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Direct evidence of recombination in the *recA* gene of *Aeromonas bestiarum*

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ABSTRACT

Two hundred and twenty-one strains representative of all *Aeromonas* species were characterized using the *recA* gene sequence, assessing its potential as a molecular marker for the genus *Aeromonas*. The interspecies distance values obtained demonstrated that *recA* has a high discriminatory power. Phylogenetic analysis, based on full-length gene nucleotide sequences, revealed a robust topology with clearly separated clusters for each species. The maximum likelihood tree showed the *Aeromonas bestiarum* strains in a well-defined cluster, containing a subset of four strains of different geographical origins in a deep internal branch. Data analysis provided strong evidence of recombination at the end of the *recA* sequences in these four strains. Intergenomic recombination corresponding to partial regions of the two adjacent genes *recA* and *recX* (248 bp) was identified between *A. bestiarum* (major parent) and *Aeromonas eucrenophila* (minor parent). The low number of recombinant strains detected (1.8%) suggests that horizontal flow between *recA* sequences is relatively uncommon in this genus. Moreover, only a few nucleotide differences were detected among these fragments, indicating that recombination has occurred recently. Finally, we also determined if the recombinant fragment could have influenced the structure and basic functions of the RecA protein, comparing models reconstructed from the translated amino acid sequences of our *A. bestiarum* strains with known *Escherichia coli* RecA structures.

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Introduction

The main goal of bacterial phylogenetic studies is to reconstruct the correct genealogical relationships among the strains analyzed, estimate when their divergence occurred, and determine the sequence of events along the evolutionary lineages. Nevertheless, not all the genes commonly used for this purpose are always suitable, and their properties need to be considered before their application in a phylogenetic study [11]. The gene should be conserved, encode essential cell functions, have only a single copy, and be present in all species of the genus [52].

Bacteria reproduce asexually, giving two identical individuals after their division, with the exception of changes produced by mutation or recombination. Although this reproduction process is not associated with recombination, in contrast with eukaryotes, bacteria have acquired three basic mechanisms by which they can incorporate genes from other bacterial species. Nevertheless, their genomes are not simply arbitrary assortments of genes of mixed heritage. Recombination in bacteria is always restricted to small DNA fragments, is unidirectional and independent of reproduction, and occurs with a relatively low frequency, although genes codifying virulence factors or antibiotic resistance experiment more frequent recombination changes [10,12].

The impact of recombination on bacterial phylogenies has been the subject of considerable discussion [10,13,17,36,41,56]. Recently, with the availability of sequencing techniques and the analytical power of new programmes, the detection of recombination events has increased dramatically. This has led to the questioning of existing phylogenies and the methods used for their construction, such as Maximum Likelihood (ML) and Maximum Parsimony (MP), which assume that the analyzed sequences have the same evolutionary history. Due to the importance of recombination in evolutionary analysis, it is essential to be able to identify whether a given set of sequences has undergone recombination

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events, define the boundaries of the recombinational units, and evaluate the impact of recombination on our ability to reconstruct evolutionary histories and estimate population genetic parameters [12,27].

Traditionally, recombination in a given set of sequences has been identified by the incongruence of the different gene trees analyzed, the presence of mosaic structures, and variations in the G+C content or the codon bias. Several new methods have been developed to test the presence of recombination, as well as to identify the parental and recombinant individuals or the recombination break-points. Those methods can be classified in different categories: similarity, distance, phylogenetic, compatibility, and nucleotide substitution distribution [23,40]. Their performance varies, depending on the level of recombination, but in general most of them are efficient, and although they can have trouble in detecting recombination when the level of divergence is low, their discriminatory power increases when the level of recombination is high [41].

The genus *Aeromonas* Stanier 1943 belongs to the family *Aeromonadaceae* within the class *Gammaproteobacteria* [29]. Aeromonads are autochthonous inhabitants of aquatic environments, including chlorinated and polluted waters, although they can also be isolated from a wide variety of environmental and clinical sources. They are usual microbiota (as well as primary or secondary pathogens) of fish, amphibians and other animals. Some species, mainly *Aeromonas caviae*, *Aeromonas hydrophila* and *Aeromonas veronii* bv. Sobria, are opportunistic pathogens of humans [19]. Several attempts have been made to generate phylogenies using DNA gene sequences to reconstruct the correct genealogical ties among species in *Aeromonas* [9,11,30,46]. However, the genes chosen for this purpose are not always suitable, and do not necessarily give congruent phylogenies [21,51].

In our study we investigated the discriminatory power of the *recA* gene sequences at inter- and intra-specific levels for application in *Aeromonas* phylogenetic studies. Any cluster showing incongruences was analyzed looking for the presence of potential recombinant fragments in their *recA* gene sequences.

