

Virulence of Capsulated and Noncapsulated Isolates of *Pasteurella multocida* and Their Adherence to Porcine Respiratory Tract Cells and Mucus

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The virulence and the adherence to porcine respiratory tract cells and mucus of three toxigenic, capsular type D *Pasteurella multocida* isolates and their noncapsulated variants were evaluated in the present study. Loss of capsule by *P. multocida*, verified by transmission electron microscopy after polycationic ferritin labeling, was associated with a massive reduction in virulence of the organisms in mice. Specific-pathogen-free piglets inoculated intranasally with one of the capsulated isolates or its noncapsulated variant developed turbinate lesions characterized by bone resorption and by an inflammation of the mucosa associated with hyperplasia and squamous metaplasia of the epithelium. Infection with the capsulated isolate led to more severe lesions and atrophy of turbinates. The interactions of these *P. multocida* isolates with porcine respiratory tract cells and mucus were studied *in vitro*. The presence of capsule resulted in a decrease in binding of respiratory tract mucus to *P. multocida* isolates as determined by a dot blot assay. The presence of capsule also resulted in a significant decrease in adherence to porcine tracheal rings maintained in culture. The capsule seemed to mask outer membrane components which are involved in adherence. One of these components might be lipopolysaccharide since purified lipopolysaccharide bound respiratory tract mucus and blocked adherence of this microorganism to porcine tracheal rings. Our data indicate that capsular material does not seem to be involved in adherence of *P. multocida* to respiratory tract cells and mucus, but capsulated isolates are more virulent in mice and also in piglets.

Swine atrophic rhinitis is a multifactorial disease complex characterized by turbinate atrophy (7, 36). *Bordetella bronchiseptica* and *Pasteurella multocida* are each capable of causing atrophy of nasal turbinates in pigs, but the severity and persistence of the changes they induce are different (36). Infection with both *B. bronchiseptica* and *P. multocida* permits colonization by toxigenic *P. multocida* and leads to more severe lesions than does infection with either microorganism alone.

Isolates of *P. multocida* implicated in atrophic rhinitis produce a dermonecrotic toxin (DNT) which causes characteristic lesions when injected into the pig (6, 7, 32). Porcine isolates of *P. multocida* usually possess a carbohydrate capsule, which may vary in size (20) and in composition (e.g., capsular types A and D) (7, 32). Other important molecules, also found at the cell surface of *P. multocida*, are lipopolysaccharides (LPS) which have endotoxic activities (11) and iron-regulated proteins, usually expressed *in vivo*, which are probably implicated in iron transport (8, 37). Hemagglutinins (13) and fimbriae (39) have been reported, but it was found that these two factors were independent.

Although adhesion seems to be an important step in the pathogenesis of atrophic rhinitis (28) and several studies have evaluated the attachment of *P. multocida* to various porcine respiratory tract cells and to porcine respiratory tract mucus (4, 10, 14, 21, 24, 27, 28, 41), the adhesins of *P. multocida* have not been identified. Thus, the purpose of the present study was to examine the role of *P. multocida*

capsule in virulence and in adherence to porcine upper respiratory tract cells and mucus by using toxigenic capsular type D isolates and their noncapsulated variants.

MATERIALS AND METHODS

Bacterial isolates and growth conditions. Toxigenic, capsular type D *P. multocida* swine isolates 2884, 4275, and 4533 and their noncapsulated variants (2884B, 4275B, and 4533B) were kindly supplied by Richard B. Rimler, National Animal Disease Center, U.S. Department of Agriculture, Ames, Iowa (31). The noncapsulated variants were derived by serial passage on dextrose starch agar and selection of colonies that appeared blue in oblique-transmitted light. Bacterial isolates were grown on tryptic soy agar plates for 18 h at 37°C.

Transmission electron microscopy. Bacterial cells were prepared for transmission electron microscopy after polycationic ferritin labeling, a method which allows good preservation of capsular material, as described previously by Jacques and Foiry (20).

Protein profiles. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted by using the discontinuous system of Laemmli (23) with a 4.5% (wt/vol) polyacrylamide stacking gel and a 12.5% (wt/vol) polyacrylamide running gel as described previously (1). Gels were run on a Mini-Protean II apparatus (Bio-Rad Laboratories, Richmond, Calif.), and proteins were visualized by staining with Coomassie brilliant blue R-250.

