Genetic diversity of Pasteurella multocida fowl cholera isolates as demonstrated by ribotyping and 16S rRNA and partial atpD sequence comparisons

Kamille D. Petersen,† Henrik Christensen, Magne Bisgaard and John E. Olsen

The genetic diversity of Pasteurella multocida, the aetiological agent of fowl cholera, was investigated. The strain collection comprised 69 clinical isolates representing a wide spectrum of hosts and geographic origin. The three type strains for the subspecies of P. multocida were also included. Avian isolates of P. multocida subsp. multocida and P. multocida subsp. septica did not represent separate lines by HpaII ribotyping and the two type strains of mammalian origin (porcine and cat bite) seemed to be representative of avian strains of P. multocida subsp. multocida and septica. By ribotyping, all P. multocida subsp. gallicida strains, except one chicken isolate and the type strain, clustered together. This indicated that the bovine type strain was not representative of this subspecies and that most strains of P. multocida subsp. gallicida are genetically related and may be distantly related to other P. multocida isolates, including those of avian origin. By 16S rRNA and atpD sequence comparisons of selected strains, including both P. multocida isolated from birds and mammals and selected distantly related Pasteurella species associated with birds and mammals, it was found that P. multocida is monophyletic. Extended DNA–DNA hybridizations are highly indicated since strains may exist which would connect the existing subspecies at species level. The considerable genetic diversity of P. multocida fowl cholera isolates is probably related to the clonal nature of this organism, resulting in many divergent lines.

Keywords: phylogeny, ATP synthase, clonal lines, evolution, aetiology

INTRODUCTION

Pasteurella multocida encompasses a diverse group of bacteria with a wide disease and host spectrum, spanning from haemorrhagic septicaemia and fowl cholera in cattle and birds, respectively, where it is considered a primary pathogen, to secondary invaders of pneumonic lesions (Carter, 1984). The disease spectrum also includes dog- and cat-inflicted bites. Fowl cholera manifestations vary from being peracute to acute or chronic with pathological manifestations ranging from acute septicaemia to localized, purulent lesions (Rimler & Glisson, 1997). P. multocida is widely distributed among birds, and it would probably be unsafe to rule out any species as possible host, but the susceptibility to infection varies between different avian species (Biberstein, 1979). Like other members of the Pasteurellaceae, P. multocida has adopted a parasitic life, closely associated with its host, and can be isolated from mucous membranes or internal organs of diseased birds. Although the use of molecular methods for bacterial identification has increased, the diagnosis of fowl cholera is still mainly based upon pathological findings and identification of P. multocida by morphology, biochemical reactions (Mutters et al., 1985a) and sero-
Table 1. Origin and subspecies classification of *P. multocida* strains investigated

