Revised description and classification of atypical isolates of *Pasteurella multocida* from bovine lungs based on genotypic characterization to include variants previously classified as biovar 2 of *Pasteurella canis* and *Pasteurella avium*

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Strains deviating in key phenotypic characters, mainly isolated from cases of bovine pneumonia in five European countries, were genotyped in order to examine their genotypic relationship with *Pasteurella multocida*. Twenty-two strains of *Pasteurella avium* biovar 2, including variants in indole, xylose and mannitol, 18 strains of *Pasteurella canis* biovar 2 and variants of this taxon, five strains of *P. multocida* subsp. *septica* showing variations in indole and ornithine decarboxylase, nine strains of *P. multocida* subsp. *multocida* showing variation in ornithine decarboxylase and mannitol, and type strains of the subspecies of *P. multocida* were included. Ribotyping was used to examine the relationship of the strains, and 13 types, each containing between one and 20 isolates, were observed. Identical ribotypes were observed in some cases for *P. avium* biovar 2 and either *P. canis* biovar 2 or *P. multocida* subsp. *septica*. ITS (16S–23S rRNA internal transcribed spacer) fragment-length profiling showed identity of the majority of strains (47 of 52), representing all four taxa, with only five divergent strains. A 16S rRNA sequence comparison of 11 strains representing the main ribotype clusters showed 99–99% similarity to the type strain of *P. multocida* subsp. *multocida*, but only 97.4% similarity was obtained to *P. canis* (biovar 1) and 93.7% to *P. avium* (biovar 1). A species-specific PCR test for *P. multocida* gave a positive result with biovar 2 variants of *P. avium* and *P. canis*. DNA–DNA hybridizations between strains of *P. multocida*, biovar 2 variants of *P. avium* and *P. canis*, and *P. multocida* subsp. *septica* confirmed similarity at the species level. It is proposed, on the basis of genotypic similarity, that *P. multocida* be reclassified to include the biovar 2 variants of *P. avium* and *P. canis* and that the existence of the biovar 2 variants of *P. avium* and *P. canis* is highly questionable. It is concluded that the redefined *P. multocida* is genotypically homogeneous, although phenotypically diverse lineages exist with respect to ornithine decarboxylase, indole and mannitol, characters that have been regarded as essential for identification to the species level. A formal reclassification of the species is not possible, however, since too few strains have been found to vary in these key characters. Considering the phenotypic diversity of *P. multocida*, identification will have to depend partly on genotypic methods and the source host also seems important for safe diagnosis.

INTRODUCTION

*Pasteurella multocida* has been isolated worldwide from mammals and birds in association with various disease conditions. *P. multocida* subsp. *multocida* has been reported to have a broad host spectrum compared to *Pasteurella multocida* subsp. *gallicida*, most frequently isolated from birds, and *Pasteurella multocida* subsp. *septica*, isolated mostly from cats and dogs (Christensen & Bisgaard, 2003).
The present study has been focused on the correct identification of *P. multocida* subsp. *multocida* associated with bovine pneumonia. The clinical and diagnostic implications of *P. multocida* in bovine pneumonia have been reviewed by Frank (1989).

Significant variations in the phenotypic properties of *P. multocida* have been reported (Heddleston, 1976), leading to confusion in the definition and identification of this organism. Based upon DNA hybridization studies, the genus *Pasteurella* was reclassified by Mutters et al. (1985b). DNA-binding data for *P. multocida* identified three clusters, showing 84–100 %, 91–100 % and 89–100 % DNA binding between strains subsequently described as *P. multocida* subsp. *multocida*, subsp. *gallicida* and subsp. *septica*, respectively. However, DNA binding as low as 55 % between subsp. *multocida* and *septica*, 67 % between subsp. *septica* and *gallicida* and 77 % between subsp. *gallicida* and *multocida* was observed (Mutters et al., 1985b). The low levels of DNA binding observed between the three groups would have allowed separation at the species level; however, for clinical and epidemiological purposes, the species status of *P. multocida* was maintained (Mutters et al., 1985b). Multilocus enzyme electrophoresis and ribotyping subsequently showed a close relation between the type strains of *P. multocida* subsp. *multocida* and *gallicida*, whereas subsp. *septica* was distantly related to these taxa (Blackall et al., 1998). These results were confirmed by 16S rRNA sequence comparison (Boerlin et al., 2000; Petersen et al., 2001).

*P. multocida* has been separated from other members of the genus *Pasteurella* mainly by positive reactions for ornithine decarboxylase, indole and D-mannitol, and negative reactions for maltose and dextrin, while the subspecies of *P. multocida* can be separated by differences in acid production from D-sorbitol and dulcitol (Table 1). Variations in phenotypes within the subspecies of *P. multocida* were subsequently reported by Fegan et al. (1995), who assigned five biotypes to *P. multocida* subsp. *multocida*, two to *P. multocida* subsp. *septica*, and one to *P. multocida* subsp. *gallicida*, but two other biotypes could not be allocated to any of the recognized subspecies of *P. multocida*. The study of Blackall et al. (1998) showed by genotyping that these biovars were indeed related to *P. multocida*, although they could not be related to any recognized subspecies by phenotypic criteria. The study of Petersen et al. (2001) showed a great diversity of ribotypes among strains classified as *P. multocida* subsp. *multocida*, subsp. *gallicida* and subsp. *septica*. However, 16S rRNA and atpD gene (encoding the β subunit of ATP synthase) sequence comparisons confirmed the homogeneity of *P. multocida*. The study of Kuhnert et al. (2000) also showed that variant phenotypes of *P. multocida* shared at least 98.5 % 16S rRNA sequence similarity with the recognized subspecies of this species.