LPS profiles. Boiled bacterial cells in solubilization buffer were treated with proteinase K (1 mg/ml; Sigma Chemical

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Co., St. Louis, Mo.) (30). Samples were then separated by SDS-PAGE as described previously (1). The silver staining procedure of Tsai and Frasch (40) was used. LPS from *Escherichia coli* O111:B4 and *Salmonella typhimurium* TV119 Ra mutant (Sigma) were used as standards.

CR binding to whole cells. Bacterial cells were resuspended to an optical density at 540 nm (OD_{540}) of 1.0 in Tris buffer (100 mM, pH 7.0) containing 2 M ammonium sulfate in 1.5-ml Eppendorf tubes (22). Congo red (CR; 10 μ g/ml) was added and allowed to bind for 10 min at room temperature. Cells were pelleted by centrifugation (16 000 $\times g$, 1 min). CR binding to cells was determined spectrophotometrically at 480 nm as the residual dye in the supernatant. All samples were tested in duplicate with cell-free controls.

Bacterial cell surface hydrophobicity. The cell surface hydrophobicity was measured by the salt aggregation test described by Lindahl et al. (25). Serial dilutions were made in sodium phosphate (0.002 M, pH 6.8) to obtain $(NH_4)_2SO_4$ concentrations ranging from 4 to 0.05 M. A volume (25 μ l) of dense bacterial suspensions prepared in 0.002 M sodium phosphate buffer was mixed with an equal volume of salt solution in the depression of a glass slide. The bacterium-salt solution mixture was gently rocked for 2 min at room temperature, and visual reading was performed against a black background. *Staphylococcus aureus* Cowan 1 strain was used as a positive control.

Polymyxin B-gold labeling. The procedure used for polymyxin B-gold labeling was described by Morioka et al. (26). Briefly, polymyxin B was conjugated with bovine serum albumin (BSA) by using 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide. Colloidal gold particles (15 nm) adjusted to pH 5.5 were then coated with polymyxin B-BSA. Gold-labeled BSA was prepared as a control. A drop of a bacterial suspension was placed on a Formvar-coated electron microscope grid for 1 min. Grids with bacteria were placed on drops of polymyxin B-BSA-gold for 30 min. After being washed, the grids were negatively stained with 0.2% phosphotungstate and examined with an electron microscope (Philips 201) at an accelerating voltage of 60 kV.

DNT production. DNT production was evaluated by an enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies M815 and E816 (Dakopatts, Glostrup, Denmark) as described by Foged et al. (12).

Virulence in mice. Tests for virulence were performed in 19- to 21-g CD1 male mice by using the intraperitoneal route (1). Bacteria were grown overnight, harvested, and suspended in phosphate-buffered saline to a concentration of approximately 10^8 CFU/ml. Virulence was estimated by determining the 50% lethal dose (LD_{50}) with groups of five mice (29).

Experimental piglet infection. Twenty-four specific-pathogen-free 3-week-old piglets reared in a pathogen-free environment were used. Two groups of eight piglets received *P. multocida* 4275 (group A) and 4275B (group B), respectively. Piglets were challenged four times intranasally (0.5 ml per nostril) with a suspension containing 10^6 CFU/ml (first inoculation) or 10^9 CFU/ml (three other inoculations) 4 days in a row. Eight uninoculated piglets, maintained in a separate unit, were used as controls. Animals were monitored daily for a rise in body temperature and sneezing and weekly for growth. Pigs were killed 2 weeks after the last inoculation. Postmortem examinations were carried out on each animal, and turbinates were prepared for histopathology (3).

Affinity for porcine respiratory tract mucus. To screen for mucus binding by *P. multocida* isolates, dot blots were performed (2). Tracheas were removed from healthy adult

pigs at the slaughterhouse, and crude mucus (containing approximately 1 mg of protein per ml) was obtained by the procedure previously described by Letellier et al. (24). A rabbit antiserum against the porcine crude respiratory tract mucus was prepared as described by Rozee et al. (33). Ten-microliter aliquots of the bacterial suspensions were placed on a nitrocellulose membrane. All incubation steps were performed at room temperature; four 3-min washes with a Tris-saline buffer (10 mM Tris, 150 mM NaCl [pH 7.4]) followed. The membranes were first incubated for 1 h with 2% casein, a blocking solution, and for 1 h with the filtered mucus preparation diluted 1/5. The membranes were then incubated for 2 h with a 1/50 dilution of an antiserum against the mucus preparation and then for 1 h with a goat anti-rabbit immunoglobulin G (IgG) conjugated to peroxidase diluted 1/10,000 (Bio-Rad). The reaction was revealed by the addition of 4-chloro-1-naphthol. Reference strain *P. multocida* 4-4056 (capsular type D) was used as a control with affinity for porcine respiratory tract mucus (24), whereas *E. coli* O111 was used as a negative control.

