RESEARCH ARTICLE

Vibrio vulnificus produces *quorum sensing* signals of the AHL-class

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Abstract

Vibrio vulnificus is an aquatic pathogenic bacterium that can cause vibriosis in humans and fish. The species is subdivided into three biotypes with the fish-virulent strains belonging to biotype 2. The quorum sensing (QS) phenomenon mediated by furanosyl borate diester or autoinducer 2 (AI-2) has been described in human strains of biotype 1, and here we show that the *luxS* gene which encodes AI-2 is present in all strains of *V. vulnificus* regardless of origin, biotype or serovar. In this study, we also demonstrate that *V. vulnificus* produces QS signals of the acylated homoserine lactone (AHL) class (AI-1). AHLs were detected in strains of biotype 1 and 2 from water, fish and human wound infections but not in strains isolated from human septicaemic cases. The AHL compound was identified as *N*-butanoyl-homoserine-lactone (C_4-HL) by both reporter strains and by HPLC-high-resolution MS. C_4-HL was detected when AHL-positive strains were grown in low-nutrient medium [modified sea water yeast extract (MSWYE)] but not in rich media (tryptic soy broth or brain–heart infusion) and its production was enhanced when blood factors were added to MSWYE. C_4-HL was detected *in vivo*, in eels infected with AHL-positive biotype 2 strains. No known AHL-related gene was detected by PCR or Southern blot suggesting that AHL-related genes in *V. vulnificus* are different from those found in other Gram-negative bacteria.

Introduction

Vibrio vulnificus is an aquatic bacterium that can infect humans and fish and cause vibriosis (Tison *et al.*, 1982; Amaro & Biosca, 1996; Bisharat *et al.*, 1999; Linkous & Oliver, 1999; Strom & Paranjpye, 2000; Oliver, 2006). Both human and fish vibriosis can be transmitted by water and give rise to lethal septicaemia if the innate defenses do not act in time and properly (Strom & Paranjpye, 2000; Oliver, 2006; Valiente *et al.*, 2008b). *Vibrio vulnificus* is a heterogeneous species and has been divided into three biotypes. Biotype 2 includes fish-virulent strains grouped in different serovars (Biosca *et al.*, 1996, 1997; Hoi *et al.*, 1998; Fouz & Amaro, 2003), of which serovar E is the most virulent (Biosca *et al.*, 1997; Hoi *et al.*, 1998). This serovar is termed zoonotic because it can also sporadically infect humans (Amaro & Biosca, 1996). The genetic homology of the three biotypes is not known, although the genomes of two biotype-1 human strains have been available since 2003 (Chen *et al.*, 2003; Kim *et al.*, 2003b). A comparison of these genomes reveals that they differ in genomic islands and plasmids content (Chen *et al.*, 2003; Kim *et al.*, 2003b; Quirke *et al.*, 2006). Interestingly, the ability to infect and develop septicaemia in fish is dependent on a virulence plasmid specific to biotype 2 strains (Lee *et al.*, 2008; Valiente *et al.*, 2008b), underlining the importance of plasmids in the genetic diversity of this species.

Quorum sensing (QS) is a term used to denote a regulatory mechanism used by several bacteria especially to regulate or modulate production of extracellular compounds at high cell densities. QS relies on signalling molecules (so-called autoinducers, AI) that accumulate as bacterial population density increases, and bind to specific receptors. When a particular threshold concentration has been reached, this triggers the expression of genes involved in a different stage of the life cycle of the bacterium (Bassler, 1999). QS regulatory systems have been found in several *Vibrio* species (Stevens & Greenberg, 1997; Freeman &

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Bassler, 1999; Camara *et al.*, 2002; Kim *et al.*, 2003a), with the model QS-regulated systems being the bioluminescent phenotype of *Vibrio fischeri* and *Vibrio harveyi* in particular. The operons *luxRI* and *luxMN* code for the production of AI-1 signal molecules (or acylated homoserine lactones, AHL) and for the transcriptional regulator in *V. fischeri* and *V. harveyi,* respectively, whereas the operon *luxSP/Q* is involved in QS based on the AI-2 (or furanosyl borate diester) system in both species (Bassler *et al.*, 1993; Schauder *et al.*, 2001).

Vibrio vulnificus biotype 1 seems to produce only AI-2-type compounds (Kim *et al.*, 2003a; Bruhn *et al.*, 2005) because AI-2- but not AI-1-type compounds are produced by the type strain of the species (a biotype-1 human strain) (Kim *et al.*, 2003a) and AI-2- but not AI-1-related genes are present in the genome of the two sequenced strains (Chen *et al.*, 2003; Kim *et al.*, 2003b). In addition, mutations in *luxS* reduce virulence of *V. vulnificus* biotype 1 in mice, affecting bacterial survival in different conditions, luminescence, motility, biofilm formation, production of protease and haemolysin as well as stress responses (McDougald *et al.*, 2001; Kim *et al.*, 2003a, b; Shin *et al.*, 2005). Similar properties appear to also be influenced by *luxS* in other bacteria (Surette & Bassler, 1999; Lyon *et al.*, 2001; Sperandio *et al.*, 2002; Wen & Burne, 2002; Zhu *et al.*, 2002; McDougald *et al.*, 2003).

