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Population structure and diversity of avian isolates of *Pasteurella multocida* from Australia

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A total of 110 isolates of *Pasteurella multocida* from Australian poultry and reference strains for the 16 somatic serovars plus the three subspecies (*gallicida*, *multocida*, *septica*) were analysed to examine their population structure and diversity. The 81 field isolates examined by multilocus enzyme electrophoresis (MLEE) were diverse, being divided into 56 electrophoretic types (ETs), with the 19 reference strains in another 15 ETs. The population was clonal and somatic serotyping was not particularly useful in establishing relationships between isolates. The 71 ETs formed three distinct subclusters (A, B and C) at a genetic distance of 0·36. Biovars tended to be associated with these subclusters: A with biovars 1, 3, 4, 5 and 8 and B with biovars 2, 6, 7, 9 and 10. Ribotyping, performed on all 110 isolates using *Hpa*II, recognized 21 ribotypes forming nine clusters (R1–R9). The isolates in ribotype cluster R1 were almost identical to those in MLEE cluster B. Using both MLEE and ribotyping, the 19 non-Australian reference strains were found to be distributed over the full diversity of the Australian isolates of *P. multocida*. This study has shown that a range of *P. multocida* clones are associated with fowl cholera in Australia and that many of the Australian isolates are similar to non-Australian reference strains. Both the MLEE results and the ribotyping data identified a previously unrecognized subset of *P. multocida* strains.

Keywords: *Pasteurella multocida*, multilocus enzyme electrophoresis, ribotype, genetic diversity

INTRODUCTION

Pasteurella multocida is the causative agent of fowl cholera, a common and widely distributed disease of poultry that is of major economic importance (Rimler & Glisson, 1997). There is considerable evidence that avian isolates of *P. multocida* are diverse. Using DNA–DNA hybridization, Mutters *et al.* (1985) recognized three subspecies within *P. multocida* (*multocida*, *septica* and *gallicida*). The distribution of these subspecies in various types of birds is known to differ, e.g. it has been reported that subsp. *gallicida* is more common in waterfowl and associated wild birds in California than it is in domestic turkeys (Hirsh *et al.*, 1990). There is also considerable serological diversity with most of the 16 Heddleston somatic serovars having been isolated from birds (Rhoades & Rimler, 1987). The molecular-based techniques of restriction endonuclease analysis and ribotyping have been used extensively to demonstrate considerable diversity in avian isolates of *P. multocida* (Kim & Nagaraja, 1990; Snipes *et al.*, 1990; Carpenter *et al.*, 1991; Christiansen *et al.*, 1992a, b; Wilson *et al.*, 1993, 1995; Blackall *et al.*, 1995).

While this evidence of diversity is well known, there has been no attempt to assemble an overview of the overall population structure of avian *P. multocida* isolates in relation to this diversity. Studies on population structure in other bacterial pathogens have provided considerable insights (Musser, 1996). Such studies show that particular subgroups or clones of a species are associated with virulence, e.g. in *Streptococcus agalactiae* (Quentin *et al.*, 1995) and *Enterococcus faecalis* (Tomayko & Murray, 1995). Typically, population structure studies

Abbreviations: ET, electrophoretic type; MLEE, multilocus enzyme electrophoresis; ODC, ornithine decarboxylase.

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Table 1. Properties of isolates used in this study

Study code*	Somatic serovar†	Biovar‡	Ribotype	Ribotype cluster	ET	MLEE cluster
PM63, 66	3	3	2	R5	A1	A
PM68	4 × 12	3	2	R5	A1	A
PM70	3	3	2	R5	A1	A
PM73	4	3	2	R5	A2	A
PM46	6	1	7	R4	A3	A
PM83	Non-typeable	1	12	R7	A4	A
PM71	4	1	20	R5	A5	A
PM37	3	1	12	R7	A6	A
PM35, 40, 85, 86	3	3	5	R3	A7	A
PM31	3	3	5	R3	A8	A
PM49	1 × 15	1	14	R6	A9	A
PM88	Non-typeable	1	3	R6	A10	A
PM45	3 × 4	3	5	R3	A11	A
PM87	3	3	5	R3	A11	A
PM120, 121	12	3	11	R5	A12	A
PM51	4 × 12	3	11	R5	A13	A
PM67	3 × 12	4	13	R7	A14	A
PM76	Non-typeable	5	9	R8	A15	A
PM64	3	5	9	R8	A16	A
PM65	4	5	9	R8	A16	A
PM96	3	4	13	R7	A17	A
PM48	3 × 4	3	2	R5	A18	A
PM27	3	3	2	R5	A19	A
PM52	3 × 4 × 12	3	2	R5	A20	A
PM94	Non-typeable	3	2	R5	A20	A
PM77	3	3	10	R2	A21	A
PM18	3	3	10	R2	A22	A
PM32	3	3	10	R2	A23	A
PM78	3	3	5	R3	A24	A
PM136	3	3	5	R3	A25	A
PM80	3 × 4 × 12	3	5	R3	A26	A
PM134	4 × 7	1	3	R3	A27	A
PM95	3	3	5	R3	A28	A
PM75	3 × 4 × 12 × 14	3	5	R3	A29	A
PM74	3 × 4 × 14	3	5	R3	A30	A
PM19	3	3	5	R3	A31	A
PM69	Non-typeable	3	5	R3	A32	A
PM132	4	3	2	R5	A33	A
PM97	Non-typeable	3	11	R5	A34	A
PM133	4	3	1	R1	A35	A
PM137	4 × 7	3	1	R1	A36	A
PM84	Non-typeable	1	12	R7	A37	A
PM2, 10	4	3	7	R4	A38	A
PM3	4 × 10 × 15	3	7	R4	A38	A
PM4	3	3	7	R4	A38	A
PM5, 6 , 7, 9, 11	Non-typeable	3	7	R4	A38	A
PM8	10	3	7	R4	A38	A
PM15	11 × 12	3	7	R4	A38	A
PM16	11 × 12 × 15	3	7	R4	A38	A
PM79	1	8	15	R2	A39	A
PM130	1	8	15	R2	A40	A
PM92	3	3	5	R3	A41	A
PM1	3 × 4	2	6	R1	A42	B
PM12	3 × 4	2	6	R1	A43	B
PM13, 14	3	2	6	R1	A43	B
PM91	3	2	6	R1	A44	B
PM17	3	2	7	R4	A45	B
PM141	3	1	4	R1	A46	B
PM135	13	10	4	R1	A47	B

