NOTE

***Pasteurella skyensis* sp. nov., isolated from Atlantic salmon (*Salmo salar* L.)**

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From four separate incidents of disease in farmed Atlantic salmon over a four-year period, Gram-negative rod-shaped bacteria were consistently isolated by culture on sea-water blood agar. Biochemical and physiological tests indicated that the organism was related to the family *Pasteurellaceae*, and this was confirmed from the 16S rRNA gene sequence. Comparison of the 16S rRNA gene sequence with those of 45 members of the *Pasteurellaceae* showed that the closest phylogenetic relationship was with an organism termed '*Pasteurella phocoenarum*', isolated from a porpoise, for which the 16S rRNA gene sequence has been recorded but for which the properties have yet to be published. It is proposed that this bacterium isolated from salmon should be classified as a new species, namely *Pasteurella skyensis* sp. nov. The type strain of *Pasteurella skyensis* sp. nov. is strain 95A1^T (= NCTC 13204^T = NCIMB 13593^T).

Keywords: *Pasteurella skyensis*, *Pasteurellaceae*, Atlantic salmon (*Salmo salar*)

Marine aquaculture of salmonids has expanded rapidly in the last three decades, and the rapid growth of the industry has been paralleled by an increase in the number of recognized bacterial pathogens (Inglis *et al.*, 1993). Several of these pathogens, such as *Vibrio anguillarum* and *Aeromonas salmonicida*, had been known for a century (Inglis *et al.*, 1993), and successful vaccines have been developed to protect fish from these bacteria (Smith, 1988; Midtlyng, 1997). However, newly emerging bacterial diseases (Fryer & Bartholemew, 1996) will continue to pose problems for the industry until effective vaccines can be developed.

Here we report the isolation, from four separate outbreaks of infection in farmed Atlantic salmon in Scotland, of an organism of the family *Pasteurellaceae*. The organism, for which the name *Pasteurella skyensis* is proposed, differs from currently recognized bacteria in both phenotypic properties and 16S rRNA gene sequence. It also differs from most other *Pasteurellaceae* in lacking catalase activity and nitrate-reducing activity. Although the taxonomic position of the *Pasteurellaceae* is complex and incompletely resolved (Dewhirst *et al.*, 1992; Holmes, 1998), a recent major revision of the *Pasteurella haemolytica* complex by Angen *et al.* (1999) has clarified the position of these

organisms with their transfer to a new genus, *Mannheimia*, to include the species *Mannheimia haemolytica*, *Mannheimia granulomatis*, *Mannheimia glucosida* and *Mannheimia varigena*.

From mortalities in farmed Atlantic salmon from three geographically distinct sites in Scotland in 1995, 1997 and 1998, samples were submitted to the Fish Health and Quality Laboratory, Marine Harvest or to the Fish Veterinary Group Diagnostic Laboratory, Inverness. Fish exhibited symptoms of morbidity and loss of appetite. The pathology of affected fish has been described by Jones & Cox (1999). Primary isolation of bacteria was from kidney samples by growth on trypticase soy agar (Oxoid) supplemented with 1.5% (w/v) sea salts (Sigma) and 5% (v/v) defibrinated horse blood at 20 °C in air, resulting in pure cultures of organisms producing small grey colonies within 48 h. Later, strains were routinely subcultured on trypticase soy agar + 1.5% (w/v) sodium chloride and 10% (v/v) citrated sheep or horse blood (TSSBA) for characterization tests, or on Columbia agar supplemented as above. Marine agar (Difco) and several different agar media (brain–heart infusion, Columbia, Charcoal, Bordet–Gengou, thiosulphate citrate bile salt agar) supplemented with 1.5% (w/v) NaCl were tested but none supported growth in the absence of blood. Incorporation of 5% (v/v) fish extract (50 g Atlantic salmon flesh diced and soaked in 1 l distilled water for 2 h, then boiled 1 h before filtration), 10% (v/v) fetal

The GenBank/EMBL/DBJ accession number for the nucleotide sequence of the 16S rRNA gene of strain 95A1^T reported in this paper is AJ243202.

bovine serum, 1% (w/v) yeast extract, or combinations of these, into marine agar was also ineffective in supporting growth in the absence of blood. Growth was more rapid on salmon-blood agar than in sheep-blood agar, and slight growth occurred in trypticase soy agar + 1.5% (w/v) sodium chloride containing 10% (v/v) salmon serum or sheep plasma.