The purpose of the present work was to determine whether *V. vulnificus* strains other than biotype-1 human strains possess compounds or systems indicative of QS regulation. To this end, we screened several *V. vulnificus* strains belonging to different biotypes and serovars for AI-1 compound production (AHLs) *in vitro* and *in vivo* and also for the presence of the *luxS* gene. Indeed, an AHL compound was detected; therefore, our study also included a search for genes related to AHL production, which was performed by PCR and Southern blot with primers and probes from systems described in other bacteria.

Materials and methods

Bacterial strains and growth conditions

A selection of previously characterized *V. vulnificus* strains (Sanjuan *et al.*, 2009) was used in this study (Table 1). To detect AHL production, the modified sea water yeast extract broth or agar plus 1% NaCl (MSWYE or MSWYE agar) (Oliver & Colwell, 1973) or Brain–Heart Infusion broth or agar plus 1% NaCl (BHI-1 or BHI-1 agar) were used. These media were supplemented with $FeSO_4 \cdot 7H_2O$ (5 μM) (iron-rich medium) or 2, 2′-dipyridil (5 μM) (iron-poor medium) to test the influence of iron availability, or with eel mucus (5%, v/v), bovine mucin, eel or human blood (5%, v/v), eel or human erythrocytes (5%, v/v), eel or human lysed erythrocytes (5%, v/v), fresh or heated (56–60 °C, 0.5–1 h) human or eel serum (5%, v/v), human transferrin (0.5%, v/v), bovine haemoglobin (0.5%, v/v) or bovine fibrinogen (0.5%, v/v) to test the influence of different host factors on AHL production. All bovine and human products were purchased from Sigma while those from eels were obtained as described below.

Table 1. Origin, biotype, serovar and ribogroup of the *Vibrio vulnificus* strains used in this study

Strains	Source and country	Biotype†	Serovar†	Ribogroup†
YJ016	Human blood, Taiwan	1	No-EAI*	A
CECT 5164	Human blood, USA	1	No-EAI*	A
CG100	Oyster, Taiwan	1	No-EAI*	B
ATCC 33816	Human blood, USA	1	No-EAI*	A
CECT 4606	Healthy eel, Spain	1	No-EAI*	B
94-9-119	Human wound, Denmark	1	No-EAI*	B
CECT 4608	Fishfarm water, Spain	1	No-EAI*	B
ATCC 27562	Human blood, USA	1	No-EAI*	B
CECT 4602	Diseased eel, Spain	2	E	B
CECT 4604	Diseased eel, Spain	2	E	B
CECT 4867	Diseased eel, Spain	2	E	B
CECT 4917	Diseased eel, Spain	2	E	B
CECT 4999	Diseased eel, Spain	2	E	B
CECT 5763	Ebro Delta, Spain	2	E	B
CCUG 38521	Human wound, Sweden	2	E	B
C1	Healthy eel, Spain	2	E	B
94-9-123	Seawater, Denmark	2	E	B
CECT 5198	Diseased eel, Spain	2	A	B
CECT 5769	Diseased eel, Spain	2	A	B
CECT 7030	Diseased eel, Denmark	2	A	B
CECT 7029	Diseased eel, Denmark	2	A	B
95-8-161	Diseased eel, Denmark	2	I	B
95-8-162	Diseased eel, Denmark	2	I	B
95-8-6	Diseased eel, Denmark	2	I	B
162	Human, Israel	3	No-EAI*	B
11028	Human, Israel	3	No-EAI*	B

†Biotype, serovar and ribogroup were determined by Sanjuan *et al.* (2009).

*No serovar E, A or I.

Chromobacterium violaceum CV026 was used as reporter strain in bioassays to detect AHLs of four to eight carbon atoms (Throup *et al.*, 1995; McClean *et al.*, 1997) while *Agrobacterium tumefaciens* NT1 (pZLR4) (Cha *et al.*, 1998) was used to detect AHLs with intermediate carbon chains and 3-oxo-AHL (McClean *et al.*, 1997). *Aeromonas salmonicida* 894^T was used as positive control of AHL production in the *C. violaceum* bioassay. Finally, the strains *V. vulnificus* YJ016 (*luxS*), *V. fischeri* CECT 524^T (*luxR*, *luxI*), *Aeromonas sobria* CECT 4834 (*luxAeI*, *luxAeR*), *Serratia marcescens* SS-1 (*spnR*, *spnI*) and *V. fischeri* ES114 (*luxM*, *luxN*) were used as positive controls for PCR and Southern hybridizations. *Vibrio* spp. were routinely grown in tryptic soy broth or agar plus 1% NaCl (TSB-1, TSA-1), *A. sobria* and *A. salmonicida*

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were grown in TSB and TSA and *S. marcescens* in nutrient agar II, all of them at 28 °C, 24–48 h. *Agrobacterium tumefaciens* was grown in Luria–Bertani broth 1% NaCl (LB-1) or ABt agar (Clark & Maaløe, 1967), both supplemented with tetracycline 4.5 μg mL^{-1} and spectinomycin 50 μg mL^{-1} (ABt-AA) and *C. violaceum* was grown in LB supplemented with kanamycin 20 μg mL^{-1} (LB-AA).

All the strains were maintained both as lyophilized stock at room temperature (25 °C) and as frozen stock at − 80 °C in marine broth (Difco) plus 20% (v/v) glycerol.

Experimental fish and maintenance conditions

Eels (*Anguilla anguilla*) of 10 g were used for virulence determination, of 125 g for AHL detection *in vivo* and of 1 kg for blood and mucus extraction. Fish were held at 28 ± 2 °C in glass tanks containing dechlorinated water (1% NaCl) supplied with filtration and air systems and were fed with a commercial diet throughout the experiments.

