

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO	
RAD: 12-12902- -5	FECHA: 2013-11-07 18:18:02
DEP: 2020 DIRECCION DE NUEVAS CREACIONES	EVE: 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS: 21
ACT: 519 PRESENTACION	

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
ALVARO CORREA ORDOÑEZ

Asunto: Radicación: 12-12902- -5
Trámite: 11
Evento: 378
Actuación: 519
Oficio: 21123
Folios: 21

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/US2010/041396				
Fecha de Presentación Internacional	08/07/2010				

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	No. 12-012902-00000-0000	27/Enero/2012
Reivindicaciones:	No. 12-012902-00000-0000	27/Enero/2012
Dibujos:	No. 12-012902-00000-0000	27/Enero/2012
Secuencias:	No. 12-012902-00000-0000	27/Enero/2012
Prioridad:	US 61/223,755	08/Julio/2009

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del capítulo primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10)

reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. En el presente caso se estudiará la totalidad del capítulo reivindicatorio No. 12-012902-00000-0000 (reivindicaciones 1-57).

Título de la solicitud: “Adherencia y anti-adherencia bacteriana a mucosidad, células epiteliales y otras células”.

El problema planteado en esta solicitud consiste en la necesidad de obtener un método para detectar, identificar y medir la adherencia bacteriana a mucosidad y células epiteliales.

Para resolver este problema técnico la solicitud en estudio proporciona un método y un kit para detectar e identificar la presencia o ausencia de adherencia bacteriana a mucosidad.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C12Q 1/100; G01N 33/00.

4. Reivindicaciones relativas a un uso (Art 14 D 486)

El uso de la reivindicación 57 no es patentable, de acuerdo con el Art 14.

5. De la solicitud de patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

- 1- El kit de la reivindicación 1 debe estar definido por todos los compuestos que comprende, el envase, recipiente o contenedor y las instrucciones (sin indicar la información que contienen dichas instrucciones). La muestra que contiene bacterias no hace parte del kit, debido que corresponde a la muestra en la que se aplica el método. Se sugiere eliminar la mención de la muestra en la reivindicación.
- 2- Las reivindicaciones 2-55 no son concisas, se sugiere restringir la solicitud con los sustratos, anticuerpos, y células que identificaron la adherencia bacteriana a la mucosidad y células epiteliales y que están sustentados y probados en la descripción (reivindicaciones 2-55).
- 3- La composición de las reivindicaciones 56-57 debe ser caracterizada a partir de los ingredientes que la conforman y las proporciones de los mismos y no a partir de la identificación del agente. Se le recuerda al solicitante que la composición debe estar sustentada en la descripción. El ingrediente se está definiendo por la forma de identificarlo. Se sugiere eliminar las reivindicaciones.
 - 4- El método de las reivindicaciones 17 y 36 no es claro, en el cual se menciona que se utilizan condiciones para medir la adherencia y la antiadherencia, lo cual corresponde a un resultado a alcanzar, en donde no se definen las condiciones. Adicionalmente cada etapa se está definiendo en una reivindicación dependiente diferente se le recuerda al solicitante que cada etapa debe ser incluida en la reivindicación independiente.
 - 5- Las etapas c y d del método de la reivindicación 36 no hacen parte de las características técnicas del método. Se sugiere eliminar dichas etapas.
- 6- Las reivindicaciones 2, 4, 21, 40, 43 no son claras, la definición de los ingredientes por su funcionalidad no es aceptable (sustrato que permite la visualización del anticuerpo, en el que dicho sustrato es una composición para proporcionar una señal) debido a que no se define el ingrediente.

7– El anticuerpo IgG de cabra-AP purificado por afinidad, hace referencia a su método de obtención y no se define por su estructura. (Reivindicación 13, 32, 51). Se sugiere definir el anticuerpo por su estructura y no por el método de obtención.

Se requiere al solicitante hacer las aclaraciones necesarias.

6. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: Julio 08 de 2009, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	Ofek et al., 1986. Journal of clinical microbiology. Vol. 24, No. 4. Págs. 512-516.	“Enzyme-linked immunosorbent assay for adherence of bacteria to animal cells”	1986
D2	Jacques et al., 1993. Infection and Immunity. Vol. 61. Págs. 4785-4792.	“Virulence of capsulated and noncapsulated isolates of Pasteurella multocida and their adherence to porcine respiratory tract cells and mucus”	1993

El documento D1¹ divulga un método para medir la adherencia y anti-adherencia entre bacterias y mucosidad y/o células epiteliales (Págs. 513-514).

El documento D2 divulga un kit comprendiendo un soporte sólido fijado con bacteria, una muestra comprendiendo mucosidad, un anticuerpo primario, un anticuerpo secundario, un sustrato (Págs. 4786, 4789)

7. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La reivindicación 17 consiste en *“un método para medir la adherencia y anti-adherencia entre bacterias y mucosidad y/o células epiteliales que comprende*

- a. *Proporcionar*
 - i. *Una muestra que comprende bacterias*
 - ii. *Mucosidad y/o células epiteliales u otras*
- b. *Combinar dicha muestra que comprende bacterias y dicha mucosidad y/o células epiteliales dentro de un ensayo colorimétrico no radioactivo en condiciones tales que se mide la adherencia y la anti-adherencia entre dichas bacterias y dicha mucosidad y/o células epiteliales”.*

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1
Método para medir la adherencia y anti-adherencia entre bacterias y mucosidad y/o células epiteliales que comprende 1. Proporcionar i. Una muestra que comprende bacterias ii. Mucosidad y/o células epiteliales u otras 2. Combinar dicha muestra dentro de un ensayo colorimétrico no radioactivo	Método para medir la adherencia y anti-adherencia entre bacterias y mucosidad y/o células epiteliales que comprende 1. Proporcionar i. Una muestra que comprende bacterias (Pág. 514) ii. Mucosidad y/o células epiteliales u otras (Pág. 513) - Combinar dicha muestra dentro de un ensayo colorimétrico no radioactivo (Pág. 514)

1. <http://www.ncbi.nlm.nih.gov/pubmed/2877005>

Las características esenciales del método de la reivindicación 17 habían sido divulgadas previamente en el documento D1, el cual da a conocer un método para medir la adherencia y anti-adherencia entre bacteria y mucosidad y/o células epiteliales (Págs. 513-514). Adicionalmente, el documento D1 da a conocer que el ensayo colorimétrico no radioactivo es una ELISA (Pág. 514, Col 1, Párr. 2), la adición de anticuerpos primarios específicos para la detección de bacterias unidas con mucosidad y/o células epiteliales (Pág. 514, Col. 1, Párr. 1), la adición de anticuerpo secundario marcado (Pág. 514, Col. 1), sustrato para la visualización los anticuerpos secundarios (Pág. 514, Col. 1), mucosidad o células epiteliales fijadas en una microplaca (Pág. 513, Col. 1, Párr. 6), anticuerpo marcado que comprende un marcador enzimático (Pág. 513, Col. 1, Párr. 1), sustrato que es una composición para proveer una señal colorimétrica, fluorimétrica o quimioluminiscente en la presencia del marcador enzimático (Pág. 513, Col. 1, Párr. 1), método en el que la bacteria es E. coli (Pág. 513, Col. 1, Párr. 1) y una composición que comprende un agente (Pág. 514, Col. 2, Párr. 5).