The isolates obtained from four outbreaks of infection at three sites (A–C) in the West of Scotland from 1995 to 1998 were designated 95A1^T, 97A1, 98B1 and 98C1, the year of isolation being indicated by the first two digits.

In three separate experiments, bacterial isolates 95A1^T and 97A1 were injected intraperitoneally into Atlantic salmon post-smolts at doses of $1\text{--}4 \times 10^6$ organisms. With isolate 95A1^T, one out of two injected fish died within 4 weeks at 7.5 °C, and in two experiments with isolate 97A1, three out of five fish and two out of five fish died within 4 weeks at 15 °C. Kidney culture of the affected fish yielded pure growth of the organism inoculated, and histopathology of the fish infected with isolate 95A1^T showed clinical signs characteristic of naturally infected fish. Thus, the isolates appeared to be of relatively low virulence.

Isolates were cultured on TSSBA to provide inocula for biochemical tests. Catalase activity was tested by adding 3% hydrogen peroxide solution to bacteria resuspended from a plate culture and washed three times with sterile three-quarter-strength artificial sea water. The oxidase test was performed by smearing, with a glass rod, bacteria from a plate colony onto a filter paper moistened with a 1% (w/v) solution of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (Sigma). The reaction was recorded as positive if a purple coloration developed within 30 s. Biochemical tests were carried out as described by Cowan *et al.* (1993), except that an electrolyte supplement (1% NaCl, 0.4% MgCl₂ · H₂O, 0.4% KCl, w/v final concentrations) was added to the media and the inocula for all tests consisted of a suspension of washed bacteria (MacFarland standard no. 4) in three-quarter-strength artificial sea water. The production of urease and indole, the Voges–Proskauer reaction, and the hydrolysis of ONPG were tested as described by Cowan *et al.* (1993). Decarboxylase tests (Furniss *et al.*, 1978) were carried out with Difco decarboxylase medium and 1% (w/v) amino acid (arginine, lysine or ornithine), dispensed in 3 ml volumes and covered with liquid paraffin. Suspensions of 0.2 ml washed bacteria were added and the results determined after incubation for 4 d at 22 °C. Acid production from carbohydrates was determined by the addition of 0.2 ml of a suspension of washed bacteria to 1 ml medium (1% peptone water, 2% sodium chloride, 1% Andrade's reagent and 1% carbohydrate solution, w/v final concentrations). Results were read after incubation for 2 d at 20 °C.

Growth in different atmospheres was tested by incubation in an anaerobic jar. The reduction of nitrate

(Cowan *et al.*, 1993) was investigated by adding 0.2 ml of a suspension of washed bacteria (MacFarland standard no. 4) to 1 ml nitrate broth (0.1% potassium nitrate, 2% w/v sodium chloride in Oxoid nutrient broth). Cultures were incubated for 4 d at 22 °C before the detection of nitrite (Lee *et al.*, 1979). X- and V-factor requirements were determined on Columbia agar + 1.5% sodium chloride by using Oxoid X-, V- and X+V-factor discs. Tests for the oxidation or fermentation of glucose were done with marine OF medium (Leifson, 1963).

For determination of 16S rRNA gene sequence and phylogenetic analysis, DNA was isolated from suspensions of washed bacteria as described by Sambrook *et al.* (1989) by lysis of the cells with sodium dodecyl sulphate, EDTA and lysozyme, and extraction with phenol/chloroform solution. A fragment of > 1500 nucleotides from the 16S rRNA gene of isolate 95A1^T was amplified by using the PCR with the universal primers pA [positions 8–28 (*Escherichia coli* numbering)] and pH* (positions 1542–1522). The amplified product was cloned into pUC18 (Sureclone; Pharmacia) and used as the template for sequencing using primers for conserved regions of the 16S rRNA and the PRIDM DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems). Reaction products were electrophoresed at the Molecular Biology Support Unit, Institute of Biomedical and Life Sciences, University of Glasgow, using an Applied Biosystems 373A automatic DNA sequencer according to the manufacturer's protocols. Sequences were assembled using the SEQMAN program. For isolate 97A1, a partial sequence of the 16S rRNA gene was determined using the PCR product directly as a template for sequencing. Searches in the EMBL database were carried out using the program FASTA, and sequences of close relatives were retrieved from the EMBL database and aligned using the program PILEUP (Devereux *et al.*, 1984); the alignment was corrected manually. Programs from the PHYLIP package (Felsenstein, 1989) were used to produce a distance matrix (DNADIST) and a phylogenetic tree, using the neighbour-joining method (NEIGHBOR) and maximum-parsimony analysis (DNAPARS). The stability of the groups was assessed by bootstrapping using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE. For maximum-likelihood analysis, the FastDNAmI program of Olsen *et al.* (1994) was used; trees were drawn using TREEVIEW (Page, 1996).