Collection of blood, blood factors and mucus from eels

Blood, collected as described previously (Esteve-Gassent *et al.*, 2004), was directly added to agar plates or centrifuged at 400 ***g*** (5 min) for erythrocyte separation. Washed erythrocytes [5% in phosphate-buffered saline 1% NaCl (PBS-1), pH 7] were used for the haemolysin assay. Non-heparinized blood was allowed to clot at room temperature and serum was directly added to agar plates (5% v/v) or heated at 56 °C for 30 min to obtain inactivated eel serum. Finally, skin mucus from the surface of nonanaesthetized eels was obtained as described previously (Esteve-Gassent *et al.*, 2004).

Bioassays for AHL *in vitro* and *in vivo* production

In vitro production

The entire agar plate with bacterial growth was mixed with 30 mL of acidified ethyl acetate (0.5% formic acid), homogenized with an Ultraturex (IKA Labortechnik) and filtered through nitrocellulose filters (0.45 μm) (Millipore). The recovered ethyl acetate was dried under nitrogen flow and redissolved in 100 μL of acidified ethyl acetate (0.5% formic acid). The extracts were stored at − 20 °C until testing in AHL bioassays. In parallel, broth-grown cells were harvested (300 ***g*** × 10 min), and 6 mL of supernatant was filtered through nitrocellulose filters (0.22 μm) (Millipore). The filtrate was lyophilized and dissolved in 200 μL of distilled water, which was used in the bioassays. Before the extractions, bacterial numbers were determined by culturable counts on TSA-1 plates.

In vivo production

Groups of five eels were injected intraperitoneally with 0.1 mL of PBS (control group) or 0.1 mL of a bacterial suspension of 10^7 CFU mL^{-1} in PBS (test group) from biotype-2 strains that were positive (CECT 5763 and CECT 7029, Table 1) for AHL in the *in vitro* assay. Mortality was followed up for 10 days and samples of *c.* 2 g of mixed internal organs (liver, spleen and posterior kidney) and muscle from dead, moribund and surviving fish from control and test groups were taken for both bacterial isolation and AHL detection. The organs were mixed with 30 mL of acidified 0.5% formic acid ethyl acetate and the mixture was homogenized with an Ultraturex (IKA Labortechnik), filtered through nitrocellulose filters (0.45 μm), dried under nitrogen flow and redissolved in acidified ethyl acetate (0.5% formic acid).

Bioassay

Bioassays were performed according to Ravn *et al.* (2001). Briefly, 50 mL of ABt-broth grown with *Agr. tumefaciens* or LB broth grown with *C. violaceum* were mixed with 100 mL of ABt agar (supplemented with 75 μg mL^{-1} X-gal) or with 100 mL of LB-1 agar maintained at 46 °C, respectively. The mixtures were immediately poured as two 75-mL portions into Petri dishes (14 cm diameter) and wells of 10 mm diameter were drilled after agar solidification. Then, a volume of 60 μL of sample was added per well and plates were incubated for 48 h at 25 °C. Samples surrounded by blue (*Agr. tumefaciens*) or purple (*C. violaceum*) halos were considered positive.

Determination of the chemical structure of AHL by HPLC-high-resolution MS (HRMS) analysis

Ethyl acetate extracts were evaporated to dryness in vacuum, dissolved in 100 μL methanol : water (1 : 1), filtered through a 0.45-μm PFTE syringe filter and analysed by HPLC-positive electrospray-HRMS on an LCT orthogonal time of flight mass spectrometer (Micromass, Manchester, UK) as described previously (Nielsen & Smedsgaard, 2003). Lock mass was not used. Separation was performed on a Phenomenex (Torrance, CA) Luna II C_{18} (II), 3 μm, 50 × 2 mm column with a precolumn, using a gradient system of water (MilliQ) containing 10 mM ammonium formate and 20 mM formic acid (both analytical grade) and acetonitrile (gradient grade) containing 20 mM formic acid. A flow of 0.3 mL min^{-1} was used, starting with 5% acetonitrile for 2 min, which was increased linearly to 100% in 18 min and then held for 5 min, before returning to 5% acetonitrile in 4 min and then equilibrated for 7 min. The following reference standards were coanalysed in the sequence: *N*-butanoyl homoserine lactone, *N*-hexanoyl

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homoserine lactone, *N*-octanoyl homoserine lactone, *N*-decanoyl homoserine lactone, *N*-dodecanoyl homoserine lactone, *N*-tetradecanoyl homoserine lactone, *N*-3-oxo-hexanoyl homoserine lactone and *N*-3-oxo-octanoyl homoserine lactone.

Molecular detection and sequence analysis of *luxS* and AHL-related genes (*luxR, luxI, spnRluxM, luxN*)

DNA was isolated from pure cultures of each strain grown in 5 mL LB-1 overnight at 28 °C. Cells were harvested by centrifugation for 5 min at 300 ***g***. Genomic DNA was isolated using a DNA genomic extraction kit (Durviz). DNA manipulations were performed as described previously (Sambrook *et al*., 2001). The strains used as positive controls for PCR and Southern blot are indicated in the section 'Bacterial strains and growth conditions'.

