Use of the novel phylogenetic marker *dnaJ* and DNA–DNA hybridization to clarify interrelationships within the genus *Aeromonas*

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The interrelationships of 27 *Aeromonas* strains were investigated using *dnaJ* sequences and DNA–DNA hybridization. *dnaJ* sequence similarities showed a stronger relationship with DNA–DNA relatedness values than did 16S rRNA gene sequence similarities. Additionally, *dnaJ* sequence analysis, with interspecies divergence over 5.2 % in most cases, gave better resolution than 16S rRNA gene sequences for the differentiation of strains at the species level. Relationships among *Aeromonas* species were therefore elucidated on the basis of *dnaJ* sequences and DNA–DNA reassociation. Strains of *Aeromonas encheleia* and *Aeromonas* sp. HG11 were unquestionably grouped in the same genetic species, since they shared 98.7 % *dnaJ* sequence similarity and 82–85 % genomic relatedness. The phylogenetically close relationships obtained from *dnaJ* sequence analysis (1.7–3.3 % genetic distance) were corroborated by high DNA–DNA relatedness (73–97 %) to support the previous suggestion that *Aeromonas culicicola* and *Aeromonas allosaccharophila* are later heterotypic synonyms of *Aeromonas veronii*. Our findings will contribute to the clarification of controversial relationships in the genus *Aeromonas* and also demonstrate that analysis of *dnaJ* sequences can be a powerful tool for interspecies study of the genus.

INTRODUCTION

The taxonomy of the genus *Aeromonas* has been the subject of considerable debate. In this genus, taxonomic controversies have been attributed to the absence of sufficient phenotypic markers for the differentiation of all recognized DNA hybridization groups (HGs) and to the striking disagreement between DNA HGs and their phylogenetic positions (Martínez-Murcia, 1999). According to the second edition of *Bergey's Manual of Systematic Bacteriology*, the genus includes 14 phenospecies that correspond to at least 17 DNA HGs (Martin-Carnahan & Joseph, 2005). Recently, descriptions of novel species, reclassifications and extended descriptions of species with validly published names have resulted in ongoing changes to the taxonomy of the genus *Aeromonas*.

At present, DNA–DNA hybridization and 16S rRNA gene sequences, along with housekeeping gene sequences, are

considered crucial molecular tools for the definition of bacterial species (Stackebrandt *et al.*, 2002). In the genus *Aeromonas*, however, the high conservation of the 16S rRNA gene sequence (97.8–100 %; Martin-Carnahan & Joseph, 2005) and the existence of several copies of the gene, with intragenomic heterogeneity, in some *Aeromonas* strains (Morandi *et al.*, 2005) limit the usefulness of the 16S rRNA gene for taxonomic analysis at the species level. DNA–DNA hybridization has not been subjected to extensive analysis of interrelationships for all present species. Moreover, discordances between 16S rRNA gene analysis and DNA–DNA hybridization results (Martínez-Murcia *et al.*, 1992b) or between DNA–DNA hybridization data reported by different authors (Saavedra *et al.*, 2006) have added more fuel to the controversies regarding the sites of *Aeromonas* species boundaries. It has been reported that housekeeping gene sequences, e.g. *gyrB* and *rpoD*, could be used to clarify interspecies phylogenetic relationships within *Aeromonas* (Yáñez *et al.*, 2003; Soler *et al.*, 2004). In some cases, the different behaviour of *gyrB* and *rpoD* sequences has left species delineations (e.g. *Aeromonas culicicola* and *Aeromonas allosaccharophila*) in an ambiguous state, so they still need to be resolved (Saavedra *et al.*, 2006).

The GenBank/EMBL/DDBJ accession numbers for the partial *dnaJ* gene sequences reported in this study are AB280551–AB280578, as indicated in Fig. 1.

