NOTE

***Phocoenobacter uteri* gen. nov., sp. nov., a new member of the family Pasteurellaceae Pohl (1979) 1981 isolated from a harbour porpoise (*Phocoena phocoena*)**G. Foster,¹ H. M. Ross,¹ H. Malnick², A. Willems³, R. A. Hutson³, R. J. Reid¹ and M. D. Collins³Author for correspondence: G. Foster. Tel: +44 1463 243030. Fax: +44 1463 711103.
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Phenotypic and phylogenetic studies were performed on a Gram-negative, rod-shaped bacterium isolated from the uterus of a porpoise. 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 within this family of organisms. Based on the results of the phylogenetic analysis and phenotypic criteria, it is proposed that the bacterium be assigned to a new genus, *Phocoenobacter uteri* gen. nov., sp. nov. The type strain of *Phocoenobacter uteri* sp. nov. is NCTC 12872^T.

Keywords: *Phocoenobacter uteri* gen. nov., sp. nov., harbour porpoise, taxonomy, phylogeny, 16S rRNA gene

The harbour porpoise (*Phocoena phocoena*) is the most commonly occurring representative of the small cetaceans in the north-east Atlantic Ocean. Currently there is little information on the range of potential infectious agents occurring in populations of these animals. During a systematic investigation into the microbiological flora of harbour porpoises and other cetaceans and possible agents causing or associated with disease, we previously reported the characterization of two novel *Actinobacillus* species, *Actinobacillus delphinicola* (Foster *et al.*, 1996) and *Actinobacillus scotiae* (Foster *et al.*, 1998). In the course of this ongoing study we have isolated an unknown facultatively anaerobic Gram-negative rod-shaped bacterium following post-mortem examination of a harbour porpoise stranded on the Scottish coastline. In this article we describe the cultural and biochemical characteristics of this isolate and the results of a comparative 16S rRNA phylogenetic analysis. Based on the phenotypic and phylogenetic distinctiveness of the unknown bacterium, we propose it to be assigned

to a new genus *Phocoenobacter* within the Pasteurellaceae as *Phocoenobacter uteri* sp. nov.

Post-mortem examination was carried out in accordance with a nationally agreed protocol (Kuiken & Hartmann, 1991), and selected tissues were removed for bacteriological investigation. A uterine swab was cultured on Columbia agar (Difco) supplemented with 5% citrated sheep blood (CSBA) and incubated at 37 °C in an atmosphere containing 10% CO₂. A moderate growth of a Gram-negative rod-shaped organism designated strain M1063U/93^T was obtained after 48 h. The isolate was subcultured on CSBA to provide inocula for biochemical tests. Catalase, oxidase, X and V factor requirement, indole, nitrate reduction, arginine dihydrolase, ornithine decarboxylase, lysine decarboxylase, urease, β -galactosidase, 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, 1998). The biochemical characteristics of the strain were also examined using the API 20E, API 20NE and API ZYM systems (bioMérieux) according to the manufacturer's instructions. The G+C content of DNA was determined as described by Garvie (1978). For phylogenetic studies a large fragment of the 16S

The EMBL accession number for the 16S rRNA gene sequence of strain M1063U/93^T is X89379.

Fig. 1. Unrooted tree showing the phylogenetic position of *Phocoenobacter uteri* sp. nov. within the family Pasteurellaceae. The tree is based on a comparison of approximately 1460 16S rRNA positions and was constructed by the neighbour-joining method. Abbreviations: A., *Actinobacillus*; H., *Haemophilus*; L., *Lonepinella*; M., *Mannheimia*; and P., *Pasteurella*. Bootstrap values, expressed as a percentage of 500 replications, are given at the branching points. Bar, 1%.

rRNA gene of the unknown isolate was amplified by PCR by using universal primers pA [5'-AGAGTTT-GATCCTGGCTCAG, positions 8–28 (*Escherichia coli* numbering)] and pH* (5'-AAGGAGGTGATC-CAGCCGCA, positions 1542–1522). The amplified product was sequenced directly by using primers for conserved regions of the 16S rRNA gene. Both strands were sequenced and sequencing was performed using a PRISM DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and reaction products electrophoresed using an Applied Biosystems model 373A

automatic DNA sequencer according to the manufacturer's protocols. To establish the nearest relatives of the unknown strain, 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

Table 1. Levels of 16S rRNA sequence similarity between *Phocoenobacter uteri* gen. nov., sp. nov. and some representatives of the family *Pasteurellaceae*

