracción cultivable de microorganismos de la costa mediterránea no era fiable y estaba influenciada por los métodos de cultivo y el rendimiento de la PCR. Por ejemplo, no se cultivaron cianobacterias cuando eran los taxones más abundantes en metagenómica, probablemente por los medios de cultivo utilizados (Publicación I). Después, en el caso de Tabernas y las cabinas UV revelaron que taxones que no se habían cultivado, como *Hymenobacter* o *Rubellimicrobium*, característicos de placas solares, también habitaban estos ambientes (Latorre-Pérez *et al*., 2021; Publicación V). Además, Latorre-Pérez *et al*. (2021) establecieron una colección de microorganismos paralela a partir de muestras de *biocrust* del desierto de Tabernas. Encontraron una diversidad similar a la descrita en la Publicación II y analizaron las diferencias entre los datos de cultivo y secuenciación. Su comparación reveló que, aunque había sido posible identificar la mayoría de los microorganismos cultivables a través de la secuenciación del gen de la subunidad 16S ribosómico, algunos de los taxones diana altamente resistentes identificados por metataxonomía no se cultivaron en el laboratorio. De manera similar, *Rubellimicrobium*, *Paracoccus* y *Corynebacterium* eran abundantes en las muestras de las cabinas UV y solo se detectaron mediante técnicas de secuenciación (Publicación V).

Tal y como se ha comentado en la introducción, ambas estrategias tienen limitaciones en el análisis de la diversidad microbiana, pero es la combinación de ellas la que hace posible determinar su composición de manera más fiable.

3. Potencial biotecnológico y biomédico de los microorganismos extremófilos de ambientes

altamente irradiados y baja disponibilidad de agua

El potencial biotecnológico de los extremófilos ha sido profundamente estudiado dadas sus aplicaciones en la biotecnología industrial, biomédica y medioambiental, entre otras (Dumorné *et al*., 2017; Raddadi *et al*., 2015). En este trabajo, hemos explorado el potencial y la resistencia de microorganismos sometidos principalmente a dos estreses abióticos (la radiación UV y la desecación) pero también a otros como antibióticos (Publicaciones I, V y VII).

Las colecciones de microorganismos establecidas a partir de los ambientes muestreados (i.e., la zona supralitoral de la costa mediterránea, el *biocrust* del desierto de Tabernas y las cabinas UV de un hospital valenciano) revelaron una amplia diversidad de taxones biotecnológicamente relevantes (Publicaciones I, II y V). Concretamente, especies de *Arthrobacter*, *Sphingomonas*, *Micrococcus*, *Microbacterium*, *Kocuria, Bacillus, Roseomonas*, *Streptomyces*, *Staphylococcus* y *Massilia* estaban presentes tanto en las colecciones del desierto de Tabernas como en la del Hospital General. Además, los aislados marinos de *Bacillus* formaban también parte de la colección del litoral rocoso mediterráneo. Los usos de los microorganismos de todos estos géneros varían desde el favorecimiento del crecimiento vegetal (bacterias promotoras del crecimiento vegetal), el uso como factorías celulares biosintéticas para aplicaciones industriales (e.g., producción de metabolitos secundarios y degradación de compuestos) o la producción de pigmentos con efectos antioxidante, antitumoral y antimicrobiano (Tabla 2). De manera destacable, la empresa escandinava Promar AS (Noruega) solicitó la patente del uso de pigmentos bacterianos que prevenían los efectos de la radiación. El pigmento sarcinaxantina, aislado de una cepa de *M. luteus* del fiordo Trondheim (Noruega), y carotenoides relacionados se propusieron para ser usados como protectores solares en preparaciones farmacéuticas y cosméticas (Goksøyr, Solicitud de Patente Pub. No. US 2013/0078203 A1, 2013). De todos los usos descritos, el efecto antioxidante de los compuestos pigmentados ha sido de interés en este trabajo.

Las definiciones recientes de los antioxidantes se refieren a ellos como sustancias que previenen o retrasan las reacciones indeseadas de oxidación, que pueden estar causadas por ROS o RNS (especies reactivas de nitrógeno) (Apak, 2019; Halliwell y Gutteridge, 2015). A este respecto, el potencial antioxidante de cepas aisladas de la zona supralitoral mediterránea se analizó mediante ensayos *in vitro* e *in vivo* en un modelo de *C. elegans*.

Nuestras comparaciones, sin embargo, revelaron problemas en la traslación y discrepancias ente métodos *in vitro*. Tal y como mencionan Apak (2019) y Apak *et al*. (2013), no solo falta estandarización en los protocolos de medida de antioxidantes, sino también los resultados de dos ensayos pueden no ser idénticos para la misma muestra dadas las peculiaridades termodinámicas y cinéticas de cada reactivo (e.g., DPPH). Por tanto, las medidas de la actividad o el poder antioxidante son cortrovertidas. Tal y como se relata en la Publicación I, una combinación de métodos (i.e., ensayos de H_2O_2 y DPPH) sería más informativa para tener una perspectiva general del potencial antioxidante de la muestra.

Además, una de las principales desventajas del testado *in vitro* es el fallo en reproducir las condiciones dadas en un organismo vivo (ensayos *in vivo*). En el caso del ensayo de DPPH, la evaluación del poder antioxidante de las moléculas pigmentadas se analizó en un ambiente orgánico (i.e., metanol), que difiere de las condiciones fisiológicas dadas en un organismo (en este caso, *C. elegans*) (Publicación I). Además, Xie y Schaich (2014) destacaron algunas limitaciones a los métodos de medida de antioxidantes basados en DPPH en muestras complejas, como los extractos bacterianos estudiados, dado que molélulas diferentes pueden tener comportamientos distintos al reaccionar con los radicales de DPPH. Nuestros resultados, de hecho, confirman que cepas cuyo papel protector frente al estrés oxidativo se ha probado en un modelo de *C.*

elegans dan falsos resultados negativos en un ensayo basado en DPPH.

Teniendo todo esto en mente, decidimos explorar la potencial aplicación biomédica de algunas de las cepas aisladas en un modelo de enfermedad mitocondrial (MD) humana (Publicación VII). La evidencia previa de que algunos compuestos antioxidantes mejoran la sintomatología del Síndrome de Leigh (LS) apoyaba nuestra hipótesis de que bacterias productoras de compuestos antioxidantes, como carotenoides, podrían tener un impacto similar (Enns, 2014; Martinelli *et al*., 2012). En base a esos estudios previos, se estableció una colaboración entre nuestro grupo en la Universitat de València (UV) y el grupo de Neuropatología Mitocondrial en la Universitat Autònoma de Barcelona (UAB). Nuestra colaboración tenía como objetivo estudiar si bacterias ambientales capaces de sintetizar carotenoides podrían mejorar el fenotipo de la enfermedad en el modelo murino Ndufs4KO.

Aunque los probióticos han sido de manera tradicional microorganismos relacionados con los alimentos o comensales, en la última década el número de cepas probióticas aisladas o descubiertas de otras fuentes esta creciendo. Además, el uso de estrategias metagenómicas para comparar, por ejemplo, microbiomas de pacientes con y sin condiciones específicas (e.g., alérgicos y no alérgicos) permite la identificación de diferencias en la microbiota y taxones diana que pueden ser propuestos como probióticos (Papadimitriou *et al*., 2015).

A este respecto, decidimos evaluar el potencial de la administración oral de un preparado microbiano basado en la cepa CR10 identificada como *M. luteus*, con la esperanza de que su capacidad antioxidante pudiera tener efectos beneficiosos en los ratones enfermos (Publicación I). Sin embargo, la serendipia nos llevó a identificar que el criocorservante (maltodextrina, MDX) era el responsable de los efectos observados, que eran principalmente una reducción de la neuroinflamación y un aumento de la esperanza de vida (Figura VII.1, Figura VII.2 y Figura VII.4). Además, los ensayos preliminares y el análisis de la microbiota intestinal revelaron que, pese a que no había perfiles microbianos altamente diferenciados, el género *Akkermansia* podría estar favorecido por la MDX (Figura VII.7). Esto es interesante porque el género *Akkermansia* se ha asociado recientemente con cambios en la inflamación y propuesto como un potencial probiótico (Wang *et al*., 2022 b; Zhang *et al*., 2019). Irónicamente, después de haber descartado el efecto de los microorganismos de las preparaciones en el fenotipo observado, surge una posible 'conexión microbiana' mediada por la MDX como un potencial factor clave en el LS. Esto podría abordarse algún día mediante la modicación de la microbiota intestinal, tal y como podría estar haciéndolo la MDX.

La hipótesis de que la MDX tiene un efecto en la microbiota, que a su vez modula la respuesta a la enfermedad es muy tentadora. El uso de pre- y probióticos en otros desórdenes neurodegenerativos, como el Párkinson o el Alzhéimer, ha sido descrito. Dado que los microbios del intestino modulan los neurotransmisores y la respuesta inmune del hospedador, las alteraciones en la microbiota podrían también contribuir a desarrollar estos desórdenes. Por tanto, tanto probióticos como prebióticos son efectivos en mejorar los síntomas y prevenir la progresión de las enfermedades (Abraham *et al*, 2019; Tamtaji *et al*., 2019). Además, otros problemas crecientes de salud pública como la obesidad se están abordando a través de la suplementación microbiana (Green *et al*., 2020). Por ejemplo, cepas de *Limosilactobacillus* se han explorado como probióticos por la protección antioxidante que confieren en el intestino. Sin embargo, se necesita más investigación en este campo para determinar el potencial antioxidante real de cada cepa, las dosis y los tiempos de tratamiento necesarios para obtener efectos específicos y encontrar evidencia fiable de su efectividad en ensayos clínicos (Paulino do Nascimento *et al*., 2022).

Finalmente, el potencial de las levaduras como factorías celulares en las industrias biotecnológica y alimentaria está bien documentado (Karim *et al*., 2020; Wagner y Alper, 2016). Dada la urgente

necesidad de desarrollar sistemas de producción más sostenibles, es interesante el uso de microorganismos que de alguna forma aumenten sus capacidades biosintéticas con menos agua o con menores requerimientos energéticos. A este respecto, las estrategias de SSF tienen ventajas respecto a las de SmF, como menores costes de producción (e.g., menor equipamiento, menor precio de los sustratos, requerimientos mínimos de esterilidad) y mayores rendimientos (Doriya *et al*., 2016; Hölker y Lenz, 2005). Pero por encima de todo, la SSF cambia los flujos metabólicos y las capacidades biosintéticas de los microorganismos, lo que puede constituir de hecho una oportunidad para explorar nuevos usos biotecnológicos de los microorganismos (Publicación VI; Hölker y Lenz, 2005).

Nuestro trabajo en SSF tenía como objetivo estudiar si las matrices sólidas podían influir en el crecimiento de las levaduras y su impacto en la biotecnología. De manera específica, nuestro objetivo principal era proponer un método estandarizado para la SSF dado que la falta de reproducibilidad y estandarización en estos procesos de fermentación ha sido criticada recurrentemente (Hölker y Lenz, 2005; Singhania *et al*., 2009). Aunque nosotros no intentamos escalar los resultados, encontramos que el uso de las matrices estándar da resultados consistentes y reproducibles (Publicación VI).

El trabajo posterior en esta área puede centrarse en determinar el impacto de las matrices en rutas que podrían ser de interés para los procesos biosintéticos industriales, caracterizar los flujos metabólicos y modificarlos, así como trasladar estos resultados a volúmenes de fermentación mayores, ya que también se ha descrito que algunos metabolitos secundarios solo se sintetizan durante el crecimiento sobre sustratos sólidos (Hölker y Lenz, 2005).

4. Comentarios finales y perspectivas futuras

A lo largo de esta tesis hemos descrito el potencial biotecnológico de cepas microbianas ambientales aisladas de ambientes extremos. Sin embargo, el desarrollo de herramientas biotecnológicas basadas en microorganismos necesita más trabajo en la caracterización en profundidad de sus características genotípicas y fenotípicas. Además, el uso de microorganismos y sus productos requiere una comprensión detallada de sus habilidades biosintéticas, con especial atención a su comportamiento en procesos de producción a gran escala. A este respecto, no solo sería necesaria una caracterización completa de los microorganismos, sino también experimentos de escalado para determinar su potencial industrial (Linke *et al*., 2023).

Por tanto, se requeriría más investigación del potencial de los taxones identificados para definir los usos reales de los aislados de las colecciones microbianas. Por ejemplo, la biosíntesis de compuestos (e.g., carotenoides) o la tolerancia a estrés (e.g., resistencia a sequía y biorremediación de contaminación) son áreas de trabajo de relevancia en biotecnología microbiana (de Lorenzo, 2022; Revuelta *et al*., 2016). Las aplicaciones biotecnológicas requieren experimentos que vayan más allá de definir los posibles usos de cada cepa y el testado de sus habilidades bajo condiciones de laboratorio (e.g., medidas de actividad antioxidante o resistencia a la desecación). Por ello, se necesitarían experimentos que permitieran la traslación de resultados a aplicaciones reales, que son un campo de trabajo infraexplorado en esta tesis (e.g., testado *in vivo* de compuestos antioxidantes en líneas celulares o modelos experimentales, mejora de la síntesis de productos en ambientes de baja actividad de agua mediante ingeniería genética, experimentos de escalado y pruebas de fermentación en media y gran escala) (Dassprakash *et al*., 2012; López *et al*., 2021).

En cuanto a la evaluación del potencial biomédico de especies microbianas potencialmente antioxidantes, este trabajo solo ha descrito el posible uso de bacterias extremófilas en terapia dada su habilidad de sintetizar compuestos antioxidantes y explorado de manera preliminar su efecto. Tal y como revisa Gulcin (2020), entender los mecanismos antioxidantes y la cinética de las reacciones de moléculas específicas es importante en el cribado de antioxidantes. Por ello, se necesita más investigación para identificar qué moléculas y mecanismos son responsables de los

efectos observados y las condiciones bajo las cuales las cepas, o sus productos, son efectivos. Además, investigar las rutas biosintéticas y las condiciones más favorables para su producción en factorías celulares es crucial para su aplicación real.

Respecto a los estudios en el LS, nuestra hipótesis de que cepas microbianas aisladas de nichos extremos pudieran ser utilizadas en terapia para enfermedades mitocondriales no ha sido confirmada hasta la fecha. Sin embargo, nuestros descubrimientos acerca del posible papel de la MDX modulando la microbiota intestinal y la inflamación sugieren un posible rol de este azúcar como prebiótico, sean cuales sean los mecanismos subyacentes. Por tanto, hay todavía campo de investigación abierto para desvelar la función de este compuesto en el modelo animal, así como de estudiar una posible estrategia microbiana basada en *Akkermansia* spp. De todos modos, otros microorganismos productores de antioxidantes podrían seguir siendo investigados en esta área.

Adicionalmente, el conocido potencial biosintético de las factorías celulares basadas en levaduras podría ser explotado testando nuevas técnicas de cultivo, como las anteriormente descritas matrices 3D de SSF. No solo experimentos de escalado, sino también ingeniería genética y modelización de flujos metabólicos podrían ser áreas de investigación relevantes en la síntesis de compuestos valiosos para la biotecnología. Por ejemplo, algunos autores han descrito la deleción del gen *ADH2*, entre otros, para aumentar los rendimientos de producción de etanol en la fermentación de *S. cerevisiae*, que está sobreexpresado en condiciones de SSF (Yang *et al*., 2022; Publicación VI). Además, se podrían explorar materiales alternativos (e.g., plásticos autoclavables que redujeran la carga de trabajo) y otros diseños geométricos.

Aunque la identificación y caracterización de taxones microbianos solo el primer paso en el desarrollo de procesos biotecnológicos, es todavía una tarea relevante en ecología y taxonomía microbiana, dado que la aproximación a la materia oscura microbiana para el desarrollo de nuevas herramientas biotecnológicas solo se puede abordar mediante el descubrimiento de nuevos microorganismos. De hecho, los microbios nuevos y no convencionales tienen un enorme potencial biotecnológico a través de la ingeniería genética para aplicaciones industriales (Blombach *et al*., 2022; Vartoukian *et al*., 2010).

Por último, y como comentario final, los ambientes altamente irradiados y secos albergan, a su vez, microorganismos conocidos y todavía desconocidos con suficiente potencial como para ser explorados en busca de aplicaciones biotecnológicas creativas e innovadoras. La materia oscura microbiana revelada será, sin duda, una fuente de desarrollos nuevos y eficientes para los sectores biomédico y medioambiental, que son extraordinariamente necesarios en la situación actual de crisis climática y ambiental.

Conclusiones

En la presente tesis, se ha explorado la ecología y el potencial biotecnológico de ambientes altamente irradiados naturales y artificiales. Las conclusiones generales sacadas de este trabajo se detallan a continuación:

- La combinación de estrategias de cultivo (combinación de medios, tiempos de incubación extendidos y temperaturas de incubación diferentes) resulta en la obtención de colecciones microbianas altamente diversas, lo cual tiene un profundo impacto en su potencial biotecnológico.
- Las diferentes localizaciones a lo largo de la costa rocosa mediterránea albergan una biocenosis rica en cepas microbianas biotecnológicamente relevantes con potencial biomédico.
- El *biocrust* del desierto de Tabernas alberga comunidades microbianas diversas en las cuales una fracción significativa de los aislados podrían representar nuevos taxones bacterianos. De entre ellos, se han descrito y caracterizado por completo tres nuevas especies del género *Kineococcus* y dos de *Belnapia*.

- Las comunidades microbianas asociadas a las cabinas UV de las instalaciones de un hospital están compuestas por microorganismos típicamente medioambientales adaptados a ambientes altamente irradiados (e.g., placas solares) y de hospitales, y el análisis de sus actividades biológicas revela que no hay amenazas significativas por la selección de microorganismos resistentes a UV y antibióticos.
- La aplicación de NGS en diferentes ambientes extremos, como la zona supralitoral y las cabinas UV de fototerapia, ha permitido la comparación de los microbiomas sujetos a irradiación y desecación, y revela diferencias y similitudes respecto a la presencia de microorganismos altamente resistentes como *Truepera* spp. o *Deinococcus* spp., entre otros.
- Las levaduras muestran patrones diferentes de crecimiento y expresión de proteínas cuando se cultivan bajo condiciones de SSF mediante el uso de matrices impresas en 3D, en comparación con el cultivo tradicional en SmF, lo que tiene implicaciones en los procesos biosintéticos llevados a cabo por células de levadura y abre la investigación a mejorar la producción de metabolitos y/o la ingeniería genética. El uso de estas matrices sólidas permite gran flexibilidad en cuanto al testeo de diseños alternativos.
- La administración de preparaciones microbianas resultó en el descubrimiento fortuito de los efectos beneficiosos de la MDX en mejorar la sintomatología del LS, que son el incremento en la esperanza de vida y la reducción de la neuroinflamación. Además, la MDX podría jugar un papel en modular la microbiota intestinal, tal y como evidencia el incremento en la variabilidad de la abundancia de *Akkermansia* spp.

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Appendix A: Original publication reprints

microbial biotechnology

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Microbial communities of the Mediterranean rocky shore: ecology and biotechnological potential of the sea-land transition

Esther Molina-Menor,[1,†] Kristie Tanner,[1,2,†] Àngela Vidal-Verdú,[1] Juli Peretó[1,2,3] and Manuel Porcar[1,2,*,†]

[1]*Institute for Integrative Systems Biology I[2]SysBio, Universitat de València-CSIC, Paterna 46980, Spain.*
[2]*Darwin Bioprospecting Excellence S.L. Parc Científic Universitat de València, Paterna 46980, Spain.*
[3]*Departament de Bioquímica i Biologia Molecular, Universitat de València, Burjassot 46100, Spain.*

Summary

Microbial communities from harsh environments hold great promise as sources of biotechnologically relevant strains and compounds. In the present work, we have characterized the microorganisms from the supralittoral and splash zone in three different rocky locations of the Western Mediterranean coast, a tough environment characterized by high levels of irradiation and large temperature and salinity fluctuations. We have retrieved a complete view of the ecology and functional aspects of these communities and assessed the biotechnological potential of the cultivable microorganisms. All three locations displayed very similar taxonomic profiles, with the genus *Rubrobacter* and the families *Xenococcaceae*, *Flammeovirgaceae*, *Phyllobacteriaceae*, *Rhodobacteraceae* and *Trueperaceae* being the most abundant taxa; and *Ascomycota* and halotolerant archaea as members of the eukaryotic and archaeal community respectively. In parallel, the culture-dependent approach yielded a 100-isolates collection, out of which 12 displayed high antioxidant activities, as evidenced by two *in vitro* (hydrogen peroxide and DPPH) and confirmed *in vivo* with *Caenorhabditis elegans* assays, in which two isolates, CR22 and CR24, resulted in extended survival rates of the nematodes. This work is the first complete characterization of the Mediterranean splash-zone coastal microbiome, and our results indicate that this microbial niche is home of an extremophilic community that holds biotechnological potential.

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*For correspondence. E-mail manuel.porcar@uv.es; Tel. +34 963544473; Fax +34 96 3543576.
[†]These authors contributed equally to this work.
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Introduction

The interphase between marine and land environments is an ecologically complex habitat in which selection pressures from both environments can co-occur. Some of those pressures are high salinity, dehydration, wind and sun exposition, extreme temperature oscillations and mechanical stress associated with seawater splash, often with sand or pebbles, with strong abrasive effects. The aquatic to land transition has been reported to be linked to a narrow gradient in species distribution in function of the distance to the water line, as for example in cyanobacteria in an English lake (Pentecost, 2014). Regarding marine environments, the microbial ecology of rocky shores has previously been analysed (Chan *et al.*, 2003; Langenheder and Ragnarsson, 2007; Pinedo *et al.*, 2007; Brandes *et al.* 2015), including its links with oil spills and biodegradation (Alonso-Gutiérrez *et al.*, 2009). However, and in contrast with the well-studied microbial ecology of the intertidal zone (for a review, see Mitra *et al.*, 2014), a holistic study on the microbial ecology of the marine supralittoral Mediterranean rocky shore has not been addressed previously.

Harsh, extremophilic environments can be sources of biotechnologically relevant bacteria and therefore hold great promise for the biotechnological industry (Raddadi *et al.*, 2015). For example, extremophilic microorganisms can yield enzymes such as lipases and esterases that can be used under a wide range of conditions and may have relevant applications in the food, detergent and

biofuel industries (Fuciños *et al*., 2012). There are many other examples of biotechnologically relevant microorganisms from extreme environments, including the well-known case of *Thermus aquaticus*, which produces the widely used *Taq* polymerase; or the hyperthermophilic biofuel-producing archaea that live in deep-sea hydrothermal vents (Chien *et al*., 1976; Nishimura and Sako, 2009).

The present study focuses on the microorganisms that inhabit the rocky areas of the supralittoral zone (the area just above the tide line that is subjected regularly to splash but is not permanently underwater) of the Mediterranean coast. Surface-associated microbial communities that are sun-exposed are often rich in microorganisms that produce pigments, including carotenoids (Dorado-Morales *et al*., 2015; Kumar *et al*., 2015; : Shindo and Misawa, 2014; Tanner *et al*., 2017). These pigments play a key role in radiation tolerance (Tian and Hua, 2010; Klindworth *et al*., 2013; Sandmann, 2015; Tanner *et al*., 2018), and they are valuable for the food, pharmacological and cosmetic industries as colourants, antioxidants and protectors against solar radiation respectively (Sandmann, 2015). Therefore, we hypothesized that rough conditions of the supratidal zone may be associated with the presence of biotechnologically relevant microbial taxa. From this hypothesis, we have, in the present work, compared three different supralittoral coastal locations of the Mediterranean West coast and combined culturing techniques and high throughput sequencing data (16S rRNA amplicon and metagenomic sequencing) in order to shed light on the taxonomic composition of these communities, and to explore the biotechnological potential of the culturable strains.

Results

High-throughput 16S rRNA analysis

High-throughput 16S rRNA sequencing of the samples revealed that, based on the comparison of the richness value (number of different species; Fig. 1A) and the diversity (Shannon index; Fig. 1B), the alpha diversity was not significantly different among the locations. Moreover, the shape of the rarefaction curve at OTU level (Operational Taxonomic Unit) showed that the sequences covered the majority of taxa present in the samples (Fig. S1).

However, the composition of the bacterial communities varied depending on the location, as represented in the Principal Coordinates Analysis (PCoA; Fig. 2A). Samples from Dénia showed the highest intragroup homogeneity, whereas samples from Vinaròs and Cullera displayed higher differences between replicates. Nevertheless, samples from all three locations could be distinguished in the plot. The variability explained by both axes is high enough to conclude that the microbial communities among the three locations are different. Moreover, the representation of the relative abundances (TSS) of the top 30 most abundant genera showed that the microbial composition was generally similar along the locations (Fig. 2B), although some taxa such as the genus *Rubrobacter* in Vinaròs or the genus *Rubricoccus* in Dénia allowed the differentiation of specific regions (Table 1). Eleven out of the 30 most abundant genus were significantly different at least in one location. A list of the 30 more significantly different genus is shown in (Table S1). The original data have been deposited with the NCBI SRA accession number PRJNA556782.

Shotgun metagenomic analysis

The three locations exhibited similar taxonomic profiles according to the metagenomics analysis. The most abundant bacterial phyla were the same ones observed with high-throughput 16S rRNA sequencing, with *Cyanobacteria* being the most abundant in all three locations. Moreover, other taxa, such as the families *Rhodobacteraceae*, *Flammeovirgaceae*, *Trueperaceae* and the genus *Rubrobacter*, belonging to the phyla *Proteobacteria*, *Bacteroidetes*, *Deinococcus-Thermus* and *Actinobacteria* respectively, were also detected (Figs S2A, S3A and S4A). Metagenomic sequencing allowed the identification of abundant taxa in the *Cyanobacteria* phylum, including the genera *Staniera, Pleurocapsa*, *Myxosarcina* and *Xenococcus*, in contrast to the high-throughput 16S rRNA, which mainly showed unclassified *Xennococcaceae* taxa.

Archaeal and eukaryotic communities proved very diverse, with a high number of salt-adapted microorganisms in the former and a large fraction of *Ascomycota* in the latter (Figs S2, S3, S4B and C). Salt-adapted archaea included members of *Halococcus*, *Halobacteriaceae* (*Haladaptatus* and *Halalkalicoccus*), *Haloarculaceae*, *Haloferaceae*, *Halorubraceae* and *Natrialbaceae* families, as well as methanogenic archaea (members of the *Methanosarcinaceae* family; Figs S2B, S3B and S4B). Among the diversity of *Ascomycota*, the most abundant taxa were *Glonium stellatum*, *Cenococcum geophilum*, *Coniosporium apollinis* and *Lepidopterella palustris* (Figs S2C, S3C and S4C).

The functional analysis of the samples revealed a high representation of enzymes related to oxidative stress, being peroxiredoxin (EC 1.11.1.15) and peroxidase (EC 1.11.1.7) the most abundant activities, and displaying the highest values in Cullera and Vinaròs respectively. Thioredoxin-related enzymatic activities (EC 1.8.4.8; EC 1.8.1.9; EC 1.8.4.10) were homogeneously represented in all three samples, as well as superoxide dismutase (EC 1.15.1.1). Other enzymes

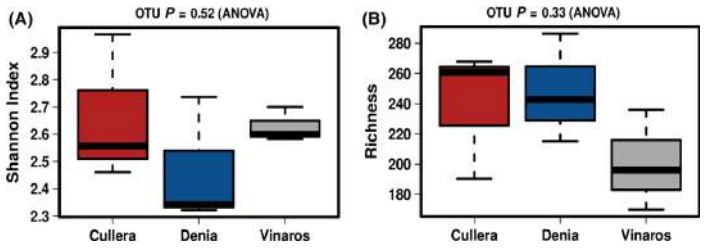

Fig. 1. Box plots showing the values of alpha diversity indexes in the sampled locations on the Mediterranean rocky-shore. (A) Observed richness at OTU level (number of OTUs). (B) Shannon index of diversity.

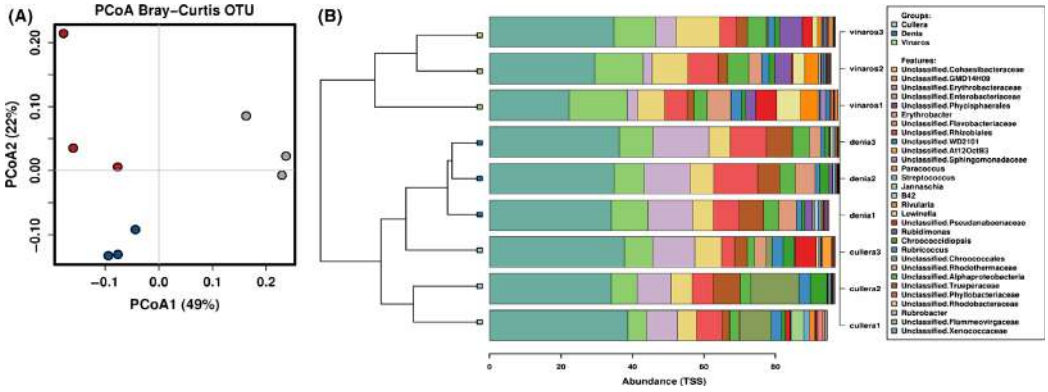

Fig. 2. (A) Principal coordinates analysis (PCoA) based on Bray–Curtis distances between OTUs in bacterial communities of three different locations. (B) Clustered-Barchart showing the top 30 most abundant genera in terms of relative abundance.

such as glutathione transferase (EC 2.5.1.18) or glutathione peroxidase (EC 1.11.1.9) varied among locations, with the former being more represented in Vinaròs and Cullera than in Dénia, and the latter being more abundant in Vinaròs. Among the genes related to carotenoid biosynthetic routes, the abscisic acid 8′-hydroxylase (EC 1.14.14.137) was particularly represented in Cullera, whereas sphingolipid-related genes such as glucosylceramidase proved to be frequent in Vinaròs (EC 3.2.1.45; Fig. 3). The original data have been deposited with the NCBI SRA accession number PRJNA556786.

Strain collection and identification

Culturing the samples on LB and Marine Agar yielded a large diversity of colonies in terms of colour, shape and morphology. A total of 100 strains were isolated and named with a code, after the location (C: Cullera, D: Dénia, V: Vinaròs) and the origin (M: Marine water, R: Rock surface). In our conditions, there was no significant fungal growth in any of the samples. The colonies observed on Marine Agar displayed the widest range of colours (wine-red, red, pink and orange, among others) in comparison with the ones observed on LB media, which were mostly yellowish and cream-coloured. Due to the known relation between the presence of pigments and antioxidant power, the main criterion for colony selection was the colour (Pawar *et al.*, 2015).

A collection of the 100 selected isolates in pure culture was established. A total of 34 isolates were initially identified through colony PCR and 16S rRNA Sanger sequencing. Although an initial step of incubation at 100°C was added to the PCR protocol of the isolates whose amplification had failed, some remained non-identified and therefore their total DNA was extracted to repeat the PCR. Finally, 56 of the isolates remained unidentified. Among the identified isolates, there were many *Bacillus* spp. (*B. oleronius*, *B. licheniformis*, *B. marisflavi*, *B. salsus* and *B. altitudinis*) and *Halobacillus* spp. (*H. trueperi* and *H. faecis*) as well as other species such as *Micrococcus antarcticus*, *Micrococcus luteus*, *Staphylococcus pasteuri*, *Vibrio tubiashii* and *Virgibacillus halodenitrificans* (Table S2).

Table 1. Top 30 most abundant genera and *P*-values for the One-Way ANOVA statistical analysis of their distributions among the three sampled locations

Taxa	*P* labelA	*P* (Tukeys) Dénia-Cullera	*P* (Tukeys) Vinaròs-Cullera	*P* (Tukeys) Vinaròs-Dénia
Rubrobacter	0.0011*	0.083	0.0096*	0.00091*
Rubricoccus	0.0018*	0.0014*	0.057	0.026*
Unclassified *Flammeovirgaceae*	0.006*	0.22	0.0051*	0.04*
Rubidimonas	0.0075*	0.58	0.0078*	0.024*
Unclassified *Erythrobacteraceae*	0.0085*	0.011*	0.018*	0.9
Unclassified *Alphaproteobacteria*	0.019*	0.052	0.02*	0.71
Unclassified *Cohaesibacteraceae*	0.021*	0.021*	0.062	0.66
Rivularia	0.026*	1	0.04*	0.038*
Unclassified *Rhodobacteraceae*	0.037*	1	0.058	0.052
Unclassified WD2101	0.039*	1	0.06	0.054
Unclassified *Chroococcales*	0.045*	0.063	0.068	1
Lewinella	0.052	1	0.073	0.075
Unclassified *Trueperaceae*	0.066	0.29	0.45	0.056
Unclassified *Phyllobacteriaceae*	0.091	0.094	0.87	0.18
Unclassified *Xenococcaceae*	0.1	0.85	0.1	0.21
Unclassified GMD14H09	0.12	1	0.17	0.16
Unclassified *Sphingomonadaceae*	0.13	0.88	0.24	0.13
Unclassified *Flavobacteriaceae*	0.13	1	0.16	0.17
B42	0.18	0.19	0.95	0.28
Unclassified *Rhodothermaceae*	0.24	0.23	0.47	0.82
Unclassified *Rhizobiales*	0.25	0.72	0.55	0.22
Chroococcidiopsis	0.28	0.4	0.3	0.97
Erythrobacter	0.28	0.29	0.42	0.95
Unclassified *Phycisphaerales*	0.32	0.29	0.66	0.73
Unclassified *Pseudanabaenaceae*	0.34	0.51	0.93	0.33
Jannaschia	0.39	0.44	0.47	1
Paracoccus	0.44	0.78	0.41	0.78
Unclassified At12OctB3	0.46	0.49	0.55	0.99
Streptococcus	0.54	0.91	0.52	0.76
Unclassified *Enterobacteriaceae*	0.77	0.91	0.75	0.95

Global *P*-values and *P*-values for the comparison by pairs is shown. Significant results are marked by an asterisk.

Antioxidant activity

In order to select and establish a collection of isolates with antioxidant properties, a high-throughput screening of the 100 isolates was performed by growing them on solid media containing H_2O_2. *Planomicrobium glaciei* and *E. coli* JM109 were used as positive and negative controls respectively. Strain JM109, with no known reports of antioxidant effect, exhibited a weak growth in the first (OD_{600} 1) and, sometimes, second dilution (OD_{600} 10^{-1}). This led us to the criterion to consider positive antioxidant producers those strains able to grow on H_2O_2-containing plates at least up to threefold dilutions (OD_{600} 10^{-2}). A total of 12 isolates were thus selected (Table 2) based on their ability to grow on 1 mM H_2O_2 plates as described above.

DPPH-based assays are widely used to detect and quantify the antioxidant power of plants or bacterial extracts. These assays are based on the decrease of DPPH absorbance at 517 nm in presence of antioxidant factors. The oxidative stress-resistant isolates selected from the H_2O_2 assay (shown in Table 2) were further tested using this method. CR17, CR21 and CR57 could not be tested due to poor growth in liquid culture, which made it impossible to obtain a concentrated extract, prepared as described in Experimental Procedures. 16S rRNA sequences were compared using NCBI BLAST tool. Isolates CR10-VR2 and CR22-CR28 were 100% identical in their 16S rRNA sequence, and therefore only one of them was selected for further assays (CR10 and CR22 respectively).

The test resulted in a general decrease in absorbance in all the samples, suggesting that the extracts were able to scavenge the DPPH. The isolates that proved more effective as antioxidants were CR22, CR24 and CR28, with values of scavenged DPPH over 30% (Fig. 4A). DR12 displayed low DPPH scavenging values maybe due to failure of the pigment extraction. Surprisingly, the control samples *P. glaciei* and JM109 did not display the expected effect. A set of three strains that had previously shown a protective effect against oxidative stress in a *Caenorhabditis elegans* model and a set of three *E. coli* strains (JM109, HB101 and DH5α) were also tested (Fig. 4D).

The two strains with the best results in the *in vitro* assays (CR22 and CR24) were selected for further *in vivo* antioxidant assays in the model organism *C. elegans,* where both proved able to display an important

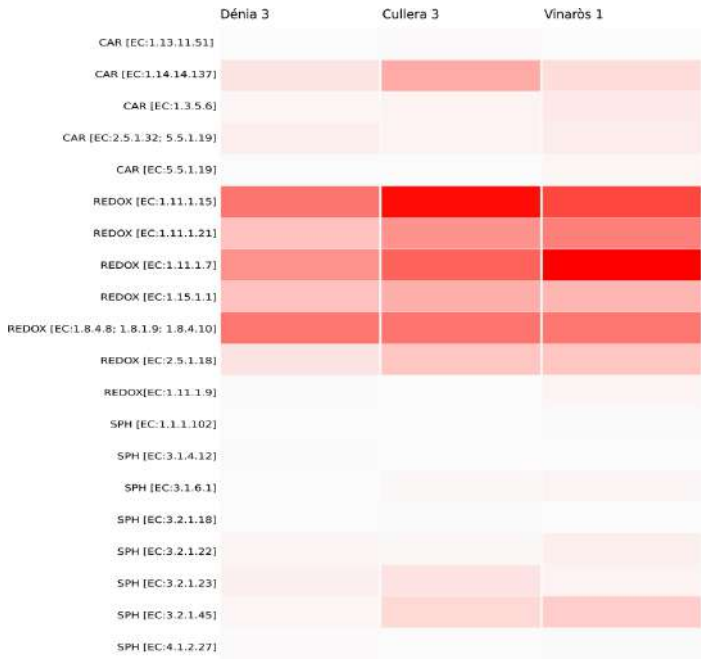

Fig. 3. Heatmap representing the functional analysis carried out through metagenomics sequencing. Enzymes related to carotenoid biosynthesis (CAR), oxidative stress (REDOX) and sphingolipid biosynthesis (SPH) are shown in the *Y*-axis.

antioxidant activity (Fig. 4B). Nematodes subjected to oxidative stress after being treated with isolates CR22 and CR24 displayed survival rates higher than the untreated worms and similar to those observed in the worms treated with vitamin C (survival rates of around 55%–65%).

Discussion

We report here, for the first time, and by using culture-dependent and independent (NGS) techniques, the microbiomes of the rocky-coastal surface of the supralittoral zone in three regions on the Mediterranean western coast. The three sampled sites, covering a coast line of about 260 km, displayed remarkably similar taxonomic profiles in terms of richness and microbial diversity, but still could perfectly be differentiated thanks to the significant difference in abundances of specific taxa, which suggest that the microbial composition of the Mediterranean supratidal zone, at least in eastern Spain, is stable but not identical within rocky locations. The studied communities were particularly dominated by bacterial strains previously described as thermophilic, halotolerant or radioresistant, such as the species within the genus *Rubrobacter* (Jurado *et al.*, 2012), and pigmented isolates, as is the case of species within the *Flameovirgaceae* family, like *Tunicatimonas pelagia* and *Porifericola rhodea* (Yoon *et al.*, 2011, 2012).

Truepera radiovictrix, characterized by an optimum growth temperature of 50°C and an extreme resistance to ionizing radiation, was first isolated from a hot spring in a geothermal area close to the Azores (Albuquerque *et al.*, 2005; Ivanova *et al.*, 2011). Moreover, the *Truepera* genus has been previously found in Lake Lucero Playa (New Mexico, USA), a particularly hostile environment as the lake dries periodically (Sirisena *et al.*, 2018). This is, to the best of our knowledge, the first report of sea-inhabiting *Truepera* in a non-thermal environment, and it is tempting to hypothesize that the genus *Truepera* might have a similar ecological niche (radiation- and desiccation-resistance) than *Deinococcus*, but in saline environments, as a consequence of both its radiation resistance and halotolerance (Albuquerque *et al.*, 2005).

Shotgun metagenomic analysis confirmed the similarity between the communities of the three sampled locations, as discussed above from the high-throughput 16S rRNA results, particularly at higher taxonomic (i.e. family) levels. Nevertheless, the results at lower taxonomic levels varied considerably among sequencing techniques. One of the largest differences at the species level was observed

Table 2. List of selected isolates, percentage of identity with the closest type strain, sequence similarity and results obtained in the H_2O_2 assay

Sample	Closest type strain	%	H_2O_2 Assay (dilution at which the isolate remains viable)
CR10	*Micrococcus luteus* (CP001628)	99.77	3
CR17	*Virgibacillus halodenitrificans* (AY543169)	99.58	7
CR21	Non-identified	–	4
CR22	*Virgibacillus halodenitrificans* (AY543169)	99.37	4
CR24	*Halobacillus trueperi* (AJ310349)	98.31	6
CR28	*Virgibacillus halodenitrificans* (AY543169)	100	6
CR37	*Bacillus marisflavi* (LGUE01000011)	100	4
CR44	Non-identified	–	3
CR67	*Bacillus oleronius* (X82492)	97.32	4
DM10	Non-identified	–	3
DR12	Non-identified	–	3
VR1	*Bacillus altitudinis* (ASJC011000029)	100	6
VR2	*Micrococcus luteus* (CP001628)	99.35	3
Positive control	*P. glaciei*		8
Negative control	*E. coli* (JM109)		1

within the Cyanobacterial group. In particular, high-throughput 16S rRNA revealed a large abundance of *Xennococcaceae*, whereas shotgun metagenomic sequencing revealed a more diverse population including members of *Pleurocapsa*, *Myxosarcina, Stanieria* and *Xenococcus*, as previously reported for marine environments (Burns *et al*., 2004; Alex *et al*., 2012; Yu *et al*., 2015; Brito *et al*., 2017).