En consecuencia, la reivindicación 17 carece de novedad, según lo divulgado en el documento D1.

Las reivindicaciones dependientes 18 – 24, 27 no mencionan alguna característica que haga nuevo el método de la reivindicación 17, por lo cual se considera que no son nuevas.

La reivindicación 36 consiste en *“un método para identificar un agente que modula la adherencia entre bacterias y mucosidad y/o células epiteliales, que comprende*

- a. Proporcionar*
 - i. Una muestra que comprende bacterias*
 - ii. Mucosidad y/o células epiteliales*
 - iii. Un agente*
- b. Combinar dicha muestra que comprende bacterias y dicha mucosidad y/o células epiteliales dentro de un ensayo colorimétrico no radioactivo en condiciones tales que se mide la adherencia y la anti-adherencia entre dichas bacterias y dicha mucosidad y/o células epiteliales.*
- c. Comparar dicha adherencia bacteriana en presencia y ausencia de dicho agente.*
- d. Identificar dicho agente como un modulador de adherencia entre dichas bacterias y dicha mucosidad y/o células epiteliales si dicha adherencia es superior o inferior a la adherencia entre dicha bacterias y dicha mucosidad y/o células epiteliales en ausencia de dicho agente”.*

La reivindicación 56 consiste en: *“composición que comprende un agente, en la que dicho agente es modulador de adherencia bacteriana con mucosidad (...)”*

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1
Método para identificar un agente que modula la adherencia entre bacterias y mucosidad y/o células epiteliales, que comprende	Método para identificar un agente que modula la adherencia entre bacterias y mucosidad y/o células epiteliales, que comprende
a. Proporcionar - i.Una muestra que comprende bacterias - ii.Mucosidad y/o células epiteliales - iii.Un agente	a. Proporcionar - i.Una muestra que comprende bacterias (Pág. 514) - ii.Mucosidad y/o células epiteliales (Pág. 513) - iii.Un agente (Pág. 514)
b. Combinar dicha muestra dentro de un ensayo colorimétrico no radioactivo	b. Combinar dicha muestra dentro de un ensayo colorimétrico no radioactivo (Págs. 513-514)

Las características esenciales de la composición de la reivindicación 36 habían sido divulgadas previamente en el documento D1, el cual da a conocer un método para

identificar un agente que modula la adherencia entre bacterias y mucosidad y/o células epiteliales (Págs. 513-514), el método también se compara la adherencia bacteriana en presencia y ausencia del agente y se identifica el agente como modulador de la adherencia entre las bacterias y la mucosidad y/o células epiteliales (Pág. 514, Tabla 2).

Adicionalmente, el documento D1 da a conocer que el ensayo colorimétrico no radioactivo es una ELISA (Pág. 514, Col 1, Párr. 2), la adición de anticuerpos primarios específicos para la detección de bacterias unidas con mucosidad y/o células epiteliales (Pág. 514, Col. 1, Párr. 1), la adición de anticuerpo secundario marcado (Pág. 514, Col. 1), sustrato para la visualización los anticuerpos secundarios (Pág. 514, Col. 1), mucosidad o células epiteliales fijadas en una microplaca (Pág. 513, Col. 1, Párr. 6), anticuerpo marcado que comprende un marcador enzimático (Pág. 513, Col. 1, Párr. 1), sustrato que es una composición para proveer una señal colorimétrica, fluorimétrica o quimioluminiscente en la presencia del marcador enzimático (Pág. 513, Col. 1, Párr. 1), método en el que la bacteria es E. coli (Pág. 513, Col. 1, Párr. 1) y un agente que se selecciona de la lista que consiste en una molécula natural, molécula derivada de manera sintética o de manera recombinante (Pág. 514, Col. 2, Párr. 5).

En consecuencia, la reivindicación 36 no es nueva o carece de novedad, según lo divulgado en el documento D1.

Las reivindicaciones dependientes 37- 43, 46, 55 no mencionan alguna característica que haga nueva a la composición de la reivindicación 36, por lo cual se considera que no son nuevas. La reivindicación 56 carece de novedad según lo divulgado en el documento D1.

Nivel inventivo (Art18 D486)

La reivindicación 1 consiste en *“un kit que comprende un ensayo de inmunoabsorción ligado a enzimas no radioactivo (ELISA) para someter a ensayo la adherencia y anti-adherencia bacteriana con mucosidad y/o células epiteliales que comprende un soporte sólido que tiene mucosidad y/o células epiteliales recubiertas sobre el mismo, una muestra que comprende bacterias, un anticuerpo primario específico para dichas bacterias, y un anticuerpo secundario marcado de forma detectable específico para dicho anticuerpo primario unido a dichas bacterias”*.

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1
Kit ELISA para someter a ensayo la adherencia y anti-adherencia bacteriana con mucosidad	Método para someter a ensayo la adherencia y anti-adherencia bacteriana con mucosidad
Soporte sólido que tiene mucosidad	Soporte sólido que tiene mucosidad (Pág. 513)
Muestra que comprende bacterias	Muestra que comprende bacterias (Pág. 514)
Anticuerpo primario específico para dichas bacterias	Anticuerpo primario específico para dichas bacterias (Pág. 514)
Anticuerpo secundario marcado	Anticuerpo secundario marcado (Pág. 514)

El documento D1 se considera el estado de la técnica cercano a la invención definida en la reivindicación 1 porque divulga un método para someter a ensayo la adherencia y anti-adherencia bacteriana con mucosidad (Págs. 513-514)

La diferencia entre la invención, definida en la reivindicación 1 y el procedimiento de D1 consiste en que la invención se presenta en forma de kit

Esta diferencia no parece conceder un efecto técnico inesperado o una ventaja al kit reclamado en esta solicitud.

El problema técnico objetivo de esta solicitud consiste en la necesidad de un kit que incluya los elementos del método para someter a ensayo la adherencia y anti-adherencia bacteriana con mucosidad del documento D1.

Sin embargo, el documento D2 divulga un kit para medir la adherencia y anti adherencia entre bacteria y mucus (Págs. 4786, 4789). De manera que el estado de la técnica sugiere a la persona del oficio normalmente versada en la materia la forma en que se resuelve el problema en esta reivindicación. Por lo cual no se considera obvia.

De manera que la solución propuesta por esta solicitud en la reivindicación 1 y sus dependientes 2 – 16, es obvia y no tiene Nivel Inventivo. Las reivindicaciones 25, 26, 28-35, 44, 45, 47-54 no cumplen con el requisito de Nivel Inventivo.

8. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	Ofek et al., 1986. Journal of clinical microbiology. Vol. 24, No. 4. Págs. 512-516.	17-56	Novedad
D1	Ofek et al., 1986. Journal of clinical microbiology. Vol. 24, No. 4. Págs. 512-516.	1-16, 25, 26, 28-35, 44, 45, 47-54	Nivel inventivo
D2	Jacques et al., 1993. Infection and Immunity. Vol. 61. Págs. 4785-4792.	1-16, 25, 26, 28-35, 44, 45, 47-54	Nivel inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2013-11-12

Elaboró: Lina Maria Lizaraso Trespalacios
Revisó: Jeny Paola Villate Becerra

**Enzyme-linked immunosorbent assay for
adherence of bacteria to animal cells.**

I Ofek, H S Courtney, D M Schifferli and E H Beachey
J. Clin. Microbiol. 1986, 24(4):512.

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Enzyme-Linked Immunosorbent Assay for Adherence of Bacteria to Animal Cells

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Epithelial cells scraped from human oral mucosa and from pig intestines were immobilized onto the flat bottom surfaces of microtiter plates to study the adherence of various bacterial species to host cells. Bacterial adherence was quantitated either by an enzyme-linked immunosorbent assay technique with specific antibacterial serum as the first antibody followed by peroxidase-conjugated second antibody or by using biotinylated bacteria and avidin-peroxidase as the detecting agent. Unlabeled *Escherichia coli* and purified *E. coli* 987P fimbriae inhibited the adherence of biotinylated *E. coli* to immobilized enterocytes. The adherence of a mannose-sensitive strain of *E. coli* to immobilized oral epithelial cells was inhibited by mannose derivatives. The adherence of fimbriated *E. coli* 987P to immobilized enterocytes was approximately four times higher than the adherence of a nonfimbriated variant of the same strain. The adherence of *Streptococcus pyogenes* to oral cells was detected in the range of 10 to 150 bacteria per cell and was inhibited by lipoteichoic acid and albumin. The data suggest that the putative receptors which bind bacteria on the immobilized cells retain a functional form similar to that of native cells in suspension. The proposed adherence assay is easy to perform, allows the detection of specific adherence of test bacteria, and provides objective quantitation of adherence with a sensitivity of 10 bacteria per cell. Most importantly, the assay allows the testing of many variables in the same day.

In the last decade, various methods have been devised to study the adherence of bacteria to various nonphagocytic cells. Three types of animal cells have been used: cells from intact excised tissue (20), tissue culture cells (13, 18), and cells in suspension obtained from scraped tissues or from exfoliated cells (16) as initially described by Duguid and Gillies (7). The binding of bacteria to these cells has been quantitated by direct microscopic counting of adherent bacteria, counting of radiolabeled bacteria, and enumeration of CFU associated with the cells. Adherence assays are performed by mixing bacterial suspensions with the animal cells and then separating the cells with adherent bacteria from the nonadherent bacteria. Various techniques have been used for separation, such as differential centrifugation, density gradient centrifugation, and filtration through 8- μ m-pore-size filters or, in the case of tissue culture cells and intact excised tissues, by sequential washing.

Two major issues must be considered in selecting the method to be used: the availability of the test animal cells and the reliability of the technique used to separate cells with attached bacteria from the nonadherent bacteria. The tissue culture method can achieve adequate separation but requires tissue culture facilities that frequently are not available in bacteriological laboratories, and the cell types available are not always germane to the particular interest of the investigator. The excised tissue method can also achieve adequate separation but has limited sources and has been confined primarily to the intestinal tract. In contrast, scraping cells from tissues or collecting exfoliated cells can provide many cells from a wide variety of tissues. The separation of animal cells with adherent bacteria from nonadherent bacteria by either differential centrifugation or filtration through >8- μ m-pore-size filters has been difficult to achieve. In addition,

reproducible, quantitative recovery of the animal cells containing adherent bacteria is virtually impossible. Most investigators, therefore, have used the tedious and somewhat subjective microscopic counting of the average number of adherent bacteria per animal cell or the percentage of animal cells containing a minimum number of adherent bacteria. Although each of the above methods has been useful in obtaining information on the mechanisms of bacterial adherence to animal cells (17), none of them is practical for the quantitation of bacterial adherence on a large scale. For example, to test the effect of five concentrations of each of five different potential inhibitors on the adherence of two bacterial strains to a desired tissue cell requires 50 test samples.

In this study, we developed a method by which cells from various mucosal tissues were immobilized on the bottoms of microtiter plates to quantitate bacterial adherence. After the cells were exposed to bacteria, the nonadherent bacteria were washed off and an enzyme-linked immunosorbent assay (ELISA) with specific antibacterial serum was used to determine the total number of bacteria adherent to the immobilized cells. Alternatively, adherence was estimated by using biotinylated bacteria. The assays were reproducible and easy to perform and, most importantly, allowed the handling of many test samples on the same day.

MATERIALS AND METHODS

Reagents. Unless specified otherwise, all reagents and chemicals were purchased from Sigma Chemical Co., St. Louis, Mo. Peroxidase-conjugated goat anti-rabbit immunoglobulin G was obtained from Cooper Biomedical Inc., West Chester, Pa. Lipoteichoic acid was prepared from *Streptococcus pyogenes* 1RP41 by extraction with phenol and purification on a Sepharose 6B column as described previously (4).

* Corresponding author.

Bacterial strains. *S. pyogenes* M type 5 was grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) for 16 h at 37°C. The bacteria were harvested by centrifugation and washed in phosphate-buffered saline (PBS; 0.15 M NaCl, 0.02 M PO₄ [pH 7.2]). Stock solutions of *S. pyogenes* were stored at -20°C and were thawed and washed once with PBS before use in the adherence assays. A urinary tract isolate of a type 1 fimbriated, mannose-sensitive strain of *Escherichia coli* M (16) was grown in brain heart infusion broth (Difco) for 24 h at 37°C, washed in PBS, and suspended to the desired concentration. A diarrheagenic *E. coli* strain, 987P (O9:K103:H⁻) was a gift from R. E. Isaacson (15). Fimbriated and nonfimbriated variants of *E. coli* 987P were selected from fresh blood agar plates as described by Nagy et al. (15). The bacteria were grown overnight in tryptic soy broth (Difco) at 37°C, washed in PBS, and suspended to the indicated concentration.