*P. avium* and *P. canis* were reported as new species by Mutters et al. (1985a, b). *P. canis* (formerly Frederiksen birov 6 of *P. multocida*) can be separated from *P. multocida* by a negative reaction for D-mannitol. At least 80 % DNA binding was observed within *P. canis*, and the closest level of DNA binding with other species was with *P. avium* strain K117 (69 %), originally reported as taxon 13 of Bisgaard (Mutters et al., 1985b). Three characters separate *P. avium* from *P. multocida* (Table 1) (Christensen & Bisgaard, 2003). DNA binding within *P. avium* was at least 88 % and the highest DNA binding with other species was to *P. stomatis*, at 81 % (Mutters et al., 1985b). *P. avium* and *P. canis* were both separated into two biovars. *P. canis* biovar 1 is indole positive and of canine origin, whereas biovar 2 is indole negative and of bovine origin (Mutters et al., 1985b). DNA binding of 80 % was observed between

<table>
<thead>
<tr>
<th></th>
<th>V-factor (NAD)</th>
<th>Ornithine decarboxylase</th>
<th>Indole</th>
<th>β-Xylose</th>
<th>D-Mannitol</th>
<th>Dulcitol</th>
<th>D-Sorbitol</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. multocida</em> subsp. <em>multocida</em></td>
<td>−</td>
<td>+*</td>
<td>+*</td>
<td>v</td>
<td>+*</td>
<td>−</td>
<td>+</td>
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<td>−</td>
<td>+</td>
<td>+</td>
<td>v</td>
<td>+*</td>
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<td>+</td>
</tr>
<tr>
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<td>−</td>
<td>+*</td>
<td>+*</td>
<td>v</td>
<td>+</td>
<td>−</td>
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<tr>
<td>Bisgaard taxon 16</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
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<td>−</td>
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<tr>
<td><em>P. canis</em> biovar 1 (canine)</td>
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<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td><em>P. canis</em> biovar 2 (bovine)</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>v*</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td><em>P. stomatis</em></td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
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<td>−</td>
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<tr>
<td><em>P. avium</em> biovar 1 (avian)</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>v</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td><em>P. avium</em> biovar 2 (bovine)</td>
<td>−</td>
<td>−</td>
<td>−*</td>
<td>v</td>
<td>−*</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Gallibacterium (<em>P. anatis</em>)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
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</table>

*Variant phenotypes of the present investigation.*
biovar 1 (type strain) and biovar 2 (strain K267) of \textit{P. canis} (Mutters et al., 1985b). \textit{P. avium} was separated into two biovars based on V-factor requirement, biovar 1 being V-factor dependent (Mutters et al., 1985a). Biovar 1 strains have been isolated from chickens and biovar 2 from cattle (Mutters et al., 1985a). DNA–DNA hybridization experiments showed 88% binding between the type strain of \textit{P. avium} (biovar 1) and strain K117 of biovar 2 (Mutters et al., 1985a). Strains of the biovar 2 variants of \textit{P. canis} and \textit{P. avium} were both originally outlined on the basis of the unnamed taxon 13 of Bisgaard (Madsen et al., 1985, 1985a, b). Crossed immunoelectrophoresis subsequently indicated a similarity between \textit{P. multocida} and the biovar 2 strains of \textit{P. canis} and \textit{P. avium} (Schmid et al., 1991).

Since, as stated above, routine characterization and identification of the redefined taxa of \textit{Pasteurella} presently rest on a single or a few phenotypic characters, and strains aberrant for these characters have been reported (Bisgaard et al., 1991a, b), specific genetic tools or improved phenotypic tests are urgently needed to improve diagnostic methods within this area of bacteriology, which is of importance for both the veterinary and medical professions.

PCR tests specific for the detection of \textit{P. multocida} have been reviewed (Christensen et al., 2003a). \textit{P. multocida} can be detected by a PCR test targeting the 23S rRNA gene (Miflin & Blackall, 2001) or an unknown gene (Townsend et al., 1998). Specific detection is also possible based on the \textit{psl} gene combined with hybridization (Kasten et al., 1997). Problems with the non-specificity of PCR and \textit{in situ} hybridization tests for \textit{P. multocida} have recently been reported for biovar 2 of \textit{P. avium} and biovar 2 of \textit{P. canis} (Mbuthia et al., 2001; Miflin & Blackall, 2001; Townsend et al., 2001).

In the present study, genotypic methods were used to evaluate key characters employed for phenotypic characterization and identification of \textit{P. multocida}. The study has focused on phenotypically variant strains of \textit{P. multocida} and related taxa, mainly isolated from cases of bovine pneumonia. The study has aimed at a more strict definition of \textit{P. multocida}, leading to improved identification and consequently better understanding of epidemiology and clinical implications.

