Phylogenetic positions of *Aeromonas encheleia*, *Aeromonas popoffii*, *Aeromonas* DNA hybridization Group 11 and *Aeromonas* Group 501

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The 16S rDNA sequences of the recently described *Aeromonas encheleia* and *Aeromonas popoffii*, were determined and compared with data from all known *Aeromonas* sp. Diagnostic 16S rDNA regions were also sequenced for some strains previously considered as an extension of *A. encheleia* and a strain of *Aeromonas* Group 501 (formerly Enteric Group 501). Results indicated that *A. encheleia* and *A. popoffii* are phylogenetically separated species as originally described. A conclusion about HG11 taxonomic status is not recommended until previous discrepancies are clarified by further DNA–DNA hybridization and sequencing studies.

Keywords: *Aeromonas encheleia*, *Aeromonas popoffii*, *Aeromonas* sp. HG11, *Aeromonas* Group 501, rRNA

The taxonomy of the genus *Aeromonas* has undergone major changes since its description in *Bergey's Manual of Systematic Bacteriology* (Popoff, 1984). Extensive DNA–DNA hybridization studies (Popoff *et al.*, 1981; Hickman-Brenner *et al.*, 1987, 1988; Kuijper *et al.*, 1989; Carnahan *et al.*, 1991b) have resulted in the recognition of fourteen so-called DNA homology groups (HG): *Aeromonas hydrophila* (HG1), *Aeromonas* sp. (unnamed HG2), *Aeromonas salmonicida* (HG3), *Aeromonas caviae* (HG4), *Aeromonas media* (HG5), *Aeromonas eucrenophila* (HG6), *Aeromonas sobria* (HG7), *Aeromonas veronii* biogroup *sobria* (HG8), *Aeromonas jandaei* (HG9), *Aeromonas veronii* biogroup *veronii* (HG10), *Aeromonas* sp. (unnamed; HG11), *Aeromonas schubertii* (HG12), *Aeromonas* Group 501 (HG13; formerly Enteric Group 501) and *Aeromonas trota* (HG14). The name *Aeromonas bestiarum* has been proposed for strains included in HG2 (Ali *et al.*, 1996). Recently, three new descriptions of species include: *Aeromonas allosaccharophila* (Martínez-Murcia *et al.*, 1992b), *Aeromonas encheleia* (Esteve *et al.*, 1995) and *Aeromonas popoffii* (Huys *et al.*, 1997b). Discrepancies remain between observed HGs and groups derived from phenotypic analysis. The species *Aeromonas ichthiosmia* (Schubert *et al.*, 1990b) and *Aeromonas enteropelogenes* (Schubert

et al., 1990a) are now considered to be synonyms of *A. veronii* and *A. trota*, respectively, as they were identical by 16S rDNA sequencing (Collins *et al.*, 1993), phenotyping, fatty acid profile by gas liquid chromatography, and nearly identical by ribotyping (Carnahan, 1993). DNA probes and RFLP profiles designed from 16S rDNA diagnostic regions have served to identify most species of the genus *Aeromonas* (Martínez-Murcia, 1993; Ash *et al.*, 1993a, b; Dorsch *et al.*, 1994; Khan & Cerniglia, 1997; Borrell *et al.*, 1997). A chemotaxonomic and genotypic study carried out by Huys *et al.* (1996a, b) have questioned the original species description of *A. encheleia* as some *A. eucrenophila* and *Aeromonas* sp. HG11 strains were affiliated to *A. encheleia*. Further, the inclusion of *Aeromonas* sp. HG11 in *A. encheleia* and extended descriptions of *A. eucrenophila* and *A. encheleia* were proposed (Huys *et al.*, 1997a), which is in contrast to the original description based on phenotype and DNA–DNA pairing (Esteve *et al.*, 1995). The 16S rDNA sequence of *A. encheleia* type strain (S181^T, CECT 4342^T) was not included in either the original or the extended descriptions of *A. encheleia* (Esteve *et al.*, 1995; Huys *et al.*, 1997a). A phylogenetic analysis of all known *Aeromonas* spp., including *A. encheleia*, *A. popoffii* and *Aeromonas* Group 501 is reported in the present study.

The *A. encheleia* type strain (S181) was obtained in 1991 from Esteve's personal collection and later, a duplicate CECT 4342^T and *Aeromonas* Group 501

Abbreviation: HG, hybridization group.