Adherence to porcine tracheal rings. Adherence of *P. multocida* to porcine tracheal rings maintained in culture was performed as described previously (1, 10). Adherence was measured after 6 h of incubation with an inoculum of approximately 10^8 CFU/ml. An adherence index (1), which takes into account the inoculum size, was calculated as the anti-log₁₀ of [(log₁₀ CFU per ring) - (log₁₀ CFU per ml of inoculum) + 5]. Results were compared for statistical significance by Student's *t* test.

Extraction and isolation of LPS. LPS from *P. multocida* were extracted and isolated by the method of Darveau and Hancock (9). In brief, disrupted cells were treated with DNase, RNase, pronase, and SDS and subjected to $MgCl_2$ precipitation and high-speed centrifugation. A colorimetric assay for the 2-keto-3-deoxyoctulosonic acid released after hydrolysis of the LPS was performed on the LPS preparation (15).

Adherence inhibition experiments. Porcine tracheal rings were preincubated for 15 min with purified LPS containing 0.025 μ M 2-keto-3-deoxyoctulosonic acid diluted 1/4 in minimal essential medium (1). Adherence of *P. multocida* in the presence of purified LPS (also diluted 1/4) was evaluated after 1 h of incubation at 37°C.

RESULTS

We examined all isolates by electron microscopy after polycationic ferritin labeling to confirm the presence or the absence of capsular material. The three parent isolates were, as expected, covered with a dense layer of ferritin granules of 40 to 60 nm, whereas their noncapsulated variants were totally devoid of capsular material (Fig. 1). The monolayer of ferritin granules seen around the noncapsulated variants indicated the net negative charge of the bacterial cell surface. The noncapsulated variants were stable; we never observed a reversion to the original phenotype.

Whole-cell protein profiles of isolates were examined after SDS-PAGE and staining with Coomassie brilliant blue R-250. Whole-cell lysates were also treated with proteinase K, run on an SDS-polyacrylamide gel, and silver stained for examination of the LPS profiles. We found that noncapsulated isolates had profiles identical to the profiles of the capsulated parent isolates. The LPS profiles of all isolates were characteristic of a semirough type of LPS (Fig. 2). We determined the ability of all isolates to bind CR in the presence of ammonium sulfate. As can be seen in Table 1,

