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Value of *recN* sequences for species identification and as a phylogenetic marker within the family "*Leuconostocaceae*"

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Summary. The genera *Leuconostoc*, *Oenococcus*, and *Weissella* (family "*Leuconostocaceae*") constitute a group of lactic acid bacteria of great interest in food microbiology. From the taxonomic point of view, they are considered phylogenetically coherent according to their 16S rRNA gene sequences and other macromolecules. These three genera were the focus of the present study; specifically, the resolution and discriminatory power of *recN* (encoding a DNA repair and genetic recombination protein) as a molecular marker at the species level were investigated. For this purpose, partial sequences (about 1200 nt) were obtained from 23 type strains and from several additional strains following direct amplification of *recN* and subsequent sequencing. Phylogeny was evaluated according to different treeing methods (neighbor joining, maximum likelihood, and maximum parsimony) and the inclusion of variability filters. The results showed that *recN*, used either alone or in combination with 16S rRNA data, can serve as a phylogenetic marker as well as a tool for species identification.[**Int Microbiol** 2008; 11(1): 33-39]

Key words: *Leuconostoc* · *Oenococcus* · *Weissella* · gene *recN* · phylogeny

Introduction

The family "*Leuconostocaceae*" (name without standing in the nomenclature at the time of submission) comprises three genera of lactic acid bacteria—*Leuconostoc*, *Oenococcus* and *Weissella*—with quite different relevancies in food microbiology. *Oenococcus oeni* is the major bacterial species used to induce malolactic fermentation during wine making. Species of *Leuconostoc* and *Weissella* are present in meat and meat products, dairy foods, and other fresh and fermented food habitats. Selected strains (mainly *Leuconostoc* spp.) are used as starter cultures for fermented milk and vegetable products, whereas others may act as food spoilers, causing sour off-odors, off-tastes, package loosening due to gas production, and other undesirable effects [3].

Several food preservation strategies have been developed to prevent bacterial spoilage and thereby extend product shelf-life [10]; however, this is not always easily achieved. A better understanding of the spoilage process and, as a result, the development of more effective preservation methods require identification of those species primarily responsible for spoilage. Nonetheless, identification to the species level

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and the recognition of new taxa among isolates poses many challenges, particularly when phenotypic means [3] or combined methodologies [8] are used. While many approaches have been employed, including those based on specific macromolecules, numerical analysis, and DNA–DNA reassociation techniques [3 and references therein; 6,17], clarification of the taxonomy of *Leuconostoc*, *Oenococcus*, and *Weissella* remains problematic. For this reason, these three genera were considered to serve as a suitable benchmark to test the value of *recN* sequences as a species identification tool and phylogenetic marker. This work gives additional support to the use of *recN* sequences as a phylogenetic marker in prokaryotic systematics, following the proposal of Ziegler [19] and as applied in the analysis of *Geobacillus* [20], *Pasteurellaceae* [9], and to the proposal of a new species in this last family [1].

Materials and methods

Bacterial strains. The strains included in this study are shown in Table 1. All were obtained as type strains from the Spanish Type Culture Collection (CECT) and grown using the recommended media and incubation conditions described in the online catalogue [http://www.cect.org]. Cell pellets were collected for each strain and DNA was extracted using a microbial DNA isolation kit (Ultra Clean, Mo Bio Laboratories, Carlsbad, CA, USA). The DNA was checked for purity using standard methods [13].

Gene *recN* database. A dataset of *recN* sequences from members of the order *Lactobacillales* (including the family "*Leuconostocaceae*") was imported from GenBank/EMBL/DDBJ into the software package ARB [11], [http://www.mikro.biologie.tu-muenchen.de] and aligned both as nucleotide and as amino acid sequences. The organisms represented in this dataset were: *Enterococcus faecalis* V583, *Lactobacillus acidophilus* NCFM, *Lactobacillus brevis* ATCC 367, *Lactobacillus casei* ATCC 334, *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842^T and ATCC BAA-365, *Lactobacillus gasseri* ATCC 33323^T, *Lactobacillus johnsonii* NCC 533, *Lactobacillus plantarum* WCFS1, *Lactobacillus sakei* subsp. *sakei* 23K, *Lactobacillus salivarius* subsp. *salivarius* UCC118, *Lactococcus lactis* subsp. *cremoris* SK11, *Lactococcus lactis* subsp. *lactis* Il1403, *Pediococcus pentosaceus* ATCC 25745, *Streptococcus agalactiae* 2603V/R and A909, *Streptococcus mutans* UA159, *Streptococcus pneumoniae* D39, R6 and TIGR4, *Streptococcus pyogenes* M1 GAS, MGAS10270, MGAS10394, MGAS2096, MGAS315, MGAS5005, MGAS6180, MGAS8232, MGAS9429 and SSI-1, and *Streptococcus thermophilus* CNRZ1066, LMD-9 and LMG 18311.

