Otariodibacter oris gen. nov., sp. nov., a member of the family Pasteurellaceae isolated from the oral cavity of pinnipeds

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A total of 27 bacterial isolates from California sea lions and a walrus tentatively classified within the family Pasteurellaceae was further characterized by genotypic and phenotypic tests. Phylogenetic analysis of partial 16S rRNA and rpoB gene sequences showed that the isolates investigated formed a monophyletic group, tentatively designated Bisgaard taxon 57. According to 16S rRNA gene sequences, the most closely related species with a validly published name was Bisgaardia hudsonensis and the most closely related species based on rpoB sequence comparison was Pasteurella multocida subsp. multocida; highest similarities between the isolates and the type strains of B. hudsonensis and P. multocida subsp. multocida were 95.0 and 88.2%, respectively. All isolates of Bisgaard taxon 57 exhibit the phenotypic characters of the family Pasteurellaceae. Members of Bisgaard taxon 57 can be separated from existing genera of the Pasteurellaceae by the following tests: positive reactions for catalase, oxidase, Voges–Proskauer and indole; no X- or V-factor dependency; and acid production from L-arabinose (slow), L-fucose, maltose and trehalose, but not from dulcitol, d-mannitol, d-mannose or sucrose. The main fatty acids of Bisgaard taxon 57 (CCUG 59994T) are C14:0, C16:0, C16:1v7c and the summed feature C14:0 3-OH/iso-C15:0 i 1. This fatty acid profile is characteristic of members of the Pasteurellaceae. The quinone profile of Bisgaard taxon 57 (DSM 23800T) was similar to that of other genera in the Pasteurellaceae. The DNA G+C content of strain Baika1T is 36.2 mol%, which is at the lower end of the range for members of the family Pasteurellaceae. On the basis of both phylogenetic and phenotypic evidence, it is proposed that members of Bisgaard taxon 57 should be classified as representatives of a novel species in a new genus, Otariodibacter oris gen. nov., sp. nov. The type strain of Otariodibacter oris is Baika1T (=CCUG 59994T=DSM 23800T), which was isolated from the oral cavity of a healthy California sea lion in Copenhagen Zoo, Denmark, in 2007.

Members of the family Pasteurellaceae have been reported from diseased marine mammals, but may also be isolated from healthy individuals (Dunn et al., 2001; Higgins, 2000). Pasteurella multocida and Mannheimia haemolytica have both been obtained from septicaemia and cases of mortality in pinnipeds and cetaceans (Dunn et al., 2001; Howard et al., 1983; Sweeney, 1978; Sweeney & Ridgway, 1975). However, methods for identification were not stated in these reports and since M. haemolytica has often been misidentified (Angen et al., 1999; Collins et al., 1981), it is not unlikely that isolated bacteria belonged to other members of the Pasteurellaceae. Pasteurella species have been isolated from conjunctiva and wounds of stranded harbour seals (Phoca vitulina) (Lockwood et al., 2006). Within the family Pasteurellaceae, three species with validly published names, [Actinobacillus] delphinicola (Foster et al., 1996), [Actinobacillus] scotiae (Foster et al., 1998) and Phocoenobacter uteri (Foster et al., 2000), have been isolated.
exclusively from cetaceans. Attempts to isolate [Actinobacillus delphinicola from a large number of seals of different species (>100 individuals) have been unsuccessful (Foster et al., 1996). Recently, members of a new genus consisting of one species, Bisgaardia hudsonensis, and one genomospecies, Bisgaardia genomospecies 1, have been isolated from seals (Foster et al., 2011).

Pasteurellaceae have been associated with bite-wound infections in animals and man, often resulting in subsequent generalized infections (Holst et al., 1992; Smith et al., 2000). Such bite wounds associated with social aggression are well documented in both wild and captive marine mammals (Campagna, 2002; Chilvers et al., 2005; Scott et al., 2005). Nevertheless, only a few cases in the literature report on bacteria isolated from the oral cavity of marine mammals, and none of these are members of the family Pasteurellaceae (Baker et al., 1998; Madoff et al., 1991; Suer & Vedros, 1988). However, recently Bisgaardia hudsonensis has been isolated from a case of seal finger caused by a seal bite in a man (Sundeep & Cleeve, 2011). Finally, as an increasing number of people get exposed to potential zoonoses through contact with both wild and captive marine mammals during professional and leisure activities (Cowan et al., 2001; Hunt et al., 2008), knowledge about the bacterial flora of marine mammals becomes increasingly important from a medical perspective.