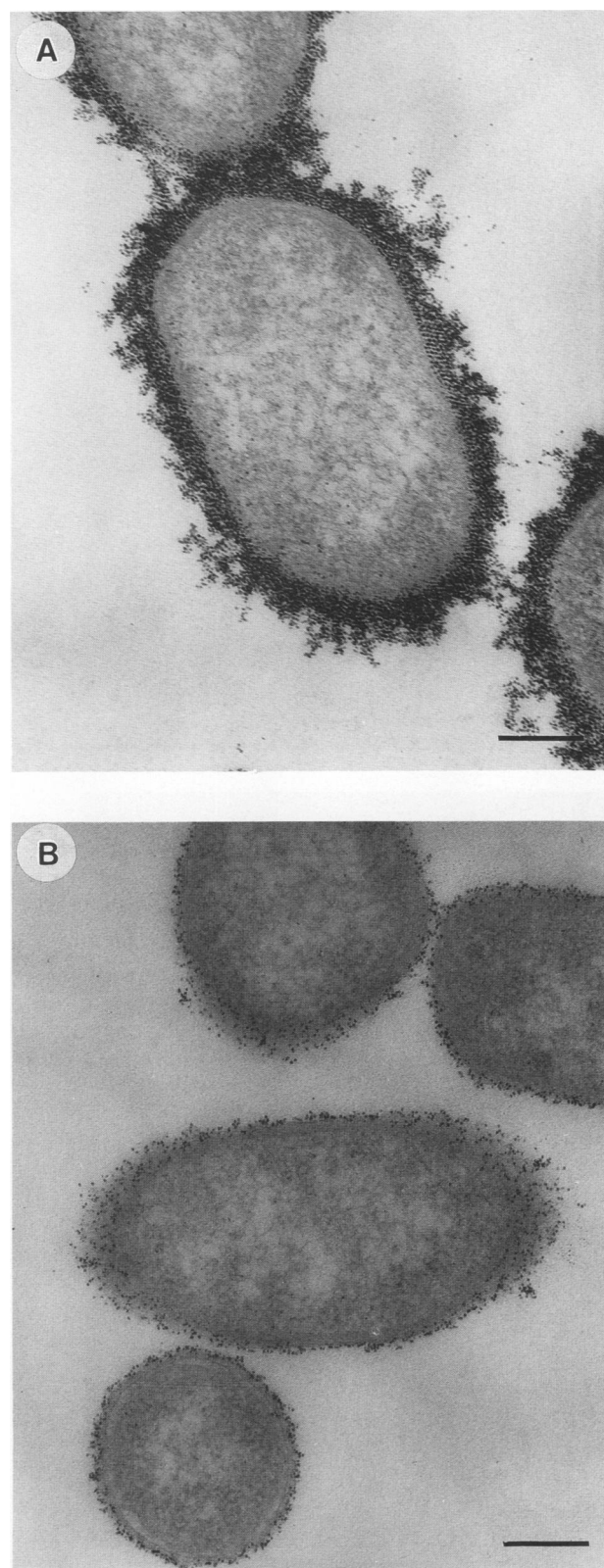


FIG. 1. Transmission electron micrographs of thin sections of cells of *P. multocida* isolates 4533 (A) and 4533B (B) labeled with polycationic ferritin. Bars, 200 nm.

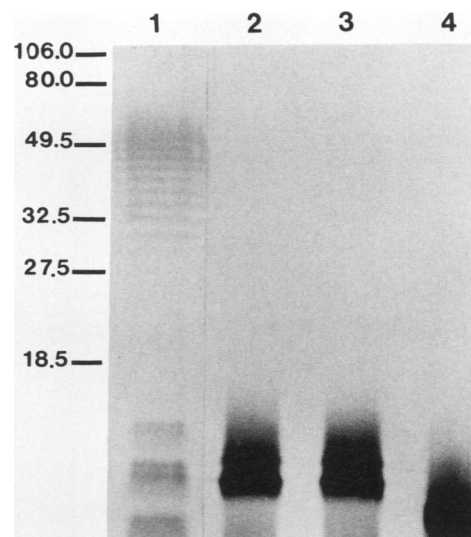


FIG. 2. Silver-stained SDS-PAGE profiles of whole-cell, proteinase K-treated preparations of *P. multocida* 4533 (lane 2) and 4533B (lane 3). LPS from *E. coli* O111:B4 (lane 1) and *S. typhimurium* TV119 Ra mutant (lane 4) were used as standards. The positions of the molecular mass markers are indicated to the left of the gel in kilodaltons.

CR bound strongly to the noncapsulated isolates and not at all to the capsulated isolates. The salt aggregation test was used to compare the hydrophobic surface properties of all isolates. The lowest concentrations of ammonium sulfate which gave visual aggregation were >4 M for the capsulated isolates and 4 M for the noncapsulated isolates.

We found that all noncapsulated isolates produced the DNT (Table 1) and in amounts similar (i.e., similar OD_{414} readings) to those of their capsulated parent isolates.

The virulence of all isolates was evaluated in mice (Table 1). The noncapsulated isolates were markedly less virulent in mice than the parent isolates, as their LD_{50} s increased by a magnitude of 6 to 7 log units. The pair of isolates showing the largest difference in virulence for mice, isolates 4275 and 4275B, were then inoculated to specific-pathogen-free piglets. Sneezing was observed in both groups 1 week after the last inoculation. The body temperature, normally around

TABLE 1. CR binding, DNT production, virulence in mice (LD_{50}), and adherence to porcine tracheal rings of capsulated and noncapsulated isolates of *P. multocida*

Isolate	Capsule	CR ^a	DNT ^b	LD_{50} ^c	Adherence index (mean $\pm$ SD) ^d
2884	+	0	0.45	4.2×10^1	951 ± 86
2884B	-	71 ± 9	0.47	4.3×10^7	$1,606 \pm 139^e$
4275	+	0	0.32	7.1×10^1	138 ± 10
4275B	-	78 ± 9	0.40	7.6×10^8	201 ± 12^e
4533	+	0	0.42	8.1×10^1	40 ± 39
4533B	-	74 ± 1	0.37	2.3×10^7	250 ± 27^e

^a CR binding is expressed as the OD_{480} of residual dye in the supernatant divided by the OD_{480} of control without bacterial cells multiplied by 100.

^b DNT production was determined by ELISA and is expressed as the OD_{414} ; negative control OD values were less than 0.05.

^c Virulence in mice (LD_{50}) is expressed as the number of CFU.

^d Determined after 6 h of incubation.

^e Significant differences ($P < 0.04$) were observed between adherence of a capsulated isolate and adherence of its noncapsulated variant.

