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* (Yañez 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 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 (Takekawi 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 subs. 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’-GCAGATCAAGAAGGGCTACAAG-3’) and Aero-dnaJR3 (5’-CACCACCTTGACATCAGACTC-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

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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 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. ichthioseinia, 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 2880T, which showed far larger distances (4.7 and 5.1 %, respectively) from A. hydrophila subsp. hydrophila GTC 2793T and A. hydrophila subsp. ranaei GTC 2877T. 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 (Minàna-Galbis 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. ichthiosnia and Aeromonas enteropelogenes are respectively considered

<table>
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<tr>
<th>Strain</th>
<th>Hybridization (%) with labelled reference DNA from strain:</th>
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<tr>
<td>1. A. culicicola GTC 2882T</td>
<td>100 97 88 85 81 51 54 55 45 43 49 44 45 41 54 48 45 43 38 32 32</td>
</tr>
<tr>
<td>2. A. veronii bv. Veronii GTC 2800T</td>
<td>94 100 94 90 82 64 67 61 59 55 52 48 60 46 61 51 58 61 41 35 38</td>
</tr>
<tr>
<td>3. A. veronii bv. Sobria GTC 2938</td>
<td>91 93 100 83 73 61 63 66 47 49 49 43 51 42 60 50 55 52 46 25 35</td>
</tr>
<tr>
<td>4. A. ichthiosnia GTC 2766T</td>
<td>75 94 83 100 70 62 67 48 49 48 60 50 54 35 53 44 55 60 35 37 29</td>
</tr>
<tr>
<td>5. A. allosaccharophila GTC 2879T</td>
<td>87 86 75 67 100 59 50 53 47 38 54 47 32 42 38 48 37 48 39 35 31 27</td>
</tr>
<tr>
<td>6. A. sobria GTC 2798T</td>
<td>66 61 64 63 64 100 62 68 57 47 50 51 64 38 57 48 53 60 39 38 32</td>
</tr>
<tr>
<td>7. A. jandaei GTC 707T</td>
<td>59 67 62 60 53 48 100 64 47 49 55 46 52 40 53 43 52 55 39 35 34</td>
</tr>
<tr>
<td>8. A. enteropelogenes GTC 2764T</td>
<td>55 58 63 50 44 50 49 100 87 47 55 46 49 51 57 56 52 47 50 39 44</td>
</tr>
<tr>
<td>9. A. trota GTC 2767T</td>
<td>58 60 60 53 58 47 56 82 100 52 44 45 58 45 59 50 58 59 40 37 43</td>
</tr>
<tr>
<td>10. A. bestiarum GTC 2790T</td>
<td>49 57 57 43 45 46 48 55 59 100 81 72 61 53 62 53 56 58 42 37 37</td>
</tr>
<tr>
<td>11. A. popoffii GTC 2881T</td>
<td>45 54 56 64 46 44 51 63 42 71 100 64 56 46 65 50 60 55 41 34 35</td>
</tr>
<tr>
<td>12. A. salmonicida subsp. salmonicida GTC 2802T</td>
<td>37 42 45 40 46 34 47 56 35 68 61 100 52 37 57 53 48 51 41 30 32</td>
</tr>
<tr>
<td>13. A. encheleia GTC 2786T</td>
<td>42 47 46 36 39 39 39 39 52 44 51 53 54 100 82 66 52 57 47 39 35 34</td>
</tr>
<tr>
<td>14. Aeromonas sp. HG11 GTC 2939</td>
<td>42 47 51 45 46 37 46 51 43 57 51 48 85 100 62 56 59 56 51 30 36</td>
</tr>
<tr>
<td>15. A. eucrenophila GTC 2797T</td>
<td>54 56 52 53 39 46 50 66 55 54 54 54 67 63 100 59 60 48 52 38 39</td>
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<td>16. A. caviae GTC 2793T</td>
<td>51 52 56 48 46 47 53 54 56 57 55 52 60 64 60 100 64 56 39 46 47</td>
</tr>
<tr>
<td>17. A. media GTC 2768T</td>
<td>50 51 49 43 44 38 45 59 42 47 58 52 62 60 54 61 100 51 39 37 35</td>
</tr>
<tr>
<td>18. A. hydrophila subsp. hydrophila GTC 2793T</td>
<td>55 58 64 55 45 45 54 61 53 62 65 66 68 57 67 55 62 100 45 44 44</td>
</tr>
<tr>
<td>19. A. molluscorum GTC 2878T</td>
<td>38 42 46 34 33 32 40 53 36 37 48 46 48 41 60 41 62 40 100 31 34</td>
</tr>
<tr>
<td>20. A. simiae GTC 2791T</td>
<td>30 40 32 29 30 26 31 44 33 30 37 32 36 27 45 36 42 38 37 100 44</td>
</tr>
<tr>
<td>21. A. schuberti GTC 2769T</td>
<td>45 51 46 34 32 33 38 50 43 29 36 40 38 36 49 36 40 36 35 100 55</td>
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to be synonyms of *A. veronii* and *Aeromonas trota* (Huys et al., 2001, 2002), high genomic relatedness was observed between *A. ichthiosmia* GTC 2766<sup>T</sup> and *A. veronii* strains (83–94%) as well between *A. enteropelogenes* GTC 2764<sup>T</sup> and *A. trota* GTC 2767<sup>T</sup> (82–87%) (Table 1). DNA–DNA relatedness values in reciprocal hybridizations between *A. encheleia* GTC 2788<sup>T</sup> 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 DNA–DNA relatedness values. Regression analysis showed a stronger relationship between DNA–DNA reassociation values and *dnaJ* sequence similarities (*r<sup>2</sup> = 0.706, *P* < 0.001) than between the former and 16S rRNA gene sequence similarities (*r<sup>2</sup> = 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 (Laganowska & Kaznowski, 2005). On the other hand, they were found to share similar combined intergenic spacer region (ISR)-RFLP patterns and to constitute one group (Laganowska & 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 among the five strains A. ichthiosmia GTC 2766T, A. culicicola GTC 2882T, A. allosaccharophila GTC 2879T, A. veronii bv. Veronii GTC 2800T 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 2790T and A. popoffii GTC 2881T 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.

REFERENCES


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