PCR

The sequences used to design the primers, the primers and the amplicon size are indicated in Table 3. The PCR conditions were the following: 50 μL reaction mixture containing 1.2 μL of each primer (10 mM), 0.3 μL of Taq DNA polymerase (GoTaq, Promega; 5 U μL^{-1}), 10 μL of 5 × Taq reaction buffer (Promega, Gotaq Green), 3.0 μL of 50 mM $MgCl_2$, 0.5 of dNTP mix (Promega, 10 mM of each) and 2 μL of template DNA (50 ng μL^{-1}). The PCR was performed in a Techne thermocycler (TC-412). The reaction started with 10 min of denaturation at 94 °C, which was followed by 45 cycles of 40 s of denaturation at 94 °C, 45 s of annealing at 45 °C and 90 s of extension at 72 °C and an additional extension at 72 °C for 10 min.

Southern blot

The amplified products from the positive control strains with the primers showed in Table 3 were labelled with digoxigenin as described previously (Roig & Amaro, 2009) and were used as probes for Southern hybridization. Southern blot was performed essentially as described by Roig & Amaro (2009). Digested genomic DNA of positive control strains for each gene and those of AHL-positive and AHL-negative *V. vulnificus* strains were used as template. Approximately 1 μg of genomic DNA was digested overnight with an excess of restriction enzyme (30 U) of HincII and HincIII (Takara).

Gene sequencing

It was carried out using the dideoxy-chain termination method (Sambrook *et al*., 2001) with the ABI Prism dye terminator cycle sequencing kit (Perkin-Elmer). Oligonucleotides were purchased from Pharmacia LKB Biotechnology. The multiple sequence alignment was processed using CLUSTAL W software (Thompson *et al*., 1994).

Results

Detection of AHL and determination of its chemical structure

The *V. vulnificus* strains used in this study belong to the biotypes, serovars and ribogroups described in the species (Table 1). None of the strains were positive in the AHL bioassay performed with *Agr. tumefaciens*, which detects intermediate-sized AHL (from 4 to 12 carbon atoms in the chain with oxo substitution) while nine strains (35%) were positive in the bioassay with *C. violaceum* CV026 (Table 2) which detects short–medium-sized AHL. There were positive strains among biotype 1 (25%) and 2 (34%) isolates, but not among biotype 3 isolates. AHL-positive biotype 2 strains included all three serovars. Finally, all AHL-positive strains belonged to ribogroup B (Table 1), a ribogroup that clusters biotypes 2 and 3 strains together with environmental and human wound isolates of biotype 1 according to Sanjuan *et al*. (2009).

All AHL-positive strains produced AHLs when grown in the low-nutrient medium MSWYE supplemented with blood additives (Table 2) but not when grown in the rich medium BHI-1 supplemented with the same additives (data not shown). In addition, the AHL compounds were only detected by the bioassay when the population size in MSWYE or supplemented MSWYE exceeded 10^9 CFU mL^{-1}. Under these conditions, the AHLs were detected after 24 and 48 h of incubation. Iron addition, i.e. comparing AHL detection from iron-rich (MSWYE plus ferric sulphate) or iron-poor medium (MSWYE plus an iron chelator), did not appear to influence AHL production as indicated by the halo size (Table 2). However, the AHL induction zone increased when host factors from blood were added to the low-nutrient medium MSWYE, with the largest positive zones being around extracts from medium supplemented with inactivated serum or transferrin (Table 2 and Fig. 1). AHL halos also increased when cultures had been grown with purified haemoglobin and fibrinogen but not with mucin, a nonblood additive (Table 2). AHLs were detected in extracts from *A. salmonicida* (positive control) but not from any of the supplements tested directly on the plates (negative control). None of the strains were positive when fresh eel or human sera were used as supplement for the growth media (Table 2).

To determine whether *V. vulnificus* produced AHLs *in vivo*, we selected one AHL-positive biotype-2 strain, strain CECT 5763, infected eels intraperitonially and analysed

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Table 2. Size of AHL-induced halos (in cm) in the AHL bioassay with the reporter bacterium *Chromobacterium violaceum*