The eukaryotic fraction of the samples was mainly composed of *Ascomycota*, such as *Glonium stellatum*. The genus *Glonium* includes saprophytic *Dothideomycetes* that produce darkly pigmented apothecia, which could contribute to the dark colour of the sampled rocks (Spatafora *et al*., 2012). Other species detected in the samples included as follows: *Cenococcum geophilum*, an ectomycorrhizal fungus previously described in coastal forest soils (Matsuda *et al*., 2015) and previously demonstrated to grow at up to 100 mM of NaCl (Obase *et al*., 2010); *Coniosporium apollinis*, a rock-inhabiting fungi previously isolated from the Mediterranean basin (Sterflinger *et al*., 1997); and *Lepidopterella palustris*, typically a freshwater fungus (Shearer *et al*., 2009), with this being, to the best of our knowledge, the first description of this species in a salt water habitat.

Taken together, the results obtained from both high-throughput 16S rRNA and metagenomic sequencing suggest that the sampled communities are composed of a diverse array of fungi (mainly belonging to the phylum Ascomycota), cyanobacteria (mainly *S. cyanosphaera* and *Pleurocapsa* spp., but also *Myxosarcina* spp. and *Xenococcus* spp.) and salt-adapted archaea, which remain rather stable among the three different sampled locations.

From the functional point of view, metagenomics sequencing showed abundance of enzymes involved in oxidative stress, mainly peroxidase, peroxiredoxin and thioredoxin, but also catalase and glutathione transferase. In contrast with this, enzymes involved in carotenoid or sphingolipid biosynthesis, which also play a role in the protection against oxidative stress, were less abundant and varied among locations, being abscisic acid 8′-hydroxylase (EC 1.14.14.137) in Cullera and glucosylceramidase (EC 3.2.1.45) in Vinaròs the ones with the highest values.

From the collection of cultured microorganisms, a total of 12 isolates were selected for their high antioxidant activity as measured by the oxidative stress assay performed with H_2O_2. Of those, *M. luteus* has been reported to encode genes related to resistance and tolerance to oxidative stress (superoxide dismutase and NADP reductase; Lafi *et al*., 2017). The DPPH assay was performed to dismiss false positives through the H_2O_2 assay. In general, the results correlated well with the ones previously observed in the H_2O_2 assay. It is important to note that, although DR12 displayed low scavenging in the DPPH assay, the extraction of pigments from this isolate was sub-optimal, since the pellet remained pink-coloured after the extraction process. Surprisingly, the control samples *P. glaciei* and JM109 did not display the expected effect in terms of antioxidant activity. On one hand, *P. glaciei* was expected to be one of the most antioxidant isolates, as its antioxidant activity was demonstrated in previous *in vivo* assays in *C. elegans* (Tanner *et al*., 2019) and in the H_2O_2 assay. Nevertheless, it was the worst strain in terms of DPPH scavenging. On the other hand, *E. coli* JM109, with no previous reports on antioxidant activity, resulted in high DPPH scavenging. This raises concerns on the suitability of DPPH-methods in bioprospecting for the determination of antioxidant activity and highlights the importance of using several alternative methods as the best option to have a proxy of the *in vivo* antioxidant effects. Nevertheless, the *in vivo*

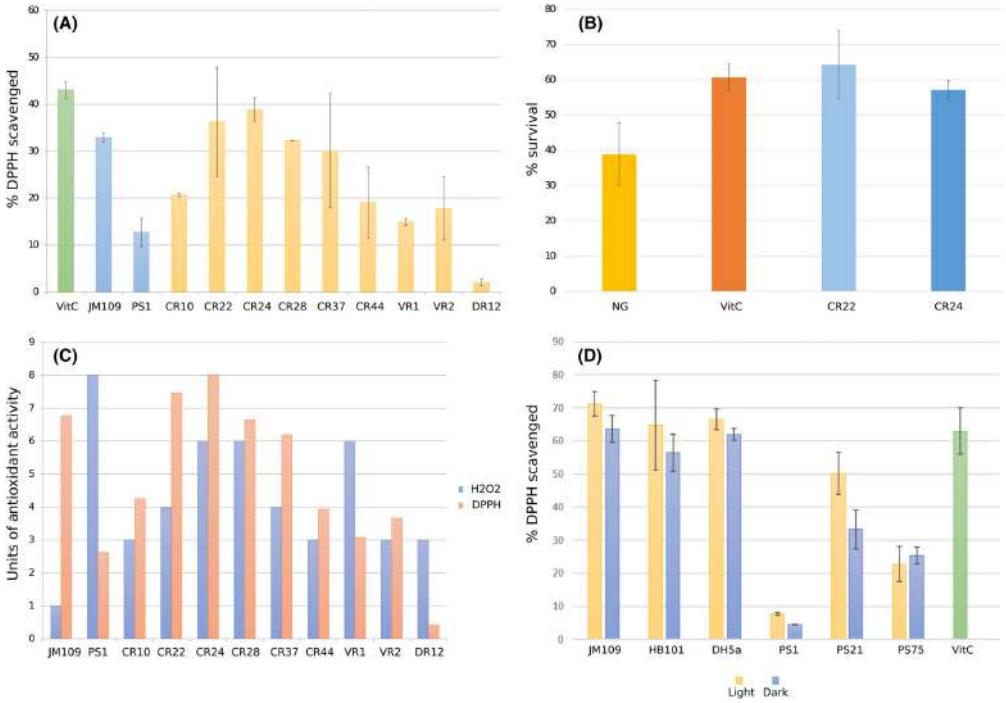

Fig. 4. (A) Antioxidant activity as measured through DPPH assay as described in EP. Absorbance was measured at 517 nm after 30 min of incubation with DPPH 50 μM. DPPH scavenged (%) is represented in *Y*-axis. VitC, vitamin C (0.5 μg ml^{-1} solution). (B) Antioxidant activity *in vivo* (using the model organism *C. elegans*). *Y*-axis indicates percentage of surviving worms after 5 h of incubation under oxidative stress (H_2O_2). Worms were treated with either a control diet (NG), a diet supplemented with the known antioxidant vitamin C as a positive control (VitC), or a diet supplemented with the selected strains CR22 and CR24. (C) Comparative analysis of the results obtained with H_2O_2 and DPPH assays. Values in *Y*-axis are normalized with respect to the highest value obtained in both assays. (D) DPPH assay with positive and negative controls. Absorbance was measured at 517 nm after 30 min of incubation with DPPH 50 μM. DPPH scavenged percentage is represented in *Y*-axis. VitC, Vitamin C, 0.5 μg ml^{-1} solution. Light and dark conditions are represented.

antioxidant assay performed in *C. elegans* allowed to confirm the antioxidant activity detected in the DPPH and H_2O_2 tests. Specifically, CR22 and CR24 displayed an antioxidant activity similar to the one observed in Vitamin C (Fig. 4B).

In general, though, the correlation between both methods was good, as the isolates with higher survival in the presence of H_2O_2 also displayed higher DPPH-scavenging ability (Fig. 4C). Nevertheless, there were some isolates that displayed different results depending on the method, in particular VR1 and CR37. Differences in VR1 could be the result of catalase activity, which may have enhanced its growth on the H_2O_2-supplemented plates. On the contrary, differences between both methods for CR37 could be caused by a deficient growth in solid medium. Once again, these results highlight the limitation of using a single screening technique for the selection of microbial strains with antioxidant activities.

A collection of both positive and negative controls (in terms of theoretical antioxidant activity) were tested using both assays (H_2O_2 and DPPH). PS1, PS21 and PS75 (*P. glaciei* 423, 97.38% ID; *Rhodobacter maris* JA276, 98.89% ID; and *Bacillus megaterium* NBRC 15308, 100% ID respectively) were the three control strains selected, all of them recovered from solar panels and previously tested in *C. elegans* for *in vivo* protection against oxidative stress (Tanner *et al.*, 2019). Three different strains of *E. coli* were chosen as negative controls (JM109, BH101, DH5α). For the DPPH assay, the isolates were grown under both light and dark conditions, in order to determine whether the light had a negative impact on the production of pigments or other antioxidant factors, as it is known that many pigments, particularly carotenoids, are prone to photodegradation (Boon *et al.*, 2010). For the *E. coli* strains, no significant differences were observed between growth in dark and light

conditions, whereas PS21 proved very sensitive to light (Fig. 4D). Moreover, the scavenging effect of the JM109 strain was also observed in the other two *E. coli* strains, confirming that the extracts obtained from *E. coli* contain compounds that are indeed able to react with DPPH. Even though *R. maris* and *B. megaterium* displayed better antioxidant properties than *P. glaciei*, which was again comparable to the negative control of methanol, they yielded lower DPPH-based activity than *E. coli* strains.

The biotechnological potential of extremophiles is well known, and saline environments are no exception to this rule (de Lourdes Moreno *et al*., 2013). However, and in contrast with the well-studied intertidal zone (Mitra *et al*., 2014), the supralittoral zone has been poorly studied to date. Interestingly, this zone experiences much higher selection pressures than the intertidal zone since while the intertidal zone is basically a marine environment which is only transiently and partially exposed to land conditions, the supralittoral zone forces organisms to adapt to a sea/land intermediate habitat where both marine and land stresses are present.

This work is the first holistic (using culture-dependent, culture-independent and biological activity assays) approach studying the microbial ecology and biotechnological potential, in terms of antioxidant properties, of the supralittoral zone of the Mediterranean rocky shore. Our results suggest that the western coastline of the Mediterranean Sea harbours a stable microbial community that is conserved among different locations, with cyanobacteria as the majoritarian bacterial taxon, followed by members of the *Flameovirgaceae* family and members of the *Rubrobacter* genus, as well as eukaryotic and archaeal members, such *Ascomycota* and halotolerant archaea. Furthermore, *in vitro* and *in vivo* assays demonstrate that this environment is a potential source of microorganisms with antioxidant activities that could hold potential for a wide range of applications in the food, cosmetic or pharmacological industries.

Experimental procedures

Sampling

Samples were collected from three different locations on the Mediterranean Western coast, in Eastern Spain: Vinaròs (Castelló), Cullera (València) and Dénia (Alacant). Three samples of dark-stained rock, at least two metres apart from each other and thus considered as biological replicates, were collected from the supralittoral (splash) zone of each location by scraping the surface with a sterile blade. Samples of the adjacent marine water were also taken, and both types of samples (scrapped rock and sea water samples) were separately stored in Falcon tubes in 15% glycerol, transported to the laboratory on ice and then stored at −20°C until required.

High-throughput rRNA and metagenomic sequencing

Total DNA was isolated from the samples with the PowerSoil DNA Isolation kit (MO BIO laboratories, Carlsbad, CA, USA) following the manufacturer's instructions. The quantity and quality of the isolated DNA was assessed using a Nanodrop-100 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and purified DNA samples were sequenced by Life Sequencing SL (València, Spain). On one hand, the hypervariable V3-V4 regions of the 16S rRNA gene was amplified as described by Klindworth *et al*. (2013) and sequenced on the high-throughput NextSeq 500 (Illumina) platform. Greengenes database was used for the taxonomic analysis. The statistical analysis was carried out with Calypso web tool (version 8.84; http://cgenome.net). The statistical comparison of the relative abundances between locations at the genus level was calculated through One-Way Anova test (Tables 1 and S1). Richness and Shannon index box plots, PCoA, relative abundances clustering and rarefaction curve were also constructed with Calypso.

On the other hand, shotgun metagenomic sequencing was performed on the NextSeq500 Illumina platform, with paired-end sequences and reads of 150 base pairs. The obtained sequences were filtered by using 'BBtools' version 37.28 (https://jgi.doe.gov/data-and-tools/bbtools/) in order to avoid ends holding quality values under the Q20 standards. Lectures coming from human contamination were also dismissed by mapping them against the reference human genome (GRCh37d5) version 0.7.15. Assembly was carried out with 'SPAdes' (Bankevich *et al*., 2012) version 3.9. ORFs prediction was carried out by 'MegaGeneMArk' (Zhu *et al*., 2010) version 3.38 and rRNA prediction, by 'RNAmmer' (Lagesen *et al*., 2007) version 1.2. Functional annotation of the predicted CDS was carried out with *BLAST2go* (Conesa *et al*., 2005) version 4.1.9.

The Clustergrammer on-line software (Fernández *et al*., 2017) was used for the functional analysis heatmap construction, by using a correlation type distance and average linkage.

Isolation and identification of bacterial strains

Three different growth media were used for this study: Lysogenic Broth (LB, composition in g l^{-1}: 10 tryptone, 10 NaCl, 5.0 yeast extract, 15 agar); Reasoner's 2A agar (R2A, composition in g l^{-1}: peptone 0.5, casaminoacids 0.5, yeast extract 0.5, dextrose 0.5, soluble starch 0.5, K_2HPO_4 0.3, $MgSO_4$ 0.05, sodium pyruvate 0.3, 15 agar); and Marine Agar (composition in g l^{-1}: peptone 5.0, yeast extract 1.0, ferric citrate 0.1, NaCl 19.45, $MgCl_2$ 5.9, Na_2SO_4 3.24, $CaCl_2$ 1.8, KCl 0.55,

$NaHCO_3$ 0.16, KBr 0.08, $SrCl_2$ 0.034, H_3BO_3 0.022, Na_4O_4Si 0.004, NaF 0.024, NH_4NO_3 0.0016, Na_2HPO_4 0.008, 15 agar). The scraped rock samples were homogenized in the Falcon tube by vigorously mixing with a vortex, and serial dilutions were cultured on the different media and incubated at room temperature for 7 days. Marine water samples were also cultured in the same conditions. After 1 week of incubation, individual colonies were selected based on colony pigmentation and isolated by independent re-streaking on fresh medium. Pure cultures were then cryo-preserved at −80°C in 20% glycerol (vol:vol) until required.

Colony PCR and, were needed, DNA extracts of each of the isolated strains, were used for taxonomic identification through 16S rRNA gene sequencing using universal primers 28F (5′-GAG TTT GAT CNT GGC TCA G-3′) and 519R (5′-GTN TTA CNG CGG CKG CTG-3′). Colony PCR was performed with an initial step of incubation at 95°C for 5 min to lyse cells followed by PCR amplification (30 cycles of 30 s at 95°C, 30 s at 54°C, 30 s at 72°C, followed by 10 min at 72°C). The DNA extraction was done following the Latorre *et al*. (1986) protocol. Amplifications were verified by electrophoresis in a 0.8% agarose gel and then amplicons were precipitated overnight in isopropanol 1:1 (vol:vol) and potassium acetate 1:10 (vol:vol; 3 M, pH 5). DNA pellets were washed with 70% ethanol and resuspended in 30 μl Milli-Q water. BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) was used to tag amplicons, which were sequenced with the Sanger method by the Sequencing Service (SCSIE) of the University of Valencia (Spain). All sequences were manually edited with Pregap4 (Staden Package, 2002) to eliminate low-quality base calls, and final sequences were compared by EzBioCloud 16S tool (https://sourceforge.net/projects/staden/).

Antioxidant activity

Hydrogen peroxide assay. The collection of isolates was initially screened for antioxidant activity by applying oxidative stress to the isolated colonies through the addition of hydrogen peroxide (H_2O_2) to the growth medium. In order to do so, isolates were grown on solid media for 4 days or until reaching enough biomass. Then, the optical density at 600 nm (OD_{600}) was measured, adjusted to a value of 1, and serial dilutions prepared up to seven times fold. Two microlitres of each dilution were placed on a LB or Marine Agar place, to which 1 mM H_2O_2 had been previously added. The plates were incubated at room temperature and in the dark to avoid degradation of the H_2O_2, and results were recorded after two, four and six days. Two strains were used as controls for the assay: PS1 (*Planomicrobium glaciei* 423, 97.38% ID) and *Escherichia coli* JM109 as a positive and negative control for antioxidant activity respectively. *Planomicrobium glaciei* is a pigmented microorganism whose antioxidant activity has previously been reported *in vivo* using a *Caenorhabditis elegans* model (Tanner *et al*., 2019).

DPPH assay. Since the H_2O_2 assay can result in false-positive results due to catalase activity, a second assay using 2,2-diphenyl-1-picrylhydrazyl (DPPH) was performed to dismiss false positives in the H_2O_2 assay and to confirm the antioxidant activity of the selected strains (the ones with the best antioxidant activity according to the previous assay). Pigments were extracted from the isolates based on the protocols described by Brand-Williams *et al*. (1995), von Gadow *et al*. (1997) and Su *et al*. (2015), with the modifications suggested by Sharma and Bhat (2009). Briefly, the isolates were grown overnight in liquid LB medium and OD_{600} was measured and normalized at a value of 1.2. Cells were then harvested by centrifugation at 11,300 *g* for 3.5 min, and the pellets resuspended in 500 μL of methanol, vigorously vortexed and sonicated for 5 min (Ultrasonic bath XUBA1, Grant Instruments, Royston, UK). The supernatant was collected after centrifugation at 11,300 *g* for 3 min and kept in the dark until the assay was performed. The extraction was repeated as described until a colourless pellet was obtained.

For the DPPH assay, 600 μl of the extract in methanol were mixed with 400 μl of DPPH solution (50 μM in methanol) and incubated for 30 min in the dark. The negative control sample consisted of DPPH mixed with methanol. Absorbance was measured at 517 nm (Ultrospec 200 UV/V Visible Spectrophotometer, Pharmacia Biotech, Piscataway Township, NJ, USA).

A standard curve with a control antioxidant, ascorbic acid (vitamin C) was performed at 10, 5, 1, 0.5, 0.1, 0.05 and 0.01 μg ml^{-1} concentrations in methanol. The detection threshold was established at 0.5 μg ml^{-1} of vitamin C, as lower concentrations of vitamin C did not change DPPH absorbance (data not shown).

DPPH scavenging ability was quantified by measuring the decrease in the absorbance of this compound at 517 nm, and the percentage of scavenged DPPH was calculated using the following formula:

$$\%\,\mathrm{DPPH} = \left(1 - \frac{\mathrm{Abs\,517\,Extract}}{\mathrm{Abs\,517\,Control}}\right) \times 100.$$

In vivo *oxidative stress assays with* C. elegans

Wild-type *C. elegans* strain N2 (Bristol, UK) was routinely propagated at 20°C on Nematode Growth Medium

(NGM) plates supplemented with *E. coli* strain OP50 as the regular food source.

Nematodes were synchronized by isolating eggs from gravid adults at 20°C. Synchronization was performed on NGM plates with different treatments: *E. coli* OP50 was supplied as a negative control; *E. coli* OP50 plus vitamin C (vitC) at 10 μg ml^{-1} as a positive control; and, finally, *E. coli* OP50 plus one of the selected isolates was used in order to test the effect of administrating the selected strains. Duplicates were performed for every condition. Bacterial strains were grown overnight in liquid LB medium at 28°C and 11,300 *g*. Then, OD_{600} was adjusted to 30 and 50 μl of the bacterial suspension were added to the plates.

The synchronized worms were incubated for 3 days on the previously described plates, until reaching young adult stage. Then, young adult worms were selected for each treatment ($n = 50$) and incubated at 20°C on the corresponding treatment, until reaching 5-day adult stage. The selected worms were then transferred to plates containing basal medium supplemented with 2 mM H_2O_2 and incubated for 5 h at 20°C. After incubation, survival rates for each condition (negative control, positive control and bacteria-fed worms) were recorded by manually counting the number of living versus dead worms.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

MP conceived the work. MP, KT and EMM collected the samples. EMM, KT and ÀVV performed the culture-based characterization, and KT carried out the bioinformatic analysis. All authors (MP, KT, ÀVV, EMM and JP) analysed the results, wrote and approved the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Rarefaction curve at OTU level.
Fig. S2. Main bacterial (A), archaeal (B) and eukaryotic (C) groups identified in the sample obtained from Vinaròs and analysed through metagenomics sequencing.
Fig. S3. Main bacterial (A), archaeal (B) and eukaryotic (C) groups identified in the sample obtained from Cullera and analysed through metagenomics sequencing.
Fig. S4. Main bacterial (A), archaeal (B) and eukaryotic (C) groups identified in the sample obtained from Dénia and analysed through metagenomics sequencing.

Table S1. Top 30 most significant genera and *P*-values for the One-Way ANOVA statistical analysis of their distributions among the three sampled locations. Global *P*-values and *P*-values for the comparison by pairs is shown. Significant results are marked by an asterisk.
Table S2. List of the strains identified in the collection, with the closest type strain, accession number, ID percentage and the GenBank accession number for the 16S rRNA sequences. The identification code of the strains corresponds to the location from which it was isolated (V: Vinaròs, C: Cullera, D: Dènia), the sample type (R: rock, M: marine water) and a number.

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High Culturable Bacterial Diversity From a European Desert: The Tabernas Desert

*Esther Molina-Menor[1], Helena Gimeno-Valero[2], Javier Pascual[2], Juli Peretó[1,2,3] and Manuel Porcar[1,2]**

[1] Institute for Integrative Systems Biology I[2]SysBio (University of València-CSIC), Paterna, Spain, [2] Darwin Bioprospecting Excellence S.L., Parc Científic Universitat de València, Paterna, Spain, [3] Departament de Bioquímica i Biologia Molecular, Universitat de València, Burjassot, Spain

One of the most diverse ecological niches for microbial bioprospecting is soil, including that of drylands. Drylands are one of the most abundant biomes on Earth, but extreme cases, such as deserts, are considered very rare in Europe. The so-called Tabernas Desert is one of the few examples of a desert area in continental Europe, and although some microbial studies have been performed on this region, a comprehensive strategy to maximize the isolation of environmental bacteria has not been conducted to date. We report here a culturomics approach to study the bacterial diversity of this dryland by using a simple strategy consisting of combining different media, using serial dilutions of the nutrients, and using extended incubation times. With this strategy, we were able to set a large (254 strains) collection of bacteria, the majority of which (93%) were identified through 16S ribosomal RNA (rRNA) gene amplification and sequencing. A significant fraction of the collection consisted of Actinobacteria and Proteobacteria, as well as Firmicutes strains. Among the 254 isolates, 37 different genera were represented, and a high number of possible new taxa were identified (31%), of which, three new *Kineococcus* species. Moreover, 5 out of the 13 genera represented by one isolate were also possible new species. Specifically, the sequences of 80 isolates held a percentage of identity below the 98.7% threshold considered for potentially new species. These strains belonged to 20 genera. Our results reveal a clear link between medium dilution and isolation of new species, highlight the unexploited bacterial biodiversity of the Tabernas Desert, and evidence the potential of simple strategies to yield surprisingly large numbers of diverse, previously unreported, bacterial strains and species.

Keywords: microbial diversity, Tabernas Desert, drylands ecology, biocrust, Actinobacteria

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***Correspondence:**
Manuel Porcar
manuel.porcar@uv.es

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INTRODUCTION

Only a small fraction of the microbial diversity of our planet can be cultured under traditional microbiological tools, media, and strategies (Overmann et al., 2017; Steen et al., 2019). The use of ribosomal RNA (rRNA) genes and other genomic sequences as key markers in microbial ecology led to the discovery of a previously unknown microbial diversity (Rappé and Giovannoni, 2003).

Traditional culturing techniques have allowed the isolation of rapidly-growing, easy-to-culture microbial taxa, thus relegating the vast majority of the microbial world to the imprecise group of "unculturable" organisms, which has been addressed as the "microbial dark matter" (Bernard et al., 2018; Bowman, 2018).

The generally accepted "1% culturability paradigm," which refers to the fact that around 99% of environmental microorganisms are in fact unculturable, was recently revised by Martiny (2019). The evidence based on the study of different environments no longer supports some of the interpretations. However, even though this "1%" is probably no longer acceptable, as discussed by Steen et al. (2019), microbiologists are still far from culturing most of the existing bacterial diversity.

One of the reasons behind the obstacles in culturing environmental bacteria is the difficulty in mimicking the particular environmental conditions that certain microbes require for growth, as standard laboratory conditions may differ from the natural habitats (Stewart, 2012). Small variations in a wide variety of parameters make almost impossible to cover the immense number of different micro-environments that can be found in nature. Moreover, not only abiotic factors but also the interactions between species and metabolic cooperation are critical for the development of specific taxa that may be reluctant to grow under laboratory conditions (Vartoukian et al., 2010).

Multi-omic data have broadened our knowledge on previously unknown microbial communities and taxa (Vilanova and Porcar, 2016). Specifically, metataxonomics, which refers to the high-throughput characterization of the microbiota emphasizing on the taxonomic status and relationships between taxa (Marchesi and Ravel, 2015), has shed light on the actual composition of microbial communities, whereas metagenomics has partially unveiled some key ecological interactions, with no need for cultivating microorganisms (Lok, 2015).

Metagenomic data have been used in order to design tailor-made culture media for specific samples (Gutleben et al., 2020), but the inference of metabolic features and the functional analysis depends on the availability of complete annotated genes or genomes in public databases, what leads to failure in function assignment due to mislead annotations in genomes (Choi et al., 2016). All possible novel metabolic pathways are as diverse as the microorganisms that feature them, highlighting the countless potential applications that can derive from the uncultured microbial world (Overmann et al., 2017).

Sophisticated culture-dependent techniques have already been developed, such as the so-called ichip device, which is based on the cultivation of microorganisms by incubating the chips *in situ*, overcoming the problem of mimicking the environmental conditions (Berdi et al., 2017), or reverse genomics, which uses genomic data in order to obtain targeted antibodies to capture and sort specific cell types (Cross et al., 2019). Moreover, culturing gut microbes through techniques, such as "dilution to extinction" (Gross et al., 2015), in complete anaerobic flow work (Browne et al., 2016) and the selection of micro colonies in combination with multiple incubation conditions (Lagier et al., 2016), among other efforts in cultivating "uncultured" bacteria, have resulted in a considerable increase in the number of identified species (Martiny, 2019). Moreover, increasing the incubation times and diluting media have also proven to be useful for this purpose (Gutleben et al., 2020).

Soils are known to harbor a wide bacterial diversity with an immense potential in biotechnology and biomedicine. Actinobacteria are the major inhabitants of soil environments and one of the most ancient groups of bacteria (Battistuzzi et al., 2004), which play an important role from the ecological point of view in maintenance processes (Lewin et al., 2016). They are well-known because of their ability to produce a wide range of bioactive secondary metabolites with immense potential and applications, in particular due to their role in the synthesis of antibiotics (Zhao et al., 2018), but also enzymes, such as cellulases, which can be used in the industrial-scale breakdown of cellulosic plant biomass into simple sugars that can then be converted into biofuels (Lewin et al., 2016).

Among the different terrestrial ecosystems and soil environments, drylands have been discovered as a source of biotechnologically-relevant bacterial strains (Azua-Bustos and González-Silva, 2014; Mohammadipanah and Wink, 2016). Extreme environments, such as deserts, have been found to host a wide diversity of microbial taxa adapted to live under such harsh conditions of temperature, desiccation, and radiation, among which low water and nutrient availability are the main limiting factors for organisms (Saul-Tcherkas et al., 2013). These factors shape the local biocenosis and act as a selection pressure toward interesting mechanisms to overcome the hurdles of living in an extreme ecological niche. Both culture-dependent and -independent studies have been performed in the Atacama, the Sahara, or the Gibson deserts (Lester et al., 2007; Azua-Bustos et al., 2014; Belov et al., 2018). However, European arid lands have been poorly studied from both points of view.

The Tabernas Desert, located in the province of Almeria (southeastern Spain), is a particular, not very much studied region to date in terms of bacterial culturability. It is formed by marls, sandstone, and scarce vegetation. The annual rainfall is below 250 mm, its climate is considered hot semiarid to desert climate, depending on the altitude, and it has interesting, well-developed biocrusts.

Biological soil crusts are multi-organism consortia worldwide—spread in dryland landscapes, being estimated that around 12% of the total terrestrial surface is covered by biocrust (Rodríguez-Caballero et al., 2018). Specifically, Miralles et al. (2020) recently reported how biocrusts influenced the microbial communities in the surrounding soil, which also depend on multiple environmental factors, such as pH, temperature, or salinity, and other biotic interactions. Moreover, Maestre et al. (2013) have studied in detail the effect of climate change on the microbial biocenosis associated with biocrust in different areas, including Sorbas, which is in the vicinities of the Tabernas Desert. The studies revealed that changes in biocrusts play a major role in further modeling the composition of microorganisms.

In the present work, we have characterized the microbial communities of the Tabernas Desert by using improved culturomics techniques that have allowed the identification of an unprecedented rich diversity of bacterial strains from this unique European dryland. We hypothesized that by combining

culture laboratory techniques and increasing incubation times, a higher diversity of bacterial taxa would be isolated. The results obtained confirm that simple strategies in culturomics can lead to a better understanding of the culturable fraction of microbial communities, including the isolation of new and potentially new microbial taxa.

MATERIALS AND METHODS

Sample Collection

Biocrust samples were obtained in September 2018 at the Tabernas Desert, in the vicinity of the Natural Park. Sampling was carried out by taking in Falcon tubes the upper 1–2 cm of biocrust-covered surfaces in three different locations (geolocation 1: 37.0083240, −2.4532390; geolocation 2: 37.0215300, −2.4323020; geolocation 8: 37.0226290, −2.4295450), and a total of eight samples were collected of different biocrusts in terms of color and appearance (**Figure 1**), which were further used for culturing assays. Sample 1.1.1 corresponded to a black stained biocrust; sample 1.2.1 was from a white-pink biocrust; samples 1.3.1, 2.1.1, and 8.1.1 were all white biocrust with similar appearance; sample 1.4.1 was a yellowish biocrust; sample 2.5.1 was a white smooth biocrust; and sample 8.3.1 was a dark yellow biocrust (**Table 1**).

Isolation of Bacterial Strains

The samples were homogenized by mixing 1 g of biocrust with 1 ml of sterile phosphate buffer saline (PBS) 1×. Then, serial dilutions of the suspensions were prepared, and 50 μl of the 10^{-3}–10^{-5} samples was spread on Petri dishes containing either Tryptic Soy Agar (TSA) medium (composition in g/L: 15.0 tryptone, 5.0 soya peptone, 5.0 sodium chloride, 15.0 agar) or Reasoner's 2A (R2A) medium (composition in g/L: 1 peptone, 0.5 yeast extract, 0.5 dextrose, 0.5 soluble starch, 0.3 dipotassium phosphate, 0.05 magnesium phosphate, 0.3 sodium pyruvate, 15.0 agar) at concentrations 1 × (standard concentration), 0.1×, and 0.01 × (10 and 100 times diluted, respectively). TSA is a rich media used for general purposes, whereas R2A is a nutrient-poor media that favors the isolation of slow-growing and oligotrophic bacteria. Media were sterilized by autoclaving at 121°C for 20 min. Agar was autoclaved separately to the nutrient solution of each medium and added just before pouring the media into the plates. After 2 weeks of incubation at room temperature (23°C), individual colonies were selected based on their color and morphology and isolated by independent re-streaking on fresh media in order to obtain them in pure culture. New colonies were also selected a month of incubation, in order to identify, small, slow-growing microorganisms. A total of 254 isolates were cryo-preserved as a glycerol stock (20% glycerol in PBS, vol:vol) at −80°C until required.

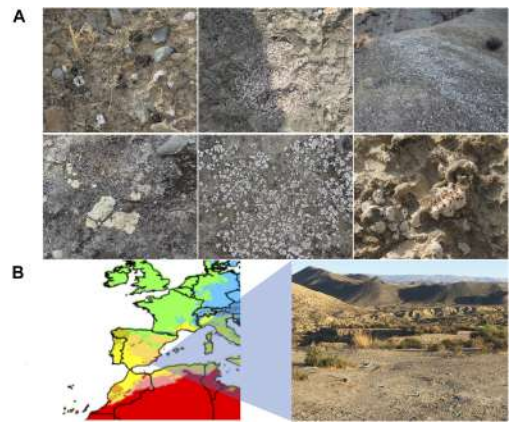


FIGURE 1 | (A) Biocrust sampling sites in the Tabernas Desert. Six different biocrusts are shown. **(B)** Climate map zoom of Spain in the Köppen–Geiger climate classification map (1980–2016) from Beck et al. (2018). The different climate areas are represented in colors. Warm-arid regions, corresponding to desert environments, are represented in red. Picture of the sampling area in the vicinity of the natural Park of the Tabernas Desert (Almeria, southeastern Spain).

Colony Identification Through 16S rRNA Sequencing

A loopful of 3 μl of microbial biomass from grown plates was suspended in 100 μl of Milli-Q sterile water and then boiled for 10 min before the PCR in order to ensure cellular lysis. Colony PCR was used for taxonomic identification through 16S rRNA gene sequencing by using the universal primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) (Edwards et al., 1989) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Stackebrandt and Liesack, 1993). PCR was carried out with a step of incubation at 94°C for 5 min, then 24 cycles of denaturation at 94°C for 15 s, annealing at 48°C for 15 s, elongation at 72°C for 5 min, and a final elongation step at 72°C for 5 min.

Amplifications were visualized by electrophoresis in a 1.2% agarose gel stained with GelRed nucleic acid gel stain (Biotium, CA, United States) (100 V for 30 min). Amplicons were precipitated overnight in isopropanol 1:1 (vol:vol) and potassium acetate 1:10 (vol:vol) (3 M, pH 5) at −20°C. DNA was pelleted by centrifugation at 12,000 rpm for 10 min, then washed with ethanol 70%, and resuspended in the required 15 μl of Milli-Q water. BigDye® Terminator v3.1 Cycle Sequencing

TABLE 1 | List of biocrust samples.

	Sample	Appearance
Geolocation 1	1.1.1	Black
	1.2.1	White-pink
	1.3.1	White
	1.4.1	Pale yellow
Geolocation 2	2.1.1	White
	2.5.1	White smooth
Geolocation 8	8.1.1	White
	8.3.1	Dark yellow

Kit (Applied Biosystems, Carlsbad, CA, United States) was used to tag amplicons, which were sequenced with the Sanger method by the Sequencing Service (Servei Central de Suport a la Investigació Experimental SCSIE) of the University of Valencia (Spain). All sequences were manually edited with Trev (Bonfield and Staden, 2002) to eliminate low-quality base calls, and final sequences were compared by EzBioCloud 16S identification BLAST tool to nucleotide databases. Primers 341R (5′-CTGCTGCCTCCCGTAGG-3′) (Muyzer et al., 1996) and 1055F (5′-ATGGCTGTCGTCAGCT-3′) (Harms et al., 2003) were used for whole 16S rRNA sequencing in order to fully identify the isolates holding an identity lower than 98.7% with the closest type strain by sequencing partially the 16S rRNA gene. The MEGA7 tool was used to assemble the whole 16S rRNA gene sequence. The quality of the chromatograms was checked with the Sequence Scanner software. The quality value (QV) threshold was 20. In the case of competing peaks, the highest peak in both forward and reverse sequences after the assembly was chosen. The sequences were manually revised afterward and have been deposited under the GenBank/EMBL/DDBJ accession numbers MT749781–MT750013 and MN069869–MN069868.

In order to analyze the closest environmental clone or isolates of our strains, an extensive blast was carried out against the NCBI Nucleotide collection (nr/nt) databases optimized for highly similar sequences.

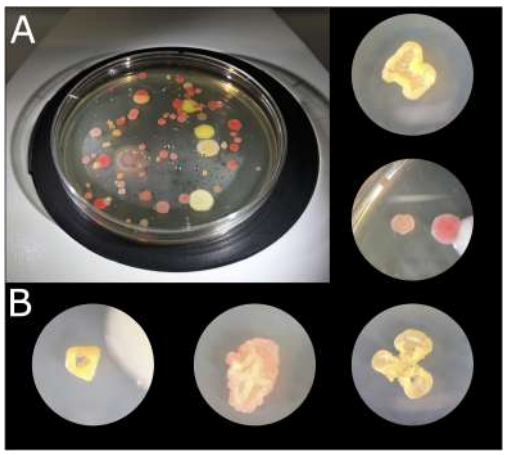


FIGURE 2 | Microbial colonies obtained after culturing biocrust samples at 23°C for 2 weeks. **(A)** TSA 1× plate of sample 1.4.1. **(B)** Microbial colonies under the binocular loupe.

RESULTS

Isolation of Bacterial Strains

Culturing different biocrust samples from the Tabernas Desert yielded a large number of different colonies in terms of color, shape, and morphology (**Figure 2**). A total of 254 strains were isolated in pure culture, which were named as T or R depending on the medium from which they had been isolated (TSA or R2A, respectively) and numbered consecutively. There was no significant fungal growth, and most of the bacterial colonies displayed bright colors, being the most abundant those of red, pink, orange, and yellow-pigmented bacteria. There were also dark stained isolates, and some others changed from orange to purple with time. The morphology of the isolates was also diverse, being particularly curious those growing in 3D structures and cell clumps (**Figure 2B**). Isolates T1 to T159 were isolated from TSA plates after 2 weeks of incubation, of which 131 were obtained in pure culture and cryopreserved for further use; isolates R1 to R115 were selected from R2A plates, of which only 76 were obtained in pure culture. It was not possible to obtain all the selected colonies in pure culture due to crossed contamination or growth failure after several re-streaking steps. After a month of incubation of the plates, small colonies were also selected, and 47 of them were obtained in pure culture, being 20 of them originally from TSA and 27 from R2A plates.

Colony Identification Through 16S rRNA Sequencing

From the 254-strains collection, a total of 236 isolates (93%) were identified, belonging to 37 different genera, and only 18 isolates remained not identified due to either a lack of amplification in PCR or failure in sequencing. A total of 62 isolates were identified as *Arthrobacter* spp., which was the most abundant genus representing 24.8% of the collection. The second most represented genus was *Mycolicibacterium*, holding a 7.09% of abundance, similar to the abundances of *Microbacterium* and *Roseomonas*. In contrast, the less abundant genera were *Agrococcus*, *Amycolatopsis*, *Azospirillum*, *Bacillus*, *Caulobacter*, *Enterovirga*, *Flaviflagellibacter*, *Herbaspirillum*, *Micrococcus*, *Noviherbaspirillum*, *Paenarthrobacter*, *Staphylococcus*, and *Variovorax*, being represented only by one isolate each (**Figure 3**).

A total of 80 isolates in the collection showed a percentage of identity lower than 98.7%, suggesting that they could represent new species within that genus (Chun et al., 2018). Out of the 80 possible new species, 26 isolates were identified as *Arthrobacter* spp., whereas the rest were distributed among half of the genera detected. Interestingly, between the genera that had at least one possible new species, five of them were represented by just one isolate (*Caulobacter*, *Herbaspirillum*, *Azospirillum*, *Enterovirga*, and *Flaviflagellibacter*) (**Figure 3**). For example, we isolated five *Kineococcus* strains as being possible new species, and three were further identified and described. The three new species have recently been published with valid names *Kineococcus vitellinus* sp. nov., *Kineococcus indalonis* sp. nov., and *Kineococcus siccus* sp. nov. (Molina-Menor et al., 2020). Moreover, the isolates identified as *Homoserinibacter* and *Ramlibacter*, which were 10 and 7, respectively, were all potential new species, and one isolate in the collection showed an ID value lower than 95%, which is the threshold value for new genus description (Chun et al., 2018). This possible new genus, represented by the isolate named as T16, would be closely related to *Roseomonas*, within the Acetobacteraceae family.

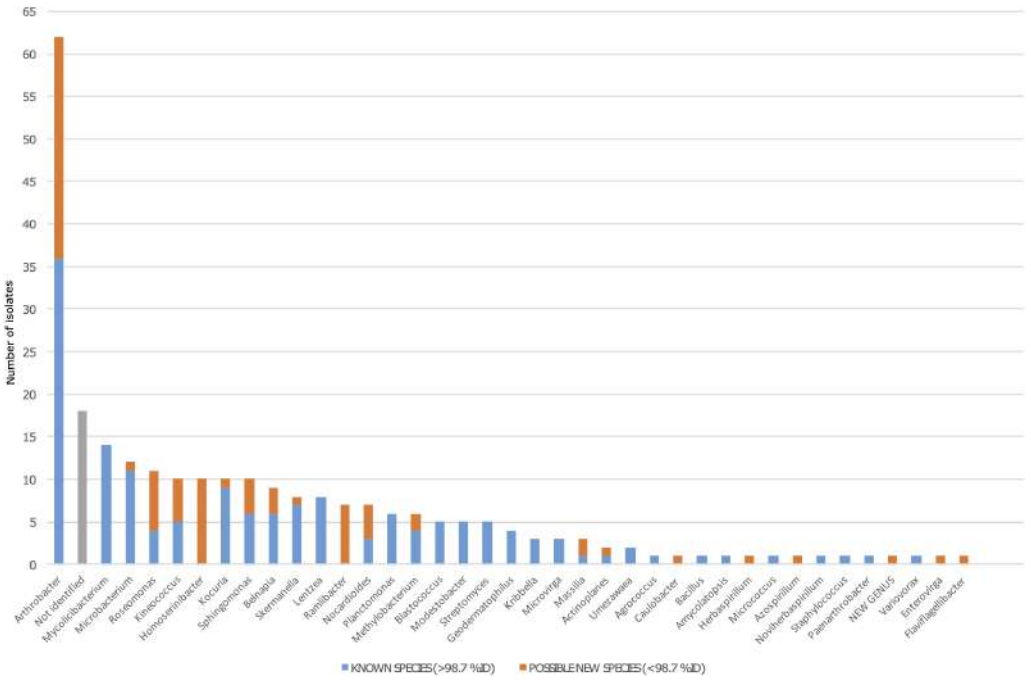


FIGURE 3 | Bacterial diversity among the 254 isolates from the Tabernas Desert collection. The blue bar represents the number of fully identified isolates in each genus (>98.7% of identity against 16S rRNA databases). The orange bar represents the number of possible new species in each genus (as defined by <98.7% of identity against 16S rRNA databases). Not identified isolates are shown in gray.

Other genera identified in the collection were *Actinoplanes*, *Belnapia*, *Blastococcus*, *Geodermatophilus*, *Kocuria*, *Kribbella*, *Lentzea*, *Massilia*, *Methylobacterium*, *Microvirga*, *Modestobacter*, *Nocardioides*, *Planctomonas*, *Skermanella*, *Sphingomonas*, *Streptomyces*, and *Umezawaea* (**Figure 3**).

