NOTE

***Atopobacter phocae* gen. nov., sp. nov., a novel bacterium isolated from common seals**

Paul A. Lawson,¹ Geoffrey Foster,² Enevold Falsen,³ Maria Ohlén³ and Matthew D. Collins¹

Author for correspondence: Matthew D. Collins. Tel: +44 118 935 7000. Fax: +44 118 926 7917.
e-mail: m.d.collins@reading.ac.uk

¹ Department of Food Science and Technology, University of Reading, Reading RG6 6AP, UK

² SAC Veterinary Services, Inverness, UK

³ Culture Collection, Department of Clinical Bacteriology, University of Göteborg, Göteborg, Sweden

Two strains of a Gram-positive, catalase-negative, facultatively anaerobic, rod-shaped bacterium isolated from common seals were characterized using phenotypic and molecular taxonomic methods. The two strains closely resembled each other based on their biochemical characteristics, and PAGE analysis of whole-cell protein patterns confirmed their close phenotypic affinity. 16S rRNA gene sequencing showed that the two strains were genetically highly related (99.8% sequence similarity) and that they constitute a new line of descent within the lactic acid group of bacteria. The nearest phylogenetic neighbours of the unknown bacterium were *Granulicatella* spp., with related taxa such as enterococci, carnobacteria, *Desemzia incerta*, *Lactosphaera pasteurii*, *Melissococcus plutonius*, tetragenococci and vagococci more distantly related. Based on phylogenetic and phenotypic evidence it is proposed that the unknown bacterium from seals be classified in a new genus as *Atopobacter phocae* gen. nov., sp. nov. The type strain of *Atopobacter phocae* is CCUG 42358^T (= CIP 106392^T).

Keywords: *Atopobacter phocae*, 16S rRNA, taxonomy, phylogeny

The lactic acid bacteria constitute a phylogenetically heterogeneous assortment of Gram-positive, facultatively anaerobic, mostly catalase-negative, rod- or coccus-shaped organisms which belong to the *Clostridium* branch of the Gram-positive bacteria. The genus *Lactobacillus* constitutes the largest group of rod-shaped organisms within the lactic acid bacteria and currently over 50 species are recognized (Hammes *et al.*, 1992). Other rod-shaped genera within the lactic acid group of bacteria include *Carnobacterium* and *Desemzia*. The genus *Carnobacterium* was described to accommodate the species *Lactobacillus divergens* and *Lactobacillus piscicola* and some atypical lactobacilli which were isolated from refrigerated meats (Collins *et al.*, 1987). The genus *Carnobacterium* originally contained four species but two further species from Antarctic lakes have subsequently been added to the genus (Franzmann *et al.*, 1991). Recently, the genus *Desemzia* was described for the species previously designated *Brevibacterium incertum* (Stackebrandt *et al.*, 1999). Although both carnobacteria and *Desemzia incerta* consist of Gram-positive, rod-shaped

organisms, numerous studies have shown these taxa are only remotely related to lactobacilli, and in fact possess a closer phylogenetic affinity with several catalase-negative coccus-shaped organisms such as *Granulicatella* spp., enterococci, vagococci, tetragenococci, *Melissococcus plutonius* and *Lactosphaera pasteurii* (e.g. Collins & Lawson, 2000; Lawson *et al.*, 1999a,b; Stackebrandt *et al.*, 1999).

During the course of an investigation into the Gram-positive flora of aquatic mammals, we have isolated a hitherto unknown rod-shaped bacterium from two dead seals which displays a phylogenetic association with *Carnobacterium*, *Desemzia* and the aforementioned catalase-negative, coccus-shaped organisms. The unknown bacterium was readily distinguished from other described Gram-positive, catalase-negative organisms by biochemical tests and electrophoretic analysis of whole-cell proteins. Based on phylogenetic and phenotypic evidence it is proposed that the unidentified bacterium be classified as *Atopobacter phocae* gen. nov., sp. nov.

