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Genetic diversity among *Pasteurella multocida* strains of avian, bovine, ovine and porcine origin from England and Wales by comparative sequence analysis of the 16S rRNA gene

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Genetic diversity among 86 *Pasteurella multocida* isolates was investigated by comparative sequence analysis of a 1468 bp fragment of the 16S rRNA gene. The strains included 79 field isolates recovered from birds (poultry) (22), cattle (21), pigs (26) and sheep (10) within England and Wales, four Asian isolates associated with bovine haemorrhagic septicaemia, and the type strains of the three subspecies of *P. multocida*. Dulcitol and sorbitol fermentation patterns were also determined to establish correlations between subspecies status and phylogenetic relatedness. Nineteen 16S rRNA types were identified, but these were clustered into two distinct phylogenetic lineages, A and B. Sequences within lineages A and B had a mean number of nucleotide differences of 21·12 ± 3·90. Isolates within lineage A were associated with birds, cattle, pigs and sheep, whereas those belonging to lineage B were recovered from birds and a cat. Eighty-seven per cent of the isolates were classified as *P. multocida* subsp. *multocida* by dulcitol and sorbitol fermentation patterns, but these have diverse 16S rRNA gene sequences that were represented in both lineages A and B. Avian *P. multocida* subsp. *septica* isolates were associated exclusively with lineage B, but bovine *P. multocida* subsp. *septica* isolates were present in lineage A. *P. multocida* subsp. *gallicida* isolates of avian, bovine and porcine origin represent a homogeneous group within lineage A, but they have the same 16S rRNA type as certain *P. multocida* subsp. *multocida* isolates. These findings provide strong support for the view that dulcitol and sorbitol fermentation patterns are inaccurate indicators of genetic relatedness among *P. multocida* strains. Avian capsular type B isolates and capsular type B and E isolates associated with haemorrhagic septicaemia of cattle and water buffaloes are closely related and form a distinct cluster within lineage A. The current subspecies nomenclature of *P. multocida* neither accurately reflects the 16S rRNA-based phylogenetic relationships among isolates nor does it adequately encompass the full range of diversity within the species. The study provides a 16S rRNA-based evolutionary framework that will form the basis of further studies into the genetic diversity of *P. multocida* and will also help in the reclassification of the species.

INTRODUCTION

Pasteurella multocida represents a heterogeneous group of Gram-negative bacteria that are commensals in the upper respiratory tract of many mammals and birds (Rimler & Rhoades, 1989). The organism is also a primary or secondary pathogen and is responsible for a wide range of economically important diseases in domesticated animals throughout the world. Infections caused by *P. multocida* include fowl cholera of poultry (Rhoades & Rimler, 1989; Rimler & Glisson, 1997), progressive atrophic rhinitis of pigs (Chanter & Rutter, 1989), pneumonia of cattle, sheep and pigs (Chanter & Rutter, 1989; Frank, 1989), and haemorrhagic septicaemia of cattle and water buffaloes in certain enzootic areas of Asia and Africa (Carter & de Alwis, 1989). The pathogen has also been associated with atrophic rhinitis and septicaemia of sheep (Krametter *et al.*, 2004; Watson & Davies, 2002). In addition, *P. multocida* is responsible for infections in deer (Aalbaek *et al.*, 1999; Rimler *et al.*, 1987), causes respiratory tract disease in rabbits (Lu *et al.*, 1988; Rimler & Brogden, 1986), and is associated with human infections resulting from cat and dog bites (Holm & Tarnvik, 2000; Westling *et al.*, 2000).

P. multocida strains express a polysaccharide capsule on

Abbreviations: MLEE, multilocus enzyme electrophoresis; OMP, outer-membrane protein.

their cell surfaces and the antigenic specificity of the capsule determines the organism's serogroup: A, B, D, E or F (Rimler & Rhoades, 1989). It has long been recognized that strains of certain capsular serogroups are associated with specific diseases and animal species, which suggests that the capsular polysaccharide type plays a role in host and disease specificity. For example, the majority of cases of fowl cholera are caused by capsular type A strains (Rhoades & Rimler, 1989), atrophic rhinitis of pigs is associated predominantly with capsular type D isolates (Chanter & Rutter, 1989), bovine and porcine pneumonia are associated mainly with capsular type A strains (Chanter & Rutter, 1989; Frank, 1989), and haemorrhagic septicaemia of cattle and water buffaloes is caused exclusively by capsular type B and E isolates (Carter & de Alwis, 1989). However, the underlying genetic relationships of isolates representing different capsular types and associated with different host species and diseases is poorly understood.

P. multocida is classified into three subspecies based on DNA–DNA hybridization, namely *P. multocida* subsp. *gallicida*, *P. multocida* subsp. *multocida* and *P. multocida* subsp. *septica* (Mutters *et al.*, 1985). The DNA–DNA hybridization study of Mutters *et al.* (1985) indicated that the three subspecies could be classified as distinct species, but this was considered pointless from the clinical viewpoint. These authors also demonstrated that fermentation of dulcitol and sorbitol could be used to differentiate between the three subspecies: *P. multocida* subsp. *gallicida* isolates fermented both dulcitol and sorbitol; *P. multocida* subsp. *multocida* isolates fermented sorbitol but not dulcitol; and *P. multocida* subsp. *septica* isolates fermented neither sugar alcohol. However, more recent data based on multilocus enzyme electrophoresis (MLEE) and ribotyping indicate that the three *P. multocida* subspecies do not represent distinct genotypic groups (Blackall *et al.*, 1998; Muhairwa *et al.*, 2001; Petersen *et al.*, 1998, 2001). Furthermore, Kuhnert *et al.* (2000) showed that only two of six isolates that clustered as *P. multocida* subsp. *septica* by 16S rRNA sequencing gave negative sorbitol reactions. Thus, the dulcitol and sorbitol fermentation patterns that define the three subspecies of *P. multocida* according to Mutters *et al.* (1985) do not accurately reflect the genetic relatedness of isolates. These conflicting results clearly indicate that the precise phylogenetic relationships of isolates representing each of these subspecies is complex and has yet to be satisfactorily resolved.

The genetic diversity of avian, bovine and porcine isolates of *P. multocida* have been investigated separately using a variety of molecular techniques, including restriction endonuclease analysis (Blackall *et al.*, 2000; Rimler, 2000; Wilson *et al.*, 1993), ribotyping (Blackall *et al.*, 2000; Dabo *et al.*, 1999; Petersen *et al.*, 2001; Zhao *et al.*, 1992), pulsed field gel electrophoresis (Gunawardana *et al.*, 2000), repetitive sequence-based PCR and amplified fragment length polymorphism (Amonsin *et al.*, 2002), and MLEE (Blackall *et al.*, 1998). However, detailed comparative investigations of *P. multocida* isolates from poultry, cattle, sheep and pigs have not been undertaken and very little is known about the genetic relationships of such isolates. Strain diversity among a large collection of avian, bovine, ovine and porcine isolates of *P. multocida* has previously been examined by analysis of capsular polysaccharide and outer-membrane protein (OMP) variation (Davies *et al.*, 2003a, b, c, 2004). Although these methods yield useful epidemiological data, they provide little information about the underlying genetic relatedness of isolates. The aim of the present study was to investigate the phylogenetic relationships of representative *P. multocida* isolates from these four host species by comparative sequence analysis of the 16S rRNA gene. Dulcitol and sorbitol fermentation patterns were also determined to establish correlations between these phenotypic characteristics and the phylogeny of *P. multocida*.