As for the isolation conditions, it was possible to analyze the bacterial diversity according to the media from which the genera had been isolated, as well as the incubation time required for their isolation. Almost 60% of the genera were isolated from at least two different media (TSA or R2A at three concentrations), whereas 15 genera were isolated just from one of the tested conditions (**Supplementary Table 1**). The quantitative distribution of genera among the different isolation media is shown in **Figure 4A**. In the case of *Arthrobacter* and *Microbacterium*, isolates were identified in all six media but, most frequently, in the most concentrated ones. Others, such as *Actinoplanes*, were isolated from the most diluted concentration on TSA and R2A, and the possible new genus was isolated from TSA 0.01×. Non-identified strains were also isolated from all six media, although the higher abundance was detected in the less concentrated ones, especially on R2A 0.01×. Interestingly, the five *Blastococcus* isolates grew on R2A 1× (**Figure 4A**).

The co-isolation of genera in the different nutrient conditions was compared through Venn diagram representations, which are shown in **Figures 4B,C**, listed in **Tables 2, 3,** and **in Figure 5**. In the case of TSA media, 11 different genera were detected in the three concentrations assayed. The concentration that allowed the isolation of more exclusive taxa was the intermediate 0.1×, with eight unique taxa, in contrast with two on TSA 1× and four on TSA 0.01× (**Figure 4B**). On the contrary, for R2A media, the concentrated R2A 1× seemed the best media for isolating different genera, being only five of them common for the three concentrations. Only two exclusive taxa were isolated from 0.1 and 0.01 × R2A (**Figure 4C**). In general, thus, bacterial genera tended to be shared by the TSA media, regardless of the dilution. In contrast, genera isolated from R2A tended to concentrate in the non-diluted medium.

The comparison, regardless of the dilution factor, of TSA and R2A media in terms of genera diversity revealed that one fourth of the groups (as non-identified isolates and the possible new genus were included) had been exclusively isolated in TSA, whereas around 20% were unique taxa for R2A (**Figure 5**). This revealed a clear preference of some genera to grow under specific environments, as almost half of them were only isolated from one of the media used. Interestingly, all the genera that were isolated exclusively in TSA were represented by one isolate, except for *Planctomonas*, which was represented by six (five from TSA 1× and one from TSA 0.01×). In the case of the unique R2A taxa,

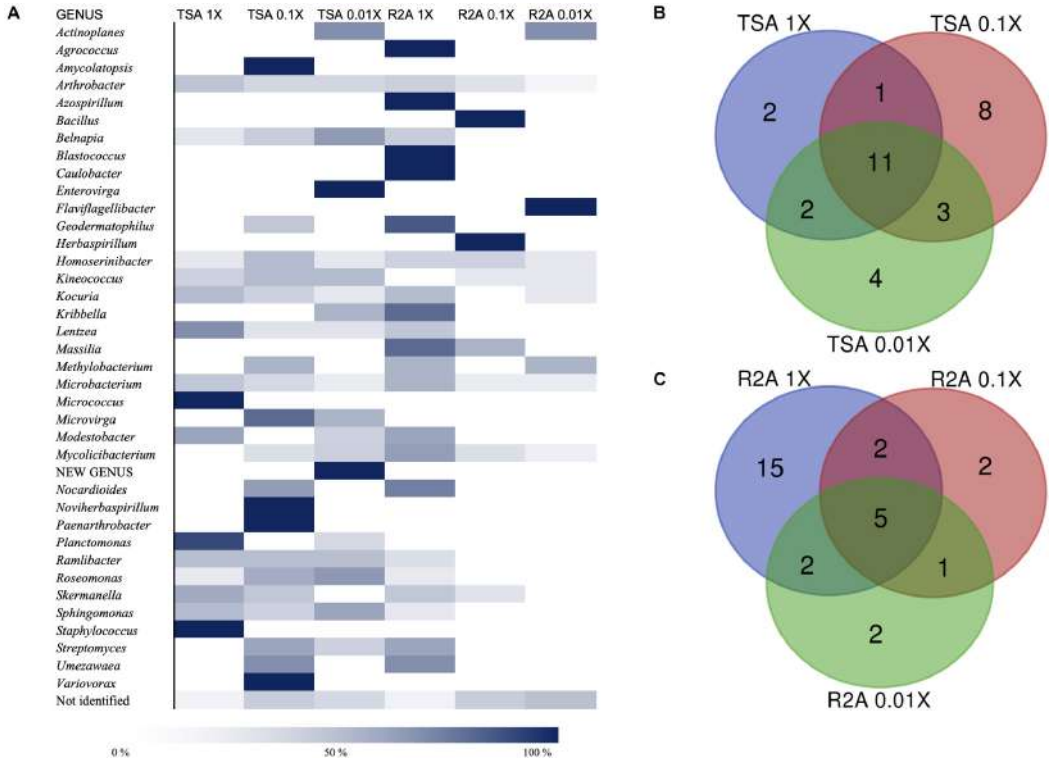


FIGURE 4 | Taxonomic diversity as a function of the culture media and dilutions used. **(A)** Heatmap of relative abundances of the different genera, possible new genera, and non-identified isolates among media (TSA at 1, 0.1, and 0.01×; R2A at 1, 0.1, and 0.01×). The genera are listed in alphabetical order. Possible new genera and non-identified isolates are included. **(B)** Venn diagram of shared and exclusive taxa at the genus level in TSA media at three different concentrations (1, 0.1, and 0.01×). Possible new genera and non-identified groups are included. **(C)** Venn diagram of shared and exclusive taxa at the genus level in R2A media at three different concentrations (1, 0.1, and 0.01×). Possible new genera and non-identified groups are included.

apart from the five *Blastococcus* and three *Massilia* strains, the rest were also represented by one isolate.

Moreover, the distribution of the possible new species and the non-identified isolates was different among the media dilution used (**Figure 6**). The ones that gave the higher fraction of potential new taxa were the lowest nutrient concentrations (media TSA 0.1 and 0.01× and all the R2A combinations). Specifically, the best results for new taxa isolation were obtained in R2A 0.1×, whereas the highest fraction of non-identified ones was obtained in R2A 0.01×. Interestingly, the sum of both groups of isolates (potential new species and unidentified ones) comprised a similar percentage in these two media, being almost 65%, which is approximately a threefold increase with respect to the TSA 1× group.

Regarding the differences in diversity depending on the incubation time after which each bacterial strain was isolated, the comparison of the groups, at the genus level, isolated after 2 weeks and after 1 month of incubation revealed that most of the taxa had been already detected in the first selection (**Figure 7A**). However, four genera were exclusively isolated after 1 month, which were *Caulobacter*, *Geodermatophilus*, *Azospirillum*, and *Blastococcus* (**Figure 7B**). Moreover, there was no significant difference in isolating potentially new taxa, as the percentage of isolates showing an identity percentage below 98.7 with the closest blast was similar between both selection times.

Finally, the differences in diversity observed depending on the biocrust sample and sampling site were analyzed (**Supplementary Tables 2–4**). The geolocation that yielded the highest number of colonies was geolocation 1, with as much as 60% of the total isolates. On the contrary, geolocations 2 and 3 yielded a total of 51 isolates each. In terms of number of different genera identified, the most diverse sample was 1.4.1, from which 23 genera were isolated in pure culture. Although sample 1.4.1 was also the one that yielded the highest number of colonies, 30% of them corresponded to *Arthrobacter* spp.

Arthrobacter spp., which was the most abundant genus, was isolated from seven out of the eight samples, with different abundances. Particularly, in sample 2.1.1, half of the

TABLE 2 | List of coincident genera in the TSA media as it is compared in the Venn diagram in **Figure 4B**.

Media	N.	Genera
TSA 1×, TSA 0.1×, TSA 0.01×	11	*Lentzea*, *Belnapia*, *Kocuria*, *Arthrobacter*, *Ramlibacter*, *Homoserinibacter*, *Sphingomonas*, *Roseomonas*, *Kineococcus*, *Microbacterium*, not identified
TSA 1×, TSA 0.1×	1	*Skermanella*
TSA 0.1×, TSA 0.01×	3	*Streptomyces*, *Mycolicibacterium*, *Microvirga*
TSA 1×, TSA 0.01×	2	*Planctomonas*, *Modestobacter*
TSA 1×	2	*Micrococcus*, *Staphylococcus*
TSA 0.1×	8	*Methylobacterium*, *Geodermatophilus*, *Nocardioides*, *Variovorax*, *Umezawaea*, *Amycolatopsis*, *Paenarthrobacter*, *Noviherbaspirillum*
TSA 0.01×	4	New genus, *Enterovirga*, *Actinoplanes*, *Kribbella*

isolates belonged to this genus. Interestingly, 8 of the 10 *Homoserinibacter* spp. were from sample 1.4.1, but none had been isolated from geolocations 2 and 3, and the *Kineococcus* genus was also unique for the four samples of geolocation 1.

Ecological Novelty

Many of our strains were not phylogenetically related with any previously cultured strain. Specifically, 10.6% of the isolates shared less than 98.7% of 16S rRNA gene sequence similarity against any isolate previously studied, and 5.5% of the strains shared less than 98.7% regarding any environmental clone. Moreover, 24.8% of our isolates showed similarities with, to date, uncultured microorganisms (**Supplementary Table 5**).

Up to 40.5% of the closest neighbors of our strains were environmental clones or isolates associated with soil environments, being 4.7% of them specifically from deserts around the globe, such as the Mojave Desert (Southwestern United States) and the Badain Jaran Desert (China), among others. Others (8.66%) were closely linked to clones or isolates inhabiting extreme environments, such as oil or heavy metal-contaminated soils, or cold environments, such as glaciers and permafrost samples. Furthermore, 8.3% of our strains were related to environmental clones or isolates found in aquatic habitats, mainly rainwater but also lakes, fish ponds, and freshwater sediments. Interestingly, 8.7% of the closest relatives corresponded to plant-associated clones, either associated with the rhizosphere, phyllosphere, or endophytic bacteria, and a small fraction representing 4.7% of the collection was similar to animal-associated bacteria, such as the gut of insects or corals.

DISCUSSION

The Tabernas Desert Soils Harbor a Wide Bacterial Diversity

Although drylands and cold and hot deserts are a source of biotechnologically-relevant bacterial strains (Azua-Bustos and González-Silva, 2014; Mohammadipanah and Wink, 2016), the unique ecosystem of the Tabernas Desert remained poorly unexplored from the culturomics point of view. In the present study, we established a large collection consisting of, at least, 254 bacterial strains from 37 genera, in addition to one possible new genus, which belonged to three phyla.

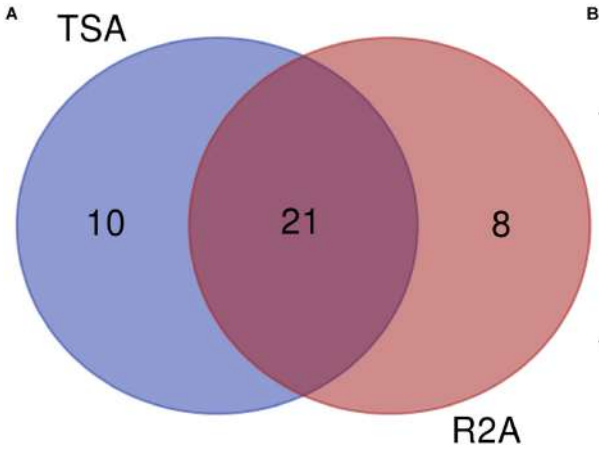
The distribution of phyla in the collection resembles the microbial communities studied in other desert environments, such as the Atacama, the Gibson, or the Sahara Desert, with Actinobacteria as the most abundant phylum, followed by Proteobacteria and, to less extent, Firmicutes, among the culturable fraction (Belov et al., 2018; Schulze-Makuch et al., 2018). These taxa are also the ones previously detected in airborne sand particles from the Sahara Desert (Meola et al., 2015). Moreover, 19 different families were identified in the present work within those phyla: ten Actinobacteria families, five α-Proteobacteria, two β-Proteobacteria, and two Firmicutes. Actinobacteria, as reported by many authors, are one of the most common inhabitants in soil, but also particularly abundant in what is considered the extremobiosphere (Bull, 2011; Mohammadipanah and Wink, 2016).

The role of Actinobacteria in the synthesis of bioactive microbial metabolites with high pharmacological and commercial interests, such as antibiotics, is well-known. In particular, *Microbacterium* and *Lentzea* isolates, as well as other represented genus to lesser extent, such as *Streptomyces*, *Amycolatopsis*, and *Actinoplanes*, may be playing a role in the modulation of communities through competitors' growth inhibition (Tao et al., 2016; Zhao et al., 2018). Nithya et al. (2015) reported the isolation of 16 actinobacterial strains from the desert in Arabia Saudi that exhibited antimicrobial potential, and Hoshino et al. (2018) described for the first time a new type of macrolactams named as umezawamides as they are synthetized by the genus *Umezawaea*. Moreover, Wichner et al. (2017) reported the isolation of six new metabolites that had never been traced before from microbial sources by a novel strain of *Lentzea* sp. from the Atacama Desert, which confirms the potential that these bacteria hold in the synthesis of interesting secondary metabolites.

Other groups within the Actinobacteria clade have been described as important complex organic matter-degrading

TABLE 3 | List of coincident genera in the R2A media as it is compared in the Venn diagram in **Figure 4C**.

Media	N.	Genera
R2A 1×, R2A 0.1×, R2A 0.01×	5	*Mycolicibacterium*, *Arthrobacter*, *Homoserinibacter*, *Microbacterium*, not identified
R2A 1×, R2A 0.1×	2	*Skermanella*, *Massilia*
R2A 0.1×, R2A 0.01×	1	*Kineococcus*
R2A 1×, R2A 0.01×	2	*Kocuria*, *Methylobacterium*
R2A 1×	15	*Lentzea*, *Agrococcus*, *Umezawaea*, *Nocardioides*, *Geodermatophilus*, *Blastococcus*, *Modestobacter*, *Curvibacter*, *Sphingomonas*, *Caulobacter*, *Roseomonas*, *Azospirillum*, *Belnapia*, *Streptomyces*, *Kribbella*
R2A 0.1×	2	*Bacillus*, *Herbaspirillum*
R2A 0.01×	2	*Actinoplanes*, *Flaviflagellibacter*



B

TSA	*Enterovirga*	*Planctomonas*
	Variovorax	*Microvirga*
	Noviherbaspirillum	*Paenarthrobacter*
	New genus	*Staphylococcus*
	Micrococcus	*Amycolatopsis*
Shared	*Lentzea*	*Methylobacterium*
	Nocardioides	*Kineococcus*
	Kocuria	*Geodermatophilus*
	Arthrobacter	*Mycolicibacterium*
	Ramlibacter	*Umezawaea*
	Homoserinibacter	*Actinoplanes*
	Sphingomonas	*Modestobacter*
	Not identified	*Skermanella*
	Roseomonas	*Microbacterium*
	Belnapia	*Kribbella*
	Streptomyces	
R2A	*Caulobacter*	*Flaviflagellibacter*
	Herbaspirillum	*Azospirillum*
	Massilia	*Agrococcus*
	Bacillus	*Blastococcus*

FIGURE 5 | Taxonomic diversity as a function of the culture media and dilutions used. **(A)** Venn diagram comparing the genera isolated in TSA medium and R2A medium, considering all the three dilutions used for microbial growth (1, 0.1, and 0.01×). Possible new genera and non-identified groups are included. **(B)** List of genera represented in the Venn diagram.

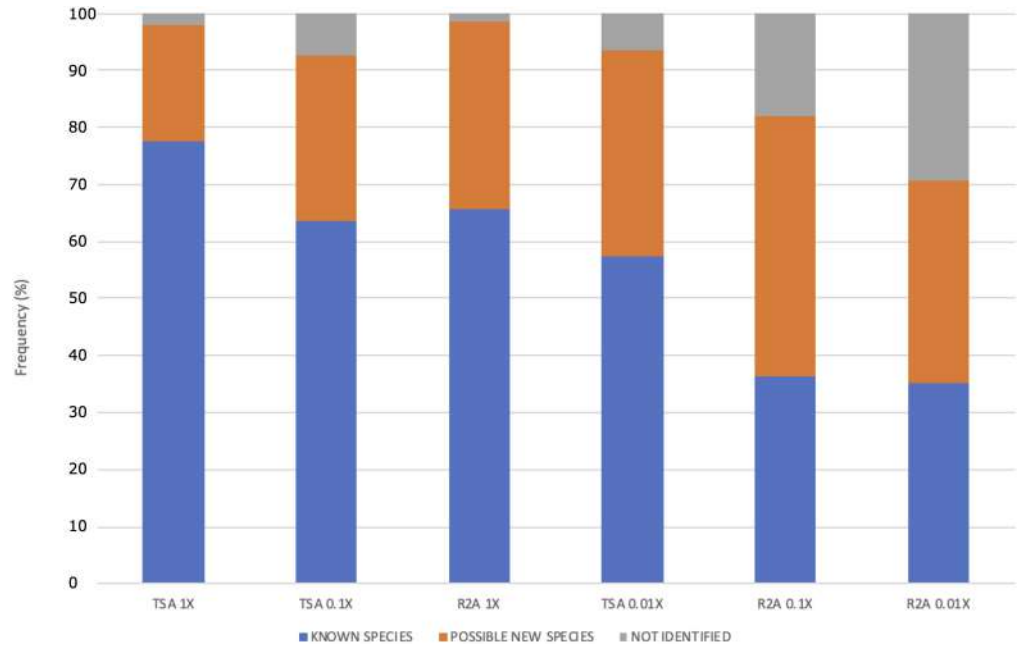


FIGURE 6 | Frequency (%) of possible new species, known species, and non-identified isolates as function of the culture media and the dilution used. The media are ordered according to the concentration of protein hydrolysate. Two different media, TSA and R2A, were used at three different concentrations: 1, 0.1, and 0.01×.

bacteria, such as Actinomycetales, which are proficient degraders of polysaccharide of plant, animal, or fungal origin in soils. This polysaccharide degradation has a crucial impact in the carbon sources reuse cycles (Yeager et al., 2017). Glucosidases, glycoside hydrolases, carbohydrate esterases, and other related enzymatic activities are abundant in the analyzed genomes of the

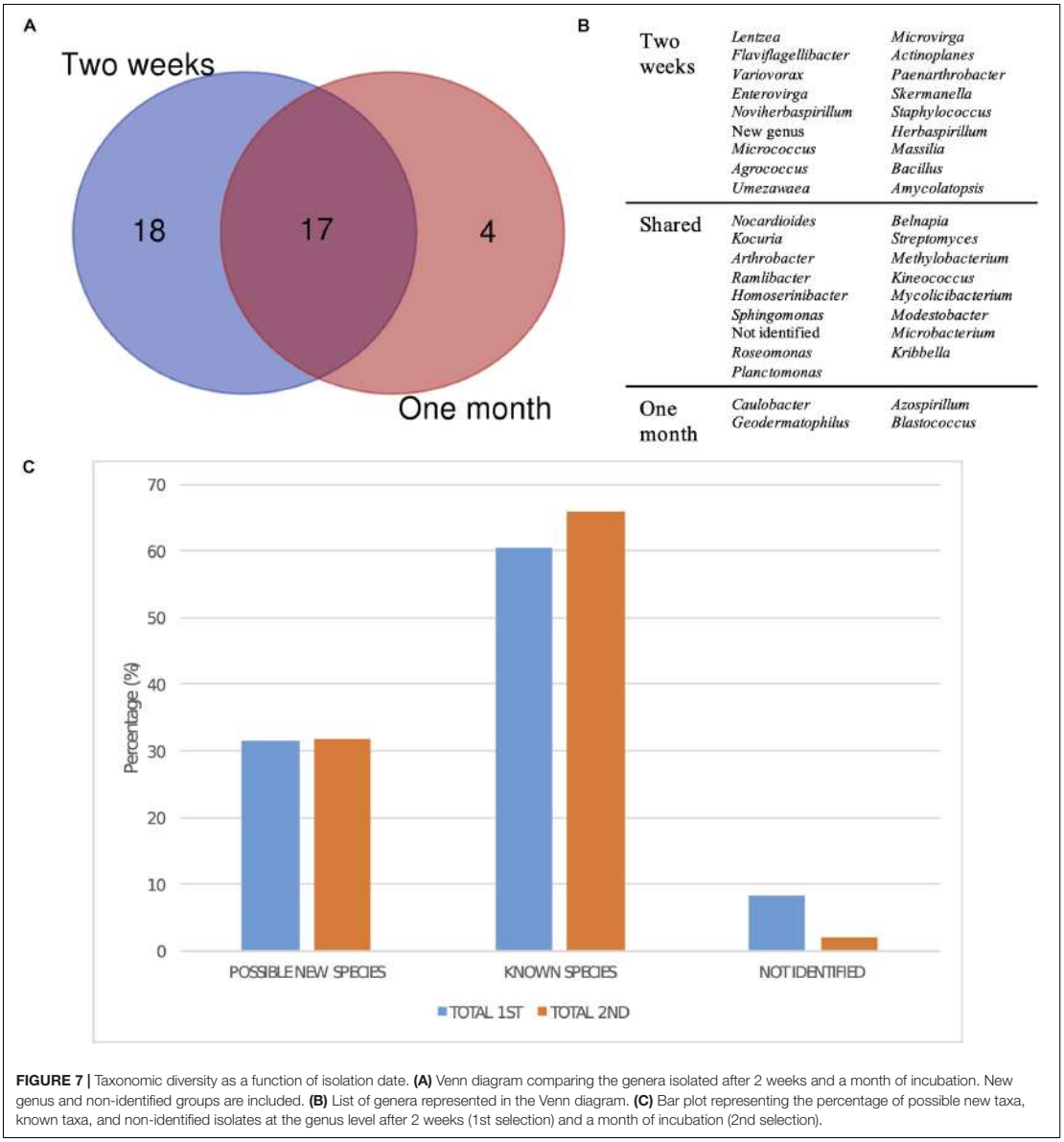


FIGURE 7 | Taxonomic diversity as a function of isolation date. **(A)** Venn diagram comparing the genera isolated after 2 weeks and a month of incubation. New genus and non-identified groups are included. **(B)** List of genera represented in the Venn diagram. **(C)** Bar plot representing the percentage of possible new taxa, known taxa, and non-identified isolates at the genus level after 2 weeks (1st selection) and a month of incubation (2nd selection).

three new *Kineococcus* strains described from Tabernas indeed (Molina-Menor et al., 2020).

Actinobacterial species can play also an important role in bioremediation. The ability to degrade recalcitrant compounds and other environmental contaminants, such as heavy metals, has been reported for species within the Actinomycetales clade, what would have an application in the bioconversion of wastes into high-value products (Masuda et al., 2012; Inoue et al., 2016; Alvarez et al., 2017; Herrero et al., 2018). *Kocuria* and *Microbacterium* species isolated from rhizosphere have been used for the bioremediation of pesticides, such as lindane (Abhilash et al., 2011), and *Microbacterium* spp. have also been found to potentially degrade organophosphorus pesticides and polycyclic aromatic hydrocarbons (PAHs) (Panneerselvan et al., 2018). Moreover, some of the *Roseomonas* species have been first isolated from oil-contaminated soils (Chaudhary and

Kim, 2017; Subhash and Lee, 2018), and *Arthrobacter* species have demonstrated their potential to degrade polycyclic aromatic compounds (Gil et al., 2000; Muangchinda et al., 2017), which is interesting as they are one of the main inhabitants of soil environments (Mongodin et al., 2006).

In fact, *Arthrobacter* is the most abundant taxa in the collection established from the Tabernas Desert, with as many as 62 strains assigned, 26 of which being possible new species. *Arthrobacter* spp. do not only play a major role in the maintenance of natural processes in soils, such as the degradation of organic matter (Yeager et al., 2017) or the recycling of different carbon sources (Adams et al., 2011; Anderson et al., 2012; Berlemont and Martiny, 2013), but also in their recovery after fires (Fernández-González et al., 2017) and in the rhizosphere as common plant growth-promoting bacteria, preventing other bacteria and nematodes infections (Topalovic et al., 2020). They are common inhabitants of extreme environments, such as warm or cold arid deserts, and the robustness of this genus makes it interesting from the biotechnological point of view as it can be a source of bioactive compounds, such as the highly valued carotenoids (Fong et al., 2001). This, summed up to the diversity that has been found in the collection, opens up possible new biotechnological applications in several areas for the isolates within this genus.

The samples from the Tabernas Desert display a wide range of colors, probably due to the presence of these carotenoids, which may be playing a major role in their natural protection against oxidative stress and other consequences of sun exposure. The relationship between the synthesis of carotenoids, among other pigments, and the resistance to UV radiation in highly irradiated environments has been well described (Villareal et al., 2016; Peyrat et al., 2019). Not only *Arthrobacter* but also the genus *Microbacterium*, the second most abundant one with 14 representatives, is also known for its ability to synthetize carotenoids. Han et al. (2016) and Reis-Mansur et al. (2019) reported the UV resistance of two carotenoid-producing *Microbacterium* spp., both isolated from Antarctica. Furthermore, the genus *Methylobacterium*, represented by six isolates in the collection, has been described to synthetize pink carotenoids, which presumably act as antioxidants against reactive oxygen species (ROS) generated by UVA and UVB stresses (Sundin et al., 2002; Gao and Garcia-Pichel, 2011). Moreover, Yoshida et al. (2017) described an avobenzone-like compound isolated from several strains within the genus *Methylobacterium* with UVA-absorption and photostability activities, whose similarity to avobenzone supports its potential use as a commercial sunscreen ingredient.

The resistance to a wide range of stressors, particularly the resistance to gamma or UV radiation, has been largely described for the genera identified in the collection. Species within the genera *Roseomonas* (Kim et al., 2018), *Kocuria* (Gholami et al., 2015; Mehrabadi et al., 2016), or *Geodermatophilus* (Montero-Calasanz et al., 2013; Hezbri et al., 2016), among others, have been described as highly resistant to radiation. Moreover, *Sphingomonas* species, which represent almost 4% of the collection, have been reported to be one of the main inhabitants of solar panels. These artificial devices, exposed to the maximum sun radiation and distributed worldwide, are colonized by a stable and diverse microbial community dominated by highly resistant bacteria (Dorado-Morales et al., 2016). Interestingly, Yu et al. (2015) reported for the first time the resistance to radiation for bacteria that had been isolated from the Taklamakan Desert belonging to the genera *Nocardioides* and *Microvirga*, among others. These two genera are represented by seven and three isolates in the collection, respectively.

As expected according to the origin of the samples, most of the taxa have been previously described as soil inhabitants, with representatives having been isolated from desert environments indeed. Not only *Arthrobacter* and *Microbacterium*, as mentioned above, but also many other species identified come from similar ecological niches. *Roseomonas* and *Kineococcus* species (Yokota et al., 1993; Lee, 2009; Liu et al., 2009; Nie et al., 2012b; Ramaprasad et al., 2015; Chaudhary and Kim, 2017; Kim et al., 2017), the only species in the genus *Homoserinibacter* (Kim et al., 2014) or *Lentzea terrea*, *Lentzea soli*, and *Lentzea jiangxiensis*, among other representatives of the genus *Lentzea*, are examples of taxa that have been described as members of soil microbial communities (Li et al., 2012, 2018a,b). Moreover, other genera, such as *Skermanella*, *Belnapia*, or *Blastococcus*, which are comprised by few members, have also representatives that come from soil or desert ecosystems (Reddy et al., 2006; Zhang et al., 2015; Castro et al., 2018; Yang et al., 2019).

Although culture-independent approaches have led to the discovery of large unknown microbial diversity, these techniques can only predict to a certain extent the biotechnological potential of the identified microorganisms. The isolation of culturable, novel bacterial or fungal taxa under laboratory conditions is thus a crucial step for the development of innovative and new whole cell-based applications. For example, desert-inhabiting extremophilic bacteria have demonstrated to be useful in improving plant toleration to drought (Marasco et al., 2012; Rolli et al., 2015). Moreover, Sibanda et al. (2017) reported a culture-based screening to identify strains able to degrade some of the compounds present in carwash effluents, such as PAHs, heavy metals, and other pollutants.

Extremophilic bacteria have proven to be an excellent source of highly resistant proteins for several industries, such as in biofuel production, biorefinery, bioremediation, or the revalorization of agricultural waste products, such as lignocellulosic material (Zhu et al., 2020). However, many of these applications rely on genetic engineering techniques and synthetic biology and represent only a fraction of their possibilities.

Simple Culture-Based Strategies Rescue Significant Fractions of Previously Unknown Bacterial Diversity

The Tabernas Desert is particularly diverse in novel bacteria. Many of the closest relatives of our strains are environmental clones that inhabit a wide range of environments, mainly terrestrial ecosystems, including extreme environments, such as hot and cold deserts. These results highlight the importance of our collection of microorganisms from an ecological point of view (**Supplementary Table 5**) since we have been able to

access many bacteria never before studied, including through molecular tools.

Our methodology and results have, thus, important implications in terms of defining the best strategies for the discovery of new genetic variants of the soil bacteriome. We found a clearly different pattern in the two media we analyzed. Indeed, the composition in terms of nutrients and concentration is considerably different between TSA and R2A media. TSA is a rich media, which differs from the natural environments in terms of nutrient availability as these compounds are not usually abundant, whereas R2A is a poor media with limited amount of nutrients and carbon sources. TSA media seem to be equally good for yielding different isolates, according to the high number of shared taxa (**Figure 4B**), whereas for R2A, it is clear that 1× combination, which is already a diluted media, is a suitable concentration for microbial growth because further dilutions yielded a very low number of colonies after 1 month (**Figure 4C**). However, the highest number of non-identified isolates was isolated from R2A 0.01×, with as much as five isolates that represented 27.8% of them (**Figure 4A**).

Arthrobacter, *Homoserinibacter*, and *Microbacterium* were identified in all of the tested media, whereas *Actinoplanes* or *Microvirga*, among others, was only found in diluted media (**Figure 4A** and **Supplementary Table 1**). The ability of *Microvirga* species to grow on different media has been previously reported. Species within this genus are able to grow both in concentrated media, such as TSA, and in diluted ones, such as R2A (Dahal and Kim, 2017), but others, such as *Microvirga*, are unable to grow in nutrient abundance (Huq, 2018). *Nocardioides* spp., *Geodermatophilus* spp., and *Umezawaea* spp. have been identified in TSA 0.1× and R2A 1×, in which the nutrients are at the same order of magnitude, suggesting a more specific requirement, even though there are reports of the isolation and growth of species within these genera in a broader number of media (Nie et al., 2012a; Montero-Calasanz et al., 2013; Hezbri et al., 2016).

In contrast with this, the genera *Kocuria*, *Kineococcus*, and *Mycolicibacterium* were isolated from five out of the six media used. In the case of *Kineococcus* spp., there are previous reports on their isolation from a wide diversity of media and their ability to grow on them too, including diluted TSA 0.1× (Liu et al., 2009), the rich starch-casein agar medium (Duangmal et al., 2008; Li et al., 2015), or pure TSA (Xu et al., 2017), which also happens for *Mycolicibacterium* species, as reported by Nouioui et al. (2019). Moreover, Kaewkla and Franco (2013) reported a similar strategy of isolating novel culturable strains from trees by extending incubation times and using diluted media, which resulted in the isolation of a wide diversity of genera and new species, including several *Nocardioides*, *Kribbella*, and *Amycolatopsis* isolates, which have grown preferably in intermediate to low concentrations in the case of the samples from the Tabernas Desert.

Of the 39 groups, including non-identified isolates and the possible new genus, 15 were identified in just one media, which represents almost 40% of the diversity at the genus level. This confirms that the diversity observed is highly dependent on the use of a combination of culture media, which supports the idea that combining media and diluting them increase the chances of finding different and new taxa.

It is interesting to note that the potentially new genus we identified, which is closely related to the *Roseomonas* clade according to the 16S rRNA sequencing analysis, was isolated from the most diluted TSA media. Moreover, the strain R60, isolated from the lower concentration of R2A, was initially identified as another possible new genus and is now classified within the genus *Flaviflagellibacter*, as it has been recently published by Dong et al. (2019), after the beginning of this work. Thus, isolate R60 is now a possible new species in this novel genus, which includes only one species, *Flaviflagellibacter deserti*, that was first isolated from R2A media plates that had been incubated for 10 days, although it showed the ability to grow also in more concentrated media, such as TSA and LB, suggesting a flexible behavior in terms of nutrient availability for this genus.

The fact that around 25% of the taxa at the genus level were exclusively isolated in TSA and 20% in R2A suggests that even applying dilution factors to the media and increasing the incubation times, culturing samples in a selection of media is worth it in order to obtain a higher diversity (**Figure 5**), and that the selection of the proper media is critical for it. Moreover, only 3 out of the 18 exclusive groups, *Planctomonas* in TSA and *Massilia* and *Blastococcus* in R2A, are represented by more than one isolate, which suggests that the less abundant taxa in culture collections are the ones with more specific requirements for growth. Interestingly, the only species described within *Planctomonas* was first isolated after 4 weeks of incubation (Liu et al., 2019).

The differences observed in the distribution of potential new species depending on the isolation media suggest that low nutrient concentrations increase the chances of identifying new taxa (**Figure 6**), which is in accordance with the results observed in terms of diversity at the genus level (**Figures 4B,C**). The percentage of possible new species increases, whereas the concentration of nutrients decreases. The concentrations in media TSA 0.1× and R2A 1× as well as and the concentrations in TSA 0.01× and R2A 0.1× are in the same order of magnitude. Interestingly, the most diluted media, R2A 0.01× , was not the best one in terms of possible new taxa, but yet exhibited the highest fraction of non-identified isolates, which can at least partially correspond to new species (**Figure 6**). The results obtained support the idea that traditional, concentrated media have favored the isolation of well-known, specific groups of fast-growing taxa. Almost 80% of the isolated strains in TSA 1× have been already described, whereas the fraction of unknown isolates (possible new species and non-identified ones) comprises more than 60% of the total in highly diluted media (R2A 0.1 and 0.01×) (**Figure 6**).

Finally, the moment in which the different genera were isolated reveals that most of them could already be detected after 2 weeks of growth (**Figure 7A**). However, four genera were exclusively isolated after a month, confirming that extending incubation times is important in order to obtain a higher microbial diversity, especially for isolating slow-growing and oligotrophic bacteria. One of these genera was *Blastococcus*, which is in accordance with the long incubation times reported

by Yang et al. (2019) for the isolation of *Blastococcus deserti* in diluted media.

Surprisingly, the fraction of possible new species was similar after 15 days and 1 month of growth, but the percentage of non-identified isolates was higher in the first sampling (**Figure 7C**). Song et al. (2016) studied the effect of nutrient availability and incubation times in the development of soil microbial communities, revealing that the highest bacterial diversity is found in intermediate nutrient concentrations, as copiotrophic bacteria rapidly grow in concentrated media, whereas only resistant oligotrophic strains develop in highly diluted ones, which is in accordance with the results described above. Moreover, they observed that the peak in diversity is reached at the same time regardless of the nutrient concentration, which may explain the constant fractions of known and unknown diversity discussed at two sampling times.

Taken together, our results reveal that simple culturomics approaches, such as using combinations of media, long incubation times, and extreme dilutions of the nutrients, can be useful to yield a large diverse set of culturable microbial strains if applied to biodiverse natural samples, since we were able to build a diverse collection of bacterial strains from a few samples using these simple techniques. Our findings, combined with similar efforts, can help to fill the gap between the high numbers of bacterial genetic variants identified in culture-independent next generation sequencing (NGS) studies and the–up to date–still very low fraction of culturable microorganisms. This has, well beyond microbial ecology, important biotechnological implications.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, MT749781–MT750013 and MN069867–MN069869.

AUTHOR CONTRIBUTIONS

MP conceived the work. EM-M, HG-V, and MP carried out the sampling. EM-M and HG-V performed all the experiments. EM-M, HG-V, JuP, JaP, and MP analyzed the results, wrote, and approved the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.583120/full#supplementary-material

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The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

microorganisms

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Article

Kineococcus vitellinus sp. nov., *Kineococcus indalonis* sp. nov. and *Kineococcus siccus* sp. nov., Isolated Nearby the Tabernas Desert (Almería, Spain)

Esther Molina-Menor [1], Helena Gimeno-Valero [2], Javier Pascual [2], Juli Peretó [1,2,3] and Manuel Porcar [1,2,*]

[1] Institute for Integrative Systems Biology I2SysBio (Universitat de València-CSIC), Calle del Catedràtic Agustin Escardino Benlloch 9, 46980 Paterna, Spain; esther.molina@uv.es (E.M.-M.); juli.pereto@uv.es (J.P.)
[2] Darwin Bioprospecting Excellence SL. Parc Científic Universitat de València, Calle del Catedràtic Agustin Escardino Benlloch 9, 46980 Paterna, Spain; hgimeno@darwinbioprospecting.com (H.G.-V.); jpascual@darwinbioprospecting.com (J.P.)
[3] Departament de Bioquimica i Biologia Molecular, Universitat de València, Calle del Dr. Moliner 50, 46100 Burjassot, Spain
* Correspondence: manuel.porcar@uv.es

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Abstract: Three novel Gram-positive, aerobic, chemoheterotrophic, motile, non-endospore-forming, orange-pigmented bacteria designated strains T13^T, T90^T and R8^T were isolated from the Tabernas Desert biocrust (Almería, Spain). Cells of the three strains were coccus-shaped and occurred singly, in pairs or clusters. The three strains were oxidase-negative and catalase-positive, and showed a mesophilic, neutrophilic and non-halophilic metabolism. Based on the 16S rRNA gene sequences, the closest neighbours of strains T13^T, T90^T and R8^T were *Kineococcus aurantiacus* IFO 15268^T, *Kineococcus gypseus* YIM 121300^T and *Kineococcus radiotolerans* SRS 30216^T (98.5%, 97.1% and 97.9% gene sequence similarity, respectively). The genomes were sequenced, and have been deposited in the GenBank/EMBL/DDBJ databases under the accession numbers JAAALL000000000, JAAALM000000000 and JAAALN000000000, respectively, for strains T13^T, T90^T and R8^T. The average nucleotide identity (ANIb) and digital DNA-DNA hybridization (dDDH) values confirmed their adscription to three new species within the genus *Kineococcus*. The genomic G + C content of strains T13^T, T90^T and R8^T ranged from 75.1% to 76.3%. The predominant fatty acid of all three strains was anteiso-$C_{15:0}$. According to a polyphasic study, strains T13^T, T90^T and R8^T are representatives of three new species in the genus *Kineococcus*, for which names *Kineococcus vitellinus* sp. nov. (type strain T13^T = CECT 9936^T = DSM 110024^T), *Kineococcus indalonis* sp. nov. (type strain T90^T = CECT 9938^T = DSM 110026^T) and *Kineococcus siccus* sp. nov. (type strain R8^T = CECT 9937^T = DSM 110025^T) are proposed.

Keywords: new taxa; biocrust; Kineococcus indalonis; Kineococcus siccus; Kineococcus vitellinus; Tabernas Desert

1. Introduction

The genus *Kineococcus*, belonging to the family *Kineosporiaceae* [1], was first proposed by Yokota et al. in 1993 [2]. At the time of writing, the genus *Kineococcus* is comprised of 11 species with valid published names (http://lpsn.dsmz.de/) which were isolated from soil [2–5], saline environments [6–8], plants [9,10] and radioactive work areas [11]. This genus includes species with the ability to grow in a wide range of temperatures [5] and high salt concentrations [8,9]. Their resistance to multiple

stresses and extreme conditions has also been reported by several authors, with *Kineococcus radiotolerans* standing out among them because of its high resistance to radiation [11].

The genus *Kineococcus* unites Gram-stain-positive, aerobic, catalase-positive and oxidase-negative microorganisms. Colonies are cream to orange in colour, and cells are motile and occur singly, in pairs or in clusters. The major cellular fatty acid is $C_{15:0}$ anteiso, whereas diphosphatidylglycerol and phosphatidylglycerol are the major polar lipids. The genomic DNA G + C content of the *Kineococcus* species ranges from 73.9% to 74.2% [12].

During a study on the culturable microbial diversity of biocrust in dry environments exposed to high solar radiation, three different strains, T13^T, T90^T and R8^T, were isolated. The objective of the study was to characterize in detail the microbial communities from the Tabernas Desert (Almería, Spain), with particular emphasis in the isolation of extremophilic bacteria. The bacterial strains isolated from similar environmental niches, such as solar panels or the intertidal coastal zone, are able to tolerate UV radiation, and could thus be potentially useful in the synthesis of antioxidant compounds [13,14]. Among them, pigments such as carotenoids are particularly valuable for the biotechnological and pharmaceutical industries. In this paper, we describe the isolation and polyphasic characterization of these bacteria, and propose them as new species of the genus *Kineococcus*, for which the names *Kineococcus vitellinus* sp. nov., *Kineococcus indalonis* sp. nov. and *Kineococcus siccus* sp. nov. are proposed.