Primer design. Degenerate *recN*-specific primers for PCR amplification and sequencing reactions were designed using the dataset mentioned above. Primers were located within the gene (but close to the ends), with an expected amplicon size of about 1200 nucleotides (nt; the length of the complete gene is around 1660 nt). The primers were recN-F (forward): 5′-GCAGGA AARTCTATTATYATTGATGC-3′ and recN-R (reverse): 5′-CWCCTGTAT CAACTTCATCAAA-3′, both obtained from Invitrogen/Life Technologies (Carlsbad, CA, USA).

PCR amplification. DNA templates were PCR-amplified on a Genius thermocycler (Techne, Burlington, NJ, USA). The amplification mixture (100 µl) comprised 2 µl (50 pmol/µl) each of recN-F and recN-R primers, 0.5 µl (2U/µl) of Taq DNA polymerase (Finnzymes, Espoo, Finland), 10 µl of 10× reaction buffer (Finnzymes), 10 µl of dNTP mixture (containing 1 mM each of dATP, dGTP, dCTP and dTTP) (Roche, Basel, Switzerland), 70 µl of sterile filtered water (Milli-Q purification system, Millipore, Billerica, MA, USA), and 5.5 µl of DNA template. The DNA templates were amplified by initial denaturation at 94ºC for 10 min, followed by 40 cycles of denaturation at 94ºC for 1 min, annealing at 55ºC for 1 min, extension at 72ºC for 1 min, and a final extension at 72ºC for 10 min. Negative controls, devoid of DNA, were simultaneously included in the amplification process. The integrity of the PCR products was confirmed by the appearance of single bands following electrophoresis for 1 h at 100 V in 2% (w/v) agarose gels in Tris-borate EDTA buffer. The bands were visualized by ethidium bromide staining and illumination with UV light.

As control, all DNA samples were also tested in parallel using universal primers amplifying a 1000-bp region of the 16S rRNA gene 616V (forward): 5′-AGAGTTTGATYMTGGCTC AG-3′ and 699R (reverse): 5′-RGGGTTG CGCTCGTT-3′ (from Invitrogen/Life Technologies) under the same conditions. The positions for these primers are 8–25 and 1099–1113, respectively (*Escherichia coli* numbering).

Sequencing and phylogenetic analyses. Although the targets of the *recN* primers were located in conserved regions, five strains failed repeatedly to yield an amplicon: *L. citreum* CECT 4025^T, *L. pseudomesenteroides* CECT 4027^T, *L. ficulneum* CECT 5747^T, *L. fructosum* CECT 7088^T, and *W. paramesenteroides* CECT 4268^T. This situation was also noted by Ziegler [20]. It is possible that sequence variation in the target regions for these species is too high to allow the annealing of one or both primers. For all other strains, amplicons were obtained and purified using an UltraClean PCR clean-up kit (Mo Bio Laboratories). Subsequent sequencing reactions were done on an Abi Prism 3730 automated sequencer using the Big Dye Terminator v3.1 cycle sequencing kit, premixed format. Sequencing primers were the same ones used in the amplification reaction but diluted ten-folds (5 pmol). Corrected sequences were incorporated into the *recN* database in ARB software and aligned using the integrated alignment tool. Final alignments were inspected visually. In addition to the sequences obtained in this study three more, retrieved from genome sequencing projects, were added (Table 1) during the course of this study: *O. oeni* PSU-1 (CP000411), *L. mesenteroides* subsp. *mesenteroides* ATCC 8293^T (CP000414), and *O. oeni* ATCC BAA-1163 (AAUV00000000).

Nucleotide sequence data reported herein are available in the DDBJ/EMBL/GenBank databases and their accession numbers (AM698012 to AM698034) are listed in Table 1.

