Phylogenetic analysis of vibrios and related species by means of *atpA* gene sequences

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We investigated the use of *atpA* gene sequences as alternative phylogenetic and identification markers for vibrios. A fragment of 1322 bp (corresponding to approximately 88 % of the coding region) was analysed in 151 strains of vibrios. The relationships observed were in agreement with the phylogeny inferred from 16S rRNA gene sequence analysis. For instance, the *Vibrio cholerae*, *Vibrio halotocoli*, *Vibrio harveyi* and *Vibrio splendidus* species groups appeared in the *atpA* gene phylogenetic analyses, suggesting that these groups may be considered as separate genera within the current *Vibrio* genus. Overall, *atpA* gene sequences appeared to be more discriminatory for species differentiation than 16S rRNA gene sequences. 16S rRNA gene sequence similarities above 97 % corresponded to *atpA* gene sequences similarities above 80 %. The intraspecies variation in the *atpA* gene sequence was about 99 % sequence similarity. The results showed clearly that *atpA* gene sequences are a suitable alternative for the identification and phylogenetic study of vibrios.

INTRODUCTION

Vibrios are ubiquitous in the marine environment and are found in high abundance, particularly in association with animals. Interactions with hosts can range from mutualistic, e.g. *Vibrio fischeri*–squid, to pathogenic, e.g. *Vibrio harveyi*–shrimps. The genus *Vibrio* is the largest genus within the family *Vibrionaceae*, comprising 81 species. Thompson *et al.* (2004) suggested that this genus could be split in several genera to encompass recognizable species groups (*Vibrio cholerae*-, *Vibrio halotocoli*-, *Vibrio harveyi*- and *Vibrio splendidus*-species clades). The different species groups found within the genus *Vibrio* may have different ecological niches. For instance, the *V. harveyi*-species group is frequently associated with diseases in various aquatic organisms worldwide, notably in shrimp. *Vibrio coralliilyticus* is one of the aetiological agents involved in the phenomenon of coral bleaching (Ben-Haim *et al.*, 2003).

Abbreviations: DDH, DNA–DNA hybridization; MP, maximum-parsimony; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession numbers for the *atpA* gene sequences determined in this study are EF601226–EF601373.

The strains used in this study are listed in Supplementary Table S1 which is available with the online version of this paper. Figures showing a regression curve between *atpA* and 16S rRNA pairwise gene sequence similarity and a phylogenetic tree constructed with the maximum-parsimony method from *atpA* gene sequences are also available as supplementary material.

The *V. splendidus*-species group is frequently involved with diseases in molluscs, while the *V. halotocoli*-species group comprises species that are potentially mutualistic organisms in abalones and also act as probionts in abalone rearing. *V. cholerae*, the aetiological agent of cholera, is considered one of the most serious human pathogens within the genus *Vibrio*.

The identification of vibrios at the family and genus levels can be achieved by comparative analysis of the 16S rRNA, *recA*, *rpoA* and *pyrH* gene sequences (Thompson *et al.*, 2005), whereas identification at the species and strain levels requires the application of several genomic techniques, including DNA–DNA hybridization (DDH), repetitive extragenic palindromic PCR (REP-PCR), amplified fragment length polymorphism (AFLP) analysis and multi-locus sequence analysis (MLSA) (Thompson *et al.*, 2004, 2005). Identification of vibrios at the species level is not always trivial because several species of the genus *Vibrio*, e.g. *V. cholerae*–*Vibrio mimicus*, have almost identical gene sequences in several loci and around 70 % DDH similarity (Thompson *et al.*, 2005). In order to find new identification markers and to improve our knowledge of the phylogeny and taxonomy of vibrios, it is essential to analyse alternative loci.