The G+C contents of DNA preparations were determined from thermal denaturation midpoints (*T*_m) (Marmur & Doty, 1962) and were calculated according to Johnson (1985).

The characteristics of the isolates are given in the description of *Pasteurella skyensis* sp. nov. (see below). The inability to culture the isolates in blood-free media meant that many biochemical characterization tests could not be carried out by conventional methods, so the small-volume heavy-inoculum method described by Foster *et al.* (1996) was used. Tests for catalase

Table 1. Levels of 16S rRNA identity between *Pasteurella skyensis* strain 95A1^T and representative members of the *Pasteurellaceae*

Species	Strain	16S rRNA sequence identity with <i>P. skyensis</i> (%)
<i>Actinobacillus actinomycetemcomitans</i>	ATCC 33384 ^T	90.3
<i>Actinobacillus capsulatus</i>	NCTC 11408 ^T	90.1
<i>Actinobacillus delphinicola</i>	NCTC 12871	92.3
<i>Actinobacillus equuli</i>	NCTC 8529 ^T	90.6
<i>Actinobacillus hominis</i>	CCUG 19800	91.0
<i>Actinobacillus indolicus</i>	46KC2	91.6
<i>Actinobacillus lignieresii</i>	NCTC 4189 ^T	89.9
<i>Actinobacillus minor</i>	NM 305	91.6
<i>Actinobacillus pleuropneumoniae</i>	ATCC 27088 ^T	90.8
<i>Actinobacillus porcinus</i>	NM 319	91.5
<i>Actinobacillus scotiae</i>	M2000/95/1	91.7
<i>Actinobacillus seminis</i>	ATCC 15768 ^T	90.0
<i>Actinobacillus suis</i>	ATCC 15557	90.3
<i>Haemophilus aegyptius</i>	NCTC 8502 ^T	90.3
<i>Haemophilus aphrophilus</i>	ATCC 33389 ^T	89.9
<i>Haemophilus ducreyi</i>	CPI 542 ^T	92.6
<i>Haemophilus haemoglobinophilus</i>	NCTC 1659 ^T	91.5
<i>Haemophilus haemolyticus</i>	NCTC 10659 ^T	89.7
<i>Haemophilus influenzae</i>	NCTC 8143 ^T	91.5
<i>Haemophilus paracuniculus</i>	ATCC 29986	91.2
<i>Haemophilus paragallinarum</i>	NCTC 11296 ^T	89.5
<i>Haemophilus parahaemolyticus</i>	NCTC 8479 ^T	90.2
<i>Haemophilus paraphrohaemolyticus</i>	NCTC 10670 ^T	90.9
<i>Haemophilus parainfluenzae</i>	ATCC 33392 ^T	90.6
<i>Haemophilus paraphrophilus</i>	ATCC 29242	90.3
<i>Haemophilus parasuis</i>	NCTC 4557 ^T	90.9
<i>Haemophilus segnis</i>	ATCC 33393 ^T	90.0
<i>Haemophilus somnus</i>	OVCG 43826	89.0
<i>Mannheimia haemolytica</i>	NCTC 9380 ^T	91.7
<i>Pasteurella aerogenes</i>	ATCC 27883 ^T	90.2
<i>Pasteurella anatis</i>	ATCC 43329 ^T	89.1
<i>Pasteurella avium</i>	NCTC 11297 ^T	90.8
<i>Pasteurella bettyae</i>	CCUG 2042 ^T	90.4
<i>Pasteurella canis</i>	ATCC 43326 ^T	90.3
<i>Pasteurella dagmatis</i>	ATCC 43325 ^T	91.3
<i>Pasteurella gallinarum</i>	NCTC 11188 ^T	90.5
<i>Pasteurella langaaensis</i>	ATCC 43328 ^T	90.9
<i>Pasteurella multocida</i> subsp. <i>multocida</i>	NCTC 10322 ^T	90.7
<i>Pasteurella mairii</i>	CCUG 27189	91.2
<i>Pasteurella phocoenarum</i>	NCTC 12872	97.1
<i>Pasteurella pneumotropica</i>	NCTC 8141 ^T	91.0
<i>Pasteurella stomatis</i>	ATCC 43327 ^T	90.1
<i>Pasteurella testudinis</i>	CCUG 19802	90.7
<i>Pasteurella trehalosi</i>	NCTC 10624	92.3
<i>Pasteurella volantium</i>	NCTC 3438 ^T	90.7