**METHODS**

**Selection of bacterial strains and phenotypic characterization.** A total of 61 bacterial strains were investigated, including reference strains and strains previously described (Bisgaard et al., 1991a, b; Madsen et al., 1985) (Table 2). The strains were selected to represent the phenotypic and geographical diversity of bacteria characterized as \textit{P. multocida}, biovar 2 of \textit{P. canis} and biovar 2 of \textit{P. avium}. Phenotypic characterization was performed as previously reported (Bisgaard et al., 1991a).

**Ribotyping.** Ribotyping of 55 strains, including reference strains, was done as described by Christensen et al. (1993). Briefly, 6 μg DNA was digested with \textit{HpaII} at 37 °C for 2 h. Digests were separated on 0-8% agarose gel for 16 h at 35 V. The gel was stained with ethidium bromide and the DNA vacuum-blotted onto a nitrocellulose filter. The filter was hybridized with a 16S–23S rRNA probe overnight at 56 °C. Ribotype patterns were observed using the DIG wash and block buffer kit (Roche), according to the manufacturer’s instructions. \(\lambda\)-DNA digested with HindIII was included as size marker. Ribotype patterns were analysed by Gelcompar 4.0 (Applied Maths) and Dice-coefficients used to construct a neighbour-joining dendrogram.

**The 16S–23S rRNA internal transcribed spacer (ITS).** ITS was amplified for 58 strains, including reference strains, by three nested primer-sets, as recently described (Christensen et al., 2003b). Fragments generated with the three primer-sets were respectively 149, 189 and 337 bp longer than the 16S–23S ITS. PCR amplification and denaturing PAGE were performed as described in Christensen et al. (1999). Mean lengths of ITS fragments were calculated for each strain.

**PCR.** The procedure of Miflin & Blackall (2001) was followed. Briefly, a loopful of an overnight culture was taken from the surface of a blood agar plate and suspended in 200 μl sterile water. The suspension was boiled for 10 min, the cells were spun down at 13 000 g for 2 min and the supernatant was used as template for PCR. PCR was performed in a total volume of 50 μl, containing 10 mM Tris/HCl (pH 8.3), 3·0 mM MgCl\(_2\), 50 mM KCl, 200 μM of each dNTP, 25 pmol of each of the primers PM23F1 (5’-ggc tgg gaa gcc aaa tca aag-3’) and PM23R2 (5’-cga ggg act aca att gta-3’), 1·25 U Taq polymerase (Perkin-Elmer) and 1 μl lysate. DNA was amplified for 30 cycles, using the following settings: denaturation at 94 °C for 1 min, annealing at 61 °C for 1 min and extension at 72 °C for 1 min, followed by 10 °C. After electrophoresis and ethidium bromide staining, an amplicon of about 1400 bp could be visualized under UV light in positive strains.

Capsular type was determined by the PCR method of Townsend et al. (2001).

**Sequencing of 16S rRNA genes.** The 16S rRNA gene sequence was determined for 11 strains: 160, 214, RA12/2, 5, A284/86, A285/86, 25, X225, K323, W208 and 16 (Table 2). Bacteria were cultured overnight in brain heart infusion broth (Difco) at 37 °C. PCR-amplified fragments were purified and cycle sequenced, as recently reported (Christensen et al., 2002). Searches for 16S rRNA sequences were performed by FastA and BLAST in GenBank by the Wisconsin Sequence Analysis Package (GCG). Pairwise similarities were calculated by Bestfit (GCG).

**DNA–DNA hybridization.** DNA binding was investigated by the micro-well method (Christensen et al., 2000) for 11 strains, including type strains. To represent pheno- and genotypic diversity, DNA preparations were only used if they were of high concentration (> 150 μg ml\(^{-1}\)). To limit the amount of capsular material formed, cells were cultured in brain heart infusion broth with 1 mM EDDA (ethylenediamine-N,N’-diacetic acid; Sigma-Aldrich) (Ogunnariwo & Schyvers, 1990) and 1 mg hyaluronidase ml\(^{-1}\) (from bovine testes; Sigma-Aldrich), since the capsular material of type A is susceptible to hyaluronidase treatment (Rimler, 1994).

**GenBank accession numbers.** The 16S rRNA gene sequences of strains 5, 214, RA12/2, W208 and X225 were deposited with GenBank under the accession numbers AY316314, AY316315, AY316316, AY316317 and AY507110, respectively. The sequence of strain 5 was identical to that of strains 25, A285/86 and K323, the sequence of strain W208 was identical to that of strains 16 and A284/86 and the sequence of strain 160 was identical to that of strain RA12/2.
Table 2. List of strains used for investigation of *P. multocida*