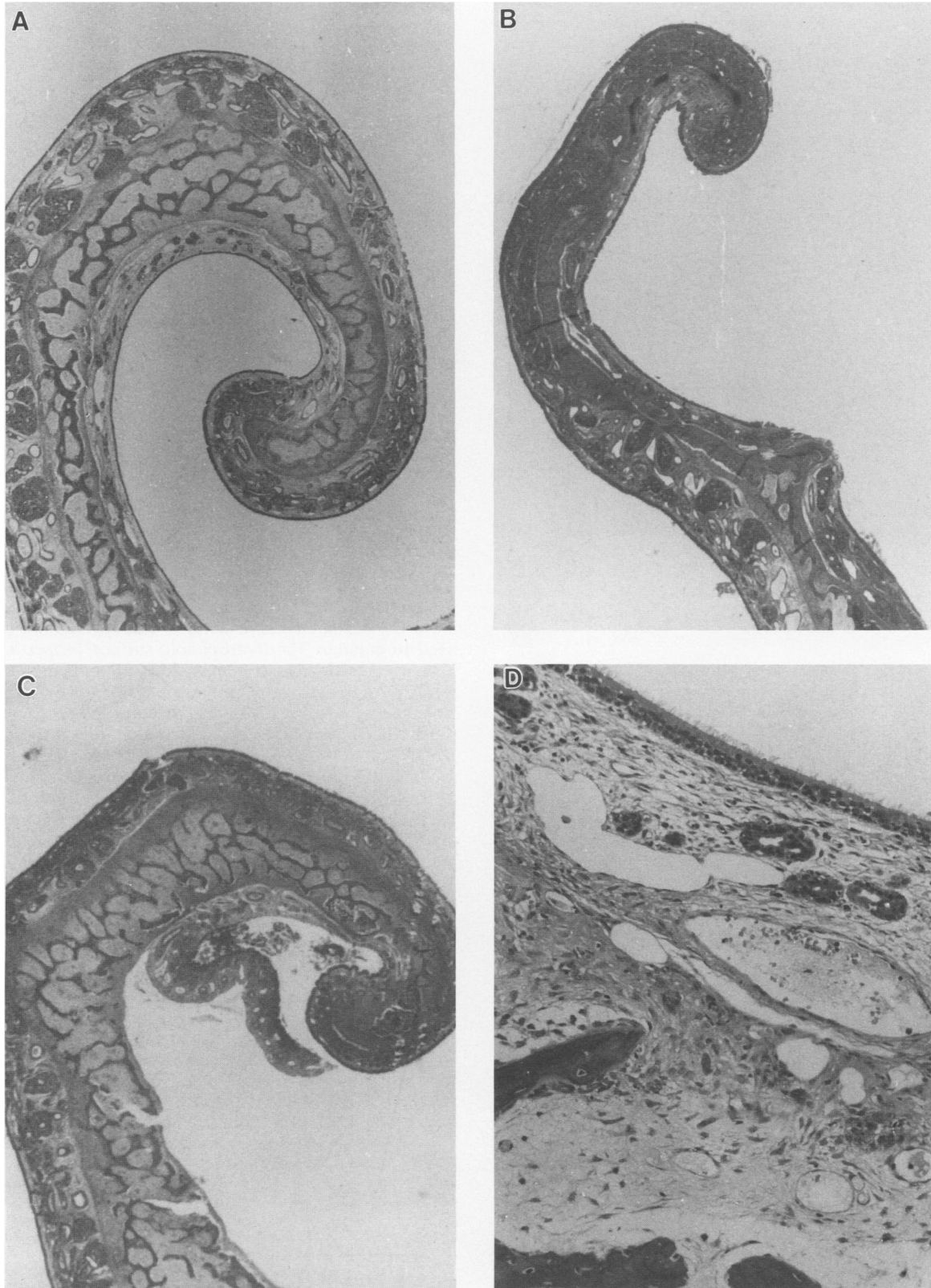


FIG. 3. Histological sections of nasal turbinates from a control pig (A and D) or from pigs inoculated with capsulated isolate *P. multocida* 4275 (B and E) or with noncapsulated isolate *P. multocida* 4275B (C). Note in panel B the turbinate atrophy observed in pigs inoculated with the capsulated isolate and in panel E the bone resorption and inflammation of the mucosa associated with hyperplasia and squamous metaplasia of the epithelium seen in all pigs inoculated with either the capsulated or the noncapsulated isolates. Stain, hematoxylin and eosin. Magnification, $\times 20$ (A, B, and C) or $\times 100$ (D and E).