Fig. 1. Phylogenetic relationships of described *Aeromonas* species (or DNA Homology Groups) as determined by a continuous 1502-nucleotide 16S rDNA sequence comparison using the neighbour-joining method. Strains and sequence accession numbers (in parentheses) are: *Aeromonas hydrophila* ATCC 7966^T, (X60404); *A. bestiarum* CIP 7430^T, (X60406); *A. salmonicida* NCIMB 1102^T, (X60405); *A. caviae* NCIMB 13016^T, (X60408); *A. media* ATCC 33907^T, (X60410); *A. eucrenophila* NCIMB 74^T, (X60411); *A. sobria* NCIMB 12065^T, (X60412); *A. veronii* ATCC 35624^T, (X60414); *A. jandaei* ATCC 49568^T, (X60413); *Aeromonas* sp. DHG11 ATCC 35941^T, (X604417); *A. schubertii* ATCC 43700^T, (X60416); *A. trota* ATCC 49657^T, (X60415); *A. allosaccharophila* CECT 4199^T, (S39232); *A. encheleia* CECT 4342^T, (AJ224309); *A. popoffii* LMG 17541^T, (AJ224308); *Aeromonas* Group 501 CECT 4254, (U88663).

strain CECT 4254 (ATCC 43946), from the Colección Española de Cultivos Tipo (CECT; Universidad de Valencia, Spain). All other strains, listed in Table 2, were provided in 1995 by M. Altwegg (Institute of Medical Microbiology, Zürich, Switzerland). Strain *A. popoffii* (LMG 17541^T) was a gift from M. J. Figueras, Universidad Rovira i Virgili, Reus, Spain. Strains were cultivated in trypticase soy broth (Oxoid) at 30 °C for 24 h. Cultures were harvested and washed in deionized sterile water. Total DNA was extracted and purified, PCR performed and 16S rDNA sequence (continuous stretch of 1502 nucleotides) of *A. encheleia* CECT 4342^T determined following the methods described in Martínez-Murcia *et al.* (1992a). The homologous sequence (1502 nucleotides) of *A. popoffii* LMG 17541^T and the diagnostic 16S rDNA nucleotides located at the V2 and V3 regions (ca a continuous stretch of 300 bp ranging from positions 230 to 530, approximately; Martínez-Murcia *et al.*, 1992a) of the remaining strains (Table 2) were sequenced using the Cy5 AutoRead Sequencing Kit (Pharmacia) on the ALFexpress DNA sequencer (Pharmacia), according to the manufacturer's indications. After this work was completed, partial 16S rRNAs of *A. encheleia* and *A. popoffii* (1407 nucleotides each) were found submitted in the GenBank in February 1998 by Demarta and co-workers (accession numbers AJ224309 and AJ224308, respectively). Except for size, sequences were compared and no difference was found. Newly determined sequences at both ends (95 nucleotides) of the gene were found to be conserved to all these known for *Aeromonas*. Sequences of *A. encheleia*, *A. popoffii* and *Aeromonas* Group

501, were aligned to other previously published sequences of *Aeromonas* (Martínez-Murcia *et al.*, 1992a, b) and a phylogenetic tree produced by the neighbour-joining method (Saitou & Nei, 1987) using the MEGA Program version 1.01 (Fig. 1).

The original description of *A. schubertii* was based on seven highly related strains with the vernacular name Enteric Group 501 (Hickman-Brenner *et al.*, 1988). This group included an eighth strain, CDC 2478-85 (ATCC 43946), that was less related to *A. schubertii* by DNA hybridization (70% at 60 °C, 61% at 75 °C, with a divergence of 5%). This result and phenotype suggest that this strain is close to *A. schubertii* but has diverged somewhat and authors kept it, together with a new strain CDC 2555-87, in the so-called *Aeromonas* Group 501 (Hickman-Brenner *et al.*, 1988). A specific HG number, HG13, was later assigned (Carnahan *et al.*, 1991a). Sequencing of about 450 bp of the 16S rDNA 5'-end of strain CECT 4254 (CDC 2478-85) indicated a close relationship to *A. schubertii* but four nucleotide differences were detected. This stretch was compared with the sequence (1467 nt) determined from the same strain and deposited by A. M. Whitney in the databases (accession no. U88663) in August 1997. Two inconsistencies were found: the sequence from the GeneBank showed variation at two positions (202 and 203; numbering as by Martínez-Murcia *et al.*, 1992a) that are conserved for all *Aeromonas* (guanine at both sites). Further sequencing confirmed our previous result with no ambiguities. Excluding these two heterogeneities, the sequence (1467 bp) of *Aeromonas* Group 501 was different from that of *A.*

