The Capsule Is a Virulence Determinant in the Pathogenesis of *Pasteurella multocida* M1404 (B:2)

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Capsules from a range of pathogenic bacteria are key virulence determinants, and the capsule has been implicated in virulence in *Pasteurella multocida*. We have previously identified and determined the nucleotide sequence of the *P. multocida* M1404 (B:2) capsule biosynthetic locus (J. D. Boyce, J. Y. Chung, and B. Adler, Vet. Microbiol. 72:121–134, 2000). The *cap* locus consists of 15 genes, which can be grouped into three functional regions. Regions 1 and 3 contain genes proposed to encode proteins involved in capsule export, and region 2 contains genes proposed to encode proteins involved in polysaccharide biosynthesis. In order to construct a mutant impaired in capsule export, the final gene of region 1, *cexA*, was disrupted by insertion of a tetracycline resistance cassette by allelic replacement. The genotype of the *tet*(M) Ω*cexA* mutant was confirmed by Southern hybridization and PCR. The acapsular phenotype was confirmed by immunofluorescence, and the strain could be complemented and returned to capsule production by the presence of a cloned uninterrupted copy of *cexA*. Wild-type, mutant, and complemented strains were tested for virulence by intraperitoneal challenge of mice; the presence of the capsule was shown to be a crucial virulence determinant. Following intraperitoneal challenge of mice, the acapsular bacteria were removed efficiently from the blood, spleen, and liver, while wild-type bacteria multiplied rapidly. Acapsular bacteria were readily taken up by murine peritoneal macrophages, but wild-type bacteria were significantly resistant to phagocytosis. Both wild-type and acapsular bacteria were resistant to complement in bovine and murine serum.

Pasteurella multocida is the causative agent of a wide range of diseases in both wild and domestic animals, and the diseases which affect livestock cause significant economic losses worldwide. Many *P. multocida* strains express a polysaccharide capsule on their surface, and isolates can be differentiated serologically by capsular antigens into serogroups A, B, D, E, and F (5, 26). The disease caused by the organism is generally dependent on capsular type, since serogroups B and E cause hemorrhagic septicemia in cattle and buffalo, serogroup A causes fowl cholera in poultry, and serogroup D causes atrophic rhinitis in pigs.

Polysaccharide capsules are found on the surface of a wide range of bacteria. With gram-negative bacteria, the capsule lies outside the outer membrane and is composed of highly hydrated polyanionic polysaccharides (27). Capsules have a significant role in determining access of certain molecules to the cell membrane, mediating adherence to surfaces, and increasing tolerance of desiccation. Furthermore, capsules of many pathogenic bacteria impair phagocytosis (22, 29, 30) and reduce the action of complement-mediated killing (7, 31, 35). Thus, capsules are likely to be major virulence determinants, and indeed, genetically defined acapsular mutants of a number of organisms have been shown to have reduced virulence (*Vibrio vulnificus* [38], *Streptococcus* spp. [30, 36], *Staphylococcus aureus* [32], *Actinobacillus pleuropneumoniae* [35], *Haemophilus influenzae* [17, 20], *Klebsiella pneumoniae* [9], and *Cryptococcus neoformans* [8]). The capsule has been implicated in virulence in *P. multocida*, since encapsulated strains have been shown to be more virulent (15) and able to resist complement-mediated killing (10, 31) than spontaneous acapsular strains. However, no genetically defined acapsular strains have

been constructed to allow unequivocal demonstration of the *P. multocida* capsule as a virulence determinant. Experiments with purified *P. multocida* B:6 capsular extract have indicated that it has significant antiphagocytic activity, although it should be noted that the extract used contained small amounts of nucleic acid and protein contaminants (21).

We have recently determined the nucleotide sequence of the *P. multocida* M1404 capsule biosynthetic locus (4). Like other gram-negative group II-like polysaccharide capsule biosynthetic loci, the genes can be grouped into three functional regions. Regions 1 and 3 contain a total of six genes, which are involved in transport of the polysaccharide, while region 2 contains nine genes, which are postulated to be involved in the biosynthesis of the polysaccharide. The proteins encoded by regions 1 and 3 showed significant similarity to those encoded by capsule export genes. Using this information, we have constructed isogenic strains impaired in capsule export to investigate the contribution to virulence of the *P. multocida* M1404 capsule.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are shown in Table 1. *P. multocida* B:2 strain M1404 and *Escherichia coli* DH5α were grown with aeration at 37°C in brain heart infusion (BHI) or 2YT (Oxoid, Hampshire, England), respectively. Kanamycin (50 µg/ml) and tetracycline (5 µg/ml for *P. multocida* and 15 µg/ml for *E. coli*) were added to solid and liquid media when required.