Species	Similarity (%)	Species	Similarity (%)
<i>Actinobacillus capsulatus</i>	92.3	<i>Haemophilus parainfluenzae</i>	92.7
<i>Actinobacillus delphinicola</i>	93.5	<i>Haemophilus paraphrohaemolyticus</i>	93.0
<i>Actinobacillus equuli</i>	92.3	<i>Haemophilus paraphrophilus</i>	91.7
<i>Actinobacillus hominis</i>	92.4	<i>Haemophilus parasuis</i>	92.6
<i>Actinobacillus indolicus</i>	92.6	<i>Haemophilus segnis</i>	92.4
<i>Actinobacillus ligniersii</i>	92.8	' <i>Haemophilus somnus</i> '	91.7
<i>Actinobacillus minor</i>	93.3	<i>Mannheimia haemolytica</i>	92.3
<i>Actinobacillus pleuropneumoniae</i>	92.7	<i>Pasteurella aerogenes</i>	93.0
<i>Actinobacillus porcinus</i>	93.9	<i>Pasteurella anatis</i>	90.2
<i>Actinobacillus scotiae</i>	93.0	<i>Pasteurella avium</i>	92.1
<i>Actinobacillus seminis</i>	92.5	<i>Pasteurella bettyae</i>	92.6
<i>Actinobacillus suis</i>	92.4	<i>Pasteurella canis</i>	92.4
<i>Haemophilus actinomycetemcomitans</i>	91.1	<i>Pasteurella dagmatis</i>	92.9
<i>Haemophilus aegyptius</i>	92.7	<i>Pasteurella gallinarum</i>	92.0
<i>Haemophilus aphrophilus</i>	91.8	<i>Pasteurella langaaensis</i>	92.3
<i>Haemophilus ducreyi</i>	94.6	<i>Pasteurella mairii</i>	92.6
<i>Haemophilus haemoglobinophilus</i>	92.5	<i>Pasteurella multocida</i>	93.0
<i>Haemophilus haemolyticus</i>	92.4	<i>Pasteurella pneumotropica</i>	92.5
<i>Haemophilus influenzae</i>	92.8	<i>Pasteurella stomatis</i>	92.9
<i>Haemophilus paracuniculus</i>	93.9	<i>Pasteurella testudinis</i>	91.9
<i>Haemophilus paragallinarum</i>	91.9	<i>Pasteurella trehalosi</i>	93.2
<i>Haemophilus paraahaemolyticus</i>	92.4	<i>Pasteurella volantium</i>	92.0

using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE (Felsenstein, 1989).

Visible colonies took 48 h to appear on CSBA incubated in 10% CO₂ at 37 °C. Colonies were circular, entire, low convex, smooth, grey and non-haemolytic. Microscopic examination showed cells consisted of pleomorphic Gram-negative rods. The bacterium was catalase-negative, oxidase-positive and non-motile. Growth was obtained under anaerobic conditions and in air without added CO₂ and also at 22 °C but not at 42 °C. Growth did not occur on Columbia agar without added blood or serum but a scanty growth did occur on tryptic soy agar without blood or serum. X and V factors did not produce growth. No growth occurred on MacConkey agar with or without NaCl. The strain was negative for urease and indole, arginine dihydrolase, ornithine decarboxylase and lysine decarboxylase. Nitrate was reduced to nitrite, but nitrite was not reduced. Acid was produced from glucose but not from adonitol, dulcitol, galactose, inositol, inulin, lactose, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose or xylose using a small-volume-heavy-inoculum method (Foster *et al.*, 1996). Voges-Proskauer and nitrate reduction were the only positive reactions observed with the API 20E and API 20NE systems, respectively. Using the API ZYM system strong positive reactions were obtained for alkaline phosphatase, esterase-lipase, acid phosphatase and naphthol-AS-BI-phosphohydrolase and weak

positive reactions were obtained with esterase and leucine arylamidase.

To investigate the phylogenetic relationships of the isolate, genes encoding 16S rRNA were amplified by PCR and subjected to direct sequence analysis. The almost complete 16S rRNA gene sequence of strain M1063U/93^T was determined. Sequence searches of the EMBL database revealed that the determined 16S rRNA sequence was most closely related (sequence similarity generally >91%) to those of members of the family *Pasteurellaceae*. The levels of 16S rRNA sequence relatedness with other Gram-negative taxa were significantly lower (data not shown). The 16S rRNA sequence of strain M1063U/93^T and those of reference *Actinobacillus* spp., *Haemophilus* spp., *Pasteurella* spp. and *Mannheimia* 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 M1063U/93^T within the family *Pasteurellaceae*. Levels of 16S rRNA sequence relatedness are shown in Table 1. Isolate M1063U/93^T formed a distinct subline within the *Pasteurellaceae*. Distance comparison indicated *Haemophilus ducreyi* was the closest genealogical relative of the porpoise uterine isolate, although a sequence divergence of >5% showed the affinity between these organisms was not particularly close. The distinction between the unknown isolate and *H. ducreyi* was reinforced by the treeing analysis. In the

Table 2. Differential characteristics of *Phocoenobacter uteri* gen. nov., sp. nov. and other genera within the *Pasteurellaceae*

Data from Angen *et al.* (1999) and Osawa *et al.* (1995). Abbreviations: +, only positive reactions occur; –, only negative reactions occur; d, positive or negative; NK, not known.