Strain M1590/94/2^T was isolated following a post-mortem examination of a common seal pup. The animal had general lymphadenopathy and acutely

The GenBank accession number for the 16S rRNA gene sequence of strain CCUG 42358^T is Y16546.

congested lungs with pulmonary haemorrhaging. The rod-shaped bacterium was recovered from the small intestine and from the mesenteric, external iliac, pre-scaphular and pancreatic lymph nodes. Additionally it was co-isolated with *Arcanobacterium phocae* from the lungs (Pascual Ramos *et al.*, 1997). Strain M955/98/1 was isolated from an adult common seal that had died from gun-shot wounds. It was isolated from a mixed culture with an unidentified *Actinomyces* sp. obtained from the liver, spleen and blood. All of the rod-shaped isolates recovered from these multiple sites of the respective seals were found to possess identical cellular and colonial morphologies and biochemical profiles. Strains M1590/94/2^T and M955/98/1 have been deposited in the Culture Collection of the University of Göteborg (CCUG) under accession numbers 42358^T and 42577, respectively.

Both strains were cultured on Columbia agar supplemented with 5% defibrinated horse blood (Oxoid Unipath) and incubated in air plus 5% CO₂ at 37 °C. The strains were biochemically characterized by using the API rapid ID32S and API ZYM systems according to the manufacturer's instructions (API bioMérieux). PAGE analysis of whole-cell proteins was performed as described by Pot *et al.* (1994). The GelCompar GCW 3.0 software package (Applied Maths) was used for densitometric analysis, normalization and interpretation of protein patterns. The cell wall murein structure of strain CCUG 42358^T was determined by the method of Schleifer & Kandler (1972) except that ascending TLC on cellulose sheets (Merck) was used. The 16S rRNA genes of the isolates were amplified by PCR and directly sequenced using a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems) and an automatic DNA sequencer (Applied Biosystems; model 373A). The closest known relatives of the new isolates were determined by performing database searches. These sequences and those of other known related strains were retrieved from the GenBank or Ribosomal Database Project (RDP) databases and aligned with the newly determined sequences using the program PILEUP (Devereux *et al.*, 1984). The resulting multiple sequence alignment was corrected manually and a distance matrix was calculated using the programs PRETTY and DNADIST (using the Kimura two-correction parameter) (Felsenstein, 1989). A phylogenetic tree was constructed according to the neighbour-joining method with the program NEIGHBOR (Felsenstein, 1989). The stability of the groupings was estimated by bootstrap analysis (500 replications) using the programs DNABOOT, DNADIST, NEIGHBOR and CONSENSE (Felsenstein, 1989). The GenBank accession number for the 16S rRNA gene sequence of strain CCUG 42358^T is Y16546.

The two isolates were Gram-positive, irregular-rod-shaped cells which formed smooth, grey-coloured, non-haemolytic, pin-sized colonies on Columbia agar supplemented with 5% defibrinated horse blood when incubated at 37 °C in air plus 5% CO₂ for 24 h. Growth was also observed without the addition of

blood to the basal medium exhibiting the same colony morphology as above. Both strains were facultatively anaerobic and catalase-negative. Growth was observed at 25 but not at 42 °C. Both strains produced acid from cyclodextrin, glucose, glycogen, maltose, pullulan and ribose, but failed to produce acid from D-arabitol, L-arabinose, mannitol, melibiose, melezitose, methyl β -D-glucopyranoside, raffinose, sorbitol, tagatose and D-xylose. The production of acid from lactose, sucrose and trehalose was variable. Both isolates showed acid phosphatase, alkaline phosphatase, arginine dihydrolase, esterase C-4 (weak reaction), ester lipase C-8 (weak reaction), pyroglutamic acid arylamidase (weak reaction), pyrazinamidase (weak reaction) and pyrrolidonyl arylamidase activity. Activity of alanine phenylalanine proline arylamidase and leucine arylamidase was either weak or negative. Neither of the strains however displayed activity for *N*-acetylglucosaminidase, chymotrypsin, cystine arylamidase, α -fucosidase, α -galactosidase, β -glucosidase, β -glucuronidase, glycine tryptophan arylamidase, α -mannosidase, β -mannosidase lipase C-14, trypsin or valine arylamidase. Neither of the strains hydrolysed aesculin, gelatin, hippurate or urease. They were Voges-Proskauer-negative and did not reduce nitrate.