Table 1. (cont.)

Study code*	Somatic serovar†	Biovar‡	Ribotype	Ribotype cluster	ET	MLEE cluster
PM140	13 × 14 × 15	7	4	R1	A48	B
PM44	1	9	4	R1	A49	B
PM36	14	9	4	R1	A50	B
PM90, 93	Non-typeable	9	4	R1	A50	B
PM72	3 × 14	6	4	R1	A51	B
PM81	Non-typeable	6	4	R1	A52	B
PM139	1 × 15	2	4	R1	A53	B
PM138	1 × 15	2	4	R1	A54	B
PM131	1	2	4	R1	A55	B
PM82	1 × 15	8	15	R2	A56	C
ATS16	16	3	18	R9	R1	A
ATS15	15	2	19	R3	R2	A
ATS10	10	3	11	R5	R3	A
ATS4	4	3	7	R4	R4	A
ATS13	13	1	16	R7	R5	A
ATS14	14	11	16	R7	R5	A
ATS1	1	1	16	R7	R6	A
ATS5	5	3	18	R9	R7	A
ATS3	3	3	5	R3	R8	A
ATS6	6	1	21	R4	R9	A
ATS11	11	1	11	R5	R10	A
ATS12	12	3	19	R3	R10	A
ATS2	2	3	8	R3	R11	A
P. multocida subsp. *multocida*	NK	11	17	R6	R12	A
P. multocida subsp. *gallicida*	NK	2	13	R7	R13	A
ATS7	7	7	4	R1	R14	B
ATS8	8	7	4	R1	R14	B
ATS9	9	2	4	R1	R14	B
P. multocida subsp. *septica*	NK	7	6	R1	R15	B
PM204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214	NK	3	2	R5	NK	NK
PM89	Non-typeable	9	4	R1	NK	NK
PM119	3	3	5	R3	NK	NK
PM147	7	3	5	R3	NK	NK
PM149, 150	3	3	5	R3	NK	NK
PM224	NK	3	5	R3	NK	NK
PM225	NK	3	5	R3	NK	NK
PM226	NK	3	5	R3	NK	NK
PM142	3	2	6	R1	NK	NK
PM146	7	5	6	R1	NK	NK
PM148	1 × 10 × 12	5	6	R1	NK	NK
PM145	NK	1	7	R4	NK	NK
PM144	NK	2	8	R3	NK	NK
PM221, 222, 223	NK	3	10	R2	NK	NK
PM143	NK	8	15	R2	NK	NK
PM151	1 × 15	8	15	R2	NK	NK

* Australian field isolates were given PM codes. The somatic serovar reference strains were given ATS codes where ATS1 means the reference strain for somatic serovar 1.

† Cross reactions are indicated by ×, e.g. 3 × 4 indicates cross-reacting with serovars 3 and 4. NK, not known.

‡ Biovars as defined by Fegan *et al.* (1995).

are based on the technique known as multilocus enzyme electrophoresis (MLEE) (Musser, 1996). As well as insights into pathogenicity, MLEE studies have also provided considerable new knowledge on the epidemiology of disease outbreaks associated with a range of bacteria, e.g. *Mycobacterium avium* (Feizabadi *et al*., 1996) and *Neisseria meningitidis* (Weis & Lind, 1996).

In this study, we have described a polyphasic approach that attempts to generate an overall view of the population structure of a collection of avian *P. multocida* isolates. We analysed an extensive collection of Australian avian isolates plus type strains for the three subspecies and 16 somatic serovars using a range of techniques – extended phenotypic characterization, serological characterization, MLEE and ribotyping.