Test	<i>Phocoenobacter</i>	<i>Actinobacillus</i>	<i>Haemophilus</i>	<i>Pasteurella</i>	<i>Mannheimia</i>	<i>Lonepinella</i>
Haemolysis	–	d	d	–	d	–
Catalase	–	d	d	+*	+†	–
Oxidase	+	d	d	d	+‡	–
X and/or V-factor dependency	–	d	+§	–	–	–
Voges–Proskauer, 37 °C	+	–	–	–	–	+
Urease	–	+	d	d	–	–
Ornithine decarboxylase	–	–	d	d	d	–
Indole	–	–	d	+	–‡	–
Mannitol	–	d	–	d	+	–
D-Mannose	–	d	d	+	–	NK
D-Melibiose	–	d	–	–	–	d
meso-Inositol	–	–	–	–	d	–
D-Sorbitol	–	–¶	–	d	d	NK
Trehalose	–	d	–	d	–	NK

* Undescribed genomospecies designated *Pasteurella* sp. B reported to be catalase-negative.

† Result for *M. glucosida*, *M. ruminalis* and *M. varigena* not stated.

‡ Deviating strains occur.

§ *H. ducreyi* and *H. haemoglobinophilus* are reported to lack requirements for X and V factors.

|| *Pasteurella avium* indole-negative.

¶ Bisgaard taxon 9 late positive.

latter analysis the porpoise bacterium clustered, albeit loosely, with *Pasteurella testudinis*. The sequence divergence between these organisms was however >8%. Bootstrap resampling also showed the association between the unknown bacterium and *Pasteurella testudinis* was statistically not significant.

It is clear from the phylogenetic analysis that isolate M1063U/93^T from a harbour porpoise represents a hitherto unknown subline within the *Pasteurellaceae* Pohl (1981). Phylogenetically the bacterium does not show a particularly close affinity with any described species, displaying sequence divergence values of >5% and clearly represents a distinct species (Stackebrandt & Goebel, 1994). It has long been recognized that the taxonomy of the *Pasteurellaceae* is unsatisfactory. 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 new species to specific genera within the *Pasteurellaceae* has often proved to be problematic given the phylogenetic complexity of this family and the intermixing of species of its constituent genera. The *Pasteurellaceae* is thus in need of extensive taxonomic restructuring, consistent with phylogenetic knowledge. Some progress in this direction has been initiated with the proposal of a new genus, *Lonepinella* for a tannin-protein complex reducing bacterium (Osawa *et al.*, 1995). More recently a

new genus, *Mannheimia* has been created to accommodate the trehalose-negative [*Pasteurella*] *haemolytica* complex (Angen *et al.*, 1999). It is evident from the present phylogenetic analysis, that the unknown bacterium from a porpoise constitutes a new line within the *Pasteurellaceae*, equivalent in rank to *Lonepinella* and *Mannheimia*. Therefore, we propose the novel porpoise bacterium be classified in a new genus *Phocoenobacter*, as *Phocoenobacter uteri* sp. nov. The newly reported bacterium can be readily distinguished from currently described members of *Pasteurella*, *Haemophilus*, *Actinobacillus*, *Mannheimia* and *Lonepinella* by the characteristics shown in Table 2.

Description of *Phocoenobacter* gen. nov.

Phocoenobacter (Pho.coe.no.bac'ter. M.L. n. *phocoena* derived from Gr. n. *phokaina* porpoise; M.L. masc. n. *bacter* rod; M. L. masc. n. *phocoenobacter* a rod from a porpoise).

Cells consist of pleomorphic Gram-negative rods. Non-motile and facultatively anaerobic. Neither X nor V factors are required for growth. Does not grow on MacConkey agar. Catalase-negative and oxidase-positive. Acid is produced from glucose. Arginine dihydrolase, ornithine decarboxylase, lysine decarboxylase, urease and indole are negative. Nitrate is reduced to nitrite but nitrite is not reduced. Voges–

Proskauer-positive. The G + C content of the DNA is 41.5 mol%. The type species is *Phocoenobacter uteri*. The genus is a member of the family *Pasteurellaceae*.

Description of *Phocoenobacter uteri* sp. nov.

Phocoenobacter uteri (u'te.ri. L. masc. n. *uteris* uterus).

Cells consist of pleomorphic Gram-negative rods. Non-motile and facultatively anaerobic. Colonies on CSBA are circular, entire, low convex, smooth, grey and attain a diameter of 0.5 mm after 48 h aerobic incubation at 37 °C. Non-haemolytic. Growth is poor or absent on media without blood or serum. No growth on MacConkey agar. Growth occurs at 22 °C but not at 42 °C. Catalase-negative and oxidase-positive. Arginine dihydrolase, ornithine decarboxylase, lysine decarboxylase, urease and indole-negative. Nitrate is reduced to nitrite but nitrite is not reduced. Voges-Proskauer- and β -galactosidase-positive. Acid is produced from glucose but not from adonitol, dulcitol, galactose, inositol, inulin, lactose, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose or xylose. Alkaline phosphatase, esterase lipase, acid phosphatase and naphthol-AS-BI-phosphohydrolase-positive. Leucine arylamidase is weakly positive. The G + C content of the DNA is 41.5 mol%. Isolated from the uterus of a harbour porpoise. Pathological significance is unknown. The type strain is NCTC 12872^T (= M1063U/93^T).

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