Systematic and Applied Microbiology 38 (2015) 161–168

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.de/syapm

Aeromonas aquatica sp. nov., *Aeromonas finlandiensis* sp. nov. and *Aeromonas lacus* sp. nov. isolated from Finnish waters associated with cyanobacterial blooms☆

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ARTICLE INFO

Article history:
Received 31 March 2014
Received in revised form 18 February 2015
Accepted 27 February 2015

Keywords:
Aeromonas
New taxa
Polyphasic
ANI
Water

ABSTRACT

Three groups of *Aeromonas* strains isolated from Finland lakes experiencing cyanobacterial blooms could not be assigned to any known species of this genus on the basis of 16S rRNA and *rpoD* gene sequences. The Multilocus Phylogenetic Analysis (MLPA) of the concatenated sequence of seven genes (*gyrB*, *rpoD*, *recA*, *dnaJ*, *gyrA*, *dnaX* and *atpD*; 4093 bp) showed that the three groups of strains did not cluster with any known *Aeromonas* spp. and formed three independent lineages. This was confirmed by performing the analysis with their closest relatives using 15 genes (the latter 7 and *cpn60*, *dnaK*, *gltA*, *mdh*, *radA*, *rpoB*, *tsf*, *zipA*; 8751 bp). Furthermore, ANI results between the genomes of the type strains of the three potential new species and those of their close relatives were all <96% which is the previously proposed cutoff value for differentiating species within this genus. The *in silico* DDH values of the three type strains of the new species also showed a similarity <70% with the most closely related species indicating they belong to different taxa. The three groups of strains could be differentiated from each other and from other known *Aeromonas* species on the basis of several phenotypic characters. This polyphasic study revealed that the 3 groups of strains represent 3 novel *Aeromonas* species for which the names *Aeromonas aquatica* sp. nov. (type strain AE235^T = CECT 8025^T = LMG 26712^T), *Aeromonas finlandiensis* sp. nov. (type strain 4287D^T = CECT 8028^T = LMG 26709^T) and *Aeromonas lacus* sp. nov. (type strain AE122^T = CECT 8024^T = LMG 26710^T) are proposed.

The genus *Aeromonas* belongs to the domain Bacteria, Phylum *Proteobacteria*, Class *Gammaproteobacteria*, Order *Aeromonadales* and Family *Aeromonadaceae* and include oxidase-positive, facultatively anaerobic, Gram-negative bacilli [26]. Key phenotypic characteristics for the recognition of *Aeromonas* are resistance to the vibriostatic agent O/129 (2,4-diamino-6,7-diisopropyl pteridine at concentrations of 150 mg/disk) with the exceptions of *Aeromonas cavernicola* and *Aeromonas australiensis* that are sensitive [5,27], inability to ferment inositol and to grow on media containing 6% NaCl although they grow on nutrient agar at 0% NaCl [26].

Since the last review of the genus performed by Janda and Abbott [23], 8 new species have been described: *Aeromonas piscicola* isolated from diseased salmon [7], *Aeromonas fluvialis* [4], *Aeromonas rivuli* [15], *A. australiensis* [5] and *A. cavernicola* [27] (the latter species has not yet been validly published) from water and 3 new clinical species recovered from wound infections: *Aeromonas taiwanensis*, *Aeromonas sanarellii* [3] and *Aeromonas diversa* (previously Enteric group 501) [32]. Furthermore *Aeromonas aquariorum* [30] and *A. hydrophila* subsp. *dhakensis*

☆ The GenBank/EMBL/DDBJ accession numbers of the 16S rRNA gene sequences of strains 4287D^T (=CECT 8028^T = LMG 26709^T), AE235^T (=CECT 8025^T = LMG 26712^T) and AE122^T (=CECT 8024^T = LMG 26710^T) are LM654283, HG970952 and HG970953, respectively. The *gyrB*, *rpoD*, *recA*, *dnaJ*, *gyrA*, *dnaX* and *atpD* of the other strains of the three novel species have also been deposited under the accession numbers HG970918–HG970928, HG970940–HG970950, HG970929–HG970939, HG970885–HG970895, HG970907–HG970917, HG970896–HG970906, HG970874–HG970883, respectively.