PAGE analysis of whole-cell proteins confirmed the close phenotypic resemblance of the two bacterial seal isolates as they formed a tight cluster (approx. 78% similarity), which was separate from all other Gram-positive, catalase-negative reference organisms examined (see Fig. 1). Taxa recovered close to the unknown strains on PAGE analysis included *Tetragenococcus halophilus*, *Aerococcus viridans* and some *Leuconostoc* spp., although their association was not particularly close (joining the unknown bacterium cluster at less than 60% similarity). An examination of the cell wall of strain CCUG 42358^T revealed a murein based on L-Orn-D-Asp (type A4 β) (nomenclature of Schleifer & Kandler, 1972). Within the Gram-positive, catalase-negative, non-spore-forming, rod-shaped taxa this murein type is relatively uncommon, occurring in some bifidobacteria, *Lactobacillus fermentum*, *Lactobacillus pontis*, *Lactobacillus vaginalis* and *Lactobacillus uli*. Similarly, amongst coccus-shaped taxa this murein type is to our knowledge found only in *Granulicatella balaenopterae*, '*Peptostreptococcus glycinophilus*', *Sporosarcina halophila* and *Halobacillus* spp. It is pertinent to note that carnobacteria and *D. incerta*, the nearest rod-shaped phylogenetic relatives of the unknown seal bacterium, possess a directly cross-linked wall based on meso-diaminopimelic acid (type A1 γ) and a L-Lysine-D-Glu-type murein, respectively (Collins *et al.*, 1987; Stackebrandt *et al.*, 1999).

To investigate the genealogical affinity between the two isolates and their relationship with other Gram-positive, catalase-negative taxa, comparative 16S rRNA gene sequence analyses were performed. Pair-wise analysis of the almost complete gene sequence (> 1400 nucleotides) of the two isolates from seals were

Fig. 1. Similarity dendrogram based on whole-cell protein patterns of *Atopobacter phocae* sp. nov. and related species. Levels of correlation are expressed as percentages of similarity for convenience.

determined and revealed 99.8 % 16S rRNA similarity. This close genealogical affinity between the isolates was consistent with the results of whole-cell protein PAGE analysis and their close biochemical resem-

blance. Sequence searches of GenBank and RDP databases revealed the unknown isolates were phylogenetically most closely associated with the lactic acid group of bacteria, particularly *Granulicatella* spp.,

Fig. 2. Unrooted tree showing the phylogenetic relationships of *Atopobacter phocae* sp. nov. and some other low G+C content, Gram-positive bacteria. The tree, constructed using the neighbour-joining method, was based on a comparison of approximately 1320 nucleotides. Bootstrap values, expressed as a percentage of 500 replications, are given at branching points.

Lactosphaera pasteurii, *M. plutonius*, enterococci, carnobacteria, vagococci and tetragenococci (data not shown). A tree constructed by the neighbour-joining method depicting the phylogenetic affinity of the unknown rod as exemplified by strain CCUG 42358^T is shown in Fig. 2. The treeing analysis confirmed the association of the unknown bacterium with the lactic acid group of bacteria and showed that it represents a previously unknown line, close to but distinct from, *Granulicatella adiacens* and related species.

It is evident from the findings of the investigation that the two rod-shaped strains originating from seals represent a hitherto unrecognized bacterium within the low G+C content, Gram-positive, catalase-negative group of organisms. Phylogenetically, the rod-shaped bacterium shows a loose association with a cluster of coccus-shaped species which includes *G. adiacens*, *G. balaenopterae* and *Granulicatella elegans* (approx. 93.0–93.7% similarity) although bootstrap resampling showed this relationship is not statistically significant. Other taxa displaying a somewhat looser affinity with the unknown bacterium included the coccus-shaped *Lactosphaera pasteurii*, *M. plutonius*,

tetragenococci and vagococci, and the rod-shaped *D. incerta* and carnobacteria (Fig. 2). Based on tree topology considerations and sequence divergence values of 6% or greater with the aforementioned taxa, the unknown bacterium merits classification as a new genus. Chemotaxonomic data (whole-cell protein patterns and wall murein composition) reinforce the separateness of the unknown rod from all currently described Gram-positive, catalase-negative reference taxa. Therefore, based on both phenotypic and phylogenetic findings, we consider the unknown catalase-negative, Gram-positive rod from seals merits classification in a new genus, for which the name *Atopobacter phocae* gen. nov., sp. nov. is proposed. Characteristics which are useful in distinguishing *Atopobacter phocae* from its closest phylogenetic relatives and the rod-shaped carnobacteria and *D. incerta* are shown in Table 1.

Description of *Atopobacter* gen. nov.

Atopobacter (A.to.po.bac'ter. Gr. adj. *atopos* having no place, strange; M.L. masc. n. *bacter* rod; M.L. masc. n. *Atopobacter*, strange rod).

