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# *Actinobacillus scotiae* sp. nov., a new member of the family *Pasteurellaceae* Pohl (1979) 1981 isolated from porpoises (*Phocoena phocoena*)

G. Foster,[1] H. M. Ross,[1] I. A. P. Patterson,[1] R. A. Hutson[2] and M. D. Collins[2]

Author for correspondence: G. Foster. Tel: +44 1463 243030. Fax: +44 1463 711103.
e-mail: vcinverness@ed.sac.ac.uk

[1] SAC Veterinary Services, Drummondhill, Inverness IV2 4JZ, UK

[2] BBSRC Institute of Food Research, Reading RG6 6BZ, UK

**Phenotypic and phylogenetic studies were performed on a Gram-negative, rod-shaped bacterium isolated from three porpoises. Biochemical and physiological studies indicated that the bacterium was related to the family *Pasteurellaceae*. Comparative 16S rRNA gene sequencing studies confirmed these findings and demonstrated that the bacterium represents a hitherto unknown subline. The nearest phylogenetic relative of the unknown bacterium was *Actinobacillus delphinicola*, an organism also originating from sea mammals, although a sequence divergence of 3% demonstrated that the newly isolated bacterium is a distinct species. On the basis of the results of the phylogenetic analysis and phenotypic criteria, it is proposed that the bacterium should be classified as a new species, *Actinobacillus scotiae* sp. nov. The type strain of *Actinobacillus scotiae* sp. nov. is NCTC 12922$^{T}$ (=M2000/95/1$^{T}$).**

**Keywords:** *Actinobacillus scotiae*, *Pasteurellaceae*, porpoise (*Phocoena phocoena*)

## INTRODUCTION

The range of infectious agents occurring among North-East Atlantic populations of cetaceans is poorly documented. Considerable concern has been expressed regarding the influence of man's activities on the marine environment and on the possible effects on marine life, particularly cetaceans. An improved understanding of naturally occurring infectious agents among cetacean populations is seen as crucial to the study of environmental influences on these animals.

During a systematic investigation into the microbiological flora of cetaceans and also possible agents causing or associated with disease, we recently reported the characterization of a novel *Actinobacillus* species, *Actinobacillus delphinicola* (Foster *et al.*, 1996). In the course of this on-going study, we subsequently isolated three identical strains of an unknown Gram-negative rod-shaped bacterium following post-mortem examination of three harbour porpoises (*Phocoena phocoena*) stranded at different locations on the Scottish coastline. On the basis of biochemical and physiological criteria, the unknown isolates could not be assigned to any described species of the genera *Actinobacillus*, *Haemophilus* or *Pasteurella*. In this paper we describe the cultural and biochemical characteristics of these bacteria and the results of a phylogenetic analysis based on 16S rRNA gene sequencing. Based on the phenotypic and phylogenetic distinctiveness of the unknown bacterium from porpoises, a new species, *Actinobacillus scotiae*, is described.

The EMBL accession number for the nucleotide sequence of the 16S rRNA gene of strain M2000/95/1$^{T}$ reported in this paper is Y09653.

## METHODS

**Samples.** Porpoises were reported as having been stranded on the Scottish coastline and were subjected to post-mortem examination. Examinations were carried out in accordance with a nationally agreed protocol (Kuiken & Hartmann, 1991) and selected tissues were removed for bacteriological investigation.

**Isolation and cultivation.** Primary isolations were made on Columbia agar (Difco) supplemented with 5% citrated sheep blood (CSBA) that was incubated at 37 °C in an atmosphere containing 10% $CO_2$ for 24 h. Strains were subcultured to CSBA for characterization tests.

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**Biochemical characterization.** Isolates were routinely grown on CSBA to provide inocula for biochemical tests. Indole and nitrate reduction were tested using Rosco tablets (Bioconnections). Nitrate was also tested by making a heavy inoculum in nutrient broth (Oxoid) containing 0·1 % $KNO_3$ and incubating overnight. In both methods, after incubation 0·8 % sulfanic acid (Rosco) and 0·6 % *N*-*N*-dimethyl-α-naphthylamine (Rosco) were added and development of a red colour indicated a positive result. Catalase, oxidase, X and V factor requirement, arginine dihydrolase, ornithine decarboxylase, lysine decarboxylase, urease, acid production from carbohydrates and ability to grow in different atmospheres, at selected temperatures, without blood or serum and on MacConkey agar were determined as described previously (Foster *et al*., 1996). The biochemical characteristics of the strains were also examined with the API 20E system (bioMérieux) according to the manufacturer's instructions.

