Use of *recA* as an alternative phylogenetic marker in the family *Vibrionaceae*

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This study analysed the usefulness of *recA* gene sequences as an alternative phylogenetic and/or identification marker for vibrios. The *recA* sequences suggest that the genus *Vibrio* is polyphyletic. The high heterogeneity observed within vibrios was congruent with former polyphasic taxonomic studies on this group. *Photobacterium* species clustered together and apparently nested within vibrios, while *Grimontia hollisae* was apart from other vibrios. Within the vibrios, *Vibrio cholerae* and *Vibrio mimicus* clustered apart from the other genus members. *Vibrio harveyi*- and *Vibrio splendidus*-related species formed compact separated groups. On the other hand, species related to *Vibrio tubiashii* appeared scattered in the phylogenetic tree. The pairs *Vibrio coralliilyticus* and *Vibrio neptunius*, *Vibrio nereis* and *Vibrio xuii* and *V. tubiashii* and *Vibrio brasiliensis* clustered completely apart from each other. There was a correlation of 0·58 between *recA* and 16S rDNA pairwise similarities. Strains of the same species have at least 94 % *recA* sequence similarity. *recA* gene sequences are much more discriminatory than 16S rDNA. For 16S rDNA similarity values above 98 % there was a wide range of *rec*A similarities, from 83 to 99 %.

Cultivation-dependent and -independent studies have shown that vibrios are abundant worldwide in aquatic environments, including estuaries, marine coastal waters and sediments, and aquaculture settings (Heidelberg *et al.*, 2002a, b; Suantika *et al.*, 2001; Urakawa *et al.*, 2000). Vibrios are particularly abundant in and/or on marine organisms (Gomez-Gil *et al.*, 1998; Nishiguchi, 2000; Rosenberg & Ben-Haim, 2002). Several *Vibrio* species are serious pathogens of aquatic animals (Austin & Austin, 1999; Lightner & Redman, 1998). *Vibrio anguillarum*, *Vibrio salmonicida* and *Vibrio vulnificus* are important bacterial pathogens of several fish species, while *Vibrio splendidus*-related species represent a threat to bivalves, and *Vibrio harveyi* and *Vibrio campbellii* to shrimps (Austin & Austin, 1999; Le Roux *et al.*, 2002). *Vibrio cholerae*, *Vibrio parahaemolyticus* and *V. vulnificus* are pathogens of man (Farmer & Hickman-Brenner, 1992; Wachsmuth *et al.*, 1994). *V. cholerae*, the causative agent of cholera, has killed thousands of people worldwide in the last decade (WHO, 2001, 2002; http://www.who.int/en/). In the last 3 years alone, about 10 000 people have died of cholera, mainly in developing countries (WHO, 2001, 2002).

According to *Bergey's Manual of Systematic Bacteriology* (2002) (see http://dx.doi.org/10.1007/bergeysoutline200210), there are six genera within the current family *Vibrionaceae*: *Allomonas* (one species), *Enhydrobacter* (one species), *Listonella* (two species), *Photobacterium* (six species), *Salinivibrio* (one species) and *Vibrio* (44 species). The genera *Allomonas* (Kalina *et al.*, 1984) and *Enhydrobacter* (Staley *et al.*, 1987) were tentatively allocated to the family *Vibrionaceae* based on phenotypic characteristics, but it is now known that *Allomonas* belongs to *Vibrio* and *Enhydrobacter* to *Moraxella* (Thompson *et al.*, 2003a). Several novel *Vibrio* species isolated mainly from the aquatic environment and marine organisms have been described in the last few years, including species related to *Vibrio tubiashii*, i.e. *Vibrio brasiliensis*, *Vibrio coralliilyticus*, *Vibrio neptunius*, and *Vibrio xuii* (Ben-Haim *et al.*, 2003; Thompson *et al.*, 2003b); species related to *Vibrio splendidus*, i.e. *Vibrio tasmaniensis*, *Vibrio kanaloae*, *Vibrio pomeroyi* and *Vibrio chagasii* (Thompson *et al.*, 2003c, d); species related to *Vibrio halioticoli*, i.e. '*Vibrio ezurae*', '*Vibrio gallicus*' and *Vibrio superstes* (Hayashi *et al.*, 2003; Sawabe *et al.*, 2003); species related to *V. harveyi*, i.e. *Vibrio rotiferianus* (Gomez-Gil *et al.*, 2003a) and species related to *Vibrio furnissii*, i.e. *Vibrio pacinii* (Gomez-Gil *et al.*, 2003b). The identification of *Vibrio* species requires the application of genomic analyses, including AFLP, rep-PCR, DNA–DNA hybridization and 16S rDNA gene sequencing (Thompson *et al.*, 2001a).