Phylogenetic analyses were carried out on the nucleotide sequences and on their corresponding amino acid translations. The appropriate ARB tool [11] was used to create conservation profiles of both types of sequences to reduce the influence of highly variable positions. The three most commonly used treeing methods—distance matrix, maximum parsimony, and maximum likelihood—were applied on all combinations of nucleotide/amino acid raw/filtered sequence data to evaluate the robustness of the topologies. As an additional confidence test, bootstrap values were calculated.

Results and Discussion

Phylogeny. A general agreement between *recN* and 16S rRNA phylogenies was obtained (Fig. 1). In both cases, the three genera could be well-resolved and most nodes were supported by high bootstrap values or reproducibility according to different treeing methods. The use of variability filters on both the nucleotide and the amino acid alignments permitted further evaluation of the robustness of the topologies inferred.

Table 1. Strains used in this study. The abbreviated codes displayed in Fig. 1 for each strain, the *recN* sequence accession numbers, and the 16S rRNA sequence accession number from the same or an equivalent strain are shown

Strains[a]	Code	*recN*	16S rRNA
Leuconostoc carnosum CECT 4024^T	Lcar	AM698023	AB022925
Leuconostoc durionis CECT 7089^T	Ldur	AM698024	AJ780981
Leuconostoc fallax CECT 7087^T	Lfal	AM698025	AF360738
Leuconostoc gasicomitatum CECT 5767^T	Lgas	AM698026	AF231131
Leuconostoc gelidum CECT 4026^T	Lgel	AM698027	AF175402
Leuconostoc inhae CECT 7026^T	Linh	AM698028	AF439560
Leuconostoc lactis CECT 4173^T	Llac	AM698029	M23031
Leuconostoc lactis CECT 5746[b]	Larg	AM698030	AF175403
Leuconostoc mesenteroides subsp. *cremoris* CECT 872^T	Lmcr	AM698031	M23034
Leuconostoc mesenteroides subsp. *dextranicum* CECT 912^T	Lmde	AM698032	AB023244
Leuconostoc mesenteroides subsp. *mesenteroides* ATCC 8293^T	Lmm1	Note[c]	Note[c]
Leuconostoc mesenteroides subsp. *mesenteroides* CECT 219^T	Lmm2	AM698033	M23035
Leuconostoc pseudoficulneum CECT 5759^T	Lpsf	AM698034	AY169967
Oenococcus oeni CECT 217^T	Ooe1	AM698012	AB022924
Oenococcus oeni PSU-1	Ooe2	Note[c]	Note[c]
Oenococcus oeni ATCC BAA-1163	Ooe3	Note[c]	Note[c]
Weissella cibaria CECT 7032^T	Wcib	AM698013	AJ295989
Weissella confusa CECT 4707^T	Wcon	AM698014	M23036
Weissella halotolerans CECT 573^T	Whal	AM698015	M23037
Weissella hellenica CECT 7033^T	Whel	AM698016	S67831
Weissella kandleri CECT 4307^T	Wkan	AM698017	M23038
Weissella koreensis CECT 7110^T	Wkor	AM698018	AY035891
Weissella minor CECT 572^T	Wmin	AM698019	M23039
Weissella soli CECT 7031^T	Wsol	AM698020	AY028260
Weissella thailandensis CECT 7052^T	Wtha	AM698021	AB023838
Weissella viridescens CECT 283^T	Wvir	AM698022	M23040

[a]The following strains yielded no amplicon: *L. citreum* CECT 4025^T, *L. ficulneum* CECT 5747^T, *L. fructosum* CECT 7088^T, *L. pseudomesenteroides* CECT 4027^T, and *W. paramesenteroides* CECT 4268^T.
[b]Strain CECT 5746 is the type of *L. argentinum* (later heterotypic synonym of *L. lactis* [17]).
[c]Retrieved from genome sequencing projects CP000414 (Lmm1), CP000411 (Ooe2) and AAUV00000000 (Ooe3).

Figure 2 summarizes these results. Note that although the three genera—*Leuconostoc*, *Oenococcus* and *Weissella*—are clearly separated from each other, *recN* does not clearly support their unity as a family. Similarly, Ziegler [20] observed that *recN* had poor resolving power for higher-order taxa (family, order, class), probably related to the mutational saturation that occurs in the rapidly diverging *recN* gene.