METHODS

Bacterial strains and growth conditions. Seventy-nine British field isolates of *P. multocida* were investigated in this study. These included 22 avian (poultry), 21 bovine, 10 ovine and 26 porcine isolates that were obtained from regional laboratories of the Veterinary Laboratories Agency (VLA) and originated from widespread geographic locations within England and Wales over a 13-year period (1987–99). The isolates were recovered mainly from diseased animals and were selected to represent the major variants associated with each host species as defined by their capsular polysaccharide and OMP types (Davies *et al.*, 2003a, b, c, 2004). Two or more isolates were examined in those groups that represented the more common capsule/OMP type combinations. Four isolates originating from suspected cases of haemorrhagic septicaemia in cattle were also included in the study. Two of these (PM30 and PM36) were obtained from the National Collection of Type Cultures, whereas the other two (PM1192 and PM1200) originated from Pakistan and were provided by Dr R. Parton, University of Glasgow. In addition, the type strains of *P. multocida* subsp. *gallicida* (NCTC 10204), *P. multocida* subsp. *multocida* (NCTC 10322) and *P. multocida* subsp. *septica* (NCTC 11995) (obtained from the National Collection of Type Cultures) were included in the study. Properties of these isolates are presented in Table 1.

The isolates were stored at −85 °C in 50 % (v/v) glycerol in brain heart infusion broth (BHIB; Oxoid). Bacteria from −85 °C stock cultures were streaked onto blood agar [brain heart infusion agar containing 5 % (v/v) defibrinated sheep's blood] and incubated aerobically overnight at 37 °C. For preparation of DNA, a few colonies were inoculated into 15 ml volumes of BHIB and grown overnight at 37 °C with shaking at 120 r.p.m.

Dulcitol and sorbitol fermentation. Fermentation studies were performed using peptone water (Oxoid) containing 1 % (w/v) of either dulcitol or sorbitol and 18 mg phenol red l^{-1} as indicator. A few colonies of overnight cultures were resuspended in 3 ml volumes of PBS (pH 7·2) and 50 µl of this suspension was inoculated into 3 ml volumes of dulcitol or sorbitol peptone water. The cultures were incubated at 37 °C and the results recorded after 24, 48 and 120 h.

Preparation of chromosomal DNA. Cells from 1·0 ml of overnight cultures were harvested by centrifugation for 1 min at 13 000 ***g*** and washed once in sterile, distilled H_2O. DNA was prepared with the InstaGene Matrix (Bio-Rad) according to the manufacturer's instructions and stored at −20 °C.

Table 1. Properties of *Pasteurella multocida* isolates

Unless otherwise stated, all strains are *P. multocida* subsp. *multocida*. OMP types are unique for strains from each host species. AR, atrophic rhinitis; HS, haemorrhagic septicaemia; UT, untypable.

Strain designation (subspecies in parentheses)	Host of origin	Capsular type	OMP type	16S type	Disease status	GenBank accession no.
PM210	Avian	A	2.1	1	Anorexic	
PM86 (subsp. *gallicida*)	Avian	A	3.1	1	Fowl cholera	
PM70	Avian	A	4.1	1	Oedema of head	
PM386	Bovine	A	1.1	1	Pneumonia	AY299304
PM646	Bovine	A	1.1	1	Mastitis	
PM344	Bovine	A	3.1	1	Pneumonia	
PM384	Bovine	A	3.1	1	Pneumonia	
PM586	Bovine	A	3.1	1	Pneumonia	
PM414	Bovine	A	4.1	1	Respiratory distress	
PM426	Bovine	A	4.1	1	Coughing/nasal discharge	
PM526	Bovine	A	4.1	1	Not known	
PM632	Bovine	A	4.1	1	Pneumonia	
PM402 (subsp. *septica*)	Bovine	A	5.1	1	Pneumonia	
PM430	Bovine	A	8.1	1	Pneumonia	
NCTC 10204^T (subsp. *gallicida*)	Bovine	A	9.1	1	Pneumonia	AY078998
PM486 (subsp. *gallicida*)	Bovine	A	9.1	1	Pneumonia	
PM966	Ovine	A	1.1	1	Pneumonia	
PM974	Ovine	A	1.1	1	Pneumonia	
PM26	Ovine	A	1.2	1	Asymptomatic (vagina)	
PM982	Ovine	D	3.1	1	Pneumonia	
PM986	Ovine	D	3.1	1	Pneumonia	
PM54	Porcine	A	1.1	1	Pneumonia	
PM120	Porcine	A	1.1	1	Pneumonia	
NCTC 10322^T (subsp. *multocida*)	Porcine	A	1.2	1	Pneumonia	AY078999
PM666	Porcine	A	2.1	1	Pneumonia	
PM914	Porcine	A	2.1	1	Pneumonia	
PM116	Porcine	A	3.1	1	Pneumonia	
PM656	Porcine	A	3.1	1	Pneumonia	
PM830	Porcine	A	3.1	1	AR	
PM708	Porcine	A	3.2	1	Pneumonia	
PM702	Porcine	A	5.1	1	Pneumonia	
PM934 (subsp. *gallicida*)	Porcine	A	5.1	1	Pneumonia	
PM954 (subsp. *gallicida*)	Porcine	A	5.1	1	Pneumonia	
PM144	Avian	A	1.1	2	Septicaemia	
PM288	Avian	A	1.2	2	Pneumonia	
PM148	Avian	F	2.2	2	Eye infection	
PM246	Avian	F	2.2	2	Septicaemia	
PM282	Avian	F	2.2	2	Septicaemia	
PM336	Bovine	A	6.1	2	Pneumonia	
PM368	Bovine	A	6.1	2	Pneumonia	
PM306	Bovine	F	7.1	2	Oedema of head	
PM994	Ovine	F	1.1	2	Pneumonia	
PM734	Porcine	A	1.1	2	Pneumonia	AY299305
PM820	Porcine	A	1.1	2	Pneumonia	
PM850	Porcine	A	1.1	2	Pneumonia	
PM952	Porcine	F	3.2	2	Pneumonia	
PM382	Porcine	A	4.1	2	Respiratory problem	
PM716	Porcine	D	4.1	2	Suspected AR	
PM706	Porcine	UT	4.1	2	Pneumonia	
PM684	Porcine	A	6.1	2	Suspected AR	

Table 1. cont.