Clusters I–VI correspond to ribotype clusters obtained using *Hpa*II for digestion.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Year of isolation</th>
<th>Country</th>
<th>Source</th>
<th>Subspecies</th>
<th>Cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-1059</td>
<td>1962</td>
<td>Maryland, USA</td>
<td>Turkey</td>
<td>multocida</td>
<td>I</td>
</tr>
<tr>
<td>40605-1</td>
<td>1996</td>
<td>Denmark</td>
<td>Eider</td>
<td>multocida</td>
<td>I</td>
</tr>
<tr>
<td>P 25</td>
<td>Unknown</td>
<td>Zimbabwe</td>
<td>Chicken</td>
<td>multocida</td>
<td>II</td>
</tr>
<tr>
<td>P 34</td>
<td>Unknown</td>
<td>Zimbabwe</td>
<td>Chicken</td>
<td>multocida</td>
<td>II</td>
</tr>
<tr>
<td>P 44</td>
<td>Unknown</td>
<td>Zimbabwe</td>
<td>Chicken</td>
<td>multocida</td>
<td>II</td>
</tr>
<tr>
<td>277</td>
<td>1990</td>
<td>Denmark</td>
<td>Starling</td>
<td>multocida</td>
<td>II</td>
</tr>
<tr>
<td>NCTC 10322T</td>
<td>1962</td>
<td>Canada</td>
<td>Pig</td>
<td>multocida</td>
<td>II</td>
</tr>
<tr>
<td>B01007</td>
<td>1997</td>
<td>England</td>
<td>Turkey</td>
<td>multocida</td>
<td>II</td>
</tr>
<tr>
<td>B973/2/98-1</td>
<td>1998</td>
<td>England</td>
<td>Turkey</td>
<td>multocida</td>
<td>II</td>
</tr>
<tr>
<td>B529-152</td>
<td>1998</td>
<td>England</td>
<td>Turkey</td>
<td>multocida</td>
<td>II</td>
</tr>
<tr>
<td>B237/1/98-1196</td>
<td>1998</td>
<td>England</td>
<td>Turkey</td>
<td>multocida</td>
<td>II</td>
</tr>
<tr>
<td>VB151</td>
<td>1996</td>
<td>England</td>
<td>Duck</td>
<td>multocida</td>
<td>II</td>
</tr>
<tr>
<td>B237-1174</td>
<td>1998</td>
<td>England</td>
<td>Turkey</td>
<td>multocida</td>
<td>II</td>
</tr>
<tr>
<td>24609/1</td>
<td>1984</td>
<td>Denmark</td>
<td>Chicken</td>
<td>gallicida</td>
<td>Separate</td>
</tr>
<tr>
<td>NCTC 10204T (SSI P426T)</td>
<td>1945</td>
<td>UK</td>
<td>Cow</td>
<td>gallicida</td>
<td>Separate</td>
</tr>
<tr>
<td>33886/1L</td>
<td>1987</td>
<td>Denmark</td>
<td>Turkey</td>
<td>multocida</td>
<td>III</td>
</tr>
<tr>
<td>B1233-1071</td>
<td>1997</td>
<td>England</td>
<td>Turkey</td>
<td>multocida</td>
<td>III</td>
</tr>
<tr>
<td>B01008</td>
<td>1997</td>
<td>England</td>
<td>Turkey</td>
<td>multocida</td>
<td>III</td>
</tr>
<tr>
<td>48545</td>
<td>1990</td>
<td>Denmark</td>
<td>Turkey</td>
<td>multocida</td>
<td>III</td>
</tr>
<tr>
<td>PD97/872</td>
<td>1997</td>
<td>England</td>
<td>Turkey</td>
<td>multocida</td>
<td>III</td>
</tr>
<tr>
<td>R260/89</td>
<td>1989</td>
<td>Denmark</td>
<td>Turkey</td>
<td>multocida</td>
<td>III</td>
</tr>
<tr>
<td>B827/1/98</td>
<td>1998</td>
<td>England</td>
<td>Turkey</td>
<td>septica</td>
<td>IV</td>
</tr>
<tr>
<td>86773</td>
<td>1994</td>
<td>Denmark</td>
<td>Chicken</td>
<td>septica</td>
<td>IV</td>
</tr>
<tr>
<td>OM6955</td>
<td>1995</td>
<td>N Ireland</td>
<td>Turkey</td>
<td>septica</td>
<td>IV</td>
</tr>
<tr>
<td>2168/52/89</td>
<td>1989</td>
<td>Germany</td>
<td>Blackbird</td>
<td>septica</td>
<td>IV</td>
</tr>
<tr>
<td>2261/S12</td>
<td>Unknown</td>
<td>Germany</td>
<td>Pigeon</td>
<td>septica</td>
<td>IV</td>
</tr>
<tr>
<td>2631/S12/88</td>
<td>1988</td>
<td>Germany</td>
<td>Blackbird</td>
<td>septica</td>
<td>IV</td>
</tr>
<tr>
<td>B924/9/97/1</td>
<td>1997</td>
<td>England</td>
<td>Broiler breeder</td>
<td>septica</td>
<td>IV</td>
</tr>
<tr>
<td>NCTC 11619T (CIP A125T)</td>
<td>Unknown</td>
<td>France</td>
<td>Human cat bite</td>
<td>septica</td>
<td>IV</td>
</tr>
<tr>
<td>4147/57/88</td>
<td>1988</td>
<td>Germany</td>
<td>Blackbird</td>
<td>septica</td>
<td>IV</td>
</tr>
<tr>
<td>61445</td>
<td>1992</td>
<td>Denmark</td>
<td>Turkey</td>
<td>septica</td>
<td>IV</td>
</tr>
<tr>
<td>B00697</td>
<td>1996</td>
<td>England</td>
<td>Broiler breeder</td>
<td>septica</td>
<td>IV</td>
</tr>
<tr>
<td>3035/S4/88</td>
<td>1988</td>
<td>Germany</td>
<td>Great spotted woodpecker</td>
<td>septica</td>
<td>IV</td>
</tr>
<tr>
<td>46069/1L</td>
<td>1990</td>
<td>Denmark</td>
<td>Goose</td>
<td>multocida</td>
<td>IV</td>
</tr>
<tr>
<td>2586/52/90</td>
<td>1990</td>
<td>Germany</td>
<td>Great spotted woodpecker</td>
<td>multocida</td>
<td>IV</td>
</tr>
<tr>
<td>46896/5L</td>
<td>1990</td>
<td>Denmark</td>
<td>Goose</td>
<td>multocida</td>
<td>IV</td>
</tr>
<tr>
<td>47490/1L</td>
<td>1990</td>
<td>Denmark</td>
<td>Goose</td>
<td>multocida</td>
<td>IV</td>
</tr>
<tr>
<td>M76</td>
<td>Unknown</td>
<td>Sweden</td>
<td>Goose</td>
<td>septica</td>
<td>IV</td>
</tr>
<tr>
<td>46896/2</td>
<td>1990</td>
<td>Denmark</td>
<td>Duck</td>
<td>multocida</td>
<td>IV</td>
</tr>
<tr>
<td>46001/1L</td>
<td>1990</td>
<td>Denmark</td>
<td>Duck</td>
<td>multocida</td>
<td>IV</td>
</tr>
<tr>
<td>1721/52/90</td>
<td>1990</td>
<td>Germany</td>
<td>Pigeon</td>
<td>multocida</td>
<td>IV</td>
</tr>
<tr>
<td>40534-3</td>
<td>1989</td>
<td>Denmark</td>
<td>Chicken</td>
<td>septica</td>
<td>IV</td>
</tr>
<tr>
<td>72808</td>
<td>1993</td>
<td>Denmark</td>
<td>Chicken</td>
<td>septica</td>
<td>IV</td>
</tr>
<tr>
<td>77989</td>
<td>1993</td>
<td>Denmark</td>
<td>Chicken</td>
<td>septica</td>
<td>IV</td>
</tr>
<tr>
<td>87860</td>
<td>1994</td>
<td>Denmark</td>
<td>Chicken</td>
<td>septica</td>
<td>IV</td>
</tr>
<tr>
<td>86122</td>
<td>1994</td>
<td>Denmark</td>
<td>Chicken</td>
<td>septica</td>
<td>IV</td>
</tr>
<tr>
<td>B0-1004</td>
<td>1997</td>
<td>England</td>
<td>Broiler breeder</td>
<td>septica</td>
<td>IV</td>
</tr>
<tr>
<td>1781/87</td>
<td>1987</td>
<td>Germany</td>
<td>Blackbird</td>
<td>multocida</td>
<td>IV</td>
</tr>
<tr>
<td>1649/87</td>
<td>1987</td>
<td>Germany</td>
<td>Wood duck</td>
<td>septica</td>
<td>IV</td>
</tr>
<tr>
<td>4269/510</td>
<td>Unknown</td>
<td>Germany</td>
<td>Unknown bird sp.</td>
<td>septica</td>
<td>IV</td>
</tr>
<tr>
<td>2598/518/86</td>
<td>1986</td>
<td>Germany</td>
<td>Fieldfare</td>
<td>septica</td>
<td>IV</td>
</tr>
<tr>
<td>VB134</td>
<td>1996</td>
<td>England</td>
<td>Duck</td>
<td>multocida</td>
<td>IV</td>
</tr>
</tbody>
</table>
typing, including capsular antigens (Carter, 1955; Rimler & Rhoades, 1987) and/or LPS (Heddlestone et al., 1972).

