Psychrobacter pulmonis sp. nov., isolated from the lungs of lambs

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Unusual Gram-negative, catalase- and oxidase-positive, coccus-shaped bacteria isolated from the lungs of two lambs were characterized by phenotypic and molecular-genetic methods. Comparative 16S rRNA gene sequencing studies demonstrated that the unknown isolates were genealogically highly related to each other (99·8% sequence similarity) and represent a novel subline within the genus Psychrobacter. The unknown bacterium was phylogenetically closely related to, but distinct from, Psychrobacter phenylpyruvicus, Psychrobacter immobilis, Psychrobacter glacincola and Psychrobacter urativorans. The novel Psychrobacter isolates were readily distinguished from all other Psychrobacter species and other Gram-negative, oxidase-positive bacteria usually responsible for lung infections in sheep by physiological and biochemical tests. Based on molecular-genetic and phenotypic evidence, it is proposed that the unknown Psychrobacter isolates from lambs be classified as Psychrobacter pulmonis sp. nov. The type strain is strain S-606T (= CECT 5989T = CCUG 46240T).

During the past two decades, there has been increasing awareness among clinical microbiologists of the importance of members of the family Moraxellaceae as emerging pathogens. Acinetobacter species are becoming increasingly important nosocomial pathogens and Moraxella species have been implicated in different human infections and are well-established pathogens of animals. Acinetobacter baumannii is the main species associated with outbreaks of nosocomial infection (Bergogne-Bérézin & Towner, 1996), but other Acinetobacter species have also been implicated in human infections (Chang et al., 2000; Kappstein et al., 2000; Ku et al., 2000). In veterinary medicine, A. baumannii is also considered a nosocomial pathogen for small animals (Boerlin et al., 2001; Francey et al., 2000) and Acinetobacter lwoffii has been associated with arthritis in pigeons (Duchatel et al., 2000). Various species of Moraxella, particularly Moraxella bovis, have traditionally been considered as animal pathogens, being responsible for eye and respiratory infections (Brown et al., 1998; Dubay et al., 2000; Lavin et al., 2000). In humans, Moraxella catarrhalis is of clinical relevance, being responsible for respiratory infections, endocarditis and meningitis, especially in immuno-compromised patients (Verduin et al., 2002); other species have also been implicated in human infections (Berrocal et al., 2001). Members of the genus Psychrobacter resemble Moraxella and Acinetobacter microscopically and are close phylogenetic relatives of these genera (Bowman et al., 1996). Psychrobacters are characteristically halotolerant and psychrophilic and have been isolated from diverse habitats such as the skin, gills and intestines of fish, sea water, cold-storage meat products and ornithogenic soils (Bowman et al., 1996, 1997a, b; Denner et al., 2001; González et al., 2000; Maruyama et al., 2000; Prieto et al., 1992). Little is known about the clinical significance of psychrobacters, although these organisms have been isolated from a variety of human sources such as brain tissue, eye, urethra, blood and cerebrospinal fluid (Hudson et al., 1987; Juni & Heym, 1986). Human infection by psychrobacters is very rare and only a limited number of case reports of infection by Psychrobacter immobilis and Psychrobacter phenylpyruvicus (previously Moraxella phenylpyruvica; Bowman et al., 1996) have been reported (Gini, 1990; Guttigoli & Zaman, 2000; Lloyd-Puryear et al., 1991; Lozano et al., 1994). P. phenylpyruvicus has been found in the urogenital and intestinal tracts of animals but, as far as we know, there have been no reports of the isolation of psychrobacters from animal clinical specimens. In this article, we report the phenotypic and phylogenetic characterization of two strains of an unusual Psychrobacter-like species isolated in pure...
culture from lung clinical specimens of two lambs. Based on the findings presented, a novel species of the genus *Psychrobacter, Psychrobacter pulmonis* sp. nov., is described.

