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NOTE

Small subunit rRNA gene sequences of *Aeromonas salmonicida* subsp. *smithia* and *Haemophilus piscium* reveal pronounced similarities with *A. salmonicida* subsp. *salmonicida*

Jacinta M. Thornton[1], Dawn A. Austin[2], Brian Austin[2], Richard Powell[1,*]

[1]Department of Microbiology, National University of Ireland, Galway, Ireland
[2]Department of Biological Sciences, Heriot-Watt University, Riccarton, Edinburgh EH14 4AS, Scotland, UK

ABSTRACT: The small subunit ribosomal RNA (SSU rRNA) encoding genes from reference strains of *Aeromonas salmonicida* subsp. *smithia* and *Haemophilus piscium* were amplified by polymerase chain reaction and cloned into *Escherichia coli* cells. Almost the entire SSU rRNA gene sequence (1505 nucleotides) from both organisms was determined. These DNA sequences were compared with those previously described from *A. salmonicida* subsp. *salmonicida*, subsp. *achromogenes* and subsp. *masoucida*. This genetic analysis revealed that *A. salmonicida* subsp. *smithia* and *H. piscium* showed 99.4 and 99.6% SSU rRNA gene sequence identity, respectively, with *A. salmonicida* subsp. *salmonicida*.

KEY WORDS: *Aeromonas salmonicida* · *Haemophilus piscium* · Ribosomal RNA genes

In the 1950s, phenetic analysis resulted in a detailed description of *Aeromonas salmonicida*, the bacterium associated with furunculosis disease of salmonids (Griffin 1953, Snieszko 1957). Subsequently, analysis of strains with some aberrant traits redefined this taxon into 3 subspecies (Schubert 1974), i.e. *A. salmonicida* subsp. *salmonicida*, subsp. *achromogenes* (Smith 1963), and subsp. *masoucida* (Kimura 1969). More recently, a further subspecies, subsp. *smithia* (Austin et al. 1989), has been described. Also of note, *Haemophilus piscium*, the etiological agent of ulcer disease in salmonid species (Snieszko et al. 1950), is commonly thought to represent another aberrant member of *A. salmonicida* (Paterson et al. 1980, Trust et al. 1980), a view reinforced by Austin et al. (1998).

In a recent characterization study that included reference strains for *Aeromonas salmonicida* subsp. *salmonicida*, subsp. *achromogenes*, subsp. *masoucida*, and subsp. *smithia* and *Haemophils piscium*, all strains were separated on the basis of ribotype; only subsp. *achromogenes* and *H. piscium* were grouped together in a biochemical analysis, and only subsp. *masoucida* and subsp. *achromogenes* were grouped together in a polymerase chain reaction (PCR) analysis (Austin et al. 1998). In light of these results, we sought to examine these strains by determining the nucleotide sequences of their respective small subunit ribosomal RNA (SSU rRNA) encoding genes. SSU rRNA gene sequences have been shown to be useful for the determination of either close or distant genealogical relationships (Woese 1987), and the SSU rRNA gene sequences for *A. salmonicida* subsp. *salmonicida*, subsp. *achromogenes* and subsp. *masoucida* have already been described (Martinez-Murcia et al. 1992). Here, we report the SSU rRNA gene sequences of *A. salmonicida* subsp. *smithia* and *H. piscium* and a comparative sequence analysis with the SSU rRNA genes of *A. salmonicida* subsp. *salmonicida*, subsp. *achromogenes*, and subsp. *masoucida*.