In 1985, the genus Pasteurella Trevisan 1887 was reclassified on the basis of DNA–DNA hybridization studies. Eleven species were defined and P. multocida was divided into three subspecies (subsp. multocida, subsp. septica and subsp. gallicida) (Mutters et al., 1985a, b). DNA–DNA hybridization experiments showed 84–100%, 89–100% and 91–100% DNA binding within P. multocida subsp. multocida, P. multocida subsp. septica and P. multocida subsp. gallicida, respectively, whereas DNA bindings were as low as 55–82% between P. multocida subsp. multocida and septica, 67% between P. multocida subsp. septica and gallicida and 77% between P. multocida subsp. gallicida and multocida. Although the DNA–DNA hybridization results obtained justified separation into three species, P. multocida was kept as a single species. Phenotypically, the three subspecies could be separated by differences in fermentation of sorbitol and dulcitol. P. multocida subsp. multocida and P. multocida subsp. septica are dulcitol-negative (sorbitol-positive and sorbitol-negative, respectively) and isolated from mammals and birds, while the epithet gallicida was preserved for dulcitol-positive P. multocida, which has been described as characteristic for strains isolated from avian sources (Mutters et al., 1985a). Strains in the above-mentioned study included a limited number of avian P. multocida subsp. multocida (4/11) and P. multocida subsp. septica (1/4), just as the source of the three P. multocida subsp. gallicida strains included was not specified. In addition, only selected DNA–DNA hybridizations were performed. All three subspecies of P. multocida have subsequently been isolated from outbreaks of fowl cholera (Snipes et al., 1989; Hirsh et al., 1990; Fegan et al., 1995) and a considerable heterogeneity seems to exist among these isolates as determined by DNA-based typing methods (Snipes et al., 1989; Christiansen et al., 1992; Wilson et al., 1993, 1995). Different genotypic methods have been used to estimate the phylogenetic relationship between bacterial taxa, including rRNA–DNA hybridizations, and rRNA sequence and atpD DNA sequence comparisons (Amann et al., 1988; Ludwig et al., 1993; Vandamme et al., 1996). According to De Ley et al. (1990), strains belonging to a well-defined genus are all on the same rRNA branch. However, the rRNA branch of the type strain of P. multocida NCTC 10322T only contained two additional strains (Pasteurella species A, strain HIM 789-5, and Pasteur species B, strain SSI P683), were located outside the Pasteurella branch, indicating heterogeneity of the genus Pasteurella sensu stricto as well as the species P. multocida.

