Genetic Organization of *Pasteurella multocida* cap Loci and Development of a Multiplex Capsular PCR Typing System†

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Current serotyping methods classify *Pasteurella multocida* into five capsular serogroups (serogroups A, B, D, E, and F) and 16 somatic serotypes (serotypes 1 to 16). In the present study, we have developed a multiplex PCR assay as a rapid alternative to the conventional capsular serotyping system. The serogroup-specific primers used in this assay were designed following identification, sequence determination, and analysis of the capsular biosynthetic loci of each capsular serogroup. The entire capsular biosynthetic loci of *P. multocida* A:1 (X-73) and B:2 (M1404) have been cloned and sequenced previously (J. Y. Chung, Y. M. Zhang, and B. Adler, FEMS Microbiol. Lett. 166:289–296, 1998; J. D. Boyce, J. Y. Chung, and B. Adler, Vet. Microbiol. 72:121–134, 2000). Nucleotide sequence analysis of the biosynthetic region (region 2) from each of the remaining three serogroups, serogroups D, E, and F, identified serogroup-specific regions and gave an indication of the capsular polysaccharide composition. The multiplex capsular PCR assay was highly specific, and its results, with the exception of those for some serogroup F strains, correlated well with conventional serotyping results. Sequence analysis of the strains that gave conflicting results confirmed the validity of the multiplex PCR and indicated that these strains were in fact capsular serogroup A. The multiplex PCR will clarify the distinction between closely related serogroups A and F and constitutes a rapid assay for the definitive classification of *P. multocida* capsular types.

*Pasteurella multocida* is a heterogeneous species that produces septicemic or respiratory diseases in domesticated and wild animals (12). Considerable variation has been observed among strains with respect to host predilection, pathogenicity, carbohydrate fermentation, colonial morphology, and antigenic specificity (6). Serological differences were also observed in the type-specific capsular antigen of *P. multocida* identified by Carter (4). This resulted in the development of the indirect hemagglutination test, which now recognizes five distinct capsular serogroups (serogroups A, B, D, E, and F) (12).

Until recently, little was known of the composition of capsular material from *P. multocida* serogroups other than that of serogroup A, which is known to be sensitive to the action of hyaluronidase (3, 5). Nuclear magnetic resonance studies confirmed that the major polysaccharide component of the capsule was hyaluronic acid (13). The capsular material of serogroups D and F has been identified primarily through the action of mucopolysaccharidasises (11). On the basis of the decapsulation profiles of *P. multocida* by these enzymes, it was proposed that serogroups D and F produced capsular material that contained heparin and chondroitin sulfate, respectively (11). The production of a chondroitin or chondroitin-like polysaccharide capsule by *P. multocida* serogroup F was recently confirmed by carbohydrate analysis (9). The monosaccharide analysis of a serogroup B *P. multocida* strain determined that the purified capsular polysaccharide was composed of arabinose, mannose, and galactose in a ratio of 0.5:2.0:0.8 (N. Muniandy, J. Edgar, J. B. Woolcock, and T. K. S. Mukkur, Int. Workshop Pasteurellosis Prod. Anim., 1992). The chemical composition of the serogroup E capsule remains unknown.

The biosynthetic loci of the complete capsules of *P. multocida* serotypes A:1 (7) and B:2 (1) have recently been identified, with gene identification within the serogroup-specific region 2 of both loci supporting previous data regarding capsule composition. Two genes within this region of the B:2 locus, *bcbA* and *bcbB*, were similar to *Escherichia coli wecB* and *wecC*, which catalyze the conversion of UDP-N-acetylgalactosamine to *N*-acetyl-D-mannosaminuronic acid (1). The presence of these homologs in the serogroup B cap locus suggests that the mannoside identified as the major type B capsular component by Muniandy et al. (Int. Workshop Pasteurellosis Prod. Anim.) exists as *N*-acetyl-D-mannosaminuronic acid in situ (1). Region 2 has also been partially cloned and sequenced from serogroup F, with the identification of a glycosyltransferase involved in elongation of chondroitin polymers (9). A detailed review of the composition, function, and genetics of the *P. multocida* A:1 and B:2 capsules has recently been published (2).