2. Materials and Methods

2.1. Isolation of Strains and Culture Conditions

Biocrust samples were collected in the vicinity of the Tabernas Desert (37.002404 N, 2.450655 W) in September 2018. One gram of each biocrust sample was resuspended in one millilitre of phosphate-buffered saline (PBS) pH 7.4. The suspensions were vigorously shaken, spread on 1X, 0.1X and 0.01X trypticase soy agar (TSA; in g/L: 15 tryptone, 5 NaCl, 5 soya peptone), and Reasoner's 2A agar (R2A; in g/L: 1 peptone, 0.5 yeast extract, 0.5 dextrose, 0.5 soluble starch, 0.3 dipotassium phosphate, 0.05 magnesium sulphate heptahydrate, 0.3 sodium pyruvate) media and incubated at 23 °C for one week. T13^T and T90^T strains were isolated from 0.1X and 0.01X TSA plates, respectively, whereas R8^T was isolated from a 0.1X R2A plate. All three strains were purified and cultivated by re-streaking on fresh media. For cryopreservation at −80 °C, cell suspensions in trypticase soy broth (TSB) were supplemented with 15% (*v/v*) glycerol. To determine the taxonomic status of strains T13^T, T90^T and R8^T, six reference type strains were used in this study: *Kineococcus radiotolerans* DSM 14245^T, *Kineococcus aurantiacus* DSM 7487^T, *Kineococcus gypseus* DSM 27627^T and *Kineococcus aureolus* DSM 102158^T from the DSMZ—German Collection of Microorganisms and Cell Cultures (Leibniz Institute, Braunschweig, Germany), and *Kineococcus gynurae* NBRC 103943^T and *Kineococcus mangrovi* NBRC 110933^T from the NITE Biological Resource Center (Tokyo, Japan). The selection of reference strains was based on the comparison of 16S rRNA gene sequences of the three isolates against the EzBioCloud database (http://www.ezbiocloud.net/) as detailed below. The comparative analysis conditions were selected according to the available literature on the species within this genus. Unless otherwise specified, all nine strains were grown on TSA media at 30 °C.

2.2. DNA Extraction and Sequencing

Extraction of genomic DNA was carried out using the DNeasy PowerSoil kit (QUIAGEN, Hilden, Germany) according to the manufacturer's instructions. A PCR of the whole 16S rRNA gene was performed with universal primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') [15] and 1492R (5'-GGTTACCTTGTTACGACTT-3') [16] (94 °C for 5 min, 24 cycles of denaturation at 94 °C for 15 s, annealing at 48 °C for 15 s and elongation at 72 °C for 5 min, and a final elongation step at 72 °C for 5 min). The 16S rRNA gene sequence length of the strains T13^T, T90^T and R8^T were 1415 bp (accession number MN069869), 1425 bp (MN069867) and 1404 bp (MN069868), respectively. The draft genome of

strains T13^T, T90^T and R8^T was sequenced using a MiSeq sequencer (Illumina, San Diego, CA, USA), and the Nextera XT Prep Kit protocol was used for library preparation.

2.3. Phylogenetic Analysis

We identified the closest relatives of the three strains T13^T, T90^T and R8^T by comparing their 16S rRNA gene sequences against the EzBioCloud database update 2020.05.13 (http://www.ezbiocloud.net/). Maximum-likelihood (ML) (Figure 1) [17] and neighbour-joining trees (NJ) (Figure 2) [18] were inferred with the software MEGA X v.10.1.7 (https://www.megasoftware.net/). The Tamura–Nei G+I evolutionary model was used for the ML tree, whereas the Kimura two-parameter model was used for the NJ tree. The reliability of the branch patterns was assessed using bootstrap analysis based on 500 replicates for the ML tree and on 1000 replicates for the NJ tree [19]. *Acidimicrobium ferrooxidans* DSM 10331^T was used as an outgroup for the phylogenetic analyses.

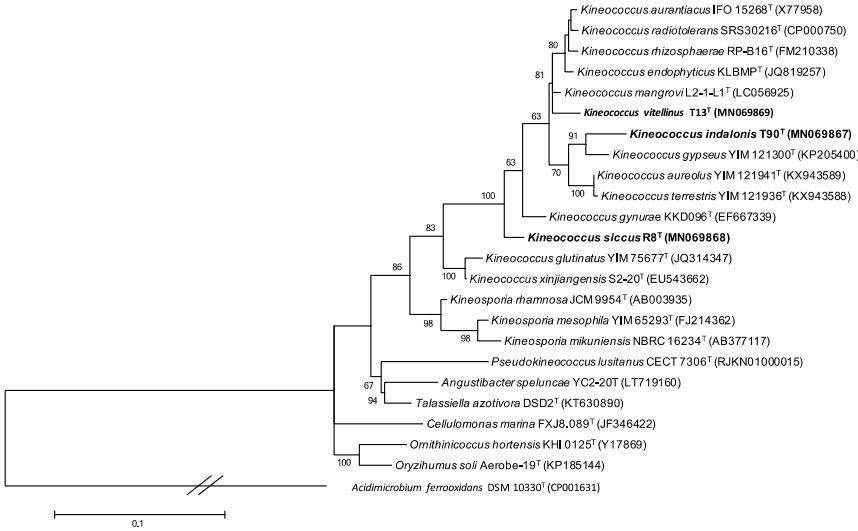

Figure 1. Maximum likelihood phylogenetic tree showing the relationships between strains T13^T, T90^T, R8^T and other members of the family *Kineosporiaceae* based on 16S rRNA sequences. The optimal evolutionary model of nucleotide substitution applied was Tamura–Nei G+I. Numbers at branch points refer to bootstrap percentages based on 500 replicates (values under 50% are not indicated). *Acidimicrobium ferrooxidans* DSM 10331^T (CP001631) was used as an outgroup. Bar 0.1 fixed nucleotide substitutions per site.

2.4. Genomic Analysis

The FastQC tool [20] was used to assess the quality of the sequence reads. Genome assembly of paired reads was performed using SPAdes 3.12.0 [21]. The draft genomes were annotated using the RAST tool kit (RAStk) [22] integrated in PATRIC v.3.5.41 (https://www.patricbrc.org). The circular genomic maps were also obtained from PATRIC v.3.5.41. The whole-genome shotgun projects of strains T13^T, T90^T and R8^T have been deposited in GenBank/EMBL/DDBJ under accession numbers JAAALL000000000, JAAALM000000000 and JAAALN000000000, respectively. The completeness and contamination of the genomes was analysed with the bioinformatic tool CheckM v.1.0.6 [23].

Pairwise average nucleotide identity values (ANIb) [24] were calculated between strains T13^T, T90^T and R8^T and their closest type strains whose genomes were publicly available, by using the JSpeciesWS online tool [25]. Additionally, digital DNA-DNA hybridization (dDDH) pairwise values were also

obtained using the Genome-to-Genome Distance Calculator 2.1 (GGDC) tool [26]. As recommended for incompletely sequenced genomes, formula 2 was used for calculating the digital DDH values [26].

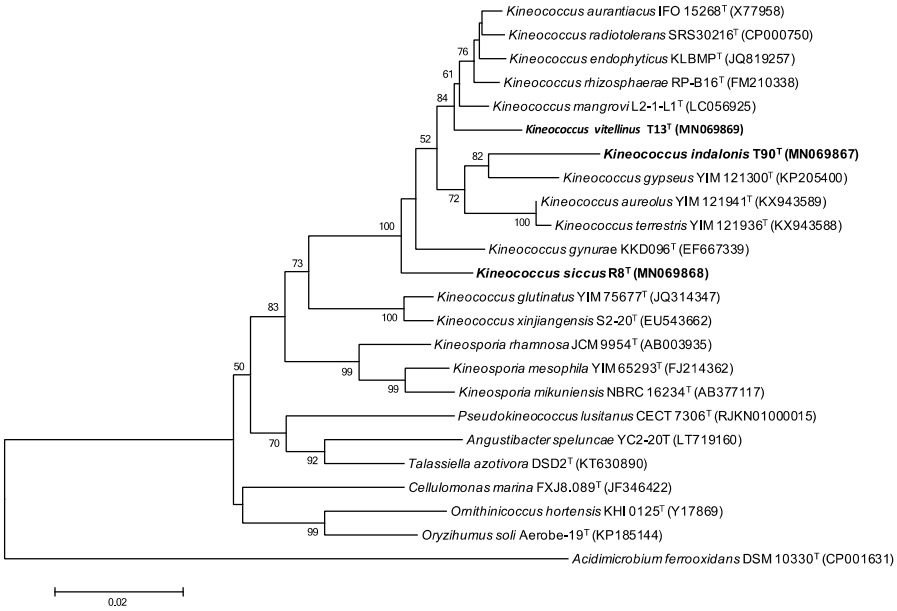

Figure 2. Neighbour-joining phylogenetic tree showing the relationships between strains T13^T, T90^T, R8^T and other members of the family *Kineosporiaceae* based on 16S rRNA sequences. The evolutionary model of nucleotide substitution applied was the Kimura two-parameter model (K2P). Numbers at branch points refer to bootstrap percentages based on 1000 replicates (values under 50% are not indicated). *Acidimicrobium ferrooxidans* DSM 10331^T (CP001631) was used as an outgroup. Bar 0.02 fixed nucleotide substitutions per site.

2.5. Morphological and Biochemical Characteristics

The phenotypic characteristics of the bacterial cultures were determined after one week of growth at 30 °C on TSA medium following the procedures outlined previously by other authors [27,28]. Cell morphology was observed under the microscope with crystal violet glass stain. Motility was analysed on wet mounts. The presence of flagella was determined with the staining method described by Heimbrook et al. [29].

Growth under microaerophilic conditions was tested by incubating the plates in a candle jar. The ability to grow in anaerobiosis was determined with the BD GasPak^{TM} EZ pouch system (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Catalase activity was determined by using 30% (*v*/*v*) H_2O_2, recording bubble production as a positive result. Oxidase activity was determined with Oxidase Test Stick for microbiology (PanReac AppliChem, Barcelona, Spain). Gram type was determined by using KOH 3% (*w*/*v*), with cell lysis as a positive result for Gram-negative bacteria. Growth at different temperatures (4, 10, 15, 25, 30, 37, 40 and 45 °C) and at various final NaCl concentrations (0.0–4.0% at intervals of 0.5%, and 4.0–10.0% at intervals of 1.0%) was examined by cultivating the isolates on TSA medium. The ability to grow at different pH values (4.0–11.0 at intervals of 1.0 pH unit) was examined in TSB using the MES (pH 4–6), HEPES (pH 7–8) and CHES (pH 9–11) buffers at 10 mM. Carbon source assimilation and enzymatic activities were determined using the API 20NE and API ZYM system strips (bioMérieux, Marcy-l'Étoile, France) according to manufacturer's instructions, as well as BIOLOG GEN III MicroPlates (BIOLOG Inc., Hayward, CA, USA).

2.6. Chemotaxonomic Analysis

For fatty acid analysis, strains T13^T, T90^T, R8^T and their closest relatives were grown on TSA at 26 °C for 72 h. Analysis of cellular fatty acids was carried out using the Sherlock Microbial Identification System (version 6.1, MIDI, Inc., Newark, DE, USA) [30]. Fatty acids were analysed on an Agilent 6859 gas chromatography system and using the MIDI method TSBA6 [31], according to the manufacturer's instructions.

3. Results and Discussion

3.1. Phylogenetic Analysis

The almost-complete 16S rRNA gene sequences showed that the three strains were phylogenetically related to representatives of the genus *Kineococcus*. According to the EzBioCloud database tool (http://www.ezbiocloud.net/), the closest type strains of T13^T, T90^T and R8^T are *K. aurantiacus* IFO 15268^T (98.5% 16S rRNA gene sequence similarity), *K. gypseus* YIM 121300^T (97.1%) and *K. radiotolerans* SRS 30216^T (97.9%), respectively. Since these 16S rRNA gene sequence similarities are lower than 98.7%, we have evidence that suggests that the strains T13^T, T90^T and R8^T may belong to new species [32,33]. According to these results, *K. aurantiacus* DSM 7487^T and *K. radiotolerans* DSM 14245^T were selected as comparative reference strains for T13^T; *K. gypseus* DSM 27627^T, *K. aureolus* DSM 102158^T and *K. mangrovi* NBRC 110933^T for T90^T; and *K. radiotolerans* DSM 14245^T, *K. aurantiacus* DSM 7487^T and *K. gynurae* NBRC 103943^T for R8^T.

The three isolates are classified into the genus *Kineococcus* in both 16S-rRNA-based phylogenetic trees constructed by ML and NJ. Specifically, T13^T showed an external position in the cluster formed by *K. aurantiacus* IFO 15268^T, *K. mangrovi* L2-1-L1^T, *K. endophyticus* KLBMP^T, *K. rhizosphaerae* RP-B16^T and *K. radiotolerans* SRS30216^T (Figures 1 and 2). Strain T90^T formed a monophyletic group with *K. gypseus* YIM 121300^T (Figures 1 and 2) while strain R8^T appeared as an external taxon within the genus *Kineococcus* (Figures 1 and 2). The phylogenetic inference of the three strains based on the 16S rRNA was supported by high bootstrap values.

The ANI and digital DDH values between strains T13^T vs. *K. radiotolerans* SRS 30216^T and R8^T vs. *K. radiotolerans* SRS 30216^T were 80.6–24.20% and 77.40–41.73%, respectively. As the values were higher than the threshold established to circumscribe prokaryotic species, namely 95% for ANI values [34] and 70% for dDDH [26], both genome-related indexes [35] confirmed the adscription of strains T13^T and R8^T to hitherto unknown species. On the other hand, the closest relative of T90^T is *K. gypseus* YIM 121300^T. It must be noted that the genome of *K. gypseus* YIM 121300^T (97.1%) was not publicly available at the time of writing the manuscript. However, since the 16S rRNA gene sequence similarity between T90^T and its closest relative *K. gypseus* YIM 121300^T is <98.7%, it is not necessary to calculate any overall genome relatedness index (OGRI) to propose T90^T as an independent genomospecies [32].

3.2. Genomic Characteristics

The circular map highlighting the main genomic features of the three strains is shown in Figures S2–S4. The draft genome of strains T13^T, T90^T and R8^T consisted of 705, 1067 and 502 contigs, yielding a total length of 4,857,076, 4,498,067 and 4,581,425 bp, respectively. The genomic G + C content of strains T13^T, T90^T and R8^T was 75.4%, 76.3% and 75.1%, respectively. This genomic G + C content is in accordance with other *Kineococcus* species, and further confirms their adscription to the *Kineococcus* genus [2,7,8]. A total of 5039, 4690 and 4633 coding sequences (CDSs) were predicted for strains T13^T, T90^T and R8^T, respectively, of which 1894, 2906 and 2832 were proteins with functional assignments. In the case of tRNA genes, a total of 45 were predicted for strains T13^T and R8^T, and 46 were predicted for strain T90^T. Regarding rRNA genes, six of them were identified in T13^T; whereas five genes were found for T90^T and R8^T. The genome completeness values of strains T13^T, T90^T and R8^T were 98.9%, 98.1% and 99.2%, respectively; and the levels of contamination were 1.1%, 1.7% and 0%, respectively. Therefore, the draft genomes showed high enough quality for further analysis [32].

By analysing the draft genome of strains T13 ^{T}, T90^{T} and R8^{T}, their ability to synthesise diphosphatidylglycerol and phosphatidylglycerol was predicted based on the presence of genes coding for cardiolipin synthase A/B (EC:2.7.8.-) and ribosomal-protein-serine acetyltransferase (EC 2.3.1.-). However, the synthesis of phosphatidylinositol (EC 2.7.8.11), a major polar lipid of several *Kineococcus* species, could not be predicted in any of the three genomes [2,8,11]. Furthermore, the synthesis of meso-diaminopimelic acid in the three strains was predicted based on the presence of the enzyme diaminopimelate epimerase (EC 5.1.1.7), and the synthesis of menaquinones was predicted based on the identification of the enzyme demethylmenaquinone methyltransferase (EC 2.1.1.163). Therefore, the chemotaxonomic profile of the three new strains is in accordance with other *Kineococcus* species, corroborating their inclusion into the genus *Kineococcus* [2,7,8].

3.3. Phenotypic Characterization

Strains T13^{T}, T90^{T} and R8^{T} proved to be aerobic, Gram-positive, motile and coccus-shaped (1 µm in diameter). Like other members of the genus *Kineococcus*, cells of the three strains occur singly, in pairs or in clusters (Figure S1). Cell motility was confirmed by the presence of flagella. Colonies were orange-coloured, with irregular margins and a rough surface. T13^{T} colonies were paler than other *Kineococcus* strains. T90^{T} colonies changed in colour to dark orange-greenish after incubation at low temperatures (below 15 °C). The colonies displayed a diameter of around 1–2 mm after 3–4 days of growth, although T90^{T} colonies displayed a larger diameter.

All three strains were able to grow between 10 and 30 °C (optimum 25–30 °C). Furthermore, strain R8^{T} was able to grow at 4 °C, while strains T13^{T} and T90^{T} could grow up to 37 and 40 °C, respectively (Table 1). Strains T90^{T} and R8^{T} could grow at NaCl concentrations up to 4%, while T13^{T} could grow at concentrations up to 5% (optimum for the three strains, 0% NaCl). *K. radiotolerans* DSM 14245^{T}, *K. aurantiacus* DSM 7487^{T}, *K. gypseus* DSM 27627^{T} and *K. gynurae* NBRC 103943^{T} showed a salinity tolerance range similar to our strains, while *K. aureolus* DSM 102158^{T} and *K. mangrovi* NBRC 110933^{T} were able to grow at concentrations up to 8% and 9% NaCl, respectively (Table 1). Even though the three strains were found to be neutrophilic (optimum growth at pH 7), strains T13^{T} and T90^{T} were able to grow at alkaline values (up to pH 9 and 10, respectively), whereas R8^{T} could grow weakly at pH 5. Other *Kineococcus* species such as *K. aureolus* DSM 102158^{T}, *K. aurantiacus* DSM 7487^{T}, *K. gypseus* DSM 27627^{T}, *K. mangrovi* NBRC 110933^{T} and *K. gynurae* NBRC 103943^{T} were able to tolerate alkaline pH (Table 1). All three strains were able to grow under microaerophilic conditions, but no growth was observed for any of them in anaerobiosis.

Strains T13^{T}, T90^{T} and R8^{T}, like their closest relatives, showed a positive response for esculin hydrolysis, β-galactosidase, esterase (C4), esterase lipase (C8), leucine arylamidase, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and β-glucosidase. On the other hand, strains T13^{T}, T90^{T} and R8^{T} and their closest neighbours showed a negative response for nitrate reduction, indole production, glucose fermentation, arginine dihydrolysis and N-acetyl-β-glucosaminidase. Strains R8^{T} and *K. gynurae* NBRC 103943^{T} showed urease activity, while strain T90^{T}, *K. gynurae* NBRC 103943^{T}, *K. aureolus* DSM 102158^{T} and *K. gypseus* DSM 27627^{T} were able to hydrolyse gelatine (Table 1). In API 20 NE strips, strain R8^{T} proved able to use all the saccharides tested (Table 1). On the contrary, strain T13^{T} was only able to grow weakly with glucose, while T90^{T} could not grow in any of the carbon sources tested. Furthermore, BIOLOG assays revealed that strains T13^{T}, T90^{T} and R8^{T} were able to oxidize 31, 62 and 26 out of the 71 tested carbon sources, respectively, and these were mainly organic acids and amino acids (Table S1). This suggests that strain T90^{T} displays a more polytrophic metabolism than strains T13^{T} and R8^{T}.

Table 1. Differential characteristics between strains T13^T, T90^T, R8^T and related type strains of the genus *Kineococcus*.

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Isolation source	Biocrust	Biocrust	Biocrust	Radioactive work area [11]	Saline sediment [8]	Soil [1]	Saline sediment [6]	Mangrove sediment [7]	Medicinal plant [9]
Growth at/in:									
Temperature range (°C)	10–37	10–40	4–30	4–37	10–45	4–40	15–37	10–30	4–30
pH range	6–9	6–10	5–8	6–8	7–11	5–9	5–10	5–9	5–10
NaCl tolerance (%, *w*/*v*)	0–5	0–4	0–4	0–4	0–8	0–4	0–4	0–9	0–6
Carbon source utilization (API 20NE)									
D-Glucose	W	-	+	+	+	+	+	+	+
L-Arabinose	-	-	+	+	+	+	+	+	+
D-Mannose	-	-	+	+	+	+	+	+	+
D-Mannitol	-	-	+	+	+	+	W	+	+
N-Acetyl-glucosamine	-	-	+	W	-	+	-	W	-
D-Maltose	-	-	+	W	+	+	W	+	+
Potassium gluconate	-	-	W	+	+	W	-	+	W
Adipic acid	-	-	W	W	W	-	-	-	-
Malic acid	-	-	-	W	+	-	W	+	W
Trisodium citrate	-	-	-	-	W	-	-	-	W
Phenylacetic acid	-	-	-	-	W	-	-	-	-
Enzymatic activity (API 20NE)									
Urease	-	-	+	-	-	-	-	-	+
Gelatinase	-	+	-	-	+	-	+	-	+
Enzymatic activity (API ZYM)									
Alkaline phosphatase	-	+	+	+	-	+	-	+	+
Lipase (C14)	-	-	-	+	-	-	-	-	-
Valine arylamidase	+	+	+	+	+	+	+	-	-
Cystine arylamidase	-	-	+	+	-	+	+	-	-
Trypsin	-	-	-	+	-	-	-	-	-
α-Galactosidase	+	+	+	+	+	+	-	+	+
β-Glucuronidase	-	-	-	+	-	-	-	-	-
α-Glucosidase	+	+	-	+	-	-	+	+	+
α-Manosidase	+	+	-	-	+	+	-	-	+
α-Fucosidase	+	-	+	-	+	-	-	-	-

Strains: 1, T13^T; 2, T90^T; 3, R8^T; 4, *Kineococcus radiotolerans* DSM 14245^T; 5, *Kineococcus aureolus* DSM 102158^T; 6, *Kineococcus aurantiacus* DSM 7487^T; 7, *Kineococcus gypseus* DSM 27627^T; 8, *Kineococcus mangrovi* NBRC 110933^T; 9, *Kineococcus gynurae* NBRC 103943^T. Data in the present study were obtained from experiments carried out under identical conditions. All strains were positive for the following characteristics: Gram reaction, catalase activity, esculin hydrolysis, β-galactosidase, esterase (C4), esterase lipase (C8), leucine arylamidase, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and β-glucosidase. All strains were negative for the following characteristics: oxidase, nitrate reduction, indole formation, glucose fermentation, arginine dihydrolysis, capric acid assimilation and N-acetyl-β-glucosaminidase. +, positive; -, negative; W, weak reaction.

The major fatty acids for strains T13^T, T90^T and R8^T were anteiso-$C_{15:0}$ (96.1%, 73.9% and 94.0%, respectively). Similarly, their closest relatives showed high amounts of anteiso-$C_{15:0}$ (Table 2), corroborating the inclusion of the three strains in the genus *Kineococcus* [2,8,11]. The presence of anteiso-$C_{15:0}$ fatty acid in the cell membranes has been associated with resistance to low temperatures [36,37]. In fact, this fatty acid has been found in the psychrophilic members of the genus *Flavobacterium* [38] isolated from a cold desert environment. Moreover, anteiso-$C_{15:0}$ fatty acid can play an important role in biofilm formation [39,40], which is in accordance with the biofilms and cell clumps observed for the three *Kineococcus* strains under liquid growth conditions.

Table 2. Cellular fatty acid composition of strains T13^T, T90^T and R8^T and their closest relatives.

Fatty Acids	1	2	3	4	5	6	7	8	9
Saturated									
$C_{14:0}$	tr	1.2	1.37	-	1.8	1.3	2.1	3.9	1.6
iso-$C_{14:0}$	-	1.7	1.34	-	2.0		1.8	7.1	2.9
$C_{15:0}$	-	tr	-	-	1.7	1.0	2.6	-	2.2
iso-$C_{15:0}$	tr	1.3	-	-	tr	-	-	-	-
anteiso-$C_{15:0}$	96.1	73.9	94.0	97.9	78.8	75.9	73.0	84.4	74.2
$C_{16:0}$	-	tr	tr	-	2.0	1.8	2.2	tr	1.8
$C_{18:0}$	-	-	tr	-	tr	1.5	1.2	-	-
Unsaturated									
AT 12–13 $C_{13:1}$	-	-	-	-	-	3.4	-	-	3.4
anteiso-$C_{15:1}$ A	2.6	16.9	-	tr	6.7	-	12.3	-	-
Hydroxylated									
$C_{14:0}$ 2-OH	-	-	-	-	-	5.4	-	tr	4.0
$C_{17:0}$ 2-OH	-	-	-	-	-	-	-	2.8	-
$C_{17:0}$ 3-OH	-	3.4	tr	1.2	3	2.4	3.5	-	1.3
Summed *									
Sum in feature 2	-	-	-	-	-	2.7	-	-	2.8
Sum in feature 3	-	-	-	-	-	2.7	-	-	2.4
Sum in feature 5	-	-	-	-	-	2.0	-	-	-

* Summed features represent groups of fatty acids that cannot be separated with the chromatographic system. Sum in feature 2 corresponds to $C_{13:0}$ 3-OH/iso-$C_{15:1}$ I/iso-$C_{15:1}$ H. Sum in feature 3 corresponds to iso-$C_{16:1}$ I/$C_{14:0}$ 3-OH/$C_{12:0}$ alde? Sum in feature 5 corresponds to iso-$C_{17:1}$ I/anteiso-$C_{17:1}$ B.; Strains: 1, T13^T; 2, T90^T; 3, R8^T; 4, *Kineococcus radiotolerans* DSM 14245^T; 5, *Kineococcus aureolus* DSM 102158^T; 6, *Kineococcus aurantiacus* DSM 7487^T; 7, *Kineococcus gypseus* DSM 27627^T; 8, *Kineococcus mangrovi* NBRC 110933^T; 9, *Kineococcus gynurae* NBRC 103943^T. Data for reference strains were obtained in the present study. Values are shown as percentages. – means not detected; tr, <1.0% trace.

Unlike their closest relatives, strains T90^T and *K. gypseus* DSM 27627^T also showed high amounts of anteiso-$C_{15:1}$ (16.9%) (Table 2). Furthermore, the presence or absence of other minor fatty acids allowed us to differentiate the new strains from their closest neighbours (Table 2).

4. Conclusions

During a study on the microbial communities associated with biocrust in sun-exposed and dry surfaces, the three strains T13^T, T90^T and R8^T were isolated in pure culture. All three strains, which were phylogenetically related to the genus *Kineococcus*, were characterized using a polyphasic approach. According to the phenotypic, genomic and phylogenetic analyses, strains T13^T, T90^T and R8^T should be considered as new species within the *Kineococcus* genus, for which the names *Kineococcus vitellinus* sp. nov., *Kineococcus indalonis* sp. nov. and *Kineococcus siccus* sp. nov., respectively, are proposed.

4.1. Description of Kineococcus vitellinus sp. nov.

Kineococcus vitellinus (vi.tel.li'nus. N.L. masc. adj. Vitellinus Egg-Yolk-Coloured)

Cells are Gram-positive, motile, non-endospore-forming, catalase-positive, oxidase-negative cocci (1 µm in diameter). Cells occur singly, in pairs or in clusters. Colonies are 1–1.5 mm in diameter, circular, rough and pale orange. Temperature range for growth is 10–37 °C, with an optimum at 25–30 °C. Growth occurs

at pH 6–9 (optimum pH 7.0) and tolerates up to 5% NaCl (*w*/*v*), with optimum at 0% NaCl (*w*/*v*). Esculin hydrolysis, β-galactosidase, esterase (C4), esterase lipase (C8), leucine arylamidase, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-glucosidase, valine arylamidase, α-galactosidase, α-glucosidase, α-mannosidase and α- fucosidase activities are detected. Nitrate reduction, indole production, glucose fermentation, arginine dihydrolysis, urease, gelatinase, alkaline phosphatase, lipase (C14), cystine arylamidase, trypsin, β-glucuronidase and N-acetyl-β-glucosaminidase are not detected. Using API 20NE test kit, this species is weakly positive for the assimilation of glucose and negative for arabinose, mannitol, N-acetyl-glucosamine, maltose, potassium gluconate, malic acid, mannose, capric acid, adipic acid, trisodium citrate and phenylacetic acid. Using BIOLOG GENIII MicroPlates, this species is positive for the utilization of D-raffinose, α- D-glucose, D-sorbitol, pectin, Tween 40, dextrin, α-D-lactose, D-mannose, D-mannitol, D-maltose, D-melibiose, D-fructose, L-alanine, D-trehalose, β-methyl-D-glucoside, D-galactose, myo-inositol, D-cellobiose, D-salicin, glycerol, gentiobiose, N-acetyl-D-glucosamine, glucuronamide, acetoacetic acid, sucrose, D-fructose-6PO_4, D -turanose, L-rhamnose, L-pyroglutamic acid, stachyose and L-serine; and negative for the utilization of gelatine, p-hydroxy-phenylacetic acid, glycyl- L-proline, D-galacturonic acid, methyl pyruvate, γ-amino-butyric acid, D-arabitol, L-galactonic acid lactone, D-lactic acid methyl ester, α-hydroxy-butyric acid, L-arginine, D-gluconic acid, L-lactic acid, β-hydroxy- D, L-butyric acid, 3-methyl-D-glucoside, L-aspartic acid, D-glucuronic acid, citric acid, α-keto-butyric acid, D-fucose, D -glucose-6-PO_4, L-glutamic acid, β-keto-glutaric acid, N-acetyl-β-D-mannosamine, L-fucose, L-histidine, mucic acid, D-malic acid, propionic acid, N-acetyl-D-galactosamine, D-aspartic acid, quinic acid, L-malic acid, acetic acid, N-acetyl neuraminic acid, inosine, D-serine, D-saccharic acid, bromo-succinic acid and formic acid. The major fatty acid is anteiso-$C_{15:0}$. The type strain T13^T (CECT 9936^T = DSM 110024^T) was isolated nearby the Tabernas Desert in Almería (Spain), from biocrust samples. The G + C content of the type strain is 75.4%. The GenBank/EMBL/DDBJ accession number for the 16S rRNA sequence is MN069869, and the genome accession number is JAAALL000000000.

4.2. Description of Kineococcus indalonis sp. nov.

Kineococcus indalonis (in.da.lo'nis. N.L. gen. n. *indalonis* of Indalo, Which Is a Prehistoric Symbol Found in Rock Paintings in Almería (Spain), Referring to the Place Where the Microorganism Was Isolated)

Cells are Gram-positive, motile, non-endospore-forming, catalase-positive, oxidase-negative cocci (1 μm in diameter). Cells occur singly, in pairs or in clusters. Colonies are small (1 mm in diameter), circular with irregular margins, rough and pale orange, but below 20 °C the colour changes from orange to dark greenish. Temperature range for growth is 10–40 °C, with an optimum at 25–30 °C. No growth is observed below 10 or above 40 °C. Growth occurs at pH 6–10 (optimum, 6–9) and tolerates up to 4% NaCl (*w*/*v*), with optimum at 0% (*w*/*v*). Esculin hydrolysis, β-galactosidase, gelatinase, esterase (C4), esterase lipase (C8), leucine arylamidase, α-chymotrypsin, acid phosphatase, alkaline phosphatase, valine arylamidase, α-galactosidase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, α-mannosidase and β-glucosidase activities are detected. Nitrate reduction, indole production, glucose fermentation, arginine dihydrolysis, urease, lipase (C14), cystine arylamidase, trypsin, β-glucuronidase, α-fucosidase and N-acetyl-β-glucosaminidase are not detected. Using API 20NE test kit, this species is negative for the assimilation of glucose, arabinose, mannitol, N-acetyl-glucosamine, maltose, potassium gluconate, malic acid, mannose, capric acid, adipic acid, trisodium citrate and phenylacetic acid. Using BIOLOG GENIII MicroPlates, this species is positive for the utilization of D-raffinose, α-D-glucose, D-sorbitol, gelatine, pectin, Tween 40, dextrin, α- D-lactose, D-mannose, D-mannitol, glycyl-L-proline, D-galacturonic acid, γ-amino-butyric acid, D-maltose, D-melibiose, D-fructose, D-arabitol, L-alanine, L-galactonic acid lactone, D-lactic acid methyl ester, D-trehalose, β-methyl-D-glucoside, D-galactose, myo-inositol, L-arginine, D-gluconic acid, β-hydroxy-D,L-butyric acid, D-cellobiose, D-salicin, 3-methyl-D-glucoside, glycerol, L-aspartic acid, D-glucuronic acid, citric acid, α-keto-butyric acid, gentiobiose, N-acetyl-D-glucosamine, D-fucose, D-glucose-6-PO_4, L-glutamic acid, glucuronamide, α-keto-glutaric acid, acetoacetic acid, sucrose, N-acetyl-β-D-mannose, L-fucose, D-fructose-6PO_4, L-histidine, mucic acid, D-malic acid, propionic acid, D-turanose, N-acetyl-D-galactosamine, L-rhamnose, D-aspartic acid,

L-pyroglutamic acid, L-malic acid, acetic acid, stachyose, D-serine, L-serine and bromo-succinic acid; and negative for the utilization of p-hydroxy-phenylacetic acid, methyl pyruvate, α-hydroxy-butyric acid, L-lactic acid, quinic acid, N-acetyl neuraminic acid, inosine, D-saccharic acid and formic acid. The major fatty acids for strain T90^T are anteiso-$C_{15:0}$ and anteiso-$C_{15:1}$ A. The type strain T90^T (CECT 9938^T = DSM 110026^T) was first isolated nearby the Tabernas Desert in Almería (Spain), from biocrust samples. The G + C content of the type strain is 76.3%. The GenBank/EMBL/DDBJ accession number for the 16S rRNA sequence is MN069867, and the genome accession number is JAAALM000000000.

4.3. Description of Kineococcus siccus sp. nov.

Kineococcus siccus (*siccus*. L. masc. adj. *Siccus*, Dry)

Cells are Gram-positive, motile, non-endospore-forming, catalase-positive, oxidase-negative cocci (1 μm in diameter). Cells occur singly, in pairs or in clusters. Colonies are orange, circular, with irregular margins and variable size (1–2 mm diameter). Temperature range for growth is 4–30 °C with an optimum at 25–30 °C. No growth is observed at 40 °C. Growth occurs at pH 5–8 (optimum 6–7) and tolerates up to 4% NaCl (*w*/*v*), with optimum at 0% (*w*/*v*). Esculin hydrolysis, β-galactosidase, urease, esterase (C4), esterase lipase (C8), leucine arylamidase, α-chymotrypsin, acid phosphatase, alkaline phosphatase, valine arylamidase, cystine arylamidase, α-galactosidase, naphthol-AS-BI-phosphohydrolase, α-fucosidase and β-glucosidase activities are detected. Nitrate reduction, indole production, glucose fermentation, arginine dihydrolysis, gelatinase, lipase (C14), trypsin, α-glucosidase, β-glucuronidase, α-mannosidase and N-acetyl-β-glucosaminidase are not detected. Using API 20NE test kit, this species is positive for the assimilation of glucose, arabinose, mannose, mannitol, N-acetyl-glucosamine and maltose; weak for the assimilation potassium gluconate and adipic acid and negative for the assimilation of malic acid, capric acid, trisodium citrate and phenylacetic acid. Using BIOLOG GENIII MicroPlates, this species is positive for the utilization of pectin, Tween 40, dextrin, D-fructose, D-arabitol, L-alanine, D-galactose, D-gluconic acid, L-lactic acid, D-cellobiose, 3-methyl-D-glucoside, glycerol, D-glucuronic acid, gentiobiose, glucuronamide, acetoacetic acid, L-fucose, D-fructose-6-PO_4, mucic acid, D-malic acid, D-turanose, D-aspartic acid, quinic acid, L-malic acid, acetic acid and D-serine; and negative for the utilization of D-raffinose, α-D-glucose, D-sorbitol, gelatine, p-hydroxy-phenylacetic acid, α-D-lactose, D-mannose, D-mannitol, glycyl-L-proline, D-galacturonic acid, methyl pyruvate, γ-amino-butyric acid, D-maltose, D-melibiose, L-galactonic acid lactone, D-lactic acid methyl ester, α-hydroxy-butyric acid, D-trehalose, β-methyl-D-glucoside, myo-inositol, L-arginine, β-hydroxy-D,L-butyric acid, D-salicin, L-aspartic acid, citric acid, α-keto-butyric acid, N-acetyl-D-glucosamine, D-fucose, D-glucose-6-PO_4, L-glutamic acid, α-keto-glutaric acid, sucrose, N-acetyl-β-D-mannose, L-histidine, propionic acid, N-acetyl-D-galactosamine, L-rhamnose, L-pyroglutamic acid, stachyose, N-acetyl neuraminic acid, inosine, L-serine, D-saccharic acid, bromo-succinic acid and formic acid. The major fatty acid is anteiso-$C_{15:0}$. The type strain R8^T (CECT 9937^T = DSM 110025^T) was first isolated nearby the Tabernas Desert in Almería (Spain), from biocrust samples. The G + C content of the type strain is 75.1%. The GenBank/EMBL/DDBJ accession number for the 16S rRNA sequence is MN069868, and the genome accession number is JAAALN000000000.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2607/8/10/1547/s1, Table S1: Carbon source utilization comparison using GENIII MicroPlates of strains T13^T, T90^T, R8^T and the type strains of closely related *Kineococcus* species. Figure S1: Cell morphology of strains T13^T (A, B), T90^T (C, D) and R8^T (E, F) under the optical microscope at 100× magnification, Figure S2: *Kineococcus vitellinus* sp. nov. T13^T circular genomic map. Figure S3: *Kineococcus indalonis* sp. nov. T90^T circular genomic map. Figure S4: *Kineococcus siccus* sp. nov. R8^T circular genomic map.

Author Contributions: M.P. conceived the work. M.P., E.M.-M. and H.G.-V. carried out the sampling. E.M.-M. and H.G.-V. performed the identification. E.M.-M. carried out the phenotypic characterization. J.P. (Javier Pascual) supervised the bioinformatic and phylogenetic analysis. E.M.-M., H.G.-V., J.P. (Javier Pascual), J.P. (Juli Peretó) and

M.P. analysed the results, wrote and approved the manuscript. All authors have read and agreed to the published version of the manuscript.

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Belnapia mucosa sp. nov. and *Belnapia arida* sp. nov., isolated from desert biocrust

Esther Molina-Menor[1], Àngela Vidal-Verdú[1], Leila Satari[1], Alba Calonge-García[1], Javier Pascual[2], Juli Peretó[1,2,3] and Manuel Porcar[1,2,*]

Abstract

Two novel Gram-staining-negative, aerobic, cocci-shaped, non-motile, non-spore forming, pink-pigmented bacteria designated strains T6^T and T18^T, were isolated from a biocrust (biological soil crust) sample from the vicinity of the Tabernas Desert (Spain). Both strains were catalase-positive and oxidase-negative, and grew under mesophilic, neutrophilic and non-halophilic conditions. According to the 16S rRNA gene sequences, strains T6^T and T18^T showed similarities with *Belnapia rosea* CGMCC 1.10758^T and *Belnapia moabensis* CP2C^T (98.11 and 98.55% gene sequence similarity, respectively). The DNA G+C content was 69.80 and 68.96% for strains T6^T and T18^T, respectively; the average nucleotide identity by BLAST (ANIb) and digital DNA–DNA hybridization (dDDH) values confirmed their adscription to two novel species within the genus *Belnapia*. The predominant fatty acids were summed feature 8 ($C_{18:1}\omega7c/C_{18:1}\omega6c$), $C_{16:0}$, $C_{18:1}$ 2-OH and summed feature 3 ($C_{16:1}\omega7c/C_{16:1}\omega6c$). According to he results of the polyphasic study, strains T6^T and T18^T represent two novel species in the genus *Belnapia* (which currently includes only three species), for which names *Belnapia mucosa* sp. nov. (type strain T6^T = CECT 30228^T=DSM 112073^T) and *Belnapia arida* sp. nov. (type strain T18^T=CECT 30229^T=DSM 112074^T) are proposed, respectively.

The genus *Belnapia* was first described by Reddy *et al.* [1] and it is, at the time of writing, comprised of three species, which were all isolated from soil samples: *Belnapia moabensis* [1], *Belnapia rosea* [2] and *Belnapia soli* [3]. In this study we describe the polyphasic characterization of two strains, namely T6^T and T18^T, which were isolated from biocrust (biological soil crust) samples from south-eastern Spain during a study on the microbial diversity of European arid regions.

Strains T6^T and T18^T were isolated in the vicinity of the Tabernas Desert (Almería, Spain) during a study on the culturable microbial diversity in European drylands [4]. The Tabernas Desert is considered the only arid desert in Europe. Biocrust samples were obtained from near the Tabernas Desert Parc (37.002404° N, 2.450655° W) and homogenized in phosphate buffered saline (PBS; NaCl 8.0 g l^{-1}, KCl 0.2 g l^{-1}, Na_2HPO_4 1.44 g l^{-1}, KH_2PO_4 0.24 g l^{-1}) pH 7.4 (1 g in 1 ml). The suspensions were then spread on 1, 0.1 and 0.01× trypticase soy agar (TSA; 15 g l^{-1} tryptone, 5 g l^{-1} NaCl, 5 g l^{-1} soya peptone), and Reasoner's 2A Agar (R2A; 1 g l^{-1} peptone, 0.5 g l^{-1} yeast extract, 0.5 g l^{-1} dextrose, 0.5 g l^{-1} soluble starch, 0.3 g l^{-1} dipotassium phosphate, 0.05 g l^{-1} magnesium sulphate heptahydrate, 0.3 g l^{-1} sodium pyruvate). Agar was autoclaved separately and added before plating at a final concentration of 15 g l^{-1}. The plates were incubated at 23 °C for 1 week. Strain T6^T was isolated from 0.1× TSA plates, whereas T18^T was isolated from a 0.01× TSA plate. The isolation of the strains was carried out by re-streaking on fresh media until a pure culture was obtained. Cell suspensions in TSA and R2A were cryopreserved at −80 °C with 15% glycerol (v/v). Their taxonomic status was determined by a polyphasic approach. On the basis of the results from phylogenetic, phenotypic and chemotaxonomic analysis, it is concluded that strains T6^T and T18^T are related to members of the genus *Belnapia* and representatives of two novel species. In the present work,

Author affiliations: [1]Institute for Integrative Systems Biology I2SysBio (Universitat de València-CSIC), Calle del Catedràtic Agustin Escardino Benlloch 9, 46980 Paterna, Spain; [2]Darwin Bioprospecting Excellence SL. Parc Científic Universitat de València, Calle del Catedràtic Agustin Escardino Benlloch 9, 46980 Paterna, Spain; [3]Departament de Bioquimica i Biologia Molecular, Universitat de València, Calle del Dr. Moliner 5046100 Burjassot, Spain.
***Correspondence:** Manuel Porcar, manuel.porcar@csic.es
Keywords: *Belnapia*; Tabernas Desert; biocrust; novel species; *Alphaproteobacteria*.
Abbreviations: ANIb, average nucleotide identities according to BLAST; dDDH, digital DNA hybridization; ML, maximum likelihood; NJ, neighbour joining.
The 16S rRNA gene sequences of strains T6^T and T18^T have been deposited in DDBJ/ENA/GenBank under the accession numbers MW960268 and MW960269, respectively. The genomic sequences of strains T6^T and T18^T have been deposited under the DDBJ/ENA/GenBank accession numbers JAEUXJ000000000 and JAETWB000000000, respectively.
One supplementary figure and two supplementary tables are available with the online version of this article.

the reference strains *B. rosea* DSM 23312^T, *B. moabensis* DSM 16746^T and *B. soli* DSM 28067^T, from the DSMZ (German Collection of Microorganisms and Cell Cultures, Leibniz Institute, Braunschweig, Germany), and strains T6^T and T18^T were all grown in parallel on R2A media at 30 °C, unless otherwise specified.