Materials and methods. ***Origin and growth of strains:*** The type strain *Aeromonas salmonicida* subsp. *smithia* (CCM 4103) was obtained from the Czechoslovakian Collection of Microorganisms, Brno, Czech Republic. The *Haemophilus piscium* strain (NCIMB 1952) was obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland. It should be noted that an authentic type strain of *H. piscium* is not available in any culture collection. CCM 4103 was cultivated on colombia base agar (Oxoid, Unipath Ltd, Hampshire, UK) supplemented with 7% human blood, whereas the *H. piscium* strain was grown on tryptone soya agar supplemented with 1%

*Addressee for correspondence.
E-mail: richard.powell@nuigalway.ie

NaCl and 0.0002% cocarboxylase (Sigma Chemical Co., St. Louis, USA). Both strains required 2 to 3 d growth at 22°C.

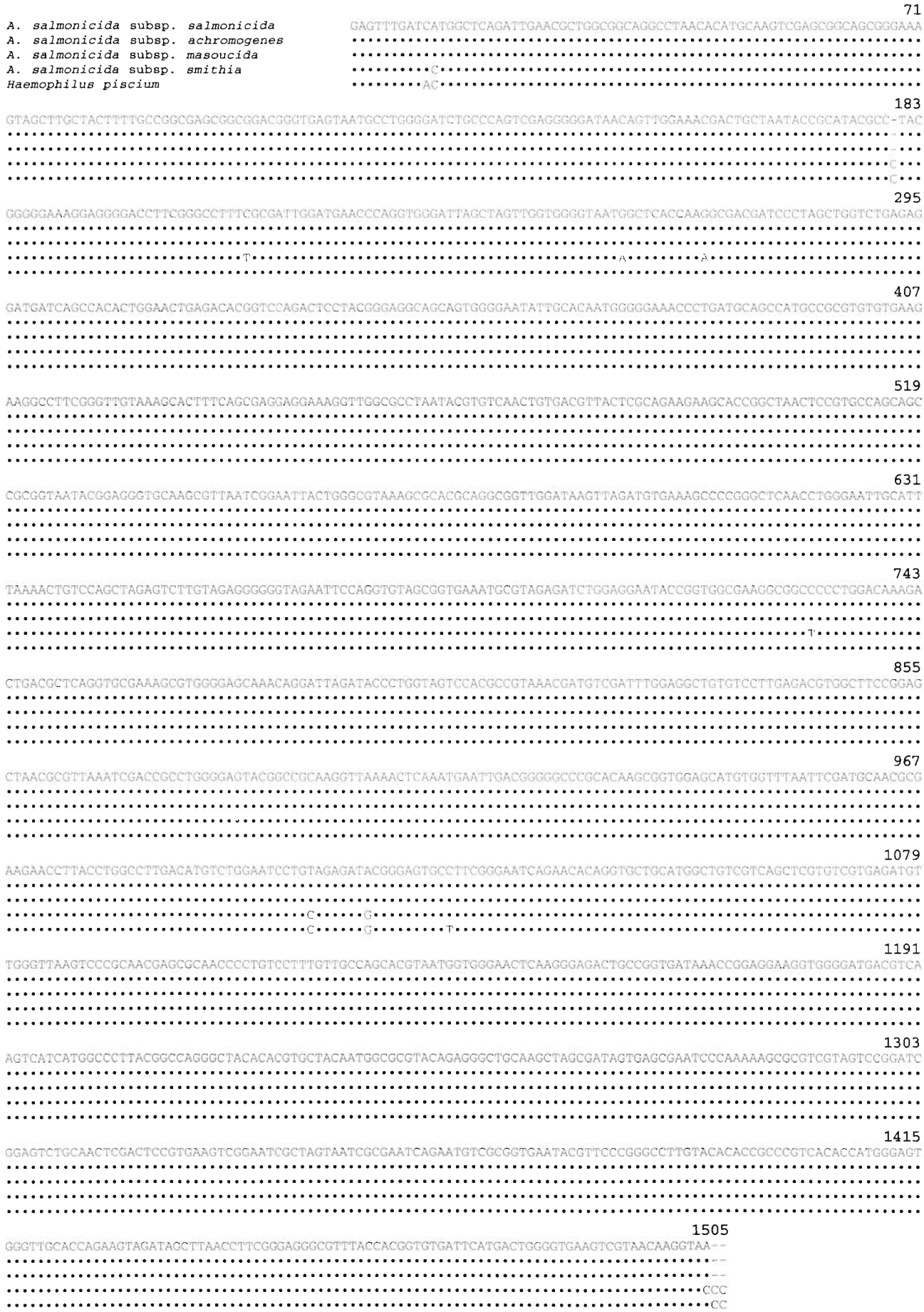
Bacterial DNA extraction: Bacterial biomass from 10 ml cultures was collected by centrifugation and washed in 1 ml of sterile H_2O, followed by centrifugation for 5 min at 9650 × *g* (MSE Micro Centaur, Sanyo, UK) and resuspension in 400 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The cells were then lysed by the addition of 200 µl of 10 mg ml^{-1} lysozyme (Boehringer Mannheim GmbH, Germany) and incubation at 37°C for 60 min. The preparation was then incubated for 10 min with 40 µl of 10 mg ml^{-1} proteinase K (Boehringer Mannheim) at room temperature, followed by the addition of sodium dodecyl sulphate (SDS) to a final concentration of 1% and incubation at room temperature until the preparation was clear. Eighty µl of 0.5 M EDTA was added, and mixed by gentle agitation and the solution was deproteinated by sequential phenol and chloroform isoamyl alcohol (24:1 vol/vol) extraction. The genomic DNA was precipitated in ethanol and resuspended in sterile H_2O. The DNA concentration was estimated by visual comparison with the standard DNA size markers after electrophoresis through 1% agarose (BioGene Ltd, Cambridgeshire, UK) TAE (tris-acetate EDTA) gels stained with 0.5 µg ml^{-1} ethidium bromide (Sigma Chemical Co.).

