Aeromonas australiensis sp. nov., isolated from irrigation water

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A Gram-negative, facultatively anaerobic bacillus, designated strain 266T, was isolated from an irrigation water system in the south-west of Western Australia. Analysis of the 16S rRNA gene sequence confirmed that strain 266T belonged to the genus Aeromonas, with the nearest species being Aeromonas fluvialis (99.6 % similarity to the type strain, with 6 nucleotide differences) followed by Aeromonas veronii and Aeromonas allosaccharophila (both 99.5 %). Analysis of gyrB and rpoD sequences suggested that strain 266T formed a phylogenetic line independent of other species in the genus. This was confirmed using the concatenated sequences of six housekeeping genes (gyrB, rpoD, recA, dnaJ, gyrA and dnaX) that also indicated that A. veronii and A. allosaccharophila were the nearest relatives. DNA–DNA reassociation experiments and phenotypic analysis further supported the conclusion that strain 266T represents a novel species, for which the name Aeromonas australiensis sp. nov. is proposed, with type strain 266T (= CECT 8023T = LMG 2670T).

The genus Aeromonas consists of Gram-negative, non-sporo-forming bacilli or coccobacilli, motile by polar flagella, that are oxidase- and catalase-positive, reduce nitrate to nitrite and are usually resistant to the vibriostatic agent O/129. The genus Aeromonas resides within the family Aeromonadaceae that belongs to the class Gamma-proteobacteria (Abbott et al., 2003; Martin-Carnahan & Joseph, 2005). Aeromonas species are found globally in water and soil environments and are often associated with fish and human disease (Janda & Abbott, 1998, 2010; Figueras, 2005). At the time of writing, the genus includes 25 species with validly published names: Aeromonas hydrophila, A. bestiarum, A. salmonicida, A. caviae, A. media, A. eucnemophila, A. sobria, A. veronii, A. jandaei, A. schuberti, A. trota, A. allosaccharophila, A. encheleia, A. popoffii (Martínez-Carnahan & Joseph, 2005), A. molluscorum (Miñana-Galbis et al., 2004), A. simiae (Harf-Monteil et al., 2004), A. bivalvium (Miñana-Galbis et al., 2007), A. aquariorum (Martínez-Murcia et al., 2008), A. tecta (Demarta et al., 2008), A. piscicola (Beaz-Hidalgo et al., 2009), A. fluvialis (Alperi et al., 2010a), A. tainwensis and A. sanarellii (Alperi et al., 2010b), A. diversa (formerly Aeromonas group 501) (Miñana-Galbis et al., 2010) and A. rivuli, isolated from a karst water rivulet in Germany (Figueras et al., 2011a).

The complex taxonomy of this genus has been attributed to a lack of definitive biochemical markers and poor congruence between genotypic and phenotypic methods (Soler et al., 2003; Figueras, 2005; Ørmen et al., 2005; Beaz-Hidalgo et al., 2009; Figueras et al., 2011b). In addition, the 16S rRNA gene sequences, which are classically considered an important taxonomic tool, do not have the resolving power to discriminate between closely related Aeromonas species. This is due to the high interspecies sequence similarity of this gene, ranging from 96.7 to 100 % (Martínez-Murcia et al., 2007; Figueras et al., 2011b), and...
the existence of sequence variation in copies of the gene (microheterogeneities) in some species (Morandi et al., 2005; Alperi et al., 2008; Roger et al., 2012a). Dependence on the 16S rRNA gene sequence to differentiate between Aeromonas species has been superseded by the use of single-copy genes that perform essential functions in bacteria, e.g. rpoD, gyrB, dnaJ, recA and cpn60, which are known as housekeeping genes (Yáñez et al., 2003; Soler et al., 2004; Küpfer et al., 2006; Nhung et al., 2007; Sepe et al., 2008; Miñana-Galbis et al., 2009). In this sense, the rpoD and gyrB genes were employed for the first time in the definition of the species A. tecta and A. aquariorum (Demarta et al., 2008; Martínez-Murcia et al., 2008), while four concatenated housekeeping gene sequences were used in the description of A. piscicola and A. diversa (Beaz-Hidalgo et al., 2009; Miñana-Galbis et al., 2010). In the case of A. piscicola, the four genes employed were rpoD, gyrB, dnaJ and recA (Beaz-Hidalgo et al., 2009), while, in the case of A. diversa, recA was replaced by cpn60 (Miñana-Galbis et al., 2010). According to the ad hoc committee for the re-evaluation of the species definition in bacteriology, there is a current consensus that ‘an informative level of phylogenetic data would be obtained from the determination of a minimum of five genes under stabilizing selection for encoded metabolic functions (housekeeping genes) (Stackebrandt et al., 2002). A. fluvialis, A. taiwanensis, A. sanarellii and A. rivuli are the first species of Aeromonas that included in their description the concatenated sequences of five genes (rpoD, gyrB, dnaJ, recA and gyrA) (Alperi et al., 2010a, b; Figueras et al., 2011a). Recently, a multilocus phylogenetic analysis (MLPA) of the complete genus with information derived from seven concatenated genes (gyrB, rpoD, recA, dnaJ, gyrA, dnaX and atpD) demonstrated concordance with the species delineation based on DNA–DNA results (Martínez-Murcia et al., 2011). Almost the same phylogenetic conclusions were recently inferred by Roger et al. (2012b) using an MLPA, also based on seven housekeeping genes (dnaK, gltA, gyrB, radA, rpoB, tsf and zipA), six of which were different from those employed by Martínez-Murcia et al. (2011).