Purification of *E. coli* 987P fimbriae. Fimbriae were purified from *E. coli* 987P by a modification of the technique of Korhonen et al. (12). The *E. coli* cells were grown in tryptic soy broth overnight at 37°C with agitation, harvested by centrifugation, and washed in 10 mM Tris hydrochloride (pH 7.8). The *E. coli* cells were subjected to maximum speed in a blender (Oster, Milwaukee, Wis.) for 2 min. This was repeated five times with intermittent cooling periods. The particulate fraction was removed by centrifugation, and the fimbriae were precipitated with 12% ammonium sulfate. The precipitate was dialyzed against 10 mM Tris hydrochloride (pH 7.8)-0.5% deoxycholate and centrifuged on a 10 to 60% sucrose gradient for 20 h at 22,000 rpm on an SW28 rotor (64,000 × *g*). The fractions containing fimbriae were pooled and dialyzed against 10 mM Tris hydrochloride. Deoxycholate was removed by the batch technique with Amberlite XAD-2 beads (Polysciences Inc., Warrington, Pa.). The fimbriae were judged to be pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by electron microscopy.

Antisera. Rabbit antisera against pepsin-extracted M5 protein from group A streptococci and against *E. coli* were prepared as previously described (5, 16). O9 antiserum was purchased from Difco.

Biotinylation of *E. coli*. *E. coli* 987P was grown as described above, washed in PBS, and suspended in 0.1 M carbonate (pH 8.2) to an optical density at 550 nm (OD₅₅₀) of 0.7. A sample (12.5 μl) of biotin-*n*-hydroxysuccinimide (10 mg/ml in dimethyl sulfoxide) was added to 1 ml of the bacterial suspension and incubated for 2 h at ambient temperature. The *E. coli* cells were then washed three times with PBS and suspended in PBS to the indicated concentration.

Preparation of epithelial cells. Oral epithelial cells (OEC) were collected from human donors by swabbing the buccal epithelium with cotton swabs. The swabs were vigorously stirred in PBS to release the epithelial cells. The epithelial cells were pooled, washed, and resuspended in PBS to a concentration of 1.5 × 10⁵/ml. Enterocytes were isolated by scraping the pig small intestines by the method of Dean and Isaacson (6). The cells were suspended in PBS at a concentration of 2 × 10⁶/ml.

Immobilization of cells onto microtiter plates. To each well (96-well, flat-bottomed microtiter plates; Linbro/Titertek; Flow Laboratories, McLean, Va.), 100 μl of a 1 M lysine solution in distilled water was added and incubated for at least 10 min and up to 2 h at room temperature. The plates were then washed three times with distilled water, and 100 μl of a 1.25% glutaraldehyde solution in distilled water was immediately added. The plates were incubated for exactly 5

TABLE 1. Blocking by various agents of nonspecific binding of *S. pyogenes* to activated plates

Blocking agent ^a	Concn	% Inhibition of binding ± SD ^b
Gelatin	2 mg/ml	99 ± 0.5
Amino acid mixture ^c	1 mg/ml	10 ± 5.6
Tween 20	0.01%	95 ± 2.1
Horse serum	10%	39 ± 5.2
BSA	50 mg/ml	84 ± 1.5
Hemoglobin	20 mg/ml	94 ± 1.9
Casein	20 mg/ml	43 ± 4.8

^a All agents were dissolved in PBS.

^b The ELISA values for binding to activated plastic in the absence of blocker ranged from 1.77 to 2.30. Percentage of inhibition = [1 - (values of blocked wells/values of unblocked wells)] × 100.

^c A mixture of 20 amino acids, each at 1 mg/ml.

min and then were washed two times with distilled water. Microtiter plates at this stage were termed activated. PBS (100 μl) was immediately added to each well, followed by 50 μl of the epithelial cell suspension. The cells were gently mixed and allowed to settle for 10 min at ambient temperature. The plates were then centrifuged for 10 min at 650 × *g*. The supernatant was aspirated with caution so as not to disrupt the cell monolayer. The plates were then dried overnight at 37°C and stored at room temperature until used. Control plates were prepared as described above but without epithelial cells.

Assay of blocking agents. Various agents were tested for their ability to block nonspecific binding of *S. pyogenes* to activated plastic. Various agents (Table 1) were dissolved in PBS at the indicated concentrations, and 100 μl of each was added to the wells of activated plates. The plates were incubated at 37°C for 1 h and washed five times with PBS, followed by the addition of 100 μl of the streptococcal suspension and adjustment to an OD₅₃₀ of 0.4 in PBS. The plates were rotated horizontally for 1 h at ambient temperature, washed five times with PBS, and heat-fixed for 10 min at 65°C. The bound bacteria were enumerated by the ELISA technique as described below.

Adherence assay with biotinylated bacteria. The unoccupied plastic space in wells containing immobilized enterocytes was blocked by adding 100 μl of a 5% solution of bovine serum albumin (BSA). The plates were incubated for 3 h at 37°C and washed three times in PBS. A sample (50 μl) of biotinylated *E. coli* (approximately 2 × 10⁸ ml) was added to appropriate wells and incubated for 40 min at 37°C. The plates were washed three times with PBS and heat-fixed for 10 min at 65°C. Avidin-peroxidase conjugate (100 μl; 200 ng/ml in 5% BSA) was added to each well and incubated for 30 min at 37°C. The plates were then washed three times with PBS, and 100 μl of *O*-phenylenediamine solution was added to each well. The color was allowed to develop for 15 to 30 min, and the A₄₅₀ was measured with a microtiter plate reader (Dynatech Laboratories, Inc., Alexandria, Va.). The controls consisted of wells treated as described above but without immobilized enterocytes or without biotinylated bacteria to determine nonspecific binding. To determine whether biotinylation of the bacteria affected the adhesive properties of the *E. coli* cells, serial twofold dilutions of unlabeled bacteria were added to each well before the addition of the biotinylated bacteria. The specificity of the binding of biotinylated bacteria to the immobilized enterocytes was determined by mixing increasing concentrations of purified fimbriae with the biotinylated *E. coli* cells.

Adherence assay with antibodies. Before the adherence

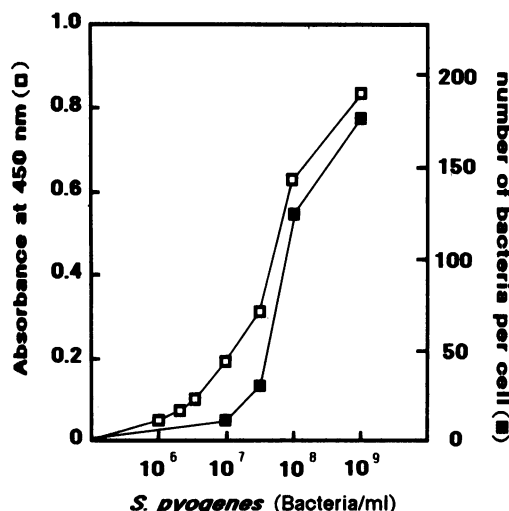


FIG. 1. Correlation between ELISA values for adherence and the number of bacteria attached to epithelial cells. Increasing numbers of *S. pyogenes* cells were added to the wells of a microtiter plate as described in Materials and Methods. The adherent bacteria were enumerated by an ELISA (□) or by direct microscopic count (■).