activity and nitrate-reduction activity were all consistently negative, and oxidase activity was very weakly positive in all isolates. In the API ZYM reactions, all strains reacted identically, having pronounced alkaline phosphatase, acid phosphatase and naphthol-ASBI-phosphohydrolase activities, and detectable esterase–lipase and leucine arylamidase activities. The

pattern of reactivity was similar to that reported for *Actinobacillus delphinicola* (Foster *et al.*, 1996), although the latter organism lacked naphthol-ASBI-phosphohydrolase activity. However, the API ZYM profile was significantly different from that of *Photobacterium damsela* subsp. *piscicida* NCIMB 2058^T, formerly termed *Pasteurella piscicida*, which possessed

Fig. 1. Unrooted tree, based on maximum likelihood (fastDNAmI), showing the phylogenetic position of *Pasteurella skyensis* in the *Actinobacillus*-*Haemophilus*-*Pasteurella* species complex. The tree is based on approximately 1459 16S rRNA positions, and the scale bar indicates 50 substitutions. Bootstrap values, expressed as percentages of 500 replications, are given at the branching points. Only bootstrap values greater than 50 % are shown.

strong *N*-acetyl- β -glucosaminidase activity, but the above activities were very weak or undetectable.

To determine the relationship between isolate 95A1^T and other micro-organisms, a sequence (> 1500 nucleotides) of the 16S rRNA gene was obtained and compared, using the FASTA program, with sequence data in the EMBL and GenBank libraries. The closest similarity, 97.1 %, was found with '*Pasteurella phocoenarum*', an organism for which the 16S rRNA sequence has been documented (GenBank EMBL accession no. PPS16SM106) but for which no other data have been published. Only three other sequences, *Actinobacillus delphinicola*, *Haemophilus ducreyi* and *Pasteurella trehalosi*, showed > 92 % similarity. The sequence similarity between isolate 95A1^T and 45 members of the *Pasteurellaceae* is shown in Table 1. Comparison of the sequence with those in the Ribosomal Database Project database, using Sequence Match Version 2.7 of the SIMRANK package (N. Larssen), generated a Ribosomal Database Project score of 0.89 with '*Pasteurella phocoenarum*', no other scores being above 0.75. The phylogenetic relationships between 95A1^T and the above 45 members of the

Pasteurellaceae complex were investigated using the PHYLIP package (Felsenstein, 1989) to determine evolutionary distances. These were used to construct a dendrogram showing the phylogenetic position of strain 95A1^T within the *Pasteurellaceae*. By using the neighbour-joining, maximum-parsimony and maximum-likelihood methods, strain 95A1^T was shown to have low sequence relatedness to all the recognized members of the *Pasteurellaceae*, except in the case of '*Pasteurella phocoenarum*', indicating a distinct lineage within the *Pasteurellaceae*. Bootstrap resampling confirmed that the relationship between strain 95A1^T and '*Pasteurella phocoenarum*' was significant (100 %); the same conclusion was reached using the maximum-likelihood and maximum-parsimony methods. An unrooted phylogenetic tree based on maximum-likelihood analysis of strain 95A1^T (= NCTC 13204^T = NCIMB 13593^T), type species of the genera *Pasteurella*, *Actinobacillus* and *Haemophilus*, and nine other members of the *Pasteurellaceae* associated with strain 95A1^T in the above analyses is shown in Fig. 1.

To determine whether the 16S rRNA gene sequence of

isolate 95A1^T was typical of the group of isolates, a partial sequence of approximately 600 bases (30–634, *E. coli* numbering) of isolate 97A1 was determined. Two base differences were found (T for C, and T for A at positions 170 and 187, respectively).

Biochemically, the strains described here appeared to be identical and characteristic of members of the *Pasteurellaceae*, being non-motile, facultatively anaerobic, pleomorphic, Gram-negative rods that were indole-positive, ONPG-negative and Voges–Proskauer-negative. Oxidase activity was very weak for all isolates. All lacked catalase activity and nitrate-reducing activity, activities which are normally possessed by the *Pasteurellaceae*, although many species lack one of these activities. All isolates in the present study required blood for growth and this could not be substituted by X and V factors. The requirement for X and V factors may not delineate the genera of the *Pasteurellaceae* (Dewhirst *et al.*, 1992), but the lack of a requirement for X and/or V factors indicates that strain 95A1^T is not, by conventional criteria, a member of the *Haemophilus* genus.