Material and methods

Data set

221 *Aeromonas* strains were analyzed based on the nucleotide sequences of the *recA* gene: 125 belonged to the "*A. hydrophila* Species Complex" (study 1), and 150 (54 strains from study 1) represented all the species and subspecies of this genus described to date, including the type strains as well as some strains considered as synonymous (study 2) (Table S1). The strains were obtained from several culture collections (CECT, Colección Española de Cultivos Tipo, Universitat de València, Valencia, Spain; CIP, Collection de l'Institut Pasteur, Paris, France; JCM, Japan Collection of Microorganisms, RIKEN BioResource Center, Ibaraki, Japan; LMG, Culture Collection of the Laboratorium voor Microbiologie Gent, Universiteit Gent, Ghent, Belgium), kindly supplied by different authors or research groups (Katri Berg, University of Helsinki, Helsinki, Finlandia; Yogesh Shouche, Molecular Biology Laboratory, National Centre for Cell Science, Pune, India; Margarita Gomila, Universitat de les Illes Balears, Palma de Mallorca, Spain; Mª José Figueras, Universitat Rovira i Virgili, Reus, Spain; Antonio Martínez-Murcia, Universidad de Alicante, Spain), or obtained by our group from freshwater and food samplings [35]. Strains were grown aerobically on tryptone soy agar (TSA; Pronadisa, Laboratorios Conda) supplemented with 1% (w/v) NaCl for 24–48 h at their optimum temperature, which ranged between 25 and 30 °C. For long-term storage, pure cultures were stored frozen at −40 and −80 °C in triptone soy broth (TSB; Oxoid, Thermo Fisher Scientific Inc.) containing 20% glycerol and on Protect™ cryobeads (Technical Service Consultants Ltd.). Species affiliation, source, geographical origin and the type of phylogenetic study of all the strains analyzed are listed in Table S1.

DNA extraction, primers, and PCR conditions

Genomic DNA was extracted and purified with the REALPURE® Genomic DNA extraction kit (Durviz) and stored at −20 °C until use. Primer3 software was used to design PCR and sequencing primers (http://primer3.sourceforge.net/, [55]). Oligonucleotide primers were designed from published genome sequences of *A. hydrophila* ATCC 7966^T (GenBank accession number: CP000462, [50]) and *Aeromonas salmonicida* subsp. *salmonicida* A449 (GenBank accession number: CP000644, [43]). All primers used in this study are shown in Table S2. Six different primer sets were used to amplify by PCR the complete *recA* gene and its flanking regions (Table S2). Additional internal primers were designed for the sequencing of *recA*. The oligonucleotides were synthesized by Invitrogen™ (Thermo Fisher Scientific). The conditions of amplification by PCR were optimized in a 50 μL final volume reaction, containing 0.5–10 μL of genomic DNA as the template, 0.2–2 μM each primer, 0.2 mM each dNTP, 0.5 mM $MgCl_2$, 5% dimethyl sulfoxide (DMSO) (optional), 1X Buffer I (10X Buffer I: 100 mM Tris–HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% (w/v) gelatin) and 1.25 U of AmpliTaq Gold® DNA polymerase (Applied Biosystems). Amplifications were performed in Veriti® (Applied Biosystems) and Applied Biosystems® 2720 thermal cyclers using the following programme: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 53–58 °C for 1 min and elongation at 72 °C for 2 min, and a final extension at 72 °C for 10 min. The PCR products were resolved by electrophoresis in 1% agarose gels stained with GelRed™ (Biotium) and visualized by UV transillumination. Amplicons were purified with a MSB® Spin PCRapace kit (STRATEC Molecular). Purified PCR products were directly sequenced on both strands using either the PCR or internal primers. Sequencing reactions were performed with the BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and analyzed on an ABI PRISM® 3700 DNA sequencer (Applied Biosystems) by the Genomics Unit of the Scientific and Technological Centers of the University of Barcelona (CCiTUB).

Phylogenetic and sequence analysis

Multiple sequence alignments were performed using the ClustalW program [22] implemented in MEGA6 software [54]. We also applied the graphical dots plot method for the exploratory sequence analysis. This method was carried out with functions included in *phyclust* [7] and *ape* [38] packages using the R programming language [42]. Maximum likelihood (ML) phylogenetic analyses were performed using the PhyML 3.1 program [16] with 1000 bootstrap replicates to assess tree topology robustness. Phylogenetic trees were reconstructed based on the best fit model of evolution for each dataset estimated in the MEGA6. Phylogenetic trees were visualized using the NJPlot program [39].

Recombination detection methods

To detect potential recombination events, we analyzed the incongruences in the tree topology, examined the sequence alignments and the dots plots, determined the G+C content (mol %) with the DnaSP program (version 5, [24]), and calculated the codon usage bias with MEGA6 software. In addition, we evaluated the possible recombination events, and identified potential major and

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