The phylogenetic structure of the *Leuconostoc–Oenococcus–Weissella* clade was extensively evaluated in a recent study [4] through sequence analyses of the 16S rRNA gene as well as the genes *dnaA*, *gyrB*, *rpoC*, and *dnaK* , including the all concatenated gene sequences (accounting for 7672 nt sites). That study included 18 strains of "*Leuconostocaceae*" whereas our study examined 26 strains, of which only 12 are common; therefore, any comparison of the results is limited. Yet, by examining the tree topologies (in terms of the number of common nodes) it can be concluded that the best agreement occurred between *recN* and the concatenated sequences reported in [4]. This can be taken as another indication of the adequacy of *recN* as a phylogenetic marker.

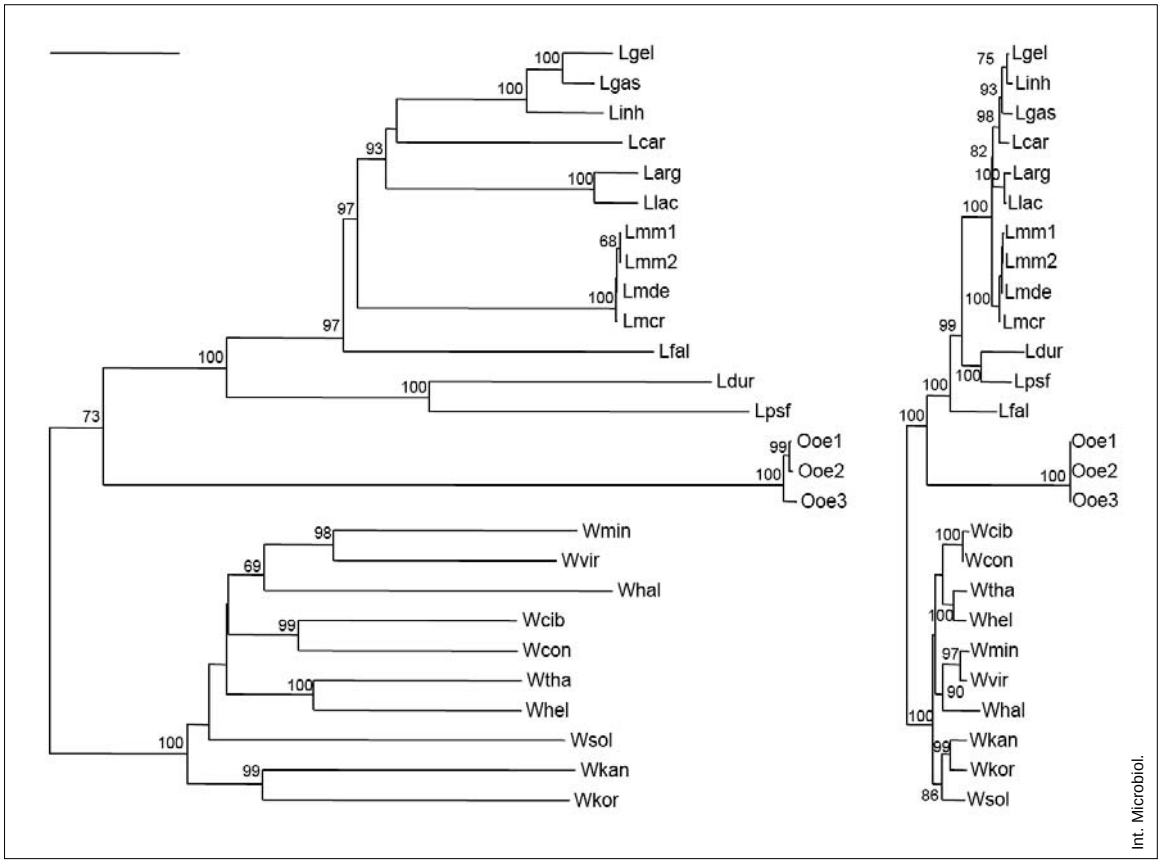

Fig. 1. Neighbor-joining trees from members of the family '*Leuconostocaceae*'. Outgroup not shown. Bar (same scale on both trees), 10 estimated substitutions per 100 bases positions. Only bootstrap values >60% are shown (1000 resamplings) at the branching points. Bold-face bootstrap values indicate that the corresponding nodes are also recovered in maximum-likelihood and maximum-parsimony trees. Left tree based on *recN* gene sequences (ca. 1200 nt); right tree based on public 16S rRNA sequences (ca. 1500 nt) from the same strains shown in the left tree or from known equivalent strains. Accession numbers and strain codes are listed in Table 1.