The lack of genetic knowledge regarding the capsular material of serogroups D, E, and F and the increasing need for a simple, DNA-based capsular typing method provided the impetus for the investigation described here. Oligonucleotide

† This paper is dedicated to the memory of our colleague, the late Rick Rimler, whose contribution to *P. multocida* research is gratefully acknowledged.

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primers designed during sequencing of the biosynthetic loci of the capsules of serogroups A and B (1, 7) were used to determine the nucleotide sequences of the region 2 genes from the remaining three capsular serogroups (serogroups D, E, and F). Serogroup-specific sequences were then identified for use as primers in a multiplex PCR assay. This assay represents a rapid and reproducible alternative to the serological and nonsero-

### RESULTS

**Bacteria.** The *P. multocida* strains used in this study are described in Table 1. The *P. multocida* isolates from the Veterinary Pathology Laboratory were serotyped by the Veterinary Research Institute, Peradeniya, Sri Lanka. All other *P. multocida* serotype designations were determined by the National Animal Disease Center (NADC), Ames, Iowa (R. B. Rimler and K. A. Brogden, personal communication), or from previous publications (6, 10, 15).

**PCR primers.** All primers used in the study either were synthesized by Life Technologies (Gaithersburg, Md.) or Genset Pacific (Lismore, New South Wales, Australia) or were previously synthesized for sequence determination of the *P. multocida* A1 and B2 cap loci (1, 7). The primer sequences used in the multiplex *P. multocida* capsular typing PCR assay are listed in Table 2.

**PCR conditions.** Initial PCR amplification was carried out with single primer sets from within the region 2 genes of the serogroup A and B capsule biosynthetic loci. For ease and rapidity, whole cells obtained from single colonies grown on 8% sheep blood agar were used as the template in the amplification reactions. A pipette tip was lightly touched onto a bacterial colony, and the colony was resuspended in the PCR amplification mixture (25 μl) containing each primer at a concentration of 3.2 μM, each deoxynucleoside triphosphate at a concentration of 200 μM, 1× PCR buffer, 2 mM MgCl₂, and 0.5 U of Taq DNA polymerase (Gibco BRL Life Technologies). All amplifications were performed with the GeneAmp PCR system 2400 (Perkin-Elmer, Branchburg, N.J.). For products with an expected size of ≤1 kb, the following standard cycling procedure was used: an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 5 min. The same cycling procedure was used for products of an expected size between 1 and 3 kb, except that the extension time was increased by 30 s for each additional 1 kb.

Long-template (≥3 kb) amplification used for sequence analysis of the region 2 genes from serogroups D, E, and F was performed with the tLONGase Enzyme Mix (Gibco BRL Life Technologies).

**Restriction endonuclease analysis of regions demonstrating similarity between serogroups.** PCR-restriction fragment length polymorphism analysis was performed on regions 1 and 3 of the cap loci from reference type strains of all five capsular serogroups. These regions were amplified with the tLONGase Enzyme Mix (Gibco BRL Life Technologies) and oligonucleotide primers from serogroup A and B cap loci. The PCR products were then purified with the QIAquick PCR purification kit (QIAGEN Pty. Ltd., Clifton Hill, Victoria, Australia) and digested with a restriction enzyme, BamHI, EcoRI, EcoRV, HinfI, HindIII, PstI, or SacI. Digestion products were visualized following agarose gel electrophoresis.

**Determination of nucleotide sequences of region 2 genes of serogroups D, E, and F.** Strains P934 (serogroup D), P1234 (serogroup E), and P4218 (serogroup F) were used to determine the nucleotide sequences of the region 2 genes from the *cap* loci of serogroups D, E, and F. Following long-template amplification, the PCR products were purified with the QIAquick PCR purification kit (QIAGEN) and cloned into the pGEM-T Easy Vector (Promega, Madison, Wis.) for sequence analysis.