In order to find new identification markers and improve our knowledge of the phylogenetic structure of vibrios, it is essential to analyse other genes apart from 16S rDNA. *recA*

The GenBank/EMBL/DDBJ accession numbers for the *recA* sequences reported in this study are AJ580845–AJ580909.

Details of strains used in the study and the 16S rDNA/*recA* regression curve are available as supplementary material in IJSEM Online.

has been suggested as a potential marker to unravel phylogenetic relationships among higher taxonomic ranks such as families, classes and phyla because of its ubiquity and house-keeping function in bacteria (Eisen, 1995; Ludwig & Klenk, 2001; Zeigler, 2003). *rec*A is a multifunctional protein contributing to homologous recombination, DNA repair and the SOS response, and specifically binds stretches of single-stranded DNA, unwinds duplex DNA, and finds regions of homology between chromosomes in homologous recombination (Cox, 2003; Lloyd & Sharp, 1993). To date, *recA* gene sequences have been used only to analyse *V. cholerae* strains (Byun *et al.*, 1999; Stine *et al.*, 2000), and their value in discriminating closely related species within the family *Vibrionaceae* is not known. Our aim in the present study was to analyse the usefulness of *recA* gene sequences as an alternative phylogenetic and identification marker for vibrios.

The strains used in this study are listed in Supplementary Table A in IJSEM Online. Strains were grown aerobically on marine agar 2216E (Difco) at 27 °C for 24 h. All strains included in this study are deposited in the BCCM^{TM}/LMG Bacteria Collection or in the research Collection at Ghent University (Ghent, Belgium). Bacterial genomic DNA was extracted following the methodology described by Pitcher *et al.* (1989).