Recombinant DNA techniques. Genomic DNA was prepared by cetyltrimethylammonium bromide precipitation (2), and plasmid DNA was prepared by alkaline lysis (3). Plasmid DNA was further purified by polyethylene glycol precipitation (2) or by purification on Qiagen (Hilden, Germany) anion-exchange columns. DNA restriction and ligation reactions were carried out using enzymes obtained from Roche Molecular Biochemicals (Basel, Switzerland) or New England Biolabs (Beverly, Mass.), and reactions were performed according to the manufacturers' instructions. DNA was introduced into *E. coli* and *P. multocida* by electroporation as previously described (2, 14). DNA sequencing was carried out using the BigDye Ready Reaction DyeDeoxy Terminator cycle sequencing kits (Perkin-Elmer, Foster City, Calif.), and the reactions were analyzed with a 373A DNA sequencing system.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
E. coli DH5α	F⁻ *endA1 hsdR17* (r$_k$⁻ m$_k$⁺) *thi-1* λ⁻ *recA1 gyrA96 relA1* φ80d*lacZ*ΔM15	Bethesda Research Laboratories, Rockville, Md.
P. multocida		
M1404	Serotype B:2 wild-type strain	K. R. Rhoades, National Animal Disease Center, Ames, Iowa
PBA875	M1404 *tet*(M) Ω*cexA* mutant containing pPBA1100; Kan^r Tet^r	This study
PBA1514	M1404 *tet*(M) Ω*cexA* mutant, complemented with pPBA1621; Kan^r Tet^r	This study
Plasmids		
pPBA1100	*P. multocida-E. coli* shuttle vector; Kan^r	12
pPBA1620	pWSK129 containing the *tet*(M) Ω*cexA* mutagenesis cassette; Kan^r Tet^r	This study
pPBA1621	*cexA* cloned in pPBA1100 under control of the *lacZ* promoter; Kan^r	This study
pWSK129	Low-copy-number *E. coli* cloning vector; Kan^r	34
pVB101	pBR322 containing the *tet*(M) gene from Tn*916*; Ap^r Tet^r	Vickers Burdett, Duke University, Durham, N.C.

PCR amplification was performed with the Expand high-fidelity PCR kit, using the reaction conditions specified by the manufacturer (Roche Molecular Biochemicals). Oligonucleotides used in this investigation are listed in Table 2. Prior to sequencing or cloning, PCR fragments were purified by polyethylene glycol precipitation. Southern hybridizations were carried out as described previously (4).

Construction of acapsular *P. multocida* M1404 (B:2) by allelic exchange. A DNA fragment containing a *tet*(M) insertion in *cexA* was constructed by ligating two PCR-generated fragments to the *tet*(M) gene from pVB101 (Fig. 1A). The oligonucleotides BAP521 and BAP520 (Table 2) were used to amplify by PCR a 1,100-bp fragment containing the 178 codons of *cexA* corresponding to the C terminus and 615 bp of downstream DNA. The oligonucleotides BAP519 and BAP518 (Table 2) were used to amplify a 1,800-bp fragment containing the 36 codons of *cexA* corresponding to the N terminus, all of *cexB*, and the 329 codons of *cexC* corresponding to the C terminus. These PCR-generated fragments were ligated to either end of the *Bam*HI fragment from pVB101, which contains *tet*(M), and were cloned into pWSK129 to generate pPBA1620 (Table 1). The cloned mutagenesis cassette was further amplified by PCR, using pPBA1620 as a template and using oligonucleotides from the pWSK129 polylinker. Approximately 3 μg of each of the linear DNA fragments was used to transform *P. multocida* M1404 by electroporation (14), and the transformants were selected on NA (2.5% nutrient broth no. 2 [Oxoid], 0.3% tryptone, 1.0% agar) plates containing 5 μg of tetracycline/ml.