assay, all wells were incubated with 100 μ l of blocking agent for 1 h at 37°C. The wells were then washed four times with PBS. The bacteria were adjusted to the indicated concentration in PBS, and 100 μ l of the solution was added to each well. The microtiter plates were rotated horizontally for 1 h at ambient temperature and then washed five times with PBS. After the last wash was removed, the plates were fixed by heating at 65°C for 10 min. A sample (100 μ l) of the indicated dilution of rabbit antibacterial serum in 5% BSA was added to each well, incubated for 1 h at 37°C, and washed five times with PBS. Peroxidase-labeled goat anti-rabbit immunoglobulin G (100 μ l) diluted 1/1,000 in PBS–5% BSA was then added to each well and incubated for 1 h at 37°C. After five washes with PBS, 100 μ l of a solution of 5-aminosalicylic acid was added to each well. The color was allowed to develop for 15 to 30 min and was then measured at 450 nm with a microtiter plate reader (Dynatech). Controls consisted of wells without bacteria to ensure that the primary antibody did not cross-react with the endogenous bacteria or with the host cells and wells without immobilized cells to determine the amount of nonspecific binding of the bacteria to plastic.

RESULTS

The number of cells immobilized on the flat bottoms of microtiter plates was approximately 7×10^3 to 8×10^3 for OEC and approximately 13×10^3 to 14×10^3 for enterocytes. The OEC were approximately twice the size of enterocytes, and this seemed to affect the cell density, which was calculated to be 60 to 70/mm² and 120 to 140/mm² for OEC and enterocytes, respectively. Cell density was an important factor in the assay because of the possible nonspecific adherence of bacteria to unoccupied plastic space. We estimated that in the case of OEC about half of the plastic surface was occupied by the immobilized cells. Therefore, the ELISA for wells without epithelial cells overestimated by approximately 50% the nonspecific binding of bacteria to plastic. Thus, any difference in ELISA values

between wells containing immobilized cells and those without cells is a reflection of specific adherence to host cells.

The choice of a blocking agent is crucial. We tested various agents for the ability to block nonspecific binding of bacteria to activated plastic (Table 1). Gelatin, BSA, Tween 20, and hemoglobin were the best blocking agents tested. The choice of blocking agent for adherence assays was guided by the ability of the agent to block more than 80% of nonspecific binding to plastic and by consideration of any effects that the agent may have on specific adherence to the immobilized cells.

We used hemoglobin for streptococcal adherence assays and BSA for assays of gram-negative bacteria. By using these two proteins as blockers for nonspecific adherence of bacteria to plastic, we obtained low background ELISA readings in wells without epithelial cells. These background readings were subtracted from the higher values to obtain a quantitative measure of bacterial adherence to epithelial cells. Such an approach probably overestimates the values for nonspecific adherence of bacteria to plastic in wells containing immobilized cells, especially in wells containing a confluent monolayer of cells, but it seems to be the most practical approach to rule out any possible effect of nonspecific adherence of bacteria to plastic.

The adherence of *S. pyogenes* to immobilized OEC was dose dependent from 10^6 to 10^9 bacteria per ml (Fig. 1). To determine whether the ELISA readings corresponded to bacterial adherence to cells, the bottoms of the wells were removed and the number of adherent bacteria per epithelial cell was counted microscopically. There was a good correlation between the ELISA readings for adherence and those obtained by direct microscopic counts, and adherence as low as 10 bacteria per cell was detected by the ELISA technique (Fig. 1). This suggests that the ELISA is reliable for measuring the adherence of bacteria to animal cells.

To determine whether the mechanisms of bacterial adherence to immobilized cells are the same as those involved in the adherence of bacteria to cells in suspension, we tested the inhibitory activity of various agents that were previously demonstrated to specifically block the adherence of bacteria to cells in suspension. Methyl- α -D-mannoside and *p*-nitrophenyl-mannoside were used as specific inhibitors for the mannose-sensitive adherence of *E. coli* (8), and BSA and lipoteichoic acid were used as inhibitors of streptococcal adherence (3, 17). These inhibitors blocked the adherence of the corresponding bacteria, suggesting that the molecular mechanisms of adherence of *E. coli* and *S. pyogenes* to immobilized cells and cells in suspension are similar (Table 2).

Cells other than OEC may be immobilized onto microtiter plates. We immobilized enterocytes from the intestinal mucosa of pigs and assayed the adherence of *E. coli* 987P. The immobilized enterocytes bound significantly more of the fimbriated *E. coli* 987P than of the nonfimbriated phenotype (Table 2). Similar results were obtained previously by using cells in suspension (10). In addition, biotinylated *E. coli* was used to study bacterial adherence to enterocytes. Biotinylated *E. coli* bound to immobilized enterocytes in a dose-dependent manner, and the binding was inhibited by unlabeled bacteria (Fig. 2), suggesting that the biotinylation of the bacteria did not alter the binding properties of the *E. coli*. Purified 987P fimbriae inhibited the attachment of the biotinylated *E. coli* to immobilized enterocytes in a concentration-dependent manner (Fig. 3). Isaacson et al. (10) obtained similar results with native *E. coli* and enterocytes in suspension. These results suggest that biotinylation may be

TABLE 2. Inhibition of adherence of different bacterial strains to cells from various tissues immobilized onto microtiter plates

Bacterium	Cell type	Inhibitor	Concn (mg/ml)	Adherence (ELISA units) ^a
<i>S. pyogenes</i> ^b	OEC			0.354
	OEC	Lipoteichoic acid	1	0.061
	OEC	BSA	1	0.055
<i>E. coli</i> M ^c	OEC			0.389
	OEC	Methyl- α -D-glucoside	25	0.316
	OEC	p-Nitrophenyl- α -D-mannoside	0.5	0.056
	OEC	Methyl- α -D-mannoside	25	0.041
<i>E. coli</i> 987P+ ^d	Enterocytes			0.817
<i>E. coli</i> 987P- ^d	Enterocytes			0.240

^a ELISA units, A_{450} of the reduced substrate.

^b *S. pyogenes* cells were used at an OD_{540} of 0.4, and anti-pepsin-extracted M5 protein serum was used at a 1:400 dilution. Hemoglobin (20 mg/ml) was the blocking agent.

^c *E. coli* with mannose-sensitive type 1 pili was determined by yeast cell agglutination. The *E. coli* M was used at an OD_{540} of 0.4, and anti-*E. coli* M serum was used at a 1:400 dilution. BSA (50 mg/ml) was the blocking agent.

^d Fimbriation (P+) or lack of fimbriation (P-) was determined by electron microscopic examination of negatively stained samples. *E. coli* 987P was used at an OD_{540} of 0.5. BSA (50 mg/ml) was the blocking agent. Antisera to *E. coli* O9 were used at a dilution of 1:1,000.

used in lieu of bacterium-specific antibodies and that immobilization of enterocytes on microtiter plates does not alter the functional properties of their receptor.