PCR mixtures were composed of 33·5 µl sterile MilliQ water, 5·0 µl 10 × PCR buffer, 5·0 µl dNTPs, 0·5 µl forward primer (10 µM; 5′-TGGACGAGAATAAACAGAAGGC-3′; Byun *et al.*, 1999), 0·5 µl reverse primer (10 µM; 5′-CCGTTATAGCTGTACCAAGCGCCC-3′; Stine *et al.*, 2000), 0·5 µl AmpliTaq DNA polymerase and 5·0 µl template DNA (0·01µg µl^{-1}). PCR was performed using a GeneAmp PCR System 9600 thermocycler (Applied Biosystems). The thermal program consisted of 5 min at 95 °C, 3 cycles of 45 s at 95 °C, 2 min at 55 °C and 1 min at 72 °C, 30 cycles of 20 s at 95 °C, 1 min at 55 °C and 1 min at 72 °C and a final extension of 7 min. at 72 °C. Positive PCRs, giving a product with the expected size and intensity, were purified using the Nucleofast 96 PCR clean up membrane system (Macherey-Nagel), which is based on ultrafiltration membranes. Purified PCR products were eluted in sterile MilliQ water. Subsequently, 3·0 µl purified PCR product was mixed with 1·0 µl ABI Prism Big Dye Terminator ready reaction mix, 3·0 µl forward and/or reverse primer (4 µM), 1·5 µl 5 × buffer and 1·5 µl MilliQ water. The thermal program consisted of 30 cycles of 15 s at 96 °C, 1 s at 35 °C and 4 min at 60 °C. Sequencing products were purified using the Montage SEQ$_{96}$ sequencing reaction cleanup kit (Genomics), which removes contaminating salts and unincorporated dye terminators from DNA sequencing reactions. Purified sequencing products were then dried in an Eppendorf Concentrator 5301 at room temperature for 40 min. Purified sequencing reactions were mixed with 20 µl deionized formamide and heated at 95 °C for 2 min. Subsequently, the mixture was chilled on ice for 2–3 min. Separation of the DNA fragments was carried out in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Time and voltage of sample injection were 22 s at 1 kV. Each run was performed at 50 °C, for 6500 s, at 0·1 mA and 12·2 kV. Raw sequence data were transferred to AutoAssembler software (Applied Biosystems), where consensus sequences were determined. Consensus sequences were imported into BioNumerics 2.5 software, where a similarity matrix and phylogenetic trees were created, based on the neighbour-joining method (Saitou & Nei, 1987) and maximum-parsimony. Pearson's product-moment correlation coefficient and a regression curve were calculated between *recA* and 16S rDNA data, based on 43 strains. The 16S rDNA data were obtained from EMBL. Splits tree decomposition analysis was carried out using software available online (http://www.mlst.net; Huson, 1998) while Sawyer's test was calculated using the software package START obtained from http://pubmlst.org/software/analysis/start/ (Jolley *et al.*, 2001). EMBL accession numbers for the *rec*A sequences are AJ580845–AJ580909.

In this study we sequenced a 739 bp fragment of *recA* of 62 *Vibrionaceae* strains, corresponding to approximately 70 % of the coding region of this locus. We compared *recA* and 16S rDNA pairwise similarities between 43 strains using Pearson's product-moment correlation coefficient. The correlation of *recA* and 16S rDNA data was found to be relatively good, at 0·58 (see Supplementary Fig. A in IJSEM Online). The data fitted well in a polynomial regression of second degree ($r=0·8$). Overall, *recA* gene sequences were much more discriminatory than 16S rDNA. For 16S rDNA similarity values above 98 %, there was a wide range of *recA* similarities, from 83 to 99 %. The mean ($\pm$SD) G+C content of the *recA* gene sequences was 46·9$\pm$2·2 mol%. This value is in agreement with the G+C content of the whole genome of vibrios (Thompson *et al.*, 2003b, c), suggesting that this locus has not been affected by horizontal gene transfer. In addition, splits tree decomposition analysis and Sawyer's test did not indicate any evidence for recombination within the *recA* sequences in this study.

A neighbour-joining (NJ) tree with the estimated positions of the 71 representative *Vibrionaceae* strains based on 673 bp is presented in Fig. 1. We also built a maximum-parsimony tree that confirmed the grouping obtained by NJ. *Vibrio* species appear to be polyphyletic. *Photobacterium* species had at most 84 % *recA* sequence similarity towards *Vibrio* species, but are apparently nested within vibrios. *Photobacterium* species did not form a homogeneous group, having *recA* similarity values ranging from 82 to 96 %. Baumann *et al.* (1983) suggested that *Photobacterium* and *Vibrio* are two distinct phylogenetic groups. The *recA* sequence similarities of *Vibrio* species were between 74 and 99·6 %. Several *Vibrio* species formed deep branches e.g. *V. gallicus*, *V. pacinii*, *Vibrio cincinnatiensis*, *Vibrio rumoiensis* and *Vibrio aestuarianus*.