Table 1. Characteristics differentiating *Atopobacter phocae* sp. nov. from its nearest phylogenetic relatives and the rod-shaped carnobacteria and *D. incerta*

Data from Janssen *et al.* (1995), Stackebrandt *et al.* (1999) and this study. Biochemical tests were determined using API rapid ID32S and ZYM systems. v, Variable; w, weak; ND, not determined.

Characteristic	<i>Atopobacter phocae</i>	<i>G. adiacens</i>	<i>G. balaenopterae</i>	<i>G. elegans</i>	<i>D. incerta</i>	<i>C. alterfundium</i>	<i>C. divergens</i>	<i>C. funditum</i>	<i>C. gallinarum</i>	<i>C. mobile</i>	<i>C. piscicola</i>
Production of acid from:											
Cyclodextrin	+	—	—	—	—	—	+	—	—	—	+
Glycogen	+	—	—	—	—	—	—	—	—	—	—
Lactose	v	—	—	—	+	—	—	—	+	—	—
Pullulan	+	—	+	—	—	—	—	—	—	—	—
Ribose	+	—	—	—	—	—	+	—	+	—	+
Trehalose	v	—	+	—	+	+	+	w	+	+	+
Sucrose	v	v	—	+	+	w	+	w	+	+	+
Production of:											
Alkaline phosphatase	+	—	—	+	—	—	—	—	—	—	—
Arginine dihydrolase	+	—	+	+	—	—	+	—	+	+	+
β -Glucosidase	—	v	—	—	+	+	+	—	+	w	—
β -Galactosidase	v	—	—	—	+	—	—	—	—	—	—
β -Glucuronidase	—	v	—	—	+	+	+	+	+	+	+
Murein type	A4 β	A3 α	A4 β	ND	A4 α	A1 γ	A1 γ	A1 γ	A1 γ	A1 γ	A1 γ

Cells consist of Gram-positive, short, non-spore-forming, irregular rods. Non-haemolytic, small pin-sized, grey-coloured, smooth colonies are formed on horse blood agar after incubation at 37 °C for 24 h. Growth is observed without the addition of blood or serum to the basal medium. Facultatively anaerobic and catalase-negative. Growth is observed at 25 but not at 42 °C. Acid but no gas is produced from D-glucose. Acid phosphatase, arginine dihydrolase, alkaline phosphatase and pyrrolidonyl arylamidase are produced. Aesculin, gelatin, hippurate and urea are not hydrolysed. Nitrate is not reduced to nitrite. Voges–Proskauer-negative. The cell wall contains an L-Orn-D-Asp-type murein (A4 β). Isolated from dead common seals. Habitat is not known. The type species of the genus is *Atopobacter phocae*. As determined by 16S rRNA gene sequence analysis, the genus *Atopobacter* belongs to the lactic acid group of bacteria and is phylogenetically most closely related to *G. adiacens*, *G. balaenopterae* and *G. elegans*.

Description of *Atopobacter phocae* sp. nov.

Atopobacter phocae (pho'cae. N.L. gen. n. *phocae* of or pertaining to the common seal *Phoca vitulina*, from which the organism was isolated).

On blood agar cells are short, irregular rods that form small, smooth, grey, pin-sized colonies after 24 h incubation at 37 °C. Haemolysis is not observed. Cells stain Gram-positive. Facultatively anaerobic and catalase-negative. Growth is observed at 25 but not at 42 °C. Using commercial API systems, acid is produced from cyclodextrin, glucose, glycogen, maltose, pullulan and ribose, but not from D-arabitol, L-arabinose, mannitol, melibiose, melezitose, methyl β -D-glucopyranoside, raffinose, sorbitol, tagatose and D-xylose. Acid from lactose, sucrose and trehalose may

or may not be produced. Activity for acid phosphatase, alkaline phosphatase, arginine dihydrolase, esterase C-4 (weak reaction), ester lipase C-8 (weak reaction), pyroglutamic acid arylamidase (weak reaction), pyrazinamidase (weak reaction) and pyrrolidonyl arylamidase is detected. No activity for *N*-acetylglucosamidase, chymotrypsin, cystine arylamidase, α -fucosidase, α -galactosidase, β -glucosidase, β -glucuronidase, glycine tryptophan arylamidase, β -mannosidase, lipase C-14, trypsin or valine arylamidase is detected. Activity for β -galactosidase may or may not be detected and β -galacturonidase activity is either weak or negative. Aesculin, gelatin, hippurate and urease are not hydrolysed. Acetoin is not produced. Nitrate is not reduced to nitrite. The cell wall contains an L-Orn-D-Asp-based murein (type A4 β). Isolated from dead common seals. Habitat is not known. The type strain is CCUG 42358^T (= CIP 106392^T).