The purpose of the present study was to use a polyphasic approach to characterize the taxonomic position of strain 266T. The results revealed that this strain represents an independent phylogenetic line within the genus Aeromonas.

Strain 266T was isolated by membrane filtration on an enriched lauryl sulfate agar (50 mm) plate while determining a total coliform count from a treated effluent used for irrigation at a sports ground in the south-west of Western Australia.

Cell size, morphology and the presence of flagella were determined by electron microscopy following procedures described previously (Collado et al., 2009).

Biochemical and physiological tests for the characterization of strain 266T were performed at 30 and 35 °C. All type strains of species belonging to the genus Aeromonas were tested in parallel under identical conditions in two laboratories (in Spain and in Australia). A total of 36 phenotypic tests for the characterization of strain 266T were selected from those performed by Abbott et al. (2003) following the procedure described in Alperi et al. (2010a) with some exceptions, i.e. utilization of citrate, which was determined by the method of Hänninen (1994) and using Simmon’s method (Cowan & Steel, 1993), oxidation of potassium gluconate, production of lipase, urease, Jordan’s turtrate, malonate, phenylalanine deaminase, nitrate reduction (MacFaddin, 1976) and bacteriolytic activity (expression of a staphylosin) (Satta et al., 1977). Acid production from carbohydrate was performed in broth (Excel) at a final concentration of 1% (w/v) of the desired sugar and 1% (v/v) Andrade’s indicator as well as by the method described by Alperi et al. (2010a). The following carbohydrates were used: adonitol, amygdalin, L-arabinose, cellobiose, dulcitol, glucose, glucose 1-phosphate, glucose 6-phosphate, glycerol, myo-inositol, lactose, lactulose, maltose, mannanose, D-mannitol, melibiose, methyl 1,2-D-glucoside, raffinose, L-rhamnose, ribose, salicin, D-sorbitol, sucrose and trehalose. Additional carbohydrate fermentation was investigated with the API 20E and API CH50 systems (bioMérieux).

The ability to grow at different temperatures was assayed on TSA supplemented with sheep blood agar at 4, 25, 30, 35 and 44 °C. Acid production from carbohydrates, hydrolysis of aesculin, urea and DNA and production of hydrogen sulfide from cysteine were evaluated for 7 days. Other tests were read as described by Abbott et al. (2003). Appropriate positive and negative controls were included.

The inability of strain 266T to produce acid from D-mannitol was a significant phenotypic marker, as the majority of Aeromonas species can produce acid from this carbohydrate, with the exception of A. schubertii, A. simiae and A. diversa and some strains of A. trota (Table 1 and Table S1, available in IJSEM Online). Key differentiating phenotypic features between these D-mannitol-negative species and strain 266T are indicated in Table S1. Strain 266T can be differentiated from A. schubertii by producing indole from tryptophan and acid from sucrose; from A. simiae by being haemolytic (strain 266T exhibited β-haemolysis, while A. simiae did not) and positive for Voges–Proskauer and indole reactions; from A. diversa by its ability to decarboxylate lysine and produce acid from sucrose and from A. trota by being positive for the Voges–Proskauer reaction but negative for the utilization of citrate (Tables 1 and S1). A small zone of inhibition (~10.8 mm in diameter) with a 150 μg disc containing the vibriostatic agent O/129 was observed after overnight incubation on blood agar. This susceptibility to the vibriostatic agent is uncommon in the genus and was previously reported for two strains of A. eucrenophila and one of A. veronii by Abbott et al. (2003), and recently for the novel species ‘Aeromonas cavernicola’ (Martínez-Murcia et al., 2013).
Hydrolysis of aesculin