Vibrio diazotrophicus LMG 7893^{T} and LMG 11217 were found in two different AFLP clusters, A57 and A34, respectively (Thompson *et al.*, 2001a), and were later

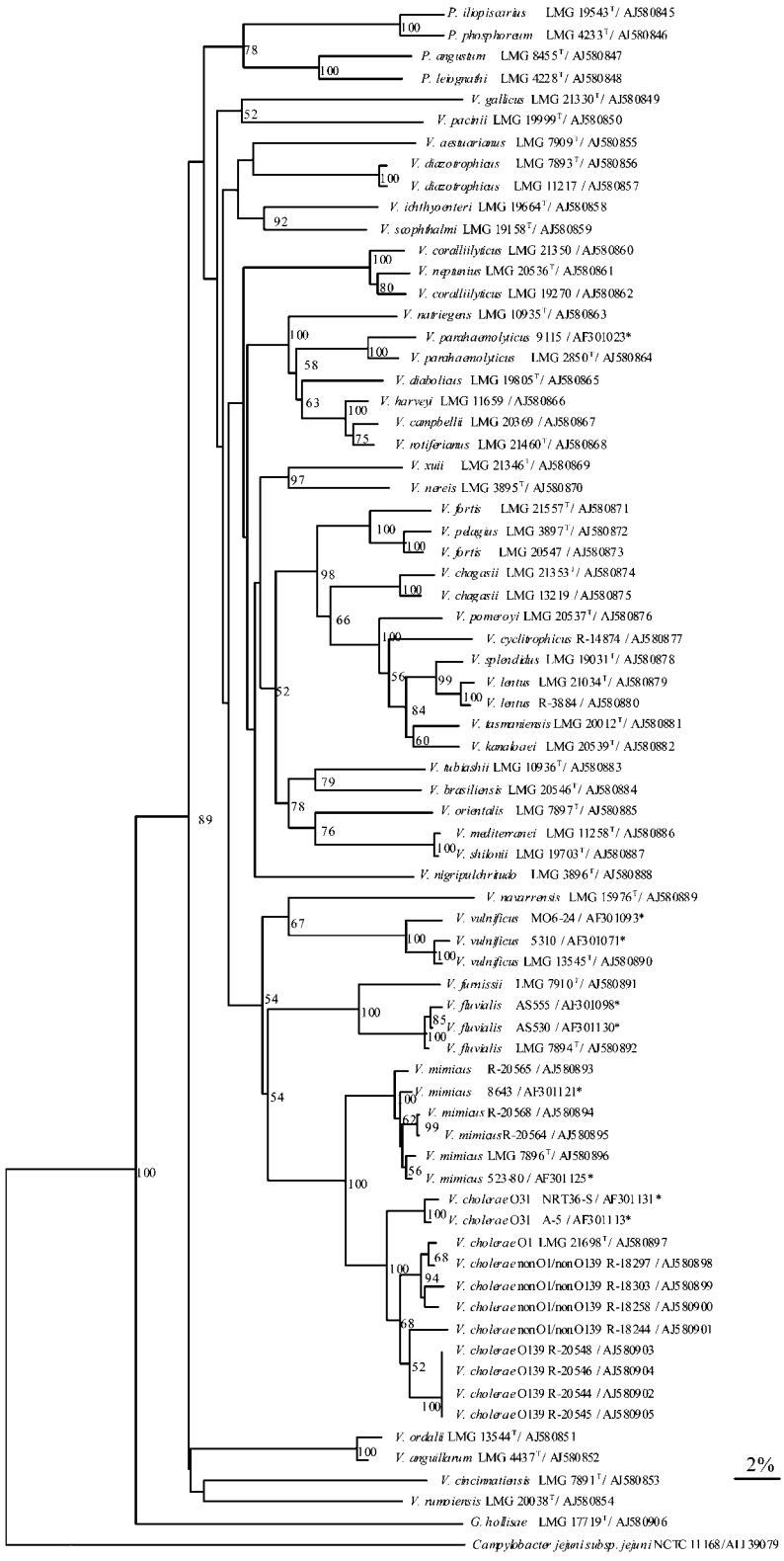

Fig. 1. Phylogenetic tree based on the neighbour-joining method, using *recA* sequences (673 bp) of 71 representative *Vibrionaceae* strains in the genera *Grimontia*, *Photobacterium* and *Vibrio*. Distance estimations were obtained by the model of Jukes & Cantor (1969). Bootstrap percentages after 500 simulations are shown. Bar, 2 % estimated sequence divergence. *, Sequences obtained from Stine *et al.* (2000). The *Campylobacter* sequence was used as an outgroup.

confirmed by DNA–DNA hybridization to belong to the same species (unpublished data). *Vibrio ichthyoenteri* and *Vibrio scophthalmi* shared only 90 % *recA* sequence similarity, but 99 % similarity for the 16S rDNA (Thompson *et al.*, 2002).