Sequence similarities and predictive value. The application of the analysis of *recN* sequence similarities to the identification of bacterial species was tested recently within the genus *Geobacillus* [20]. Those results showed that the resolving power of this approach, at either the species or subspecies level, is nearly an order of magnitude greater than that of 16S rRNA analysis. In a previous study [19], it was estimated that at a *recN* sequence similarity of <84% two strains can be considered as separate species, whereas a similarity >96% indicates that they are the same species, leaving a zone of uncertainty for similarities between 84 and 96%. In this study, these three situations were encountered and are illustrated in Fig. 3. Since strain selection consisted mainly of type strains from established species, it can be expected that most pair-wise comparisons correspond to *recN* similarities of <84%.

All values <84% obtained in our study corresponded to interspecies comparisons. These ranged between 53.1 and 73.5% (average 64.1%) for the genus *Leuconostoc* and between 57.4 and 73.9% (average 69.6%) for the genus *Weissella*. Species allocated in separate genera had even lower ranges of similarities (43.0–52.2%, average 48.4%), which suggested that *recN* sequence comparison alone (without reconstruction of phylogenetic trees) may be sufficient to classify strains at the genus level.

As shown in Fig. 3, a few pair-wise comparisons yielded values in the range of 84–96%. These strains would require DNA reassociation assays to elucidate whether they should be considered as a single species. A separate analysis of these cases follows:

(i) *L. gasicomitatum*, *L. gelidum*, and *L. inhae*. The genome similarity values estimated according to *recN* similarities using the method of Zeigler [19] for these organisms are close to the outer limits generally accepted for species differentiation (81% for *L. gasicomitatum–L. gelidum*, 69% for *L. inhae* with any of the other two), whereas those