Strain designation (subspecies in parentheses)	Host of origin	Capsular type	OMP type	16S type	Disease status	GenBank accession no.
PM762	Porcine	D	6.1	2	AR	
PM230	Avian	A	9.1	3	Septicaemia	
PM316	Bovine	A	1.1	3	Pneumonia	
PM564	Bovine	A	2.1	3	Pneumonia	AY299306
PM600	Bovine	A	2.1	3	Pneumonia	
PM628	Bovine	A	2.1	3	Pneumonia	
PM776	Porcine	A	3.2	3	Pneumonia	
PM44	Ovine	F	1.1	4	Asymptomatic (vagina)	
PM998	Ovine	F	1.1	4	Pneumonia	
PM2	Ovine	F	2.1	4	Severe peritonitis	AY078996
PM8	Ovine	F	2.1	4	Asymptomatic (vagina)	
PM296	Avian	D	13.1	5	Pneumonia	
PM52	Porcine	D	1.2	5	Pneumonia	
PM758	Porcine	D	4.2	5	AR	
PM714	Porcine	D	6.1	5	Pneumonia	AY299307
PM752	Porcine	D	6.1	5	Pneumonia	
PM818	Porcine	D	6.1	5	Pneumonia	
PM338	Bovine	A	4.1	6	Pneumonia	AY299308
PM548	Bovine	A	5.3	7	Vaginal discharge	AY299309
PM62	Avian	B	12.1	8	Respiratory symptoms	AY299310
PM104	Avian	A	4.1	9	Septicaemia	AY299311
PM36 (NCTC 10326)	Bovine	E	Not done	10	HS	AY324032
PM30 (NCTC 10323)	Bovine	B	Not done	11	HS	AY299312
PM1192	Bovine	B	Not done	11	HS	
PM1200	Bovine	B	Not done	11	HS	
PM74	Avian	UT	5.1	12	Fowl cholera	AY299313
PM152 (subsp. *septica*)	Avian	A	6.1	13	Swollen joints	AY299314
PM214	Avian	A	8.1	13	Septicaemia	
NCTC 11995^T (subsp. *septica*)	Feline	A	Unclassified	14	Abscess/cat bite	AY079000
PM64	Avian	A	11.1	15	Fowl cholera	AY299315
PM106 (subsp. *septica*)	Avian	UT	10.2	16	Fowl cholera	AY299316
PM60 (subsp. *septica*)	Avian	UT	10.1	17	Septicaemia	AY299317
PM80 (subsp. *septica*)	Avian	A	14.1	18	Swollen heads	AY299318
PM82	Avian	A	7.1	19	Swollen heads	AY299319
PM88	Avian	A	7.1	19	High mortality	
PM96	Avian	A	7.1	19	Respiratory problems	

Amplification of the 16S rRNA gene and DNA sequence analysis. PCR fragments corresponding to nucleotides 20–1487 of the published 16S rRNA gene sequence of *P. multocida* subsp. *gallicida* strain MCCM 00021 (GenBank accession no. AF224297) were amplified from the chromosomal DNA of all isolates with the universal primers 5′-AGAGTTTGATYMTGGC-3′ (forward; positions 4–19) and 5′-GYTACCTTGTTACGACTT-3′ (reverse; 1505–1488) (Davies *et al.*, 1996). The 16S rRNA gene fragments were amplified with a *Taq* DNA polymerase kit (Boehringer Mannheim) according to the manufacturer's instructions. PCR errors were shown to be negligible by amplifying and sequencing the 16S rRNA gene twice in a small number of randomly selected isolates. PCRs were carried out in a Perkin Elmer 480 DNA thermal cycler using the following amplification parameters: denaturation at 94 °C for 45 s, annealing at 50 °C for 45 s and extension at 72 °C for 45 s. Thirty cycles were performed and a final elongation step of 72 °C for 10 min was used. Production of a PCR amplicon of the expected size was confirmed by agarose gel electrophoresis and the DNA purified with a QIAquick PCR purification kit (Qiagen). The DNA was finally eluted in 30 µl sterile distilled H_2O and stored at −20 °C. Sequence reactions were performed with the ABI Prism Big Dye Terminator cycle sequencing kit (Applied Biosystems) in a GeneAmp PCR System 9700 (Applied Biosystems) thermal cycler and sequence analysis carried out with an Applied Biosystems 377 DNA Sequencer (University of Glasgow Sequencing Service). Both strands of the gene were sequenced in three overlapping segments using the following additional internal primers: 5′-AAGAAGCAC-CGGCTAACT-3′ (forward/2; nucleotides 493–510), 5′-GCGAAGA-ACCTTACCTAC-3′ (forward/3; nucleotides 967–984), 5′-GTAAG-GTTCTTCGCGTTG-3′ (reverse/2; nucleotides 980–963) and 5′-CCGGTGCTTCTTCTGTAA-3′ (reverse/3; nucleotides 504–487). The primers were designed using the computer program Primer Designer (Version 2.0) and synthesized by Sigma-GenoSys.

Analysis of nucleotide sequence data. Nucleotide sequence data were analysed, edited and assembled with SEQED (Applied

Biosystems) and the Lasergene (DNASTAR) sequence analysis software. Complete assembled sequences were aligned with CLUSTALX and statistical and phylogenetic analyses were conducted using MEGA version 2.1 (Kumar *et al.*, 2001). The GenBank accession numbers for the 16S rRNA gene sequences corresponding to 16S types 1–19 are provided in Table 1.

RESULTS

Dulcitol and sorbitol fermentation

Seventy-five isolates fermented sorbitol but not dulcitol and were classified as *P. multocida* subsp. *multocida* (Table 1). Five isolates fermented both dulcitol and sorbitol and were classified as *P. multocida* subsp. *gallicida*. These included isolates PM86 (avian), NCTC 10204 and PM486 (bovine), and PM934 and PM954 (porcine) (Table 1). The avian isolate represented capsule/OMP types A/3.1, the bovine isolates capsule/OMP types A/9.1 and the porcine isolates capsule/OMP types A/5.1 (Table 1). However, it has previously been shown that the avian OMP type 3.1, bovine OMP type 9.1 and porcine OMP type 5.1 profiles are very similar (they represent OMP group 4 – see Davies *et al.*, 2004), suggesting that these isolates are closely related. Six isolates were unable to ferment either sugar alcohol and were classified as *P. multocida* subsp. *septica*. These included isolates PM60, PM80, PM106 and PM152 (avian), PM402 (bovine), and NCTC 11995 (feline) (Table 1). The avian isolates represented capsule/OMP types UT (untypable)/10.1 (PM60), A/14.1 (PM80), UT/10.2 (PM106) and A/6.1 (PM152), and the bovine isolate represented capsule/OMP types A/5.1 (Table 1).