Identification of the surface exported capsule by immunofluorescence. The *P. multocida* M1404 capsule was visualized by immunofluorescence as previously described (6). To maximize capsule production, cells were grown on dextrose starch agar (Difco, Detroit, Mich.) containing 6% avian serum (Monash University Animal Services, Clayton, Australia) prior to fixation. The primary antibody was *P. multocida* serogroup B capsular typing serum (kindly supplied by Thula Wijewardana, Veterinary Research Institute, Peradeniya, Sri Lanka), and the secondary antibody was fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulin. FITC-labeled preparations were visualized with a Zeiss (Oberkochen, Germany) IM35 epifluorescence inverted microscope, using a 100× oil immersion objective. Kodak Elite chrome 200 ASA film (Eastman Kodak, Rochester, N.Y.) was used to record the images, and the processed slides were scanned with a Nikon (Tokyo, Japan) Coolscan II. Minor adjustments to the scanned images, which did not affect the data integrity, were completed in Adobe Photoshop (Adobe, San Jose, Calif.).

Assessment of virulence of acapsular *P. multocida*. *P. multocida* strains were grown in BHI to an optical density at 650 nm of 0.6 and diluted in BHI to obtain cultures of approximately 10² to 10⁹ CFU/ml. Exact bacterial numbers in each dilution were checked by direct-plate viable counts. Groups of at least six mice were injected intraperitoneally (i.p.) with aliquots of 0.1 ml of the appropriate dilutions. Mice were observed for signs of disease and were killed by cervical dislocation when deemed moribund, in accordance with animal ethics requirements. A control group of six mice injected with BHI showed no effects.

Kinetics of *P. multocida* infection of mice. Groups of three mice were injected i.p. with 5 × 10⁴ CFU, and bacterial counts were determined from the blood, liver, and spleen at 1, 4, 24, and 48 h after injection. Blood samples were taken from the retro-orbital plexus and diluted in BHI containing heparin prior to plating on NA. Liver and spleen were removed aseptically, homogenized in 4 ml of BHI, and, where necessary, diluted further in BHI prior to plating on NA.

Uptake of bacteria and survival inside macrophages. Bacterial uptake and survival within mouse peritoneal macrophages were determined by using gentamicin to kill extracellular bacteria as described previously (25). Mouse peritoneal macrophages were harvested as described previously (33), except that an influx of macrophages was stimulated 48 h prior to harvest with 1 ml of 2% starch. The

cells were resuspended in 3 ml of M199 (CSL Ltd., Melbourne, Australia) containing 10% fetal calf serum (M199-FCS), dispensed into Lab Tek Slide chambers (Nunc, Naperville, Ill.), and incubated at 37°C in a 5% CO$_2$ humidified incubator for 16 h to allow cells to adhere to the slide surface. The medium was removed, and the cells were washed three times in M199-FCS. The cells were incubated for a further 48 h, with a medium change and wash at 24 and 48 h. Bacteria (10⁷ CFU) were resuspended in M199-FCS, added to the macrophages, and incubated at 37°C for 30 min in a 5% CO$_2$ humidified incubator. The slides were then washed five times in M199-FCS containing 200 μg of gentamicin/ml, and incubation was continued for a further 30 min. The supernatant was removed, and the macrophages were lysed by addition of 0.4 ml of cold BHI. The numbers of released bacteria were determined by direct plate counts. Strains were tested in triplicate slide wells.

Bacterial interaction with murine peritoneal phagocytes. Mouse peritoneal macrophages were harvested and grown as described above. Bacteria were grown in BHI to an optical density at 650 nm of 0.3, washed once in phosphate-buffered saline, and resuspended in M199-FCS at 10⁸ CFU/ml. Approximately 3 × 10⁷ bacteria were added to the slide chambers containing the murine macrophages and were incubated for 90 min at 37°C in a 5% CO$_2$ humidified incubator. Slides were then washed three times in phosphate-buffered saline and stained with Giemsa stain for 20 min. Cells were visualized with a Zeiss IM35 inverted microscope using a 100× oil immersion objective, and images were recorded as described previously. A total of 100 (PBA875 and PBA1514) or 150 (*P. multocida* M1404) macrophages was observed across multiple randomly chosen microscope fields, with the presence of interacting bacteria recorded. Strains were tested in triplicate or quadruplicate slide wells.