Superscripts denote the following earlier investigations: Ba, Bisgaard *et al.* (1991a); M, Madsen *et al.* (1985); Bb, Bisgaard *et al.* (1991b); P, Petersen *et al.* (2001); R, reference strain. +, Positive reaction in the *P. multocida*-specific PCR of Miflin & Blackall (2001); −, negative reaction; ND, not done.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Country of isolation</th>
<th>Animal, disease and organ</th>
<th>Phenotypic variant character</th>
<th>Capsular type</th>
<th>Ribotype</th>
<th>PCR</th>
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<tr>
<td><strong>Reference strains</strong></td>
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<td>NCTC 10322&lt;sup&gt;T&lt;/sup&gt; (＝ATCC 43137&lt;sup&gt;T&lt;/sup&gt;)†</td>
<td></td>
<td>A 10 +</td>
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<td>CIP A125&lt;sup&gt;T&lt;/sup&gt; (＝NCTC 11995&lt;sup&gt;T&lt;/sup&gt;)†</td>
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<td><em>P. canis</em></td>
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<td></td>
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<td><em>P. gallinarum</em></td>
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<td></td>
<td>ND ND ND</td>
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<td><strong>Strains now classified with <em>P. multocida</em></strong></td>
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<td>Calf pneumonia</td>
<td>Mannitol negative</td>
<td>A 3 +</td>
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<tr>
<td></td>
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<td>164&lt;sup&gt;Ba&lt;/sup&gt;</td>
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<td>DK</td>
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<td>F ND +</td>
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<td>Calf pneumonia</td>
<td>Xylose negative</td>
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<td>Xylose negative</td>
<td>A 5 +</td>
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<td><em>P. avium</em> biovar 2</td>
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<td>Calf pneumonia</td>
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<td>A 5 +</td>
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<td>A 5 +</td>
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<td></td>
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<td><em>P. avium</em> biovar 2</td>
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<td>UK</td>
<td>Source unknown</td>
<td>Late indole positive, xylose negative</td>
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<td></td>
</tr>
<tr>
<td><em>P. avium</em> biovar 2</td>
<td>79</td>
<td>B</td>
<td>Bovine lung</td>
<td>Xylose negative</td>
<td>A 5 +</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. avium</em> biovar 2</td>
<td>85</td>
<td>B</td>
<td>Bovine lung</td>
<td>Xylose negative</td>
<td>A 1 +</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. avium</em> biovar 2</td>
<td>A283/86&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>D</td>
<td>Calf pneumonia</td>
<td>Mannitol positive</td>
<td>A 4 +</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. avium</em> biovar 2</td>
<td>A284/86&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>D</td>
<td>Calf pneumonia</td>
<td>Mannitol positive</td>
<td>A 4 +</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. avium</em> biovar 2</td>
<td>A285/86&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>D</td>
<td>Calf pneumonia</td>
<td>Mannitol positive</td>
<td>A 4 +</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. avium</em> biovar 2</td>
<td>Hom.4</td>
<td>B</td>
<td>Bovine lung</td>
<td>Xylose negative</td>
<td>A 1 +</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. avium</em> biovar 2</td>
<td>Hom.5</td>
<td>B</td>
<td>Bovine lung</td>
<td>Xylose negative</td>
<td>A 1 +</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. avium</em> biovar 2</td>
<td>Hom.22</td>
<td>B</td>
<td>Bovine lung</td>
<td>Xylose negative</td>
<td>A 5 +</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. avium</em> biovar 2</td>
<td>K117&lt;sup&gt;M&lt;/sup&gt; (=CCUG 16497)</td>
<td>DK</td>
<td>Calf pneumonia</td>
<td>A 2 +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. avium</em> biovar 2</td>
<td>K223</td>
<td>DK</td>
<td>Calf pneumonia</td>
<td>A 2 +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. avium</em> biovar 2</td>
<td>K417</td>
<td>DK</td>
<td>Calf pneumonia</td>
<td>A 2 +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. avium</em> biovar 2</td>
<td>K461</td>
<td>DK</td>
<td>Calf pneumonia</td>
<td>A 2 +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. canis</em> biovar 1</td>
<td>X225&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>UK</td>
<td>Bovine pneumonia</td>
<td>A 7 +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. canis</em> biovar 2</td>
<td>8&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>D</td>
<td>Calf pneumonia</td>
<td>A 9 +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. canis</em> biovar 2</td>
<td>25&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>D</td>
<td>Calf pneumonia</td>
<td>A 5 +</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
RESULTS

Phenotypic characterization

All nine strains of *P. multocida* subsp. *multocida* were D-mannitol-negative variants, two of which were also ornithine decarboxylase negative, while the five strains of *P. multocida* subsp. *septica* deviated from the type strain of the subspecies by being negative in ornithine decarboxylase and indole. Five out of the 22 *P. avium* biovar 2 strains characterized showed atypical reactions. Two strains were late indole-positive (positive within 3–14 days) and D-xylose negative, two strains were D-xylose negative, and a single strain was D-mannitol positive. Three out of the 18 strains of *P. canis* biovar 2 were D-xylose negative (Table 2). The impact of these deviations may be deduced from Table 1.

Ribotyping

Between 6 and 11 bands were registered at 20 positions in 13 ribotypes (Fig. 1). The most conserved ribotype (RT5) was shared between 20 strains of *P. avium* biovar 2 and *P. canis* biovar 2 from Germany and Belgium. All 11 Danish isolates of *P. avium* biovar 2 and *P. canis* biovar 2 investigated also belonged to the same ribotype (RT2). Ornithine decarboxylase- and indole-negative strains of *P. multocida* subsp. *septica* belonged to RT1 and RT4. These two clusters also included strains of *P. avium* biovar 2. Phenotypic variants of *P. multocida* subsp. *multocida* belonged to three different ribotypes. Seven out of 18 strains of *P. canis* biovar 2 were D-xylose negative (Table 2). The impact of these deviations may be deduced from Table 1.