The phenotypic characteristics of T6^T and T18^T were analysed after 1 week of growth at 30 °C. A Gram staining test was carried out with KOH 3 % (w/v), recording viscosity as a negative result. Oxidase activity was tested by using the commercial Oxidase Test Stick for microbiology (PanReac AppliChem). Catalase activity was tested with hydrogen peroxide 30% (v/v), recording bubble formation as a positive result. Cell morphology was observed under an optical microscope with crystal violet glass stain. Growth at different temperatures (4, 10, 15, 20, 23, 30, 37, 40, 42 and 45 °C) and NaCl concentrations (0.0–4.0% at intervals of 0.5%) was checked on R2A. Growth at different pH values (4.0–10.0 at intervals of 1.0 pH unit) was examined by growing the strains in liquid R2A using the buffers MES (pH 4–6), HEPES (pH 7–8) and CHES (pH 9–10) at a final concentration of 10 mM. Growth under microaerophilic and anaerobic conditions was tested by incubating the plates in a candle jar and with the BD GasPak EZ pouch system (Becton, Dickinson), respectively. Carbon source assimilation and enzymatic activities were checked using the API 20NE and API ZYM system strips (bioMérieux) according to manufacturer's instructions. BIOLOG GEN III MicroPlates (BIOLOG) were also used to determine carbon source assimilation.

Strains T6^T, T18^T and the reference strains *B. rosea* DSM 23312^T, *B. moabensis* DSM 16746^T and *B. soli* DSM 28067^T were grown on R2A medium at 30 °C for 72 h for analysis of cellular fatty acids. The analysis was carried out following the protocol recommended by MIDI Microbial Identification System (version 6.1, MIDI, Inc, Newark, DE, USA) [5]. The fatty acids content was analysed on a 6850 gas chromatography system (Agilent) using the TSBA6 method [6].

Genomic DNA extraction was carried out using the DNeasy Power Soil kit (Qiagen), according to the manufacturer's instructions, but incubating at 65 °C after the addition of C1. Whole 16S rRNA gene PCR was carried out with universal primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) [7] and 1492R (5′-GGTTACCTTGTTACGACTT-3′) [8] following procedures described previously [4]. Phylogenetic trees based on the 16S rRNA gene sequences were reconstructed using the maximum-likelihood (ML) [9] and neighbour-joining (NJ) [10] methods with the software MEGA X v.10.1.7. The TamuraNei G+I evolutionary model and the Kimura two-parameter model were used for the ML and NJ trees, respectively. The reliability of the branch patterns was assessed using bootstrap analysis based on 500 and 1000 replicates, respectively, for the ML and the NJ trees [11].

The draft genome of strains T6^T and T18^T were sequenced with the NovaSeq 6000 system (Illumina; 2×150 bp paired-end sequencing). The genomic DNA was randomly fragmented by sonication, then DNA fragments were end polished, A-tailed and ligated with the full-length adapters for Illumina sequencing. Further PCR amplification was carried out with P5 and indexed P7 oligonucleotides, and PCR products for the final construction of the libraries were purified with an AMPure XP system. Libraries were then checked for size distribution by Agilent 2100 Bioanalyzer (Agilent Technologies), and quantified by real-time PCR. The FastQC tool (v0.11.5) [12] was utilized to assess the quality of the sequence reads. There were 14 375 848 and 12 879 132 paired-end reads for strains T6^T and T18^T, respectively before filtering. After quality filtering, there were considered to be 13 106 155 and 11 792 897 paired-end sequences the genomes of strains T6^T and T18^T, respectively. Genome assembly of paired reads was performed using the '--isolate' mode in SPAdes (3.14.1) [13]. Assembly statistics were calculated with QUAST (v.5.0.2) [14] and the completeness and contamination levels were evaluated with CheckM (v.1.1.3) [15]. The draft genomes were annotated using the RAST tool kit (RAStk) [16] integrated in PATRIC v.3.6.8. The draft genomes were analysed with the TYGS tool [17] in order to identify the most closely related type strains to T6^T and T18^T with publicly available genomes and to calculate digital DNA–DNA hybridization (dDDH) indexes. JSpecies [18] was used for calculating the average nucleotide identities according to BLAST (ANIb) between genome pairs. UBCG (v.3.0) [19] was used for reconstructing the phylogenomic tree among the selected strains based on a multiple alignment of a set of 92 housekeeping genes. We selected the alignment method codon and inferred the phylogenetic relationships with FastTree. The reliability of the branch patterns was assessed using bootstrap analysis based on 100 replicates.

Strains T6^T and T18^T were aerobic, Gram-staining-negative, non-motile and coccus-shaped (0.8–1.0 µm in diameter). The cells of both strains occurred singly, as in other members of the genus *Belnapia*. Colonies were pink, irregular and mucous. T6^T colonies were paler than those of the rest of the members of the genus *Belnapia*. After 3–5 days of growth at 30 °C, the colonies of both strains displayed a diameter of around 3–4 mm.

Both strains were able to grow at between 4 and 40 °C (optimum at 30 °C). Moreover, T6^T was able to grow at up to 42 °C. Both strains showed tolerance to up to 1.5% (w/v) of NaCl (optimum 0–1%). *B. moabensis* DSM 16746^T and *B. rosea* DSM 23312^T showed similar NaCl tolerances, in contrast with *B. soli* DSM 28067^T, which was able to grow at concentrations of up to 3%. All five strains were able to grow at between pH 5 and 9, with an optimum at 6–7 (Table 1).

Strains T6^T and T18^T showed, like the other members of the genus *Belnapia*, a positive response for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase and napthol-AS-BI-phosphohydrolase. In contrast, T6^T and T18^T and their relatives of the genus *Belnapia* showed a negative response for lipase (C14), valine arylamidase, cystine arylamidase, α-chymotripsin, α-galactosidase, *β*-galactosidase, *β*-glucuronidase, α-glucosidase, *β*-glucosidase, *N*-acetyl-*β*-glucosaminidase, α-manosidase, α-fucosidase, fermentation

Table 1. Differential phenotypic characteristics between strains T6^T, T18^T and the other members of the genus *Belnapia*

Strains: 1, T6^T; 2, T18^T; 3, *Belnapia moabensis* DSM 16746^T; 4, *Belnapia rosea* DSM 23312^T; 5, *Belnapia soli* DSM 28067^T. Data for reference strains were obtained in the present study. +, Positive; –, negative; w, weak reaction. All strains are positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase and napthol-AS-BI-phosphohydrolase. All strains are negative for lipase (C14), valine arylamidase, cystine arylamidase, α-chymotrypsin, α-galactosidase, *β*-galactosidase, *β*-glucuronidase, α-glucosidase, *β*-glucosidase, *N*-acetyl-*β*-glucosaminidase, α-mannosidase, α-fucosidase, fermentation of glucose, arginine dihydrolysis, aesculin hydrolysis and the assimilation of D-mannitol, *N*-acetyl-glucosamine, maltose, capric acid, trisodium citrate and phenylacetic acid.

Characteristic	**1**	**2**	**3**	**4**	**5**
Isolation source	Biocrust	Biocrust	Soil crust	Forest soil	Grass soil
Growth at/in					
Temperature range (°C)	4–42	4–40	4–40	4–40	4–40
pH range	5–9	5–9	5–9	5–9	5–9
NaCl tolerance (%, w/v)	0–1.5	0–1.5	0–2	0–1.5	0–3
Carbon source utilization (API 20NE)					
D-glucose	–	–	–	–	w
L-arabinose	–	–	w	w	w
D-mannose	–	–	–	w	–
Potassium gluconate	w	–	–	w	–
Adipic acid	w	–	–	+	+
Malic acid	w	–	–	–	w
Enzymatic activity (API 20NE)					
Nitrate reduction	–	–	–	+	–
Indole production	–	–	–	+	–
Urease	–	w	+	w	w
Gelatin	–	–	+	–	–
Enzymatic activity (API ZYM)					
Trypsin	–	–	+	–	–

of glucose, arginine dihydrolysis and aesculin hydrolysis. *B. moabensis* DSM 16746^T showed positive response for trypsin and gelatin, while *B. rosea* DSM 23312^T showed positive response for reduction of nitrate and indole production. T6^T was negative for urease, in contrast to the other four strains, which were positive or weakly positive for this activity. In API 20 NE strips, T18^T was not able to assimilate any of the saccharides tested, whereas T6^T could grow weakly with potassium gluconate, adipic acid and malic acid (Table 1). Furthermore, the results of the BIOLOG GENIII plates indicated that strains T6^T and T18^T were only able to oxidize five and four out of the 71 carbon sources, respectively (Table S1, available in the online version of this article). In contrast, the reference strains *B. moabensis* DSM 16746^T, *B. rosea* DSM 23312^T and *B. soli* DSM 28067^T were able to oxidize 41, 25 and 16 carbon sources, respectively. This indicates that the reference strains present a more polytrophic metabolism than strains T6^T and T18^T.

Almost complete 16S rRNA gene sequences were obtained. The 16S rRNA gene sequence lengths of strains T6^T and T18^T are 1392 (accession number MW583035) and 1383 bp (MW583036), respectively. According to the EzBioCloud database tool, the most closely related type strains of T6^T were *B. rosea* CGMCC 1.10758^T (98.11%), *B. moabensis* CP2C^T (97.38%) and *B. soli* PB-K8^T (96.80%); whereas the closest relatives of T18^T were *B. moabensis* CP2C^T (98.55%), *B. soli* PB-K8^T (97.54%) and *B. rosea* CGMCC 1.10758^T (97.40%). The type strains *B. rosea* DSM 23312^T, *B. moabensis* DSM 16746^T and *B. soli* DSM 28067^T were, thus, selected as comparative reference strains, which were obtained from the DSMZ-German Collection of Microorganisms and Cell Cultures (Leibniz Institute, Braunschweig, Germany).

The phylogenetic positions of strains T6^T and T18^T within the genus *Belnapia* were confirmed by both 16S-rRNA-based ML and NJ phylogenetic tress (Figs 1 and S1). T6^T grouped with *B. rosea* CGMCC 1.10758^T, whereas T18^T showed an external position in the cluster formed by *Belnapia moabensis* CP2C^T and *Belnapia soli* PB-K8^T in both trees. This phylogenetic inference was supported by high bootstrap values.

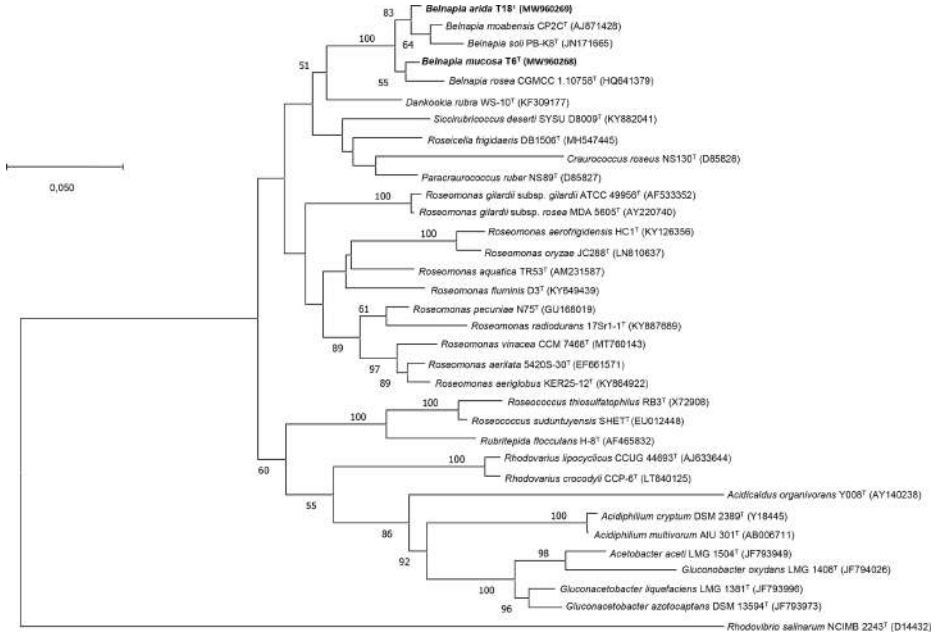

Fig. 1. Maximum likelihood phylogenetic tree showing the relationships between strains T6^T, T18^T and other members of the family *Acetobacteraceae* based on 16S rRNA gene sequences. The optimal evolutionary model of nucleotide substitution applied was Tamura–Nei G+I. Numbers at branch points refer to bootstrap percentages based on 500 replicates (values under 50% are not indicated). *Rhodovibrio salinarum* NCIMB 2243^T (D14432) was used as an outgroup. Bar, 0.05 fixed nucleotide substitutions per site.

The draft genomes of strains T6^T and T18^T consisted of 220 and 355 contigs, respectively, which constituted a total length of 6449681 and 6937094 bp, respectively. The N50 values were 328 210 and 194 160 for T6^T and T18^T respectively. The genomic DNA G+C contents were 69.80 and 68.96% for T6^T and T18^T respectively, which is in accordance with the values previously described for the rest of the species within the genus *Belnapia* and further confirms their adscription to this genus [1–3]. A total of 6369 and 7450 coding sequences (CDSs) were predicted for strains T6^T and T18^T, of which 3380 and 3480, respectively, corresponded to proteins with functional assignment. Regarding the prediction of tRNA and rRNAs, a total of 49 and 47 tRNAs, and 3 and 2 rRNAs were predicted for strains T6^T and T18^T, respectively. The 16S rRNA gene sequences of strains T6^T and T18^T were also extracted from the genome, which were 1496 and 1494 bp long, respectively (accession numbers MW960268 and MW960269, respectively). According to the EzBioCloud database tool, the most closesly related type strains to T6^T were *B. rosea* CGMCC 1.10758^T (98.16%), *B. moabensis* CP2C^T (97.45%) and *B. soli* PB-K8^T (96.80%); whereas the most closely related type strains to T18^T were *B. moabensis* CP2C^T (98.58%), *B. soli* PB-K8^T (97.54%) and *B. rosea* CGMCC 1.10758^T (97.45%). The completeness values of genomes were 100 and 99.5 % for strains T6^T and T18^T, respectively; and the levels of contamination were 0.5 and 2.49%, respectively. The draft genomes showed, thus, sufficient quality for further analysis.

In order to obtain a more accurate phylogenetic inference of novel strains, a phylogenomic tree based on nucleotide sequences was reconstructed (Fig. 2). The phylogenomic tree corroborated that the two strains represent members of the genus *Belnapia*. Strain T18^T was most closely related to *B. moabensis* DSM 16746T, while T6^T showed an external position to the rest of the members of the genus *Belnapia*. The type strain of *B. soli* was not included in this analysis because its genome was not publicly available at the time of writing.

The ANIb and digital DDH values between strains T6^T and T18^T and other related species were calculated (Table S2). The ANIb and dDDH values of strain T6^T vs. *Belnapia rosea* CGMCC 110758^T were 83.26 and 29 %, respectively; the ANIb and dDDH values of strain T18^T vs. *Belnapia moabensis* DSM 16746^T were 88.47 and 40.5 %, respectively. Moreover, both genome indexes were calculated between strains T6^T and T18^T, which were 82.96 and 28.5 % for ANIb and dDDH, respectively. As the values were below the thresholds established to circumscribe prokaryotic species, namely 95 % for ANI values [20] and 70 % for dDDH [21], both genome indexes confirmed the classification of strains T6^T and T18^T as representing novel species [22].

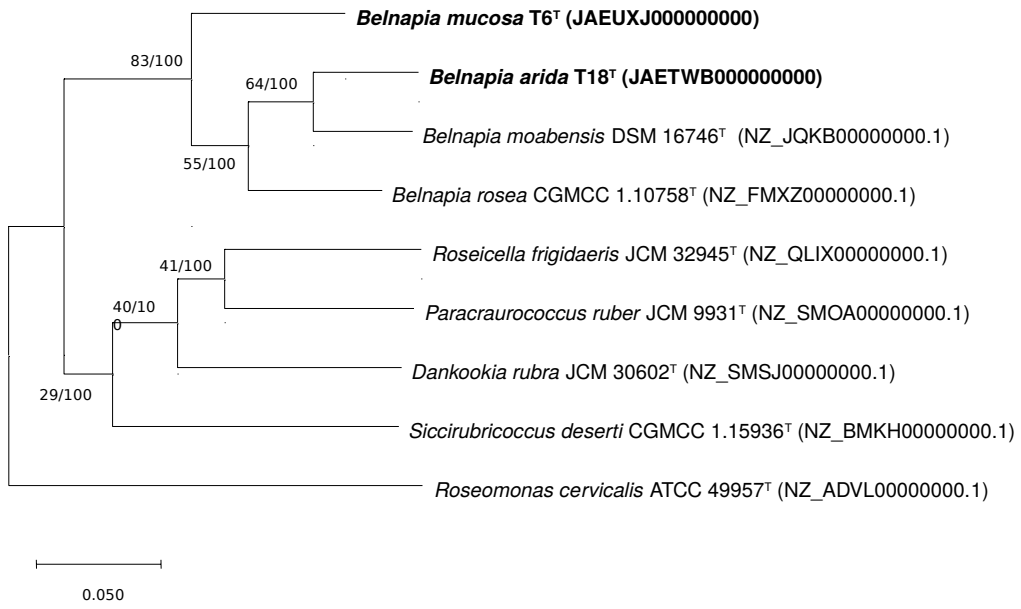

Fig. 2. Phylogenomic tree showing the relationships between strains T6^T, T18^T and other members of the family *Acetobacteraceae* based on a multiple alignment of a set of 92 gene sequences using the UBCG (v.3.0) [19] pipeline. Bootstrap analysis was carried out using 100 replicates. Gene support indices (maximum value; 92 genes) and percentage bootstrap values (maximum value; 100%) are given at the branch points. *Roseomonas cervicalis* ATCC 49957^T was used as an outgroup. Bar 0.050 substitutions per position.

The analysis of the genome of strains T6^T and T18^T allowed the prediction of their ability to synthesize phosphatidylglycerol, phosphatidylcholine and diphosphatidylglycerol on the basis of the presence of genes coding for phosphatidylglycerol phosphatase (EC 3.1.3.27), phosphatidylethanolamine *N*-methyltransferase (EC 2.1.1.17) and cardiolipin synthase (EC 2.7.8.-) respectively. This polar lipids' profile is in agreement with the polar lipid analyses results available for other species of the genus *Belnapia* [1–3]. Furthermore, their ability to synthesize phosphatidylethanolamine was predicted on the basis of the presence of the genes coding for phosphatidylserine synthase (EC 2.7.8.8) and phosphatidylserine decarboxylase (EC 4.1.1.65), which had been previously described for *B. soli* [3].

The major fatty acid for strains T6^T and T18^T was summed feature 8 ($C_{18:1}\omega7c/C_{18:1}\omega6c$) (41.4 and 51.5%, respectively). However, there were also high amounts of $C_{16:0}$ (15.7 and 12.9%, respectively for T6^T and T18^T), $C_{18:1}$ 2-OH (12.2 and 10.0%, respectively) and summed feature 3 ($C_{16:1}\omega7c/C_{16:1}\omega6c$) (10.3 and 12.2%, respectively) (Table 2). This is in accordance with the profiles obtained for the members of the genus *Belnapia*, which also showed high amounts of summed feature 8, summed feature 3, $C_{18:1}$ 2-OH and $C_{16:0}$, thus confirming the inclusion of both strains within the genus *Belnapia*.

The results of the phenotypic, chemotaxonomic, genomic and phylogenetic analyses confirm that strains T6^T and T18^T should be considered as each representing a novel species within the genus *Belnapia*, for which the names *Belnapia mucosa* sp. nov. and *Belnapia arida* sp. nov., respectively, are proposed.

DESCRIPTION OF *BELNAPIA MUCOSA* SP. NOV.

Belnapia mucosa (mu.co'sa. L. fem. adj. *mucosa*, mucous, slimy).

Colonies are circular, smooth, mucous, convex and pale-pink. Cells are Gram-reaction-negative, coccoid (0.8–1.0 µm) and non-motile. Growth occurs at 4–42 °C (optimum at 30 °C) and pH 5–9 (optimum 6–7), and it can tolerate up to 1.5 % (w/v) NaCl (optimum 0–1%). This species grows under aerobic and microaerophilic conditions, no growth is observed under anaerobic conditions. Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities are detected. Lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, *β*-galactosidase,

Table 2. Cellular fatty acid composition (percentages) of strains T6^T, T18^T and the members of the genus *Belnapia*

Strains: 1, T6^T; 2, T18^T; 3, *Belnapia moabensis* DSM 16746^T; 4, *Belnapia rosea* DSM 23312^T; 5, *Belnapia soli* DSM 28067^T. TR, trace (<1.0%); –, not detected.

	1	2	3	4	5
Saturated					
$C_{9:0}$	TR	TR	–	TR	TR
$C_{10:0}$	–	TR	–	TR	TR
$C_{14:0}$	1.9	TR	–	TR	1.2
$C_{16:0}$	15.7	12.9	13.2	11.3	16.8
$C_{18:0}$	3.6	1.8	1.9	1.2	2.6
$C_{19:0}$cyclo ω8*c*	–	–	–	1.8	2.3
Unsaturated					
$C_{16:1}$ω5*c*	–	TR	–	1.0	TR
$C_{18:1}$ω5*c*	–	–	–	TR	–
$C_{18:1}$ω9*c*	5.4	3.5	1.7	1.2	2.4
Hydroxylated					
$C_{12:0}$ 2-OH	6.4	3.9	2.0	1.6	2.3
$C_{16:0}$ 3-OH	–	TR	–	TR	1.4
$C_{18:1}$ 2-OH	12.2	10.0	8.2	9.0	10.7
$C_{18:0}$ 3-OH	1.4	–	–	–	TR
Summed Features*					
Summed feature 2	1.1	TR	TR	TR	1.3
Summed feature 3	10.3	12.2	10.2	19.2	16.5
Summed feature 8	41.4	51.5	62.0	50.6	40.4

*Summed features are fatty acids that cannot be resolved reliably from another fatty acid using the chromatographic conditions chosen. The MIDI system groups these fatty acids together as one feature with a single percentage of the total. Summed feature 2 corresponds to $C_{14:0}$ 3-OH/iso-$C_{16:1}$I/$C_{12:0}$ aldehyde; summed feature 3 corresponds to $C_{16:1}\omega7c/C_{16:1}\omega6c$; summed feature 8 corresponds to $C_{18:1}\omega7c/C_{18:1}\omega6c$.

β-glucuronidase, α-glucosidase, *β*-glucosidase, *N*-acetyl-*β*-glucosaminidase, α-mannosidase, α-fucosidase, nitrate reduction, indole production, glucose fermentation, arginine dihydrolysis, urease, aesculin hydrolysis and gelatinase are not detected. According to API 20 NE test kits, this species is weakly positive for the assimilation of potassium gluconate, adipic acid and malic acid and negative for the assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, maltose, capric acid, trisodium citrate and phenylacetic acid. According to BIOLOG GENIII MicroPlates, this species is positive for the utilization of L-galactonic acid lactone, *β*-hydroxy-DL-butyric acid, D-glucuronic acid, glucuronamide and acetoacetic acid; and negative for raffinose, α-D-glucose, D-sorbitol, gelatin, pectin, *p*-hydroxy-phenylacetic acid, Tween 40, dextrin, lactose, D-mannose, D-mannitol, glycyl-L-proline, D-galacturonic acid, methyl pyruvate, γ-aminobutyric acid, maltose, melibiose, D-fructose, D-arabitol, L-alanine, D-lactic acid methyl ester, α-hydroxybutyric acid, trehalose, methyl *β*-D-glucoside, D-galactose, *myo*-inositol, L-arginine, D-gluconic acid, L-lactic acid, cellobiose, D-salicin, 3-methyl-D-glucoside, glycerol, L-aspartic acid, citric acid, α-ketobutyric acid, gentiobiose, *N*-acetyl-D-glucosamine, D-fucose, D-glucose-6-phosphate, L-glutamic acid, α-ketoglutaric acid, sucrose, *N*-acetyl-*β*-D-mannosamine, L-fucose, D-fructose-6-phosphate, L-histidine, mucic acid, D-malic acid, propionic acid, turanose, *N*-acetyl-D-galactosamine, L-rhamnose, D-aspartic acid, L-pyroglutamic acid, quinic acid, L-malic acid, acetic acid, stachyose, *N*-acetyl neuraminic acid, inosine, D-serine, L-serine, D-saccharic acid, bromo-succinic acid and formic acid. The major fatty acids are summed feature 8 ($C_{18:1}\omega7c/C_{18:1}\omega6c$), $C_{16:0}$, $C_{18:1}$ 2-OH and summed feature 3 ($C_{16:1}\omega7c/C1_{6:1}\omega6c$).

The type strain T6^T (CECT 30228^T=DSM 112073^T) was first isolated from the Tabernas Desert in Almería (Spain) from a biocrust sample. The DNA G+C content of the type strain is 69.80%. The DDBJ/ENA/GenBank accession number for the 16S rRNA gene sequence is MW960268 and the genome accession number is JAEUXJ000000000.

DESCRIPTION OF *BELNAPIA ARIDA* SP. NOV.

Belnapia arida (a'ri.da. L. fem. adj. *arida*, dry, referring to the isolation of the strain from an arid soil)

Colonies are circular, smooth, mucous, convex and pink. Cells are Gram-reaction-negative, coccoid-shaped (0.8–1.0 µm) and non-motile. Growth occurs at 4–40 °C (optimum at 30 °C) and pH 5–9 (optimum 6–7), and it can tolerate up to 1.5% (w/v) NaCl (optimum 0–1%). This species grows under aerobic and microaerophilic conditions, no growth is observed under anaerobic conditions. Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and urease activities are detected. Lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, *β*-galactosidase, *β*-glucuronidase, α-glucosidase, *β*-glucosidase, *N*-acetyl-*β*-glucosaminidase, α-mannosidase, α-fucosidase, nitrate reduction, indole production, glucose fermentation, arginine dihydrolysis, aesculin hydrolysis and gelatinase are not detected. According to API 20 NE test kits, this species is negative for the assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid. According to BIOLOG GENIII MicroPlates, this species is positive for the utilization of D-gluconic acid, *β*-hydroxy-DL-butyric acid, L-pyroglutamic acid and L-malic acid; and negative for the utilization of raffinose, α-D-glucose, D-sorbitol, gelatin, pectin, *p*-hydroxyphenylacetic acid, Tween 40,

dextrin, lactose, D-mannose, D-mannitol, glycyl-L-proline, D-galacturonic acid, methyl pyruvate, γ-aminobutyric acid, maltose, melibiose, D-fructose, D-arabitol, L-alanine, L-galactonic acid lactone, D-lactic acid methyl ester, α-hydroxybutyric acid, trehalose, methyl *β*-D-glucoside, D-galactose, *myo*-inositol, L-arginine, L-lactic acid, cellobiose, D-salicin, 3-methyl-D-glucoside, glycerol, L-aspartic acid, D-glucuronic acid, citric acid, α-ketobutyric acid, gentiobiose, *N*-acetyl-D-glucosamine, D-fucose, D-glucose-6-phosphate, L-glutamic acid, glucuronamide, α-ketoglutaric acid, acetoacetic acid, sucrose, *N*-acetyl-*β*-D-mannosamine, L-fucose, D-fructose-6-phosphate, L-histidine, mucic acid, D-malic acid, propionic acid, turanose, *N*-acetyl-D-galactosamine, L-rhamnose, D-aspartic acid, quinic acid, acetic acid, stachyose, *N*-acetyl neuraminic acid, inosine, D-serine, L-serine, D-saccharic acid, bromosuccinic acid and formic acid. The major fatty acids are summed feature 8 ($C_{18:1}\omega7c/C_{18:1}\omega6c$), $C_{16:0}$, summed feature 3 ($C_{16:1}\omega7c/C_{16:1}\omega6c$) and $C_{18:1}$ 2-OH.

The type strain T18^T (CECT 30229^T=DSM 112074^T) was first isolated from the Tabernas Desert in Almería (Spain) from a biocrust sample. The DNA G+C content of the type strain is 68.96%. The DDBJ/ENA/GenBank accession number for the 16S rRNA gene sequence is MW960269 and the genome accession number is JAETWB000000000.

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Author contributions
E.M.M., sampling, experimental procedures, bioinformatic analysis, writing and approving the manuscript. À.V.V., experimental procedures, bioinformatic analysis, writing and approving the manuscript. L.S., experimental procedures, bioinformatic analysis, writing and approving the manuscript. A.C., experimental procedures, bioinformatic analysis, writing and approving the manuscript. J. Pascual, supervising experimental work, bioinformatic analysis, writing and approving the manuscript. J. Peretó, writing and approving the manuscript. M.P., conceived the work, sampling, writing and approving the manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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Appendix B: Supplementary material

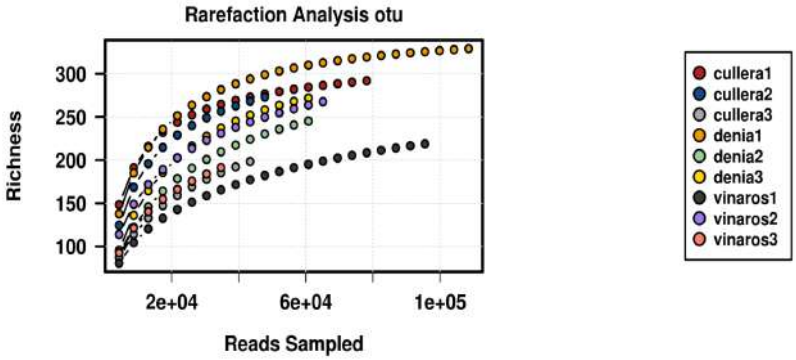


Figure I.S1. Rarefaction curve at OTU level.

Table I.S1. Top 30 most significant genera and P-values for the One-Way ANOVA statistical analysis of their distributions among the three sampled locations. Global P-values and P-values for the comparison by pairs is shown. Significant results are marked by an asterisk.

TAXA	P labelA	P (Tukeys) Denia-Cullera	P (Tukeys) Vinaros-Cullera	P (Tukeys) Vinaros-Denia
Flexithrix	0.0000037*	0.68	0.0000054*	0.0000075*
Unclassified.Saprospiraceae	0.000026*	0.38	0.000034*	0.000068*
Rubrobacter	0.0011*	0.083	0.0096*	0.00091*
Rhodovulum	0.0012*	1	0.0021*	0.0021*
Rubricoccus	0.0018*	0.0014*	0.057	0.026*
Halalkalicoccus	0.0039*	0.0056*	0.0076*	0.95
Acaryochloris	0.0039*	0.0065*	0.0065*	1
Unclassified.Flammeovirgaceae	0.006*	0.22	0.0051*	0.04*
Staphylococcus	0.0066*	0.48	0.0065*	0.024*
Rubidimonas	0.0075*	0.58	0.0078*	0.024*
Unclassified.Erythrobacteraceae	0.0085*	0.011*	0.018*	0.9
Unclassified.Acidimicrobiales	0.011*	0.68	0.012*	0.031*
Pseudoruegeria	0.011*	0.77	0.013*	0.028*
Oceanicella	0.011*	0.55	0.011*	0.038*
Unclassified.Gammaproteobacteria	0.014*	0.013*	0.44	0.058
Unclassified.Alphaproteobacteria	0.019*	0.052	0.02*	0.71
Unclassified.Cohaesibacteraceae	0.021*	0.021*	0.062*	0.66
Rivularia	0.026*	1	0.04*	0.038*
Unclassified.TM71	0.027*	0.11*	0.48	0.024*
Glaciecola	0.027*	0.11*	0.48	0.024*
Alteromonas	0.028*	0.041*	1	0.041*
Tunicatimonas	0.031*	0.68	0.031*	0.09
Maritimibacter	0.031*	0.68	0.031*	0.09
Unclassified.Rhodobacteraceae	0.037*	1	0.058	0.052
Synechococcus	0.037*	0.27	0.27	0.031*
Unclassified.WD2101	0.039*	1	0.06	0.054
Unclassified.Chroococcales	0.045*	0.063	0.068	1
Lewinella	0.052	1	0.073	0.075
Unclassified.Trueperaceae	0.066	0.29	0.45	0.056
Anaerospora	0.068	0.16	0.069	0.81

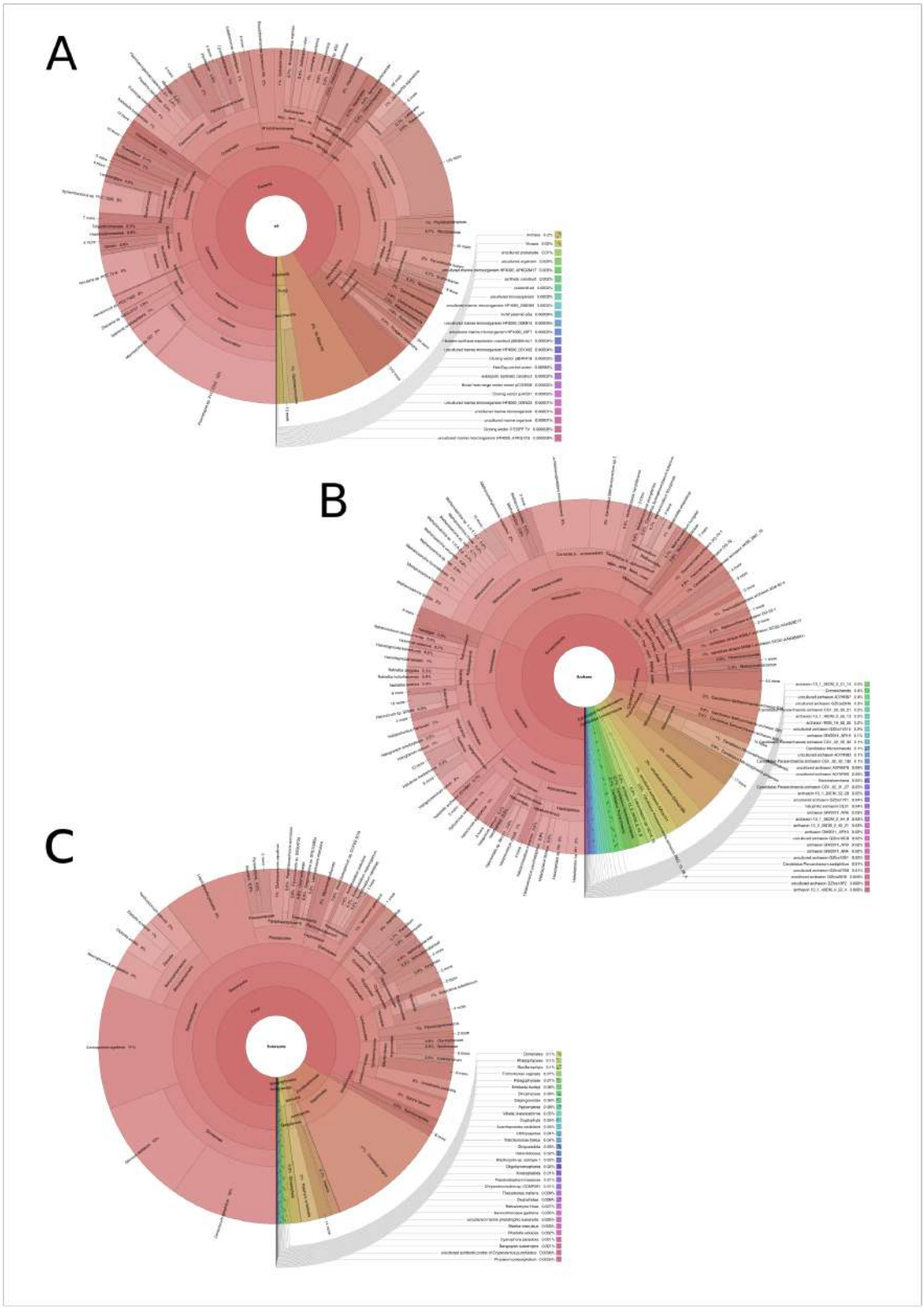


Figure I.S2. Main bacterial (A), archaeal (B) and eukaryotic (C) groups identified in the sample obtained from Vinaròs and analysed through metagenomics sequencing.

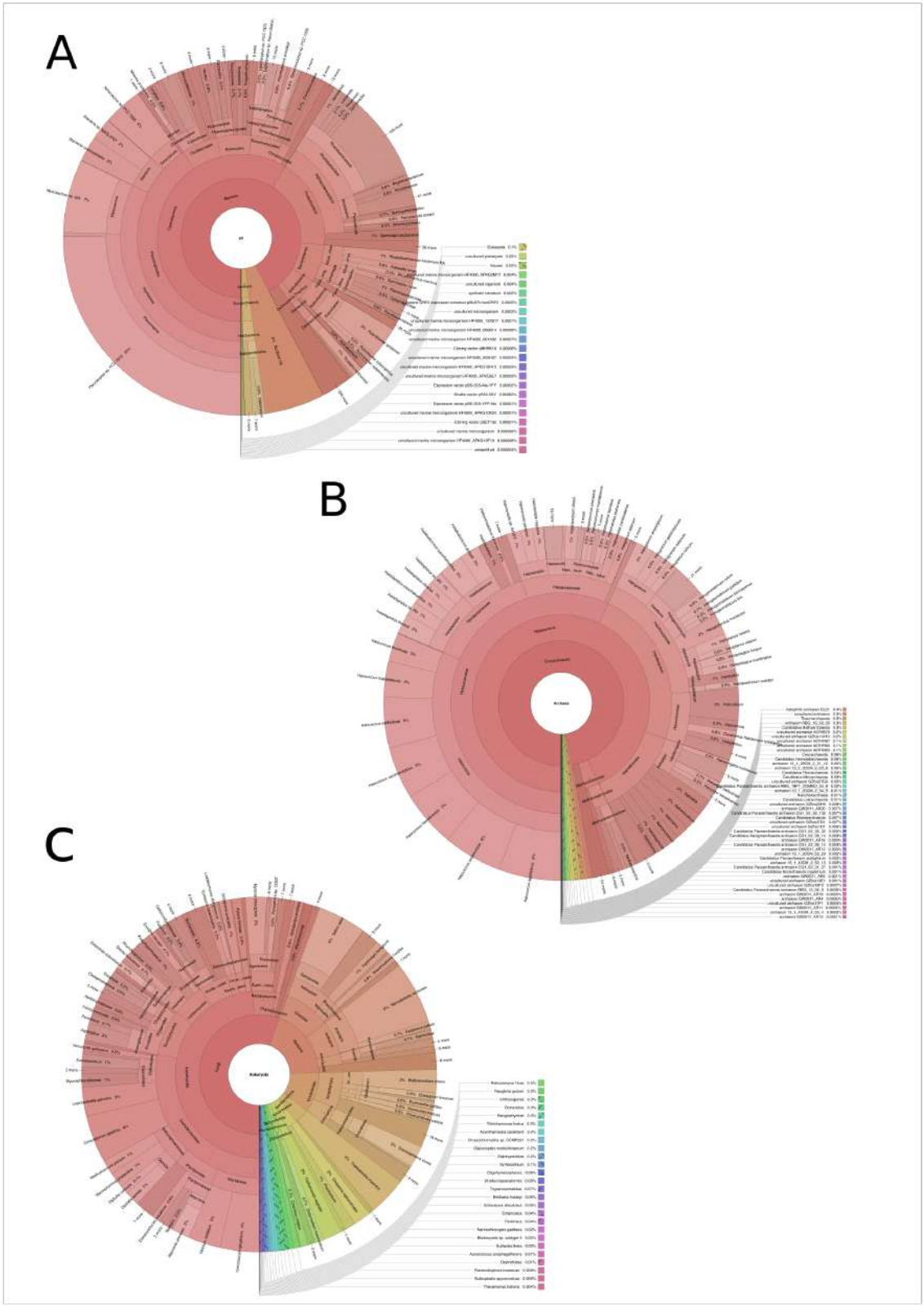


Figure I.S3. Main bacterial (A), archaeal (B) and eukaryotic (C) groups identified in the sample obtained from Cullera and analysed through metagenomics sequencing.