Serum sensitivity assays. The sensitivity of *P. multocida* strains to the bactericidal complement activity of bovine (AMRAD, Adelaide, Australia) or murine (Monash University Animal Services) serum was determined by direct plate counts after incubation of approximately 10⁴ bacteria in 90% serum for 4 h at 37°C with shaking. Complement activity was inactivated in control samples by heating at 56°C for 30 min.

TABLE 2. Oligonucleotides used in this study

Name	Sequence	Position[a]
BAP474	5′-ATTCAACAGATAAAGAGAGAG-3′	284–304
BAP494	5′-CTACCAATTAGAGATTTTTACG-3′	2325–2304
BAP518	5′-NNNNNNGAATTCATGCGATCTCGTG-3′	2376–2352
BAP519	5′-CGCCCGAGGATCCCTACTTTCTC-3′[b]	589–611
BAP520	5′-GCGTAATGGATCCGGGAAATCAAC-3′[b]	591–568
BAP521	5′-CGTCATCAGTCGACGTCACG-3′	—[c]
BAP745	5′-GTTACAAAGCTTTTAGCTGATG-3′[b]	—[d]
BAP747	5′-GGGAATGTTTGCTATCGG-3′	1028–1011

[a] Position relative to the sequence deposited in GenBank (accession no. AF169324).

[b] Does not match the wild-type sequence exactly due to the incorporation of restriction sites.

[c] This sequence is approximately 550 bp 5′ of bp 1 (GenBank accession no. AF169324).

[d] This sequence is approximately 50 bp 5′ of bp 1 (GenBank accession no. AF169324).
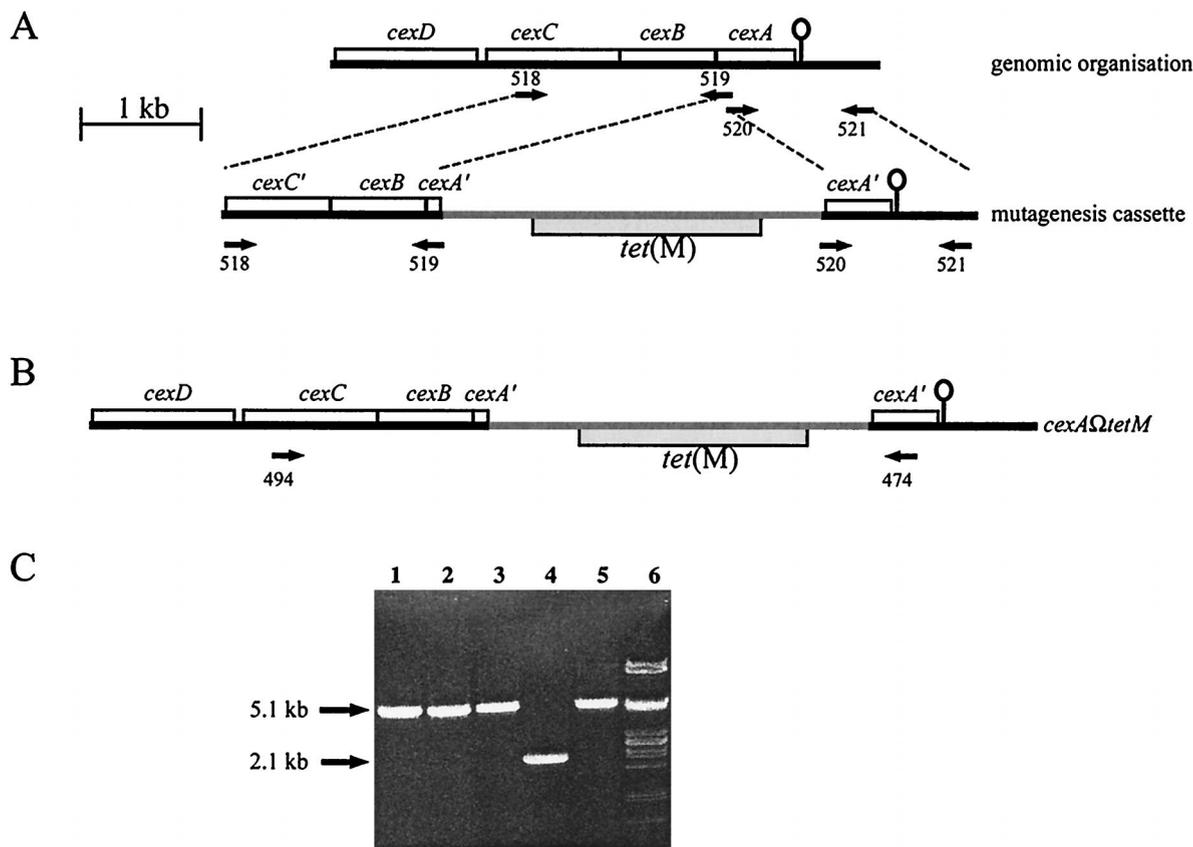
FIG. 1. Schematic representation of construction of the capsule-deficient mutant of *P. multocida* M1404. (A) A 1,800-bp fragment containing all of *cexB* and part of *cexA* and *cexC* was amplified by PCR using the oligonucleotides BAP518 and BAP519 (designated by arrows labeled 518 and 519). Similarly, a 1,100-bp fragment containing part of *cexA* and downstream DNA was amplified by using the oligonucleotides BAP520 and BAP521 (designated by arrows labeled 520 and 521). These fragments were ligated to either end of the *tet*(M) gene to obtain the linear DNA (mutagenesis cassette) used for mutagenesis by allelic exchange. The line-and-ball characters indicate the location of a putative transcriptional terminator, proposed to constitute the end of the *cap* locus (4). (B) Integration of the fragment by double-crossover homologous recombination would give rise to Tet^r bacteria with inactive *cexA*. (C) The genotypes of three putative mutants were investigated by PCR. Genomic DNA was isolated from each mutant (lanes 1 to 3) and *P. multocida* M1404 (lane 4) and was used as a template for PCR with oligonucleotides BAP474 and BAP494. For comparison, the mutagenesis cassette DNA was also used as a template for PCR with the oligonucleotides BAP474 and BAP494 (lane 5). Lane 6 contains λ DNA digested with *Pst*I. The sizes of the PCR products are indicated.