V. coralliilyticus LMG 21350 and LMG 19270 shared 96·8 % *recA* sequence similarity. These strains appeared within the former AFLP clusters A1 and A3, respectively (Thompson *et al.*, 2001a). *V. coralliilyticus* and *V. neptunius* were phylogenetic neighbours, sharing 96 % *recA* similarity. According

to Ben-Haim *et al.* (2003), *V. neptunius*, *V. tubiashii*, *V. nereis* and *Vibrio shilonii* were the species most closely related to *V. coralliilyticus*, having 97 to 98 % 16S rDNA sequence similarity.

V. harveyi-related species, i.e. *V. campbellii*, *Vibrio natriegens*, *V. parahaemolyticus* and *V. rotiferianus*, had *recA* sequence similarities between 89 and 98 %. *V. parahaemolyticus* strains LMG 2850^T and 9115 had 96 % *recA* sequence similarity. Their closest neighbour was *V. harveyi* (92 % similarity). *V. campbellii*, *V. harveyi* and *V. rotiferianus* had about 97 % *recA* sequence similarity. These species are highly related on the basis of 16S rDNA sequences (>98 %), DNA–DNA hybridization (>65 %), Biolog and FAME (Gomez-Gil *et al.*, 2003a).

V. nereis and *V. xuii* had 99 % 16S rDNA sequence similarity, but only 90 % *recA* gene sequence similarity. *Vibrio fortis* and *Vibrio pelagius* were closely related by 16S rDNA (98·8 %) and DNA–DNA hybridization (65 %) (Thompson *et al.*, 2003e), but the *recA* sequence similarity between these two species was only 94·5 %. *V. fortis* LMG 20547 was closer to *V. pelagius* (98 % *recA* sequence similarity) than to *V. fortis* LMG 21557^T, suggesting that strain LMG 20547 belongs to the species *V. pelagius*. LMG 20547 and the type strain of *V. pelagius* had 66 % similarity by DNA–DNA hybridization (Thompson *et al.*, 2003e).

V. chagasii LMG 21353^T and LMG 13219 had 97 % *recA* sequence similarity. These strains were found in the former FAFLP clusters A52 and A53, respectively (Thompson *et al.*, 2001a). The closest neighbours of *V. chagasii* strains were *V. fortis* and *V. pomeroyi*, having at most 91 % *recA* sequence similarity. *V. pomeroyi*, *V. splendidus* and *Vibrio cyclitrophicus* shared only 92 % *recA* sequence similarity. *V. splendidus* had 93·7 and 95 % *recA* sequence similarity towards *V. kanaloae* and *V. tasmaniensis*, respectively. *V. tasmaniensis*, *V. pomeroyi* and *V. kanaloae* shared at most 95·7 % *recA* sequence similarity. *Vibrio lentus* LMG 21034^T and R-3884 had 99 % *recA* sequence similarity, but only 97 % towards *V. splendidus*. *V. lentus* had 94 and 92 % similarity towards *V. tasmaniensis* and *V. cyclitrophicus*, respectively. The *V. splendidus*-related group is very homogeneous on the basis of 16S rDNA sequences (>98·7 % similarity), DNA–DNA hybridization (>50 %) and phenotype (Macian *et al.*, 2001; Thompson *et al.*, 2003c, d), suggesting that *recA* sequences are indeed valuable for the discrimination of these species.