16S rRNA sequence comparisons have subsequently shown that members of the genus Pasteurella sensu

<table>
<thead>
<tr>
<th>Strain</th>
<th>Year of isolation</th>
<th>Country</th>
<th>Source</th>
<th>Subspecies</th>
<th>Cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>VB136</td>
<td>1996</td>
<td>England</td>
<td>Duck</td>
<td>multocida</td>
<td>IV</td>
</tr>
<tr>
<td>B847/1/98-1</td>
<td>1998</td>
<td>England</td>
<td>Broiler breeder</td>
<td>multocida</td>
<td>IV</td>
</tr>
<tr>
<td>VB37</td>
<td>1995</td>
<td>England</td>
<td>Duck</td>
<td>septica</td>
<td>V</td>
</tr>
<tr>
<td>1800/S1/89</td>
<td>1989</td>
<td>Germany</td>
<td>Blackbird</td>
<td>septica</td>
<td>V</td>
</tr>
<tr>
<td>Pm 130</td>
<td>Unknown</td>
<td>Australia</td>
<td>Chicken</td>
<td>gallicida</td>
<td>VI</td>
</tr>
<tr>
<td>1917</td>
<td>Unknown</td>
<td>Germany</td>
<td>Pig</td>
<td>gallicida</td>
<td>VI</td>
</tr>
<tr>
<td>1915</td>
<td>Unknown</td>
<td>Germany</td>
<td>Pig</td>
<td>gallicida</td>
<td>VI</td>
</tr>
<tr>
<td>Pm 82</td>
<td>Unknown</td>
<td>Australia</td>
<td>Chicken</td>
<td>gallicida</td>
<td>VI</td>
</tr>
<tr>
<td>Pm 197</td>
<td>Unknown</td>
<td>Australia</td>
<td>Pig</td>
<td>gallicida</td>
<td>VI</td>
</tr>
<tr>
<td>Pm 451</td>
<td>Unknown</td>
<td>Australia</td>
<td>Chicken</td>
<td>gallicida</td>
<td>VI</td>
</tr>
<tr>
<td>C14</td>
<td>Unknown</td>
<td>Belgium</td>
<td>Partridge</td>
<td>gallicida</td>
<td>VI</td>
</tr>
<tr>
<td>41779</td>
<td>1989</td>
<td>Denmark</td>
<td>Chicken</td>
<td>gallicida</td>
<td>VI</td>
</tr>
<tr>
<td>69550</td>
<td>1992</td>
<td>Denmark</td>
<td>Chicken</td>
<td>gallicida</td>
<td>VI</td>
</tr>
<tr>
<td>77179</td>
<td>1993</td>
<td>Denmark</td>
<td>Chicken</td>
<td>gallicida</td>
<td>VI</td>
</tr>
<tr>
<td>71304</td>
<td>1993</td>
<td>Denmark</td>
<td>Chicken</td>
<td>gallicida</td>
<td>VI</td>
</tr>
<tr>
<td>Pm 238</td>
<td>Unknown</td>
<td>Australia</td>
<td>Pig</td>
<td>gallicida</td>
<td>VI</td>
</tr>
<tr>
<td>H49</td>
<td>Unknown</td>
<td>Belgium</td>
<td>Rabbit</td>
<td>gallicida</td>
<td>VI</td>
</tr>
<tr>
<td>2474/3L</td>
<td>1985</td>
<td>Denmark</td>
<td>Duck</td>
<td>gallicida</td>
<td>VI</td>
</tr>
<tr>
<td>M3771/85</td>
<td>1985</td>
<td>Denmark</td>
<td>Duck</td>
<td>gallicida</td>
<td>VI</td>
</tr>
<tr>
<td>2913S5/1</td>
<td>1985</td>
<td>Denmark</td>
<td>Duck</td>
<td>gallicida</td>
<td>VI</td>
</tr>
</tbody>
</table>
stricto were contained in three disconnected clusters (Dewhirst et al., 1993), underlining that the genus Pasteurella may represent more than one genus. Dewhirst et al. (1993) found that Pasteurella species A, Pasteurella avium, Pasteurella volantium and Pasteurella gallinarum, all bird-associated species, were included in cluster 3A. Cluster 3B contained the type strains for P. multocida subsp. multocida, Pasteurella canis, Pasteurella stomatis, Pasteurella dagmatis and Pasteurella species B. Thus all strains included in cluster 3B are characterized by having a mammalian host, except for P. multocida, which can be isolated from both birds and mammals (Bisgaard, 1993). The rRNA sequence of P. multocida subsp. septica and P. multocida subsp. gallicida was not included in the study by Dewhirst et al. (1993). Cluster 3D contained Pasteurella langaensis and Pasteurella anatis, two species also found to be associated with birds (Bisgaard, 1993).

Since indications have been published that P. multocida might represent different species and most species of the Pasteurellaceae seem to have a restricted host reservoir, the aim of the present study was to investigate the genetic diversity of avian strains of all three subspecies of P. multocida, one of which, the dulcitol-positive P. multocida subsp. gallicida, seems mainly associated with avian hosts. The hypothesis was that P. multocida subsp. gallicida and fowl-cholera-associated P. multocida subsp. multocida and septica might represent different phylogenetic lines, some of which might show a relationship with the bird-associated rRNA sequence clusters 3A and 3D published by Dewhirst et al. (1993). Initially the correlation between phenotypic classification of strains into subspecies and genotype clusters obtained by ribotyping was investigated. Based upon ribotyping results, strains were selected for 16S rRNA and atpD sequence comparison.