DISCUSSION

Numerous studies (for reviews, see references 1, 2, 8, 11, and 17) have confirmed that bacterial adherence to target tissue cells is an important initial step in the infectious process and involves a stereospecific interaction between bacterial adhesins and complementary receptor molecules on the animal cell surface. The identification of an adhesin and its receptor have allowed the design of in vivo experiments aimed at preventing infection. For example, experimental infections in animals have been prevented by using soluble analogs of the receptor or the adhesin that competitively inhibited adherence or by using antibodies raised against the bacterial adhesin or the host cell receptor (1, 2, 8). Usually, the identity of a receptor or adhesin is deduced from information obtained from experiments in which potential inhibitors or chemical and physical treatments of cells are tested for their effects on bacterial adherence. Such testing would be facilitated by an adherence assay that is

easily performed, provides objective results, and is readily available to most research and clinical laboratories.

The method described in this study for assaying bacterial adherence to animal cells has some advantages which make it suitable as the assay of choice in many laboratories. First, and probably most important, many variables may be tested on the same day, i.e., type of host target cell, type of bacterial strain, and various treatments or potential inhibitors. Second, the adherence is evaluated by the objective criteria of an ELISA, which avoids the use of radioactivity yet can achieve similar sensitivity. Third, the sources of the immobilized cells can be diverse, and the immobilized cells can be kept for relatively long periods. Immobilized enterocytes or OEC that were stored for 3 to 4 weeks exhibited adherence properties similar to those of cells immobilized on day 1 (data not shown). The method can be used to study not only bacterial adherence but also the binding of bacterial products to tissue cells. In preliminary experiments, we found that the B fragment of the *E. coli*

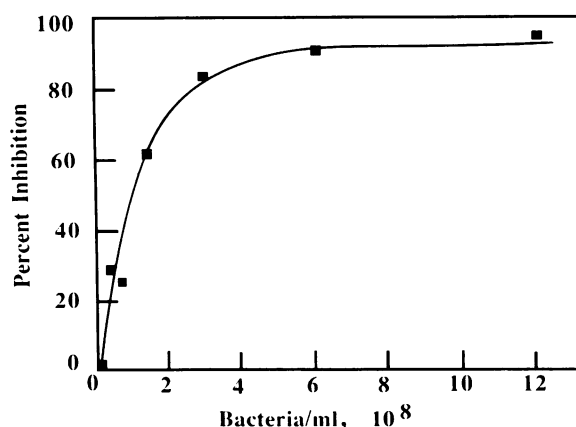


FIG. 2. Inhibition of adherence of biotinylated *E. coli* to enterocytes by unlabeled *E. coli*. Serial twofold dilutions of unlabeled *E. coli* 987P were added to biotinylated *E. coli* 987P (approximately 2×10^8 /ml), and the mixtures were added to the wells of a microtiter plate containing immobilized enterocytes. The adherence assay was performed as described in Materials and Methods. The percentage of inhibition was calculated by the formula $[1 - (A_{450} \text{ of the test well} / A_{450} \text{ of the control well})] \times 100$.

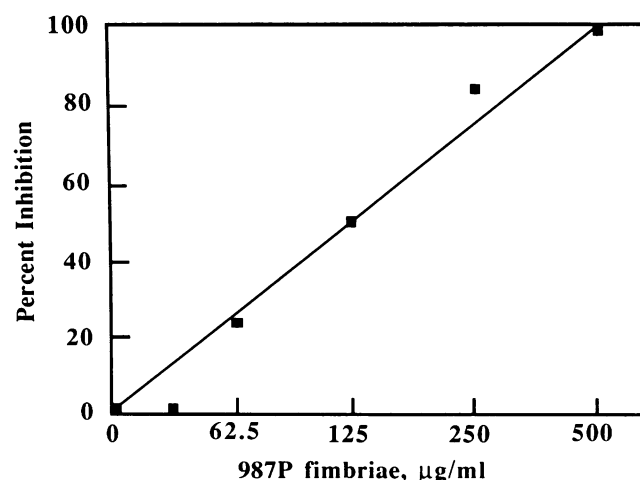


FIG. 3. Inhibition of adherence of biotinylated *E. coli* by purified 987P fimbriae. Increasing concentrations of purified 987P fimbriae were added to the wells of a microtiter plate containing immobilized enterocytes. Biotinylated *E. coli* 987P (approximately 2×10^8 /ml) was added, and the adherence assay was performed as described in Materials and Methods. The percentage of inhibition was calculated by the formula $[1 - (A_{450} \text{ of the test well} / A_{450} \text{ of the control well})] \times 100$.

labile toxin was able to bind to immobilized enterocytes (unpublished observation).

Other assays for the rapid detection of bacterial adhesins have been described. Hemagglutination of bacteria is a simple and convenient method that can be used in most laboratories to monitor bacteria for adhesive activities and allows the screening of many potential inhibitors (14). However, the test may lead to erroneous conclusions, because many strains of bacteria cause hemagglutination yet adhere poorly to epithelial cells (9, 11, 19). Furthermore, bacteria may produce two adhesins, only one of which is involved in hemagglutination reactions (9, 11, 19). Clearly, there is a need for a convenient and reliable assay for adherence which uses desired host cells to screen the adherence of bacterial pathogens, especially when the bacterial receptor is confined to a particular tissue.

In this study we used three different bacterial strains, each of which binds to a different receptor. Our results with specific inhibitors or adherent and nonadherent variants of the same strain were compatible with results obtained by the standard assay with cells in suspension. These results suggest that the receptors on the immobilized cells retain their functional form. As with other in vitro assays, including hemagglutination, the functional activity of these putative receptors must be confirmed in vivo. However, as a rule, such confirmation is not done until some information about the receptor and the corresponding adhesin is obtained by in vitro techniques.

In summary, the proposed assay employing immobilized animal cells on the flat bottoms of microtiter plates and ELISA techniques allows for reproducible quantitation for screening as well as for characterizing the molecular mechanism of bacterial adherence of many bacterial pathogens to diverse types of tissue cells.