The novel species *V. neptunius*, *V. brasiliensis* and *V. xuii* were closely related to *V. tubiashii* (98–99 % 16S rDNA gene sequence similarity) (Thompson *et al.*, 2003b), but *recA* sequence similarity among these species was below 90 %. *V. mediterranei* and *V. shilonii* were almost identical by both *recA* and 16S rDNA. These results corroborate the proposal of Thompson *et al.* (2001b) that *V. shilonii* should be considered a later synonym of *V. mediterranei*.

V. vulnificus LMG 13545^T, MO6-24S and 5310 had *recA* sequence similarity between 96 and 99 %. *Vibrio fluvialis* LMG 7894^T, AS555 and AS530 shared more than 99 % sequence similarity, whereas *V. fluvialis* and *V. furnissii* had at most 93 % similarity. Strains AS555 and AS530 were identified by Stine *et al.* (2000) as *V. parahaemolyticus*, but our results indicate that they may belong to *V. fluvialis*.

Vibrio mimicus and *V. cholerae*, the two bona fide members of the genus *Vibrio*, were separated in two different groups. *V. mimicus* LMG 7896^T, 523-80, 8643, R-20564, R-20565 and R-20568 shared at least 98 % *recA* sequence similarity, while *V. cholerae* LMG 21698^T, A-5, NRT36-S, R-18244, R-18258, R-18297, R-18303, R-20544, R-20545, R-20546 and R-20548 had at least 94 % *recA* sequence similarity. In our AFLP analysis, these R- strains were highly related and had 74–98 % pattern similarity (Thompson *et al.*, 2003f). *V. cholerae* strains included in this study represent well the currently known genomic diversity within this species (Thompson *et al.*, 2003f), suggesting that *recA* is a valuable marker for the identification of *V. cholerae* strains. The *recA* similarity values between *V. cholerae* and *V. mimicus* were at most 94 %. Strains NRT36-S and A-5 clustered in between *V. cholerae* and *V. mimicus*. These strains appear to be at the outskirts of the species *V. cholerae*. Strains of different serogroups had very similar (99 %) *recA* sequences. *V. cholerae* LMG 21698^T and R-18297 clustered together, having 99 % *recA* similarity. All *V. cholerae* O139 strains had 100 % similarity. Farfan *et al.* (2002) have highlighted that *V. cholerae* strains of different serogroups may have identical sequences. It has been argued that serogroup O139 strains have evolved from O1 strains (Faruque *et al.*, 1998).

Vibrio ordalii and *V. anguillarum* showed a close relationship, having 98 % *recA* sequence similarity. *V. diazotrophicus* LMG 7893^T and LMG 11217 had more than 99 % sequence similarity, and their closest neighbour was *V. campbellii* (86·9 % similarity). *Grimontia hollisae* was distantly related to *Vibrio* (66·3 % sequence similarity) and *Photobacterium* (70·5 % sequence similarity).

Until now, the backbone of bacterial systematics has been derived from the 16S rDNA sequence-based phylogeny (Ludwig & Klenk, 2001). 16S rDNA is indeed the most useful chronometer to allocate strains to different branches of the family *Vibrionaceae* (Thompson *et al.*, 2002). However, several vibrios have nearly identical 16S rDNA sequences and this makes it difficult to use 16S rDNA sequences to discriminate closely related species. Furthermore, phenotypic features are very similar among vibrios and so AFLP or DNA–DNA hybridization remain the techniques of choice for species identification. The grouping of the *Vibrionaceae* obtained on the basis of *recA* gene sequences reflects well the grouping based on 16S rDNA gene sequences (Thompson *et al.*, 2002). This study highlights the usefulness of *recA* gene sequences as alternative phylogenetic markers for vibrios. Our data suggest that strains of the same species have at least 94 % *recA* sequence similarity, but additional strains from each recognized species of the *Vibrionaceae* should be examined in future

studies in order to confirm this threshold. We also intend to search for other alternative chronometers (Zeigler, 2003) which, in combination with *recA*, will provide a rapid and reliable means of identifying vibrios.

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