METHODS

Bacteria. A total of 129 isolates of *P. multocida* were used in this study. They consisted of 110 isolates from Australian poultry, all previously characterized phenotypically (Fegan *et al*., 1995) and 19 reference strains. The Australian field isolates have been assigned to one of 10 biovars as well as one of the three subspecies (*multocida*, *septica* and *gallicida*) on the basis of ornithine decarboxylase (ODC) activity and ability to ferment six carbohydrates (arabinose, dulcitol, maltose, sorbitol, trehalose and xylose) (Fegan *et al*., 1995). The isolates were serotyped by the Heddleston somatic serotyping scheme (Heddleston *et al*., 1972). The Australian field isolates were obtained from chickens (44 isolates), turkeys (36 isolates) and a duck (one isolate), all having been diagnosed as suffering from fowl cholera. The isolates were collected over the period 1966–1994. The 19 reference strains consisted of the 16 reference strains for the Heddleston somatic serotyping scheme and the reference strains for the three subspecies of *P. multocida* – NCTC 10204 (*P. multocida* subsp. *gallicida*), NCTC 10322 (*P. multocida* subsp. *multocida*) and CIP A125 (*P. multocida* subsp. *septica*). The attributes of the *P. multocida* isolates and reference strains are listed in Table 1. All 19 reference strains were used in the restriction endonuclease analysis, ribotyping and MLEE work. While all 110 field isolates were used in the ribotyping study, only 81 of the field isolates were analysed in the MLEE study.

Bacterial growth, enzyme preparation and DNA extraction. Isolates were grown overnight at 37 °C in Brain Heart Infusion broth (BBL). Following this incubation, samples were plated onto blood agar to check for contamination. The bacteria were harvested by centrifugation (15000 ***g***, 20 min, 4 °C). For MLEE, the bacterial pellet was resuspended in PBS (pH 7·2), centrifuged again and the resultant pellet resuspended in 2 ml sonication buffer (10 mM Tris/HCl, 1 mM EDTA, 0·5 mM NADP, pH 6·8). The bacteria were lysed by three 1 min cycles of sonication on ice with 30 s cooling periods. The cell debris was removed by centrifugation (25000 ***g***, 20 min, 4 °C) and the supernatant was stored at −70 °C until used. For the restriction endonuclease analysis and ribotyping study, the bacterial pellet was washed in 5 ml SE buffer (150 mM NaCl, 100 mM EDTA, pH 8) and duplicate cell pellets were retained and held at −20 °C until required for extraction. DNA extraction was performed by a standard methodology as described previously (Blackall *et al*., 1995). DNA concentration and purity were determined spectrophotometrically.

MLEE. The bacterial lysates were electrophoresed in 14% horizontal starch gels (Selander *et al*., 1986). The following 18 enzymes displayed strong activity and good resolution, enabling mobility comparisons to be made between isolates: adenylate kinase (ADK), arginine phosphokinase (APK), catalase (CAT), esterase (EST), fumerase (FUM), glucose-6-phosphate dehydrogenase (G6PD), NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (GP), glutamate–oxaloacetate transaminase (GOT), NADP-dependent glutamate dehydrogenase (GDH), hexokinase (HEX), L-leucyl-L-tyrosine peptidase (LT), malate dehydrogenase (MDH), malic enzyme (ME), mannose-6-phosphate isomerase (MPI), nucleoside phosphorylase (NP), phosphoglucomutase (PGM), phosphoglucose isomerase (PGI) and 6-phosphogluconate dehydrogenase (6PGD).

Analysis of MLEE data. The banding patterns were consistent with a single locus for each enzyme and mobility variants were interpreted as the products of different alleles at the corresponding locus. Groups of one or more isolates with the same alleles at all loci were referred to as being an electrophoretic type (ET). Genetic diversity (h) at each enzyme locus was calculated as $h = (1-\mathrm{S}P_i^2)[n/(n-1)]$, where P_i is the frequency of the ith allele and n is the number of ETs or isolates in the sample (Nei, 1978). Total genetic diversity (H) was calculated as the mean of h over all loci. Diversity among isolates of serovar 3 was also calculated. Genetic distances between ETs were calculated as the proportion of fixed loci at which dissimilar alleles occurred and the unweighted pair-group method of arithmetic means clustering fusion strategy was used to create a phenogram to show the relationships between isolates (Sneath & Sokal, 1973).

The matrix of coefficients used for allelic mismatches between pairs of isolates was also used for calculating the index of association (I_A). This index, which describes multilocus linkage disequilibrium in bacterial populations (Maynard Smith *et al*., 1993), is significantly different from zero for a clonal population. I_A was calculated for the 56 ETs containing Australian isolates.

Ribotyping. Total DNA (5 mg) was digested with *Hpa*II (Boehringer Mannheim) in Tris/acetate buffer (O'Farrell *et al*., 1980) using 6·25 U enzyme per 50 µl reaction. The reaction was performed at 37 °C for 3 h and terminated by heating at 65 °C for 20 min. DNA fragments were separated by horizontal electrophoresis through a 0·7% agarose gel at 25 V for 16 h in Tris/Borate/EDTA electrophoresis buffer (Sambrook *et al*., 1989). DIG-labelled DNA Molecular Weight Marker II (Boehringer Mannheim) was used as a marker. The resultant fragment pattern was visualized under UV light and photographed.

After photography, DNA from the agarose gel was transferred to a positively charged nylon membrane (Boehringer Mannheim) with the VacuGene XL Vacuum Blotting System (Pharmacia) according to the manufacturer's instructions. DNA was bound to the membrane by UV cross-linking. The probe consisted of a PCR-generated 16S rDNA fragment of the type strain of *P. multocida* subsp. *multocida* (NCTC 10322) amplified with the universal primers 27f and 1525r (Lane, 1991). This represents an almost complete copy of the double-stranded 16S rRNA gene sequence of *P. multocida* subsp. *multocida* NCTC 10322. Before use the probe was labelled with DIG using the DIG DNA Labelling Kit (Boehringer Mannheim). The probe was used at a concentration of 15–20 ng (ml hybridization solution)$^{-1}$.