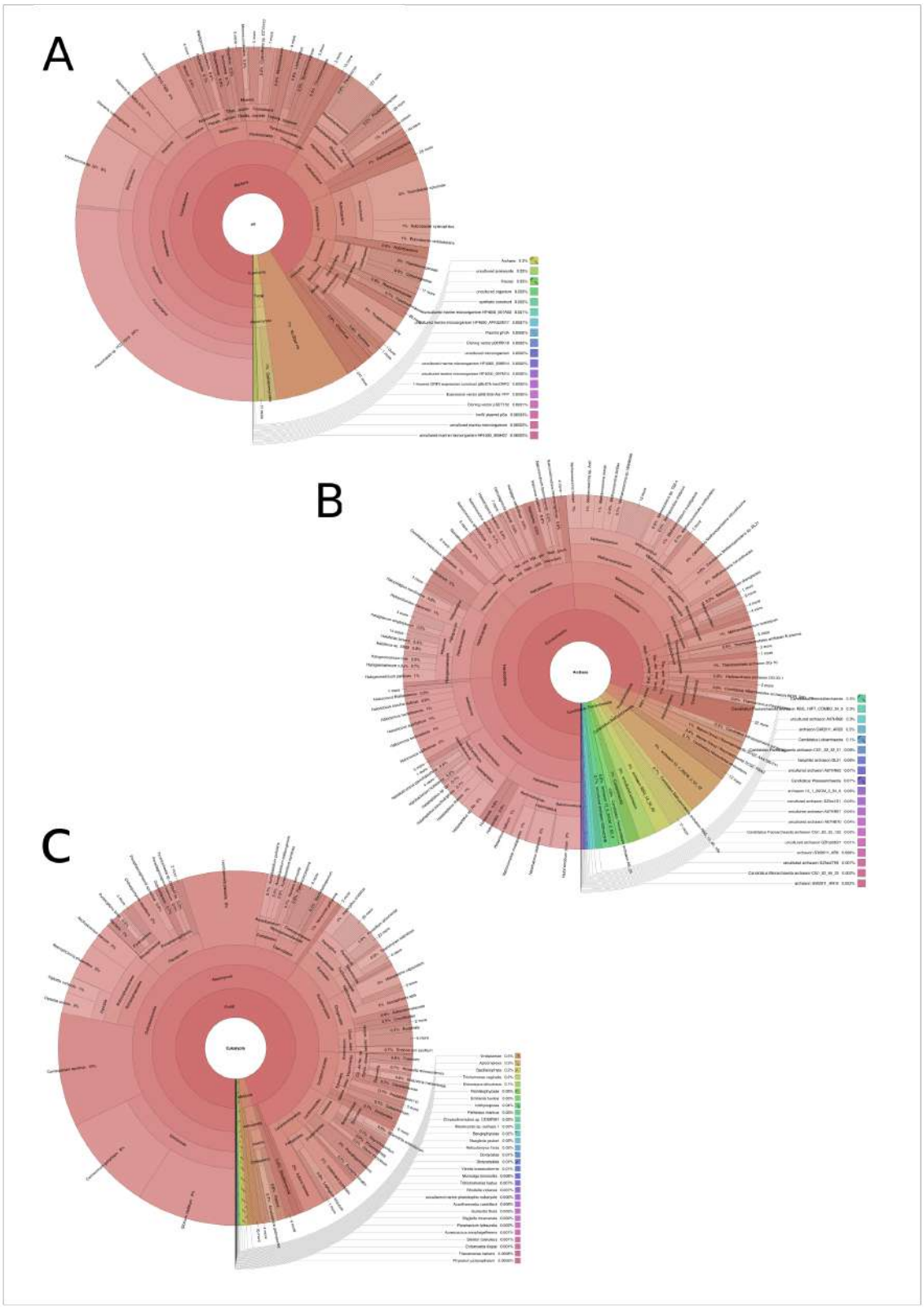


Figure I.S4. Main bacterial (A), archaeal (B) and eukaryotic (C) groups identified in the sample obtained from Dénia and analysed through metagenomics sequencing.

Table I.S2. List of the strains identified in the collection, with the closest type strain, accession number, ID percentage and the GenBank accession number for the 16S rRNA sequences. The identification code of the strains corresponds to the location from which it was isolated (V: Vinaròs, C: Cullera, D: Dénia), the sample type (R: rock, M: marine water) and a number.

STRAIN	CLOSEST TYPE STRAIN	ACCESSION NUMBER	ID (%)	GENBANK ACC. NUMBER
CM1	*Bacillus oleronius*	X82492	97,30	MN209966
CR2	*Halobacillus trueperi*	AJ310149	98,31	MN209967
CR3	*Halobacillus faecis*	AJ310149	98,66	MN209968
CR4	*Halobacillus trueperi*	AJ310149	97,69	MN209969
CR5	*Bacillus oleronius*	X82492	97,36	MN209970
CR6	*Halobacillus trueperi*	AJ310149	98,30	MN209971
CR7	*Halobacillus faecis*	AB243865	98,54	MN209972
CR8	*Bacillus oleronius*	X82492	97,30	MN209973
CR9	*Halobacillus trueperi*	AJ310149	98,31	MN209974
CR10	*Micrococcus luteus*	CP001628	99,77	MN209975
CR11	*Halobacillus trueperi*	AJ310149	98,30	MN209976
CR12	*Halobacillus trueperi*	AJ310149	98,30	MN209977
CR13	*Halobacillus trueperi*	AJ310149	98,31	MN209978
CR14	*Halobacillus trueperi*	AJ310149	98,31	MN209979
CR16	*Halobacillus trueperi*	AJ310149	98,30	MN209980
CR17	*Virgibacillus halodenitrificans*	AY543169	99,58	MN209981
CR18	*Halobacillus trueperi*	AJ310149	98,31	MN209982
CR20	*Bacillus oleronius*	X82492	97,32	MN209983
CR22	*Virgibacillus halodenitrificans*	AY543169	99,37	MN209984
CR23	*Halobacillus trueperi*	AJ310149	98,30	MN209985
CR24	*Halobacillus trueperi*	AJ310149	98,31	MN209986
CR26	*Halobacillus trueperi*	AJ310149	98,30	MN209987
CR27	*Halobacillus trueperi*	AJ310149	98,31	MN209988
CR28	*Virgibacillus halodenitrificans*	AY543169	100,00	MN209989
CR31	*Bacillus oleronius*	X82492	97,31	MN209990
CR32	*Bacillus licheniformis*	ATCC 14580	97,39	MN209991
CR33	*Bacillus marisflavi*	LGUE01000011	97,32	MN209992
CR34	*Bacillus oleronius*	X82492	97,09	MN209993
CR35	*Bacillus licheniformis*	ATCC 14580	98,08	MN209994
CR37	*Bacillus marisflavi*	LGUE01000011	100,00	MN209995
CR39	*Halobacillus trueperi*	AJ310149	98,30	MN209996
CR41	*Halobacillus trueperi*	DSM 10404	98,31	MN209997
CR43	*Bacillus oleronius*	X82492	97,31	MN209998
CR45	*Bacillus licheniformis*	AE017333	93,20	MN209999
CR47	*Bacillus oleronius*	X82493	97,31	MN210000
CM48	*Bacillus marisflavi*	LGUE01000011	96,99	MN210001
CR55	*Bacillus marisflavi*	LGUE01000011	97,80	MN210002
CR67	*Bacillus oleronius*	X82492	97,32	MN210003
DM1	*Staphylococcus pasteuri*	AF041361	100,00	MN210004
DR11	*Bacillus salsus*	A24	99,61	MN210005
DM15	*Vibrio tubiashii*	CP009354	100,00	MN210006
VR1	*Bacillus altitudinis*	ASJC011000029	100,00	MN210007
VR2	*Micrococcus luteus*	CP001628	99,35	MN210008
VR3	*Micrococcus antarcticus*	AJ005932	99,30	MN210009

Chapter 2

Table II.S1. List of coincident genera comparing all the six possible media.

MEDIA	N.	GENERA
TSA 1X; TSA 0.1X; TSA 0.01X; R2A 1X; R2A 0.1X; R2A 0.01X	4	*Arthrobacter, Homoserinibacter, Microbacterium, Not identified*
TSA 1X; TSA 0.1X; TSA 0.01X; R2A 1X; R2A 0.01X	1	*Kocuria*
TSA 1X; TSA 0.1X; TSA 0.01X; R2A 0.1X; R2A 0.01X	1	*Kineococcus*
TSA 1X; TSA 0.01X; R2A 1X; R2A 0.1X; R2A 0.01X	1	*Mycolicibacterium*
TSA 1X; TSA 0.1X; TSA 0.01X; R2A 1X	5	*Lentzea, Sphingomonas, Roseomonas, Belnapia, Ramlibacter*
TSA 1X; TSA 0.1X; R2A 1X; R2A 0.1X	1	*Skermanella*
TSA 1X; TSA 0.01X; R2A 1X	1	*Modestobacter*
TSA 0.1X; TSA 0.01X; R2A 1X; R2A 0.1X	1	*Streptomyces*
TSA 0.1X; R2A 1X; R2A 0.01X	1	*Methylobacterium*
TSA 1X; TSA 0.01X	1	*Planctomonas*
TSA 0.1X; TSA 0.01X	1	*Microvirga*
TSA 0.1X; R2A 1X	3	*Nocardioides, Geodermatophilus, Umezawaea*
TSA 0.01X; R2A 1X	1	*Kribbella*
TSA 0.01X; R2A 0.01X	1	*Actinoplanes*
R2A 1X; R2A 0.1X	1	*Massilia*
TSA 1X	2	*Micrococcus, Staphylococcus*
TSA 0.1X	4	*Variovorax, Noviherbaspirillum, Paenarthrobacter, Amycolatopsis*
TSA 0.01X	2	*Enterovirga, New genus*
R2A 1X	4	*Agrococcus, Blastococcus, Caulobacter, Azospirillum*
R2A 0.1X	2	*Herbaspirillum, Bacillus*
R2A 0.01X	1	*Flaviflagellibacter*

Table II.S2. List of genera in each biocrust sample in geolocation 1. NID means 'Not identified'. Abundance is expressed as the absolute number of strains isolated in pure culture.

GEOLOCATION	SAMPLE	GENERA	ABUNDANCE
1	1.1.1	*Arthrobacter*	6
		Kocuria	4
		NID	3
		Nocardioides	3
		Kineococcus	3
		Roseomonas	2
		Microvirga	2
		Belnapia	2
		Methylobacterium	2
		NEW GENUS	1
		Geodermatophilus	1
		Paenarthrobacter	1
		Planctomonas	1
		Homoserinibacter	1
		Streptomyces	1
		Modestobacter	1
		Blastococcus	1
	1.2.1	*Arthrobacter*	4
		Geodermatophilus	3
		Mycolicibacterium	2
		Ramlibacter	1
		Blastococcus	1
		Agrococcus	1
		Sphingomonas	1
		Skermanella	1
		Kineococcus	1
		Streptomyces	1
		Kocuria	1
		Kribbella	1
	1.3.1	*Skermanella*	4
		Sphingomonas	4
		Arthrobacter	3
		Planctomonas	2
		Mycolicibacterium	2

GEOLOCATION	SAMPLE	GENERA	ABUNDANCE
1	1.3.1	*Lentzea*	2
		Kocuria	2
		Variovorax	1
		Homoserinibacter	1
		NID	1
		Belnapia	1
		Herbaspirillum	1
		Massilia	1
		Kineococcus	1
		Microvirga	1
		Blastococcus	1
		Enterovirga	1
	1.4.1	*Arthrobacter*	21
		Homoserinibacter	8
		Kineococcus	5
		NID	5
		Lentzea	4
		Mycolicibacterium	3
		Nocardioides	3
		Planctomonas	2
		Sphingomonas	2
		Methylobacterium	2
		Microbacterium	2
		Streptomyces	1
		Massilia	1
		Roseomonas	1
		Flaviflagellibacter	1
		Modestobacter	1
		Skermanella	1
		Amycolatopsis	1
		Staphylococcus	1
		Belnapia	1
		Actinoplanes	1
		Kocuria	1
		Caulobacter	1
		Azospirillum	1

Table II.S3. List of genera in each biocrust sample in geolocation 2. NID means 'Not identified'. Abundance is expressed as the absolute number of strains isolated in pure culture.

GEOLOCATION	SAMPLE	GENERA	ABUNDANCE
2	2.1.1	*Arthrobacter*	21
		Belnapia	3
		Roseomonas	3
		NID	3
		Lentzea	2
		Umezawaea	2
		Sphingomonas	1
		Bacillus	1
		Massilia	1
		Microbacterium	1
		Blastococcus	1
		Mycolicibacterium	1
		Planctomonas	1
		Methylobacterium	1
	2.5.1	*Streptomyces*	2
		Kocuria	1
		Blastococcus	1
		Skermanella	1
		Sphingomonas	1
		Methylobacterium	1
		Kribbella	1
		Modestobacter	1

Table II.S4. List of genera in each biocrust sample in geolocation 3. NID means 'Not identified'. Abundance is expressed as the absolute number of strains isolated in pure culture.

GEOLOCATION	SAMPLE	GENERA	ABUNDANCE
3	8.1.1	*Arthrobacter*	4
		NID	2
		Roseomonas	2
		Noviherbaspirillum	1
		Belnapia	1
		Kribbella	1
		Modestobacter	1
		Microbacterium	1
	8.3.1	*Microbacterium*	8
		Mycolicibacterium	6
		Ramlibacter	6
		NID	4
		Arthrobacter	3
		Roseomonas	3
		Nocardioides	1
		Kocuria	1
		Sphingomonas	1
		Belnapia	1
		Skermanella	1
		Micrococcus	1
		Actinoplanes	1
		Modestobacter	1

Table II.S5. NCBI Blast results. The 16S rRNA sequences of the isolates were blasted against the NCBI 'non-redundant nucleotide collection' database optimised for highly similar sequences. The isolates that were not identified through molecular tools are classified as 'Not identified'. 'NA' means 'Not Available'.

STRAIN	CLOSEST RELATIVE	ACC. NUMBER	16S rRNA (%)	Origin
T1	Kocuria sp. MI-46a	DQ180950.1	99,54	Kartchner Caverns, Arizona
T2	Uncultured bacterium gene for 16S rNA, clone PEKCLN052	LC026857.1	99,33	Aerosol
T3	Arthrobacter agilis strain GE2	MT749783.1	99,56	Deteriorated Lede stone with gypsum crust
T4	Microbacteriaceae bacterium strain 13S1-3	MH287062.1	99,00	NA
T6	Alpha proteobacterium P12	FM211707.1	98,54	NA
T7	Arthrobacter sp. AA25	DQ224399.1	100,00	Radionuclide- and metal-contaminated subsurface soils
T8	Nocardioides sp. Strain zg-1308T	MT002689.1	99,52	Respiratory tract of Marmot
T9	Uncultured Balneimonas sp. clone NLS4.24	HQ397222.1	99,16	Soil
T10	Kocuria rosea strain ATCC 186	CP035103.1	99,25	NA
T11	Not identified			
T12	Uncultured bacterium clone SY201307-17	KX508700.1	99,46	Rainwater
T13	Kineococcus-like bacterium AS3381	AF060691.1	98,30	Mojave Desert
T14	Uncultured bacterium clone NTS003Fastc5_10758	JQ377241.2	98,71	FACE soil sample
T15	Uncultured bacterium clone 12A-9	DQ906897.1	99,72	Subsurface soil, Oman
T16	Roseomonas gilardii strain E9464	AY150051.1	94,71	
T17	Arthrobacter agilis strain TP-Snow-C64	KC986994.1	97,62	Snowpack from the Tibetan Plateau, China
T18	Belnapia moabensis strain TF4	KC503663.1	98,80	Tibet soil
T20	Kocuria dechangensis strain NEAU-ST5-33	NR_137239.1	98,71	Saline and alkaline soils
T22	Streptomyces sp. strain D15	MT450477.1	99,56	NA
T23	Uncultured bacterium clone SY201307-28	KX508711.1	99,22	Rainwater
T26	Lentzea sp. ML457-mF8	AB838595.1	99,77	Soil
T27	Arthrobacter agilis strain y5	JF309339.1	100,00	Soil sample from corn field
T28	Uncultured bacterium clone JSC7-58	DQ532203.1	99,49	Johnson Space Center, cleanroom
T29	Curtobacterium sp. DoB77	JQ359111.1	99,78	Endophytic of Dendrobium officinale
T30	Uncultured bacterium clone 12TCLN387	AB637313.1	98,97	Soil
T31	Herbiconiux sp. Strain YIM 130850	KX502894.1	100,00	Lichen
T32	Lentzea californiensis JCM 11305	LC214996.1	99,32	Soil
T34	Variovorax sp. Strain C1_RP_3	MT354026.1	100,00	NA
T35	Uncultured bacterium clone SY201307-28	KX508711.1	99,36	Rainwater
T36	Microvirga guangxiensis PDS_PXH_57	KY951358.1	100,00	PXH lake water
T37	Uncultured bacterium clone SY201307-28	KX508711.1	99,36	Rainwater
T39	Arthrobacter agilis strain TP-Snow-C64	KC986994.1	99,35	Snowpack from the Tibetan Plateau, China
T40	Uncultured bacterium clone TC1A_R_061	KC889229.1	99,52	Phyllosphere of Trichilia clausenii
T41	Not identified			
T42	Uncultured bacterium clone SY201307-17	KX508700.1	99,79	Rainwater
T43	Uncultured bacterium clone EDW07B003_40	HM066514.1	97,96	Texas state well #DX 68-23-618
T44	Psychroglaciecola sp. strain GI_Sw_6a_1	MG232359.1	99,65	Inner surface of termite's tapetum
T45	Mycolicibacterium arabiense JCM 18538	AP022593.1	99,55	NA
T46	Alpha proteobacterium BIWA07	LC217394.1	98,02	Surface water (Lake Biwa)
T47	Alpha proteobacterium P12	FM211707.1	98,33	NA
T48	Mycolicibacterium arabiense JCM 18538	AP022593.1	100,00	NA
T49	Kineococcus-like bacterium AS3380	AF060690.1	98,02	Mojave Desert
T50	Sphingomonas sp. GP0503	KF830230.1	99,74	Siziwang desert
T51	Uncultured bacterium clone NT54a10_20490	JQ378734.2	99,56	FACE soil sample
T52	Skermanella aerolata strain CB-282242	JX841089.1	99,33	Soil
T53	Kineosporia sp. CC-YMW-2	EU008824.1	98,59	Soil
T54	Arthrobacter sp. strain SLN-3	MK621196.1	99,77	Soil biocrust
T55	Kineococcus-like bacterium AS3381	AF060691.1	99,00	Mojave Desert
T56	Uncultured bacterium clone SY201307-17	KX508700.1	99,33	Rainwater
T57	Uncultured bacterium clone NT20f5_21705	JQ377141.2	97,07	FACE soil sample
T58	Uncultured Curtobacterium sp. clone Plot4-2F07	EU449556.1	98,88	Agricultural soil
T59	Staphylococcus pasteuri strain ZJH	MT071663.1	100,00	Fish pond
T61	Not identified			
T62	Uncultured bacterium clone ncd1997a07c1	JF173498.1	100,00	Skin, volar forearm
T64	Arthrobacter sp. strain SLN-3	MK621196.1	99,32	Biocrust
T65	Nocardioides sp. Strain zg-1308T	MT002689.1	99,93	Respiratory tract of Marmot
T68	Uncultured bacterium clone NT20f5_21705	JQ377141.2	96,60	FACE soil sample
T71	Herbiconiux sp. Strain YIM 130850	KX502894.1	100,00	Lichen
T74	Arthrobacter agilis strain TP-Snow-C64	KC986994.1	99,56	Snowpack from the Tibetan Plateau, China
T75	Arthrobacter sp. strain SLN-3	MK621196.1	100,00	Biocrust
T76	Amycolatopsis magusensis strain KT2025	NR_109060.1	99,54	Soil
T77	Uncultured Belnapia sp. clone f2SD-45	JF832329.1	99,76	Mont Blanc snow with Saharan dust layer (dust event)
T78	Microbacterium sp. strain CB2015-224-DE_0511	MH512461.1	99,55	Skin of fire salamander
T79	Kineococcus-like bacterium AS3388	AF060671.1	100,00	Mojave Desert
T81	Herbiconiux sp. Strain YIM 130850	KX502894.1	99,78	Lichen
T83	Actinobacteria bacterium P1_50_5AA2	KT906993.1	99,57	Monterey Bay, Santa Cruz Wharf surface
T84	Unidentified microorganism clone xplvp148r2a2008	MG270734.1	99,78	Air
T85	Uncultured bacterium clone 12TCLN354	AB637280.1	99,59	Soil
T86	Arthrobacter agilis strain 234	EU730943.1	99,57	Water purifier
T87	Uncultured bacterium clone SY201307-17	KX508700.1	99,86	Rainwater
T88	Lysinimonas sp. LM-2018 strain zZDQ473536zZ	MG934577.1	97,76	Cattail root soil of a tailings
T89	Bacterium ZSGR24	KC577574.1	99,21	Snow pit drilled from Zangser Kangri
T90	Kineococcus-like bacterium AS3380	AF060690.1	98,32	Mojave Desert
T91	Uncultured bacterium clone 126	GU225974.1	99,58	Rape phyllosphere
T92	Actinoplanes sp. partial 16S rRNA gene, strain 19	AJ488563.1	98,97	Soil
T93	Lentzea violacea strain 173540	EU593726.1	100,00	Xinjiang
T94	Arthrobacter sp. strain J915	MN599067.1	100,00	NA
T95	Lentzea pudingi strain DHS C021	NR_159115.1	98,87	Weathered limestone sample in a karst area
T97	Uncultured bacterium clone NT20f5_21705	JQ377141.2	99,34	FACE soil sample
T99	Lentzea pudingi strain DHS C021	NR_159115.1	99,55	Limestone
T100	Uncultured bacterium clone ncm09g04c1	KF073781.1	99,07	Skin, nare
T102	Uncultured bacterium clone FL5Bd9_13612	JQ377694.2	99,34	FACE soil sample
T103	Not identified			
T104	Roseomonas sp. ZSGR23	KC577573.1	98,73	Snow pit drilled from Zangser Kangri
T105	Arthrobacter sp. strain J915	MN599067.1	100,00	NA
T106	Uncultured bacterium clone SY201307-17	KX508700.1	99,93	Rainwater
T107	Methylobacterium sp. strain PP-WC-2A-150	MN596064.1	99,76	Phyllosphere of Arrhenatherum elatius
T108	Arthrobacter sp. MDT2-14	JX949673.2	99,93	Glacier
T109	Not identified			
T110	Arthrobacter agilis strain y5	JF309339.1	99,84	Soil sample from corn field

T111	Umezawaea sp. strain Da 62-40.2	MN810952.1	99,63	Rhizosphere soil
T112	Arthrobacter agilis strain y24	KF306346.1	99,40	Ocean sediment
T113	Uncultured bacterium clone NT15h9_18090	JQ383812.2	99,75	FACE soil sample
T114	Arthrobacter sp. strain SLN-3	MK621196.1	99,66	Biocrust
T115	Arthrobacter sp. strain SLN-3	MK621196.1	99,73	Biocrust
T116	Uncultured bacterium clone NT42e11_17557	JQ384826.2	100,00	FACE soil sample
T117	Sphingomonas yunnanensis strain MJ4B-4B	KY412157.1	99,79	Surface of cave paintings
T119	Streptomyces vinaceus strain ATCC 27476	CP023692.1	100,00	Soil
T121	Uncultured bacterium clone 12TCLN372	AB637298.1	99,60	Soil
T123	Uncultured bacterium clone HL201306-56	KU515074.1	99,31	Rainwater
T125	Roseomonas nepalensis strain G-3-5	KX129819.1	98,60	Oil-contaminated soil
T126	Roseomonas sp. strain H01Y-146	MK493569.1	98,47	Algal culture
T127	Belnapia moabensis strain TF4	KC503663.1	98,95	Tibet soil
T128	Arthrobacter sp. LMR320	KU182826.1	99,08	Heavy metal multi-polluted soil
T129	Kribbella flavida strain DSM 17836	NR_074441.1	99,55	Soil from China
T130	Modestobacter sp. UAPS01112	KT936120.1	99,78	Rhizospheric, Cactus
T131	Not identified			
T132	Uncultured bacterium clone MSD18_C12	JQ357616.1	99,76	Detritusphere of decomposing residue
T133	Microbacterium sp. TW109C	KJ716588.1	99,78	Gut of Coptotermes formosanus Shiraki
T135	Uncultured bacterium	LR639600.1	99,34	Wastewater treatment system
T136	Microbacterium sp. RI53	DQ530148.1	99,56	Soybean rhizosphere
T137	Uncultured bacterium clone SY201307-28	KX508711.1	99,44	Rainwater
T138	Actinobacteria bacterium P1_50_5AA2	KT906993.1	99,79	Monterey Bay, Santa Cruz Wharf surface water
T139	Sphingomonas sp. LK11	CP013916.1	98,08	Leaves of Tephrosia apollinea
T140	Bacterium strain MTL8-13	MH151304.1	100,00	NA
T141	Not identified			
T142	Mycolicibacterium arabiense JCM 18538	AP022593.1	98,88	NA
T143	Uncultured bacterium clone MTDH201308-36	KX508940.1	98,38	Rainwater
T144	Mycolicibacterium arabiense JCM 18538	AP022593.1	100,00	NA
T145	Nocardioides sp. strain R-68482	KY386444.1	98,31	Soil
T147	Uncultured bacterium clone S1-29	EU015090.1	98,51	Membrane bioreactor suspension and biofilm
T148	Roseomonas sp. CPCC 101081	MN883396.1	97,30	Badain Jaran Desert
T149	Microbacterium sp. strain YIM 132057	MT071614.1	99,67	NA
T151	Not identified			
T153	Uncultured bacterium clone MTDH201308-36	KX508940.1	97,77	Rainwater
T154	Microbacterium sp. B150	HQ877782.1	99,78	Soil
T155	Actinobacteria bacterium P1_50_5AA2	KT906993.1	99,78	Monterey Bay, Santa Cruz Wharf surface water
T156	Actinobacteria bacterium P1_50_5AA2	KT906993.1	99,56	Monterey Bay, Santa Cruz Wharf surface water
T157	Kocuria assamensis partial 16S rRNA gene, isolate 184	LN623593.1	100,00	Gut Rhynchophorus ferrugineus
T158	Bacterium IAD-7	LC361402.1	98,65	Bark , Hacer palmatum
T159	Uncultured bacterium clone NT15h9_18090	JQ383812.2	99,52	FACE soil sample
T165	Arthrobacter sp. strain SLN-3	MK621196.1	100,00	Biocrust
T166	Arthrobacter sp. strain SLN-3	MK621196.1	99,32	Biocrust
T167	Arthrobacter sp. MDT2-14	JX949673.2	99,93	Glacier
T168	Uncultured bacterium clone MTDH201308-36	KX508940.1	98,38	Rainwater
T169	Geodermatophilus sp. lzl_geo	KF077881.1	100,00	Mangrove sediment
T170	Kineococcus-like bacterium AS3635	AF060694.1	98,77	Endemic to Mojave Desert
T171	Mycolicibacterium arabiense JCM 18538	AP022593.1	99,77	NA
T172	Sphingomonas xinjiangensis strain 10-1-84	NR_108386.1	98,16	Desert soil, Xinjiang
T173	Microbacteriaceae bacterium strain 13S1-3	MH287062.1	100,00	NA
T174	Arthrobacter pityocampae strain HS1-84	KX809756.1	99,12	Freshwater sediment
T175	Microbacteriaceae bacterium strain 13S1-3	MH287062.1	99,80	NA
T177	Arthrobacter pityocampae strain HS1-84	KX809756.1	99,53	Freshwater sediment
T178	Uncultured bacterium clone MTDH201308-36	KX508940.1	98,37	Rainwater
T179	Roseomonas sp. BZ31r	HQ588841.1	96,87	Oil and heavy metal-contaminated soil
T180	Kineococcus sp. strain G3S-R4-3-26	MT910594.1	98,83	Rhizospheric sand Durbantunggut Desert
T185	Herbiconiux ginsengi strain wged11	NR_043879.1	97,94	Gingsen root
T186	Streptomyces spiroverticillatus strain sj33	JX013967.1	99,32	Soil
T187	Uncultured bacterium clone NDB15	HM366537.1	98,89	Urbal aerosols
T188	Methylobacterium sp. strain PP-WC-2A-150	MN596064.1	98,96	Phyllosphere of Arrhenatherum elatius
T189	Modestobacter versicolor strain FMS-37	MK396600.1	99,11	NA
R1	Nocardioides sp. Strain zg-1308T	MT002689.1	99,1	Respiratory tract of Marmot
R2	Arthrobacter agilis strain TP-Snow-C64	KC986994.1	99,56	Snowpack from the Tibetan Plateau, China
R4	Nocardioides sp. Strain zg-1308T	MT002689.1	99,1	Respiratory tract of Marmot
R7	Not identified			
R8	Kineosporiaceae bacterium B12	MN493040.1	99,50	NASA, cleanroom floor
R9	Uncultured bacterium clone C:1850:Lera1:10	HQ285123.1	100	From compacted bentonite clay exposed to groundwater from 450 m depth of the Aespoe Hard Rock Laboratory, Sweden
R10	Uncultured bacterium partial 16S rRNA gene, clone U26	HG764510.1	98,66	Soil from wall paintings
R11	Not identified			
R13	Agrococcus sp. Marseille-P2731	LT558844.1	100	Permafrost
R14	Mycobacterium arabiense strain W13104	KJ676965.1	100	Saline environment
R15	Arthrobacter sp. strain SLN-3	MK621196.1	100	Biocrust
R16	Uncultured bacterium clone NTS001Fastd9_1275	JQ386551.2	99,75	FACE soil sample
R17	Uncultured bacterium clone MSFC_2M3I	DQ447789.1	99,28	Marshall Space Flight Center, REMS facility
R18	Uncultured bacterium clone SH201205-79	KX508347.1	99,78	rainwater
R19	Uncultured bacterium clone ncd2151c08c1	JF180749.1	97,03	Skin microbiome, children with dermatitis
R20	Lysinimonas sp. LM-2018 strain zZDQ473536zZ	MG934577.1	97,75	Cattail root soil of a tailing
R22	Streptomyces altiplanensis strain HST21	KX130868.2	98,77	Salar de Huasco, arid soil sample
R24	Mycolicibacterium arabiense JCM 18538	AP022593.1	99,55	Comprehensive subspecies identification of 175 nontuberculous mycobacteria species based on 7547 genomic profiles
R25	Uncultured bacterium clone HL201307-95	KU515212.1	99,72	Rainwater in Hulunber grassland, Inner Mongolia, China
R26	Lentzea albida strain S8-TSB-7	MN179971.1	99,77	Waste site
R30	Lentzea sp. YIM 65117	FJ214358.1	99,84	Medicinal plants of tropical rain forests in Xishuangbanna, China
R31	Arthrobacter agilis strain TP-Snow-C64	KC986994.1	99,72	Snowpack from the Tibetan Plateau, China
R32	Arthrobacter sp. strain SLN-3	MK621196.1	99,93	Biocrust
R34	Arthrobacter sp. strain SLN-3	MK621196.1	100	Biocrust
R36	Nocardioides alpinus strain Cr7-14	NR_108845.1	98,67	Glacier cryoconite
R37	Arthrobacter sp. strain SLN-3	MK621196.1	99,64	Biocrust
R38	Arthrobacter sp. strain SLN-3	MK621196.1	100	Biocrust
R39	Arthrobacter agilis strain TP-Snow-C64	KC986994.1	99,56	Snowpack from the Tibetan Plateau, China
R40	Lysinimonas sp. LM-2018 strain zZDQ473536zZ	MG934577.1	97,75	Cattail root soil of a tailing
R44	Mycobacterium manitobense	AY082001.2	99,72	Soft tissue
R45	Arthrobacter sp. strain SLN-3	MK621196.1	99,93	Biocrust
R47	Lysinimonas sp. LM-2018 strain zZDQ473536zZ	MG934577.1	97,95	Cattail root soil of a tailing
R48	Arthrobacter agilis strain OAct554	KJ812397.1	99,65	Soil
R50	Arthrobacter sp. strain SLN-3	MK621196.1	99,66	Biocrust
R51	Arthrobacter sp. strain SLN-3	MK621196.1	100	Biocrust
R52	Herbiconiux sp. Strain YIM 130850	KX502894.1	99,42	Lichen
R55	Uncultured bacterium clone SH201205-77	KX508345.1	100	Rainwater
R56	Not identified			

R57	Kineosporiaceae bacterium B12	MN493040.1	99,36	NASA, Cleanroom Floor
R59	Not identified			
R60	Uncultured bacterium clone B8-186	KF494629.1	98,45	Permafrost soil
R61	Uncultured bacterium clone OTU_373	MT526654.1	100	Larval gut fruit fly
R62	Not identified			
R64	Arthrobacter agilis strain TP-Snow-C64	KC986994.1	99,33	Snowpack from the Tibetan Plateau, China
R65	Arthrobacter sp. MDT2-14	JX949673.2	99,93	Glacier
R66	Arthrobacter sp. strain SLN-3	MK621196.1	99,45	Biocrust
R67	Bacterium 7(2011)	HQ728389.1	98,64	Phyllosphere
R68	Mycolicibacterium arabiense JCM 18538	AP022593.1	100	NA
R70	Arthrobacter sp. MDT2-14	JX949673.2	99,79	Glacier
R72	Bacillus subtilis strain HFBP08	MT538260.1	100	Phosphate mines in the Khouribga, Morocco
R76	Arthrobacter sp. MDT2-14	JX949673.2	99,53	Glacier
R77	Arthrobacter sp. strain AM3-2	MN968946.1	100	Stone monunent, China
R78	Arthrobacter sp. strain SLN-3	MK621196.1	99,65	Biocrust
R80	Uncultured bacterium clone BJ201110-24	KX507404.1	98,91	Rainwater
R81	Umezawaea tangerina strain 1w5	KF554225.1	100	Rock surface
R82	Paracraurococcus sp. strain jgi-1041375	MH547450.1	99,66	NA
R83	Bacterium XD-R1	KU603612.1	100	Alpine meadow soil
R84	Not identified			
R87	Skermanella aerolata strain QT22	MH844946.1	99,86	NA
R89	Kribbella lupini strain LU14	NR_042333.1	98,85	Roots of Lupinus angustifolius
R90	Skermanella aerolata strain QT22	MN888742.1	98,86	Compost
R91	Modestobacter versicolor strain DJG-10	MF101076.1	100	Salix gordejevii
R97	Arthrobacter sp. strain J915	MN599067.1	99,55	NA
R98	Not identified			
R99	Arthrobacter sp. MDT2-14	JX949673.2	99,52	Glacier
R103	Modestobacter sp. UAPS01112	KT936120.1	100	Rhizospheric from Neobuxbaumia
R105	Mycolicibacterium arabiense strain YIM 121001	NR_109734.2	99,55	Sand sample from marine shore
R106	Uncultured bacterium clone NT32c1_18525	JQ386400.2	98,4	FACE soil sample
R107	Uncultured bacterium clone S1-29	EU015090.1	98,43	Membrane bioreactor suspension and biofilm
R109	Microbacterium paraoxydans strain DSM 1920	MN175935.1	99,71	Wound
R110	Not identified			
R111	Mycolicibacterium arabiense JCM 18538	AP022593.1	99,77	NA
R112	Microbacterium sp. strain YIM 132057	MT071614.1	99,67	NA
R113	Mycolicibacterium arabiense JCM 18538	AP022593.1	100	NA
R114	Micromonosporaceae bacterium 232006	GU130120.1	98,05	Sediment
R115	Not identified			
R116	Geodermatophilus nigrescens strain YIM 75980	NR_109505.1	100	Soil
R117	Uncultured bacterium clone MP104-SW-b1	DQ088797.1	99,31	Crustal biotome
R119	Geodermatophilus sp. strain SYSU_D00696	MT527633.1	99,16	NA
R120	Kribbella sandramycini strain QT237	MT072122.1	99,4	Soil
R124	Herbiconiux sp. Strain YIM 130850	KX502894.1	99,53	Lichen
R125	Uncultured bacterium clone NT32e8_19828	JQ378163.2	100	FACE soil sample
R126	Not identified			
R128	Kocuria rhizophila strain LAEU9	MF925210.1	100	healthy control human duodenum biopsy
R129	Belnapia rosea strain ArASBr117	MH424892.1	99,71	Hospital
R131	Uncultured bacterium clone AN0C1CG05	JQ426248.1	99,4	Soil
R133	Uncultured bacterium clone KH-T7-179	KU930413.1	98,78	Tobacco soil
R136	Mycobacterium sp. SBA 21	HG941821.1	100	Rhizosphere
R137	Uncultured bacterium clone NTS001Powerg1_11092	JQ377975.2	100	FACE soil sample
R138	Geodermatophilus sp. NEAU-TX1	KC710966.1	99,55	Soil
R139	Uncultured bacterium partial 16S rRNA gene, clone W1SH10	LN880172.1	99,78	Wheat phyllosphere
R140	Blastococcus sp. C-1-31	KT583401.1	100	Associated to coral Baltic sea
R141	Kocuria sp. strain YKFH1121	MT393631.1	99	Red Sea, El Tor in the Gulf of Suez, Suez
R143	Nocardioides sp. strain R-68482	KY386444.1	97,85	Soil Antarctica
R144	Uncultured Caulobacter sp. clone NJFU_SLX-S126	KJ127991.1	96,84	Slime from paper machine
R146	Azospirillum sp. NCCP-699	LC193946.2	98,5	Desert soil, Arabia Saudi
R147	Microbacterium sp. strain SYSU_D01038	MT527696.1	99,02	NA
R151	Microbacterium sp. strain SYSU_D01038	MT527696.1	99,38	NA
R153	Blastococcus sp. C-1-31	KT583401.1	100	Associated to coral, Baltic sea
R154	Uncultured bacterium clone NT32g3_20100	JQ377991.2	99,97	FACE soil sample
R156	Kocuria rosea strain ATCC 186	CP035103.1	99,32	NA
R158	Microbacterium pumilum strain S_S_TSA_8	KC213957.1	99,77	Roach gut Rutilus rutilus
R159	Microbacterium sp. strain SYSU_D01038	MT527696.1	99,17	NA

Table III.S1: Carbon source utilization comparison using GENIII MicroPlates of strains T13^T, T90^T, R8^T and the type strains of closely related *Kineococcus* species. Strains: 1, T13^T;2, T90^T; 3, R8^T; 4, *Kineococcus radiotolerans* DSM 14245^T; 5, *Kineococcus aureolus* DSM 102158^T; 6, *Kineococcus aurantiacus* DSM 7487^T; 7, *Kineococcus gypseus* DSM 27627^T; 8, *Kineococcus mangrovi* NBRC 110933^T; 9, *Kineococcus gynurae* NBRC 103943^T. Data for reference strains were obtained in the present study. +, positive; -, negative. All strains are positive for D-turanose. All strains are negative for p-hydroxy-phenylacetic acid, N-acetyl neuraminic acid and α-hydroxy-butyric acid.