Two bacterial strains (designated S-606T and S-1263) were isolated from the lungs of two different lambs. Strain S-606T was isolated from a 20-day-old lamb, whilst strain S-1263 was recovered from a 1-year-old lamb. In both cases, the only clinical sign observed was the sudden death of the animal. Symptoms of respiratory distress were observed only in the case of the 20-day-old lamb. Necropsy was done within 1 h of the death of the animals. The macroscopic lesions observed at the necropsy were a clear congestion in the lungs of both animals. No other macroscopic lesions were observed. Samples of lung were taken for microbiological analysis under aseptic conditions to avoid environmental contamination and kept under refrigeration until processed in the laboratory. Clinical specimens were cultured on Columbia blood agar (bioMérieux) at 37 °C and incubated under aerobic and anaerobic conditions. Incubation was extended to 48 h. Strains were isolated in pure culture from both lungs. Oxidase activity was tested with oxidase test sticks (Oxoid). The strains were characterized biochemically by using the API 20E, API 20NE and API ZYM systems according to the manufacturer’s instructions (bioMérieux). Phylogenetic characterization was performed using 16S rRNA gene sequencing. A large fragment (approx. 1450 bases) of the 16S rRNA genes of both isolates was amplified by PCR and sequenced directly using a *Tag DyeDeoxy* terminator cycle sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 373A; Applied Biosystems). The closest known relatives of the novel isolates were determined by performing database searches of the GenBank and Ribosomal Database Project libraries. 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 genotypic relatedness of the two clinical isolates was also assessed by DNA–DNA hybridization. DNA–DNA reassociation experiments were carried out according to the spectrophotometric method of De Ley et al. (1970), with the modification described by Escara & Hutton (1980) and Huß et al. (1983), using a Gilford System model 2600 spectrophotometer equipped with a Gilford model 2527-R thermal programmer. Renaturation rates were computed with the program TRANSFER.BAS (Jahnke, 1992).

Both strains grew on Columbia blood agar at 37 °C under aerobic conditions, forming non-pigmented, smooth colonies. Neither strain grew under anaerobic conditions. The two isolates consisted of Gram-negative, non-motile, coccus-shaped cells that were catalase- and oxidase-positive. Both strains produced growth in 6·5 % NaCl but failed to grow on MacConkey agar. These phenotypic characteristics are consistent with those of the genus *Psychrobacter* (Juni & Heym, 1986), but neither of the strains was identified using the commercial identification systems. Using the miniaturized kits, the two isolates displayed identical phenotypic profiles. The strains produced acetoin and reduced nitrate. Neither of the isolates produced arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, β-galactosidase or tryptophan deaminase. They failed to hydrolyse urea, gelatin and aesculin and did not produce indole or H2S. Neither of the isolates produced acid from glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin or arabinose. Citrate was not utilized. Assimilation of glucose, arabinose, mannose, mannitol, maltose, gluconate, caprate, adipate, malate, phenylacetate and citrate was not observed. Using the API ZYM system, only esterase C-4, ester lipase C8, leucine arylamidase, valine arylamidase and cystine arylamidase were detected. All other tests were negative.

To establish the phylogenetic affinities of the clinical isolates, their 16S RNA gene sequences were determined and subjected to a comparative analysis. The almost-complete sequences of the two strains were determined and pair-wise analysis revealed that they were almost identical (99-8 % sequence similarity), thereby demonstrating that the strains were phylogenetically very closely related. Searches of the GenBank and Ribosomal Database Project libraries revealed that the unknown cocci were phylogenetically most closely related to the genus *Psychrobacter* (results not shown). Highest sequence relatedness was shown to *P. phenylpyruvicus* (95·8 %), *Psychrobacter glacina* (97·4 %), *Psychrobacter urativorans* (95·8 %) and *P. immobilis* (96·1 %). Treeing analysis confirmed this affinity and a dendrogram depicting the phylogenetic relationships of the unidentified cocci [as exemplified by strain S-606T (= CECT 5989T)] within the genus *Psychrobacter* is shown in Fig. 1. To investigate the genetic relationship between the two clinical isolates further, chromosomal DNA–DNA reassociation was performed. The two isolates were found to display 71 % DNA relatedness to each other. Reassociation values between the two clinical strains (S-606T and S-1263) and the type strain of *P. immobilis* (DSM 7229T) used for comparison were respectively 36 and 38 %.

It is clear from the polyphasic taxonomic study that the two clinical isolates represent a distinct species within the genus *Psychrobacter*. Chromosomal DNA renaturation clearly showed that the two strains are members of a single genetic species and 16S rRNA sequencing demonstrated that the isolates form a hitherto unknown subline within the genus *Psychrobacter* (Fig. 1). The novel lamb isolates displayed 2·6 % or greater sequence divergence from validated *Psychrobacter* species, which is strongly indicative of a separate species. Furthermore, the novel lamb bacterium is also phenotypically very different from all described *Psychrobacter* species. In particular, the novel bacterium can be readily distinguished from its closest phylogenetic relative, *P. glacina*, by growing at 37 °C, producing cystine...
arylamidase and valine arylamidase, failing to produce lipase C14 and naphthol-AS-BI phosphohydrolase and by not using citrate or caproate as a sole energy and carbon source. *P. glacincola* shows the opposite characteristics. Tests that serve to distinguish the novel lamb bacterium from all validly described *Psychrobacter* species are shown in Table 1. It is pertinent to note that the bacterium isolated from congestive lungs in lambs can also be differentiated easily from other Gram-negative, catalase- and oxidase-positive bacteria commonly isolated from pulmonary infections in small ruminants, such as *Pasteurella haemolytica*, *Pasteurella multocida* and *Bordetella bronchiseptica* (Quinn et al., 1999a, b): *Pasteurella haemolytica* and *Pasteurella multocida* are facultatively anaerobic and produce acid from glucose and sucrose (Quinn et al., 1999a) and *B. bronchiseptica* grows on MacConkey agar and is urease-positive (Quinn et al., 1999b). Additionally, the lamb isolates are able to grow at 4°C, which is consistent with the description of the genus *Psychrobacter* (Juni & Heym, 1986; Bowman et al., 1996). This trait is useful in distinguishing the isolates from strains of the genus *Moraxella* (Bøvre, 1986). Therefore, based on both phenotypic and molecular-genetic considerations, we consider that the two unidentified coccus-shaped organisms from lambs merit classification as a novel species of the genus *Psychrobacter*, for which the name *Psychrobacter pulmonis* sp. nov. is proposed. Although the isolates could not be implicated directly in the deaths of the animals, the facts that both strains were isolated in pure culture and that