Acknowledgements

We are grateful to Professor Hans Trüper (University of Bonn, Germany) for help in coining the genus name. We also thank Anthony Patterson and Robert Reid of SAC Veterinary Science Division, Inverness. This work is part of an ongoing study of Scottish strandings and is conducted under contract to the UK Department of the Environment, Transport and the Regions as part of its co-ordinated programme of research on the North Sea.

References

- Collins, M. D. & Lawson, P. A. (2000). The genus *Abiotrophia* (Kawamura *et al.*) is not monophyletic: proposal of *Granulicatella* gen. nov., *Granulicatella adiacens* comb. nov., *Granulicatella elegans* comb. nov. and *Granulicatella balaenopterae* comb. nov. *Int J Syst Evol Microbiol* **50**, 365–369.

- Collins, M. D., Farrow, J. A. E., Phillips, B. A., Fersu, S. & Jones, D. (1987). Classification of *Lactobacillus divergens*, *Lactobacillus piscicola*, and some catalase-negative, asporogenous, rod-shaped bacteria from poultry in a new genus, *Carnobacterium*, *Int J Syst Bacteriol* **37**, 310–316.
- Devereux, J., Haeberli, P. & Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX, *Nucleic Acids Res* **12**, 387–395.
- Felsenstein, J. (1989). PHYLIP – phylogeny inference package (version 3.2), *Cladistics* **5**, 164–166.
- Franzmann, P. D., Höpfl, P., Weiss, N. & Tindall, B. J. (1991). Psychrophilic, lactic acid-producing bacteria from anoxic waters in Ace Lake, Antarctica; *Carnobacterium funditum* sp. nov. and *Carnobacterium alterfunditum* sp. nov., *Arch Microbiol* **156**, 255–262.
- Hammes, W. P., Weiss, N. & Holzapfel, W. (1992). The genera *Lactobacillus* and *Carnobacterium*. In *The Prokaryotes*, 2nd edn, pp. 1535–1594. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer.
- Janssen, P. H., Evers, S., Rainey, F. A., Weiss, N., Ludwig, W., Harfoot, C. G. & Schink, D. (1995). *Lactosphaera* gen. nov., a new genus of lactic acid bacteria, and transfer of *Ruminococcus pasteurii* Schink 1984 to *Lactosphaera pasteurii* comb. nov., *Int J Syst Bacteriol* **45**, 565–571.
- Lawson, P. A., Foster, G., Falsen, E., Sjöden, B. & Collins, M. D. (1999a). *Abiotrophia balaenopterae* sp. nov., isolated from the minke whale (*Balaenoptera acutorostrata*), *Int J Syst Bacteriol* **49**, 503–506.
- Lawson, P. A., Foster, G., Falsen, E., Ohlén, M. & Collins, M. D. (1999b). *Vagococcus lutrae* sp. nov., isolated from the common otter (*Lutra lutra*), *Int J Syst Bacteriol* **49**, 1251–1254.
- Pascual Ramos, C., Foster, G. & Collins, M. D. (1997). Phylogenetic analysis of the genus *Actinomyces* based on 16S rRNA gene sequences: description of *Arcanobacterium phocae* sp. nov., *Arcanobacterium bernardiae* comb. nov., and *Arcanobacterium pyogenes* comb. nov., *Int J Syst Bacteriol* **47**, 46–53.
- Pot, B., Vandamme, P. & Kersters, K. (1994). Analysis of electrophoretic whole-organism protein fingerprints. In *Modern Microbial Methods: Chemical Methods in Prokaryotic Systematics*, pp. 493–521. Edited by M. Goodfellow & A. G. O'Donnell. Chichester: Wiley.
- Schleifer, K. H. & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications, *Bacteriol Rev* **36**, 407–477.
- Stackebrandt, E., Schumann, P., Swiderski, J. & Weiss, N. (1999). Reclassification of *Brevibacterium incertum* (Breed 1953) as *Desemzia incerta* gen. nov., comb. nov., *Int J Syst Bacteriol* **49**, 185–188.