**Nucleotide sequence accession numbers.** The GenBank accession numbers for the region 2 *cap* loci sequences are as follows: serogroup A (strain P934), AF302465; serogroup E (strain P1234), AF302466; and serogroup F (strain P4218), AF302467.

### MATERIALS AND METHODS

**Bacteria.** The *P. multocida* strains used in this study are described in Table 1. The *P. multocida* isolates from the Veterinary Pathology Laboratory were serotyped by the Veterinary Research Institute, Peradeniya, Sri Lanka. All other *P. multocida* serotype designations were determined by the National Animal
and F, with positive reactions obtained with most primers throughout the *P. multocida* A:1 cap locus, while no amplimers were produced from serogroups B and E (data not shown). Primers specific for *hyaC-hyaE* (region 2) showed some variation in amplification of serogroups D and F, while PCR-restriction fragment length polymorphism analysis of the region 1 genes from serogroups A, D, and F, and demonstrated identical restriction profiles with *BamHI*, *EcoRI*, *EcoRV*, *HhaI*, *HindIII*, *PstI*, and *Sau96-I* (data not shown).

The majority of primers from the *P. multocida* B:2 cap locus also amplified DNA from serogroup E but not DNA from serogroup A, D, or F (data not shown). The amplification profiles also showed that while homologs of *bcbI*, *bcbB*, and *bcbA* were present in the serogroup E cap locus, a lower degree of similarity was observed in the intervening genes, with no amplimers demonstrated when primers specific for *bcbCDEFGH* were used.

Amplification of DNA across the cap regions indicated that *P. multocida* serogroups A, D, and F possess similar organization, comprising region 1 (capsule export; *hexDCBA* homologs), region 2 (capsule biosynthesis), and region 3 (phospholipid substitution; *lipA* and *lipB* homologs) (Fig. 1). As shown previously, *P. multocida* serogroup B:2 demonstrates some re-assembly of region 1 and 3 genes, with *lipA* found to be co-transcribed with *cexDCBA*. Initial amplification studies and sequence determination showed a similar organization within the serogroup E cap locus (Fig. 1). The >99% identity of sequences flanking the cap loci of serogroups A and B was also demonstrated in the other three serogroups, suggesting a common chromosomal location for the cap loci of all serogroups.

**Analysis of region 2 genes of serogroups D, E, and F.** In order to define further the genetic organization of the *P. multocida* cap loci, the region 2 genes from capsular serogroups D, E, and F were cloned and sequenced. Predicted genes from these regions were designated *dcb*, *ecb*, and *fcb* for serogroup D, E, and F capsule biosynthesis, respectively. Whenever possible, the letter designation was maintained between serogroup homologs. Open reading frame (ORF) sizes and homolog similarities between serogroups A, D, and F are shown in Table 3, with the similarities between the region 2 genes of serogroups B and E listed in Table 4.

Region 2 of serogroup D was shown to contain four genes, *dbcfECB*, in the same orientation as *hyaEDCB* of A:1 (Fig. 1). DcbB and DcbC are homologs of HyaB and HyaC, respectively, with 98 and 92% similarities, respectively (Table 3). The 501-amino-acid product of *dcbF* displayed no similarity to HyaD of *P. multocida* A:1 but showed some similarity to bacterial glycosyltransferases (Table 5). In particular, DcbF has 54% similarity to the putative glycosyltransferase KfiC from the *Escherichia coli* K5 capsule biosynthetic locus. The deduced product of *dcbE* demonstrated 56% similarity to HyaE, although DcbE (603 amino acids) is slightly shorter than HyaE (622 amino acids) (Table 3).