Nucleotide variation of the 16S rRNA gene

A 1468 bp fragment of the 16S rRNA gene was sequenced in 86 isolates of *P. multocida*, including the type strains of the three subspecies, and 19 unique sequences, designated 16S types 1–19, were identified (Fig. 1). There were 27 (1·8 %) polymorphic nucleotide sites among these sequences, pairwise differences ranged from 1 to 24 (0·07 to 1·6 %) nucleotides, and the mean number of nucleotide differences was $5{\cdot}38 \pm 1{\cdot}01$. However, visual comparison of the polymorphic nucleotide sites clearly indicates that the 16S rRNA sequences consist of two distinct groups, A and B, represented by 16S types 1–12 and 13–19, respectively (Fig. 1). There were nine (0·6 %) polymorphic nucleotide sites among the group A sequences, pairwise differences ranged from 1 to 5 (0·07 to 0·34 %) nucleotides, and the mean number of nucleotide differences was $1{\cdot}23 \pm 0{\cdot}60$. There were eight (0·54 %) polymorphic nucleotide sites among the group B sequences, pairwise differences ranged from 1 to 7 (0·07 to 0·48 %) nucleotides, and the mean number of nucleotide differences was $2{\cdot}84 \pm 1{\cdot}06$. In contrast, pairwise differences between group A and B sequences ranged from 18 to 24 (1·23 to 1·63 %) nucleotides and the mean number of nucleotide differences was $21{\cdot}12 \pm 3{\cdot}90$. Pairwise differences among the avian sequences ranged from 1 to 23 nucleotides and the mean number of nucleotide differences was $11{\cdot}75 \pm 2{\cdot}19$. In contrast, pairwise differences among the bovine, ovine and porcine sequences ranged from 1 to 3, 1 to 2, and 1 to 3 nucleotides, respectively, and the mean number of nucleotide differences was $1{\cdot}12 \pm 0{\cdot}52$, $1{\cdot}09 \pm 0{\cdot}84$ and $0{\cdot}91 \pm 0{\cdot}59$, respectively. Fifty-nine per cent of the isolates were associated with 16S types 1 (33 isolates) or 2 (18), 19 % of the isolates contained 16S types 3 (6), 4 (4) or 5 (6), and 22 % of the isolates possessed 16S types 6–19 (19) (Table 1).

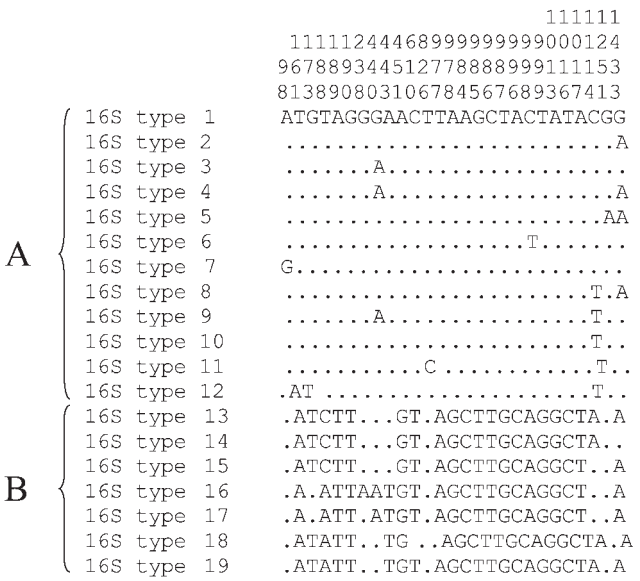
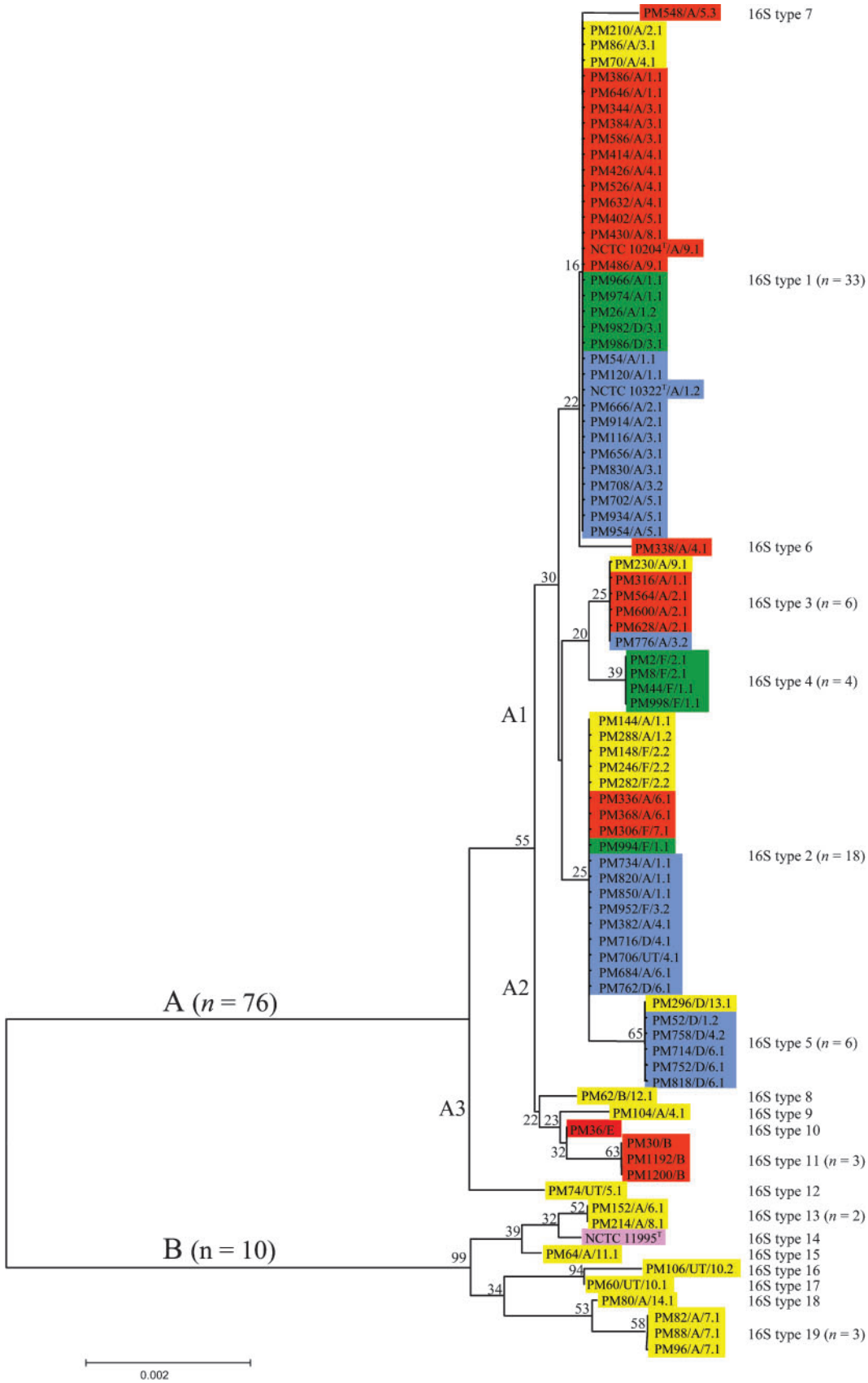


Fig. 1. Distribution of polymorphic nucleotide sites among the 19 16S rRNA alleles of *P. multocida*. 16S types are shown at the left of each sequence. The vertical numbers above the sequences represent the positions of polymorphic nucleotide sites within the 1468 bp segment. The dots represent sites where the nucleotides match those of the first (topmost) sequence.