The *dnaJ* gene, encoding heat-shock protein 40, which acts as a functional cohort of DnaK in various aspects of protein dynamics (Caplan *et al.*, 1993), has been reported to be a suitable target for phylogenetic study and identification of species of *Mycobacterium* (Takewaki *et al.*, 1994), *Legionella* (Liu *et al.*, 2003) and *Streptococcus* (Itoh *et al.*, 2006). In this study, we used *dnaJ* gene sequences and extensive DNA–DNA hybridizations in an attempt to clear up the taxonomic confusion regarding interrelationships within the genus *Aeromonas*.

METHODS

Bacterial strains. The 27 strains used were obtained from the Gifu Type Culture Collection, Gifu University, Japan, and are listed in Fig. 1. Strains were grown on tryptic soy agar (Difco) at 28 °C, except for the psychrotolerant *Aeromonas salmonicida* strains, which were grown at room temperature.

Primer design. No *dnaJ* sequences from *Aeromonas* strains were available in GenBank. Therefore, the first primers were designed from conserved regions of sequences from species related to *Aeromonas* (*Vibrio cholerae*, *Vibrio harveyi*, *Escherichia coli*, *Salmonella enterica* subsp. *enterica* and *Pseudomonas putida*; GenBank accession numbers AE004171, AY639008, M12565, U58360 and AY823737, respectively). Based on sequences of *Aeromonas* strains amplified by the first primers, primers Aero-dnaJF (5'-CGAGATCAAGAAGGC-GTACAAG-3') and Aero-dnaJR3 (5'-CACCACCTTGCACATCAGATC-3') were designed to amplify a *dnaJ* fragment of approximately 934 bp.

PCR amplification and sequencing. DNA was extracted by quick-heat lysis. One colony was suspended in 100 µl distilled water and boiled for 10 min. The supernatant served as a template. Amplification reactions contained 1 × PCR buffer, 0.2 mM of each dNTP,

0.1 U *Taq* polymerase (Takara Shuzo), 0.4 µM of each primer and 1 µl template in a final reaction volume of 20 µl. PCR amplification was carried out in a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems) as follows: a 3 min initial denaturation step at 94 °C, followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s and 74 °C for 1 min, with a final extension step of 7 min at 72 °C. Amplified products were examined by agarose gel electrophoresis (1.2 %) and ethidium bromide staining. Purified PCR products were sequenced with the use of a BigDye Terminator v3.1 cycle sequencing ready reaction kit (Applied Biosystems) in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions.

Phylogenetic data analysis. *dnaJ* sequences were aligned by the CLUSTAL W program (version 1.83) (Thompson *et al.*, 1994). Genetic distances were obtained with the MEGA3 package (Kumar *et al.*, 2004) by the neighbour-joining method (Saitou & Nei, 1987) and Kimura's two-parameter distance model (Kimura, 1980). Phylogenetic trees were constructed by the neighbour-joining and maximum-parsimony methods.

DNA–DNA hybridization. A total of 21 strains were selected for the DNA–DNA reassociation study. Genomic DNA was prepared using the protocol of Marmur (1961) with minor modifications (Ezaki *et al.*, 1988). DNA–DNA hybridizations were carried out at an optimal temperature of 45 °C in 2 × SSC buffer and 50 % formamide, using the fluorometric microplate method (Ezaki *et al.*, 1989).

RESULTS AND DISCUSSION

Analysis of *dnaJ* sequences and DNA–DNA hybridization

Partial *dnaJ* gene fragments of approximately 934 bp were amplified for all 27 *Aeromonas* strains. *dnaJ* sequences from

Fig. 1. Neighbour-joining phylogenetic tree based on partial *dnaJ* (891 bp) gene sequences of 27 *Aeromonas* strains. Bootstrap values (expressed as percentages of 1000 replicates) are shown at nodes. *Vibrio cholerae* GTC 37^T was used as an outgroup. Bar, 2% sequence divergence. Numbers in parentheses indicate the *dnaJ* accession numbers. Asterisks indicate sequences from *A. trota* and *A. ichthiosmia*, which are now considered to be synonyms of *A. enteropelogenes* and *A. veronii*.