Overall, the genotypic relationship demonstrated by ribotyping between atypical bovine strains previously reported as atypical *P. multocida* or as biovar 2 variants of *P. avium* and *P. canis* was high, compared to the reference strains included.

Table 2. cont.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Country of isolation</th>
<th>Animal, disease and organ</th>
<th>Phenotypic variant character</th>
<th>Capsular type</th>
<th>Ribotype</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. canis</em> biovar 2</td>
<td>66&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>D</td>
<td>Calf pneumonia</td>
<td>D-mannitol-negative</td>
<td>ND</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td><em>P. canis</em> biovar 2</td>
<td>217&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>D</td>
<td>Calf pneumonia</td>
<td>D-mannitol-negative</td>
<td>A</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td><em>P. canis</em> biovar 2</td>
<td>248&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>D</td>
<td>Calf pneumonia</td>
<td>D-mannitol-negative</td>
<td>A</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td><em>P. canis</em> biovar 2</td>
<td>264&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>D</td>
<td>Calf pneumonia</td>
<td>D-mannitol-negative</td>
<td>A</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td><em>P. canis</em> biovar 2</td>
<td>283&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>D</td>
<td>Calf pneumonia</td>
<td>D-mannitol-negative</td>
<td>A</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td><em>P. canis</em> biovar 2</td>
<td>286&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>D</td>
<td>Calf pneumonia</td>
<td>D-mannitol-negative</td>
<td>A</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td><em>P. canis</em> biovar 2</td>
<td>A554/86&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>D</td>
<td>Calf pneumonia</td>
<td>D-mannitol-negative</td>
<td>A</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td><em>P. canis</em> biovar 2</td>
<td>K77&lt;sup&gt;M&lt;/sup&gt;</td>
<td>DK</td>
<td>Calf pneumonia</td>
<td>Ornithine decarboxylase- and indole-negative</td>
<td>A</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td><em>P. canis</em> biovar 2</td>
<td>K103&lt;sup&gt;M&lt;/sup&gt;</td>
<td>DK</td>
<td>Calf pneumonia</td>
<td>Ornithine decarboxylase- and indole-negative</td>
<td>A</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td><em>P. canis</em> biovar 2</td>
<td>K267&lt;sup&gt;M&lt;/sup&gt;</td>
<td>DK</td>
<td>Calf pneumonia</td>
<td>Ornithine decarboxylase- and indole-negative</td>
<td>Xylose negative</td>
<td>A</td>
<td>2</td>
</tr>
<tr>
<td><em>P. canis</em> biovar 2</td>
<td>K267&lt;sup&gt;M&lt;/sup&gt; = CCUG 16498</td>
<td>DK</td>
<td>Calf pneumonia</td>
<td>Ornithine decarboxylase- and indole-negative</td>
<td>Xylose negative</td>
<td>A</td>
<td>2</td>
</tr>
<tr>
<td><em>P. canis</em> biovar 2</td>
<td>K323&lt;sup&gt;M&lt;/sup&gt;</td>
<td>DK</td>
<td>Calf pneumonia</td>
<td>Ornithine decarboxylase- and indole-negative</td>
<td>A</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td><em>P. canis</em> biovar 2</td>
<td>K323&lt;sup&gt;M&lt;/sup&gt;</td>
<td>DK</td>
<td>Calf pneumonia</td>
<td>Ornithine decarboxylase- and indole-negative</td>
<td>A</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td><em>P. canis</em> biovar 2</td>
<td>K464&lt;sup&gt;M&lt;/sup&gt;</td>
<td>DK</td>
<td>Calf pneumonia</td>
<td>Ornithine decarboxylase- and indole-negative</td>
<td>A</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td><em>P. canis</em> biovar 2</td>
<td>K618&lt;sup&gt;M&lt;/sup&gt;</td>
<td>DK</td>
<td>Calf pneumonia</td>
<td>Ornithine decarboxylase- and indole-negative</td>
<td>A</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td><em>P. canis</em> biovar 2</td>
<td>K826&lt;sup&gt;M&lt;/sup&gt;</td>
<td>DK</td>
<td>Calf pneumonia</td>
<td>Ornithine decarboxylase- and indole-negative</td>
<td>A</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td><em>P. canis</em> biovar 2</td>
<td>K2041&lt;sup&gt;M&lt;/sup&gt;</td>
<td>DK</td>
<td>Calf pneumonia</td>
<td>Ornithine decarboxylase- and indole-negative</td>
<td>A</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td><em>P. multocida</em> subsp. <em>septica</em></td>
<td>W208*†</td>
<td>D</td>
<td>Bovine organs</td>
<td>Indole and ornithine negative</td>
<td>A</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td><em>P. multocida</em> subsp. <em>septica</em></td>
<td>1018/89</td>
<td>NL</td>
<td>Bovine lesions</td>
<td>Indole and ornithine negative</td>
<td>ND</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td><em>P. multocida</em> subsp. <em>septica</em></td>
<td>239&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>D</td>
<td>Calf pneumonia</td>
<td>Indole and ornithine negative</td>
<td>A</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td><em>P. multocida</em> subsp. <em>septica</em></td>
<td>255&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>D</td>
<td>Calf pneumonia</td>
<td>Indole and ornithine negative</td>
<td>A</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td><em>P. multocida</em> subsp. <em>septica</em></td>
<td>16&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>D</td>
<td>Calf pneumonia</td>
<td>Indole and ornithine negative</td>
<td>A</td>
<td>1</td>
<td>+</td>
</tr>
</tbody>
</table>