**Determination of 16S rRNA gene sequences and phylogenetic analysis.** For phylogenetic studies, a large fragment of the 16S rRNA gene was amplified by PCR by using universal primers pA (positions 8–28, *Escherichia coli* numbering) and pH* (positions 1542–1522). The amplified product was sequenced directly using primers for conserved regions of the 16S rRNA. Sequencing was performed using a PRISM DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) and reaction products were electrophoresed using an Applied Biosystems model 373A automatic DNA sequencer according to the manufacturer's protocols. To establish the nearest relatives of the unknown strains, preliminary searches in the EMBL database were performed with the program FASTA. Sequences of close relatives were retrieved from the EMBL database and aligned by using the program PILEUP (Devereux *et al*., 1984) and the alignment was corrected manually. A phylogenetic tree was constructed by the neighbour-joining method with the program NEIGHBOR of the PHYLIP package. The stability of relationships was assessed by bootstrapping by using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE (Felsenstein, 1989).

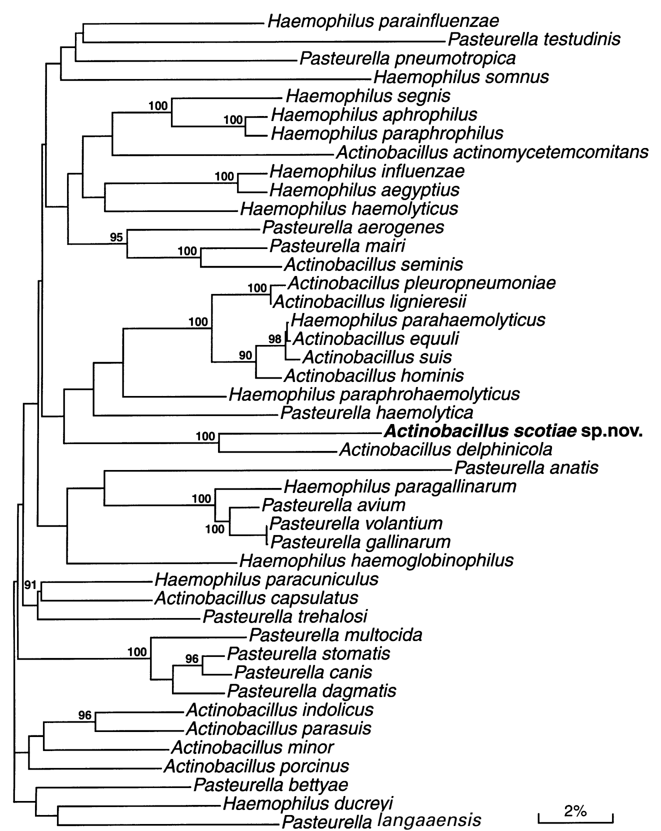


***Fig. 1.*** Unrooted tree showing the phylogenetic position of *A. scotiae* sp. nov. in the *Actinobacillus–Haemophilus–Pasteurella* species complex. The tree is based on approximately 1460 16S rRNA positions and was constructed by the neighbour-joining method. Bootstrap values, expressed as a percentage of 500 replications, are given at the branching points.