The gene that codes for the α -subunit of bacterial ATP synthase (*atpA*) functions in ATP synthesis coupled to proton transport. In organisms with a respiratory chain,

the primary role of this enzyme is to couple the electrochemical potential difference across the inner membrane with the synthesis of ATP from ADP and phosphate. In bacteria that lack a respiratory chain, the role of this enzyme is to create a proton gradient and this process is then driven by ATP hydrolysis (Kasimoglu *et al.*, 1996). Vibrios are facultative anaerobic organisms that synthesize ATP by aerobic respiration if oxygen is present but are also capable of switching to fermentation. The *atpA* gene is a housekeeping gene with ubiquitous distribution, functional constancy and is conserved among bacteria. This gene is located on chromosome I of vibrios. Studies based on whole genome sequences and on different loci of various bacterial groups have suggested that the *atpA* gene may be an alternative phylogenetic and identification marker for bacterial systematics (Zeigler, 2003; Naser *et al.*, 2005).

Previous studies on the evolutionary history of the *atpA* gene suggested that an ancient gene duplication gave rise to three types of proton pumping ATPase genes: a vacuolar type in *Eukarya* and *Archaea* and *Bacteria* types. The duplicated genes were used to root the universal tree of life. However, the archaeal type was occasionally observed in *Bacteria* and vice versa (Hilario & Gogarten, 1993). These authors suggested that horizontal gene transfer events could explain this grouping. More recently, Olendzenski *et al.* (2000) and Lapierre *et al.* (2006) showed that members of the family *Deinococcaceae* (e.g. the genera *Thermus*, *Meiothermus* and *Deinococcus*) contain ATPase genes typically found in *Archaea* or *Eukarya* which have probably been acquired by horizontal gene transfer. A Na⁺-ATPase α -subunit of the *Eukarya* type was found in *Enterococcus hirae* (Kakinuma *et al.*, 1991), but a detailed study on the identification of species of the genus *Enterococcus* based on *atpA* gene sequences (Naser *et al.*, 2005) proved that this gene can be used for identification. The aim of this study was to analyse the usefulness of *atpA* gene sequences for reliable species identification of vibrios and for inferring their evolutionary history.

METHODS

The strains used in this study are listed in Supplementary Table S1 (available in IJSEM Online). Strains were grown aerobically on tryptic soy agar (Difco) with 1.5% (w/v) NaCl at 28 °C (*V. fischeri*, *Vibrio logei*, *Vibrio tapetis* and *Vibrio wodanis* were grown at 18–19 °C) for 24 h. All strains included in this study have been deposited in the BCCM/LMG Bacteria Collection or the Research Collection at Ghent University, Belgium. Bacterial genomic DNAs were extracted according to previously described methods (Pitcher *et al.*, 1989).

The sequences of the primers used for amplification and sequencing of the *atpA* gene are listed in Table 1. These primers were designed using 17 gene sequences of the *atpA* locus including *V. cholerae* (AE003852), *Vibrio parahaemolyticus* (BA000031), *Vibrio vulnificus* (AE016795), *Escherichia coli* (AE014075, AE005174, BA000007 and U00096), *Shigella flexneri* (AE014073 and AE005674), *Salmonella enterica* serovar Typhi (AL513382 and AE014613), *S. enterica* serovar Typhimurium (AE006468), *Yersinia pestis* (AL590842 and AE009952), *Haemophilus influenzae* (L42023), *Pasteurella multocida* (AE004439)

Table 1. Amplification and sequencing primers

Primer	Sequence (5'→3')	Position
atpA-01-F	CTDAATTCHACNGAAATYAGYG	37
atpA-02-F	GCNATGGGBGAATAYTTCCG	760
atpA-03-R	CGGAARTATTCVCCCATNGC	760
atpA-04-R	TTACCARGWYTTGGGTTGC	1554
atpA-05-R	GCHAGHGC DGTACGRATACC	1204
atpA-06-F	AGCGAWCTRATYAARCARCG	55

and *Shewanella oneidensis* (AE014299). All reference sequences were acquired from publicly available data from whole genome sequencing projects.