The capsule biosynthesis genes of serogroup F showed significant similarity to those of *P. multocida* A:1, with the products of *fcbB*, *fcbC*, and *fcbE* exhibiting identical lengths and >97% similarities to their A:1 homologs (Table 3). The deduced product of *fcbD* was determined to have 90% similarity to HyaD (A:1) and pmHAS (8). In addition, FcbD had 97% similarity to the chondroitin synthase (pmCS) recently identified from the serogroup F *P. multocida* strain P4679 (9).

The region 2 genes of the serogroup E cap locus demonstrated a high level of similarity to those of serogroup B. The deduced products of *ecbI*, *ecbB*, and *ecbA* were similar in length and sequence (>92% identity) to their group B homologs (Table 4). Interestingly, there was no homolog of *bcbH* within the serogroup E cap locus, in which *ecbI* is flanked by *lipA* and *ecbG*. The sequence downstream of *ecbB* also differed from its serogroup B homolog. Two ORFs, *ecbJ* and *ecbK*, were identified that demonstrated little or no similarity to *bcbC* (Table 5). The C-terminal amino acids of EcbJ showed low levels of similarity to bacterial glycosyltransferases but no iden-

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**TABLE 2. Sequences of the oligonucleotides used in the *P. multocida* multiplex capsular PCR typing assay**

<table>
<thead>
<tr>
<th>Serogroup</th>
<th>Gene</th>
<th>Name</th>
<th>Sequence (5’→3’)</th>
<th>Amplimer size (bp)</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>KMT1</td>
<td>KMT1T7</td>
<td>ATCCGCTATTATCCACGTGG</td>
<td>460</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KMT1S6</td>
<td></td>
<td>GCTGTAAACGACACTGCCAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>hyaD</td>
<td>CAPA-FWD</td>
<td>TGCAAATCGCAGTCAG</td>
<td>1,044</td>
<td>8846–8863a</td>
</tr>
<tr>
<td></td>
<td>hyaC</td>
<td>CAPA-REV</td>
<td>TTGCCATCATTGTGAG</td>
<td>760</td>
<td>13621–13603b</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>12863–12880</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>bcbD</td>
<td>CAPB-FWD</td>
<td>CATTATCCAAGCTCCACC</td>
<td>657</td>
<td>3142–3165c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CAPB-REV</td>
<td>GCCCGAGATTTCAATCC</td>
<td>3789–3766</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>dcbF</td>
<td>CAPD-FWD</td>
<td>TTCAAAAGAAGATTAGAGGCC</td>
<td>511</td>
<td>4387–4408d</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CAPD-REV</td>
<td>CATCTACCACCTCAACCATATCAG</td>
<td>4899–4881</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>ecbJ</td>
<td>CAPE-FWD</td>
<td>TCCGCAGAAAATTATTTGACTC</td>
<td>851</td>
<td>2881–2896e</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CAPE-REV</td>
<td>GCTTGCTGCTTATTTCGTC</td>
<td>3733–3714</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>fcbD</td>
<td>CAPF-FWD</td>
<td>AATCGGAGAACCGAGAAATCAG</td>
<td>851</td>
<td>2881–2896e</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CAPF-REV</td>
<td>TTCCCGCTCAATTACTCTG</td>
<td>3733–3714</td>
<td></td>
</tr>
</tbody>
</table>

* These sequences match the coding strand of the serogroup A cap gene with GenBank accession number AF067175.

b These sequences match the coding strand of the serogroup B cap gene with GenBank accession number AF169324.

c These sequences match the coding strand of the serogroup D cap gene with GenBank accession number AF302465.

d These sequences match the coding strand of the serogroup E cap gene with GenBank accession number AF302466.