CHARACTERISTIC	1	2	3	4	5	6	7	8	9
D-Raffinose	+	+	-	+	+	-	-	+	-
α-D-Glucose	+	+	-	+	+	+	+	+	-
D-Sorbitol	+	+	-	+	+	+	-	+	-
Gelatin	-	+	-	-	+	+	-	+	-
Pectin	+	+	+	+	+	-	+	+	-
p-Hydroxy-phenylacetic acid	-	-	-	-	-	-	-	-	-
Tween 40	+	+	+	-	+	+	+	+	-
Dextrin	+	+	+	+	-	+	+	+	+
α-D-Lactose	+	+	-	+	-	-	+	+	-
D-Mannose	+	+	-	+	-	-	+	+	-
D-Mannitol	+	+	-	+	-	-	-	+	-
Glycyl-L-proline	-	+	-	-	-	-	-	-	-
D-Galacturonic acid	-	+	-	-	+	-	-	+	-
Methyl pyruvate	-	-	-	-	+	-	-	-	-
γ-Amino-butyric acid	-	+	-	-	-	-	-	-	-
D-Maltose	+	+	-	+	+	-	+	+	+
D-Melibiose	+	+	-	+	+	-	-	+	+
D-Fructose	+	+	+	+	+	-	+	+	-
D-Arabitol	-	+	+	+	+	-	-	+	-
L-Alanine	+	+	+	+	+	-	-	-	-
L-Galactonic acid lactone	-	+	-	-	-	-	-	-	-
D-Lactic acid methyl ester	-	+	-	-	-	-	-	-	-
α-Hydroxy-butyric acid	-	-	-	-	-	-	-	-	-
D-Trehalose	+	+	-	+	+	-	-	+	-
β-Methyl-D-glucoside	+	+	-	+	+	-	+	+	-
D-Galactose	+	+	+	+	+	-	+	+	-
myo-Inositol	+	+	-	+	+	-	-	+	-
L-Arginine	-	+	-	-	-	-	-	-	-
D-Gluconic acid	-	+	+	+	+	+	+	+	+
L-Lactic acid	-	-	+	-	+	-	-	-	-
β-Hydroxy-D,L-butyric acid	-	+	-	-	-	-	-	-	-
D-Cellobiose	+	+	+	+	+	+	+	+	-
D-Salicin	+	+	-	+	+	-	+	+	-
3-Methyl-D-glucoside	-	+	+	-	-	-	-	+	-
Glycerol	+	+	+	+	+	+	+	+	-
L-Aspartic acid	-	+	-	-	-	-	-	-	-
D-Glucuronic acid	-	+	+	-	+	-	-	-	-
Citric acid	-	+	-	-	-	-	-	-	-
α-Keto-butyric acid	-	+	-	+	-	-	-	-	-
Gentiobiose	+	+	+	+	+	-	+	+	-
N-acetyl-D-glucosamine	+	+	-	-	-	-	-	-	-
D-Fucose	-	+	-	+	-	-	+	-	-
D-Glucose-6-PO_4	-	+	-	+	-	+	-	-	+
L-Glutamic acid	-	+	-	+	+	-	-	+	-
Glucuronamide	+	+	+	-	-	+	+	-	-
α-Keto-glutaric acid	-	+	-	-	+	-	-	-	-
Acetoacetic acid	+	+	+	+	+	+	+	+	-
Sucrose	+	+	-	+	+	+	+	+	-
N-acetyl- β-D-mannosamine	-	+	-	-	-	-	-	-	-
L-Fucose	-	+	+	+	+	-	-	+	-
D-Fructose-6-PO_4	+	+	+	-	-	+	+	-	+
L-Histidine	-	+	-	-	-	-	-	-	-
Mucic acid	-	+	+	-	+	-	-	-	-
D-Malic acid	-	+	+	+	+	-	-	-	-
Propionic acid	-	+	-	-	+	-	-	-	-
D-Turanose	+	+	+	+	+	+	+	+	+
N-acetyl-D-galactosamine	-	+	-	-	-	-	-	-	-
L-Rhamnose	+	+	-	+	+	-	-	+	-
D-Aspartic acid	-	+	+	-	-	-	-	-	-
L-Pyroglutamic acid	+	+	-	+	+	-	-	-	-
Quinic acid	-	-	+	-	-	-	-	-	-
L-Malic acid	-	+	+	+	+	-	-	+	-
Acetic acid	-	+	+	+	+	-	-	+	-
Stachyose	+	+	-	+	+	-	-	+	-
N-Acetyl neuraminic acid	-	-	-	-	-	-	-	-	-
Inosine	-	-	-	-	+	-	-	-	-
D-Serine	-	+	+	-	-	-	-	-	-
L-Serine	+	+	-	-	-	-	-	-	-
D-Saccharic acid	-	-	-	-	+	-	-	-	-
Bromo-succinic acid	-	+	-	-	+	-	-	-	-
Formic acid	-	-	-	-	-	-	+	-	-

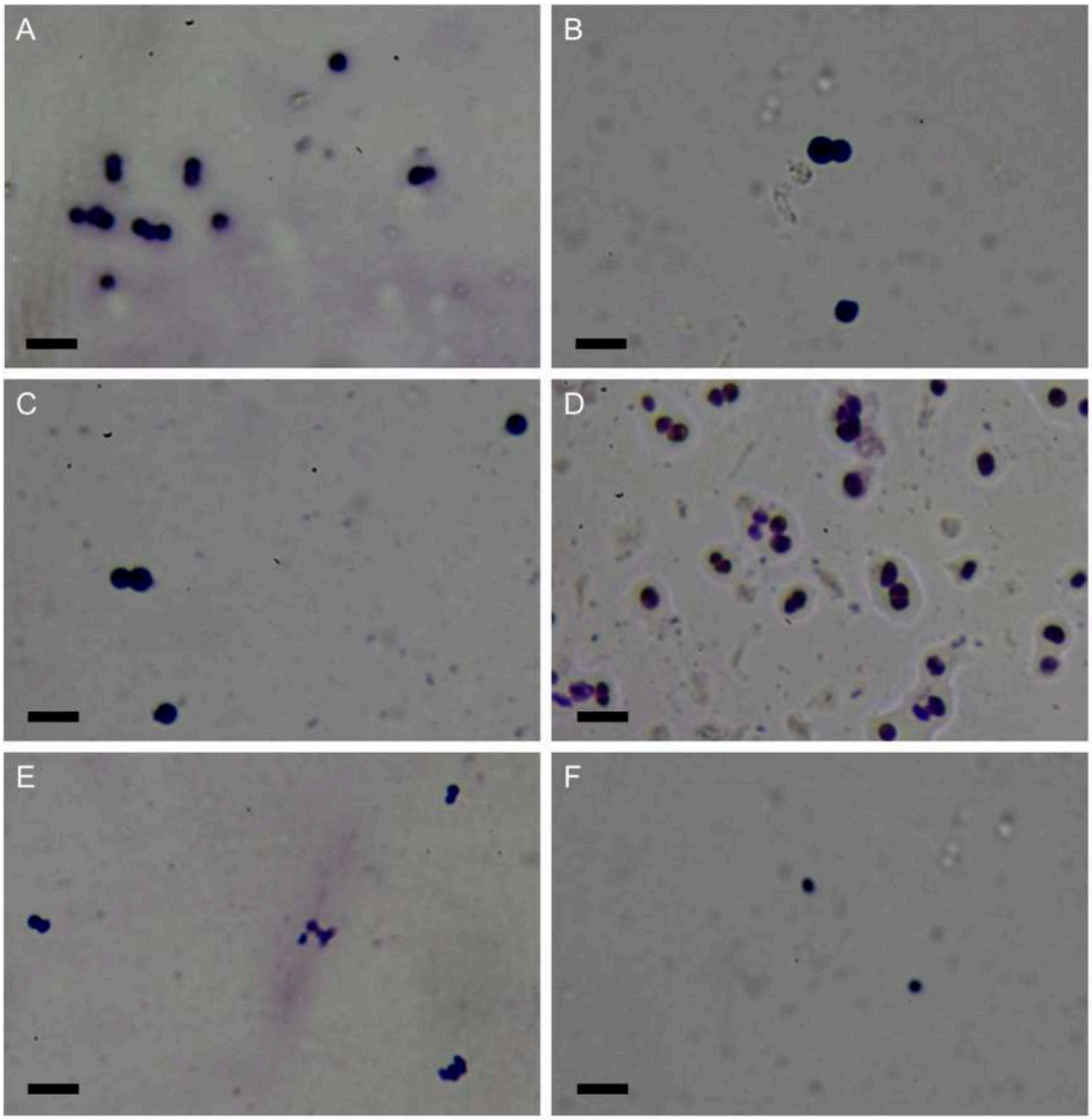


Figure III.S1: Cell morphology of strains T13^{T} (A, B), T90^{T} (C, D) and R8^{T} (E, F) under the optical microscope at 100X magnification. Cells were obtained from an overnight culture in trypticase soy broth liquid media at 30 °C and stained with crystal violet glass. Size bars correspond to 3 µm.

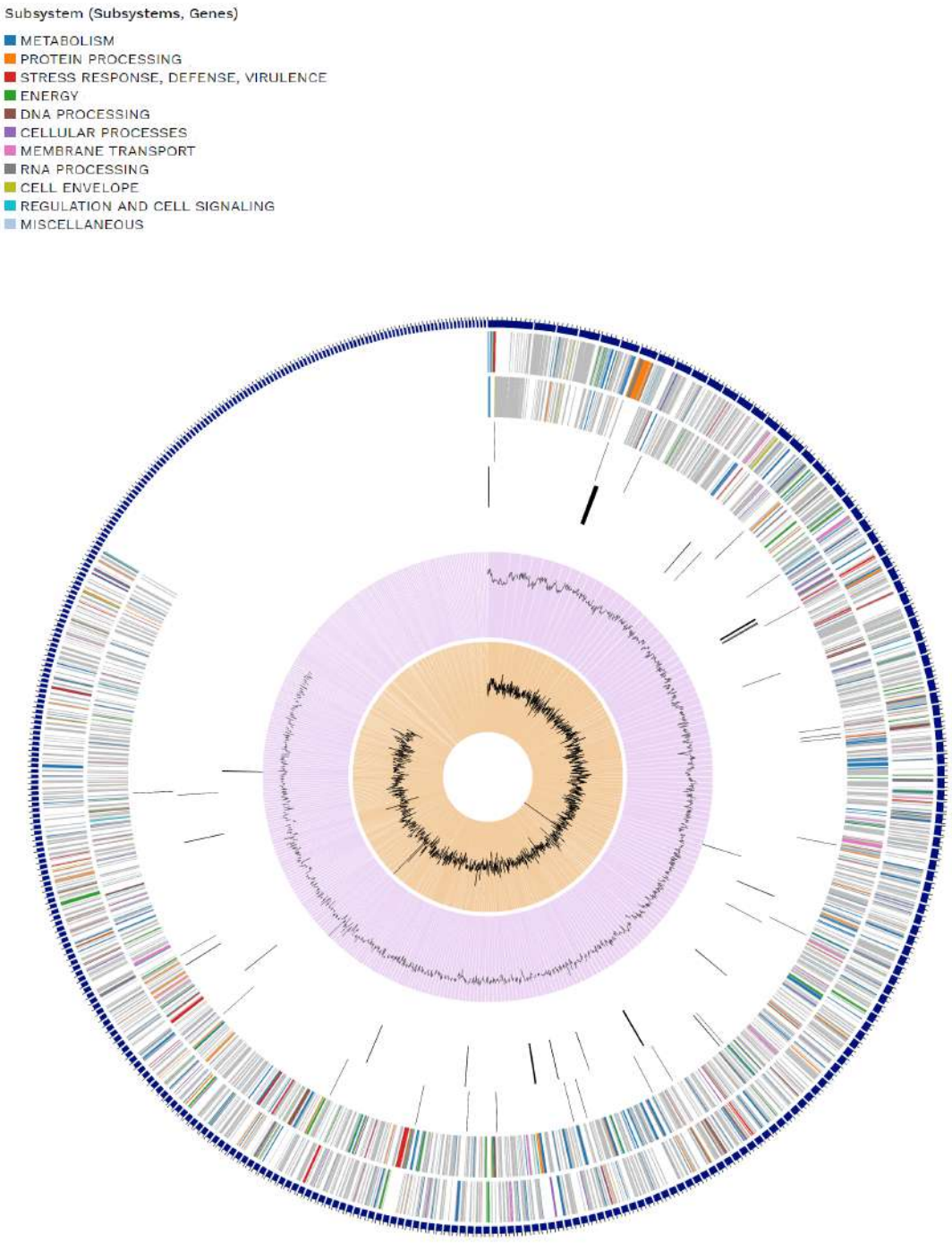


Figure III.S2: *Kineococcus vitellinus* sp. nov. T13^{T} circular genomic map.

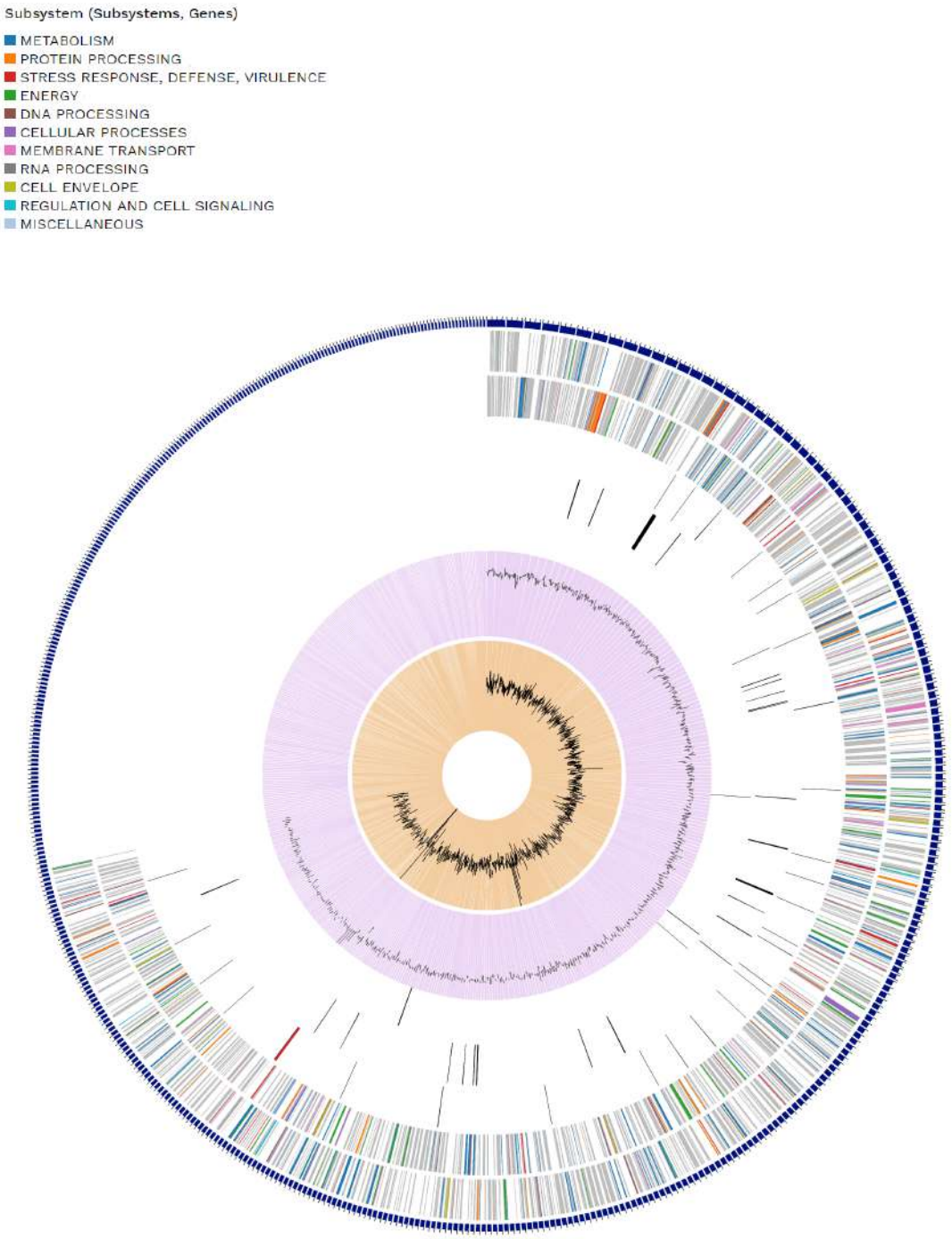


Figure III.S3: *Kineococcus indalonis* sp. nov. T90^{T} circular genomic map.

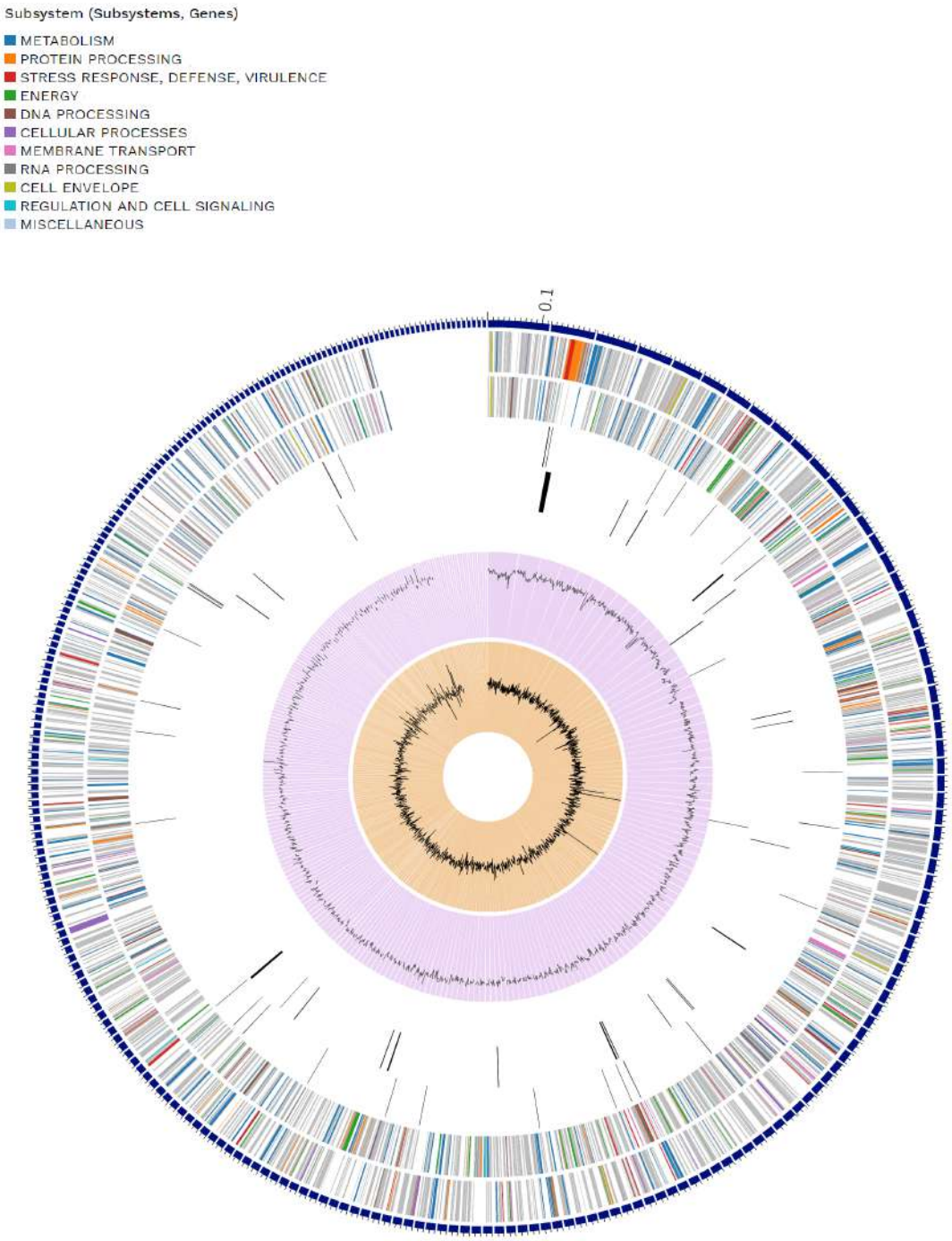


Figure III.S4: *Kineococcus siccus* sp. nov. R8^{T} circular genomic map.

Table IV.S1. Carbon source utilization comparison using Gen III Micro Plates of strains T6^T, T18^T and the type strains of closely related *Belnapia* species. Strains: 1, T6^T; 2, T18^T; 3, *Belnapia moabensis* DSM 16746^T; 4, *Belnapia rosea* DSM 23312^T; 5, *Belnapia soli* DSM 28067^T. Data for reference strains were obtained in the present study. +, positive; -, negative; W, weak reaction. All strains are positive for β-Hydroxy-D,L-butyric acid. All strains are negative for D-raffinose, pectin, dextrin, α-D-lactose, D-mannose, D-mannitol, glycyl-L-proline, D-maltose, D-melibiose, D-trehalose, β-Methyl-D-glucoside, L-arginine, D-cellobiose, D-salicin, 3-methyl-D-glucoside, L-aspartic acid, gentiobiose, D-glucose-6PO4, N-acetyl-β-D-mannosamine, D-fructose-6PO4, L-histidine, L-rhamnose, inosine, D-serine and bromo-succinic acid.

Characteristic	1	2	3	4	5
α-D-Glucose	-	-	+	-	-
D-Sorbitol	-	-	+	-	-
Gelatin	-	-	+	-	-
p-Hydroxy-phenylacetic acid	-	-	+	-	-
Tween 40	-	-	-	+	-
D-Galacturonic acid	-	-	+	+	+
Methyl pyruvate	-	-	+	-	+
γ -Amino-butyric acid	-	-	-	+	-
D-Fructose	-	-	+	-	-
D-Arabitol	-	-	+	-	-
L-Alanine	-	-	+	+	-
L-Galactonic acid lactone	+	-	+	+	+
D-Lactic acid methyl ester	-	-	+	+	-
α -Hydroxy-butyric acid	-	-	+	-	+
D-Galactose	-	-	+	-	-
myo-Inositol	-	-	+	-	-
D-Gluconic acid	-	+	+	+	-
L-Lactic acid	-	-	+	+	+
Glycerol	-	-	+	-	+
D-Glucuronic acid	+	-	+	+	+
Citric acid	-	-	-	+	-
α -Keto-butyric acid	-	-	+	-	-
N-acetyl-D-glucosamine	-	-	+	-	-
D-Fucose	-	-	+	+	+
L-Glutamic acid	-	-	+	-	+
Glucuronamide	+	-	+	+	+
α -Keto-glutaric acid	-	-	+	+	+
Acetoacetic acid	+	-	+	+	-
Sucrose	-	-	+	-	-
L-Fucose	-	-	+	-	-
Mucic acid	-	-	+	+	+
D-Malic acid	-	-	+	+	+
Propionic acid	-	-	+	+	-
D-Turanose	-	-	+	-	-
N-acetyl-D-galactosamine	-	-	+	-	-
D-Aspartic acid	-	-	+	-	-
L-Pyroglutamic acid	-	+	+	+	-
Quinic acid	-	-	-	+	-
L-Malic acid	-	+	+	+	+
Acetic acid	-	-	+	+	-
Stachyose	-	-	+	-	-
N-Acetyl neuraminic acid	-	-	-	+	-
L-Serine	-	-	+	-	-
D-Saccharic acid	-	-	+	+	+
Formic acid	-	-	+	+	-

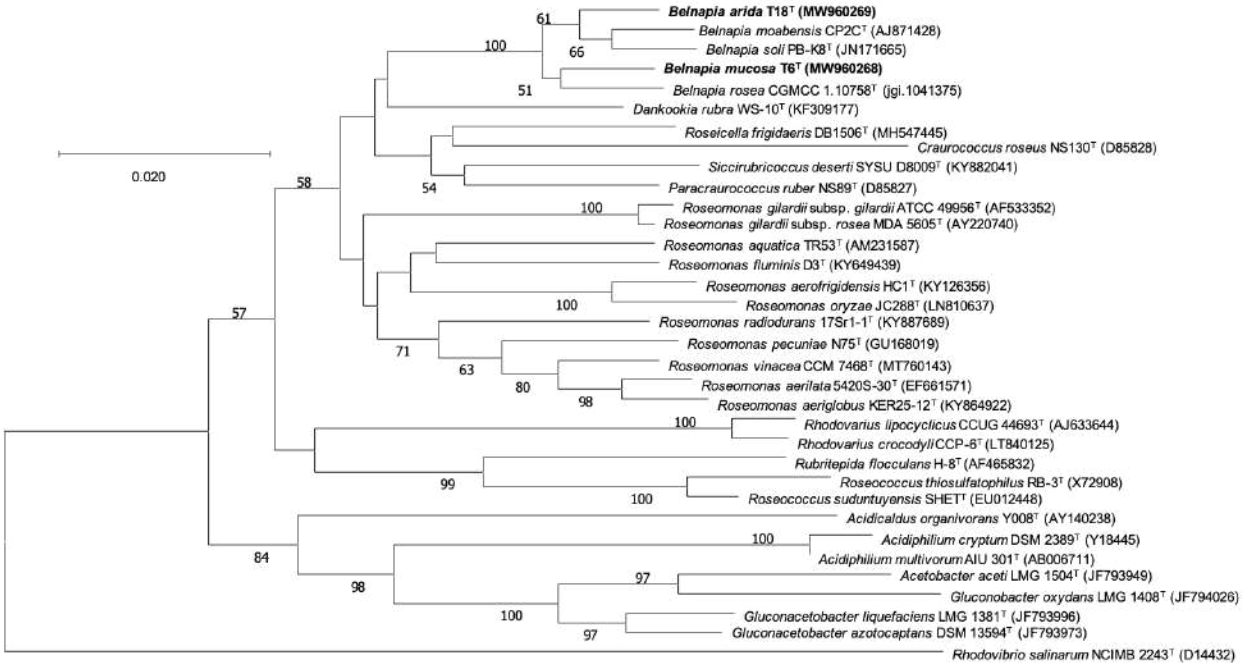


Figure IV.S1. Neighbour-Joining phylogenetic tree showing the relationships between strains T6^T, T18^T and other members of the family *Acetobacteraceae* based on 16S rRNA sequences. The evolutionary model of nucleotide substitution applied is Kimura two-parameter model (K2P). Numbers at branch points refer to bootstrap percentages based on 1000 replicates (values under 50% are not indicated). *Rhodovibrio salinarum* NCIMB 2243^T (D14432) was used as an outgroup. Bar 0.02 fixed nucleotide substitutions per site.

Table IV.S2. Genomic distance indexes for strains T6^T and T18^T, compared to other type strains of the genus *Belnapia*. Average Nucleotide Identity values (ANIb) and estimated digital DNA-DNA Hybridization (dDDH) values (%) are shown. 1.T6^T; 2. T18^T; 3. *B. moabensis* DSM 16746^T; 4. *B. rosea* CGMCC 110758^T.

ANIb	1	2	3	4
1-T6^T	*	83.07	82.86	83.26
2-T18^T	82.96	*	88.47	84.42
Estimated dDDH				
1-T6^T	*	28.5	28.3	29.0
2-T18^T	28.5	*	40.5	30.2

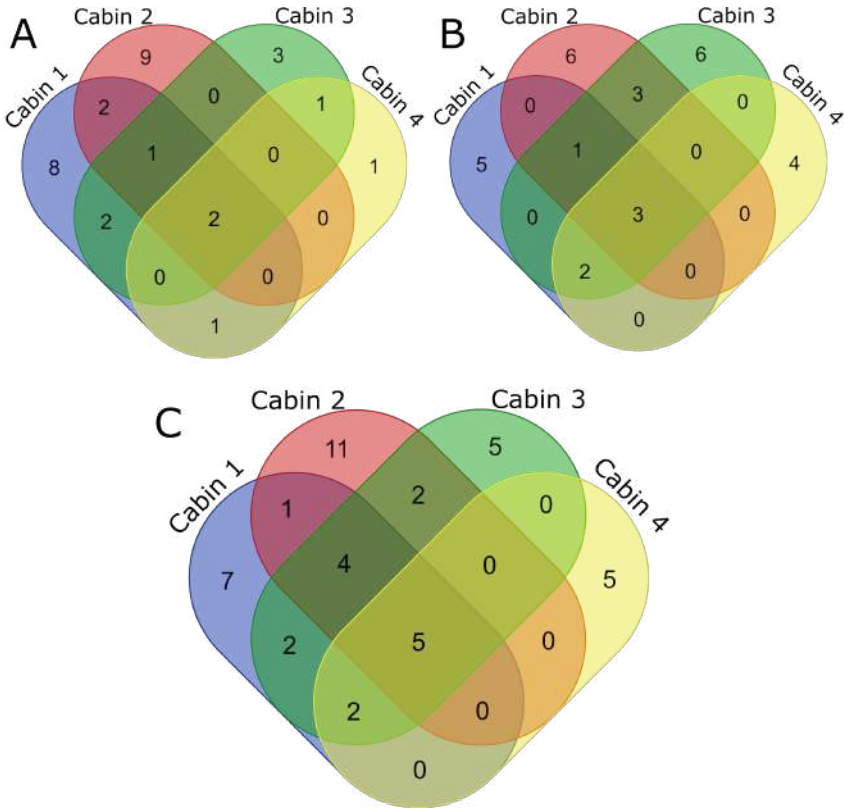


Figure V.S1. Venn diagrams showing the shared and exclusive taxa, at the genus level, in the different cabins. (A) Genera isolated from inside the cabins. (B) Genera isolated from outside the cabins. (C) Genera regardless of the sampling site.

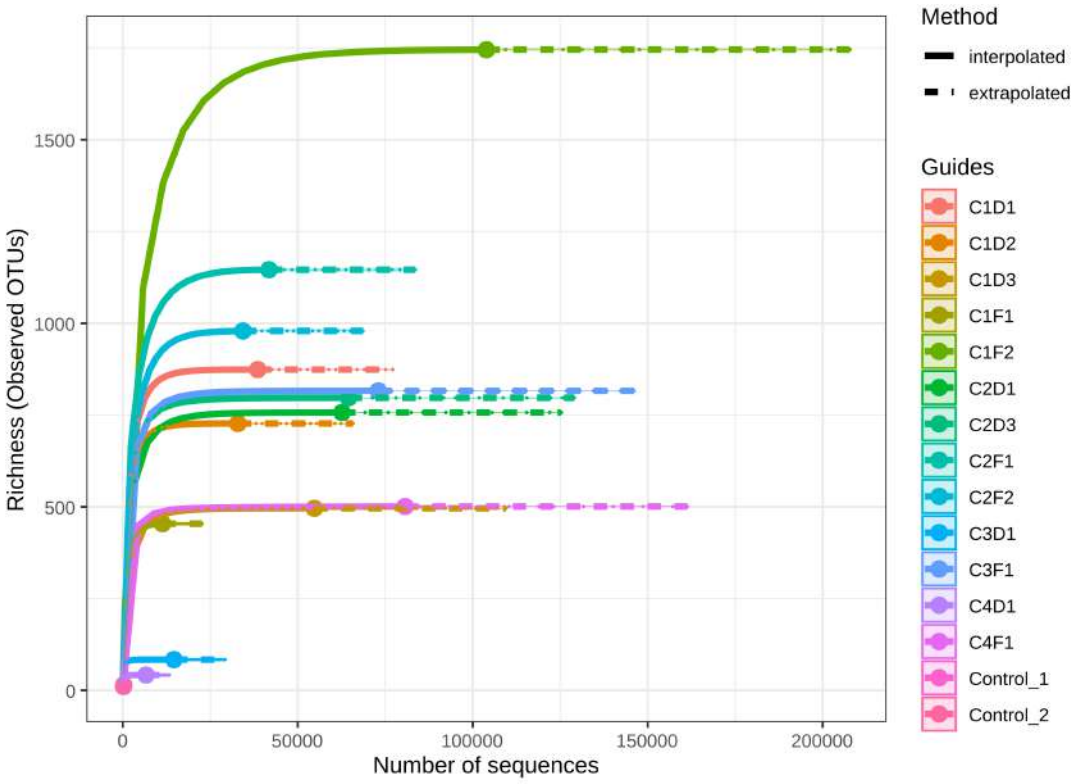
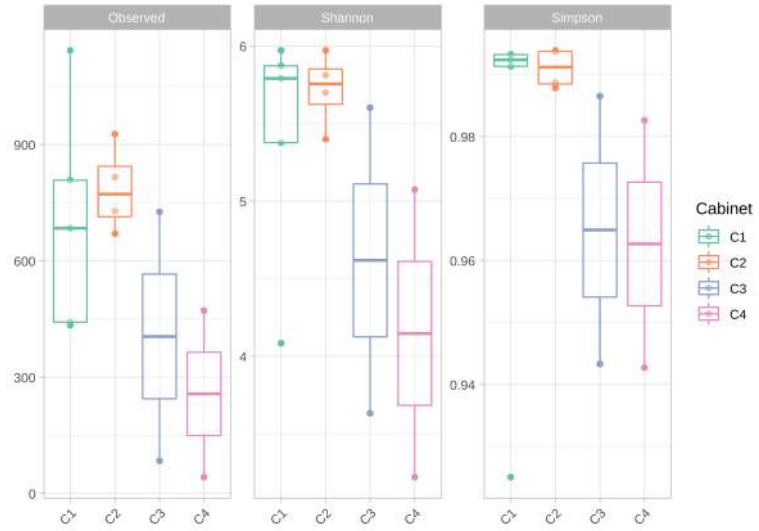


Figure V.S2. Rarefaction curve at OUT level.

A



B

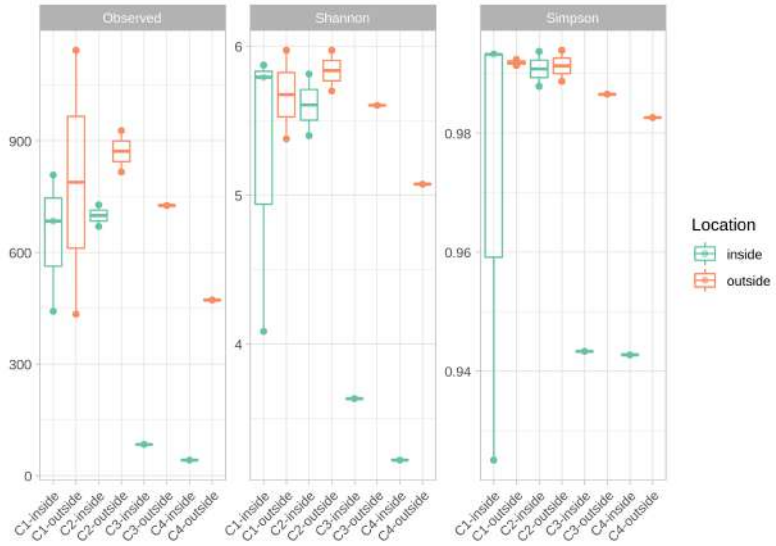
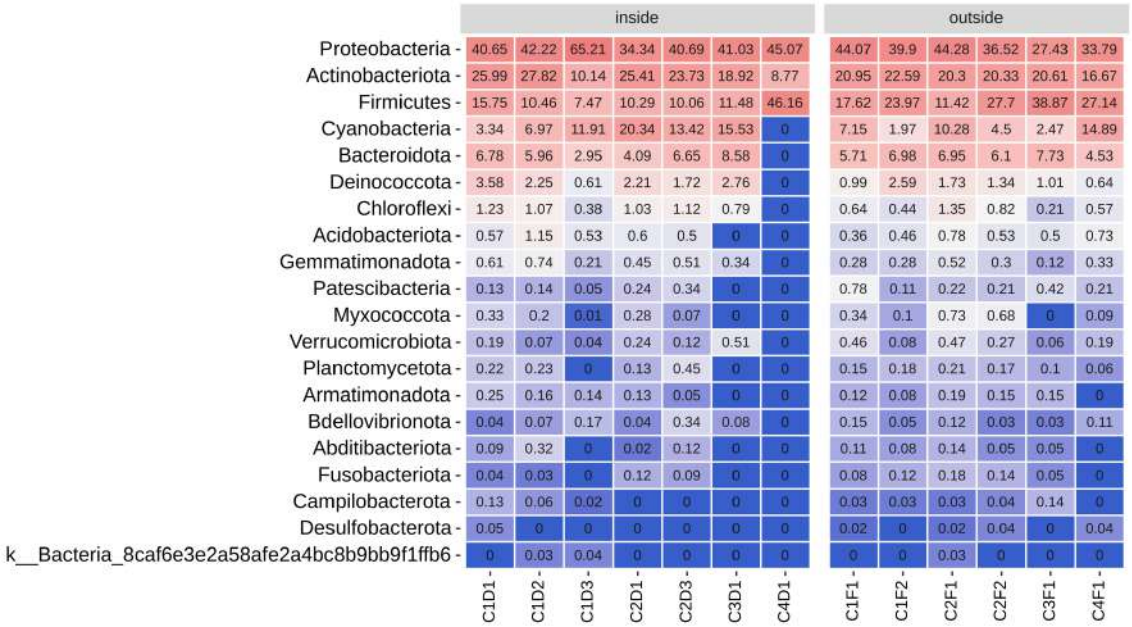


Figure V.S3. Microbial α-diversity at the ASV level (clones) observed through Wilcoxon test and measured by Shannon and Simpson indexes. (A) Diversity among cabins. (B) Diversity among cabins and sampling sites.

A



B

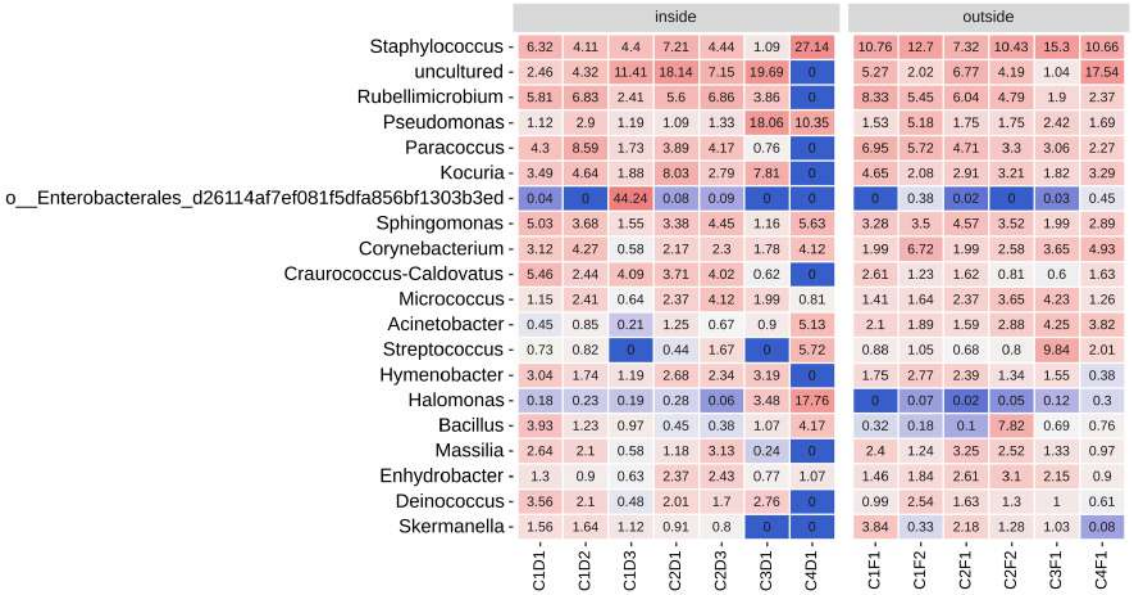


Figure V.S4. Heatmap showing the relative abundances (%) of bacteria in the sampled cabins as deduced by high-throughput 16S rRNA gene sequencing. (A) Relative abundances at the phylum level. (B) Relative abundances at the genus level.

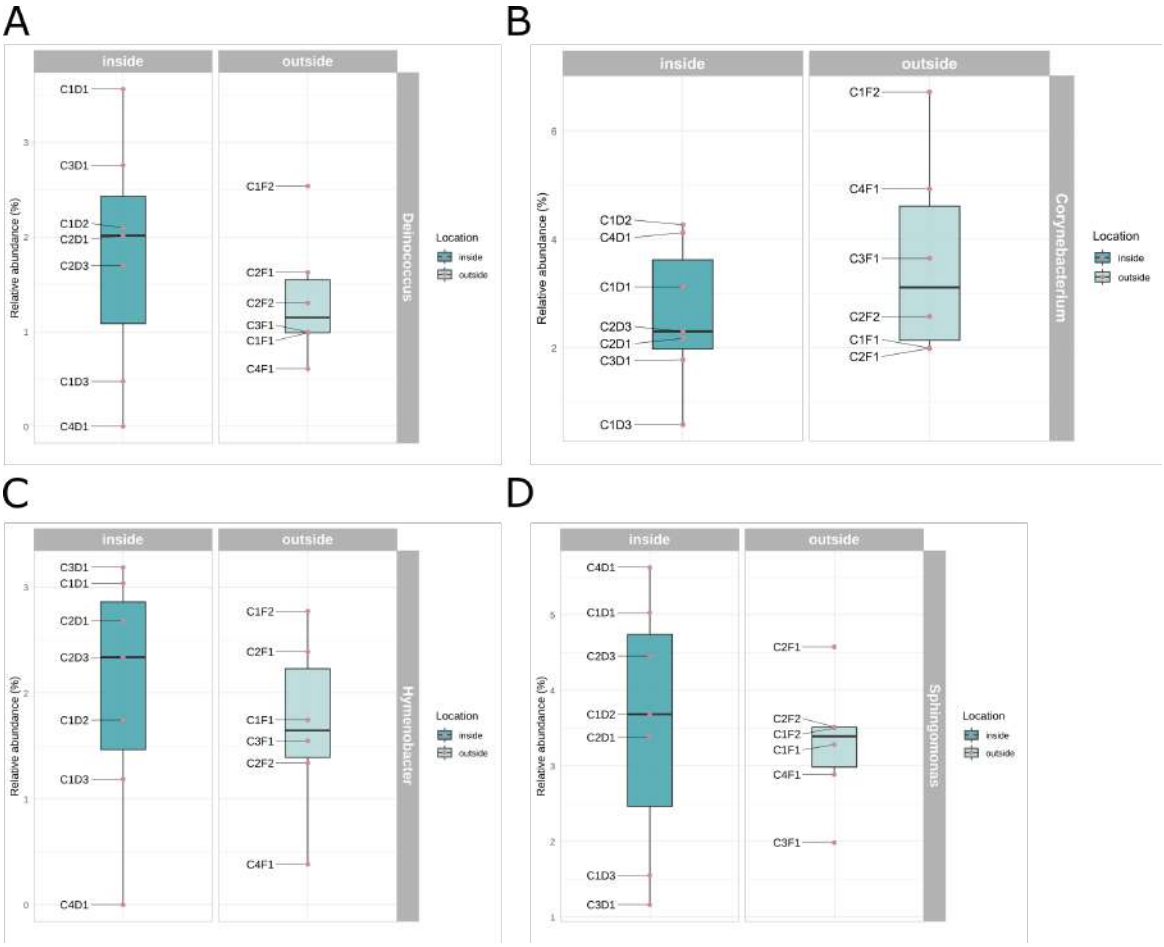


Figure V.S5. Relative abundances (%) at the genus level of specific taxa based on their abundance and relevance for the study. (A) Abundance of *Deinococcus*. (B) Abundance of *Corynebacterium*. (C) Abundance of *Hymenobacter*. (D) Abundance of *Sphingomonas*.

Table V.S1. Microbial collection identification and isolation data. Code collection: Strain Number-Isolation Media-Project. Isolation media: N, Nutrient Agar; T, TSA; R, R2A; B, Columbia Blood; Y, Yeast Mold.