Statistics. Analysis of bacterial survival within macrophages was performed using ordinary analysis of variance, followed by the Tukey-Kramer multiple-comparison test. Analyses of serum sensitivity data were performed using the Kruskal-Wallis nonparametric analysis of variance test (corrected for ties), followed by Dunn's posttest. Approximate probability values were determined using InStat, version 2.03 (GraphPad Software).

RESULTS

Construction of *P. multocida* mutants impaired in capsule polysaccharide export. The capsule biosynthetic locus of *P. multocida* has been described previously (4). To evaluate the role of the *P. multocida* capsule in virulence, we constructed an isogenic mutant in which capsule export was inactivated. A DNA fragment containing a tetracycline resistance cassette within *cexA* (at codons 36 to 40) was constructed by PCR (Fig. 1) (see Materials and Methods). Three putative mutants were identified, and the genotypes were investigated by PCR (Fig. 1C) and Southern hybridization (data not shown). All three colonies contained the *tet*(M) insertion within *cexA*, and one isolate was designated PBA875. The phenotype of PBA875 was investigated by immunofluorescence, using *P. multocida* type B antiserum as the primary antibody (Fig. 2). *P. multocida* M1404 (wild type) showed strong fluorescence completely encircling the cells, while PBA875 showed markedly polar fluorescence

(Fig. 2A and B, respectively), indicating failed capsular export. Furthermore, PBA875 cells were often significantly elongated compared to wild-type cells, especially after continued incubation on solid medium (data not shown). The immunofluorescence profile of this strain was virtually indistinguishable from that of a previously described *H. influenzae bexA* (*cexA* homologue) mutant (17). As a control, the phenotype of PBA875 was investigated by immunofluorescence using the antilipo-polysaccharide (anti-LPS) monoclonal antibodies T1C6 and T2B2 (24) as the primary antibodies. No difference was observed between the immunofluorescence profile of PBA875 and wild-type *P. multocida* M1404 with either anti-LPS antibody, indicating that LPS biosynthesis and transport had not been affected in the mutant strain.