Southern hybridization and detection were performed using the Nucleic Acid Detection Kit (Boehringer Mannheim) at a temperature of 68 °C for both pre-hybridization and hybridization steps and post-hybridization washes.

Analysis of ribotyping patterns. Fragment patterns were compared visually and distinct patterns were designated as ribotypes. All bands present in the population were identified and each ribotype assigned a band profile with a value of 1 or 0 corresponding to the presence or absence of each band in the population, respectively. A matrix describing the designated band profiles for each ribotype was converted to ASCII file data and imported into the NTSYS computer program. A similarity matrix was constructed according to the Dice co-efficient using the SIMQUAL function of the NTSYS program. A phenogram of genetic similarity was constructed according to the UPGMA clustering strategy using the SAHN clustering and TREE DISPLAY functions of NTSYS.

RESULTS

MLEE data

Only four enzymes (CAT, FUM, GP and HEX) were monomorphic. A total of 71 ETs were identified and the assignment of the isolates to these ETs is shown in Table 1. Australian field isolates were present in 56 ETs and the reference strains were located in 15 ETs. The mean number of alleles per enzyme locus was 3·39. The mean genetic diversity per locus (h) was calculated as 0·302 in relation to the number of ETs or 0·289 when the number of isolates in each ET was included in the calculations. The most variable enzyme, EST, had an h value of 0·871. Estimates of genetic relatedness are presented in the phenogram shown in Fig. 1. The phenogram revealed the existence of three distinct subclusters (A, B and C) separated by a genetic distance of 0·36. MLEE cluster C was represented by a single ET containing one Australian field isolate. A summary of the properties of the isolates within each of these clusters is provided in Table 2.

I_A was calculated as $0{\cdot}982 \pm 0{\cdot}011$ ($P < 0{\cdot}05$) for the 56 ETs containing Australian field isolates. This was significantly different from zero, suggesting that the population was not undergoing significant horizontal gene flow and was clonal.

Ribotype data

Ribotyping of the *P. multocida* isolates produced well resolved patterns that allowed distinctions to be made between strains (Fig. 2). A total of 21 different patterns or ribotypes, possessing between three and five bands each, were recognized. The allocation of the isolates to the various ribotype patterns is detailed in Table 1.

Estimates of genetic relatedness, based on the ribotyping results, are presented in the phenogram shown in Fig. 3. Nine major clusters (R1–R9) are apparent at a genetic similarity of 0·6. The properties of these nine clusters are summarized in Table 3.

Correlation between serovars, biovars, geographical origin and ET designation

No isolates of biovar 3, which was by far the most common biovar (47/81 field isolates and 7/19 reference stains) were located in MLEE cluster B and only a single isolate of biovar 1 was present in this cluster. Indeed, the biovars appeared to be grouped in specific clusters, with biovars 1, 3, 4, 5 and 8 in MLEE cluster A and biovars 2, 6, 7, 9 and 10 in MLEE cluster B.

In terms of the recognized subspecies of *P. multocida*, the MLEE analysis placed both field isolates and the three reference strains belonging to subsp. *septica* (biovars 7 and 10) and all 13 field isolates belonging to subsp. *multocida* that produce acid from sorbitol, trehalose and xylose regardless of ODC reaction (biovars 2 and 9) in MLEE cluster B. However, only one of the three reference strains that belonged to biovar 2 were allocated to MLEE cluster B, the other two being in MLEE cluster A.

In contrast, there appeared to be no obvious division of serovars between MLEE clusters A and B. Serovar 3 was the most common serovar overall and was the most common serovar in both MLEE clusters A and B. Only serovar 3 was represented by enough isolates to allow an estimation of genetic diversity (0·286).

The 15 ETs containing the 19 reference strains did not include any Australian field isolates. The 16 somatic reference strains were distributed across the diversity of the ET phenogram. The three ETs formed by the type strains for the three subspecies of *P. multocida* (ET R12, ET R13 and ET R15) formed distinct subclusters within MLEE clusters A and B.

ETs and ribotypes

There was a strong correlation between the MLEE and ribotyping results. All 81 field isolates and 19 reference strains used in the MLEE work were included in the ribotyping study. Within the nine ETs that consisted of more than one isolate (1, 7, 11, 12, 16, 20, 38, 43 and 50), all members were always allocated to a single ribotype.

At the level of the MLEE and ribotype clusters there was also good agreement. Ribotype cluster R1 contained 17 of the 18 field isolates and all four reference strains assigned to MLEE cluster B. Ribotype cluster R1 also contained only 2 of the 63 field isolates and none of the 15 reference strains assigned to MLEE cluster A. The remaining ribotype clusters (R2–R9) contained 61 of the 63 MLEE cluster A field isolates, the sole remaining MLEE cluster B field isolate and all 15 reference strains assigned to MLEE cluster A. The groupings of strains by ribotype clusters R2–R9 did not closely match the groupings within MLEE cluster A. As an example, isolates in ribotype cluster R3 were located in ETs A7, A8, A11, A24, A25, A26, A28, A29, A30, R8, R10 and R11.