METHODS

Bacterial strains. A total of 72 epidemiologically unrelated strains was investigated, representing 69 field isolates and the three type strains of the subspecies of P. multocida (Table 1). The strains were collected over the period 1945–1998. The strains were selected to represent a wide geographic distribution and avian host spectrum, and for P. multocida subsp. gallicida, mammalian strains were included as well. The field strains included 29 P. multocida subsp. multocida and 23 P. multocida subsp. septica strains, all isolated from fowl cholera. Seventeen strains of P. multocida subsp. gallicida were included: 12 were isolated from fowl cholera, 4 were of porcine origin and a single isolate was obtained from a rabbit. The P. multocida strain collection comprises fowl cholera isolates from 13 different avian species representing domestic and wild birds in nine different countries on four different continents (Table 1). All strains had been kept at $-80^\circ$C since the original isolation or as freeze-dried cultures. Type strains were received as freeze-dried cultures and subsequently kept at $-80^\circ$C. Ampoules containing freeze-dried cultures were opened under sterile conditions, and the content was dissolved in 5 ml of TGY broth (Difco), subsequently inoculated on blood agar [tryptose blood agar base (Difco) containing 5% citrated bovine blood] and incubated aerobically at 37 °C for 24 h, to ensure that they represented pure cultures. Cultures kept at $-80^\circ$C were controlled according to the same method.

Phenotypic characterization. Phenotypic characterization was done according to Bisgaard et al. (1991) to allow classification into one of the three subspecies of P. multocida (multocida, septica and gallicida) according to Mutters et al. (1985a).

Ribotyping. Ribotyping was carried out as previously described (Christensen et al., 1993) with the following modifications. Instead of 1.5 ml of overnight culture, 6 ml was centrifuged and the pellet was frozen before being resuspended in TE buffer (50 mM Tris, 50 mM EDTA). HpaII (Boehringer Mannheim Biochemicals) was used to digest DNA, and before digestion each sample was adjusted to contain 17 μg DNA by spectrophotometry (Gene Quant; Pharmacia Biotech). The probe was derived from 16S and 23S Escherichia coli rRNA (Sigma) and labelled with digoxigenin. A digoxigenin-labelled lambda phage digested with HindIII and EcoRI (Boehringer Mannheim Biochemicals) served as molecular size marker. Strain 4147/87/88 was included as a reference on every gel. Band patterns were analysed using GelCompar version 4.0 (Applied Maths, Kortrijk, Belgium). Only bands with a molecular mass of 0.8 kb or higher were included and analysed using a fine optimization and a band position tolerance of 1%. The similarity between band patterns of individual strains was estimated using the Dice coefficient, and dendrograms were derived from the similarity matrix by UPGMA.

Determination of partial 16S RNA and atpD gene sequences. To investigate the phylogenetic relationship between the clusters obtained by ribotyping, three strains were selected for 16S RNA sequencing. These strains included the type strain of P. multocida subsp. septica, NCTC 11619°, and P. multocida subsp. gallicida strains NCTC 10204° and 77179 (representing ribotype cluster VI). Together with the 16S rRNA sequence of P. multocida NCTC 1032²⁷ obtained from GenBank (accession no. M35018), these four strains represented three of the six obtained ribotype clusters. atpD DNA sequence determination included the type strains of P. multocida, P. multocida subsp. gallicida strain 77179, P. canis strain NCTC 11621° and P. gallinarum strain ATCC 13361°, the last two representing 16S rRNA clusters 3B (mammalian) and 3A (avian) of Dewhirst et al. (1993). PCR amplification was performed as described by Vogel et al. (1997). Oligonucleotides for both PCR amplification and 16S rDNA sequence determination were synthesized according to Dewhirst et al. (1985a, 1988) and Paster & Dewhirst (1988). PCR-amplified fragments were purified on Microspin columns (Pharmacia Biotech) and cycle sequenced (Thermo Sequenase fluorescent labelled primer cycle sequencing kit; Amersham) on an ALF sequencer (Pharmacia Biotech) by use of fluorescein-labeled primers. Primers for atpD gene DNA sequence determination were chosen after comparison of the published genome database (http://www.tigr.org). Gene positions are listed according to the putative atpD sequence of H. influenzae. For PCR amplification, the primers pmkatpf3w (20 nt upstream atpD, 5'-GCT CGT CAA GCA AGT ATY AC-3') and pmkatpr3w (1356 nt of atpD, 5'-CTA AYA CTG CRT CGA TKG WAC-3') were used. For DNA sequence determination, primers pmkatpf3w, pmkatpr3w, pmkatpr4w (158 nt of atpD, 5'-CGC AAT GGG ATC WTC WG-3') and pmkatp5 (338 nt of atpD, 5'-RAG YTA TGA AGA ACA AKA-3'), pmkatp7 (1057 nt of atpD, 5'-TAY RAY GTT CGT CGT GGY G-3'), pmkatp6 (1135 nt of atpD, 5'-GWA CCR TTA AAT ACT
TCM GC-3\(^{\prime}\) and pmkatpr5 (1054 nt of \textit{atpD}, 5\(^{\prime}\)-GTT CTT SAC CMA CRA C-3\(^{\prime}\)) were used with a fluorescein label at the 5\(^{\prime}\) end.