![Fig. 1. Unrooted tree based on 16S rRNA showing the phylogenetic relationships of *Psychrobacter pulmonis* sp. nov. Bootstrap values (expressed as percentages of 500 replications) are given at branching points. Bar, 1 % sequence divergence.](http://ijs.sgmjournals.org)

**Table 1. Characteristics useful in differentiating *P. pulmonis* sp. nov. from other *Psychrobacter* species**

<table>
<thead>
<tr>
<th>Test</th>
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<th>5</th>
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<tbody>
<tr>
<td>Growth at 35°C</td>
<td>+</td>
<td>V+</td>
<td>+</td>
<td>–</td>
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<td>+</td>
<td>–</td>
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<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>V+</td>
<td>–</td>
<td>–</td>
<td>(+)</td>
<td>V–</td>
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<td>Acid production from:</td>
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<td>Glucose</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<td>Rhamnose</td>
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<td>ND</td>
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<td>Arabinose</td>
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<td>+</td>
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<td>Production of:</td>
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<tr>
<td>Urease</td>
<td>–</td>
<td>+</td>
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<td>V–</td>
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<td>+</td>
<td>V+</td>
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<td>Esterase C-4</td>
<td>+</td>
<td>V+</td>
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<td>Lipase C14</td>
<td>–</td>
<td>V+</td>
<td>ND</td>
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<td>Valine arylamidase</td>
<td>+</td>
<td>–</td>
<td>ND</td>
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<td>Cystine arylamidase</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>–</td>
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<td>Alkaline phosphatase</td>
<td>–</td>
<td>V+</td>
<td>(V–)</td>
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<td>Acid phosphatase</td>
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<td>Naphthol-AS-BI phosphohydrolase</td>
<td>–</td>
<td>+</td>
<td>ND</td>
<td>(+)</td>
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<td>Use as sole carbon and energy source:</td>
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<td>Citrate</td>
<td>–</td>
<td>V–</td>
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<td>+</td>
<td>–</td>
<td>–</td>
<td>(+)</td>
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<tr>
<td>Caproate</td>
<td>–</td>
<td>+</td>
<td>(–)</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>(+)</td>
<td>–</td>
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<tr>
<td>Malate</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>V+</td>
<td>–</td>
</tr>
</tbody>
</table>

Species: 1, *P. pulmonis* sp. nov.; 2, *P. immobilis*; 3, *P. pacificensis*; 4, *P. glacincola*; 5, *P. frigidicola*; 6, *P. phenylpyruvicus*; 7, *P. urativorans*. Test results are scored as: +, positive; –, negative; V+, most strains positive; V–, most strains negative; ND, not determined. Data were taken from the present study and from Bowman et al. (1996, 1997a), Denner et al. (2001), Juni & Heym (1986) and Maruyama et al. (2000). Results in parentheses differ from those obtained in the present study and were taken from Denner et al. (2001).
numerous colonies were present at primary isolation are strongly indicative of their clinical significance. This report is the first isolation of a member of *Psychrobacter* from animal clinical specimens. We consider that the formal description of this novel species will facilitate its identification in the clinical laboratory, thereby permitting the future evaluation of its distribution, clinical prevalence and significance.

**Description of *Psychrobacter pulmonis* sp. nov.**

*Psychrobacter pulmonis* (pul.mo’nis. L. gen. n. *pulmonis* of the lung).

Cells are Gram-negative, non-motile and coccus-shaped. Catalase- and oxidase-positive and strictly aerobic. Grows at 30 and 37 °C but not at 42 °C. Grows in 6-7.5% NaCl broth at 37°C. Growth does not occur on MacConkey agar. Aesculin, gelatin and urea are not hydrolysed. Indole is not produced. Acetoin is produced. Nitrate is reduced. Aesculin, gelatin and urea are not hydrolysed. Indole is produced in adults. Infection rates.

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**References**