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http://dx.doi.org/10.1016/j.syapm.2015.02.005
0723-2020/

[22] have recently been synonymised under the name *Aeromonas dhakensis* [9].

Phenotypic characterization shows different levels of precision for the identification of *Aeromonas* species and tends to overestimate the clinical and environmental relevance of the species *A. hydrophila*, thus hampering the establishment of the true incidence of other new or rare species [14,16,23,25].

Molecular identification of *Aeromonas* species using the 16S rRNA gene can also have limitations due to the high interspecies sequence similarity of this gene (96.7–100%) and due to the presence of *rrn* microheterogeneities [2,16]. Housekeeping genes like *rpoD*, *gyrB*, *rpoB* and *dnaJ* among others are used as an alternative because they can precisely identify *Aeromonas* spp. [24,35,40]. Furthermore, a Multilocus Sequence Analysis (MLSA) or Multilocus Phylogenetic Analysis (MLPA) using the concatenated sequences of five or more housekeeping genes, as recommended by the *ad hoc* committee for the re-evaluation of the species definition in bacteriology [41] has been used for the delineation of *Aeromonas* species [3–5,15,27] and shows a good concordance with DNA-DNA hybridization (DDH) results [29,38]. Therefore such analysis has been proposed as an alternative to DDH and is being used for the description of new bacterial species [16,29,36,41]. Also the correlation found between DDH results >70% with values of 95–96% for the *in silico* determined average nucleotide identity (ANI) between two genomes has lead to the proposal of using ANI as the "gold standard" for the substitution of DDH for the description of new bacterial species [18,37]. Recently we have recommended the use of ANI between type or *bona fide* strains or the use of a MLSA in order to verify the correct taxonomic affiliation of genomes [8,17].

During a survey carried out in Finnish waters where cyanobacterial blooms were suspected to have caused adverse human health effects (fever, gastrointestinal symptoms) 116 *Aeromonas* strains were recovered and identified with partial sequences of the 16S rRNA gene [10]. A re-analysis of these strains (study in preparation) using the *rpoD* gene showed that 11 of them formed 3 independent clusters (with 7, 2 and 2 strains each) and could not be assigned to any of the known *Aeromonas* species. The aim of the present study was to use a polyphasic approach to characterize these 3 potential new species.

Genomic DNA was extracted from a single colony grown on TSA plates at 30 °C for 24 h using the Instagene Matrix (Bio-Rad) according to the manufacturer's instructions. The 11 isolates were genotyped using the enterobacterial repetitive intergenic consensus (ERIC)-PCR and all isolates showed different ERIC patterns indicating that they were different strains (Fig. S1). The 16S rRNA and the genes used in the multilocus analyses (*rpoD*, *gyrB*, *recA*, *dnaJ*, *gyrA*, *dnaX*, *atpD*) were amplified and sequenced using primers and conditions described previously [28,29,40]. Genetic distances and clustering were obtained using Kimura's two-parameter model, alignments and phylogenetic trees were constructed by the neighbour-joining method using MEGA software version 5 [42]. The percentages of the inter-species ranges of nucleotide substitution of the 3 new taxa with respect to their most closely related species were also calculated with the MEGA software.

The almost complete 16S rRNA gene (1405 bp) was obtained and the unknown strains formed three new, monophyletic phylogenetic clades when compared with those from the type strains of all known *Aeromonas* species (Fig. 1). The percentage ranges of the inter- and intra-species sequence similarities of the 16S rRNA gene of the three new *Aeromonas* species are illustrated in Table 1. The high inter-species similarity of the 16S rRNA gene (99.07–99.72%) for the new species with the closest relatives is not rare in *Aeromonas* and fall within the range described for this genus [16,39]. However, a detailed analysis of the variable regions of the 16S rRNA gene sequences of the 3 new species revealed unique nucleotides at concrete positions (Table 2). These signature nucleotides were located in the variable regions V2 and V3 which have previously been considered useful regions for the delineation of *Aeromonas* species [28].