**Analysis of sequence data.** Searches for sequences in public databases were performed by \textsc{fasta} and \textsc{blast} available in the Wisconsin Sequence Analysis Package (Genetics Computer Group, GCG, Madison). Sequences were aligned with \textsc{pilup} (GCG). Maximum-likelihood analysis including bootstrap analysis was performed by fastDNAml (Felsenstein, 1981; Olsen \textit{et al.}, 1994) run on a HP9000/890 computer (UNI-C, Lyngby, Denmark). Parsimony and neighbour-joining analysis were performed by the use of \textsc{phylip} (Phylogeny Inference Package, version 3.57c; Joseph Felsenstein, University of Washington, 1995).

**GenBank accession numbers.** The accession numbers of the 16S rRNA sequences for strain HIM 830-7\(^{\prime}\) (NCTC 10204\(^{\prime}\)) and 77179 of \textit{P}. \textit{multocida} subsp. \textit{gallicida} and HIM 746-6\(^{\prime}\) (NCTC 11619\(^{\prime}\)) of \textit{P}. \textit{multocida} subsp. \textit{septica} are AF326323, AF326324 and AF326325, respectively. The GenBank accession numbers for the \textit{atpD} nucleotide sequences determined in this investigation are listed in Fig. 3.

**RESULTS**

**Ribotyping**

Ten, eight and five different ribotypes were found within \textit{P}. \textit{multocida} subsp. \textit{multocida}, \textit{P}. \textit{multocida} subsp. \textit{septica} and \textit{P}. \textit{multocida} subsp. \textit{gallicida}, respectively. The number of bands analysed varied from a minimum of 8 to a maximum of 12. All strains investigated shared two bands of approximately 0.9 and 0.8 kb in size, which were the smallest included in the analysis (Fig. 1). Six different clusters were related with a similarity of 84% or more (Fig. 2). Clusters I, II and III contained \textit{P}. \textit{multocida} subsp. \textit{multocida}, while cluster V included \textit{P}. \textit{multocida} subsp. \textit{septica}. Cluster IV contained both \textit{P}. \textit{multocida} subsp. \textit{multocida} and \textit{P}. \textit{multocida} subsp. \textit{septica}. The type strains for \textit{P}. \textit{multocida} subspp. \textit{multocida} and \textit{septica} were included in cluster II and IV, respectively, and thus were representative of avian isolates of these two subspecies of \textit{P}. \textit{multocida}. With the exception of the type strain, NCTC 10204\(^{\prime}\), and a Danish chicken isolate, 24609/1, all strains of \textit{P}.
Fig. 2. Dendrogram based on HpaII ribotypes obtained with 69 strains of P. multocida and the type strains of the three subspecies of P. multocida.
**multocida** subsp. **gallicida** clustered together (cluster VI). This cluster included strains from chickens, partridges, ducks, boe (Javan hill Mynah), pigs and rabbits, representing four different countries and two continents. Cluster VI showed only 71% similarity with cluster II, from which the type strain of **P. multocida** subsp. **gallicida** branched deeply, indicating genotypic heterogeneity within this taxon. Some strains contained in clusters II, III, IV and VI were found to cluster with 100% similarity despite their diverse origin in terms of geography and avian host species; e.g. within cluster IV eight strains of **P. multocida** subsp. **multocida** isolated from five different hosts in two different countries and 10 strains of **P. multocida** subsp. **septica** also isolated from at least four different hosts in four different countries were found to have identical band patterns. Ribotypes representing ribotype clusters I–VI and type strains of the three subspecies of **P. multocida** are demonstrated in Fig. 1.

### Comparison of 16S rRNA gene sequences

The sequence was 1463 nt long for the three strains covering the interval 28–1491 (E. coli position) of the 16S rRNA gene. The sequences of the type strains of **P. multocida** subsp. **multocida** (porcine isolate) and both strains of **P. multocida** subsp. **gallicida** (bovine and avian) were identical, while the type strain for **P. multocida** subsp. **septica** (cat bite) diverged from these strains by 20 bases, resulting in a similarity of 98.6%. The published 16S rRNA sequences for type strains of **P. multocida** subsp. **gallicida** (accession nos AF224297, AF294412 and AF326323) were identical and so were the sequences for the type strain of **P. multocida** subsp. **septica** (accession nos AF225205, AF294411 and AF326325). When the sequences of the type strain of **P. multocida** subsp. **multocida** (accession nos M35018 and AF294410) were compared, two bases (E. coli positions 703 and 1022) differed between the published sequences, but this must be related to sequencing errors. In the present study, the **P. multocida** subsp. **multocida** sequence M35018 was used for comparison.