Utilization of:

Aztreonam, cephalothin, cefazolin, cefepime, cefoxitin, norfloxacin, pipercillin/tazobactam, tetracycline, ticarcillin/clavulanate, tobramycin, trimethoprim and trimethoprim/sulfamethoxazole.

Interpretative criteria were in accordance with the CLSI (2006). Strain 266\textsuperscript{T} was resistant to amoxicillin and cefazolin and was susceptible to the other antimicrobial agents tested.

Cellular fatty acid methyl ester (FAME) analyses were interpreted as described by Huys et al. (1994). In brief, isolates were inoculated onto trypticase soy agar (TSA) and incubated at 28 °C for 48 h in air. Approximately 20 mg biomass of each strain was harvested and extracts were prepared using the standardized protocol of the Microbial Identification System (MID; Microbial ID) and analysed with a Hewlett Packard model HP6890 series GLC as described by Osterhout et al. (1991). All cultures had similar physiological age when they were harvested and cellular fatty acid analyses were performed in triplicate.

Identification of the fatty acids was performed by the Sherlock MIDI software package (version 6). Table S2 shows cellular fatty acid profiles of strain 266\textsuperscript{T} and the type strains of species included in the genus Aeromonas. The cellular fatty acid profile of strain 266\textsuperscript{T} contained summed feature 3 (28.7 %; C\textsubscript{16:1}\textomega\textsubscript{7c} and/or C\textsubscript{16:1}\textomega\textsubscript{6c}), summed feature 8 (11.4 %; C\textsubscript{18:1}\textomega\textsubscript{7c} and/or C\textsubscript{18:1}\textomega\textsubscript{6c}), C\textsubscript{16:0} (11.3 %), C\textsubscript{16:1}\textomega\textsubscript{7c} alcohol (7.20 %), C\textsubscript{12:0} (6.0 %), summed feature 2 (5.6 %; one of more of C\textsubscript{12:0} alcohol, iso-C\textsubscript{16:1} \textomega \text{I} and C\textsubscript{14:0} 3-OH), summed feature 9 (3.7 %; 10-methyl C\textsubscript{16:0} or iso-C\textsubscript{17:1}\textomega\textsubscript{9c}), iso-C\textsubscript{15:0} (3.5 %), C\textsubscript{17:1}\textomega\textsubscript{10c} (3.3 %), iso-C\textsubscript{17:0} (3.2 %), C\textsubscript{14:0} (2.5 %) and C\textsubscript{16:0} N alcohol (1.7 %). The similarity index (SI) obtained for strain 266\textsuperscript{T} varied between 0.200 and <0.300 and, in most instances, named A. schubertii as a possible match. According to the MIDI system documentation, SI values of <0.300 may represent an atypical strain of the species named first in the chromatogram. This identification was consistent with the fact that A. schubertii showed a similar biochemical profile to strain 266\textsuperscript{T}. The FAME compositions of the Aeromonas strains analysed in the present study differed significantly from previous reports (Lambert et al., 1983; Huys et al., 1994; Kämpfer et al., 1994). Consistent with previous observations, variations in FAME data can be attributed to differences in culture conditions, sets of strains used and the type of media and equipment employed to analyse results (Huys et al., 1994). Our results indicate that subtle differences exist between strain 266\textsuperscript{T} and other D-mannitol-negative Aeromonas strains (Table S2) and that Aeromonas species belong to one of two clusters, those that produce alcohols (C\textsubscript{16:1}\textomega\textsubscript{7c} alcohol and C\textsubscript{16:0} N alcohol), and those that do not. However, identification of bacteria by analysis of their FAMEs is more suitable for slowly growing bacteria such as non-fermenters (Osterhout et al., 1991) and, in agreement with
the comments by Kämpfer et al. (1994), fatty acid patterns show limited resolution to split Aeromonas species.