Strains	MSWYE	MSWYE+ Eel blood	Eel erythrocytes	Lysed eel erythrocytes	Eel serum Fresh	Eel serum Heated	Human erythrocytes	Lysed human erythrocytes	Human serum Fresh	Human serum Heated	Human transferrin	Bovine haemoglobin	Human fibrinogen	Mucin
YJ016	–	–	–	–	–	–	–	–	–	–	–	–	–	NT
CECT 5164	–	–	–	–	–	–	–	–	–	–	–	–	–	NT
CG100	–	–	–	–	–	–	–	–	–	–	–	–	–	NT
ATCC 33816	–	–	–	–	–	–	–	–	–	–	–	–	–	NT
CECT 4606	0.10 ± 0.02	2.1 ± 0.1	2.2 ± 0.1	2.2 ± 0.1	–	4.1 ± 0.4	2.1 ± 0.1	2.3 ± 0.2	–	4.0 ± 0.4	4.0 ± 0.3	–	–	NT
94-9-119	–	2.1 ± 0.3	1.3 ± 0.1	2.3 ± 0.1	–	4.2 ± 0.3	2.2 ± 0.1	2.2 ± 0.1	–	4.1 ± 0.4	4.0 ± 0.3	1.3 ± 0.1	1.4 ± 0.2	–
CECT 4608	–	–	–	–	–	–	–	–	–	–	–	–	–	NT
ATTCC 27652	–	–	–	–	–	–	–	–	–	–	–	–	–	NT
CECT 4602	–	–	–	–	–	–	–	–	–	–	–	–	–	NT
CECT 4604	–	–	–	–	–	–	–	–	–	–	–	–	–	NT
CECT 4867	0.10 ± 0.01	2.3 ± 0.2	2.4 ± 0.3	2.2 ± 0.2	–	4.0 ± 0.3	2.2 ± 0.2	2.3 ± 0.2	–	4.0 ± 0.5	4.0 ± 0.3	2.3 ± 0.2	1.3 ± 0.1	–
CECT 4917	–	2.4 ± 0.2	2.3 ± 0.3	1.3 ± 0.3	–	1.2 ± 0.2	1.3 ± 0.1	1.2 ± 0.1	–	1.4 ± 0.1	1.2 ± 0.1	2.2 ± 0.1	1.5 ± 0.1	–
CECT 4999	–	–	–	–	–	–	–	–	–	–	–	–	–	NT
CECT 5763	0.10 ± 0.02	2.4 ± 0.3	2.5 ± 0.2	2.6 ± 0.1	–	4.0 ± 0.4	1.4 ± 0.1	2.5 ± 0.2	–	4.2 ± 0.3	4.0 ± 0.4	2.4 ± 0.2	1.3 ± 0.2	–
CCUG 38521	–	–	–	–	–	–	–	–	–	–	–	–	–	NT
C1	–	–	–	–	–	–	–	–	–	–	–	–	–	NT
94-9-123	0.10 ± 0.02	1.5 ± 0.1	2.3 ± 0.2	2.4 ± 0.2	–	4.0 ± 0.1	2.3 ± 0.2	2.4 ± 0.1	–	4.1 ± 0.4	4.1 ± 0.1	2.4 ± 0.2	1.3 ± 0.1	–
CECT 5198	–	–	–	–	–	–	–	–	–	–	–	–	–	NT
CECT 5769	–	–	–	–	–	–	–	–	–	–	–	–	–	NT
CECT7030	0.10 ± 0.03	2.4 ± 0.2	2.2 ± 0.2	2.2 ± 0.2	–	4.2 ± 0.1	2.5 ± 0.2	2.4 ± 0.3	–	4.0 ± 0.5	4.0 ± 0.3	2.3 ± 0.1	1.2 ± 0.2	–
CECT 7029	0.10 ± 0.02	2.2 ± 0.2	2.4 ± 0.1	2.4 ± 0.1	–	4.1 ± 0.1	2.4 ± 0.1	2.4 ± 0.1	–	4.0 ± 0.4	4.0 ± 0.3	2.5 ± 0.3	1.2 ± 0.1	–
95-8-161	–	–	–	–	–	–	–	–	–	–	–	–	–	NT
95-8-162	–	–	–	–	–	–	–	–	–	–	–	–	–	NT
95-8-6	0.10 ± 0.02	2.3 ± 0.2	1.4 ± 0.1	1.5 ± 0.1	–	4.2 ± 0.3	1.2 ± 0.1	1.3 ± 0.1	–	4.0 ± 0.3	4.2 ± 0.3	1.2 ± 0.1	1.3 ± 0.1	–
162	–	–	–	–	–	–	–	–	–	–	–	–	–	NT
11028	–	–	–	–	–	–	–	–	–	–	–	–	–	NT

NT, non-tested.

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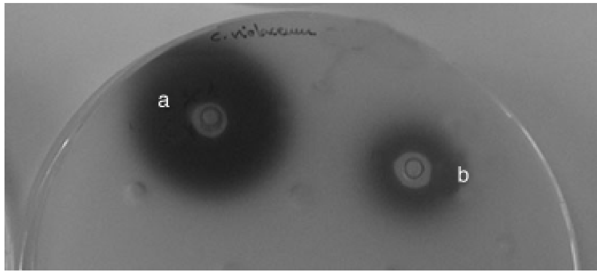

Fig. 1. Bioassay for AHL detection with the reporter strain *Chromobacterium violaceum* CV026 from culture extracts of CECT 5763 in MSWYE supplemented (a) or not supplemented (b) with inactivated serum. The halo corresponds to a positive result.

tissue extracts from moribund eels by the bioassay with *C. violaceum*. We detected AHLs after 24 and 48 h of incubation in a 20% of the samples from the analysed eels.

To determine the chemical structure of the AHL produced by *V. vulnificus* isolates AHL-positive extracts from cultures grown in MSWYE+inactivated eel serum (the optimal conditions for AHL production) and AHL-positive tissue extracts from moribund eels were analysed by HPLC-HRMS. The AHL produced in both cases was unambiguously identified as *N*-butanoyl-homoserine-lactone (Fig. 2), based on correct retention time, same MS spectrum and an accurate mass deviation of 8.7 mDa compared with a reference standard. No other even-chained AHLs with no, one hydroxy or one oxy substitutions was detected. Control samples from noninfected eels were negative.