The aim of the present investigation was to characterize a collection of 27 Pasteurellaceae isolates obtained from the oral cavity of captive California sea lions and a walrus to extend our knowledge of the phenotypic and genotypic diversity and possible host adaptation of these taxa and to improve classification and identification of the taxa demonstrated.

BBL culture swabs (BD) were used for swabbing the gingival/dental fossa in the canine teeth area. All samples originated from clinical healthy animals, trained to accept the swabbing procedure. Animals investigated originated from two captive collections in Denmark and one in Canada.

Samples were obtained from a total of 14 California sea lions (Zalophus californianus) and six walruses (Odobenus rosmarus).

Swabs were kept in Stuart’s transport medium (BD) at ambient temperatures (4–20°C) for up to 24 h before they were plated on 5% bovine blood agar (BA) (Blood agar base, CM55; Oxoid). BA plates were incubated aerobically in sealed plastic bags for 24 h at 37°C. Three California sea lions were subsequently resampled and samples were plated on the Pasteurellaceae selective medium S-MBA (Jacobsen & Nielsen, 1995) and incubated as described above. Colonies typical of Pasteurellaceae were subsequently subcultured and characterized as previously described (Bisgaard et al., 1991). Phenotypic tests included Gram staining, motility, catalase, oxidase, phosphatase, Voges–Proskauer, ONPG, indole, urease, arginine dihydrolase, ornithine decarboxylase, lysine decarboxylase, nitrate reduction, gelatin hydrolysis, X-factor and V-factor dependency, growth in the presence of 10% CO2, growth under anaerobic conditions, growth at 20, 30 and 42°C, growth on MacConkey agar and acid production from adonitol, myo-inositol, L-arginine, L-arabinose, D-ribose, D-xyllose, dulcitol, L-fucose, D-galactose, D-glucose, L-fucose, D-galactose, D-glucose, myo-inositol, D-mannitol, D-mannose, melibiose, D-sorbitol, sucrose, L-rhamnose, inulin, lactose, maltose, raffinose, salicin, trehalose and glucose in Hugh and Leifson’s medium.

Pasteurella subsp. multocida NCTC 10322T was included as a reference. All isolates were characterized using the API 20E and API ZYM (both bioMérieux) systems according to the manufacturer’s instructions. McFarland 6 standard was used for inoculation.

The DNA G+C content was determined by the Deutsche Sammlung von Microorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany) as described previously (Garvie, 1978). Fatty acid analysis of Bisgaard taxon 57 strain CCUG 59994T was performed by the Culture Collection, University of Göteborg (CCUG, Göteborg, Sweden) (Eerola & Lehtonen, 1988; Sasser, 2001; Viallard et al., 1998). Analysis of respiratory quinones was carried out by the Identification Service of the DSMZ. Quinones were extracted as described by Tindall (1990a, b). Respiratory lipoquinones were separated into their different classes by TLC on silica gel (Macherey-Nagel), using hexane/tert-butylmethyl ether (9:1, v/v) as solvent. UV absorbing bands corresponding to the different quinone classes were removed from the plate and further analysed by HPLC. This step was carried out on an LDC Analytical (Thermo Separation Products) HPLC fitted with a reverse phase column (Macherey-Nagel; 2 × 25 mm, 3 µm, RP18) using methanol/heptane (9:1, v/v) as the eluant. Respiratory lipoquinones were detected at 269 nm.

An Easy-DNA kit (Invitrogen) was used for DNA preparation according to the manufacturer’s instructions.

The partial rpoB gene sequence of the 27 isolates (Table S1) was determined as reported previously (Korczak et al., 2004; Mollet et al., 1997) and covered the region 509–680 (positions refer to Escherichia coli K-12, accession no. U00096) of the deduced protein sequence, as reported previously (Allen et al., 2003; Korczak et al., 2004). Based on results from phylogenetic analysis of the rpoB sequences, a subset of seven isolates was selected for 16S rRNA sequencing as described by Angenot et al. (2002). Sequencing was performed by Macrogen (Seoul, Korea). The resulting sequences were compared to existing gene sequences in GenBank using BLAST (Altschul et al., 1997; Benson et al., 2007). Pair-wise comparisons were performed by the program WATER included in EMBoss (Rice et al., 2000).