CODE COLLECTION	CABIN	SOURCE	GENUS	TOP HIT TYPE STRAIN
0886-N-09	1	Inside	*Peribacillus*	*Peribacillus simplex*
0887-N-09	1	Inside	*Priestia*	*Priestia megaterium*
0891-N-09	2	Inside	*Kocuria*	*Kocuria rosea*
0892-N-09	2	Inside	*Staphylococcus*	*Staphylococcus borealis*
0893-N-09	2	Inside	*Pseudoxanthomonas*	*Pseudoxanthomonas suwonensis*
0895-N-09	3	Inside	*Staphylococcus*	*Staphylococcus hominis subsp. hominis*
0900-N-09	4	Inside	*Paenibacillus*	*Paenibacillus illinoisensis*
0901-N-09	4	Inside	*Staphylococcus*	*Staphylococcus cohnii*
0903-N-09	1	Inside	*Frigoribacterium*	*Frigoribacterium endophyticum*
0904-T-09	1	Inside	*Micrococcus*	*Micrococcus luteus*
0906-T-09	2	Inside	*Micrococcus*	*Micrococcus luteus*
0907-T-09	2	Inside	*Micrococcus*	*Micrococcus luteus*
0911-T-09	3	Inside	*Staphylococcus*	*Staphylococcus hominis subsp. hominis*
0912-T-09	4	Inside	*Staphylococcus*	*Staphylococcus hominis subsp. novobiosepticus*
0914-Y-09	1	Inside	*Staphylococcus*	*Staphylococcus epidermidis*
0916-Y-09	2	Inside	*Staphylococcus*	*Staphylococcus hominis subsp. hominis*
0917-Y-09	2	Inside	*Kocuria*	*Kocuria arsenatis*
0920-Y-09	3	Inside	*Staphylococcus*	*Staphylococcus hominis subsp. hominis*
0923-N-09	2	Inside	*Acinetobacter*	*Acinetobacter variabilis*
0924-R-09	1	Inside	*Micrococcus*	*Micrococcus luteus*
0925-R-09	1	Inside	*Curtobacterium*	*Curtobacterium flaccumfaciens*
0926-R-09	1	Inside	*Deinococcus*	*Deinococcus terrestris*
0927-R-09	2	Inside	*Staphylococcus*	*Staphylococcus epidermidis*
0931-R-09	3	Inside	*Bacillus*	*Bacillus licheniformis*
0932-R-09	3	Inside	*Lysinibacillus*	*Lysinibacillus halotolerans*
0933-R-09	3	Inside	*Micrococcus*	*Micrococcus luteus*
0935-R-09	3	Inside	*Kocuria*	*Kocuria arsenatis*
0940-R-09	4	Inside	*Kocuria*	*Kocuria turfanensis*
0942-B-09	1	Inside	*Metabacillus*	*Metabacillus idriensis*
0945-B-09	2	Inside	*Planococcus*	*Planococcus glaciei*
0946-B-09	2	Inside	*Kocuria*	*Kocuria rosea*
0949-B-09	3	Inside	*Robertmurraya*	*Robertmurraya siralis*
0950-B-09	3	Inside	*Bacillus*	*Bacillus altitudinis*
0954-B-09	4	Inside	*Bacillus*	*Bacillus pseudomycoides*
0955-B-09	4	Inside	*Staphylococcus*	*Staphylococcus saprophyticus subsp. saprophyticus*
0958-B-09	1	Inside	*Kocuria*	*Kocuria rosea*
0959-B-09	1	Inside	*Micrococcus*	*Micrococcus luteus*
0960-B-09	1	Inside	*Arthrobacter*	*Arthrobacter bussei*
0961-B-09	1	Inside	*Micrococcus*	*Micrococcus luteus*
0962-B-09	2	Inside	*Arthrobacter*	*Arthrobacter bussei*
0964-B-09	2	Inside	*Acinetobacter*	*Acinetobacter variabilis*
0966-B-09	3	Inside	*Micrococcus*	*Micrococcus luteus*
0973-B-09	4	Inside	*Staphylococcus*	*Staphylococcus hominis subsp. novobiosepticus*
0975-N-09	1	Inside	*Domibacillus*	*Domibacillus indicus*
0976-N-09	1	Inside	*Streptomyces*	*Streptomyces flavovirens*
0977-N-09	1	Inside	*Frigoribacterium*	*Frigoribacterium faeni*
0981-N-09	2	Inside	*Sphingomonas*	*Sphingomonas aerolata*
0982-N-09	2	Inside	*Massilia*	*Massilia dura*
0988-N-09	4	Inside	*Staphylococcus*	*Staphylococcus cohnii*
0991-Y-09	1	Inside	*Cryptococcus*	*[Cryptococcus] albidus var. kuetzingii*
0992-Y-09	2	Inside	*Cystobasidium*	*Cystobasidium slooffiae*
0993-Y-09	2	Inside	*Massilia*	*Massilia arvi*
0994-Y-09	2	Inside	*Rhodotorula*	*Rhodotorula mucilaginosa*
0995-Y-09	2	Inside	*Saccharibacillus*	*Saccharibacillus qingshengii*
0997-Y-09	3	Inside	*Staphylococcus*	*Staphylococcus hominis subsp. hominis*
0998-Y-09	4	Inside	*Paenibacillus*	*Paenibacillus illinoisensis*
1002-R-09	1	Inside	*Aureobasidium*	*Aureobasidium namibiae*
1004-R-09	2	Inside	*Massilia*	*Massilia arvi*
1005-R-09	2	Inside	*Saccharibacillus*	*Saccharibacillus qingshengii*
1006-R-09	2	Inside	*Acinetobacter*	*Acinetobacter variabilis*
1012-R-09	4	Inside	*Curtobacterium*	*Curtobacterium citreum*
1013-R-09	4	Inside	*Curtobacterium*	*Curtobacterium oceanosedimentum*
1016-T-09	1	Inside	*Microbacterium*	*Microbacterium esteraromaticum*
1017-T-09	1	Inside	*Arthrobacter*	*Arthrobacter bussei*
1018-T-09	1	Inside	*Frigoribacterium*	*Frigoribacterium faeni*
1024-T-09	2	Inside	*Staphylococcus*	*Staphylococcus epidermidis*
1025-T-09	2	Inside	*Paracoccus*	*Paracoccus halotolerans*
1026-T-09	2	Inside	*Staphylococcus*	*Staphylococcus cohnii*
1027-T-09	2	Inside	*Arthrobacter*	*Arthrobacter bussei*

1028-T-09	2	Inside	*Kocuria*	*Kocuria polaris*
1032-T-09	3	Inside	*Microbacterium*	*Microbacterium esteraromaticum*
1033-T-09	3	Inside	*Frigoribacterium*	*Frigoribacterium faeni*
1034-T-09	3	Inside	*Rhodococcus*	*Rhodococcus corynebacterioides*
1037-T-09	4	Inside	*Staphylococcus*	*Staphylococcus cohnii*
1041-B-09	3	Inside	*Micrococcus*	*Micrococcus luteus*
1044-R-09	2	Inside	*Cellulomonas*	*Cellulomonas cellasea*
1045-R-09	1	Inside	*Paracoccus*	*Paracoccus panacisoli*
1046-Y-09	1	Inside	*Priestia*	*Priestia endophytica*
1047-Y-09	2	Inside	*Staphylococcus*	*Staphylococcus hominis subsp. hominis*
1049-T-09	3	Inside	*Frigoribacterium*	*Frigoribacterium faeni*
1050-T-09	3	Inside	*Frigoribacterium*	*Frigoribacterium faeni*
0888-N-09	1	Outside	*Pseudomonas*	*Pseudomonas stutzeri*
0889-N-09	1	Outside	*Staphylococcus*	*Staphylococcus haemolyticus*
0890-N-09	1	Outside	*Staphylococcus*	*Staphylococcus cohnii*
0894-N-09	2	Outside	*Bacillus*	*Bacillus zhangzhouensis*
0896-N-09	3	Outside	*Kocuria*	*Kocuria palustris*
0897-N-09	3	Outside	*Staphylococcus*	*Staphylococcus caprae*
0898-N-09	3	Outside	*Micrococcus*	*Micrococcus luteus*
0899-N-09	3	Outside	*Pseudomonas*	*Pseudomonas fulva*
0902-N-09	4	Outside	*Kocuria*	*Kocuria arsenatis*
0905-T-09	1	Outside	*Bacillus*	*Bacillus altitudinis*
0908-T-09	2	Outside	*Micrococcus*	*Micrococcus luteus*
0909-T-09	2	Outside	*Staphylococcus*	*Staphylococcus haemolyticus*
0910-T-09	2	Outside	*Micrococcus*	*Micrococcus luteus*
0913-T-09	4	Outside	*Micrococcus*	*Micrococcus luteus*
0915-Y-09	1	Outside	*Priestia*	*Priestia aryabhattai*
0918-Y-09	2	Outside	NID	NID
0919-Y-09	2	Outside	*Microbacterium*	*Microbacterium algeriense*
0921-Y-09	3	Outside	*Pseudomonas*	*Pseudomonas oryzihabitans*
0922-Y-09	4	Outside	*Roseomonas*	*Roseomonas mucosa*
0928-R-09	2	Outside	*Metabacillus*	*Metabacillus idriensis*
0929-R-09	4	Outside	*Staphylococcus*	*Staphylococcus hominis subsp. novobiosepticus*
0930-R-09	2	Outside	*Pseudomonas*	*Pseudomonas kuykendallii*
0934-R-09	3	Outside	*Deinococcus*	*Deinococcus ficus*
0936-R-09	3	Outside	*Paracoccus*	*Paracoccus chinensis*
0937-R-09	3	Outside	*Pantoea*	*Pantoea conspicua*
0938-R-09	3	Outside	*Bacillus*	*Bacillus manliponensis*
0939-R-09	3	Outside	*Paracoccus*	*Paracoccus niistensis*
0941-R-09	4	Outside	*Kocuria*	*Kocuria arsenatis*
0943-B-09	1	Outside	*Bacillus*	*Bacillus aerius*
0944-B-09	1	Outside	*Pseudomonas*	*Pseudomonas oryzihabitans*
0947-B-09	2	Outside	*Micrococcus*	*Micrococcus luteus*
0948-B-09	2	Outside	*Staphylococcus*	*Staphylococcus edaphicus*
0951-B-09	3	Outside	*Micrococcus*	*Micrococcus luteus*
0952-B-09	3	Outside	*Metabacillus*	*Metabacillus halosaccharovorans*
0953-B-09	3	Outside	*Staphylococcus*	*Staphylococcus equorum subsp. equorum*
0956-B-09	4	Outside	*Staphylococcus*	*Staphylococcus edaphicus*
0957-B-09	4	Outside	*Cytobacillus*	*Cytobacillus firmus*
0963-B-09	1	Outside	*Aureobasidium*	*Aureobasidium namibiae*
0965-B-09	2	Outside	*Psychrobacillus*	*Psychrobacillus psychrodurans*
0967-B-09	3	Outside	*Pseudomonas*	*Pseudomonas oryzihabitans*
0968-B-09	3	Outside	NID	NID
0969-B-09	3	Outside	*Planococcus*	*Planococcus okeanokoites*
0970-B-09	4	Outside	*Kocuria*	*Kocuria arsenatis*
0971-B-09	4	Outside	*Staphylococcus*	*Staphylococcus borealis*
0972-B-09	4	Outside	*Ustilago*	*Ustilago shanxiensis*
0974-B-09	4	Outside	*Micrococcus*	*Micrococcus luteus*
0978-N-10	1	Outside	*Streptomyces*	*Streptomyces ambofaciens*
0979-N-09	1	Outside	*Priestia*	*Priestia aryabhattai*
0980-N-09	1	Outside	*Bacillus*	*Bacillus altitudinis*
0983-N-09	2	Outside	*Pseudomonas*	*Pseudomonas oryzihabitans*
0984-N-09	2	Outside	*Staphylococcus*	*Staphylococcus argenteus*
0985-N-09	2	Outside	*Arthrobacter*	*Arthrobacter agilis*
0986-N-09	2	Outside	*Neobacillus*	*Neobacillus niacini*
0987-T-09	3	Outside	NID	NID
0989-N-09	4	Outside	*Kocuria*	*Kocuria arsenatis*
0990-N-09	4	Outside	*Pseudomonas*	*Pseudomonas kuykendallii*
0996-Y-09	2	Outside	*Erwinia*	*Erwinia endophytica*
0999-Y-09	3	Outside	*Aureobasidium*	*Aureobasidium namibiae*

1000-Y-09	4	Outside	*Kocuria*	*Kocuria arsenatis*
1001-Y-09	4	Outside	*Novosphingobium*	*Novosphingobium silvae*
1003-R-09	1	Outside	*Moraxella*	*Moraxella osloensis*
1007-R-09	2	Outside	*Massilia*	*Massilia agri*
1008-R-09	2	Outside	*Stenotrophomonas*	*Stenotrophomonas rhizophila*
1009-R-09	3	Outside	*Kosakonia*	*Kosakonia cowanii*
1010-R-09	3	Outside	*Staphylococcus*	*Staphylococcus hominis subsp. novobiosepticus*
1011-R-09	3	Outside	*Arthrobacter*	*Arthrobacter agilis*
1014-R-09	4	Outside	*Kocuria*	*Kocuria arsenatis*
1015-R-09	4	Outside	*Novosphingobium*	*Novosphingobium silvae*
1019-T-09	1	Outside	*Micrococcus*	*Micrococcus luteus*
1020-T-09	1	Outside	*Kocuria*	*Kocuria arsenatis*
1021-T-09	1	Outside	*Cellulomonas*	*Cellulomonas pakistanensis*
1022-T-09	1	Outside	*Bacillus*	*Bacillus toyonensis*
1023-T-09	1	Outside	NID	NID
1029-T-09	2	Outside	*Fredinandcohnia*	*Fredinandcohnia onubensis*
1030-T-09	2	Outside	NID	NID
1031-T-09	2	Outside	*Pseudomonas*	*Pseudomonas stutzeri*
1035-T-09	3	Outside	*Pseudomonas*	*Pseudomonas oryzihabitans*
1036-T-09	3	Outside	*Aureobasidium*	*Aureobasidium namibiae*
1038-T-09	4	Outside	*Kocuria*	*Kocuria palustris*
1039-T-09	4	Outside	*Aureobasidium*	*Aureobasidium namibiae*
1040-R-09	3	Outside	*Arthrobacter*	*Arthrobacter bussei*
1042-N-09	4	Outside	*Cytobacillus*	*Cytobacillus horneckiae*
1043-T-09	4	Outside	*Kocuria*	*Kocuria arsenatis*
1048-T-09	1	Outside	*Mixta*	*Mixta calida*
1051-T-09	2	Outside	*Staphylococcus*	*Staphylococcus edaphicus*
1052-N-09	3	Outside	*Curtobacterium*	*Curtobacterium flaccumfaciens*
1053-R-09	1	Outside	*Pseudomonas*	*Pseudomonas stutzeri*
1054-B-09	3	Outside	*Neobacillus*	*Neobacillus niacini*

Table V.S2. Selected strains for biological activity assays.

STRAIN NUMBER	ID	SOURCE
889	*Staphylococcus haemolyticus*	Outside
890	*Staphylococcus cohnii*	Outside
896	*Kocuria palustris*	Outside
901	*Staphylococcus cohnii*	Inside
906	*Micrococcus luteus*	Inside
912	*Staphylococcus hominis*	Inside
925	*Curtobacterium flaccumfaciens*	Inside
927	*Staphylococcus epidermidis*	Inside
929	*Staphylococcus hominis*	Outside
932	*Lysinibacillus halotolerans*	Inside
934	*Deinococcus ficus*	Outside
935	*Kocuria arsenatis*	Inside
950	*Bacillus altitudinis*	Inside
964	*Acinetobacter variabilis*	Inside
977	*Frigoribacterium faeni*	Inside
979	*Priestia aryabhattai*	Outside
980	*Bacillus altitudinis*	Outside
1008	*Stenotrophomonas rhizophila*	Outside
1011	*Arthrobacter agilis*	Outside
1016	*Microbacterium esteraromaticum*	Inside
1019	*Micrococcus luteus*	Outside
1020	*Kocuria arsenatis*	Outside
1027	*Arthrobacter bussei*	Inside
1028	*Kocuria polaris*	Inside
1040	*Arthrobacter bussei*	Outside
1045	*Paracoccus panacisoli*	Inside
1052	*Curtobacterium flaccumfaciens*	Outside
1053	*Pseudomonas stutzeri*	Outside

Appendix C: Other publications

environmental microbiology reports

Environmental Microbiology Reports (2018) **10**(1), 75–79 doi:10.1111/1758-2229.12608

Polar solar panels: Arctic and Antarctic microbiomes display similar taxonomic profiles

Kristie Tanner,[1] Jose Manuel Martí,[1] Josabel Belliure,[2] Mar Fernández-Méndez,[3] Esther Molina-Menor,[1] Juli Peretó[1,4,5] and Manuel Porcar[1,5]*

[1]*Institute for Integrative Systems Biology (I2SysBio, University of Valencia-CSIC), Paterna, Valencia, Spain.*
[2]*Ecology Section, Department of Life Sciences, University of Alcalá, Alcalá de Henares, Madrid, Spain.*
[3]*Norwegian Polar Institute, Tromsø, Norway.*
[4]*Department of Biochemistry and Molecular Biology, University of Valencia, Spain.*
[5]*Darwin Bioprospecting Excellence SL, Paterna, Valencia, Spain.*

Summary

Solar panels located on high (Arctic and Antarctic) latitudes combine the harshness of the climate with that of the solar exposure. We report here that these polar solar panels are inhabited by similar microbial communities in taxonomic terms, dominated by *Hymenobacter* spp., *Sphingomonas* spp. and Ascomycota. Our results suggest that solar panels, even on high latitudes, can shape a microbial ecosystem adapted to irradiation and desiccation.

Introduction

The microbial ecology of some artificial structures, including solar panels, has been poorly explored up to date (Shirakawa *et al*, 2015; Dorado-Morales *et al*, 2016). These reports suggest that photovoltaic surfaces display a diverse microbial community, highly tolerant to thermal fluctuations, ultraviolet (UV) irradiation and desiccation (Dorado-Morales *et al*, 2016). In this work, we aimed to study, through Next Generation Sequencing (NGS) and microbial culturing techniques, the microbiome of photovoltaic solar panels from two cold locations, lying close or inside the polar circles: Tromsø, Norway (69°40′N 18°56′E) and two Antarctic islands (62°0′S 58°0′W) (Fig. 1A).

Received 5 October, 2017; accepted 23 November, 2017. *For correspondence E-mail: manuel.porcar@uv.es; Tel. +34 963544473.

Tromsø is the largest urban area in northern Norway. It is located on the coast, above the Arctic Circle, and experiences a subarctic climate. Average temperatures in winter range between 0.9 and −3.6 °C and in summer between 5.3 and 12.4 °C. The South Shetlands constitute a group of islands of the Maritime Antarctica. Monthly average temperature is between −3.1 and −10.9 °C from March to October, when the sea around the islands is closed by ice, and slightly warmer from November to February, with temperatures ranging between −1.7 and 0.5 °C.

Results and discussion

A total of 14 individual solar panels were sampled in January and May 2017 in Antarctica (9 panels, 3 from Deception Island and 6 from Livingston Island) and Tromsø (5 panels), respectively (Fig. 1A). Aliquots were spread on LB and R2A media and incubated at 4 °C for three weeks to select psychrotrophic and psychrophilic microorganisms (Fig. 1B). A collection of 44 isolates was characterized by genetic identification, growth ability and UV-light and desiccation resistance (Fig. 1C). A detailed explanation of all the experimental procedures used in this study can be found on the research group website that can be accessed using the following link: http://www.uv.es/synbio/solpan. The isolates from Antarctic panels able to grow at 4 °C were identified as either *Rhodotorula mucilaginosa* (pink pigmentation) or *Alcaligenes faecalis*/*Curtobacterium* sp. (yellow pigmentation). Samples from Tromsø yielded a larger diversity of microorganisms including *Cryobacterium arcticum*, *Sphingomonas* sp., *Curtobacterium* sp., *Microbacterium* sp. and *Dioszegia fristingensis*, all of them able to grow at 15 °C. Interestingly, despite the low temperatures of their original habitats, 37 isolates were able to grow at 30 °C and 11 also grew at 37 °C. One of these was identified as *R. mucilaginosa*, a cold-adapted (with activity at up to −5 °C), carotenoid-producing, cosmopolitan, mesophilic yeast that has previously been isolated from a wide range of remote environments, including Antarctical ice cores or 11 000 m deep sea vents (Gadanho and Sampaio, 2005; Amato *et al*., 2009; Moliné *et al*., 2012; Nunes *et al*., 2013; Connell *et al*., 2014; Yu *et al*., 2015).

Regarding UV resistance, many of the isolates (a total of 30) were able to survive 30 s of irradiation with

microbial biotechnology

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Extremophilic microbial communities on photovoltaic panel surfaces: a two-year study

Kristie Tanner,[1,2,†] Esther Molina-Menor,[2,†] Adriel Latorre-Pérez,[1] Àngela Vidal-Verdú,[2] Cristina Vilanova,[1] Juli Peretó[1,2,3] and Manuel Porcar[1,2*]

[1]*Darwin Bioprospecting Excellence S.L., Calle Catedrático Agustín Escardino 9, Paterna, 46980, Spain.*
[2]*Institute for Integrative Systems Biology I2SysBio, University of Valencia – CSIC, Catedrático José Beltrán 2, Paterna, 46980, Spain.*
[3]*Department of Biochemistry and Molecular Biology, University of Valencia, Dr. Moliner 50, Burjassot, 46100, Spain.*

Summary

Solar panel surfaces can be colonized by microorganisms adapted to desiccation, temperature fluctuations and solar radiation. Although the taxonomic and functional composition of these communities has been studied, the microbial colonization process remains unclear. In the present work, we have monitored this microbial colonization process during 24 months by performing weekly measurements of the photovoltaic efficiency, carrying out 16S rRNA gene high-throughput sequencing, and studying the effect of antimicrobial compounds on the composition of the microbial biocenosis. This is the first time a long-term study of the colonization process of solar panels has been performed, and our results reveal that species richness and biodiversity exhibit seasonal fluctuations and that there is a trend towards an increase or decrease of specialist (solar panel-adapted) and generalist taxa, respectively. On the former, extremophilic bacterial genera *Deinococcus*, *Hymenobacter* and *Roseomonas* and fungal *Neocatenulostroma*, *Symmetrospora* and *Sporobolomyces* tended to dominate the biocenosis; whereas *Lactobacillus* sp or *Stemphyllium* exhibited a decreasing trend. This profile was deeply altered by washing the panels with chemical agents (Virkon), but this did not lead to an increase of the solar panels efficiency. Our results show that solar panels are extreme environments that force the selection of a particular microbial community.

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*For correspondence. E-mail manuel.porcar@uv.es; Tel. +34 628821813.
†These authors contributed equally to this work.
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Introduction

Extreme environments are characterized by their strong selective pressures, which can include physical (i.e., temperature or radiation), geochemical (i.e., desiccation or salinity) and/or biological stresses (i.e., limited nutrient availability) (Lynn and Rocco, 2001). The microorganisms that inhabit these environments, known as extremophiles or extremotolerants, are selected due a variety of mechanisms, such as biofilm formation (Flemming *et al.*, 2016; Blanco *et al.*, 2019); the production of extremolytes and extremozymes (Gabani and Singh, 2013); or highly efficient DNA repair systems (Singh and Gabani, 2011). Microorganisms inhabiting extreme environments evolve faster than those inhabiting 'benign' environments, mainly due to the high mutation rates associated to stressful environmental conditions (Li *et al.*, 2014), and this could lead to these microorganisms being rich sources of new specialized metabolites (Sayed *et al.*, 2019).

A diversity of physical, geochemical and biological extremes (solar radiation, temperature fluctuations, desiccation and limited nutrient availability) concur on solar panel surfaces. A study performed on subaerial solar panel biofilms in São Paulo revealed that dust, pollen and other debris covering the solar panel surfaces accumulated in time and included abundant fungi and pigmented bacterial genera, and this was associated with a

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Words, images and gender

Lessons from a survey on the public perception of synthetic biology and related disciplines

Manuel Porcar[1,2], Adriel Latorre-Pérez[2], Esther Molina-Menor[1] & Martí Domínguez[3]

The fast development of new research fields, such as genetic engineering or synthetic biology, is often met with public concerns or even resistance, the fate of genetically modified crops being a prime example. There are many factors at play that determine how laypeople perceive new technologies and a better understanding of these can help to inform debate. Foremost, however, it is necessary to obtain reliable information on public opinion of emerging technologies that have the potential to affect their lives. To this end, we conducted a survey to gauge public opinion on genetic engineering and biotechnology as part of a special exhibition at the CosmoCaixa Museum in Barcelona, Spain. The large sample size of 38,113 respondents allowed us to assess the effect of age, gender or education on the perception of three related terms: "biotechnology", "genetic engineering" and "synthetic biology". In addition, by randomly associating these terms with the image of either a male or a female scientist, we looked at the effect of gender on people's perception of these technologies. In short, two conclusions can be reached: the terms "biotechnology" and "genetic engineering" were preferred to "synthetic biology". Second, terms associated with an image of a female scientist were better rated compared to the same terms associated with a male researcher. These results show an interesting gender dimension of public perception of new technologies.

Public perception of biotechnology

Synthetic biology, genetic engineering and biotechnology are interrelated terms with blurred boundaries. Biotechnology uses living organisms, cells or cellular components to synthesize products for agriculture, medicine, industry and research and has been used for centuries, albeit unconsciously. Genetic engineering is one of the subdisciplines of biotechnology: it involves the manipulation of an organism's DNA sequence by addition, deletion or modification in order to expand the product range of biotechnology. While both generally are based on using organisms, genes or metabolic pathways from nature, synthetic biology aims to design novel artificial systems. Synthetic biology can thus be seen as both an extension of genetic engineering, as well as a new view on biotechnology by using engineering principles such as standardization, modularity or orthogonality [1].

> *"There are many factors at play that determine how laypeople perceive new technologies and a better understanding of these can help to inform debate."*

In the public eye, however, biotechnology, genetic engineering and synthetic biology are often reduced to genetically modified organisms (GMOs). This, combined with a critical perception of GMOs, has fuelled a generally negative attitude of biotechnology. The last Eurobarometer survey (2010) on GM food showed that only 5% of Europeans completely support it, 18% "tend to agree", but as much as 61% totally disagree, that is, are against GM food. Moreover, 83% of Europeans had not heard about synthetic biology before. The main concerns were the possible risks rather than potential benefits from these technologies (http://ec.europa.eu/commfrontoffice/publicopinion/index.cfm/Survey/index#p=1&instruments=SPECIAL&search=341). Indeed, genetic engineering is perceived with a higher degree of concern compared to other scientific fields [2].

> *"... Generation T (2011–present), also known as Generation Alpha, is growing up with an iPad or a smartphone in their hand in front of a screen."*

In relation to perceptions of gender, a number of recent studies have shown biases of how men and women are evaluated and perceived at work [3,4]. A randomized double-blind study of professors in biology, chemistry and physics showed that identical academic profiles were more positively evaluated when they belonged to a male student than a female student. The result of such biases is that women in academia have to work harder than their male peers to obtain the same recognition [5] and that males are often seen as more capable than women [6]. Just to highlight one common example of gender stereotyping, when using neutral or non-gender-specific language, people tend to assume that a specialist in question is a man [7].

The exhibition and the survey

The survey was carried out in the CosmoCaixa museum, a flagship science museum in Barcelona that is sponsored by La Caixa

1 Institute for Integrative Systems Biology (I2SysBio), Universitat de València-CSIC, Valencia, Spain. E-mail: manuel.porcar@uv.es
2 Darwin Bioprospecting Excellence SL, Valencia, Spain
3 Language Theory and Communication Sciences Department (UV), Universitat de València, Valencia, Spain
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MICROBIOLOGY SOCIETY
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Sagittula salina sp. nov., isolated from marine waste

Leila Satari[1], Esther Molina-Menor[1], Àngela Vidal-Verdú[1], Javier Pascual[2], Juli Peretó[1,2,3] and Manuel Porcar[1,2,*]

Abstract

A novel Gram-stain-negative, non-motile, halophilic bacterium designated strain M10.9X^T was isolated from the inner sediment of an aluminium can collected from the Mediterranean Sea (València, Spain). Cells of strain M10.9X^T were rod-shaped and occasionally formed aggregates. The strain was oxidase-negative and catalase-positive, and showed a slightly psychrophilic, neutrophilic and slightly halophilic metabolism. The phylogenetic analyses revealed that strain M10.9X^T was closely related to *Sagittula stellata* E-37^T and *Sagittula marina* F028-2^T. The genomic G+C content of strain M10.9X^T was 65.2mol%. The average nucleotide identity and digital DNA–DNA hybridization values were 76.6 and 20.9%, respectively, confirming its adscription to a new species within the genus *Sagittula*. The major cellular fatty acids were $C_{18:1}\,\omega7c/C_{18:1}\,\omega6c$ and $C_{16:0}$. The polar lipids consisted of phosphatidylglycerol, phosphatidylethanolamine, an unidentified aminolipid, an unidentified glycolipid, an unidentified phospholipid and an unidentified lipid. According to the resuts of a polyphasic study, strain M10.9X^T represents a novel species of the genus *Sagittula* for which the name *Sagittula salina* sp. nov. (type strain M10.9X^T=DSM 112301^T=CECT 30307^T) is proposed.

The genus *Sagittula* was first described by González *et al.* in 1997 and reclassified within the *Rhodobacteraceae* family by Lee *et al.* in 2012 [1, 2]. At the time of writing, the genus *Sagittula* is composed of only two species: *Sagittula stellata* and *Sagittula marina*, both isolated from marine environments and promising strains with bioremediation capacities [1–3]. In the present research, we describe the polyphasic characterization of strain M10.9X^T, which was isolated from the inner sediment of an aluminium can during the study on the microbial diversity of marine waste. Anthropic residue distributed worldwide represent a major environmental problem and constitute new ecological niches which may harbour potential new microbial species.

Strain M10.9X^T was isolated from the inner sediment of a can collected from Malva-rosa beach, on the western Mediterranean Coast (València, Spain; 39° 27′ 48.3″ N 0° 19′ 07.6″ E), during a study of the microbial communities associated with marine waste residues [4]. The sediment was resuspended in PBS (1×, pH 7.4) and 50 µl was then spread on marine agar (MA; Laboratorios Conda S.A. Ref: 1059). The plates were incubated at 18 °C for a week. Strain isolation was carried out by restreaking on fresh media until pure cultures were obtained. Cell suspensions in marine broth (MB; Laboratorios Conda S.A. Ref: 1217) supplemented with 15% glycerol (v/v) were cryopreserved at −80 °C. A polyphasic approach was followed in order to determine the taxonomic status of strain M10.9X^T. After isolation, analysis of the 16S rRNA gene sequence in EzBioCloud revealed that *S. stellata* DSM 11524^T and *S. marina* DSM 102235^T were closely related to strain M10.9X^T. Therefore, these strains were selected as comparative strains. Unless otherwise specified, the reference strains *S. stellata* DSM 11524^T and *S. marina* DSM 102235^T, from the DSMZ (German Collection of Microorganisms and Cell Cultures, Leibniz Institute, Braunschweig, Germany), and strain M10.9X^T were grown simultaneously on MA medium at 30 °C.

The phenotypic characterization of strain M10.9X^T was carried out after a week of growth at 30 °C. The Gram-staining test was performed with KOH 3% (w/v), recording viscosity as a positive result for Gram-negative bacteria [5, 6]. In order to test oxidase activity, a commercial oxidase test stick for microbiology (PanReac AppliChem) was used following the manufacturer's instructions. Hydrogen peroxide 30% (v/v) was used to test catalase activity, by recording bubble formation as a positive result

Author affiliations: [1]Institute for Integrative Systems Biology I2SysBio, Universitat de València-CSIC, Calle del Catedràtic Agustin Escardino Benlloch 9, 46980 Paterna, Spain; [2]Darwin Bioprospecting Excellence SL. Parc Científic Universitat de València, Calle del Catedràtic Agustin Escardino Benlloch 9, 46980 Paterna, Spain; [3]Departament de Bioquímica i Biologia Molecular, Universitat de València, Calle del Dr. Moliner 50, 46100 Burjassot, Spain.
***Correspondence:** Manuel Porcar, manuel.porcar@csic.es; manuel.porcar@uv.es; mporcar@darwinbioprospecting.com
Keywords: *Sagittula*; marine waste; new species; *Rhodobacteraceae*; *Alphaproteobacteria*.
Abbreviations: ANIb, average nucleotide identity; dDDH, digital DNA–DNA hybridization; MA, marine agar; MB, marine broth; ML, maximum-likelihood; NJ, neighbour-joining.
The 16S rRNA gene sequence of strain M10.9X^T has been deposited in DDBJ/ENA/GenBank under the accession number MW785249. The genomic sequence of strain M10.9X^T has been deposited under the DDBJ/ENA/GenBank accession number JAGISH000000000.
Two supplementary tables and one supplementary figure are available with the online version of this article.

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RESEARCH ARTICLE

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MICROBIOLOGY SOCIETY

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Maritalea mediterranea sp. nov., isolated from marine plastic residues

Àngela Vidal-Verdú[1], Esther Molina-Menor[1], Leila Satari[1], Javier Pascual[2], Juli Peretó[1,2,3] and Manuel Porcar[1,2,3,*]

Abstract

A novel Gram-reaction-negative, aerobic, motile, rod-shaped, grey bacterium, strain P4.10X^T, was isolated from plastic debris sampled from shallow waters in the Mediterranean Sea (Valencia, Spain). P4.10X^T was catalase- and oxidase-positive, and grew under mesophilic, neutrophilic and halophilic conditions. The 16S rRNA gene sequences revealed that P4.10X^T was closely related to *Maritalea myrionectae* DSM 19524^T and *Maritalea mobilis* E6^T (98.25 and 98.03% sequence similarity, respectively). The DNA G+C content of the genome sequence of P4.10X^T was 53.66%. The genomic indexes average nucleotide identity by BLAST (ANIb) and digital DNA–DNA hybridization (dDDH) confirmed its classification as representing a novel species of the genus *Maritalea*. The predominant fatty acids were summed feature 8 ($C_{18:1}\omega7c/C_{18:1}\omega6c$) and $C_{18:1}$ $\omega7c$ 11-methyl. The results of this polyphasic study confirm that P4.10X^T represents a novel species of the genus *Maritalea*, for which the name *Maritalea mediterranea* sp. nov. is proposed (type strain P4.10X^T=CECT 30306^T = DSM 112386^T).

The genus *Maritalea*, which was first described by Hwang *et al.* [1], is composed of three species with validly published names [2]. Species of the genus *Maritalea* are all Gram-negative bacillar bacteria, aerobic, motile as well as oxidase- and catalase-positive. These species were all isolated from marine environments: *Maritalea myrionectae* was isolated from a marine ciliate [1], whereas *Maritalea mobilis* (previously named *Zhangella mobilis*) and *Maritalea porphyrae* were isolated from red algae [3, 4]. We describe here the polyphasic characterization of strain P4.10X^T, which was isolated from the inner sediments of marine plastic residues (a PET bottle) retrieved from the Malva-rosa beach (València, Spain) (39° 27' 48.3"N 0° 19' 07.6"W), on the western Mediterranean coast in València (Spain). The isolation of the strain was carried out during a study on the microbial communities that inhabit the sediments trapped in plastic residues in marine environments [5].

The inner sediments of marine anthropic residues (plastic bottles and aluminium cans) were sampled and homogenized in sterile phosphate buffered saline (PBS; 8 NaCl, 0.2 KCl, 1.44 $Na_2HPO_4{\cdot}2H_2O$, 0.24 KH2PO4 g/L) pH 7.4 at a final ratio of 1:4 (v/v). Serial dilutions of the suspensions were prepared and spread on Marine Agar (MA) (Laboratorios Conda). The plates were incubated at 18 °C under aerobic conditions. The strain was re-streaked on fresh media until a pure culture was obtained and cryopreserved at −80 °C in Marine Broth supplemented with 15% glycerol (v/v) until required. The taxonomic position of this strain was determined through a polyphasic approach. According to the 16S rRNA identity, P4.10X^T was closely related to members of the genus *Maritalea*. The reference strains *Maritalea myrionectae* DSM 19524^T, *Maritalea mobilis* JCM 15144^T and *Maritalea porphyrae* LGM 25872^T, from the DSMZ (German Collection of Microorganisms and Cell Cultures, Leibniz Institute, Braunschweig, Germany), the JCM-Japan Collection of Microorganisms (RIKEN BioResource Research Centre, Japan) and the BCCM/LGM-Belgian Coordinated Collections of Microorganisms (Gent, Belgium), respectively, were used as reference strains for comparative analysis. Unless otherwise specified, P4.10X^T and the reference strains were all grown in parallel on MA media at 30 °C.

Author affiliations: [1]Institute for Integrative Systems Biology I2SysBio (Universitat de València-CSIC), Calle del Catedràtic Agustin Escardino Benlloch 9, 46980 Paterna, Spain; [2]Darwin Bioprospecting Excellence SL. Parc Científic Universitat de València, Calle del Catedràtic Agustin Escardino Benlloch 9, 46980 Paterna, Spain; [3]Departament de Bioquímica i Biologia Molecular, Universitat de València, Calle del Dr. Moliner 50, 46100 Burjassot, Spain.
***Correspondence:** Manuel Porcar, manuel.porcar@uv.es
Keywords: novel species; Alphaproteobacteria; *Maritalea*; marine plastic waste; marine sediment.
Abbreviations: ANIb, Average Nucleotide Identity by BLAST; dDDH, Digital DNA Hybridization; GSI, Gene Support Index; ML, Maximum Likelihood; NJ, Neighbour Joining.
The 16S rRNA gene sequence of strain P4.10X^T have been deposited in GenBank under the accession number MZ994596. The genomic assembly of strain P4.10X^T has been deposited under the GenBank accession number ASM2156869v1.
One supplementary figures and two supplementary tables are available with the online version of this article.

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ORIGINAL ARTICLE

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Living in a bottle: Bacteria from sediment-associated Mediterranean waste and potential growth on polyethylene terephthalate

Àngela Vidal-Verdú[1] | Adriel Latorre-Pérez[2] | Esther Molina-Menor[1] | Joaquin Baixeras[3] | Juli Peretó[1,2,4] | Manuel Porcar[1,2]

[1]Institute for Integrative Systems Biology (I2SysBio), University of Valencia-CSIC, Paterna, Spain

[2]Darwin Bioprospecting Excellence S.L., Paterna, Spain

[3]Cavanilles Institute of Biodiversity and Evolutionary Biology, University of Valencia, Paterna, Spain

[4]Department of Biochemistry and Molecular Biology, University of Valencia, Burjassot, Spain

Correspondence

Manuel Porcar, Institute for Integrative Systems Biology (I2SysBio), University of Valencia-CSIC, Catedrático José Beltrán, 2, Paterna 46980, Spain.
Email: manuel.porcar@uv.es

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Abstract

Ocean pollution is a worldwide environmental challenge that could be partially tackled through microbial applications. To shed light on the diversity and applications of the bacterial communities that inhabit the sediments trapped in artificial containers, we analyzed residues (polyethylene terephthalate [PET] bottles and aluminum cans) collected from the Mediterranean Sea by scanning electron microscopy and next generation sequencing. Moreover, we set a collection of culturable bacteria from the plastisphere that were screened for their ability to use PET as a carbon source. Our results reveal that *Proteobacteria* are the predominant phylum in all the samples and that *Rhodobacteraceae*, *Woeseia*, *Actinomarinales*, or *Vibrio* are also abundant in these residues. Moreover, we identified marine isolates with enhanced growth in the presence of PET: *Aquimarina intermedia*, *Citricoccus* spp., and *Micrococcus* spp. Our results suggest that the marine environment is a source of biotechnologically promising bacterial isolates that may use PET or PET additives as carbon sources.

KEYWORDS

bioprospecting, bioremediation, marine sediments, marine waste, plastic-degrading microorganisms, polyethylene terephthalate

1 | INTRODUCTION

Plastic production and, subsequently, plastic waste have increased exponentially through the last decades (Worm et al., 2017). The poor management of these residues, and their resistance to natural degradation (in some cases it comprises from hundreds to thousands of years) (Barnes et al., 2009), has resulted in a major, worldwide problem of plastic accumulation in all ecosystems on Earth. Even though the amount of recycled plastic has doubled from 2006 to 2018, the amount of post-consumer waste plastic that is sent to landfills in Europe was still 25% in 2018 (PlasticsEurope, 2020).

Gillisia lutea sp. nov., isolated from marine aluminum residues from the Malva-rosa beach (València, Spain)

Àngela Vidal-Verdú[1], **Esther Molina-Menor[1]**, Javier Pascual[2], Juli Peretó[1,2,3] and Manuel Porcar[1,2].

1. Institute for Integrative Systems Biology I2SysBio, Universitat de València-CSIC, Paterna 46980, Spain.
2. Darwin Bioprospecting Excellence S.L. Parc Científic Universitat de València, Paterna 46980, Spain.
3. Departament de Bioquimica i Biologia Molecular, Facultat de Ciències Biològiques, Universitat de València, Burjassot 46100, Spain.

Summary

A new Gram-negative, facultative anaerobic, rod-shaped, non-motile, non-spore forming, orange-pigmented bacterium identified as strain M10.2A^T, was isolated from marine residues submerged on the Malva-rosa beach (València, Spain), on the western coast of the Mediterranean Sea. This strain was catalase-positive and oxidase-negative, and grew under mesophilic, neutrophilic and halophilic conditions. According to the 16S rRNA gene sequences, strain M10.2A^T showed similarities with *Gillisia mitskevichiae* DSM 19839^T and *Gillisia hiemivida* IC154^T (97.57 and 97.50 % gene sequence similarity, respectively). The genome of strain M10.2A^T was sequenced and deposited in the DDBJ/ENA/GenBank databases under the accession number JAKGTH000000000. The genomic G+C content was 36.13 %. Its adscription to a new *Gillisia* species was confirmed by the genomic indexes average nucleotide identity (ANIb) and digital DNA-DNA hybridization (dDDH). The major fatty acids were iso-$C_{15:0}$, iso-$C_{15:1}$ G, iso-$C_{16:0}$ 3-OH, iso-$C_{17:0}$ 3-OH and summed feature 3 ($C_{16:1}$ ω7*c*/$C_{16:1}$ ω6*c*). According to a polyphasic study, strain M10.2A^T represents a novel species in the genus *Gillisia*, for which name *Gillisia lutea* sp. nov. (type strain M10.2A^T = CECT 30308^T = DSM 112385^T) is proposed.

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