Detection of AHL-related genes and operons by PCR and Southern Blot

We tried to determine the genetic basis for AHL production in the positive isolates by PCR using the primers in Table 3 to amplify genes homologous to AHL synthases and regulators in *V. fischeri*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio mimicus*, *Vibrio logei*, *Aeromonas* spp. and *S. marcescens*. *Serratia marcescens* is the only case of horizontal transmission of an AHL system (Wei *et al.*, 2006). PCR amplification was unsuccessful, because no amplicons were obtained, apart from those expected for the positive control

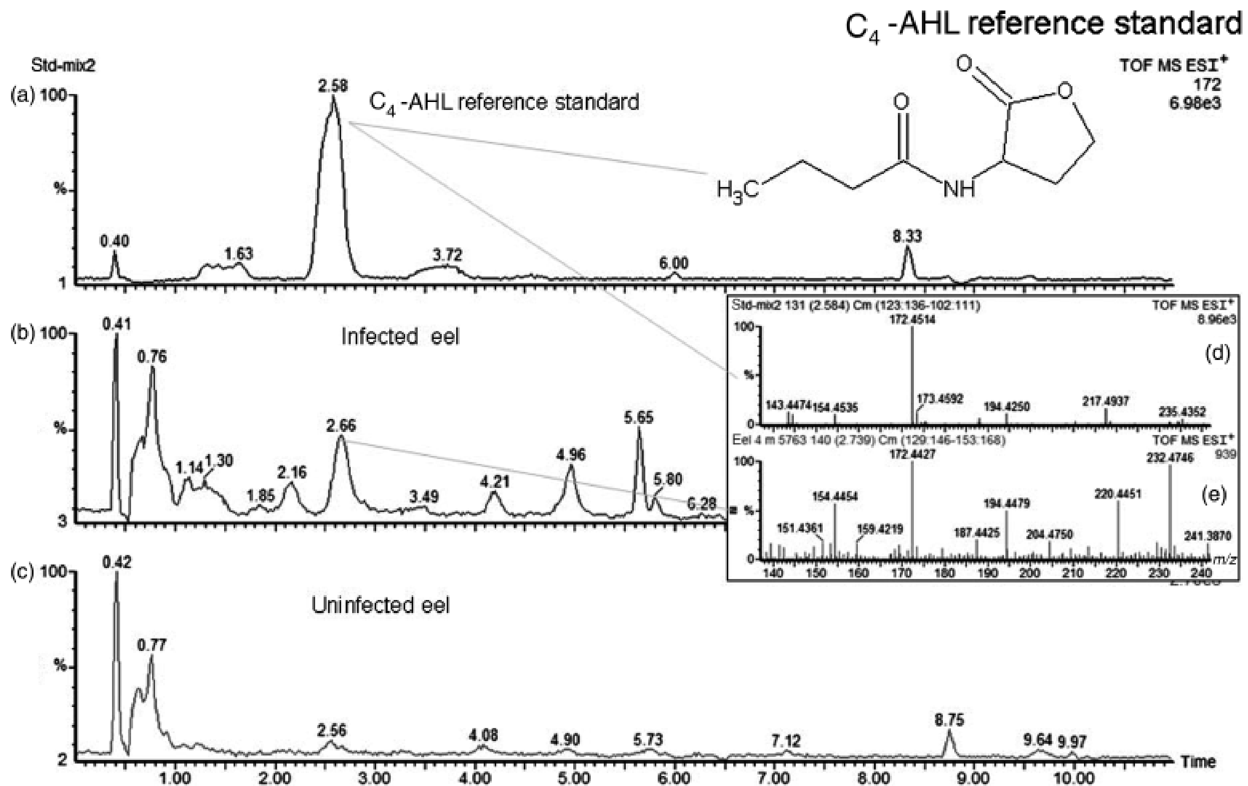

Fig. 2. HPLC-positive electrospray ionization (ESI$^+$)-HRMS detection of *N*-butanoyl-homoserine-lactone (C_4-AHL). (a) C_4-AHL reference standard showing the extracted ion chromatogram of *m/z* 172 corresponding to $[M+H]^+$ ion. (b) Sample from eel organs infected with strain CECT 5763 also showing the *m/z* 172 extracted ion chromatogram. (c) Extract from organs of noninfected eel showing no C_4-AHL. (d) ESI$^+$ spectrum of C_4-AHL reference standard showing the $[M+H]^+$ ion at *m/z* 172 and [M+Na] at 194; and (e) ESI$^+$ spectrum of C_4-AHL in the infected eel sample at the same retention time and with the same ESI$^+$ spectrum as in the reference standard (mass deviation of $[M+H]^+$ 8.7 mDa), giving an unambiguous detection of C_4-AHL in the sample.

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Table 3. Primers used in this work

Sequence name	Primer name	Sequence 5′–3′	Organisms*	Size
luxS	LuxSF	CCGTTGACCACACTCGCAT	*V. vulnificus* (AF305637)†, *V. alginolyticus* (DQ826462), *V. mimicus* (AB232376), *V. harveyi* (AF120098) and *V. schophthalmi* (EF363481)	323
	LuxSR	CCACTTTCAACACATCTTCC		
luxR	LuxRF	ATGCCCACGCTAAACATTCT	*V. fischeri* (AY292977)†, *V. logei* (AY292974), *V. mimicus* (AY292976), *V. parahaemolyticus* (AY292965) and *V. cholerae* (AY292964)	569
	LuxRR	ATGCCGACGACACTTACAGA		
luxI	LuxIF	ACGCAAGGGAGGTTGGTA		237
	LuxIR	GTAGGTAATAAACGCCAGCATCC		
spnR	SpnRF	GAGTCAGTTATTACCTGG	*S. marcescens* (AF389912)†, *S. marcescens* (AB234869), *S. liquefaciens* (AY168877), *Serratia* ATCC 39006 (AJ275980)	582
	SpnRR	CCAGGTAATAACTGACTC		
spnI	SpnIF	GCTGAAGGTGACGCACTCG		443
	SpnIR	GTCTTGATTTGCCATCCT		
luxM	LuxMF	CATTGGTTGGACTTCTGGTG	*V. fischeri* (AB090807)†, *V. splendidus* (AQ987706), *V. anguillarum* (AF288163), *V. harveyi* (AF286004) and *V. parahaemolyticus* (AY216909)	254
	LuxMR	CAGCATCTCGTACCACTT		
luxN	LuxNF	TGATCTTTGGTACTCACCACGC		567
	LuxNR	GACAAGGCTGGTGGTAACC		
luxAeR	luxAeRF	GGTCGGTTCACGCTCGGTATG	*A. sobria* (AY764307, AY987567)†, *A. hydrophila* (AY764300, AY987564), *A. salmonicida* (AY764348, AY987583), *A. bestiarium* (AY764301, AY987565)	344
	luxAeRR	CGGAGGTGATGAAGGAG		
luxAeI	luxAeIF	CTAGGCTGGGATGTCGAGTC		345
	luxAeIR	GCAGGCTGACCACGGCGA		