all the strains were aligned and DNA sequence similarities were calculated for a consecutive stretch of 891 bases. Mean sequence similarity, defined as discriminatory power (La Scola *et al.*, 2003), was 89.2 %, indicating a divergence of the *dnaJ* gene that is remarkably greater than that of the 16S rRNA gene (98.7 %), but comparable to that of *gyrB* (92.2 %) and *rpoD* (89.3 %) (Soler *et al.*, 2004). Sequence similarity among the *Aeromonas* strains ranged from 81.2 to 99.9 %, corresponding to between 167 and 1 nucleotide differences.

At the intraspecies level, the rate of nucleotide substitution did not exceed 3.3 %, except for *Aeromonas hydrophila* subsp. *dhakensis* GTC 2880^T, which showed far larger distances (4.7 and 5.1 %, respectively) from *A. hydrophila* subsp. *hydrophila* GTC 2793^T and *A. hydrophila* subsp. *ranaei* GTC 2877^T. At the interspecies level, the rate of nucleotide substitutions was greater than 5.2 % for most *Aeromonas* strain pairs but was only 1.7–3.3 % for a cluster including four species (*Aeromonas ichthiosmia*, *Aeromonas veronii*, *A. allosaccharophila* and *A. culicicola*) and 1.3 % for *Aeromonas encheleia* and *Aeromonas* sp. HG 11. These results brought into question the species delineations of *A. culicicola*, *A. allosaccharophila* and *Aeromonas* sp. HG11.

The phylogeny based on *dnaJ* sequences of 27 strains constructed by the neighbour-joining method is shown in Fig. 1. The tree generated by the maximum-parsimony method gave the same topology (data not shown). In general, the relationships based on *dnaJ* sequences were in relatively good agreement with those based on 16S rRNA gene sequences. However, some of the relationships on the 16S rRNA tree were markedly different from those on the *dnaJ* tree. In terms of the *dnaJ* gene, all *A. hydrophila* subspecies were in a monophyletic group, although their substitution rates fell in a rather wide range (1.8–5.1 %), and their allocations were reliable as supported by high bootstrap values. This was in contrast to the 16S rRNA tree, on which *A. hydrophila* subsp. *dhakensis* belonged to a separate cluster from the remaining *A. hydrophila* subspecies (Miñana-Galbés *et al.*, 2004). *A. allosaccharophila* was closely related to *A. veronii* on the *dnaJ* phylogeny but was located in a lineage far from *A. veronii* on the 16S rRNA tree.

Hybridization results are presented in Table 1. Each value was the mean of triplicate experiments. Repeated experiments exhibited a maximum standard deviation of 4 %. In agreement with the published proposals that *A. ichthiosmia* and *Aeromonas enteropelogenes* are respectively considered

Table 1. Results of DNA–DNA hybridization expressed as percentage DNA relatedness

DNA–DNA hybridization values greater than or equal to 70 % are in bold.