ACKNOWLEDGMENTS

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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- $\log_{10}$ of $[(\log_{10} \text{ CFU per ring}) - (\log_{10} \text{ CFU per ml of inoculum}) + 5]$. 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,

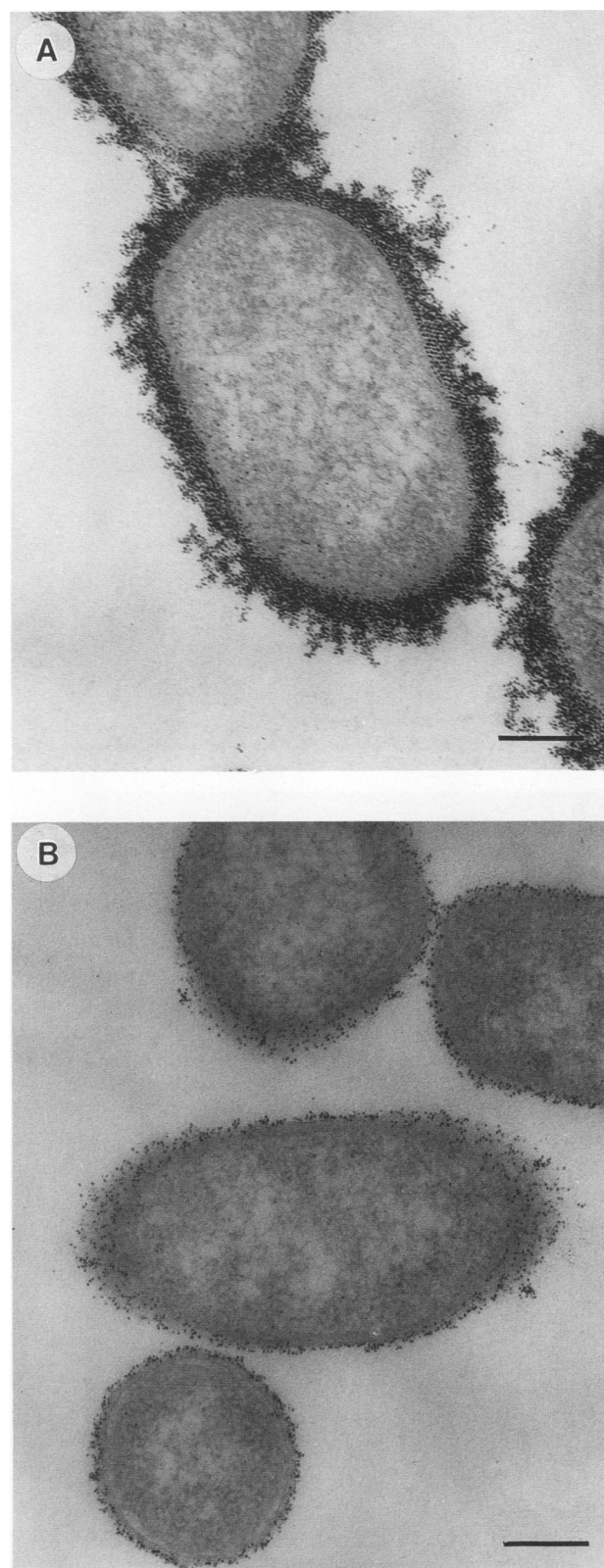


FIG. 1. Transmission electron micrographs of thin sections of cells of *P. multocida* isolates 4533 (A) and 4533B (B) labeled with polycationic ferritin. Bars, 200 nm.

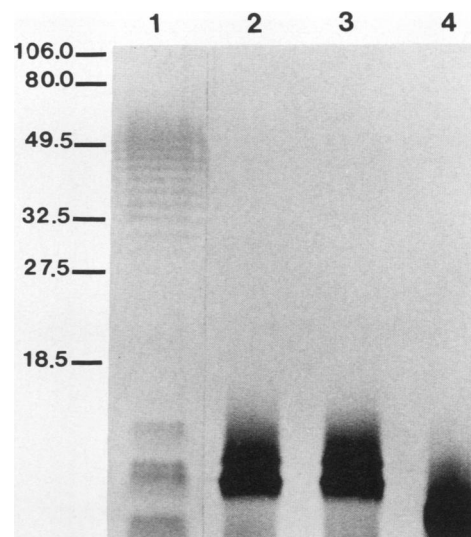


FIG. 2. Silver-stained SDS-PAGE profiles of whole-cell, proteinase K-treated preparations of *P. multocida* 4533 (lane 2) and 4533B (lane 3). LPS from *E. coli* O111:B4 (lane 1) and *S. typhimurium* TV119 Ra mutant (lane 4) were used as standards. The positions of the molecular mass markers are indicated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