Serovars, biovars, geographical origin and ribotypes

There was no obvious correlation between the serovars and ribotype clusters R1–R9. Within the 15 ribotypes that contained at least two serotyped isolates, all 15 contained at least two serovars, with ribotype 2 containing 11 different serovars.

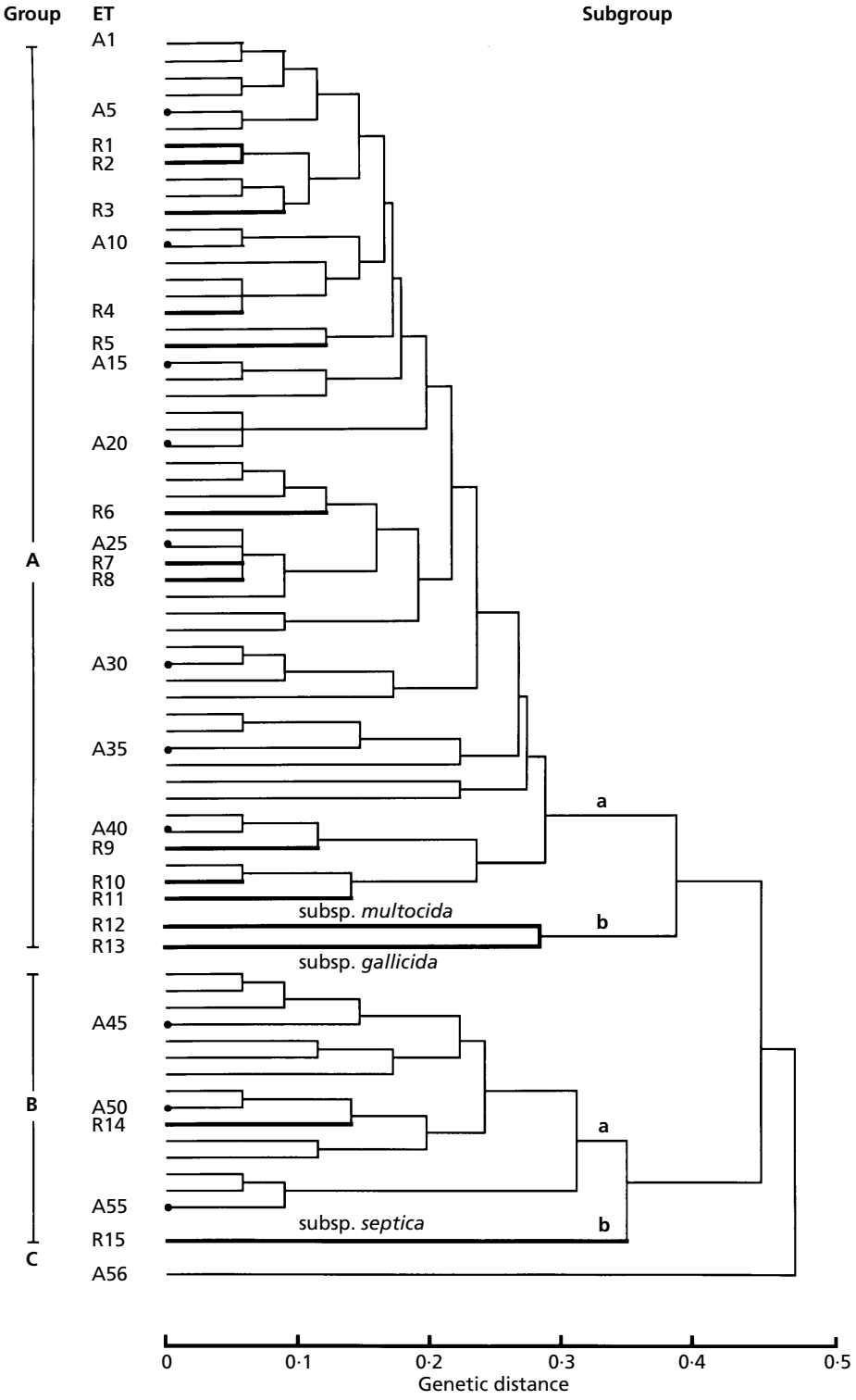

Fig. 1. Phenogram of genetic distance amongst 71 ETs of poultry isolates of *P. multocida* clustered by the UPGMA strategy. ETs marked A contain Australian field isolates; ETs marked R contain non-Australian reference strains for the 16 somatic serovars and the three subspecies *gallicida, multocida* and *septica*.

The 12 multi-isolate ribotypes in clusters R2–R9 contained only a single biovar, with two exceptions. One exception was strain PM17 in ribotype 17 which was biochemical biovar 2, while the 12 other members of the ribotype were biovar 3. The other exception was strain PM144 in ribotype 8 which was biovar 2, while the only

Table 2. Properties of *P. multocida* MLEE clusters

MLEE cluster	No. of isolates	No. of ETs	Biovar/subspecies*	Serovar*
A	62	41 (field isolates)	1 (8), 3 (46), 4 (2)/ *multocida* (56)	1 (2), 3 (22), 4 (7), 6 (1), 10 (1), 12 (2)
		13 (reference strains)	5 (3)/??? (3)	Cross-reacting with 3 and/or 4 and others (11)
			8 (3)/*gallicida* (3)	Cross-reacting with serovars other than 3 or 4 (4)
				Non-typeable (12)
B	18	14 (field isolates)	1 (1), 2 (8), 3 (1), 9 (4)/ *multocida* (14)	1 (1), 3 (5), 13 (1), 14 (1)
		2 (reference strains)		Cross-reacting with 3 and/or 4 and others (4)
			6 (2)/??? (2)	Cross-reacting with serovars other than 3 or 4 (3)
			7 (1),10 (1)/*septica* (2)	Non-typeable (3)
C	1	1 (field isolate)	2 (1)/*multocida* (1)	1 (1)

* The number of isolates is given in parentheses. ???, cannot be assigned to any of the three recognized subspecies of *P. multocida*.