PCR amplification and cloning of the SSU rRNA genes: The designation and sequences of the PCR primers and their reference positions on alignments of SSU rRNA gene sequences are EB, 5'-GAGTTTGATC-CTGGCTCAG-3' (bases 3–25) and UN, 5'-ACGGN-WACCTTGTTACGAGTT-3' (bases 1423–1402) (Standard International Union of Pure and Applied Chemistry nomenclature: N is G, A, T or C; W is A or T). Both PCR primers are described as being specific for most bacteria (Lane 1991). The 50 µl reaction contained 1 × NH_4 buffer and 1.5 mM $MgCl_2$ (Biotaq, Bioline UK Ltd), 200 µM dNTPs (BioGene Ltd), 1 U *Taq* DNA polymerase (Biotaq, Bioline UK Ltd), 170 pmol of primer EB, 157 pmol of primer UN and 20 ng of genomic DNA, overlaid with 30 µl of mineral oil. Negative controls containing no DNA were included. The reactions were then amplified using a TRIO-thermoblock thermocycler (Biometra GmbH, Göttingen, Germany) through 30 cycles of 1 min at 94°C, 1 min at 52°C and 1 min at 72°C followed by a final extension of 5 min at 72°C. Five µl aliquots of the amplification products were analysed by electrophoresis through 1% agarose TAE gels stained with 0.5 µg ml^{-1} ethidium bromide. The PCR products were purified using the Geneclean II kit (BIO 101 Inc., Vista, CA, USA) according to the manufacturers instructions. The purified PCR products were cloned using the TA cloning system according to the manufacturers protocol (Invitrogen Corp., San Diego, CA, USA).