e These sequences match the coding strand of the serogroup F cap gene with GenBank accession number AF302467.
Development of a multiplex capsular PCR typing assay for *P. multocida*. It was evident from the comparative analyses that sequences within *hyaD*, *bcbD*, *dcbF*, *ecbJ*, and *fcbD* were highly specific for their respective serogroups. As serogroups D and F possessed homologs of *hyaB*, *hyaC*, and, to some extent, *hyaE*, glycosyltransferase genes *hyaD*, *dcbF*, and *fcbD* represented potential serogroup-specific target sequences. The majority of the region 2 genes of serogroup E were similar to those of serogroup B. However, the sequence of *ecbJ* showed little similarity to sequences in GenBank and therefore represented an ideal candidate for the identification of serogroup-specific sequences. Primer sequences were then designed to generate PCR products of unique size. *P. multocida*-specific primers were also included as an internal control to confirm the species identification. The resultant multiplex PCR was shown to be highly specific among the reference capsular type strains, with only the *P. multocida*-specific product and the respective capsule serogroup-specific product being amplified (Fig. 2). However, there was some disparity between PCR type and capsular serotype within the serogroup F strains. Of the five strains previously identified as serogroup F, only strains P4218 and P5084 produced the expected serogroup F-specific PCR product. The remaining three strains (strains P4988, P5184, and P5239) were typed as serogroup A strains by the multiplex capsular PCR assay (Table 1). However, sequence analysis of the glycosyltransferase genes used for the serogroup A- and F-specific primers indicated that the

![FIG. 1. Genetic organization of region 2 of the capsule biosynthetic loci of representative isolates of the five *P. multocida* capsular serogroups (serogroups A, B, D, E, and F). The *cap* loci are shown from the following strains: *P. multocida* A:1 strain X-73 (GenBank accession number AF067175), B:2 strain M1404 (GenBank accession number AF169324), serogroup D strain P934 (GenBank accession number AF302465), serogroup E strain P1234 (GenBank accession number AF302466), and F:3 strain P4218 (GenBank accession number AF302467). Numbers above the boxes indicate the distance (in base pairs) between the last base of the preceding gene and the first base of the next gene. Genes depicted by boxes above the line are transcribed in the left-to-right direction, while those beneath the line are transcribed in the right-to-left direction. †, percent identity at amino acid level to A:1 capsule biosynthetic locus; ‡, percent identity at amino acid level to B:2 capsule biosynthetic locus.](http://jcm.asm.org/)

<table>
<thead>
<tr>
<th>Strain (serogroup)</th>
<th>Protein name, protein length (amino acids); % identity (similarity) to X-73 (A:1) homolog</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-73 (A:1)</td>
<td>HyaB, 477; HyaC, 390; HyaD, 972; HyeA, 622; DcbB, 477; DcbC, 390; DcbD, 972; DcbE, 622; DcbF, 634; DcbG, 634; DcbH, 634; DcbI, 634; LipB, 634</td>
</tr>
<tr>
<td>P934 (D)</td>
<td>HyaB, 477; HyaC, 390; DcbB, 477; DcbC, 390; DcbD, 972; DcbE, 622; DcbF, 622; DcbG, 622; DcbH, 622; DcbI, 622; LipB, 622</td>
</tr>
<tr>
<td>P4218 (F:3)</td>
<td>FcbB, 477; FcbC, 390; FcbD, 972; FcbE, 622; FcbF, 622; FcbG, 622; FcbH, 622; FcbI, 622; LipB, 622</td>
</tr>
</tbody>
</table>

*Percent identity (similarity) to serogroup A X-73 homolog. This protein has <20% identity to X-73 homolog.*
strains were serogroup A, in agreement with the multiplex PCR result.