Phylogenetic relationships of *P. multocida* isolates in relation to the host of origin and capsular polysaccharide and OMP types

A neighbour-joining dendrogram representing the phylogenetic relationships of the 86 16S rRNA gene sequences is shown in Fig. 2. The host of origin, capsular polysaccharide types and OMP types of each isolate are also shown. The 16S rRNA sequences were represented by two major lineages, A and B, which correspond to the two groups of 16S types described above. The branching of lineages A and B was extremely robust, as indicated by the high bootstrap value of 99 %. In contrast, the bootstrap values for many of the finer branches were relatively small (<50 %) because of the high similarity of the sequences. Lineage A (76 isolates) was represented by isolates from all four host species, whereas lineage B (10 isolates) was represented exclusively by isolates of avian origin as well as the feline *P. multocida* subsp. *septica* type strain. Lineage A itself consisted of three


PM548/A/5.3
PM210/A/2.1
PM86/A/3.1
PM70/A/4.1
PM386/A/1.1
PM646/A/1.1
PM344/A/3.1
PM384/A/3.1
PM586/A/3.1
PM414/A/4.1
PM426/A/4.1
PM526/A/4.1
PM632/A/4.1
PM402/A/5.1
PM430/A/8.1
NCTC 10204T/A/9.1
PM486/A/9.1
PM966/A/1.1
PM974/A/1.1
PM26/A/1.2
PM982/D/3.1
PM986/D/3.1
PM54/A/1.1
PM120/A/1.1
NCTC 10322T/A/1.2
PM666/A/2.1
PM914/A/2.1
PM116/A/3.1
PM656/A/3.1
PM830/A/3.1
PM708/A/3.2
PM702/A/5.1
PM934/A/5.1
PM954/A/5.1
PM338/A/4.1
PM230/A/9.1
PM316/A/1.1
PM564/A/2.1
PM600/A/2.1
PM628/A/2.1
PM776/A/3.2
PM2/F/2.1
PM8/F/2.1
PM44/F/1.1
PM998/F/1.1
PM144/A/1.1
PM288/A/1.2
PM148/F/2.2
PM246/F/2.2
PM282/F/2.2
PM336/A/6.1
PM368/A/6.1
PM306/F/7.1
PM994/F/1.1
PM734/A/1.1
PM820/A/1.1
PM850/A/1.1
PM952/F/3.2
PM382/A/4.1
PM716/D/4.1
PM706/UT/4.1
PM684/A/6.1
PM762/D/6.1
PM296/D/13.1
PM52/D/1.2
PM758/D/4.2
PM714/D/6.1
PM752/D/6.1
PM818/D/6.1
PM62/B/12.1
PM104/A/4.1
PM36/E
PM30/B
PM1192/B
PM1200/B
PM74/UT/5.1
PM152/A/6.1
PM214/A/8.1
NCTC 11995T
PM64/A/11.1
PM106/UT/10.2
PM60/UT/10.1
PM80/A/14.1
PM82/A/7.1
PM88/A/7.1
PM96/A/7.1
16S type 7
16S type 1 (n = 33)
16S type 6
16S type 3 (n = 6)
16S type 4 (n = 4)
16S type 2 (n = 18)
16S type 5 (n = 6)
16S type 8
16S type 9
16S type 10
16S type 11 (n = 3)
16S type 12
16S type 13 (n = 2)
16S type 14
16S type 15
16S type 16
16S type 17
16S type 18
16S type 19 (n = 3)
A (n = 76)
B (n = 10)
A1
A2
A3
16
22
30
25
20
39
55
25
65
22
23
32
63
52
32
39
99
94
34
53
58
0.002

clusters, A1–A3. Cluster A1 comprised 69 (91 %) of the lineage A isolates and included isolates from all four host species. Cluster A2 was represented by six isolates which included the four haemorrhagic septicaemia isolates as well as two avian isolates. Notably, three of the haemorrhagic septicaemia isolates and one of the avian isolates were of capsular type B; the fourth haemorrhagic septicaemia isolate was of capsular type E and the second avian isolate was of capsular type A. Cluster A3 included a single untypable avian isolate. In lineage A, 16S types 1 and 2 were associated with isolates from all four host species, 16S type 3 was present in avian, bovine and porcine isolates, and 16S type 5 occurred in avian and porcine isolates. In contrast, 16S type 4 was associated exclusively with ovine isolates, 16S types 6, 7, 10 and 11 with bovine isolates and 16S types 8, 9 and 12 with avian isolates. In lineage B, 16S types 13, 15, 16, 17, 18 and 19 were associated with avian isolates, whereas 16S type 14 was associated with the feline *P. multocida* subsp. *septica* type strain.

Avian isolates. The 16S rRNA sequences of the avian isolates were extremely diverse in comparison to those of the bovine, ovine and porcine isolates (Fig. 2). As well as being the only host species represented in lineage B (with the exception of the feline subsp. *septica* type strain), avian isolates also possessed the most divergent sequences within lineage A. Avian isolates were associated with seven 16S types in lineage A and six in lineage B. Whereas 16S types 1–3 and 5 (lineage A) were present in isolates from other host species, the remaining nine 16S types were unique to avian isolates. 16S types 8, 9, 12 and 15–19 were associated with capsule/OMP types B/12.1 (one isolate), A/4.1 (1), UT/5.1 (1), A/11.1 (1), UT/10.2 (1), UT/10.1 (1), A/14.1 (1) and A/7.1 (3), respectively. In contrast, avian isolates of 16S types 1, 2 and 13 were associated with capsule/OMP types A/2.1 (1), A/3.1 (1) and A/4.1 (1), A/1.1 (1), A/1.2 (1) and F/2.2 (3), and A/6.1 (1) and A/8.1 (1), respectively.

Bovine isolates. Bovine isolates were represented primarily by 16S types 1 (13 isolates), 2 (3), 3 (4) and 11 (3), although an additional three isolates were associated with 16S types 6, 7 and 10 (Fig. 2). 16S type 1 was associated with capsular type A isolates of OMP types 1.1 (two isolates), 3.1 (3), 4.1 (4), 5.1 (1), 8.1 (1) and 9.1 (2), whereas 16S type 2 was present in isolates of capsule/OMP types A/6.1 (2) and F/7.1 (1), and 16S type 3 occurred in capsular type A isolates of OMP types 1.1 (1) and 2.1 (3). 16S types 10 and 11 were associated with capsular type E (1) and B (3) isolates, respectively, which were isolated from cases of haemorrhagic septicaemia (the OMP types of these isolates were not determined).