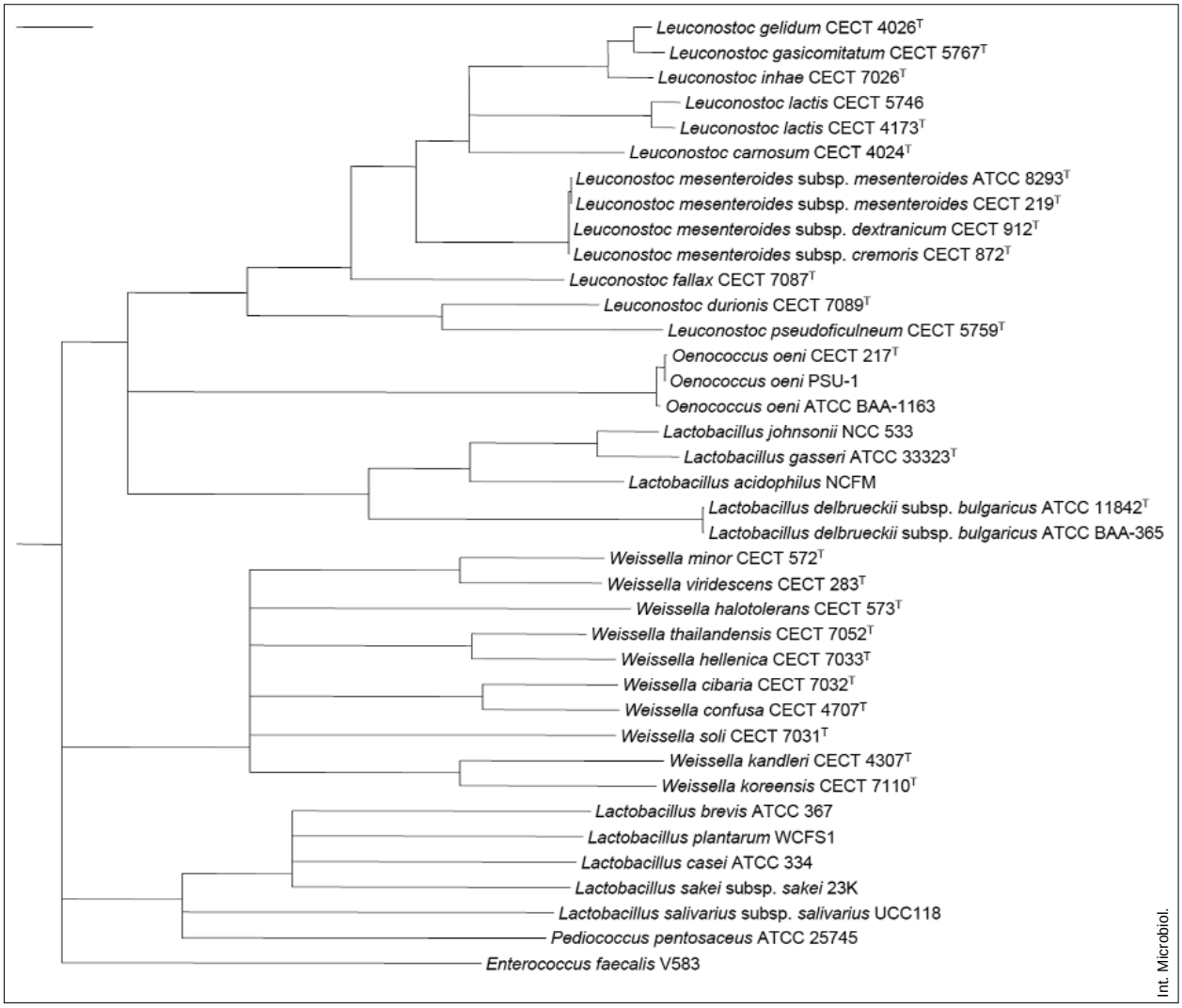

Fig. 2. The *recN* tree composed from members of the family '*Leuconostocaceae'* and other lactic acid bacteria. The outgroup (not shown) consisted of two sequences of *Lactococcus* spp. and 20 of *Streptococcus* spp. (see text). Bar, 10 estimated substitution per 100 bases positions. Treeing methods included neighbor-joining, maximum-likelihood, and maximum-parsimony (each applied to the nucleotide and the amino acid sequences in full and using a 50% conservation filter to remove variable positions). Accession numbers are listed in Table 1 or given in the text. Collapsed branches indicate that the affected nodes showed more than one possible topology; bifurcated branches are those that maintained their relative topology in all trees examined.

obtained from experimental determinations are significantly lower: 6–26% [2,6]. This illustrates perfectly the fact that, despite being a good predictor of DNA–DNA relatedness, estimations from *recN* similarities that fall within the range of uncertainty, i.e., 84–96% according to Zeigler [19], do not have a high level of confidence.

(ii) *L. argentinum* and *L. lactis.* During the course of this study, *L. argentinum* CECT 5746^T was found to represent a later heterotypic synonym of *L. lactis* (type strain CECT 4173^T) [17]. The *recN* sequences of these two strains have a similarity of 93.4%, and hence an estimated genome similarity of 80%. In contrast to the situation found with the cluster *L. gelidum–L. gasicomitatum–L. inhae*, the experimental value of DNA–DNA hybridization reported by Vancanneyt et al. [17] is virtually identical to the predicted one of 81%. Nonetheless, methodological differences in the results of DNA–DNA hybridizations are well-documented and must also be taken into account. In the case of *L. argentinum* and *L. lactis*, lower values (35–39%) were obtained by Dicks et al. [5].

In our study, *recN* similarities >96% were found in all four strains of *L. mesenteroides* (99.8–100%) and in the three *O. oeni* strains (98.2–99.8%). Although the number of strains examined was small, the results nonetheless confirm that the