Table 1. Number of nucleotide differences in the 16S rDNA sequences of *A. encheleia*, *A. popoffii* and *Aeromonas* Group 501 compared to those from all other *Aeromonas* species

1, *Aeromonas hydrophila*; 2, *A. bestiarum*; 3, *A. salmonicida*; 4, *A. caviae*; 5, *A. media*; 6, *A. eucrenophila*; 7, *A. sobria*; 8, *A. veronii*; 9, *A. jandaei*; 10, *Aeromonas* sp. DNA homology group 11; 11, *A. schubertii*; 12, *A. trota*; 13, *A. allosaccharophila*; 14, *A. encheleia*; 15, *A. popoffii*; 16, *Aeromonas* Group 501. Strains are listed in the legend to Fig. 1.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
<i>A. encheleia</i>	11	7	5	25	14	6	10	17	24	8	28	26	17	0	7	30
<i>A. popoffii</i>	12	11	9	22	15	11	10	18	21	8	28	23	16	7	0	27
<i>Aeromonas</i> Group 501	31	29	31	26	30	28	25	13	15	25	6	25	23	30	27	0

schubertii only by six nucleotides. Present phylogenetic analysis indicated that this micro-organism may belong to a different *Aeromonas* sp., closely related to *A. schubertii* (Fig. 1), which is consistent with the relationship indicated by Hickman-Brenner *et al.* (1988) by DNA–DNA hybridization data.

Sequence comparison showed that the 16S rDNA of *A. popoffii* is species-specific with an inter-species range of 7–28 nucleotide differences (Table 1). Description of this species (Huys *et al.*, 1997b) indicated some resemblances with *A. hydrophila* (HG1), *A. bestiarum* (HG2) and *A. salmonicida* (HG3) on the basis of genotype and phenotype. The highest DNA–DNA hybridization values were determined with *A. bestiarum* (51–63%; Huys *et al.*, 1997b). The 16S rDNA of *A. popoffii* exhibited a significant number of differences with the sequences of these three species (9–12 nucleotides); instead, the closest relative was *A. encheleia* (7 nucleotides) and *Aeromonas* sp. HG11 (8 nucleotides). The phylogenetic analysis (Fig. 1) indicates that *A. popoffii* belongs to a new phylogenetic line of descent within this genus, which supports the original description.

A. encheleia was more related to *A. salmonicida* and *A. eucrenophila* than to any other species on the basis of 16S rDNA comparison (5 and 6 differences, respectively; Table 1). A close relationship to *A. eucrenophila* is supported by phenotype: most key phenotypic tests for the differentiation of all known *Aeromonas* spp. do not separate *A. encheleia* from *A. eucrenophila* (see Table 3 of Huys *et al.*, 1997a, b). After alignment of all 16S rDNAs, the unnamed *Aeromonas* sp. HG11 exhibited a species-specific sequence including 'unique' nucleotides at two positions, i.e. a nucleotide composition at these sites only found in this species of the genus. The sequence of HG11 showed maximum similarity with that of *A. sobria*. Preliminarily, the tree of Fig. 1 and qualitative sequence analysis suggest the placement of HG11 as a single species phylogenetically separated from *A. encheleia*, according to previous data (Martínez-Murcia, 1993).

Diagnostic regions of the 16S rDNA (polymorphic sites at V2 and V3 regions; Martínez-Murcia *et al.*,