The tree constructed with the concatenated sequence of 7 genes (*gyrA*, *gyrB*, *rpoD*, *recA*, *dnaJ*, *atpD* and *dnaX*, 4093 bp) showed that the 3 groups of strains formed well supported (bootstrap > 94%) independent clusters from the rest of the species of the genus. The new candidate species *Aeromonas lusitana* which has not yet been formally described was included in the analysis in order to demonstrate that the three new species do not belong to this species.

The nearest phylogenetic neighbors were different than those obtained with the 16S rRNA phylogeny which is not strange considering the low bootstrap values obtained with the latter gene (Figs. 1 and 2). The most related species in the MLSA for *A. finlandiensis* sp. nov. were *A. allosaccharophila*, *A. australiensis* and *A. veronii;* for *A. aquatica* sp. nov., *A. encheleia* and *A. eucrenophila* and for *A. lacus* sp. nov., *A. jandaei* (Figs. 2 and 3). As can be seen in the present study and in many others [3,4,7,9,15,27,30] the resolution of the MLSA is better than the one provided by the 16S rRNA gene and the overall phylogenetic relatedness is more robust (bootstrap values of 100% for all the species clusters). With the 7 concatenated genes, the inter-species ranges of substitution of the new species with their closest relatives were: 2.67–3.41% for *A. finlandiensis* with *A. allosaccharophila* (3.04% between the type strains), 2.97–3.61% for *A. finlandiensis* with *A. veronii* (3.30% between the type strains), 4.10–4.61% for *A. aquatica* and *A. encheleia* (4.61% between type strains), 4.15–4.47% for *A. aquatica* and *A. eucrenophila* (4.17% between the type strains) and 2.67–3.31% for *A. lacus* and *A. jandaei* (2.87% between the type strains). Other species within the genus share similar or lower inter-species ranges like *A. allosaccharophila* and *A. veronii* (2.87–3.53%, 3.21% between type strains) or *A. bestiarum* and *A. piscicola* (2.36–3.34%, 2.63% between type strains) as has previously been demonstrated [29].

To confirm these results a more complete analysis was performed using the concatenated sequence of 15 genes of the type strains of the new species with those of their closest relatives using *A. piscicola* and *A. bestiarum* as reference of the lowest inter-species separation within the genus [29]. The additional genes *cpn60*, *dnaK*, *gltA*, *mdh*, *radA*, *rpoB*, *tsf* and *zipA* were retrieved from the genome sequences performing a BLAST search using the Basic Local Alignment Search Tool (from the NCBI web interface). The new tree confirmed that the 3 proposed species formed new independent phylogenetic lines within the genus (Fig. 3) because the length of the branches that separate them from the closest relatives were equivalent to those shown by other accepted species of the genus (i.e. *A. piscicola* and *A. bestiarum*). The inter-species nucleotide substitution rates for these 15 genes between the type strains of the new species and their close relatives were similar to those obtained with 7 genes, i.e. *A. finlandiensis* 4287D^T-*A. allosaccharophila* CECT 4199^T (3.32%), *A. finlandiensis* 4287D^T-*A. veronii* CECT 4257^T (3.60%), *A. aquatica* AE235^T-*A. encheleia* CECT 4342^T(4.9%) and *A. lacus* AE122^T-*A. jandaei* CECT 4228^T (2.85%). However, it was slightly lower (by 0.44%) for *A. aquatica* AE235^T-*A. eucrenophila* CECT 4224^T (3.73%). As in the 7 gene analysis all inter-species values between the type strains and those of their close relatives were lower than those obtained for *A. bestiarum* CECT 4227^T-*A. piscicola* CECT 7443^T (2.56%), and all except one (*A. lacus*-*A. jandaei*) were also lower than *A. allosaccharophila* CECT 4199^T – *A. veronii* CECT 4257^T (2.97%). These highly similar inter-species nucleotide substitution values could be an indication that the rate of evolution of the different genes between those species is constant.

The genome sequences of strains *A. aquatica* AE235^T and *A. lacus* AE122^T have recently been published [20] and using the same methodology we also obtained the genome of *A.*