A 50 µl PCR mixture was composed of 29.5 µl sterile MilliQ water, 5.0 µl 10 × PCR buffer, 5.0 µl deoxynucleoside triphosphates (2 mM each), 2.5 µl forward primer atpA-01F (10 µM), 2.5 µl reverse primer atpA-04R (10 µM), 0.5 µl AmpliTaq DNA polymerase (1 U µl⁻¹) and 5.0 µl template DNA (0.01 µg µl⁻¹). PCRs were performed using a GeneAmp PCR system 9600 thermocycler (Applied Biosystems). The thermal program consisted of (i) 5 min at 95 °C, (ii) 3 cycles of 1 min at 95 °C, 2 min at 57 °C and 1 min at 72 °C, (iii) 30 cycles of 35 s at 95 °C, 1 min at 57 °C, and 1 min at 72 °C, and (iv) a final 7 min at 72 °C. PCR products with the expected size (1500 bp) were purified using the Nucleofast 96 PCR cleanup membrane system (Macherey-Nagel). Subsequently, 3.0 µl purified PCR product was mixed with 1.0 µl ABI Prism BigDye terminator cycle sequencing ready reaction mix (version 3.1; Applied Biosystems), 3.0 µl sequencing primer (4 µM), 1.5 µl dilution buffer (5 ×) and 1.5 µl MilliQ water. The primers listed in Table 1 were used for sequencing. 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 with a Montage SEQ₉₆ sequencing reaction cleanup kit (Millipore). Purified products from the sequencing reaction were recovered in 20 µl injection solution and mixed with 20 µl deionized formamide. Separation of DNA fragments was performed in a genetic analyzer (ABI PRISM 3100; Applied Biosystems). Raw sequence data were transferred to the Gene Builder module within the Kodon package 2.03 (Applied Maths), where consensus sequences were determined using the six reads.

Phylogenetic analyses. Alignments were performed in CLUSTAL W (Thompson *et al.*, 1994). Phylogenetic analysis was conducted using MEGA version 3.1 (Kumar *et al.*, 2004). The phylogenetic inference was based on the neighbour-joining (NJ) distance method (Saitou & Nei, 1987) and the maximum-parsimony (MP) character method. The NJ tree was constructed using the Kimura two-parameter model (Kimura, 1980). Correlations and a regression curve between *atpA* and 16 rRNA gene sequences were generated by Pearson's product-moment correlation coefficient using BioNumerics software (Applied Maths). Recombination tests and the ratio of mean synonymous substitutions per synonymous site to mean nonsynonymous substitutions per nonsynonymous site ($d_s:d_n$) were calculated using the START software package obtained from <http://pubmlst.org/software/analysis/start> (Jolley *et al.*, 2001). Split tree decomposition analysis was performed with publicly available software (<http://bibiserv.techfak.uni-bielefeld.de/splits/>).

RESULTS AND DISCUSSION

The *atpA* gene sequences (1322 bp fragments corresponding to approximately 88 % of the coding region) of 151 strains of the family *Vibrionaceae* were analysed. In order to

Fig. 1. Phylogenetic tree based on the neighbour-joining method, using *atpA* gene sequences (1322 bp) of 151 representative strains of the family *Vibrionaceae*. Distance estimations were obtained by Kimura two-parameter model. Bootstrap percentages after 1000 simulations are shown. Bar, 2% estimated sequence divergence. *Shewanella oneidensis* LMG 19005^T was included as an outgroup.

determine the intraspecies variation within this locus, 36 species were represented by more than one strain. The DNA G+C content of the *atpA* gene (47.9 mol%) was consistent with the mean for the total genomes of vibrios (Thompson *et al.*, 2004). The $d_s:d_n$ ratio for the *atpA* gene for all strains analysed was 25.5, suggesting that this locus is under negative selective pressure. Correlations and a regression curve between pairwise similarities of *atpA* and 16S rRNA gene sequences were generated by Pearson's product-moment correlation coefficient. A significant correlation was obtained between the two genetic loci ($R^2=0.85$). The *atpA* and 16S rRNA gene sequence datasets fit a polynomial regression of the second degree. The *atpA* gene sequences are more discriminatory than the 16S rRNA gene sequences. 16S rRNA sequence similarities above 97 % corresponded to *atpA* gene sequence similarities above 80 % (see Supplementary Fig. S1 in IJSEM Online).