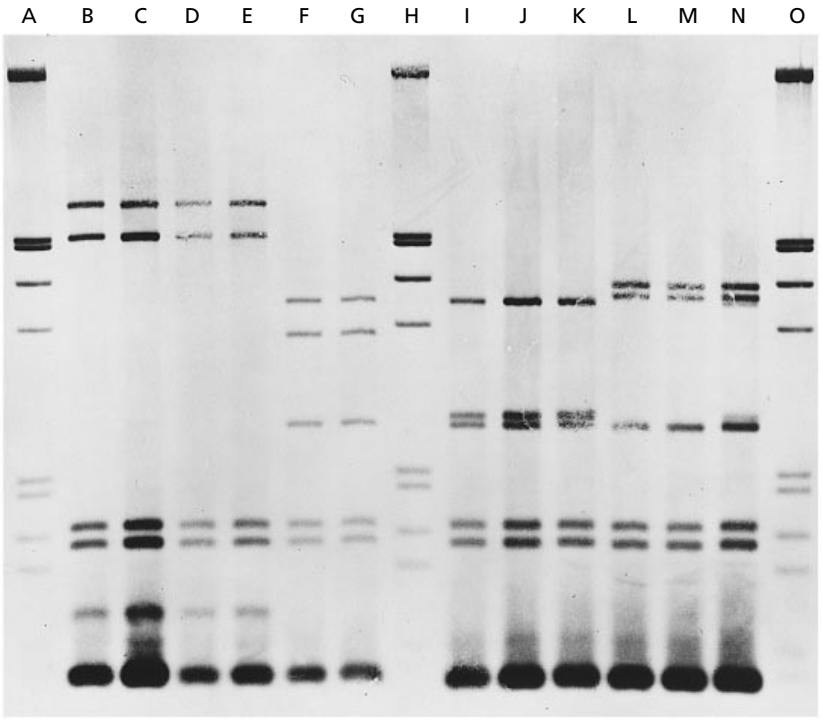

Fig. 2. Examples of ribotyping profiles generated using *Hpa*II. Lanes: A, H and O, molecular mass marker; B, PM73; C, PM132; D, PM48; E, PM52; F, PM44; G, PM139; I, PM1; J, PM12; K, PM146; L, PM2; M, PM3; N, PM17.

other member of the ribotype was the reference strain for serovar 2 which was biovar 3.

In contrast, both ribotypes 4 and 6 within ribotype cluster R1 contained multiple biovars. As an example, ribotype 4 contained six different biochemical biovars (1, 2, 6, 7, 9 and 10) while ribotype 6 contained three different biovars (2, 5 and 7).

The predominant biovar, biovar 3, was virtually absent from ribotype cluster R1, with only two of the 68 field isolates of this biovar and none of the seven reference strains occurring in this cluster. Cluster R1 also contained the sole field isolate and all three reference strains of biovar 7 and the sole field isolate of biovar 10. As both of these biovars represent subsp. *septica*, this means that cluster R1 contained all the isolates and reference strains of this rare subspecies. Also, cluster R1 contained all five field isolates of biovar 9, which were ODC-negative variants of subsp. *multocida* that produce acid from sorbitol, trehalose and xylose. Interestingly, all nine field isolates of subsp. *multocida* that produced acid from sorbitol, trehalose and xylose but were ODC-positive