Strain	Hybridization (%) with labelled reference DNA from strain:																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1. <i>A. culicicola</i> GTC 2882 ^T	100	97	88	85	81	51	54	55	45	43	49	44	45	41	54	48	45	43	38	32	32
2. <i>A. veronii</i> bv. <i>Veronii</i> GTC 2800 ^T	94	100	94	90	82	64	67	61	59	55	52	48	60	46	61	51	58	61	41	35	38
3. <i>A. veronii</i> bv. <i>Sobria</i> GTC 2938	91	93	100	83	73	61	63	66	47	49	49	43	51	42	60	50	55	52	46	25	35
4. <i>A. ichthiosmia</i> GTC 2766 ^T	75	94	83	100	70	62	67	48	49	48	60	50	54	35	53	44	55	60	35	37	29
5. <i>A. allosaccharophila</i> GTC 2879 ^T	87	86	75	67	100	59	50	53	47	38	54	47	42	38	48	37	48	39	35	31	27
6. <i>A. sobria</i> GTC 2798 ^T	66	61	64	63	64	100	62	68	57	47	50	51	64	38	57	48	53	60	39	38	32
7. <i>A. jandaei</i> GTC 707 ^T	59	67	62	60	53	48	100	64	47	49	55	46	52	40	53	43	52	55	39	35	34
8. <i>A. enteropelogenes</i> GTC 2764 ^T	55	58	63	50	44	50	49	100	87	47	55	46	49	51	57	56	52	47	50	39	44
9. <i>A. trota</i> GTC 2767 ^T	58	60	60	53	58	47	56	82	100	52	44	45	58	45	59	50	58	59	40	37	43
10. <i>A. bestiarum</i> GTC 2790 ^T	49	57	57	43	45	46	48	55	49	100	81	72	61	53	62	53	56	58	42	37	37
11. <i>A. popoffii</i> GTC 2881 ^T	45	54	56	64	46	44	51	63	42	71	100	64	56	46	65	50	60	55	41	34	35
12. <i>A. salmonicida</i> subsp. <i>salmonicida</i> GTC 2802 ^T	37	42	45	40	46	34	47	56	35	68	61	100	52	37	57	53	48	51	41	30	32
13. <i>A. encheleia</i> GTC 2788 ^T	42	47	46	36	39	39	39	52	44	51	53	54	100	82	66	52	57	47	39	35	34
14. <i>Aeromonas</i> sp. HG11 GTC 2939	42	47	51	45	46	37	46	51	43	57	51	48	85	100	62	56	59	56	51	30	36
15. <i>A. eucrenophila</i> GTC 2797 ^T	54	56	52	53	39	46	50	66	55	54	54	54	67	63	100	59	60	48	52	38	39
16. <i>A. caviae</i> GTC 2795 ^T	51	52	56	48	46	47	53	54	56	57	55	52	60	64	60	100	64	56	39	46	47
17. <i>A. media</i> GTC 2768 ^T	50	51	49	43	44	38	45	59	42	47	58	52	62	60	54	61	100	51	39	37	35
18. <i>A. hydrophila</i> subsp. <i>hydrophila</i> GTC 2793 ^T	55	58	64	55	45	45	54	61	53	62	65	66	68	57	67	55	62	100	45	44	44
19. <i>A. molluscorum</i> GTC 2878 ^T	38	42	46	34	33	32	40	53	36	37	48	46	48	41	60	41	62	40	100	31	34
20. <i>A. simiae</i> GTC 2791 ^T	30	40	32	29	30	26	31	44	33	30	37	32	36	27	45	36	42	38	37	100	44
21. <i>A. schubertii</i> GTC 2769 ^T	45	51	46	34	32	33	38	50	43	29	36	40	38	36	49	36	46	40	36	55	100

to be synonyms of *A. veronii* and *Aeromonas trota* (Huys *et al.*, 2001, 2002), high genomic relatedness was observed between *A. ichthiosmia* GTC 2766^T and *A. veronii* strains (83–94 %) as well between *A. enteropelogenes* GTC 2764^T and *A. trota* GTC 2767^T (82–87 %) (Table 1). DNA–DNA relatedness values in reciprocal hybridizations between *A. encheleia* GTC 2788^T and *Aeromonas* sp. HG11 GTC 2939, between members of the cluster including *A. veronii*, *A. ichthiosmia*, *A. allosaccharophila* and *A. culicicola* or between *Aeromonas bestiarum*, *Aeromonas popoffii* and *A. salmonicida* strains were also greater than the 70 % threshold recommended for species delineation. These DNA–DNA hybridization results were in agreement with the phylogenetic relationships observed from the *dnaJ* tree (Fig. 1).