Protein analysis of strain 266\textsuperscript{T} was performed using a Bruker microflex LT MALDI-TOF mass spectrometer (Bruker Daltonic). Protein extraction was performed as described previously (Beaz-Hidalgo et al., 2009). Peaks in the mass spectrum of strain 266\textsuperscript{T} ranged from 2000 to 11300 Da and differed from those of the closest related species, A. allosaccharophila, A. fluvialis and A. veronii (Fig. S1).

Methods of DNA extraction and the conditions and primers for amplification of the 16S rRNA, gyrB, rpoD, recA, dnaJ, gyrA and dnaX genes were as described previously (Martínez-Murcia et al., 1992, 2011). Purified PCR products were prepared for sequencing by using the BigDye Terminator version 1.1 cycle sequencing kit (Applied Biosystems) and sequencing was performed with an ABI PRISM 310 and ABI 3130XL Genetic Analyzer (Applied Biosystems). Using the CLUSTAL\_X program, version 1.8 (Thompson et al., 1997), the sequences obtained were aligned independently with sequences of type and reference strains of all members of the genus Aeromonas taken from our in-house database (Martínez-Murcia et al., 2011) and some 16S rRNA gene sequences retrieved from GenBank. Genetic distances and clustering were determined using Kimura’s two-parameter model (Kimura, 1980) and evolutionary trees were constructed by the neighbour-joining method (Saitou & Nei 1987) using the MEGA 5 program (Tamura et al., 2007). The stability of relationships was assessed by the bootstrap method (1000 replications).

Strain 266\textsuperscript{T} (1503 bp) showed the highest 16S rRNA gene sequence similarity to the type strain of A. fluvialis (99.6 %) followed by those of A. allosaccharophila and A. veronii, both with a similarity of 99.5 %, these also being the closest neighbours in the phylogenetic tree (Fig. 1). The latter two species were also the most related to strain 266\textsuperscript{T} on the basis of the MLPA constructed with the concatenated sequences of six genes (gyrB, rpoD, recA, dnaJ, gyrA and dnaX; 3606 bp) from all the type strains (Fig. 2). Strain 266\textsuperscript{T} showed the minimum interspecies divergence from A. veronii (3.2 %), which was higher than those obtained between A. piscicola and A. bestiarium (approx. 2.1 %) or A. allosaccharophila and A. veronii (approx. 2.9 %) as reported by Martinez-Murcia et al. (2011). As pointed out by Kämpfer & Glaeser (2012) and Martinez-Murcia et al. (2011), a critical comparison of the different tree topologies based on single genes is important to determine genes that may be affected by lateral gene transfer or subsequent recombination events. The trees reconstructed from the six individual genes showed that, in all them, strain 266\textsuperscript{T} formed a clearly distinct branch, but always clustered near the species A. veronii, A. fluvialis and A. allosaccharophila (Figs S2–S7).

DNA–DNA hybridization studies were performed between strain 266\textsuperscript{T} and the type strains of A. veronii (CECT 4257\textsuperscript{T}), A. piscicola CECT 7443\textsuperscript{T} (HQ832417), A. salmonicida NCIMB 1102\textsuperscript{T} (X60405), A. sobria DSM 22539\textsuperscript{T} (FJ276900), A. mullvadai CECT 5864\textsuperscript{T} (AY532691), A. popoffii CECT 5176\textsuperscript{T} (HQ832415), A. bivalvium CECT 7113\textsuperscript{T} (DQ504429), A. encheleia CECT 4934\textsuperscript{T} (HQ832414) and A. eucrenophila CECT 7403\textsuperscript{T} (FJ230077).

Accession numbers for individual sequences are provided in Figs S2–S7.
Table 2. DNA–DNA relatedness between strain 266\(^T\) and related type strains

Results are expressed as means (SD) of three determinations. –, Not determined.