*Accession number in parentheses.
†Strain used as positive control in PCR and Southern blot.

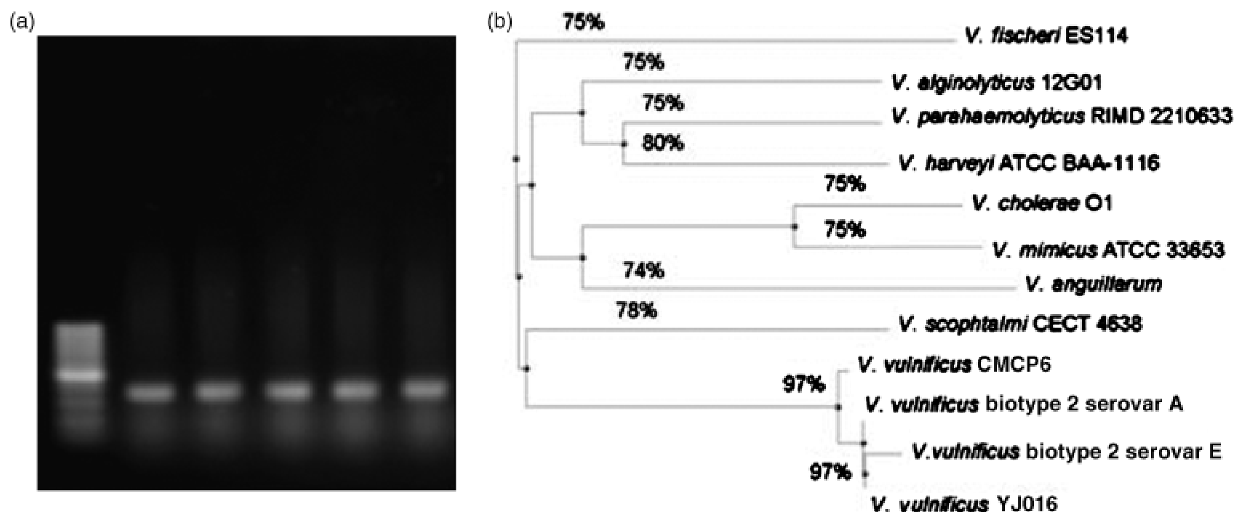

Fig. 3. *luxS* detection by PCR (a) and a distance tree for *luxS* (b) from sequence data of *Vibrio vulnificus* strains of the present work and those from databases.

strains, with only one exception that corresponding to *spnR*-homologous in which a band of the expected molecular size was obtained from both AHL-positive and AHL-negative strains. The results were confirmed by Southern blot analysis. The band was sequenced and the product showed a similarity $< 45\%$ with both *spnR* gene and *smaI* gene

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(another gene related with AHL synthesis in *S. marcencens*). Thus, the apparently positive result for *spnR* could be explained by a nonspecific amplification.

Detection of *luxS* gene by PCR

We also tested *V. vulnificus* strains for the presence of *luxS* by PCR with primers designed from strain YJ016 (Table 3). All *V. vulnificus* strains, regardless of biotype or serovar, gave an amplicon of the expected size, which was confirmed by sequencing (Fig. 3). The whole gene was sequenced for biotype 2 and serovar E strain CECT 4999 (GenBank accession no. EU643832) and for biotype-2 and serovar-A strain CECT 7029 (GenBank accession no. FJ204473) and was compared with the sequences reported for *luxS* in *V. vulnificus* biotype 1 and in other *Vibrio* species. Fig. 3 shows a distance tree based on sequences of *luxS* in which *V. vulnificus* biotypes form a compact group separated from the others, but with a similarity of around 80% with *V. parahaemolyticus*, *V. harveyi* and *Vibrio alginolyticus*.

Discussion

The *V. vulnificus* strains selected for the study are representative of the genetic variability of the species and clustered in a previous study by ribotyping in two groups, one related to environmental origin and the other to septicaemic origin (Table 1) (Sanjuan *et al.*, 2009). These two groups correlate well with the two main lineages previously reported in *V. vulnificus* populations by multilocus sequence analysis (Cohen *et al.*, 2007; E. Sanjuan, F. González & C. Amaro, unpublished data). In this work we found that all the strains, regardless of biotype, serovar and ribogroup were positive for *luxS* amplification by PCR, indicating that the gene for AI-2 biosynthesis is present at species level. The distance tree based on the homology of *luxS* showed a topology similar to the phylogenetic tree based on 16S rRNA analysis, with the two biotypes forming a single group, closely related to *V. parahaemolyticus*, *V. alginolyticus* and *V. harveyi*. This result suggests that the *luxS* gene is an ancestral gene, which has been maintained throughout the diversification of the *V. vulnificus* species into biotypes and serovars.