### Comparison of atpD gene sequences

DNA sequences were determined for the 34–1370 region of the putative sequence of **H. influenzae** strain KW20 (accession no. U32273). All sequences were of identical length with 431 amino acids translated. DNA sequence comparison showed 0–1–1% variation between **P. multocida** subsp. **multocida** and **P. multocida** subsp. **gallicida** and 2.1–2.2% variation between these two subspecies and **P. multocida** subsp. **septica**. The variations between **P. multocida** subsp. **multocida** and **P. canis**, **P. gallinarum** and **H. influenzae** were 10–2, 14–9 and 14–3%, respectively. At the amino acid level, identical sequences were observed between all three subspecies of **P. multocida**. The variations between **P. multocida** subsp. **multocida** and **P. canis**, **P. gallinarum** and **H. influenzae** were 1–6, 6–5 and 6–7%, respectively. By phylogenetic analysis of the nucleotide sequence of atpD it was possible to separate **P. multocida** subsp. **septica** from the other two subspecies, but not **P. multocida** subsp. **multocida** from **P. multocida** subsp. **gallicida** (Fig. 3). Thus the atpD sequence analysis supported the rRNA sequence analysis in that **P. multocida** is monophyletic and is more closely related to other mammal-associated *Pasteurella* species (Dewhirst cluster 3B) represented by **P. canis** than to bird-associated *Pasteurella* species (Dewhirst cluster 3A) represented by **P. gallinarum**.

### DISCUSSION

In this study, ribotyping was carried out on 69 field strains of **P. multocida** representing the three subspecies of **P. multocida** in order to investigate whether the separation into subspecies based upon differences in...
fermentation as defined by Mutters et al. (1985a) could be correlated to genotypes. The present study showed that *P. multocida* subsp. *multocida* and *P. multocida* subsp. *septica* did not represent distinct genotypic groups as defined by ribotyping. The results obtained confirmed previous investigations (Blackall et al., 1998; Petersen et al., 1998; Muhairwa et al., 2000, 2001) and it can be concluded that the differences in fermentation patterns of sorbitol and dulcitol defining the subspecies of *P. multocida* according to Mutters et al. (1983a) do not reflect separate genetic groups within the avian *P. multocida* complex as demonstrated by HpaII digestion. The restriction enzyme HpaII was used because it has been found to be highly discriminatory for typing of *P. multocida* strains from birds as well as from other animal species (Christensen et al., 1998; Petersen et al., 1998; Aalbaek et al., 1999; Muhairwa et al., 2000, 2001). Other enzymes might possibly exist which will allow separation by ribotyping, confirming the differences observed in the 16S rRNA sequencing.

All *P. multocida* subsp. *gallicida* strains investigated were included in cluster VI, with the exception of the type strain of *P. multocida* subsp. *gallicida* and a Danish strain (24609/1) obtained from chicken (Fig. 2). This indicated that most strains of *P. multocida* subsp. *gallicida* are genetically related and that this subspecies may be more distantly related to other *P. multocida* isolates, including those of avian origin. Bowles et al. (2000) ribotyped porcine *P. multocida* using the restriction enzymes HpaII and HindIII. Isolates of *P. multocida* subsp. *gallicida* investigated were contained in a separate ribotype cluster, confirming the results obtained in the present study. The type strain for *P. multocida* subsp. *gallicida*, NCTC 10204T, however, does not seem to be genetically representative of this subspecies. This supports the ribotyping results of Blackall et al. (1998), who investigated a limited number of avian *P. multocida* subsp. *gallicida* strains, some of which (Pm 130 and Pm 82) have been included in the present study. Consequently, the possible phylogenetic relationship of *P. multocida* subsp. *gallicida* represented by the present strain collection to other bird-associated *Pasteurella* species contained in the 16S rRNA cluster 3A of Dewhirst et al. (1993) was investigated. Results obtained from 16S rRNA sequence comparison showed that *P. multocida* subsp. *gallicida* strain 77179, representing ribotype cluster VI, the type strain for *P. multocida* subsp. *gallicida*, NCTC 10204T, which branched deeply with cluster II, and the type strain for *P. multocida* subsp. *multocida* and genus *Pasteurella sensu stricto*, NCTC 10322T (cluster II), had identical 16S rRNA sequences, while the type strain of *P. multocida* subsp. *septica* only showed 98% similarity. These findings contradict the rRNA–DNA hybridization results previously published by De Ley et al. (1990). Maximum-likelihood including bootstrap analysis and parsimony and neighbour-joining analysis of sequence data showed that *P. multocida*, despite the ribotyping results and the heterogeneity of the host spectrum, is monophyletic (data not shown).