Scatter plots of pairwise *dnaJ* (Fig. 2a) and 16S rRNA gene (Fig. 2b) sequence similarities and pairwise DNA–DNA relatedness values show a linear relationship between pairwise *dnaJ* or 16S rRNA gene sequence similarities and

Fig. 2. Scatter plots of DNA–DNA relatedness values against *dnaJ* (a) and 16S rRNA gene (b) sequence similarities for 21 *Aeromonas* strains. Each dot represents a pair of strains, plotted according to their genomic relatedness and sequence similarity. Solid lines represent the regression lines. Asterisks indicate DNA–DNA relatedness values greater than 70 %.

DNA–DNA relatedness values. Regression analysis showed a stronger relationship between DNA–DNA reassociation values and *dnaJ* sequence similarities ($r^2 = 0.706$, $P < 0.001$) than between the former and 16S rRNA gene sequence similarities ($r^2 = 0.347$, $P < 0.001$). As a consequence, phylogenetic relationships between *Aeromonas* species are in close agreement with the current taxonomic classification of this genus. Additionally, the discriminatory power of the *dnaJ* sequence is greater than that of the 16S rRNA gene sequence, enabling *dnaJ* sequences to differentiate clearly any two species. This highlights the fact that the *dnaJ* gene is a promising phylogenetic marker in the genus *Aeromonas*.

Clarification of controversial taxonomic issues

In the taxonomy of the genus *Aeromonas*, controversies remain regarding species delineation. Discrepancies between analyses of different phylogenetic markers or between different sets of DNA–DNA hybridization data have led to ambiguities in the allocation of members of *Aeromonas* to individual species. The present results from *dnaJ* sequence analysis and DNA–DNA hybridization can be expected to contribute to the clarification of the interrelationships between *Aeromonas* species.

Firstly, on the basis of the high DNA–DNA relatedness obtained in a previous study (75.6 %) (and corroborated in this study; 68–72 %) and the impossibility of separation by either biochemical tests or 16S rRNA gene sequence, it was suggested previously that *A. salmonicida* and *A. bestiarum* might represent a single taxon (Martínez-Murcia *et al.*, 2005). However, housekeeping gene sequence analysis revealed genetic divergence between these two species. *gyrB* showed nucleotide substitutions (2.2–3.3 %) on the borderline between the intra- and interspecies ranges (Yáñez *et al.*, 2003), but *rpoD*, with 6.8–8.7 % divergence (Soler *et al.*, 2004), and *dnaJ*, with 5.2–6.2 % divergence in this study, allowed easy differentiation of *A. salmonicida* and *A. bestiarum* as two separate taxa.

To date, one of the most debatable issues within the genus concerns *A. encheleia* and *Aeromonas* sp. HG11. Comparison of 16S rRNA gene sequences of *A. encheleia* and *Aeromonas* sp. HG11 strains showed that they were genetically different and phylogenetically separate (Martínez-Murcia, 1999). In a study on the polymorphism of tRNA intergenic spacers, these two species also presented different tDNA-PCR patterns (Łaganowska & Kaznowski, 2005). On the other hand, they were found to share similar combined intergenic spacer region (ISR)-RFLP patterns and to constitute one group (Łaganowska & Kaznowski, 2004). Divergences determined for sequences of *gyrB* (Yáñez *et al.*, 2003), *rpoD* (Soler *et al.*, 2004) and *dnaJ* (in the present study) were respectively 2.1–2.2, 1.4–1.7 and 1.3 %. Compared with the intra- and interspecies ranges of sequence divergence observed in the genus *Aeromonas*, these values fall within limits that suggest that these taxa might represent a single species. DNA–DNA reassociation, the determinate criterion, gave different results. Esteve *et al.* (1995) showed

that DNA–DNA relatedness between *A. encheleia* and *Aeromonas* sp. HG11 strains was only 12 %, whereas Huys *et al.* (1997a) reported a value of 84 %, considerably exceeding the threshold value of 70 % for membership of the same species. Our hybridization data (82–85 %) were comparable to those reported by Huys *et al.* (1997a). Thus, we suggest that *A. encheleia* and *Aeromonas* sp. HG11 belong to the same genetic group.