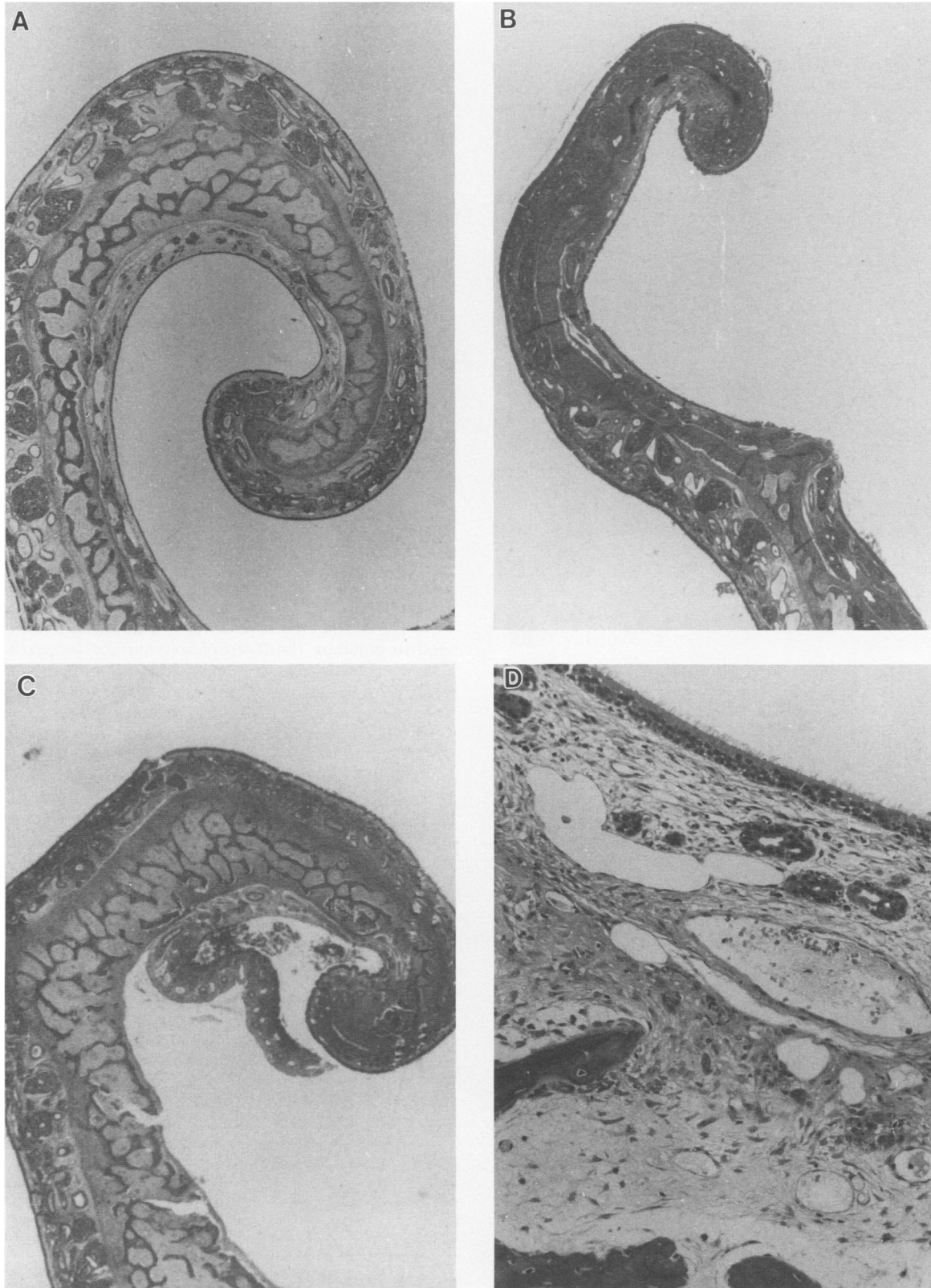


FIG. 3. Histological sections of nasal turbinates from a control pig (A and D) or from pigs inoculated with capsulated isolate *P. multocida* 4275 (B and E) or with noncapsulated isolate *P. multocida* 4275B (C). Note in panel B the turbinate atrophy observed in pigs inoculated with the capsulated isolate 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).

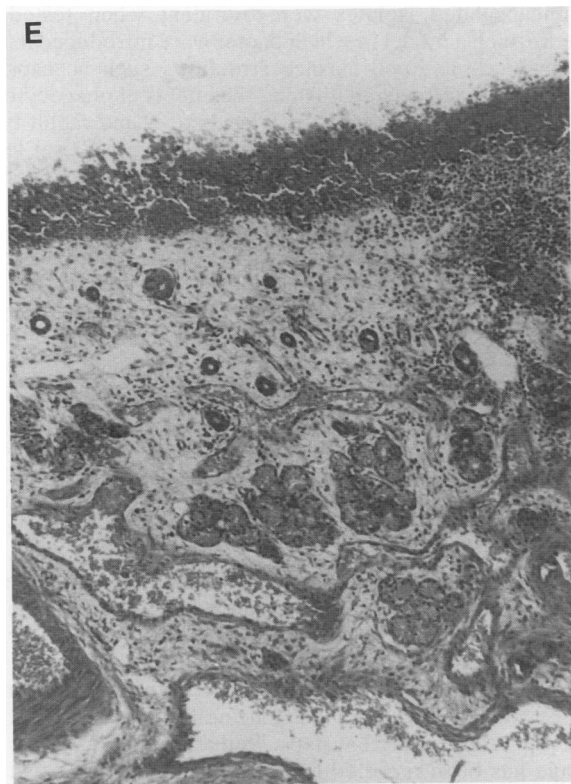


FIG. 3—Continued.

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.

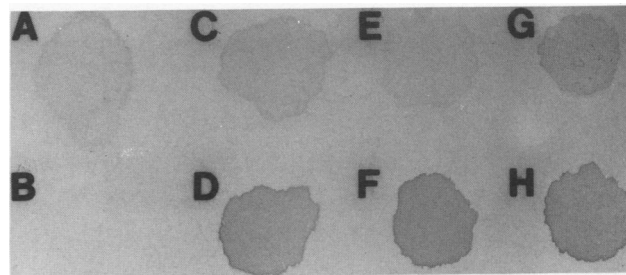


FIG. 4. Dot blot assay used to show binding of porcine respiratory tract mucus to isolates of *P. multocida*. Bacterial cells, immobilized on the membrane, were sequentially incubated with a mucus preparation and an antiserum against the mucus preparation. The following isolates were used: positive control *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.

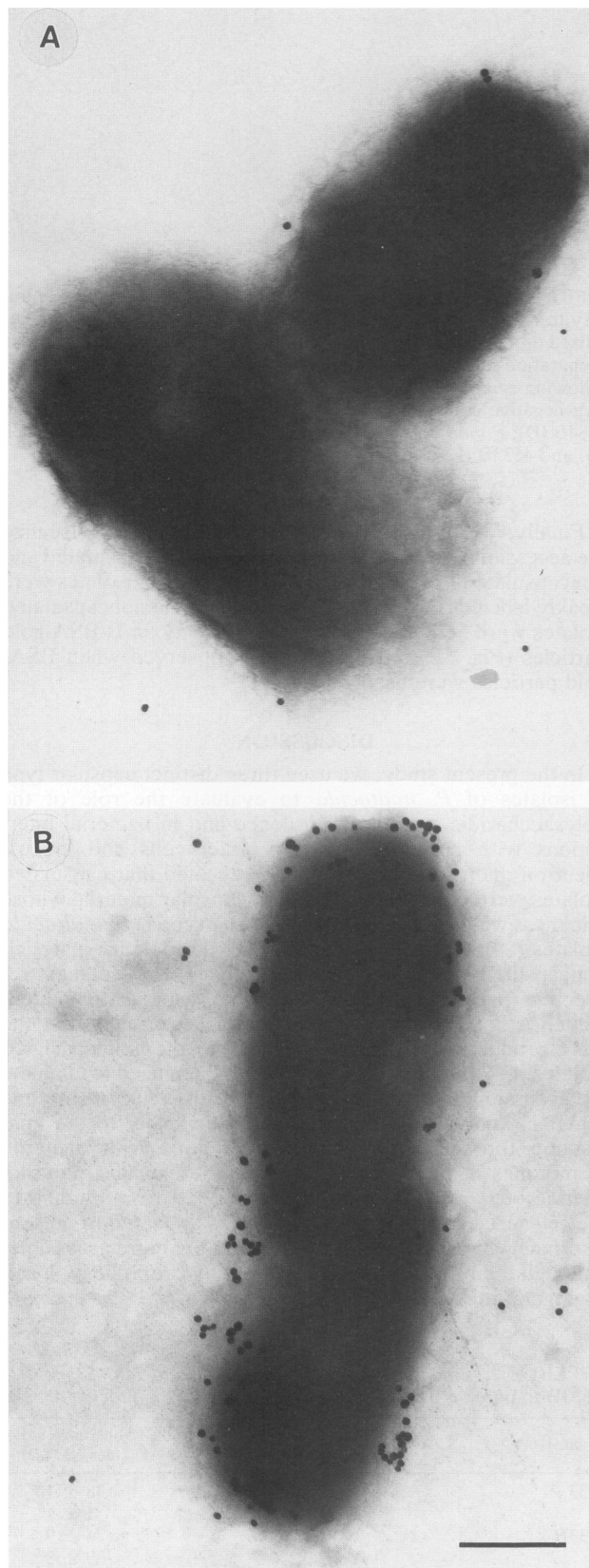


FIG. 5. Transmission electron micrographs of cells of *P. multocida* isolates 4533 (A) and 4533B (B) labeled with polymyxin B-BSA-gold particles (15 nm). Bar, 200 nm.

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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