## RESULTS AND DISCUSSION

Gram-negative, rod-shaped bacteria that were phenotypically similar to members of the *Haemophilus–Pasteurella* species complex were recovered from various tissues of three porpoises stranded at different locations on the Scottish coastline during 1995–1996. One strain was recovered from the brain, lung, spleen, liver, mesenteric lymph node, blood and small intestine of one animal (M2000/95/1$^{T}$) that died of septicaemia; a second strain was recovered from the lung, spleen, liver, kidney, mesenteric lymph node and small intestine of another (M1933/96/1), and a third strain was isolated from the spleen, liver, blood, internal iliac lymph node and small intestine of another porpoise (M1676/96/6). Colonies of the isolates grown on CSBA were circular, entire, low convex, grey, translucent or opaque and 0·5 mm in diameter after 24 h incubation at 37 °C in an atmosphere containing 10 % $CO_2$. Very slight haemolysis of sheep blood was observed. The cells were Gram-negative and rod shaped. The organisms were catalase negative, oxidase positive and non-motile. The isolates from all three porpoises grew under anaerobic conditions but not in air without added $CO_2$. Growth occurred on Columbia agar without blood or serum but was enhanced considerably by their presence. Growth did not occur on MacConkey agar with or without NaCl. They grew at 25 °C, but not at 42 °C. The organisms were urease-positive and indole-negative. Using Rosco tablets, nitrate was not reduced; however, when nitrate broth was used, nitrate was reduced to nitrite by all three isolates. Acid was produced from glucose, lactose and mannose using the small-volume, heavy-inoculum method (Foster *et al*., 1996), but not from adonitol, arabinose, dulcitol, inositol, maltose, mannitol, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose or xylose. Two strains (M1933/96/1, M1676/96/6) produced acid from galactose. Two strains (M2000/95/1$^{T}$, M1933/96/1) were positive for ornithine decarboxylase, but all three were negative for arginine dihydrolase and lysine decarboxylase. Using the API 20E system, the only positive reactions

***Table 1.*** Levels of 16S rRNA sequence similarity between *A. scotiae* sp. nov. and representatives of the *Haemophilus–Pasteurella* species complex

| Species | 16S rRNA sequence similarity to *A. scotiae* (%) |
|---|---|
| *Actinobacillus actinomycetemcomitans* | 92·0 |
| *Actinobacillus capsulatus* | 92·2 |
| *Actinobacillus delphinicola* | 96·3 |
| *Actinobacillus equuli* | 91·4 |
| *Actinobacillus hominis* | 91·8 |
| *Actinobacillus indolicus* | 92·3 |
| *Actinobacillus ligniersii* | 91·7 |
| *Actinobacillus minor* | 92·6 |
| *Actinobacillus pleuropneumoniae* | 91·5 |
| *Actinobacillus porcinus* | 93·7 |
| *Actinobacillus seminis* | 92·4 |
| *Actinobacillus suis* | 91·5 |
| *Haemophilus aegyptius* | 94·0 |
| *Haemophilus aphrophilus* | 91·7 |
| *Haemophilus ducreyi* | 92·9 |
| *Haemophilus haemoglobinophilus* | 93·5 |
| *Haemophilus haemolyticus* | 92·8 |
| *Haemophilus influenzae* | 93·8 |
| *Haemophilus paracuniculus* | 93·1 |
| *Haemophilus paragallinarum* | 93·0 |
| *Haemophilus parahaemolyticus* | 91·4 |
| *Haemophilus parainfluenzae* | 93·0 |
| *Haemophilus paraphrohaemolyticus* | 92·8 |
| *Haemophilus paraphrophilus* | 92·9 |
| *Haemophilus parasuis* | 93·5 |
| *Haemophilus segnis* | 92·0 |
| *Haemophilus somnus* | 91·6 |
| *Pasteurella aerogenes* | 93·1 |
| *Pasteurella anatis* | 91·8 |
| *Pasteurella avium* | 93·5 |
| *Pasteurella bettyae* | 92·1 |
| *Pasteurella canis* | 92·7 |
| *Pasteurella dagmatis* | 92·4 |
| *Pasteurella gallinarum* | 92·9 |
| *Pasteurella haemolytica* | 91·8 |
| *Pasteurella langaaensis* | 92·3 |
| *Pasteurella mairi* | 92·7 |
| *Pasteurella multocida* | 92·7 |
| *Pasteurella pneumotropica* | 92·8 |
| *Pasteurella stomatis* | 92·7 |
| *Pasteurella testudinis* | 91·2 |
| *Pasteurella trehalosi* | 93·0 |
| *Pasteurella volantium* | 93·0 |

observed were with ONPG, urease and Voges–Proskauer in all three strains and with ornithine decarboxylase in two strains (M2000/95/1$^{T}$, M1933/96/1).