Multiple alignment was performed by CLUSTAL_X (Thompson et al., 1997). Maximum-likelihood analysis including bootstrap analysis was performed by fastDNAML (Felsenstein, 1995; Olsen et al., 1994) on a Linux 7.2-compatible server.
The analysis was run with a transition/transversion ratio of 1.3 and 1.5 for rpoB and 16S rRNA, respectively.

Twenty-seven isolates formed a monophyletic group that was distantly related to other Pasteurellaceae (Fig. 1 and Fig. S1, available in IJSEM Online). The rpoB sequence similarity was 91.9–100 % within the group. 16S rRNA gene sequence comparisons showed 98.0–99.4 % similarity within the group. According to 16S rRNA gene sequence analysis, the most closely related species with a validly published name was B. hudsonensis and the most closely related species based on rpoB sequence comparison was P. multocida subsp. multocida; highest similarities between the isolates and the type strains of B. hudsonensis and P. multocida subsp. multocida were 95.0 and 88.2 %, respectively.

The 16S rRNA gene sequence similarities within the family Pasteurellaceae are more than 89 % and within a genus are usually more than 93 % (Korczaż & Kuhnert, 2008) but generally below 95 % (Christensen et al., 2007). Likewise, the lower similarity level of partial rpoB gene sequences for delimitating the family Pasteurellaceae has been suggested as 77 %, whereas 87 % similarity has been proposed for genera. Using these limits as criteria to define new genera of the Pasteurellaceae. Bisgaard taxon 57 is likely to represent a novel species in a new genus within the family of Pasteurellaceae.

All Bisgaard taxon 57 isolates were Gram-staining-negative, non-motile rods. All were catalase and oxidase-positive and produced acid fermentatively from glucose without gas in Hugh and Leifson’s medium. Extended phenotypic characterization was performed for seven strains: Splashsmaa, Fut3, Fut4, Baika1T, Pebblesgroenne, Docksmaagroenne and Kristina4 (Table 1). All seven strains had positive reactions for phosphatase, Voges–Proskauer, ONPG and indole. Nitrates were reduced to nitrites, but nitrites were not reduced. The strains did not require X- or V-factors and no growth occurred on MacConkey agar. Negative reactions were also obtained for gelatin hydrolysis, arginine dihydrolase, ornithine decarboxylase and lysine decarboxylase. Acid production with different carbohydrates, growth under various conditions and results obtained with the API ZYM system are stated in the genus and species descriptions.

The DNA G+C content of strain Baika1T was determined to 36.2 mol%. The DNA G+C content of strain Baika1T is one of the lowest reported for members of the family Pasteurellaceae.
Genera: 1, *Otariodibacter* gen. nov. *(this study)*; 2, *Haemophilus sensu stricto* (Kilian, 2005; Nørskov-Lauritsen et al., 2005; Winslow et al., 1917); 3, *Actinobacillus sensu stricto* (Brumpt, 1910; Christensen & Bisgaard, 2004); 4, *Lonepinella* (Osawa et al., 1995); 5, *Mannheimia* (Angen et al., 1999); 6, *Pasteurella sensu stricto* (Christensen & Bisgaard, 2003; Mutters et al., 1993; Trevisan, 1887); 7, *Phocoenobacter* (Foster et al., 2000); 8, *Gallibacterium* (Bisgaard et al., 2009); 9, *Volucribacter* (Christensen et al., 2004); 10, *Histophilus* (Angen et al., 2003); 11, *Avibacterium* (Blackall et al., 2005); 12, *Nicoletella* (Kuhnert et al., 2004); 13, *Bibersteinia* (Blackall et al., 2007); 14, *Aggregatibacter* (Nørskov-Lauritsen & Kilian, 2006; Patel et al., 2004); 15, *Basfia* (Kuhnert et al., 2010); 16, *Chelonobacter* (Gregersen et al., 2009); 17, *Necropsobacter* (Christensen et al., 2011); 18, *Bisgaardia* (Foster et al., 2011). +, Only positive reactions occur; −, only negative reactions occur; d, positive or negative; ND, no data available.

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*Not part of the formal genus description.
†Not stated if the fucose was D- and/or L-configuration.

*Pasteurellaceae*, ranging from 36–47 mol% for the genus *Actinobacillus* (Olsen & Möller, 2005) to 52.5 mol% for *Necropsobacter* (Christensen et al., 2011).