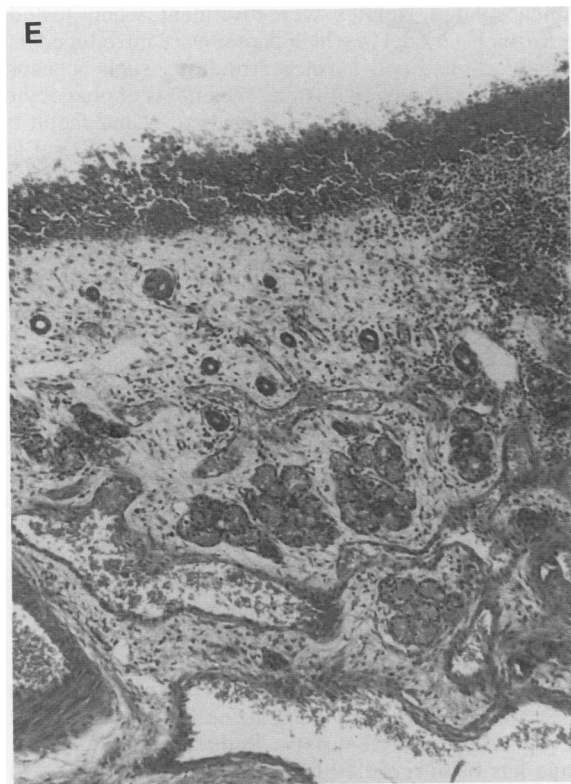


FIG. 3—Continued.

39.5°C, was between 40.5 and 41°C at 72 to 96 h after the first inoculation, in both groups. Growth was similar in both groups, with mean daily gains of 502 g (group A) and 487 g (group B). Pigs of group A developed turbinate atrophy (Fig. 3). Histologically, turbinates of control pigs were normal (Fig. 3D). Histopathological changes present in all pigs of both infected groups were bone resorption and inflammation of the mucosa associated with hyperplasia and squamous metaplasia of the epithelium (Fig. 3E); turbinate lesions were always more severe in group A infected pigs.

We used dot blotting on a nitrocellulose membrane to evaluate the binding of porcine respiratory tract mucus to *P. multocida* isolates. Bacterial cells were immobilized on the membrane and sequentially incubated with the mucus preparation and an antiserum against the mucus preparation (Fig. 4). All three noncapsulated variants showed more affinity for mucus than their capsulated parent isolate. Purified LPS from *P. multocida* dotted on a membrane were also able to bind mucus.

The adherence of all the isolates to porcine tracheal rings was also evaluated. After 6 h of incubation, the noncapsulated variants adhered in significantly ($P < 0.04$) greater numbers than their capsulated parent isolates (Table 1). The noncapsulated variants 2884B and 4275B adhered about two times better and the noncapsulated variant 4533B adhered six times better than their capsulated parent isolates. We tried to inhibit the adherence of isolates 4533 and 4533B to porcine tracheal rings with LPS purified from the noncapsulated isolate 4533B. As can be seen in Table 2, purified LPS significantly ($P < 0.05$) inhibited, by 97 and 47%, the adherence to porcine tracheal rings of isolates 4533 and 4533B, respectively.

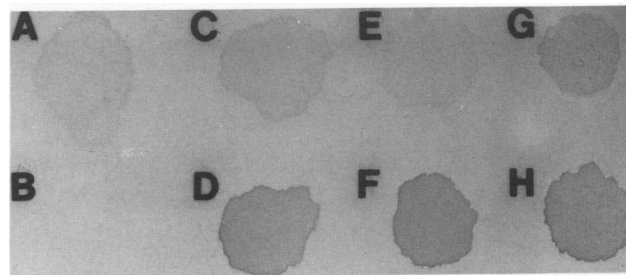


FIG. 4. Dot blot assay used to show binding of porcine respiratory tract mucus to isolates of *P. multocida*. Bacterial cells, immobilized on the membrane, were sequentially incubated with a mucus preparation and an antiserum against the mucus preparation. The following isolates were used: positive control *P. multocida* 4-4056 (A); negative control *E. coli* O111 (B); *P. multocida* 2884 (C) and 2884B (D); *P. multocida* 4275 (E) and 4275B (F); *P. multocida* 4533 (G) and 4533B (H).

Finally, we used polymyxin B-gold labeling to visualize the accessibility of LPS at the surface of both capsulated and noncapsulated isolates. Cells of the capsulated isolates were weakly labeled (Fig. 5A), whereas cells of the noncapsulated isolates were heavily labeled by the polymyxin B-BSA-gold particles (Fig. 5B). No labeling was observed when BSA-gold particles were used.