1992a) of some *A. eucrenophila* and *A. encheleia* strains, considered to be a part of the *A. encheleia* extension (Huys *et al.*, 1997a), were determined in the present work. The two known HG11 strains, A902 (a duplicate of ATCC 35941) and A926, showed the characteristic signature sequences of HG11, in agreement with published data (Martínez-Murcia *et al.*, 1992a). In fact, both strains were peripheral to cluster II by AFLP (also called cluster A2; Huys *et al.*, 1996a, b) and did not cluster to phenon 2 (Huys *et al.*, 1997a), such a clustering considered as the *A. encheleia* extended-frame. Reported chromosomal DNA relatedness added more controversy: strain A902 (reference of HG11) shows 84% DNA–DNA similarity with the type strain of *A. encheleia* (Huys *et al.*, 1997a), which is in marked disagreement to the 12% determined by Esteve *et al.* (1995). If the possibility of mislabelling or contamination of strains has been ruled out by performing experiments with duplicates from the original culture collections, this is one of the more serious discrepancies found in two sets of DNA–DNA hybridization values from different laboratories that used the same micro-organisms. Additional strains A9 and A1654, initially considered to be *A. encheleia*-like and *A. eucrenophila*, respectively, part of phenon 2 and grouped in cluster II of AFLP (Table 2), also showed the signature nucleotides of HG11. Relevant DNA–DNA hybridization values for strain A9, and the corresponding values for the pair A1654/HG11, were not reported (Huys *et al.*, 1997a). Strain A1653 (*A. eucrenophila*), having shown a really high DNA–DNA hybridization value with HG11 (96%; Table 2), revealed the diagnostic nucleotides of *A. encheleia*. This result clearly represents a new case of conflict between these two approaches as these two taxa show separated phylogenetic lines. Two strains, A1655 and A1783, also showed the 16S rDNA nucleotides characteristic of *A. encheleia*, in agreement with the species assigned by Huys *et al.* (1997a). However, strains A1653, A1654 and A1655 were related to *A. eucrenophila* at a level of 80–82% DNA–DNA hybridization (Table 2; Schubert & Hegazi, 1988) and, therefore, they were included in the original description of this species. These data make present controversy deeper. The determined sequences

Table 2. Characteristics and classification based on 16S rDNA signature sequences of some *Aeromonas* strains used in this study

LMG, BCCM/LMG Culture Collection, Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium. NR, Not reported.

Formerly classified as:*	Strain (LMG)	Phenotype*	AFLP*	DNA-DNA hybridization:†‡			Diagnostic 16S rDNA
				<i>A. encheleia</i>	<i>A. eucrenophila</i>	<i>Aeromonas</i> sp. HG11	
<i>A. encheleia</i> -like	A9 (16398)	Phenon 2	Cluster II‡	NR	NR	NR	<i>Aeromonas</i> sp. HG11
<i>Aeromonas</i> sp. HG11	A902 ^T (13075 ^T)	Unclustered	Cluster II	84 (12)	52	100	<i>Aeromonas</i> sp. HG11
<i>Aeromonas</i> sp. HG11	A926 (13076)	Unclustered	Cluster II	87	51	94	<i>Aeromonas</i> sp. HG11
<i>A. eucrenophila</i>	A1654 (13062)	Phenon 2	Cluster II	94	46 (82)	NR	<i>Aeromonas</i> sp. HG11
<i>A. eucrenophila</i>	A1653 (13061)	Peripheric	Cluster II	95	50 (80)	96	<i>A. encheleia</i>
<i>A. eucrenophila</i>	A1655 (13691)	Phenon 2	Cluster II	NR	50 (80)	NR	<i>A. encheleia</i>
<i>A. encheleia</i> -like	A1783 (16403)	Phenon 2	Cluster II	NR	NR	NR	<i>A. encheleia</i>
NR	A1784 (16404)	NR	NR	NR	NR	NR	<i>A. eucrenophila</i>
<i>A. encheleia</i> -like	A1786 (16406)	Phenon 2	Cluster II	NR	NR	NR	<i>A. eucrenophila</i>
<i>A. encheleia</i> -like	A1782 (16402)	Phenon 2	Cluster II	NR	NR	NR	<i>A. salmonicida</i> / <i>A. bestiarum</i>

* Data from Huys *et al.* (1996a, b, 1997a).

† Percentage DNA reassociated with type strains of respective species. Results in parentheses are from Esteve *et al.* (1995) and from Schubert & Hegazi (1988).

‡ Cluster II as determined by Huys *et al.* (1996b) corresponds to Cluster A2 of Huys *et al.* (1996a).

of strains A1784 and A1786 were characteristic of *A. eucrenophila*; however, that of strain A1782 was characteristic of *A. salmonicida*/*A. bestiarum*. These latter two species are only distinguishable by two paired nucleotides at positions 1011 and 1018 (Martínez-Murcia *et al.*, 1992a), consistent with difficulties found in separating strains of HG2/HG3 by DNA-DNA pairing (Hänninen, 1994). Again, DNA-DNA hybridizations have not been reported for the last three versus reference strains (Table 2). As some DNA-DNA hybridization values are unknown and, worthy of note, the thermal stability of re-associated DNA was not determined (see recommendation of Wayne *et al.*, 1987), it is better to consider these, and similar strains, in need of urgent investigation.