In order to complement the mutant strain, a plasmid was constructed which contained an uninterrupted copy of *cexA*. A fragment containing *cexA* was amplified by PCR using the oligonucleotides BAP745 and BAP747 (Table 2), digested with *Hin*dIII, and cloned into the *Hin*dIII site of pPBA1100 to generate pPBA1621 (Table 1). The plasmid pPBA1621 was transformed into PBA875 to generate the strain PBA1514. The phenotype of PBA1514 was shown by immunofluorescence to be close to that of the wild type (Fig. 2C). The growth rate of PBA1514 was indistinguishable from the growth rates of
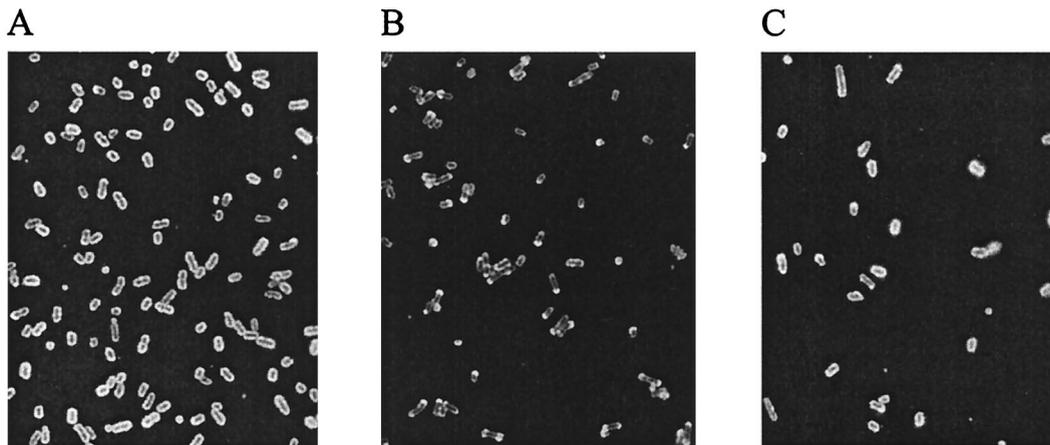
FIG. 2. Phenotypic characterization of *P. multocida* capsule expression. Photomicrographs of methanol-fixed *P. multocida* M1404 (A), PBA875 (B), and PBA1514 (C) visualized by immunofluorescence, with *P. multocida* serogroup B typing antiserum as the primary antibody and FITC-labeled anti-rabbit immunoglobulin as the secondary antibody.

PBA875 in both 2YT broth (data not shown) and bovine and murine serum (see below).

Acapsular *P. multocida* is significantly impaired in virulence. The virulence properties of *P. multocida* M1404, PBA875, and PBA1514 were investigated by i.p. challenge of mice (Table 3). Wild-type *P. multocida* had a 50% infective dose (ID$_{50}$) of <10 CFU and an ID$_{100}$ of <1,000 CFU. At a dose of 10 CFU, only 17% of mice survived the challenge. In contrast, no deaths were recorded for mice challenged with <8 × 10^5 CFU of PBA875 (acapsular mutant). However, at higher doses some mice developed fatal infections, and the ID$_{50}$ was calculated to be approximately 10^7 CFU. Bacteria isolated from the blood of mice which had received fatal doses of PBA875 were shown by PCR and immunofluorescence to be both genetically and phenotypically identical to the PBA875 mutant. The complemented mutant, PBA1514, displayed full wild-type virulence, with an ID$_{50}$ of <20 CFU and an ID$_{100}$ of <200 CFU.

Acapsular *P. multocida* is rapidly removed from the blood and other organs. The kinetics of infection of PBA875 and PBA1514 were investigated following challenge of mice (Table 4). Bacterial counts were determined from the blood, liver, and spleen after injection of 5 × 10^4 CFU. Bacteria could be isolated from all organs of mice infected with PBA1514, and the numbers of bacteria increased to approximately 5 × 10^7 CFU in all organs at 24 h (approximately the time of death). By contrast, small numbers of PBA875 (acapsular mutant) organ-

isms were isolated from all organs of the mice 1 h after infection, but none could be isolated from any of the organs 4, 24, or 48 h after infection. These data indicated that when mice were infected with 5 × 10^4 CFU of PBA875, the bacteria were cleared rapidly to low levels within the first 4 h, whereas PBA1514 could not be cleared and rapidly multiplied in all organs until lethal levels were reached.