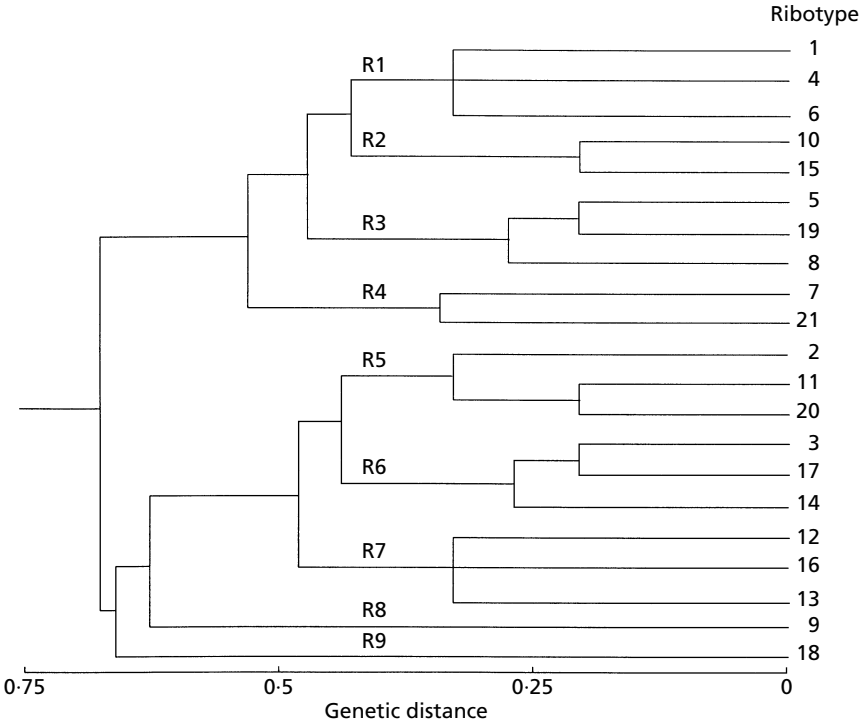

Fig. 3. Phenogram of genetic distance amongst ribotypes of 110 poultry isolates of *P. multocida* clustered by the UPGMA strategy. Nine major ribotype clusters (R1–R9) are marked. Full details are provided in Table 1.

(i.e. biovar 2) were also placed in ribotype cluster R1. However, only one of the three reference strains assigned to biovar 2 was placed in ribotype cluster R1.

The Australian field isolates of biovar 8 (subsp. *gallicida*) were quite homogeneous with all five strains falling into a single ribotype (ribotype 15 within cluster R2). This was quite different from the reference strain for this subspecies which was the sole member of ribotype 17 within cluster R6.

The 19 non-Australian isolates of *P. multocida* were distributed throughout the 21 ribotypes recognized in this study. Ribotype cluster R9 was the only one to consist solely of non-Australian isolates, while ribotype clusters R2 and R8 were the only ones to consist solely of Australian isolates. Nine of the 19 non-Australian strains formed unique ribotypes that did not contain any Australian isolates: the reference strains for serovars 1, 3 and 14 forming ribotype 16, the reference strain for subsp. *gallicida* forming ribotype 17, the reference strains for serovars 5 and 16 forming ribotype 18, the reference strains for serovars 12 and 15 forming ribotype 19 and the reference strain for serovar 6 forming ribotype 21.

DISCUSSION

This study was undertaken to develop an understanding of the population structure of Australian isolates of *P. multocida* collected from Australian poultry. Selected reference strains from outside Australia were used to validate the results of the study.

Overall, the genetic diversity of the isolates as detected by MLEE (0·302) was consistent with the results for other species of animal-pathogenic bacteria. For example, the diversity is somewhat lower than that reported for such non-avian veterinary bacterial pathogens as *Streptococcus suis* (0·512) (Hampson *et al*., 1993b) and *Escherichia coli* (0·47) (Woodward *et al*., 1993), similar to that seen in the related porcine bacterial pathogen *Actinobacillus pleuropneumoniae* (0·312) (Hampson *et al*., 1993a) and greater than that seen in another porcine pathogen, *Serpulina hyodysenteriae* (0·260) (Lee *et al*., 1993).

To date, the only published genetic diversity studies on avian bacterial pathogens have been on *Escherichia coli*. These studies (Whittam & Wilson, 1988; White *et al*., 1993a, b) have reported broadly similar genetic diversity figures (0·30, 0·355 and 0·37) to those obtained here for Australian avian *P. multocida* isolates.

Of the multiplicity of serovars included in the MLEE study, only serovar 3 was present in sufficient number to warrant a meaningful analysis. The diversity present in this serovar (0·286) approached that for the whole collection, demonstrating that serotyping is not useful in establishing relationships between isolates of avian *P. multocida*.

Of the two main clusters recognized by MLEE, biovar 3 field isolates and reference strains were virtually absent from cluster B. As biovar 3 is by far the most common biochemical type of *P. multocida* present in Australian poultry (Fegan *et al*., 1995), this absence of biovar 3 from cluster B is noteworthy. The association of biovar 3 with cluster A suggests that there is some underlying genetic background responsible for this grouping.

Considerable diversity was recognized by ribotyping with a total of 21 ribotype patterns being recognized. The level of subdivision achieved with ribotyping was