Ovine isolates. Ovine isolates were represented primarily by 16S types 1 (five isolates) and 4 (4); a single isolate was associated with 16S type 2 (Fig. 2). 16S type 1 was associated with capsular type A isolates of OMP types 1.1 (2) and 1.2 (1) and capsular type D isolates of OMP type 3.1 (2), whereas 16S type 4 was present in capsular type F isolates of OMP types 1.1 (2) and 2.1 (2). The single 16S type 2 isolate was of capsular type F and OMP type 1.1.

Porcine isolates. Porcine isolates were represented by 16S types 1 (12 isolates), 2 (9) and 5 (5); a single isolate possessed 16S type 3 (Fig. 2). 16S type 1 was associated with capsular type A isolates of OMP types 1.1 (2), 1.2 (1), 2.1 (2), 3.1 (3), 3.2 (1) and 5.1 (3), whereas 16S type 2 was present in capsular type A isolates of OMP types 1.1 (3), 4.1 (1), 6.1 (1), capsular type D isolates of OMP types 4.1 (1) and 6.1 (1), a capsular type F isolate of OMP type 3.2 and an untypable isolate of OMP type 4.1, and 16S type 5 occurred in capsular type D isolates of OMP types 1.2 (1), 4.2 (1) and 6.1 (3).

Phylogenetic relationships of *P. multocida* isolates representing 19 16S types in relation to other *Pasteurella multocida* strains and *Pasteurella* species

The phylogenetic relationships of the 19 *P. multocida* 16S rRNA sequences obtained in the present study were compared to those from biovar 2 *Pasteurella avium* and *Pasteurella canis* isolates that have recently been reclassified as *P. multocida* (Christensen *et al.*, 2004) (Fig. 3). The sequences were also compared with those from three feline *P. multocida* subsp. *septica* isolates (Kuhnert *et al.*, 2000) and from the type strains of the related species *Pasteurella dagmatis*, *P. canis*, *Pasteurella stomatis*, *P. avium*, *Pasteurella gallinarum* and *Pasteurella volantium* (Korczak *et al.*, 2004) (Fig. 3). The sequences were aligned and compared over the same 1468 bp segment corresponding to 16S types 1–19. The dendrogram was constructed using the pairwise deletion option to handle missing data because the sequences were of unequal lengths. The biovar 2 *P. avium* and *P. canis* sequences (Christensen *et al.*, 2004) were identical and together formed a lineage that was most closely related to the cluster of isolates in lineage A represented by PM386 (16S type 1), PM548 (16S type 7) and PM338 (16S type 6) (Fig. 3). The three feline *P. multocida* subsp. *septica* sequences (Kuhnert *et al.*, 2000) were associated with lineage B and were most closely related to the 16S type 13 sequence of avian isolate PM152 (Fig. 3). The 16S rRNA sequences of the type strains of *P. dagmatis*, *P. canis* and *P. stomatis*, and *P. avium*, *P. gallinarum* and *P. volantium*, represented two distinct

Fig. 2. Neighbour-joining dendrogram representing the phylogenetic relationships of the 16S rRNA gene of 86 *P. multocida* isolates. Avian, bovine, ovine, porcine and feline isolates are highlighted in yellow, red, green, blue and pink, respectively. The strain numbers, capsular types and OMP types are provided for each isolate. The OMP types are unique to strains from each host species. Bootstrap values are shown adjacent to each node.

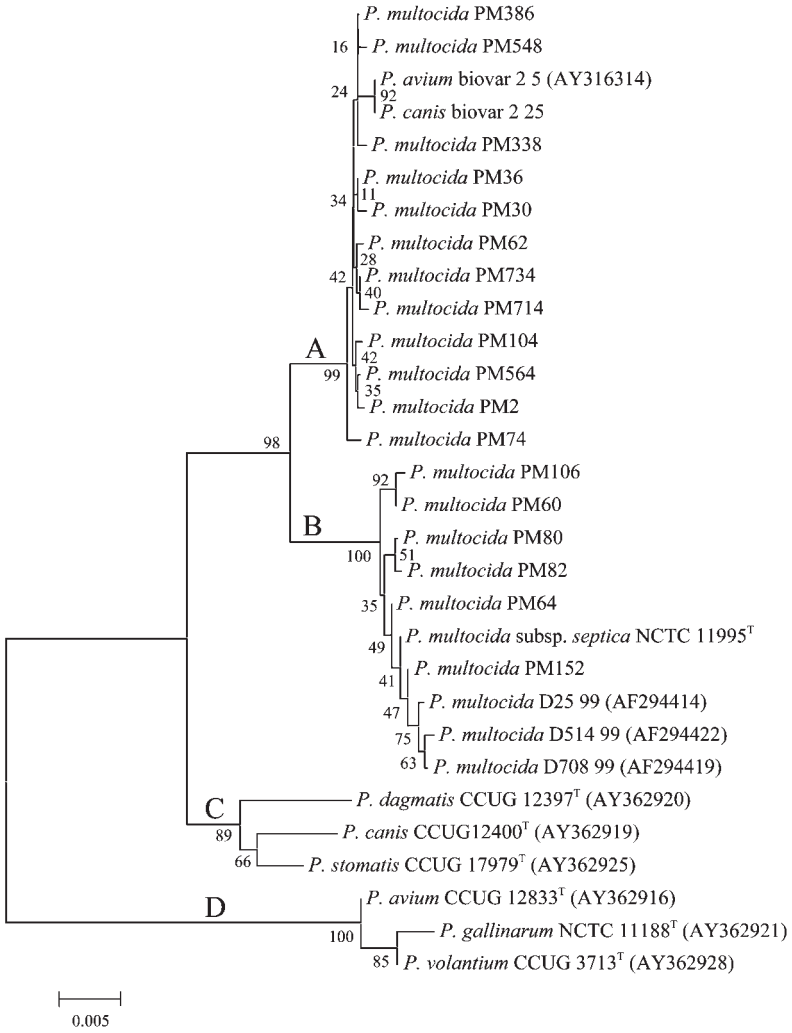


Fig. 3. Neighbour-joining dendrogram representing the phylogenetic relationships of the 16S rRNA gene of *P. multocida* strains of 16S types 1–19 in relation to the 16S rRNA gene of biovar 2 *P. avium* and *P. canis* isolates (Christensen *et al.*, 2004), feline *P. multocida* subsp. *septica* isolates (D25 99, D514 99, D708 99) (Kuhnert *et al.*, 2000), and other *Pasteurella* species (Korczak *et al.*, 2004). The dendrogram was constructed using the pairwise deletion option to handle missing data. Bootstrap values are shown adjacent to each node.

clusters, C and D, respectively. There were 28 (1·91 %) polymorphic nucleotide sites among the three sequences of lineage C, pairwise differences ranged from 14 to 25 (0·95 to 1·70 %), and the mean number of polymorphic nucleotide sites was $18{\cdot}67 \pm 3{\cdot}39$. In contrast, there were only 2 (0·14 %) polymorphic nucleotide sites among the three sequences of lineage D, pairwise differences ranged from 1 to 2 (0·07 to 0·14 %), and the mean number of polymorphic nucleotide sites was $1{\cdot}33 \pm 0{\cdot}96$.