DNA sequences and alignment: Initially the SSU rRNA gene sequences of *Aeromonas salmonicida* subsp. *smithia* and *Haemophilus piscium* were determined using the ABI 373 automatic DNA sequencer (Perkin Elmer, Applied Biosystems Inc., CA, USA) using the dye terminator kit supplied by Applied Biosystems. The DNA sequences were then aligned using the ClustalW programme (Higgins & Sharp 1988) with the SSU rRNA gene sequences retrieved from the GenBank database of *A. salmonicida* subsp. *salmonicida* NCIMB 1102 (accession no. X60405) and the identical SSU rRNA gene sequences of subsp. *achromogenes* NCIMB 1110 and subsp. *masoucida* NCIMB 2020 (X60406). All sequence variations between subsp. *smithia* and *H. piscium* and the database entries were checked by manual DNA sequencing using the T7 sequencing kit (Pharmacia, Uppsala, Sweden) and variant nucleotides were either corrected or confirmed. Both strands were sequenced in their entirety by both automated and manual methods. The sequences reported in this study have been deposited in the GenBank database under the accession nos. AJ009859 (subsp. *smithia*) and AJ009860 (*H. piscium*).

Results and discussion. Using the PCR primers UN and EB, a DNA fragment of approximately 1500 nucleotides in length was amplified from each of the 2 strains studied. Following DNA sequencing, a total of 1505 unambiguous nucleotide positions in the SSU rRNA gene from *Aeromonas salmonicida* subsp. *smithia* and *Haemophlus piscium* were determined. Fig. 1 shows a comparative nucleotide sequence alignment between these 2 sequences and the maximum available 1503 nucleotides of the SSU rRNA gene sequences of *A. salmonicida* subsp. *salmonicida* (NCIMB 1102), subsp. *achromogenes* (NCIMB 1110) and subsp. *masoucida* (NCIMB 2020). The analysis revealed very high levels of SSU rRNA gene sequence similarity. *A. salmonicida* subsp. *smithia* (CCM 4103) differed from *A. salmonicida* subsp. *salmonicida* at only 9 sites (i.e. 99.4% similarity) while *H. piscium* (NCIMB 1952) differed from the same type strain at only 6 sites (i.e. 99.6% similarity). All the reference

Fig. 1 SSU rRNA gene nucleotide sequence alignment. All sequences are compared to the SSU rRNA gene sequence of *Aeromonas salmonicida* subsp. *salmonicida* and only variant nucleotides are noted. (-) no homologous nucleotide at this position, (•) an identical nucleotide at this position


71
A. salmonicida subsp. salmonicida GAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGCAGCGGGAAA
A. salmonicida subsp. achromogenes
A. salmonicida subsp. masoucida
A. salmonicida subsp. smithia
Haemophilus piscium
183
GTAGCTTGCTACTTTTGCCGGCGAGCGGCGGACGGGTGAGTAATGCCTGGGGATCTGCCCAGTCGAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCATACGCC-TAC
295
GGGGGAAAGGAGGGGACCTTCGGGCCTTTCGCGATTGGATGAACCCAGGTGGGATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAG
407
GATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAG
519
AAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTTGGCGCCTAATACGTGTCAACTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGC
631
CGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATT
743
TAAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGA
855
CTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCCGGAG
967
CTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCG
1079
AAGAACCTTACCTGGCCTTGACATGTCTGGAATCCTGTAGAGATACGGGAGTGCCTTCGGGAATCAGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGT
1191
TGGGTTAAGTCCCGCAACGAGCGCAACCCCTGTCCTTTGTTGCCAGCACGTAATGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCA
1303
AGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGCGCGTACAGAGGGCTGCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGCGCGTCGTAGTCCGGATC
1415
GGAGTCTGCAACTCGACTCCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGT
1505
GGGTTGCACCAGAAGTAGATAGCTTAACCTTCGGGAGGGCGTTTACCACGGTGTGATTCATGACTGGGGTGAAGTCGTAACAAGGTAA--

strains are clearly very closely related in terms of shared ancestry, falling well within the 3% SSU rRNA gene sequence variation proposed as a provisional species delineator (Stackebrandt & Goebel 1994). This analysis shows that, although individual phenetic traits may show variation, in phylogenetic terms all the strains examined show insufficient SSU rRNA gene sequence variation to allow the identification of separately evolving subtaxa within the species *Aeromonas salmonicida*.

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