DISCUSSION

In the present study, we used three distinct capsular type D isolates of *P. multocida* to evaluate the role of the polysaccharidic capsule in virulence and in bacterial interactions with porcine respiratory tract cells and mucus. Electron microscopic examination revealed that capsulated isolates were covered by a layer of capsular material whose thickness was characteristic of capsular type D *P. multocida* isolates (20). Aside from the absence of capsular material, noncapsulated isolates phenotypically resembled their respective parent isolates in all other aspects evaluated, namely, the whole-cell protein and LPS profiles and the DNT production. CR bound strongly to the noncapsulated isolates. CR binding to whole cells has been used to evaluate the accessibility of the lipid fraction of the outer membrane (5). It is known that CR binds nonspecifically to the lipid fraction through hydrophobic interactions with bacterial membranes in the presence of salt. The capsular polysaccharide of *P. multocida* prevented the CR from binding to the cell surface. Noncapsulated isolates appeared to present a surface where hydrophobic molecules are more accessible, although their surface was found to be hydrophilic by a salt aggregation test.

TABLE 2. Inhibition of adherence of *P. multocida* 4533 and 4533B to porcine tracheal rings by LPS from *P. multocida* 4533B

Isolate	Incubation in the presence of LPS	Adherence index (mean $\pm$ SD) ^a
4533	No	38 $\pm$ 10
	Yes	1 $\pm$ 1 ^b
4533B	No	74 $\pm$ 9
	Yes	39 $\pm$ 3 ^b

^a Determined after 1 h of incubation.

^b Significant differences ($P < 0.05$) were observed between adherence of an isolate in the presence and in the absence of purified LPS.

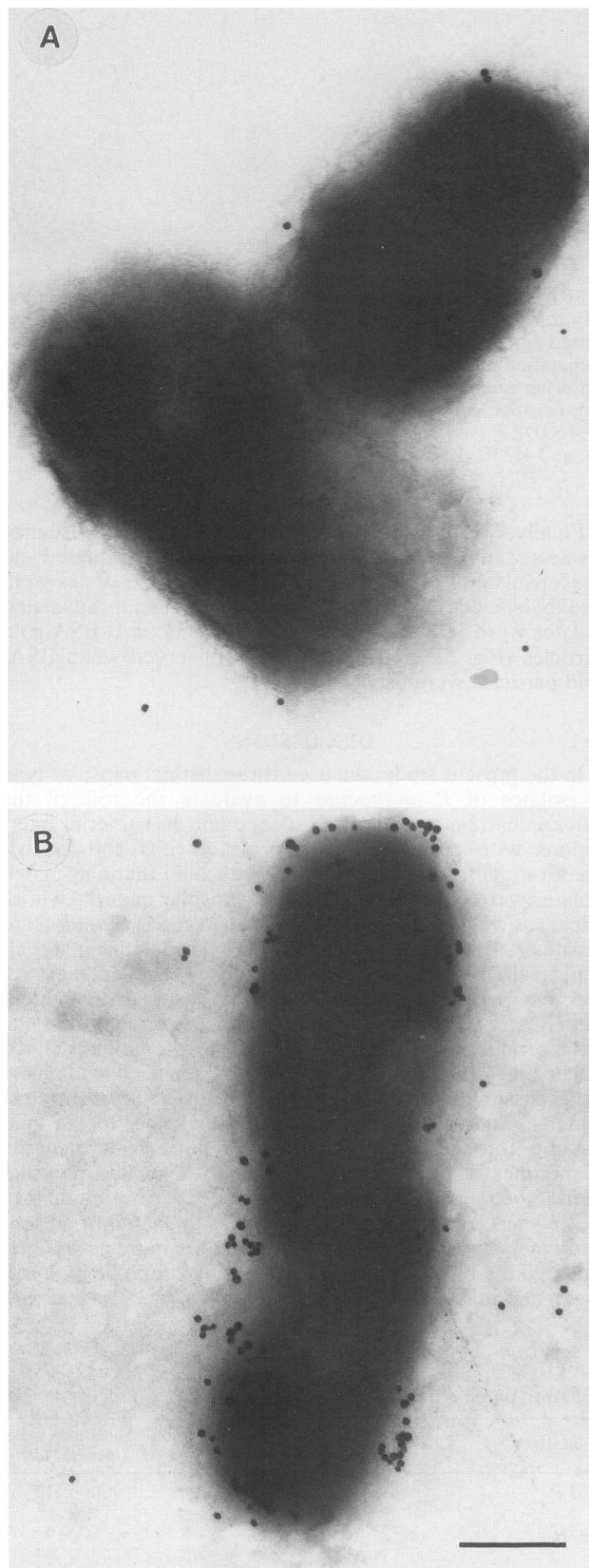


FIG. 5. Transmission electron micrographs of cells of *P. multocida* isolates 4533 (A) and 4533B (B) labeled with polymyxin B-BSA-gold particles (15 nm). Bar, 200 nm.