DISCUSSION

Previous investigations of genetic diversity and relationships among *P. multocida* isolates have usually focused on isolates originating from a single host species (Amonsin *et al.*, 2002; Blackall *et al.*, 1998, 2000; Dabo *et al.*, 1999; Gardner *et al.*, 1994; Gunawardana *et al.*, 2000; Petersen *et al.*, 2001; Rimler, 2000; Wilson *et al.*, 1993; Zhao *et al.*, 1992). Furthermore, wider taxonomic studies of the *Pasteurellaceae* have included only small numbers of *P. multocida* isolates (usually the type strains), which are not necessarily representative of natural populations of the species (Dewhirst *et al.*, 1992; Korczak *et al.*, 2004; Mutters *et al.*, 1985; Olsen *et al.*, 2003). In the present study, a 1468 bp segment of the 16S rRNA gene was sequenced in 86 isolates of *P. multocida*, including the type strains of the three subspecies. Seventy-nine of these isolates were obtained from wide geographic regions of England and Wales and were selected to represent the majority of groups previously defined by variation of capsular polysaccharide and OMP types among avian, bovine, ovine and porcine isolates (Davies *et al.*, 2003a, b, c, 2004). The present study effectively represents a comparative analysis of over 446 *P. multocida* isolates from the four major domesticated species within England and Wales.

It has previously been suggested that patterns of dulcitol and sorbitol fermentation are indicators of the three *P. multocida* subspecies (Mutters *et al.*, 1985). However, a number of studies, mostly involving avian isolates but using a variety of different techniques, suggest that the three subspecies, particularly *P. multocida* subsp. *multocida* and *P. multocida* subsp. *septica*, do not represent distinct genotypic groups (Blackall *et al.*, 1998; Muhairwa *et al.*, 2001; Petersen *et al.*, 1998, 2001). In particular, Kuhnert

et al. (2000) demonstrated that only two of six isolates classified as *P. multocida* subsp. *septica* by 16S rRNA sequence determination gave a negative reaction for sorbitol fermentation. In the present study, 87 % of the isolates were classified as *P. multocida* subsp. *multocida* by dulcitol and sorbitol fermentation patterns, but these isolates have diverse 16S rRNA gene sequences that were represented in both lineages A and B (Table 1 and Fig. 2). These isolates also have very diverse OMP profiles and were associated with all four of the host species. Avian *P. multocida* subsp. *septica* isolates were associated exclusively with lineage B, and this finding is in agreement with that of Blackall *et al.* (1998) who placed all of their avian *P. multocida* subsp. *septica* isolates into a single group (ribotype cluster R1/MLEE cluster B). However, the presence of bovine *P. multocida* subsp. *septica* isolates within lineage A indicates that this phenotype is not restricted to avian isolates of lineage B. Avian *P. multocida* subsp. *multocida* isolates were also associated with lineage B, although these could be distinguished from the *P. multocida* subsp. *septica* isolates of the same lineage by differences in their capsule/OMP and 16S types. However, a wide range of genetically diverse avian *P. multocida* subsp. *multocida* isolates was also associated with lineage A. These observations clearly support the view that dulcitol and sorbitol fermentation are not accurate indicators of genetic relatedness among *P. multocida* isolates (Blackall *et al.*, 1998; Kuhnert *et al.*, 2000; Muhairwa *et al.*, 2001; Petersen *et al.*, 1998, 2001). In general, the existence of phenotypic variation in key characters of *P. multocida* (Christensen *et al.*, 2004) does not provide a logical basis for the use of phenotypic characterization in the classification of this species.

The possession of similar OMP profiles (Davies *et al.*, 2004) and identical 16S types (Table 1) by all the isolates classified as *P. multocida* subsp. *gallicida* (recovered from birds, cattle and pigs) provides strong evidence that they represent a more homogeneous and closely related group than isolates classified as *P. multocida* subsp. *multocida* and *P. multocida* subsp. *septica*. Ribotyping studies have similarly shown that both avian (Blackall *et al.*, 1998; Petersen *et al.*, 2001) and porcine (Bowles *et al.*, 2000) isolates of *P. multocida* subsp. *gallicida* represent well-defined groups that are genetically distinct from other isolates. Together, the OMP, 16S rRNA and fermentation data suggest that *P. multocida* subsp. *gallicida* isolates represent a distinct clone or clone complex within *P. multocida*. The possession of identical 16S types (i.e. 16S type 1) by isolates phenotypically classified as *P. multocida* subsp. *gallicida* and *P. multocida* subsp. *multocida* (Table 1) does not support the view that they represent distinct species, or even subspecies (Mutters *et al.*, 1985), and raises questions about the taxonomic status of those isolates that have more divergent 16S rRNA genes. In particular, serotype B and E isolates associated with haemorrhagic septicaemia of cattle and water buffaloes possess 16S types 11 and 10 (cluster A2), respectively, and are clearly more divergent from *P. multocida* subsp. *multocida* and *P. multocida* subsp. *gallicida* isolates of 16S type 1 (cluster A1) than the latter are to each other (Fig. 2).

The 16S rRNA sequence data confirmed the conclusions of previous studies that avian *P. multocida* isolates are extremely diverse (Blackall *et al.*, 1995, 1998; Carpenter *et al.*, 1991; Christiansen *et al.*, 1992; Davies *et al.*, 2003b; Gunawardana *et al.*, 2000; Petersen *et al.*, 2001; Snipes *et al.*, 1989; Wilson *et al.*, 1993, 1995). The data also show that avian isolates are substantially more diverse than bovine, ovine and porcine isolates (Fig. 2). The avian isolates were associated with 13 16S types in lineages A (seven) and B (six), whereas the bovine, ovine and porcine isolates were associated with seven, three, and four 16S types, respectively, in lineage A. The mean number of nucleotide differences was also substantially higher for the avian ($11{\cdot}75 \pm 2{\cdot}19$) isolates than for the bovine ($1{\cdot}12 \pm 0{\cdot}52$), ovine ($1{\cdot}09 \pm 0{\cdot}84$), and porcine ($0{\cdot}91 \pm 0{\cdot}59$) isolates. Possible reasons for the greater diversity of the avian strains have been discussed previously (Davies *et al.*, 2003b). There were also marked differences in the association of 16S types with the hosts of origin between strains of lineages A and B. The 16S types of lineage A were generally associated with a wide range of host species but, with a single exception, all of the 16S types of lineage B were recovered from birds. These observations suggest that avian, bovine, ovine and porcine isolates of lineage A have evolved from a common ancestor and have become adapted to different host species, whereas avian strains of lineage B have diverged from a different common avian ancestor and have remained highly adapted to birds.