Acapsular *P. multocida* is sensitive to phagocytosis by murine peritoneal macrophages. *P. multocida* M1404 has been shown previously to be phagocytosed inefficiently but to survive inside macrophages without multiplication (25). Bacterial uptake and survival within murine peritoneal macrophages were assessed by using gentamicin to kill extracellular bacteria. PBA875 was shown to be four- to sixfold more susceptible to macrophage uptake than both wild-type and complemented bacteria. The numbers of surviving bacteria released from lysed macrophages for M1404, PBA875, and PBA1514 were 1,800 ± 400, 7,100 ± 1,800, and 1,200 ± 500 CFU/ml, respectively. The values are the sample mean ±1 standard deviation. The difference between results for PBA875 and those for M1404 or PBA1514 was determined to be highly significant

TABLE 3. Virulence of *P. multocida* M1404, PBA875, and PBA1514, as determined by i.p. challenge of female BALB/c mice

Strain	Injected dose (CFU)	% Survival (group size)
M1404	10	17 (6)
	1 × 10^2	10 (11)
	1 × 10^3	0 (6)
PBA875	8 × 10^2	100 (21)
	8 × 10^4	100 (11)
	8 × 10^5	94 (18)
	8 × 10^6	64 (11)
	8 × 10^7	33 (6)
PBA1514	20	17 (6)
	2 × 10^2	0 (6)

TABLE 4. Kinetics of infection by PBA875 and PBA1514 following i.p. challenge of female BALB/c mice

| Strain | Time (h) | CFU[a] | | |
		Blood	Liver	Spleen
PBA1514	1	3.8 (±0.3) × 10^3	9 (±6) × 10^2	5 (±3) × 10^3
	4	1 (±0.5) × 10^5	9 (±7) × 10^4	7 (±3) × 10^4
	24[b]	5 × 10^7	6 × 10^7	2 × 10^7
PBA875	1	2 (±1) × 10^2	5 (±4) × 10^2	1.3 (±1) × 10^2
	4	ND[c]	ND	ND
	24	ND	ND	ND
	48	ND	ND	ND

[a] Numbers for blood are CFU per milliliter and for the liver and spleen are CFU per total organ. Reported numbers are means (±1 standard deviation) determined from three replicates.

[b] No error limits could be determined for this time point, since only one of the group survived to 24 h.

[c] ND, no bacteria were detected for these time points. The detection limit of the experiment was estimated to be approximately 10 CFU/ml (blood) or 40 CFU/organ (liver and spleen).

TABLE 5. Sensitivity of *P. multocida* to bactericidal
activity of bovine serum

Strain	Serum heat treatment	I_{4h}[a]
PBA1514	+	36 ± 7[b]
PBA1514	−	26 ± 4[b]
PBA875	+	58 ± 5[b]
PBA875	−	33 ± 5[b]
E. coli DH5α	+	3.5 ± 0.6
E. coli DH5α	−	0.16 ± 0.02

[a] I_{4h} is defined as CFU per milliliter at 4 h divided by CFU per milliliter at 0 h. Reported numbers are means $\pm$ 1 standard deviation, determined from at least three replicates.

[b] The difference in sensitivity between PBA875 and PBA1514 in either heated or unheated serum was determined to be not significant (Kruskal-Wallis test; $P > 0.05$).

(Tukey-Kramer multiple-comparison test; $P < 0.01$), but the difference between results for M1404 and those for PBA1514 was determined to be not significant ($P > 0.05$). Therefore, acapsular bacteria are both internalized and capable of survival within murine peritoneal macrophages for at least 30 min. The interactions (macrophage uptake and adherence) of *P. multocida* M1404, PBA875, and PBA1514 with murine peritoneal macrophages were also assessed visually with Giemsa-stained preparations of bacteria and macrophages after 90 min of incubation. Only 9% of macrophages were shown to contain wild-type *P. multocida* M1404, but 64% of macrophages were observed to contain the acapsular mutant PBA875 (data not shown).

Acapsular and encapsulated *P. multocida* strains are highly resistant to complement activity in naive serum. PBA875 and PBA1514 were incubated in 90% bovine serum to investigate their sensitivity to complement-mediated killing. Both *P. multocida* strains grew rapidly in either untreated or heat-treated serum, whereas *E. coli* DH5α was rapidly killed in untreated serum but grew in heat-treated serum (Table 5). No statistically significant difference was observed between the growth rates of PBA875 in untreated and heat-treated serum, indicating that the loss of the capsule does not increase sensitivity to complement-mediated killing. Similar results were obtained for growth in murine serum (data not shown), although the level of bactericidal activity against the *E. coli* control was lower, in accordance with the lower level of bactericidal activity previously observed for murine serum (16). The growth rates of PBA875 and PBA1514 were virtually indistinguishable in both murine and bovine serum, indicating that the reduction in virulence observed for PBA875 was unlikely to be due to an altered growth rate.