The genera *Enterovibrio*, *Grimontia* and *Photobacterium* were clearly separated from the genus *Vibrio* in the NJ tree (Fig. 1). In addition, species groups were recognized within the genus *Vibrio*. These species groups may represent different genera awaiting formal taxonomic description. The *V. harveyi*-, *Vibrio tubiashii*-, *V. splendidus*- and *V. haliotocoli*-species groups formed separate cohesive groups in accordance with previous polyphasic systematic studies (Thompson *et al.*, 2004, 2005). *V. cholerae* and *V. mimicus* appeared at the outskirts of the genus *Vibrio* in the NJ tree. Several *Vibrio* species formed deep branches, e.g. *Vibrio cincinnatiensis*, *Vibrio aestuarianus*, *Vibrio rumoiensis*, *Vibrio gallicus* and *Vibrio gazogenes*, probably because these species may have accumulated a greater number of substitutions in their evolutionary history when compared with other species of vibrios. The MP tree (Supplementary Fig. S2 in IJSEM Online) based on the *atpA* gene sequences did not separate the genus *Vibrio* from the other genera of the family *Vibrionaceae*.

V. harveyi-, *V. scophthalmi*-, *V. coralliilyticus*-, *V. splendidus*-, *V. mediterranei*-, *V. diazotrophicus*-, *V. furnissii*-, *V. anguillarum*-, *V. haliotocoli*-, *V. fischeri*- and *V. cholerae*-species groups were detected in both NJ and MP trees (Fig. 1 and Supplementary Fig. S2). The *V. harveyi*-species group comprised *V. campbellii*, *V. rotiferianus*, *V. brasiliensis*, *V. parahaemolyticus*, *V. mytili*, *V. natriegens*, *V. diabolicus* and *V. alginolyticus*. These species shared at least 95 % *atpA* gene sequence similarity. The closest neighbour of *V. harveyi* was *V. campbellii* with 99 % sequence similarity, followed by *V. rotiferianus* (98 %) and *V. parahaemolyticus* (97 %). *V. harveyi* strains LMG 20370 and R-14913 clustered separately from the other *V. harveyi* strains. These two strains had about 70 % DNA–DNA similarity to *V. harveyi* LMG 4044^T and were also found in AFLP clusters A30 and A31 in a previous study (Thompson *et al.*, 2001). *V. campbellii* R-14902 clustered together with *V. harveyi* strains LMG 20370 and R-14913. In a former study, *V. campbellii* R-14902 showed a similar Biolog pattern to *V. harveyi*, suggesting that this strain belongs to *V. harveyi* (Vandenbergh *et al.*, 2003). *V. parahaemolyticus*

and *V. alginolyticus* were clearly separated, having around 97 % *atpA* gene sequence similarity.

V. scophthalmi and *V. ichthyenteri* formed a group in both NJ and MP trees, sharing around 97 % *atpA* gene sequence similarity. The pair *V. coralliilyticus* and *V. neptunius* had 95 % *atpA* gene sequence similarity, while *V. pacinii* strains formed a separate group in both NJ and MP trees. Previous analyses of 16S rRNA gene sequences of this species suggested that it was related to *V. kanaloae*, *V. pomeroyi* and *V. furnissii* (Thompson *et al.*, 2003). The group comprising *V. tubiashii*, *V. hepatarius* and *V. orientalis* had around 97 % *atpA* gene sequence similarity and appeared scattered in both NJ and MP trees. For instance, *V. brasiliensis* strains clustered with the *V. harveyi*-species clade, sharing around 96 % sequence similarity with this group. *V. splendidus*, *V. tasmaniensis*, *V. kanaloae*, *V. pomeroyi*, *V. cyclitrophicus*, *V. chagasii*, *V. pelagius* and *V. fortis* formed a group and had *atpA* gene sequence similarities of around 97.5 %, except for *V. chagasii* which had 93.7 %. *V. splendidus* strains LMG 16748 and LMG 16752 clustered with *V. kanaloae*, suggesting that these strains may belong to the latter species. *V. mediterranei* formed a group along with *V. shilonii*, sharing almost identical *atpA* gene sequences. *V. diazotrophicus* and *V. hispanicus* grouped together, having 96 % *atpA* gene sequence similarity. *V. furnissii* and *V. fluvialis* also formed a group. These two species have 94 % *atpA* gene sequence similarity. The group composed of *V. anguillarum* and *V. ordalii* had 98 % *atpA* gene sequence similarity.