*16S rRNA gene sequence determined in the present study.
†DNA–DNA hybridization performed in the present study.
Fig. 1. Genotypic relationship between atypical strains of *P. multocida*, investigated by ribotyping. Strains with identical profiles are listed at the branch tips.
ITS fragment-length typing

All strains, except five (66, 248, RA12/2, 21474/76, X225), shared an ITS profile with fragments of 388 and 612 bp (Fig. 2). Strain 248 of P. canis biovar 2 possessed four fragment lengths, two of which were also shared with the majority of strains, while the other two were also found in strain 66 of P. canis biovar 2. None of these strains had a deviating phenotype. A unique fragment profile was found in strain 21474/76 of P. avium biovar 2, which deviated in indole and D-xylose. Finally, a D-mannitol-negative strain of P. multocida subsp. multocida (RA12/2) contained an extra fragment not found in the other strains. The type strain of P. canis had ITS fragments different from the other strains investigated; however, strain X225 of P. canis biovar 1 (bovine isolate) shared a profile with the majority of strains, as did the type strain of P. gallinarum. ITS profile comparison does not therefore seem to represent a reliable criterion to separate Pasteurella species.

PCR

All 53 variant strains of P. multocida, including the biovar 2 variants of P. canis and P. avium and the reference strains, tested positive with the specific PCR test, whereas the type strain of P. canis was negative.

Of the strains tested, 52 of 53, including the type strains for the subspecies of P. multocida (Table 2), were found to be capsular type A. Strain 77179, isolated from a chicken and representing P. multocida subsp. gallicida, belonged to capsular type F.

16S rRNA sequence comparison

Eleven strains were selected for sequencing to represent isolates identified as P. multocida subsp. multocida and P. avium biovar 2 (three strains each), P. canis biovar 2 and P. multocida subsp. septica (two strains each), and strain X225, identified as P. canis biovar 1, including phenotypically aberrant strains. This selection covered 10 out of the 13 ribotypes determined for the strains characterized. For strains 5, 16, 25, 160, 214, K323, RA12/2, A284/86, A285/86 and W208, the 16S rRNA gene sequence determined covered the positions 28–1491 (Escherichia coli rrnB), resulting in 1464 bp. In strain X225, the 16S rRNA gene sequence covered the positions 28–1459 (E. coli rrnB), resulting in 1432 bp. The sequences for strains 5, 25, K323 and A285/86 were identical, just as strains RA12/2 and 160 were identical. All strains showed 99-9% similarity to the type strain of P. multocida subsp. multocida and 98-6% similarity to the type strain of P. multocida subsp. septica. Surprisingly, strains 16 and W208, phenotypically identified as P. multocida subsp. septica, showed the highest similarity to P. multocida subsp. multocida and only a more distant relationship to P. multocida subsp. septica. The highest 16S rRNA gene sequence similarity to the strains of P. multocida, biovar 2 of P. canis and biovar 2 of P. avium was found for the type strain ATCC 43327T of P. stomatis (accession no. M75050) with 97.8% similarity to strain RA12/2 and a slightly lower similarity of 97.7% to strains 5, 214 and W208. The highest similarity to the type strain of P. canis (biovar 1) was 97.8% for strain RA12/2, and the highest similarity to the type strain of P. avium (biovar 1) was 93.7% for strains 214, W208 and RA12/2.

DNA–DNA hybridization

Six strains were selected for DNA–DNA hybridization from the strains analysed by 16S rRNA sequencing to represent the taxa identified as P. avium biovar 2, P. canis biovar 2 and P. multocida subsp. septica. All investigated
strains of *P. multocida*, biovar 2 of *P. canis* and biovar 2 of *P. avium* and mannitol-negative variants of *P. multocida* subsp. *multocida* were closely related, at 79 % DNA binding, to the type strain of *P. multocida*. DNA binding between the strains of biovar 2 of *P. canis* and biovar 2 of *P. avium* ranged from 81 to 92 % (Table 3, Fig. 3). The DNA binding between the type strains of *P. multocida* and *P. canis* was only 25 %.

**DISCUSSION**

Bacterial species to be named are conventionally circumscribed by characters shared by at least 90 % of strains analysed (Krieg & Garrity, 2001). In very heterogeneous species like *P. multocida*, it becomes difficult to set a limit to the fraction of strains that share a particular character, since these bacteria are distributed worldwide and associated with diverse hosts and different disease patterns. Strict application of the 90 % rule might lead to the creation of new species, whereas a loose application of the rule will provide a weak circumscription, resulting in difficulties in the separation of species. In addition to phenotypic characters, the species should also conform to specific genotypic characteristics (Wayne et al., 1987; Stackebrandt et al., 2002).