In contrast to the report by Kim *et al.* (2003a) and Bruhn *et al.* (2005), who did not detect AHLs in *V. vulnificus*, we found that almost 30% of the strains produced C_4-AHL. Kim *et al.* (2003a) only used a *V. harveyi* reporter strain for AHL detection and Bruhn *et al.* (2005) included only four strains of *V. vulnificus* bacteria in the AHL detection study. The positive strains were all from the environmental ribogroup, belonging to biotypes 1 and 2 and originated from water, fish and superficial human wounds. The AHL was identified as *N*-butanoyl-homoserine-lactone by HPLC-HRMS, a compound that is also secreted by *V. fischeri* (Nealson *et al.*, 1970), *Serratia liquefaciens* (Eberl *et al.*, 1999) and *Aeromonas hydrophila* (Swift *et al.*, 1997). The AHLs of *V. vulnificus* were only detected when samples were concentrated by lyophilization instead of ethyl acetate extractions, which suggests that the compounds are very labile and difficult to detect by bioassays. In addition, AHL production was dependent on the growth medium and population size. Thus, AHL was detected only under conditions mimicking seawater (low nutrient medium containing sea-water salts) and when blood factors but not mucin were added to the low-nutrient medium as growth supplements. In addition, the detection was positive only when bacterial populations exceeded 10^9 CFU mL^{-1}, which suggests that the compounds were produced *in vitro* in very low quantities per cell.

To demonstrate that AHLs could be produced *in vivo* during fish infection, the internal organs of moribund eels challenged with a selected biotype-2 strain were processed for HPLC-HRMS. The same AHL compound that was detected *in vitro* was also detected *in vivo*, although only in 20% of the infected eels; hence, it is not required for *in vivo* growth. Other fish pathogenic bacteria such as *Vibrio anguillarum* and *Yersinia ruckeri* produce AHLs that are also detected *in vivo* during infection (Buch *et al.*, 2003; Bruhn *et al.*, 2005) while AHLs of other fish pathogens, such as *A. salmonicida,* are only detectable from *in vitro* cultures (Bruhn *et al.*, 2005). In this work, AHLs were only detected from *in vitro* cultures following a specific extraction and concentration procedure. When tissues are used as samples, the only published procedure is to extract AHL compounds with ethyl acetate (Buch *et al.*, 2003), a method we found to be less efficient than lyophilization. Furthermore, *V. vulnificus* biotype 2 does not achieve population sizes $> 1 \times 10^6$ CFU g^{-1} of internal organ in moribund eels (Valiente *et al.*, 2008c), levels that are quite below the minimal number for AHL detection *in vitro*. Hence, AHLs may have been present in all samples of infected eel but may have escaped detection. Previous studies have also detected the production of AHL by other fish pathogenic bacteria both *in vitro* (Milton *et al.*, 2001; Temprano *et al.*, 2001; Buch *et al.*, 2003) and *in vivo* (Milton *et al.*, 2001; Temprano *et al.*, 2001) and have suggested that AHL may play a role in fish pathogenesis or in bacterial survival. We have no evidence relating fish virulence and AHL production in *V. vulnificus*, but our results reinforce the role of blood in the lifecycle of this pathogen. *Vibrio vulnificus* is an aquatic bacterium that is chemoattracted by eel mucus, binds to it and colonizes gills and skin (Valiente *et al.*, 2008a). Following our *in vitro* data, blood released by a local inflammation reaction or a wound would increase AHL production, which could potentially trigger the next step in the infection process, i.e. invasion. Thus, AHL-positive strains could facilitate the infection of fish from mucous surfaces and, therefore, only those strains with the genetic endowment affording resistance to the innate defenses (i.e. virulence

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plasmid) (Valiente *et al.*, 2008a, b) would succeed in the invasion process and produce septicaemia.

The next objective was to detect genes homologous to those involved in C_4-AHL biosynthesis and induction/regulation in other *Vibrio*, *Aeromonas* and *Serratia* species, but we were unable to detect them either by PCR or by Southern hybridization, which suggests that the AHL genes in *V. vulnificus* are different than that reported for other Gram-negative bacteria. Further studies are underway to determine the genetic basis of AHL production in *V. vulnificus*.

In conclusion, we have demonstrated that *V. vulnificus* species can produce the AHL molecule C_4-AHL, although this ability is constrained to a group of biotype 1 and 2 strains. These strains could have a common origin as supported by previous studies on genetic diversity of *V. vulnificus* (Cohen *et al.*, 2007; Sanjuan *et al.*, 2009). These results are compatible with two possible scenarios: either the ability to produce AHL has been acquired by the ancestor of the AHL-positive group by horizontal gene transfer or, alternatively, lost by the ancestor of the AHL-negative group. AHLs were produced *in vitro* under conditions simulating the aquatic environment and production was enhanced under conditions simulating growth *in vivo* in fish. Finally, a positive amplification product for *luxS* (AI-2 biosynthetic gene) was obtained for all the strains, suggesting that the presence of *luxS* extends to all three biotypes of this species.

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