Heated discussion is also taking place with respect to *A. culicicola* and *A. allosaccharophila*. They were described as two novel *Aeromonas* species by Pidiyar *et al.* (2002) and Martínez-Murcia *et al.* (1992a). In later studies, from evidence deduced from phenotypic characteristics, amplified fragment length polymorphism and DNA–DNA hybridization results, Huys *et al.* (1996, 2001, 2005) cast doubt on the status of these species as separate from *A. veronii*. However, contradictions between hybridization data from Huys *et al.* (2001, 2005) and from the original descriptions of the three species have resulted in the exact positions of these species in the genus *Aeromonas* remaining unclear. Study of *gyrB* and *rpoD* housekeeping gene sequences (Soler *et al.*, 2004) showed that the nucleotide substitution rates of these species were slightly greater than the intraspecies substitution rate (approx. 2 %), but it is worth noting that the collection of *Aeromonas* strains used by Soler *et al.* (2004) did not include members of the *A. hydrophila* subspecies. When *A. hydrophila* subspecies were added, the highest intrasubspecies substitution rates were increased to 4.4 % for *gyrB* and 5.1 % for *rpoD*. In our study, strains of *A. culicicola* and *A. allosaccharophila*, together with two *A. veronii* biovars and *A. ichthiosmia* (a later synonym of *A. veronii*), clustered in a very close genetic group. Sequence divergences among them (1.7–3.3 %) were the lowest in comparison with interspecies divergence in the entire genus and were comparable to the intraspecies divergence. The high genomic relatedness (67–97 %; Table 1) observed between the five strains *A. ichthiosmia* GTC 2766^T, *A. culicicola* GTC 2882^T, *A. allosaccharophila* GTC 2879^T, *A. veronii* bv. *Veronii* GTC 2800^T and *A. veronii* bv. *Sobria* GTC 2938 corroborated the phylogenetic relationships obtained from housekeeping gene analysis, indicating a highly close genetic relationship between them. In terms of the status of *A. culicicola* and *A. allosaccharophila* in the genus *Aeromonas*, Huys *et al.* (2001, 2005) have endeavoured to prove the affiliation of *A. culicicola* and *A. allosaccharophila* to *A. veronii* by strong phenotypic and genotypic evidence, but the positions of these species remain debatable because of contradictions between sets of DNA–DNA hybridization data and a disagreement between recently published phylogenetic data (Soler *et al.*, 2004; Saavedra *et al.*, 2006). Our DNA–DNA hybridization results and phylogenetic analysis support the results given by Huys *et al.* (2001, 2005) to justify the recognition of *A. allosaccharophila* and *A. culicicola* as later heterotypic synonyms of *A. veronii*.

It should also be noted that, in this study, *A. bestiarum* GTC 2790^T and *A. popoffii* GTC 2881^T showed high DNA–DNA

relatedness (71–81 %), which is rather in disagreement with the result given in the original description of *A. popoffii* (53 %; Huys *et al.*, 1997b). Despite the tight relationship, these species were clearly distinguishable on the basis of housekeeping gene sequence divergence (3.0, 4.4 and 5.5 %, respectively, for *gyrB*, *rpoD* and *dnaJ*). A likely explanation for the discrepancy observed between sequencing and hybridization data in the case of *A. bestiarum* and *A. salmonicida* or *A. bestiarum* and *A. popoffii* is that the measure of DNA sequence relatedness by DNA–DNA hybridization may be too crude, or at least not adequately fine to separate highly related species (Martínez-Murcia *et al.*, 2005).

In conclusion, our findings indicate that analysis of *dnaJ* sequences offers advantages over the 16S rRNA gene in defining phylogenetic relationships within the genus *Aeromonas*. Results obtained from analyses of *dnaJ* sequences and DNA–DNA hybridization in this study provide additional information on the interrelationships of the genus *Aeromonas*, allowing greater accuracy and reliability to be obtained. However, additional reference strains for each species are required for validation of the cut-off point of *dnaJ* sequence divergence for species delineation.

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