<table>
<thead>
<tr>
<th>Source of labelled DNA</th>
<th>DNA–DNA hybridization (%) with strain:</th>
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<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1. Strain 266(^T)</td>
<td>100</td>
</tr>
<tr>
<td>2. A. fluvialis CECT 7401(^T)</td>
<td>47.6 (6.5)</td>
</tr>
<tr>
<td>3. A. veronii CECT 4257(^T)</td>
<td>62.6 (1.5)</td>
</tr>
<tr>
<td>4. A. allosaccharophila CECT 4199(^T)</td>
<td>65.5 (0.5)</td>
</tr>
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A. allosaccharophila (CECT 4199\(^T\)) and A. fluvialis (CECT 7401\(^T\)), as these were the phylogenetically closest species both in the 16S rRNA gene sequence analysis and the MLPA. DNA extraction was performed using an Easy DNA kit (Invitrogen) and DNA–DNA hybridization experiments were conducted using the methods described by Ziemke et al. (1998) and Urdiain et al. (2008). Reassociation was performed under optimal conditions at 70 °C; single- and double-stranded DNA molecules were separated by the use of hydroxyapatite and colour development was measured at 405 nm using a Biotec Power safe XS2 microplate reader. Reported mean DNA–DNA relatedness values and standard deviations were based on a minimum of three hybridizations for both direct and reciprocal reactions (Table 2). DNA–DNA hybridization between strain 266\(^T\) and the type strains of A. allosaccharophila, A. veronii and A. fluvialis was 65.3, 63.7 and 52.2 %, respectively, all below the 70 % limit for species delineation (Wayne et al., 1987; Stackebrandt & Goebel, 1994). The MLPA once more showed perfect agreement with the DNA–DNA hybridization results; both showed that strain 266\(^T\) represents a novel species within the genus Aeromonas (Fig. 2). These results were further supported by phenotypic and chemotaxonomic analysis and confirmed that strain 266\(^T\) represents a novel species, for which the name Aeromonas australiensis sp. nov. is proposed.

Description of Aeromonas australiensis sp. nov.

Aeromonas australiensis (aus.tra.li.en’sis. N.L. fem. adj. australiensis of or belonging to Australia).

Motile rods with polar flagella (Fig. S8). Cells are Gram-negative, straight, non-spore-forming and non-encapsulated rods, 0.6–0.9 μm wide and 1.8–2.7 μm long, oxidase- and catalase-positive, reduce nitrate to nitrite and are susceptible to the vibriostatic agent O/129 (150 μg). Colonies on TSA plus sheep blood are 1.5–2.0 mm in diameter, glossy, circular and beige in colour after 24 h at 35 °C. No brown diffusible pigment is produced on TSA at 35 °C. Growth occurs at 25, 30 and 35 °C, but not at 4 or 44 °C after 24 h on TSA plus sheep blood. β-Haemolysis is observed on sheep (5 %) blood agar. Grows on MacConkey (Difco) and thiosulfate-citrate-bile-sucrose agar (Difco) and in nutrient broth in 0 and 3 % NaCl, but not at 6 % NaCl. Indole is produced from tryptophan. The ONPG reaction is positive when tested by disc (Rosco) but not in the API 20E strip. Does not utilize citrate (Simmon’s and Hanninen’s methods) or malonate or produce gas from glucose, but a positive citrate reaction is observed with the API 20E strip. DL-Lactate is utilized at 30 °C but not at 35 °C. Hydrogen sulfide, urease and elastase are not produced and it does not hydrolyse aesculin. No clearing of tyrosine-containing medium, but starch hydrolysis is positive after 5 days. Produces DNase and lipase and oxidizes potassium gluconate. Dehydrolyses arginine and lysine is decarboxylated, but not ornithine. Utilizes acetate and urocanic acid and it is positive for the Voges–Proskauer reaction and hydrolysis of gelatin. No bacteriolytic activity (stapholysin) is detected. Negative for phenylalanine deaminase, alkylsulfatase, pyraziminidase and Jordan’s tartrate. Acid is produced from the following carbohydrates: fructose, galactose, glucose, glycercol, glycogen, glucose 1-phosphate, glucose 6-phosphate, maltose, mannose, N-acetylglucosamine, ribose, sucrose and trehalose, but not from adonitol, amygdalin, L-arabinose, cellobiose, dulcitol, myo-inositol, lactose, lactulose, D-mannitol, melibiose, methyl α-D-glucoside, raffinose, L-rhamnose, salicin or D-sorbitol. Acid production is observed for the following carbohydrates with the API 50CH strip: glycerol, D-ribose, D-galactose, D-glucose, D-fructose, D-mannose, N-acetylglucosamine, maltose, sucrose, trehalose, starch, glycogen and potassium gluconate.

The type strain is 266\(^T\) (=CECT 8023\(^T\) =LMG 26707\(^T\)), isolated from treated effluent in the south-west region of Western Australia.

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References