To investigate the phylogenetic relationships of the isolates, genes encoding their 16S rRNA were amplified by PCR and subjected to direct sequence analysis. Almost the complete 16S rRNA gene sequence (approx. 1505 bases) of strain M2000/95/1$^{T}$ was determined. Preliminary comparative sequence searches of the EMBL and GenBank databases using the FASTA program revealed that the newly determined 16S rRNA sequence was most similar (sequence similarity $>91$ %) to those of members of the *Actinobacillus–Haemophilus–Pasteurella* species complex. The 16S rRNA sequence was significantly less related to those of other Gram-negative taxa (data not shown). The 16S rRNA sequence of strain M2000/95/1$^{T}$ and those of reference *Actinobacillus* spp., *Haemophilus*

***Table 2.*** Differential characteristics of *Actinobacillus scotiae* sp. nov. and other members of the genus *Actinobacillus*

Data from this study and from Sneath & Stevens (1990), Møller & Kilian (1990) and Foster *et al.* (1996). *Actinobacillus* species: 1, *A. scotiae* sp. nov.; 2, *A. actinomycetemcomitans*; 3, *A. capsulatus*; 4, *A. delphinicola*; 5, *A. equuli*; 6, *A. hominis*; 7, *A. indolicus*; 8, *A. ligniersii*; 9, *A. minor*; 10, *A. muris*; 11, *A. pleuropneumoniae*; 12, *A. porcinus*; 13, *A. rossii*; 14, *A. seminis*; 15, *A. suis*. +, 90–100 % of strains are positive; (+), 80–89 % of strains are positive; d, 21–79 % of strains are positive; (–), 11–20 % of strains are positive; –, 0–10 % of strains are positive; w, weak reaction; L, late reaction.

| Characteristic | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Catalase | – | + | + | – | d | – | + | d | – | + | d | – | + | + | + |
| Oxidase | + | + | + | + | (+) | + | d | (+) | d | + | d | d | + | d | (+) |
| β-Galactosidase | + | – | + | – | d | + | + | d | + | – | + | + | (+) | – | (+) |
| Ornithine decarboxylase | d | – | – | d | – | – | – | – | – | – | – | – | – | d | – |
| Urease | + | – | + | – | + | + | – | + | + | + | + | – | + | – | + |
| Growth on MacConkey agar | – | d | + | – | + | – | – | + | – | – | – | – | (+) | – | + |
| β-Haemolysis (sheep blood) | – | – | – | – | – | – | – | – | – | – | + | – | d | – | + |
| Acid production from: | | | | | | | | | | | | | | | |
| Galactose | d | + | + | – | d | + | + | + | (+) | d,w | +,w | d | + | d, L | (+) |
| Inositol | – | – | – | – | – | – | – | – | – | d,w | – | d | + | d | – |
| Lactose | + | – | + | – | + | + | d | d,L | + | – | d | d | d | – | + |
| Maltose | – | + | + | – | + | + | + | + | + | + | + | + | (–) | d,L | + |
| Mannitol | – | (+) | + | – | + | + | – | + | – | + | + | d | + | d,L | – |
| Mannose | + | + | + | + | + | – | + | + | + | + | + | + | d | – | + |
| Melibiose | – | – | + | – | + | +,L | (–) | – | d | +,L | – | d | – | – | + |
| Raffinose | – | – | + | – | + | + | + | d | + | + | d | d | (–) | – | + |
| Salicin | – | – | + | – | – | d | – | – | – | + | – | – | – | – | + |
| Sorbitol | – | (–) | + | – | d | – | – | (–) | – | – | – | d | + | – | – |
| Sucrose | – | – | + | – | + | + | + | + | + | + | + | d | – | – | + |
| Trehalose | – | – | + | – | + | + | d | – | d | + | – | d | – | – | + |
| Xylose | – | d | + | – | + | + | d | + | d | – | + | d | + | – | + |