Noncapsulated isolates were avirulent when tested in mice. In such a model in which bacteria are introduced in the peritoneal cavity, cell surface structures such as capsule enabling the bacteria to withstand the effects of phagocytosis are known to be important. In avian type A and rabbit type D *P. multocida*, the capsule has been shown to be at least partially responsible for resistance to phagocytosis (16, 35). Inoculation of young pigs by a more natural route, the respiratory tract, enabled us to evaluate more fully the role of capsular material in virulence. The same type of lesions were observed in pigs inoculated with capsulated isolate 4275 or its noncapsulated variant 4275B, although lesions were more severe in pigs inoculated with the capsulated isolate. In addition, turbinate atrophy was observed in pigs inoculated with the capsulated isolate 4275 (group A). The production of DNT by both isolates is probably responsible for the histological findings, while the presence of capsule leads to more severe lesions. It was impossible to determine whether the noncapsulated isolate remained noncapsulated in vivo since reisolation of *P. multocida* from the nasal cavities was unsuccessful.

One consistent finding was that noncapsulated bacteria showed a greater affinity for respiratory tract mucus and also adhered in significantly higher numbers to respiratory tract cells. One possible mechanism by which the presence of capsule on a bacterium might interfere with its interactions with host cell surface is by masking adhesin(s) that might be present on the bacterial surface. Reduction of adherence by capsule has been reported before with other gram-negative bacteria, including *Haemophilus influenzae* (38, 42), enterotoxigenic *E. coli* (34), and *Actinobacillus pleuropneumoniae* (19).

Our laboratory has previously shown that porcine isolates of *P. multocida*, of capsular types A or D, had affinity for porcine respiratory mucus (24). More recently, we have shown that the ciliostasis and the concomitant accumulation of mucus induced by *B. bronchiseptica* tracheal cytotoxin might explain the predisposing effects this microorganism has on a subsequent *P. multocida* infection (10). Results of the present study suggest that *P. multocida* LPS might be one of the molecules involved in the interactions with porcine respiratory tract cells and mucus since purified LPS bound respiratory tract mucus and blocked adherence to porcine tracheal rings. The partial inhibition of adherence observed with the noncapsulated isolate 4533B suggests that there may be a non-LPS component that is also important in adherence. Results obtained in this study with polymyxin B, a substance known to interact with the lipid A-core region of LPS molecules (26), indicate that LPS is, as expected, more accessible at the surface of noncapsulated cells. Interestingly, LPS have been suggested to play a major role in adhesion of two other respiratory pathogens, both members of the *Pasteurellaceae*, i.e., *A. pleuropneumoniae* (1, 2) and *Pasteurella haemolytica* (43, 44).

It is becoming more and more apparent that, aside from the ability to recognize a receptor on a eukaryotic cell, adhesins have potential as biological effector molecules and play a substantial role in determining the outcome of a prokaryotic-eukaryotic interaction (17). They may initiate invasion by the pathogen, either by themselves or by engaging a cascade of secondary molecules, and have inherent abilities to affect host defense systems. Eukaryotic cells would be expected to experience the well-known biological effects of endotoxin in conjunction with presentation of a lipopolysaccharidic adhesin. Further studies are needed to

determine whether *P. multocida* LPS does elicit a response in the targeted cells.

The presence of capsule resulted in a decrease in adherence of *P. multocida* to porcine tracheal rings maintained in culture and to respiratory tract mucus. Results of these in vitro assays suggest that capsular material of *P. multocida* masks outer membrane components which are involved in adherence. However, data from an experimental infection in piglets suggest that capsule, although expected to interfere with colonization, is an important virulence factor as exemplified by the fact that the capsulated isolate caused more severe turbinate lesions. We have good indications that, under iron-restricted conditions such as those encountered in vivo, *P. multocida* may not be heavily encapsulated although not totally devoid of capsule (18). The decrease in capsular material production we observed under iron limitation probably results in a better exposure of the adhesin(s) at the bacterial cell surface, which could be advantageous for this microorganism during respiratory tract colonization.

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