DISCUSSION

A mutant defective in the export of the *P. multocida* capsule was constructed by allelic exchange. Using the sequence of the *P. multocida cap* locus (4), a DNA fragment was constructed with a *tet*(M) insertion within the capsule export gene *cexA*. The mutant strain (PBA875) showed markedly polar immunofluorescence (Fig. 2), which we suggest is due to a failure of capsular polysaccharide export. A *bexA* mutant (*cexA* homologue) in *H. influenzae* has previously been investigated, and this mutant appeared identical when viewed by immunofluorescence microscopy (17). Detailed analysis of the *H. influenzae bexA* mutant indicated that it was capable of synthesizing immunoreactive material but failed to export it appropriately (17). These data are in agreement with the expected phenotype of a *bexA* mutant, since *bexA* encodes the ATP binding component of the ATP binding cassette transporter complex required for export of capsular polysaccharide (18, 23). Therefore, we believe that PBA875 is also capable of synthesizing immunoreactive polysaccharide but fails to export it correctly. Complementation of PBA875 with a plasmid containing an intact copy of *cexA* returned the strain to capsule production (Fig. 2C). These data are consistent with the involvement of *cexA* in the transport of the *P. multocida* capsular polysaccharide.

It has previously been hypothesized that the *P. multocida* capsule is a virulence determinant, since spontaneous unencapsulated strains are less virulent (15) and more sensitive to complement-mediated killing (31) than encapsulated strains. However, to date no isogenic strains have been available to unequivocally demonstrate this. We infected mice with various doses of *P. multocida* M1404, PBA875, or PBA1514 and observed that PBA875 was significantly less virulent than either the wild-type or complemented strain. The ID_{50} of PBA875 was approximately 10^6-fold higher than that of *P. multocida* M1404 or PBA1514. At very high doses ($>8 \times 10^5$), PBA875 was capable of lethal infection, and bacteria isolated from mice which succumbed to challenge at these doses were shown to be acapsular.

The numbers of PBA1514 or PBA875 bacteria in various organs of mice were determined at 1, 4, 24, and 48 h after i.p. challenge. The numbers of PBA1514 organisms rose rapidly in all organs, reaching approximately 10^7 to 10^8 CFU/organ at the time of death. In contrast, PBA875 was shown to be removed rapidly from the body, with no bacteria being detected in any of the organs after 1 h. These data indicate that the reduced virulence of PBA875 was due to its rapid removal from the body after infection. It appears likely that at very high infective doses, the animals could not clear even the acapsular mutant faster than it was capable of replicating, and this may account for the virulence of PBA875 observed at doses of $>8 \times 10^5$ CFU.

Two mechanisms have been suggested for the enhanced sensitivity of acapsular bacteria to removal from the blood: (i) increased sensitivity to the bactericidal activity of complement (10, 31, 37) and (ii) increased susceptibility to phagocytosis (1, 11, 19, 30). The acapsular strain PBA875 was shown to be significantly more sensitive to phagocytosis, with four- to sixfold more PBA875 organisms internalized by mouse peritoneal macrophages than was found with either *P. multocida* M1404 or PBA1514. Acapsular bacteria were still capable of surviving within the macrophages for at least 30 min. Furthermore, the average number of macrophages observed to contain PBA875 was seven times greater than that observed for *P. multocida* M1404. Interestingly, all strains were shown to be equally resistant to the bactericidal activity of complement in either bovine or murine serum and to grow at similar rates in both sera. Therefore, we believe that the rapid reduction in the number of PBA875 organisms in the organs after infection is primarily due to increased sensitivity to phagocytosis.

Taken together, these data provide for the first time unequivocal proof that the capsule is a major virulence factor in *P. multocida*. The reduction in virulence is due primarily to the rapid removal of the acapsular bacteria from the blood and other organs, and this removal is likely due to an increased susceptibility to phagocytosis. In other species, attenuated organisms have shown potential as vaccine candidates (13, 28), and work is in progress to determine if these attenuated acapsular *P. multocida* mutants are capable of conferring a protective immune response.

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