The strains investigated in the present study were mostly isolated from cases of bovine pneumonia, and included strains that diverged from *P. multocida* in key phenotypic properties. 16S rRNA sequence-based comparisons, in addition to DNA–DNA hybridizations, were able to confirm the genotypic relationships of the strains.

Strains selected for 16S rRNA sequencing represented isolates identified as *P. multocida* subsp. *multocida*, *P. avium* biovar 2, *P. canis* biovar 1, *P. canis* biovar 2 and *P. multocida* subsp. *septica* (aberrant strains included), and allowed for geographic diversity as well as representing the major ribotypes. All strains showed at least 99·9 % similarity to the type strain of *P. multocida* subsp. *multocida*, but only 98·6 % similarity to the type strain of *P. multocida* subsp.

### Table 3. DNA–DNA hybridizations with strains of *P. multocida*

Values in square brackets are from Mutters et al. (1985b). Values in parentheses represent standard deviation (*n* = 8). ND, Not done.

<table>
<thead>
<tr>
<th>Phenotypic characterization and strain</th>
<th>DNA binding (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NCTC 10204&lt;sup&gt;T&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>P. mult. subsp. multocida</em> (NCTC10322&lt;sup&gt;T&lt;/sup&gt;)</td>
<td>82 (21) [77]</td>
</tr>
<tr>
<td><em>P. mult. subsp. gallicida</em> (NCTC10204&lt;sup&gt;T&lt;/sup&gt;)</td>
<td>ND</td>
</tr>
<tr>
<td><em>P. mult. subsp. gallicida</em> (77179)</td>
<td>ND</td>
</tr>
<tr>
<td><em>P. mult. subsp. septica</em> (CIP A125&lt;sup&gt;T&lt;/sup&gt;)</td>
<td>ND</td>
</tr>
<tr>
<td><em>P. mult. subsp. multocida</em> (214)</td>
<td>94 (12)</td>
</tr>
<tr>
<td><em>P. mult. subsp. multocida</em> (RA12/2)</td>
<td>ND</td>
</tr>
<tr>
<td><em>P. avium</em> biovar 2 (5)</td>
<td>ND</td>
</tr>
<tr>
<td><em>P. canis</em> biovar 2 (25)</td>
<td>ND</td>
</tr>
<tr>
<td><em>P. mult. subsp. septica</em> (W208)</td>
<td>ND</td>
</tr>
<tr>
<td><em>P. avium</em> biovar 2 (A285/86)</td>
<td>ND</td>
</tr>
<tr>
<td><em>P. mult. subsp. multocida</em> (W208)</td>
<td>94 (19)</td>
</tr>
</tbody>
</table>

**Fig. 3.** Relationships between strains of *P. multocida* based on pairwise DNA–DNA hybridization experiments. The names of taxa refer to previous designations. Data for DNA–DNA hybridization between type strains of *P. multocida* and *P. avium* were obtained from Mutters et al. (1985b). The dendrogram was constructed by single linkage clustering, based on the DNA-binding data shown in Table 3.
septica. Even the two strains phenotypically identified as
P. multocida subsp. septica showed the highest similarity
to P. multocida subsp. multocida. At least 98.5 % similarity
has been found between strains of P. multocida subsp.
multocida and P. multocida subsp. septica (Kuhnert et al.,
2000; Petersen et al., 2001). This indicates that the five
strains phenotypically identified as P. multocida subsp.
septica actually represented variant strains of P. multocida
subsp. multocida, not only in indole and ornithine decarbo-
ylase, used for separating species, but also in sorbitol, used
for separating the two subspecies P. multocida subsp.
multocida and P. multocida subsp. septica. For the eleven
strains sequenced, a maximum of 97-8 % similarity was
found to related species of Pasteurella such as P. stomatis
and P. canis, and only 93-7 % to P. avium.

The DNA–DNA hybridizations documented the close
genotypic relationship between the strains of P. multocida
and biovar 2 variants of P. canis and P. avium. A value
of 79 % DNA binding between the strains investigated is
only slightly lower than the 85 % species limit outlined for
Pasteurella (Maters et al., 1989). Binding as low as 49 %
was also observed. Low levels of binding between certain
strains of P. multocida have previously been reported
(Muters et al., 1985b). Strain 77149 of P. multocida subsp.
gallicida (isolated from chicken) bound at 85 % to the type
strain of P. multocida subsp. multocida.

According to the present genotypic characterization, the
strains identified as biovar 2 variants of P. canis and P.
avium all belong to P. multocida. Their classification with
taxa other than P. multocida was based on a few DNA–
DNA hybridizations (Muters et al., 1985a, b). A single
strain of P. avium biovar 2 (K117) was found to bind at
88 % to the type strain of the species (Muters et al., 1985a),
while the only strain of biovar 2 of P. canis investigated
(K267) bound at 80 % to the type strain of the species
(Mutters et al., 1985b).