spp. and *Pasteurella* spp. were subjected to a comparative analysis, and derived evolutionary distances were used to determine their phylogenetic relationships. Fig. 1 shows a dendrogram depicting the phylogenetic position of strain M2000/95/$1^T$ within the family *Pasteurellaceae*. Levels of 16S rRNA sequence relatedness are shown in Table 1. Isolate M2000/95/$1^T$ formed a distinct subline within the *Pasteurellaceae*. The closest genealogical relative of the unknown isolate was *A. delphinicola*. Bootstrap resampling confirmed the association between isolate M2000/95/$1^T$ and *A. delphinicola* to be highly significant (value 100 %). Although there are no precise definitions of a bacterial species based on 16S rRNA sequences, a sequence divergence of $> 3\%$ (corresponding to 56 mismatches for a comparison of 1505 positions) is consistent with strain M2000/95/$1^T$ being genetically a close relative of *A. delphinicola* and a separate species (see Stackebrandt & Goebel, 1994). To determine whether the sequence of strain M2000/95/$1^T$ was characteristic of the group of isolates, partial 16S rRNA sequences of strains M1993/96/1 and M1993/96/6 were determined. Comparison of short fragments of approximately 800 bases (positions 40–840, *E. coli* numbering system) that included the highly variable regions V1–V3 showed that the strains were genealogically homogeneous (100 % 16S rRNA similarity).

It is evident from both phenotypic and phylogenetic data that the three isolates from porpoises represent a hitherto unknown member of the *Pasteurellaceae* Pohl (1981). The extensive 16S rRNA sequencing studies of Dewhirst *et al.* (1992, 1993) have shown that species belonging to the genera *Actinobacillus*, *Haemophilus* and *Pasteurella* are phylogenetically intermixed. Hence, the allocation of the unknown bacterium from porpoises to a specific genus in the *Pasteurellaceae* is problematic given the phylogenetic complexity within this family. The isolates can be readily distinguished from members of the genus *Pasteurella* by their negative catalase reaction. They also differ markedly from *Haemophilus* spp. by their lack of requirements for X and V factors. Phenotypically, the porpoise bacterium most closely resembles species of the genus *Actinobacillus*. We therefore propose that the isolates be assigned to the genus *Actinobacillus* as *Actinobacillus scotiae* sp. nov. We recognize however that the assignment of the porpoise bacterium to the genus *Actinobacillus* is a placement of convenience, which in the future may require emendation as the taxonomy of the *Pasteurellaceae* is revised. The nearest phylogenetic relative of *A. scotiae* is *A. delphinicola* (Foster *et al.*, 1996), which was also isolated from cetaceans. *A. scotiae* sp. nov. can be readily distinguished from *A. delphinicola* and other *Actinobacillus* spp. by the tests shown in Table 2.

### Description of *Actinobacillus scotiae* sp. nov.

*Actinobacillus scotiae* (sco.ti.ae. L. n. *scotia* classical Latin name of Scotland; L. gen. n. *scotiae* of Scotland, where the isolates were collected).

Cells are pleomorphic non-motile Gram-negative rods. They are facultatively anaerobic, but added $CO_2$ is required for growth. Colonies on CSBA incubated in an atmosphere containing 10% added $CO_2$ at 37 °C are 0·5 mm in diameter after 24 h. Weakly haemolytic. Blood or serum enhances growth; X and V factors are not required. Growth does not occur on MacConkey agar. Growth occurs at 25 °C but not at 42 °C. Catalase-negative and oxidase-positive. Nitrate is reduced. Acid is produced from glucose, lactose and maltose, but not from adonitol, arabinose, dulcitol, inositol, maltose, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose or xylose. Some strains produce acid from galactose. Urease- and Voges–Proskauer-positive. Indole-negative. Some strains produce ornithine decarboxylase. Lysine decarboxylase and arginine dihydrolase are not produced. The G+C content of the DNA is 40·5 mol%. The bacteria are isolated from porpoises. The type strain is NCTC 12922 (=M2000/95/$1^T$). The description of the type strain corresponds to that of the species except that the type strain is positive for ornithine decarboxylase and negative for acid production from galactose.

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