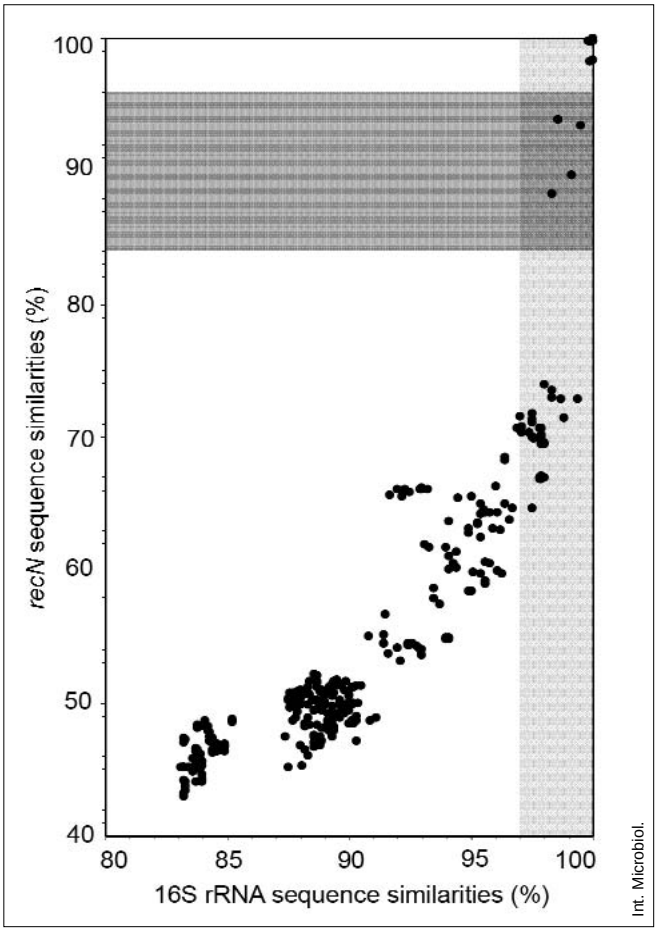

Fig. 3. Comparison of *recN* and 16S rRNA gene sequence similarities (%). Shadowed areas indicate the threshold ranges in which DNA–DNA reassociation experiments are recommended (84–96% *recN* similarities, horizontal area; 97–100% 16S rRNA similarities, vertical area). Most of the values that fall within the 16S rRNA zone of uncertainty are well-resolved in terms of *recN* similarities. Thus, the use of *recN* similarity is the only way to conclude whether two strains belong to the same species (similarities >96%) [16].

similarity of *recN* sequences is very high among strains known to represent a single species. As expected, the 16S rRNA sequence similarity for these strains was also very high: *L. mesenteroides* strains 99.8–100%, and *O. oeni* strains 99.9–100%. The difference is that based on 16S rRNA data alone; DNA–DNA hybridization assays would be mandatory but they are unnecessary if the *recN* similarity values are known.

Potential of *recN* sequences for identification. DNA–DNA hybridization has had and continues to have a crucial impact on prokaryotic taxonomy, particularly regarding species definition [12,15,16,18]. Despite its enormous importance and even though a vast array of methodological variants have been developed over nearly 50 years [for a detailed review, see 12], some common pitfalls remain that justify the search for new methods suitable for replacing DNA–DNA reassociation experiments [7,15,19].

For taxonomists, the article by Stackebrandt and Goebel [14] justified omitting DNA–DNA hybridization studies if a reassociation value of at least 70% could not reasonably be expected. Experimental observations showed that this was indeed the case for strains with <97% 16S rRNA sequence identity. In little more than a decade, the acceptance of this threshold value in prokaryote systematics has been unquestionable. Recently, Stackebrandt and Ebers [16] stated that a 16S rRNA sequence similarity of 97% as a borderline for recognizing separate species and obviating the need for DNA reassociation experiments was too conservative. To facilitate the work of taxonomists, these authors proposed raising the threshold to 98.7–99%. Yet, even at such high 16S rRNA sequence similarities it is not possible to conclude whether two strains belong to the same species. For instance, the low *recN* similarities (71.4–72.9%) of *L. gelidum–L. carnosum*, *W. cibaria–W. confusa*, and *W. minor–W. viridescens* confirm that each is a separate species, whereas the assignments would remain unresolved according to 16S rRNA sequence similarities (98.7–99.4%). Moreover, recognition of the four *L. mesenteroides* strains and the three *O. oeni* strains as single species is well-achieved by the high *recN* similarities in each case.

Our observations together with previous results [1,9,19, 20] confirm the strong potential of *recN* both as a phylogenetic marker and as a species delimiter. Used alone or, better, in combination with 16S rRNA data, this approach might well be sufficient to identify prokaryotic strains at the species level or to recognize a novel species. In addition, the sequences and primers provided in this study can be applied in other (non-sequencing) methodologies, such as DNA fingerprinting, DNA probing, and specific PCR.

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