The *V. haliotocoli*-species group, i.e. *V. neonatus*, *V. ezurae*, *V. superstes* and *V. gallicus* had *atpA* gene sequence similarities of between 89 and 99.5 %. *V. neonatus* and *V. ezurae* were the closest neighbours of *V. haliotocoli* (99.5 % and 98 %, respectively), but were separated in different clusters, suggesting that this locus can serve as an alternative means to identify this group. The *V. haliotocoli*-species group had greater than 98 % 16S rRNA gene sequence similarity. The *V. fischeri* group, e.g. *V. logei* and *V. wodanis*, had between 89 and 93 % *atpA* gene sequence similarity and was detected in both the NJ and MP trees. *V. cholerae* and *V. mimicus* were clearly separated in two groups with a high (100 %) bootstrap value. These two species formed monophyletic groups within the genus *Vibrio*. The *atpA* gene sequence similarity values between *V. cholerae* and *V. mimicus* were at most 96 %, suggesting that this locus is an alternative marker for the identification of these species.

The majority of the species groups based on the *atpA* gene sequences appeared in both the NJ and MP trees and were in agreement with 16S rRNA gene sequence analysis. Overall, the NJ trees constructed on the basis of the 16S rRNA and *atpA* gene sequences showed similar topologies. Most species groups and genera were congruent for both markers, but some differences were observed. For instance, *V. brasiliensis* appeared within the *V. harveyi* group in the *atpA* gene sequence tree and within the *V. tubiashii* group

in the 16S rRNA gene sequence tree (Thompson & Swings, 2006).

In order to check for possible recombination events in the *atpA* gene, we simultaneously analysed the 151 *atpA* gene sequences. Only Sawyer's test, based on the sum of the squares of condensed fragments ($P < 0.01$) detected possible recombination events in several strains, including *V. wodanis* strains K16 and K26, *V. fischeri* ATCC 700601, *V. neonatus* LMG 19976 and *V. scopthalmi* strains LMG 19158^T and R-15029, *V. alginolyticus* strains LMG 4407 and LMG 4409^T, *V. cholerae* R-18303 and *V. mimicus* R-20565, *Enterovibrio corallii* LMG 22228^T and *V. cholerae* R-18303, *Enterovibrio corallii* LMG 22228^T and *V. mimicus* R-20565 and *V. campbellii* LMG 20369 and *V. wodanis* K16. We also generated a split tree decomposition analysis for all strains. The split tree showed massive networks that are hallmarks of recombination, but the Phi test did not provide statistical support for this conclusion. Several different detection methods should provide evidence that recombination did occur within the *atpA* gene sequences. As the Sawyer's test only detected recombination events, we can assume that recombination is not a common evolutionary process in the *atpA* gene of vibrios.

The formal delineation of species of vibrios still relies on DDH similarity, but we have encouraged the use of gene sequences as a reliable alternative towards the establishment of an online electronic taxonomy (Thompson & Swings, 2006). This study shows the usefulness of *atpA* gene sequences for discriminating species groups and species and should lead to a refinement of the online electronic taxonomy of vibrios (<http://www.taxvibrio.lncc.br/>). Work is under way to analyse other genetic loci that will add even more discriminatory power in order to distinguish closely related species. Eventually widespread vibrio clones will be identified through the internet.

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