Table 3. Properties of the *P. multocida* isolates in the ribotype clusters

Ribotype cluster	No. of isolates*	Serovar†	Ribotype	ET	ET cluster†	Biovar†	Subspecies†
R1	27 (4)	1 (2), 3 (5), 4 (1), 7 (2), 8 (1), 9 (1), 13 (1), 14 (1)	1, 4, 6	A35–36,	A (2)	1 (1)	*multocida* (18)
		Cross-reacting with 3 and/or 4 and others (4)		A42–44,	B (21)	2 (10)	*septica* (5)
		Cross-reacting with serovars other than 3 or 4 (4)		A46–55		3 (2)	??? (4)‡
		Non-typeable (4)		R14–15		9 (5)	
						5 (2)	
						6 (2)	
						7 (4)	
						10 (1)	
R2	11 (0)	1 (2), 3 (3)	10, 15	A21–23,	A (5)	3 (6)	*multocida* (6)
		Cross-reacting with serovars other than 3 or 4 (2)		A39–40, A56	C (1)	8 (5)	*gallicida* (5)
R3	28 (4)	2 (1), 3 (15), 7 (1), 12 (1), 15 (1)	5, 8, 19	A7–8, A11,	A (20)	2 (2)	*multocida* (28)
		Cross-reacting with 3 and/or 4 and others (4)		A24–26, A28,		3 (26)	
		Non-typeable (1)		A29–32, A41			
				R2, R8,			
				R10, R11			
R4	17 (2)	3 (2), 4 (3), 6 (2)	7, 21	A3, A38, A45	A (15)	1 (3)	*multocida* (17)
		Cross-reacting with 3 and/or 4 and others (1)		R4, R9	B (1)	2 (1)	
		Cross-reacting with serovars other than 3 or 4 (2)				3 (13)	
		Non-typeable (5)					
R5	28 (2)	3 (4), 4 (3), 10 (1), 11 (1), 12 (2)	2, 11, 20	A1–2, A5,	A (17)	1 (2)	*multocida* (28)
		Cross-reacting with 3 and/or 4 and others (4)		A12–13,		3 (26)	
		Non-typeable (4)		A18–20,			
				A33–34			
				R3, R10			
R6	4 (1)	Cross-reacting with 3 and/or 4 and others (1)	3, 14, 17	A9–10, A27	A (4)	1 (3)	*multocida* (3)
		Cross-reacting with serovars other than 3 or 4 (1)		R13		11 (1)	*gallicida* (1)
		Non-typeable (1)					
R7	9 (4)	1 (1), 3 (2), 13 (1), 14 (1)	12, 13, 16	A4, A6, A14,	A (9)	1 (5)	*multocida* (8)
		Cross-reacting with 3 and/or 4 and others (1)		A17, A37		2 (1)	*gallicida* (1)
		Non-typeable (2)		R5–6, R12		4 (2)	
						11 (1)	
R8	3 (0)	3 (1), 4 (1)	9	A15–16	A (3)	5 (3)	??? (3)‡
		Non-typeable (1)					
R9	2 (2)	5 (1), 16 (1)	18	R1, R7	A (2)	3 (2)	*multocida* (2)

* The number of non-Australian reference strains is given in parentheses.

† The number of strains in the serovar, MLEE cluster, biovar or subspecies is given in parentheses.

‡???, cannot be assigned to any of the three recognized subspecies of *P. multocida*.

less than that obtained using MLEE on the subset of strains examined by both techniques (56 ETs recognized in field isolates and 15 ETs in the reference strains). This failure of ribotyping to yield as much subtyping information as MLEE is unusual as previous studies on other bacterial species have reported that ribotyping is capable of producing a finer typing of strains than MLEE (Woods *et al*., 1992; Ng & Dillon, 1993). Overall, the ribotyping results support the finding of the MLEE study that a diverse range of *P. multocida* strains are

associated with fowl cholera in Australian poultry. Although diverse, this population was also clonal with diverse ETs evolving during the course of descent rather than by convergence through extensive lateral gene flow (Maynard Smith *et al*., 1993; Musser, 1996). Thus the I_A was significantly different from zero, suggesting that recombination between the Australian field isolates was not common enough to generate a random assortment of alleles.

In general, the results of the cluster analysis performed on the ribotype patterns supported the clusters recognized in the MLEE study. In particular, ribotype cluster R1 was a very close match to MLEE cluster B. Hence, by two different methods, these groups of *P. multocida* isolates have been shown to be closely related to each other and distinguishable from other isolates. There are several unusual aspects to the organisms that make up the complex formed by ribotype cluster R1/MLEE cluster B. First, there is the virtual absence of biovar 3 isolates from the group, despite this biovar being by far the most common biovar amongst Australian isolates of *P. multocida* from poultry (Fegan *et al*., 1995). Also, biovar 3 was the most common biovar amongst the non-Australian reference strains of *P. multocida* (7/19), yet none of four non-Australian isolates of *P. multocida* assigned to ribotype cluster R1 were biovar 3. Second, both field isolates and all three reference strains of subsp. *septica* (biovars 7 and 10) were placed in this complex. These five isolates were quite diverse in their origin, consisting of two serovar reference strains (ATS7 and 8), the type strain for subsp. *septica*, an Australian isolate that has the typical properties of subsp. *septica* and another Australian isolate that is unusual in that it is a maltose-positive variant of subsp. *septica*. A third unusual aspect of this complex of organisms is the presence of all five ODC-negative variants of subsp. *multocida* (biovar 9). Biovar 9 only differs from biovar 2 in being ODC-negative, both biovars being capable of producing acid from sorbitol, trehalose and xylose. Ribotyping and MLEE placed all field isolates of both of these biovars (2 and 9) together in the single complex.

Overall, the organisms of ribotype cluster R1/MLEE cluster B make up a biochemically and serologically diverse group that are notable for the unusual biochemical patterns and the absence of the predominant biovar 3. Despite this biochemical and serological diversity, both MLEE and ribotyping have clustered these organisms together, suggesting that they have a common genetic background.

The 19 reference strains were distributed across the full diversity of the Australian isolates of *P. multocida*. Furthermore, as 10 of the 19 strains were allocated to ribotypes that also contained Australian isolates, Australian isolates of *P. multocida* are unlikely to be markedly different from those found in other countries.

In summary, this study has demonstrated for the first time that avian *P. multocida* isolates form a clonal population. Within this structure, both MLEE and ribotyping have identified a subgroup of *P. multocida* that have unusual biochemical properties. This subgrouping cuts across the currently recognized subspecies within *P. multocida* (*gallicida*, *multocida* and *septica*). We have also shown for the first time that somatic serotyping is not a useful means of establishing relationships between isolates of *P. multocida*.

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