Fatty acid analysis of Bisgaard taxon 57 (Table S2) showed a fatty acid profile very similar to that found for nine genera of the *Pasteurellaceae*, with the main fatty acids being C₁₄:₀, C₁₆:₀, C₁₆:₁₀₋₁₀, and the summed feature C₁₄:₀ 3-OH/iso-C₁₆:₁. Surprisingly, the genera *Actinobacillus* sensu stricto, *Mannheimia*, *Haemophilus*, *Histophilus*, *Aggregatibacter* and *Bibersteinia* all have high amounts of quinones with a chain length of eight (Tables S3 and S4), whereas the genera *Actinobacillus sensu stricto*, *Mannheimia*, *Haemophilus*, *Histophilus*, *Aggregatibacter* and *Bibersteinia* all express high amounts of quinones with a chain length of seven.

Bisgaard taxon 57 was isolated from all the sampled California sea lions and a single walrus investigated, suggesting that Bisgaard taxon 57 may be adapted to the families *Otariidae* (sea lions and fur seals) and *Odobenidae* (walruses). However, further studies, including a higher number of walruses in particular and more species, are needed to support these indications.

It is evident from the phylogenetic analysis presented that Bisgaard taxon 57 represents a distinct genotypic lineage within the family *Pasteurellaceae*, which can be distinguished from currently described members of *Pasteurellaceae* by phenotypic characters shown in Table 1. Based on this, we propose that Otariodibacter taxon 57 represents a novel species within the family *Pasteurellaceae*.

### Description of Otariodibacter gen. nov.

*Otariodibacter [O.ta.ri.o.di.bac‘ter].* *Otarioidae* taxonomic name for the superfamily consisting of the families *Otariidae* (sea lions and fur seals) and *Odobenidae* (walruses); N.L. masc. n. *bacter* a rod; N.L. masc. n. *Otariodibacter* rod isolated from *Otarioidae*.
Non-motile, fermentative, Gram-negative rods that are catalase- and oxidase-positive. Positive reactions are obtained for Voges–Proskauer and indole. Do not require X- or V-factors. Acid is produced from L-arabinose (slow), L-fucose, malate and trehalose, but not from dulcitol, D-mannitol, D-mannose or sucrose. The main fatty acids are C\(_{14:0}\), C\(_{16:0}\), C\(_{16:1}\), isoC\(_{15:0}\) 3-OH/iso-C\(_{16:1}\); minor fatty acids are listed in Table S2. The type species is **Ototaridibacter oris**.

**Description of Ototaridibacter oris sp. nov.**

*Ototaridibacter oris* (o’ris. L. n. os oris mouth; L. gen. n. oris of/from mouth).

Colonies of the isolates grown on BA are circular, grey, translucent to opaque, convex, entire, smooth and pinpoint to 1 mm in diameter after 24 h of incubation at 37 °C. A very slight green discoloration of the agar is often apparent, particularly around colonies incubated for more than 48 h. In addition to the phenotypic characteristics given in the genus description, positive reactions are obtained from phoshatase and ONPG. Nitrates are reduced to nitrites, but nitrates are not reduced. Growth occurs in the presence of 10% CO\(_2\), under anaerobic conditions and at 20 °C, 30 °C and 42 °C. No growth occurs on MacConkey agar. Negative reactions are obtained for gelatin hydrolysis, arginine dihydrolase, ornithine decarboxylase and lysine decarboxylase. Acid is produced from D-glucose, but not from adonitol, amygdalin, D-xylose, D-galactose, meibiose, D-ribose, D-sorbitol, inulin, L-rhamnose, lactose, raffinose and salicin. Variable reactions are obtained for urease (strains Fut3, Pebblesgroenne and Docksmaagroenne are positive) and myo-inositol (strain Docksmaagroenne is negative). The type strain is negative for urease and myo-inositol. All isolates produce strong positive reactions for alkaline phosphatase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and \(\beta\)-galactosidase. Strong or weak positive reactions are produced for esterase, esterase lipase and valine arylamidase. The main quinones are ubiquinone-8, menaquinone-8 and demethylmenaquinone-8; ubiquinone-7 and -9 are also present.

The type strain is Baika\(_{1}\)\(^T\) (=CCUG 59994\(^T\) = DSM 23800\(^T\)), isolated from the oral cavity of a healthy California sea lion in Copenhagen Zoo, Denmark, in 2007. Other strains of this species were isolated from the gingival/dental fossa in healthy California sea lions and a walrus. The pathogenic potential is unknown. The DNA G+C content of strain Baika\(_{1}\)\(^T\) is 36.2 mol%.

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