**DISCUSSION**

The identification and sequence analysis of the biosynthetic locus of the capsule of an organism can lead to a greater understanding of its capsular polysaccharide composition and can provide a genetic basis for the serological differences observed between strains. Sequence determination of the biosynthetic locus of the *P. multocida* serogroup A capsule by Chung et al. (7) identified components responsible for the synthesis of hyaluronic acid, consistent with hyaluronic acid being the principal component of the type A capsule. However, genetic analysis of the serogroup B biosynthetic locus revealed only three gene products with similarity to proteins known to be involved in polysaccharide biosynthesis, while six gene products had no similarity to known proteins (1). The structure of the type B capsule remains unknown. Until recently, very little was known about the compositions of the serogroup D and F capsular polysaccharides, with even less known about the serogroup E capsular polysaccharide. Therefore, determination of the sequences of the region 2 genes of the remaining serogroups was undertaken in order to gain information about the capsules of serogroups D, E, and F. This region was highly conserved between serogroups A, D, and F. Three of the four genes were homologous, with the unique gene for each group encoding a glycosyltransferase. The serogroup A (HyaD) and F (FcbD) genes were shown to encode hyaluronan and chondroitin synthases, respectively (8, 9). In serogroup D, this gene product (DcbF) was similar to the glycosyltransferase KfC from *E. coli* K5 that is involved in the formation of a polysaccharide similar to heparin. The genetic similarity between the capsular biosynthetic regions of *P. multocida* serogroup D and *E. coli* K5 is consistent with the possibility that the serogroup D capsule contains heparin polymers, a proposal previously based on the decapsulation of type D strains by heparinase III (11).

Determination of the nucleotide sequence of the serogroup E biosynthesis region provided little information about the capsular polysaccharide composition. Region 2 of serogroup E contains nine genes, two of which showed similarity to genes involved in polysaccharide biosynthesis. These two genes have homologs in the *P. multocida* B:2 capsular biosynthesis region, indicating that N-acetyl-d-mannosaminuronic acid is a component of both the serogroup B and the serogroup E capsules. Of the remaining seven genes, five have homologs in the B:2 capsular locus but still have no known function, one encodes a putative glycosyltransferase, and the other is unique to serogroup E. Definitive assignment of function must await further analysis and will be aided by the determination of the structures of the type B and E capsules.

Comparative analysis of the five capsular biosynthetic regions confirmed a genetic basis for the serological differences.
observed between strains. By using these genetic differences, we have developed a rational, DNA-based typing system for *P. multocida*. The multiplex capsular PCR assay provides a rapid and highly specific alternative to conventional capsular serotyping. There are currently only two laboratories worldwide that make and maintain the antisera required for capsular typing. The assay described in this report can be performed with suspected *P. multocida* colonies from primary isolation plates, thus reducing the time required for culture preparation. It is also highly specific for strains genetically capable of producing a serogroup-specific capsule. Notably, the PCR-based system was not affected by the geographical distribution of isolates. For example, isolates classified as serogroup A by conventional serotyping from Australia, Vietnam, and the United States all produced the appropriate amplimer with the serogroup A cap-specific primers. This assay will also help clarify the distinction between strains from closely related serogroups A and F. Indeed, the assay clearly identified three strains as serogroup A that had previously been serotyped erroneously as serogroup F, a finding confirmed by sequence analysis of the cap locus. Serogroups A and F are now known to have hyaluronic acid- and chondroitin-like polysaccharide capsules, respectively. As these are both nonimmunogenic polymers, it is unclear which antigens are responsible for the capsule-specific reactions observed in the indirect hemagglutination test. Despite the lack of knowledge regarding the specific immunogens, capsular serotyping has provided a useful system for *P. multocida* classification. However, with determination of the sequence of the cap locus in each serogroup, we believe that genetic cap identification by PCR will become a system for the rational and definitive typing of the *P. multocida* capsule.

**ACKNOWLEDGMENTS**

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Comparison of DNA Sequencing of the Protein A Gene Polymorphic Region with Other Molecular Typing Techniques for Typing Two Epidemiologically Diverse Collections of Methicillin-Resistant *Staphylococcus aureus*

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Volume 39, no. 3, p. 924–929, 2001. Page 927: Fig. 1 (panel E) should appear as shown below.

[Diagram showing genetic organization and development of a multiplex capsular PCR typing system.]