Sequencing of 16S rRNA is today considered a robust way to achieve bacterial taxonomy (Woese, 1987; Olsen *et al.*, 1994) and DNA-DNA hybridization has been recommended for defining species (Wayne *et al.*, 1987). Although in *Aeromonas* most rDNA derived relationships correlate well with DNA-DNA data, identical rRNA sequences but different species by DNA-DNA pairing have previously been detected (Martínez-Murcia *et al.*, 1992a). In view of these conflicts, the results of DNA reassociation have always been taken as definitive (Collins *et al.*, 1993) as the high overall conservation of the 16S rRNA may preclude the splitting of very closely related genotypes. However, at present, *Aeromonas* strains showing a DNA-DNA similarity range as high as 84–94% have

never shown a phylogenetic divergence based on 8 nucleotide positions, two of which were HG11-specific composition. Although these polymorphisms are noticeable considering the tight phylogenetic depth of this genus, the number of nucleotide differences is too low and the possibility of molecular clock 'distortions' (i.e. plesiomorphy, inter-cistronic and inter-strain diversity, etc.) has to be taken into account. Consequently, and according to the previously reported limitations of 16S rDNA analysis in the determination of relationships at the strain level (Stackebrandt & Goebel, 1994), a conclusion about the HG11 taxonomic status should be taken on the basis of DNA-DNA hybridization data.

Bacterial species definition based on chromosomal DNA hybridization (Wayne *et al.*, 1987) was empirically derived from a wide range of taxa, but values should not be treated as absolute or fixed. Concerns about variation on the level of DNA-DNA below which organisms are considered to belong to different species have been previously reported (Zakrzewska-Czerwinska *et al.*, 1988). DNA-DNA pairing may give a 'too crude' measure of DNA sequence homology or, at least, not fine enough to split highly similar species. Grimont *et al.* (1980) showed that a relative binding ratio (RBR) of 70% obtained with the hydroxyapatite method corresponds, at optimal temperature, to about 55–60% RBR obtained with the S₁-nuclease method and this correlation may vary with the temperature. Discrepancies may be important when many RBRs in *Aeromonas* are bor-

derline to the recommended value (70%) to split species. The more pronounced lack of agreement so far reported is that for *A. encheleia*/HG11/*A. eucrenophila* (Table 2) using the same reference strains but different hybridization methods: optical renaturation used by Huys *et al.* (1997a) and competitive nitrocellulose filter utilized by Esteve *et al.* (1995). Research on the correlation between several DNA-DNA procedures for these controversial strains is hereby suggested.

AFLP and ribotyping were added to support inclusion of HG11 in *A. encheleia*, commented in 'Letters to the Editor' of the *International Journal of Systematic Bacteriology* (Esteve, 1997). These grouping methods are based on fingerprints comprising chromosomal restriction fragments obtained from a limited (selected by availability) group of strains. Estimation of size-fingerprint resemblances and number of bona-fide strains included in each taxa might influence the final numerical analysis (Sneath & Sokal, 1973). Currently, difficulties caused by low intensity/background ratios are also a source of error for fingerprint scoring. As patterns are highly heterogeneous and strain-specific, there is no doubt about their usefulness for bacterial typing and epidemiological studies (Vos *et al.*, 1995). To date, however, the taxonomic value of the 'AFLP concept' (Huys *et al.*, 1996b) and similar methods (e.g. those yielding some selected information about genomic organization of unknown evolutionary nature) in a wide range of bacterial taxa, is still to be demonstrated. If a phylogenetic frame is desired, knowledge about mode and extent to which genomic organization interrelates with, and reveals final stages of, bacterial species evolution, should be required.

In view of the 16S rDNA results from the present study and all previously reported discrepancies between different sets of DNA-DNA hybridizations, a comprehensive study including all *Aeromonas* species (and HGs) is proposed by applying the same DNA-DNA hybridization method, sequencing of single 16S and 23S rDNA copies of all operons of each strain, but also presumably faster molecular clocks as the 16S-23S rDNA spacers or 'house-keeping' protein coding genes. A sequencing project with the above aim is under way.

ACKNOWLEDGEMENTS

The 16S rDNA sequence of *A. encheleia* type strain, originally strain V181, was determined in 1991 while A.J.M.-M. was a PhD student at the IFR, Reading Laboratory, UK, under supervision of Dr M. D. Collins, who was of invaluable support.

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