The closest relative to strain 95A1^T, according to 16S rRNA gene sequence comparison, is '*Pasteurella phocoenarum*', a single isolate from a porpoise (*Phocoena phocoena*) (G. Foster, SAC, Inverness), details of which have yet to be described. This organism and two others, *Actinobacillus delphinicola* and *Actinobacillus scotiae*, were isolated from cetaceans (Foster *et al.*, 1996, 1998), and the latter two organisms differ in several biochemical properties (maximum growth temperature, atmospheric requirements, requirement for blood, nitrate reduction) from strain 95A1^T.

Two fish-pathogenic bacteria have previously been classified as members of the *Pasteurellaceae*. However, *Haemophilus piscium*, described by Snieszko *et al.* (1950), is now classified as an atypical strain of *Aeromonas salmonicida* (Paterson *et al.*, 1980; Mannheim, 1984), and it has been proposed by Gauthier *et al.* (1995), on the basis of 16S rRNA sequence, that '*Pasteurella piscicida*' should be reclassified as *Photobacterium damsela* subsp. *piscicida*. Comparison of the 16S rRNA sequences of strain 95A1^T and the type strain of *Photobacterium damsela* subsp. *piscicida* (NCIMB 2058^T) showed only 85.5% sequence similarity, confirming the substantial difference between these bacteria. Similar comparisons with *V. anguillarum* (NCIMB 2130) and *Aeromonas salmonicida* (NCIMB 1102^T) showed 85.1% and 88.2% sequence similarity, respectively, these values being well below those recorded for all members of the *Pasteurellaceae* that have been examined (Table 1).

The temperature range for growth (14–32 °C) was consistent with the finding that episodes of the disease occurred in summer and ceased when water temperatures began to fall in autumn. As the upper temperature limit for growth differed from that of the cetacean isolates (*Actinobacillus delphinicola*, *Actino-*

bacillus scotiae and '*Pasteurella phocoenarum*'), all of which were capable of growth at 37 °C (Foster *et al.*, 1996, 1998) – the body temperature of these animals – they are probably not associated with these particular disease outbreaks. A possible infection source could be fish associated with warmer waters, e.g. mackerel, although there is no direct evidence to that effect. A further significant difference between the current isolates and those from cetaceans is that they did not require elevated CO₂ concentrations for growth, whereas the cetacean isolates were unable to grow in air (Foster *et al.*, 1996, 1998).

The strains described here are the first members of the *Pasteurellaceae* to have been established as causing disease in fish, and, as such, their origin, relationship to other *Pasteurellaceae*, and mode of pathogenesis are of considerable interest. It is possible that these isolates are sufficiently different, in terms of host origin, biochemical properties and 16S rRNA sequence, from other members of the *Pasteurellaceae* to justify their recognition as a new genus during further revision of the taxonomy of this family.

Description of *Pasteurella skyensis* sp. nov.

Pasteurella skyensis (skye.en'sis. N.L. fem. adj. *skyensis* pertaining to Skye, the island in Scotland, UK, where the first two isolates of this species were isolated).

Cells are pleomorphic, non-motile, Gram-negative rods that are facultatively anaerobic. Colonies on TSSBA incubated in air for 48 h at 22 °C are circular, entire, low, convex, smooth, grey and 0.5 mm in diameter. Non-haemolytic or weakly haemolytic. The addition of salt (1.5%, w/v) and blood to trypticase soy agar or Columbia agar is required for growth. X and V factors do not permit growth on blood-free media. Growth occurs at 14–32 °C but not at 37 °C. Catalase-negative and weakly positive for oxidase activity. Nitrate is not reduced. Acid is produced from glucose, lactose, maltose, mannose, mannitol and trehalose, but not from adonitol, arbutin, dulcitol, galactose, inositol, inulin, melibiose, raffinose, rhamnose, salicin, sucrose, sorbitol or xylose. Urease-negative, indole-positive, Voges–Proskauer-negative and ONPG-negative. Strains produce ornithine decarboxylase and lysine decarboxylase but not arginine dihydrolase. Alkaline phosphatase and acid phosphatase are produced. The G+C content of the DNA of the four isolates is 39–41%, as determined by the thermal method. Isolated from Atlantic salmon. The type strain is strain 95A1^T (= NCTC 13204^T = NCIMB 13593^T). The description of the type strain corresponds to that of the species, and the G+C content of the DNA is 41 mol%.

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