The high genotypic diversity among isolates of P. multocida
investigated by ribotyping (see Fig. 1) is in accordance
with previous investigations. Fussum et al. (1999), using
HindIII for digestion of DNA, found high genotypic diver-
sity among Danish porcine strains of P. multocida subsp.
multocida. P. multocida subsp. multocida isolated from fowl,
cats and dogs were separated into at least four ribotype
clusters by Muhairwa et al. (2001), and Petersen et al. (2001)
found at least six ribotype clusters among avian isolates of
P. multocida. Petersen et al. (2001) also showed that P.
multocida subsp. multocida and subsp. septica might share
ribotypes. Previous investigations by Petersen et al. (1998)
showed by ribotyping that maltose-positive strains of P.
multocida also exist. Unpublished data from the present
investigation also showed that ribotyping represents a
useful tool to separate atypical P. multocida and non-
haemolytic bovine isolates of Gallibacterium anatis.

ITS fragment-length profiling showed that 90 % of strains
shared a profile with the type strain of P. multocida. The
presence of an identical profile for the type strains of
P. multocida and P. gallinarum, however, shows that ITS
profiling cannot be used on its own for species separation
within Pasteurella as presently defined.

The existence of phenotypic variation in key characters of
P. multocida, including ornithine decarboxylase, indole and
mannitol, makes routine diagnostics based on phenotypic
characters difficult and uncertain (Table 1). Using the
source of isolation might improve correct allocation of
variant strains of P. multocida. Investigations on the
population structure and diversity of avian isolates of
P. multocida from Australia showed that a range of P.
multocida clones are associated with fowl cholera and
that many of the Australian isolates are similar to non-
Australian reference strains (Blackall et al., 1998). The
strains of biovar 2 of P. canis and P. avium investigated here
(previous taxon 13 of Bisgaard; Madsen et al., 1985) seem to
be genetically related and are exclusively associated with
pneumonia in calves, which aids in their final classification.

The present study showed that ornithine decarboxylase,
indole, mannitol and sorbitol reactions might vary for P.
multocida. In addition, maltose-positive variants have
recently been found (Petersen et al., 1998). As a conse-
quence, bovine strains negative for indole and mannitol
are members of P. multocida and should no longer be
classified as biovar 2 of P. canis. Bovine strains negative for
ornithine decarboxylase, indole and mannitol, and without
a requirement for V-factor, are members of P. multocida
and should not be classified as biovar 2 of P. avium. All
strains of biovar 2 of P. avium and P. canis investigated
have been shown to belong to P. multocida. Consequently,
the existence of P. avium biovar 2 and P. canis biovar 2
is highly questionable. Finally, the presence of sorbitol-
negative variants might lead to misidentification of P.
multocida subsp. septica when characterization is based on
a single phenotypic character. The isolation of G. anatis
from bovine lungs (Christensen et al., 2003b; Ø. Angen,
unpublished results) shows that P. multocida-like organi-
sms might still be found that are genotypically unrelated to
P. multocida. The frequency of isolation of P. multocida
divergent in key species characteristics is probably higher
from bovine lung than from other hosts, but the occurrence
of such strains is low and phenotypic identification of P.
multocida will in most cases be feasible.

Development of specific DNA tools for the detection and
identification of P. multocida has previously been hampered
by non-specific PCR and in situ detection of P. avium
biovar 2 and P. canis biovar 2 (Mbuthia et al., 2001; Miflin
& Blackall, 2001; Townsend et al., 2001). The data presented
in the present paper fully document that these organisms
should be reclassified with P. multocida, since genotypically
they represent genuine P. multocida. The existence of
sucrose-negative variant strains of P. multocida has also
been reported (Bisgaard et al., 1991a; Bus et al., 1997;
Capitini et al., 2002). Genotypic characterization is
required to document their relationship with P. multocida.
In conclusion, *P. multocida* is genotypically homogeneous, although phenotypically diverse lineages exist with respect to the ornithine decarboxylase, indole and mannitol reactions, at least in association with bovine pneumonia. A formal reclassification of the species is not possible, since only about 3% of the strains characterized during the past three decades have been found to vary in these key species characteristics, and at least 10% are needed to change the classification. This study confirmed earlier observations (Carter, 1973; Madsen et al., 1985; Frank, 1989) that bovine pneumonia is mainly caused by capsular type A of *P. multocida*. Future diagnostics should be based on extended phenotypic characterization, PCR tests and 16S rRNA sequence comparison, or equivalent methods, just as emphasis should be put on the animal species of isolation.

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REFERENCES


*Pasteurella avium* (Hinz and Kunjara 1977) comb. nov. and 

Mutters, R., Ihm, P., Pohl, S., Frederiksen, W. & Mannheim, W. 
(1985b). Reclassification of the genus *Pasteurella* Trevisan 1887 on 
the basis of DNA homology with proposals for the new species 

the Group. In *Pasteurella and Pasteurellosis*, pp. 3–34. Edited by 

*Pasteurella haemolytica*: expression and identification of a bovine-


electrophoresis applied to representative strains from 11 different *Pasteurella* species under taxonomic aspects. *Zentralbl Bakteriol* 275, 16–27.

Stackebrandt, E., Frederiksen, W., Garrity, G. M. & 10 other authors 
(2002). Report of the ad hoc committee for the re-evaluation of 


Townsend, K. M., Boyce, J. D., Chung, J. Y., Frost, A. J. & Adler, B. 
(2001). Genetic organization of *Pasteurella multocida* cap loci and 

Report of the ad hoc committee on reconciliation of approaches to 