There were strong correlations between 16S types and capsule/OMP types among isolates from each of the four host species, which suggests that isolates of the same capsule/OMP types are genetically related and represent distinct clones. In the case of the bovine isolates, the relationship between OMP type and 16S type shows partial agreement with data based on the concatenated sequences of seven housekeeping enzyme genes (Davies *et al.*, 2004). Isolates of OMP types 1.1 and 2.1, 3.1, 4.1 and 8.1, and 6.1 represent three distinct clusters for both housekeeping enzyme and 16S rRNA genes, but the housekeeping genes of isolates of OMP types 5.1, 5.3, 7.1 and 9.1 are significantly more divergent than the corresponding 16S rRNA genes (Davies *et al.*, 2004). The incongruent topologies for the 16S rRNA and concatenated housekeeping enzyme gene trees suggest that recombination has influenced the apparent phylogenies of these genes. Further evidence for recombination is provided by the association of the same OMP types with different 16S types for a given host. Recombination of the 16S rRNA gene has also been described in *Neisseria meningitidis* (Sacchi *et al.*, 2002) and *Streptococcus anginosus* (Schouls *et al.*, 2003). Sequence analysis of the slowly evolving 16S rRNA gene is incapable of detecting the finer genetic variation that exists between isolates from the same host species and which has previously been demonstrated by analysis of OMP profiles (Davies *et al.*, 2003a, b, c, 2004).

The presence of very different OMP profiles in isolates of the same 16S types indicates that cell-surface proteins are evolving more rapidly than the 16S rRNA gene. In particular, the presence of very different OMP profiles in strains of the same, or similar, 16S types, but from different host species, suggests a correlation between OMP diversification and host adaptation.

The capsular type B and E isolates associated with haemorrhagic septicaemia of cattle and water buffaloes have very similar 16S rRNA sequences (16S types 11 and 10, respectively) and are closely related to two avian capsular type A and B isolates (16S types 9 and 8, respectively). These isolates represent a distinct branch within lineage A (cluster A2) and have clearly diverged from those *P. multocida* isolates (cluster A1) that are associated with avian fowl cholera, pneumonia of cattle, sheep and pigs, and atrophic rhinitis of pigs (Table 1 and Fig. 2). These data represent the first 16S rRNA sequence comparison of capsular type B and E isolates and clearly demonstrate their phylogenetic position within the species *P. multocida*. The 16S type 8 isolate PM62 represents one of only four capsular type B (OMP type 12.1) isolates that were recovered from poultry (Davies *et al.*, 2003b). Capsular type B *P. multocida* isolates are generally recovered infrequently from birds (Rhoades & Rimler, 1987; Wilson *et al.*, 1993). The findings indicate that capsular type B isolates associated with haemorrhagic septicaemia of cattle and water buffaloes and those recovered from birds have common evolutionary origins. Capsular type F is also infrequently recovered from birds and other species (Rhoades & Rimler, 1987; Wilson *et al.*, 1993). However, with the exception of four ovine capsular type F isolates of 16S type 4, it was notable that all of the capsular type F strains examined in this study, which were isolated from all four host species, possessed 16S type 2 (Fig. 2). These findings suggest that capsular type F isolates associated with different host species also have common evolutionary origins.

Biovar 2 strains of *P. avium* and *P. canis* (formerly classified as Bisgaard Taxon 13) both gave positive reactions in a *P. multocida*-specific 23S rRNA-based PCR assay (Miflin & Blackall, 2001) and were recently reclassified as *P. multocida* by DNA–DNA hybridization and 16S rRNA sequence comparison (Christensen *et al.*, 2004). In the present study, the biovar 2 *P. avium* and *P. canis* strains were shown to be closely related to *P. multocida* isolates represented by 16S types 1 (PM386), 6 (PM338) and 7 (PM548) of lineage A (Fig. 3). Interestingly, isolates of 16S types 1, 6 and 7 are all associated with the bovine host, and this is also the case for biovar 2 strains of *P. avium* and *P. canis* (Christensen *et al.*, 2004). These findings not only provide support for the 23S rRNA-based PCR identification of *P. multocida* (Miflin & Blackall, 2001), but also provide clarification of the phylogenetic position of these strains within the species. Three 16S rRNA sequences representing feline *P. multocida* subsp. *septica* isolates (Kuhnert *et al.*, 2000) were shown to be closely related to avian *P. multocida* isolates and to the feline type strain of *P. multocida* subsp. *septica* of lineage B (Fig. 3). The emerging picture is that isolates representing lineage B of *P. multocida* are associated almost exclusively with birds and cats. Presumably, cats have acquired avian-adapted strains of *P. multocida* as a consequence of their predator–prey relationship.

The current subspecies nomenclature of *P. multocida* neither accurately reflects the 16S rRNA-based phylogenetic relationships among isolates nor does it adequately encompass the full range of diversity within the species. Kuhnert *et al.* (2000) proposed that 16S rRNA sequences, rather than sorbitol fermentation, should be used for the accurate identification of *P. multocida* subsp. *septica* isolates and their unambiguous differentiation from *P. multocida* subsp. *multocida* and *P. multocida* subsp. *gallicida*. Based on their relatively high 16S rRNA sequence divergence, these authors also suggested that isolates classified as *P. multocida* subsp. *septica* might be considered a separate species. The mean number of nucleotide differences ($21{\cdot}12 \pm 3{\cdot}90$) between the sequences of lineages A and B (*P. multocida* sequences) was marginally greater than the value ($18{\cdot}67 \pm 3{\cdot}39$) for lineage C (*P. dagmatis*, *P. canis* and *P. stomatis* sequences) but was substantially higher than the value ($1{\cdot}33 \pm 0{\cdot}96$) for lineage D (*P. avium*, *P. gallinarum* and *P. volantium* sequences) (Fig. 3). Together with the DNA–DNA hybridization data of Mutters *et al.* (1985) and the pronounced differences in host association of strains representing lineages A (birds, cattle, pigs and sheep) and B (birds and cats), these data provide strong support for the suggestion that lineage B (*P. multocida* subsp. *septica*) be reclassified as a separate species (Kuhnert *et al.*, 2000).

In conclusion, this study provides a 16S rRNA-based evolutionary framework for *P. multocida* isolates recovered from birds, cattle, pigs and sheep in England and Wales. Dulcitol and sorbitol fermentation patterns do not correlate with genetic relatedness, and the current subspecies nomenclature neither accurately reflects the 16S rRNA-based phylogenetic relationships among isolates nor adequately encompasses the full range of diversity within the species. *P. multocida* consists of two divergent lineages, A and B, and evidence is presented to support the suggestion that lineage B (*P. multocida* subsp. *septica*) might represent a separate species.

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