Comparison of *atpD* sequences at the nucleotide and amino acid levels has previously been used for phylogenetic studies of members of the *Bacteria* (Ludwig et al., 1993) and in a taxonomic study of *Salmonella* in which a genotypical resolution at the level of subspecies was found (Christensen & Olsen, 1998). In the present study, *atpD* sequence analysis of members of the *Pasteurellaceae* was introduced to investigate whether results obtained by rRNA sequence analysis could be supported. By comparing *atpD* sequences of the selected *P. multocida* strains and *Pasteurella* species representing rRNA clusters 3A (*P. gallinarum*) and 3B (*P. canis*) of Dewhirst et al. (1993), the phylogeny of the species as determined by 16S rRNA sequence comparison was overall supported. Although *P. multocida* subsp. *multocida* and *P. multocida* subsp. *gallicida* were found to have identical 16S rRNA sequences, the *atpD* sequences for these two subspecies diverged by 0–3–1.3%. This is in agreement with the *Salmonella* study mentioned above where *atpD* sequence analysis was also found to have a higher resolution compared to rRNA sequence analysis. The separation of *P. multocida* subsp. *septica* from the type strains of the other two subspecies as demonstrated in the present study by both rRNA and *atpD* sequence comparison supports the previous multilocus enzyme electrophoresis (MLEE) analysis by Blackall et al. (1998). At the amino acid level of *atpD*, no differences were observed between the three subspecies of *P. multocida*, indicating the conserved nature of this housekeeping gene within the *Pasteurellaceae*. The phylogenetic analysis of the partial *atpD* sequence also showed that *P. multocida* was more closely related to *P. canis* than *P. gallinarum* or *H. influenzae*, confirming the 16S rRNA results (Dewhirst et al., 1993). From the present investigation it can be concluded that the genus *Pasteurella sensu stricto* as defined by DNA–DNA hybridization needs to be redefined since *atpD* sequence results confirmed the existence of at least two phylogenetic clusters previously demonstrated by 16S rRNA sequence comparison. Although the three subspecies of *P. multocida* were found to differ at the species level (Mutters et al., 1985a), extended DNA–DNA hybridization is indicated because strains may exist which will connect the existing subspecies at species level and thus further support the present findings that the species *P. multocida* consists of a very diverse group of bacteria.

Knowledge of the population structure for a group of bacteria is of practical importance when diagnostic characteristics for epidemiological purposes have to be defined as well as for investigating the genetic traits of pathogenesis. The genus *Pasteurella* and its 11 species (Mutters et al., 1985a, b) were defined without knowing the degree of recombination within the population. The species proposed might consequently impose arbitrary divisions of a continuum (Smith, 1995). Evidence exists that *P. multocida* to some extent is restricted by the host; e.g. capsular type B has mainly been associated with haemorrhagic septicæmia in cattle while capsular type A is the most important cause of fowl cholera (Carter, 1984). Clones of *P. multocida* have also been
shown to be restricted to a particular host and not exchanged between different animal species, even though these were kept in close contact (Muhairwa et al., 2001). By MLEE, the population structure of avian \textit{Pasteurella multocida} isolated in Australia was found to be clonal and an overall correlation between MLEE types and ribotypes has been found for \textit{Pasteurella multocida} (Blackall et al., 1998). Correlation between MLEE types and ribotypes has also been found for other groups within the family \textit{Pasteurellaceae} (Angen et al., 1997). In the present study, strains with identical ribotypes might represent clonal lines. Within clusters II, IV and VI such strains were obtained from different avian hosts, indicating that virulence for a particular bird species is not restricted to a specific genotypic line of \textit{P. multocida}. Only turkey isolates from Denmark and England were contained in cluster III. Whether this reflects the existence of a turkey-associated \textit{P. multocida} clone, exchange of infected birds through trade or other factors is unknown.

As demonstrated in the present investigation, the genetic diversity of organisms causing fowl cholera is considerable. Previous investigations have also shown that \textit{P. multocida} associated with fowl cholera represented multiple clones (Wilson et al., 1993, 1995; Snipes et al., 1989; Christiansen et al., 1992; Blackall et al., 1998). However, clonal outbreaks of \textit{P. multocida} infections have been described in wild birds (Christensen et al., 1998), fallow deer (Aalbaek et al., 1999) and swine (Blackall et al., 2000). Isolates of \textit{P. multocida} from healthy flocks of poultry have also been shown to be clonal (Muhairwa et al., 2000). These observations seem to indicate that transmission within the flock follows infection, while recombination between different clones of \textit{P. multocida} seems rare during outbreaks. The extent of recombination and its possible contribution to the observed genetic variability within the \textit{P. multocida} complex has not been fully elucidated. The MLEE study by Blackall et al. (1998) investigating \textit{P. multocida} fowl cholera isolates showed the index of association $I_A$ for 56 electrophoretic types to be significantly different from zero, indicating that the population was not undergoing significant horizontal gene flow. However, the population structure of the \textit{P. multocida} complex remains to be further investigated.

ACKNOWLEDGEMENTS

Gitte Frederiksen, Sanne Laulund and Stina Holm provided technical assistance. The Danish Agricultural and Veterinary Research Council grant #97022797 and a PhD grant from the Royal Veterinary and Agricultural University have financed this project.

REFERENCES


Received 9 March 2001; revised 3 May 2001; accepted 9 May 2001.