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Purification and Subunit Heterogeneity of Pili of *Bordetella bronchiseptica*

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Pili were isolated and purified from *Bordetella bronchiseptica*. Electron microscopic observations revealed that pili are ubiquitous in this species. The occurrence of pili and flagella appeared to correlate with growth phase and colonial morphology. Pili were about 3 to 4 nm in diameter and morphologically similar to pili isolated from other gram-negative bacteria. Internal core structure was not evident. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified pili showed that up to three different pilus subunit variants could be observed on a single strain, depending on the colonial phase and culture condition. Enzyme immunoassay and immunoblot, however, showed that these subunit variants are serologically related. Mice vaccinated with purified pili were protected against a virulent intraperitoneal challenge of *B. bronchiseptica*. *B. bronchiseptica* pili were also found to be similar to *Bordetella pertussis* pili in morphology and in the molecular size and antigenic structure of pilus subunits. The intact pili of *B. bronchiseptica* and *B. pertussis*, however, appeared to have weak serological cross-reactivity.

Bacterial adhesion has been recognized as a critical step in mucosal infections. Certain surface antigens, such as pili, are identified as mediators in adherence of the microorganisms to the mucosal epithelial cells (2). Pili from many bacterial pathogens have been extensively studied and characterized (5, 6). *Escherichia coli* pili have been used successfully as vaccine components in the immunization of pigs against colibacillosis (12).

Pili were found on *Bordetella bronchiseptica* and *Bordetella pertussis* (1, 3, 4). Pathogens belonging to this group have been identified as causative agents in many respiratory infections such as kennel cough in dogs, atrophic rhinitis in pigs, and whooping cough in humans. In vitro studies have shown that piluslike filamentous materials might mediate the adherence of the bacteria to swine nasal epithelial cells (19). To study the protective activity of *B. bronchiseptica* pili in pigs, we purified and characterized pili from this bacterium and describe our findings here.

MATERIALS AND METHODS

Strains, growth media, and culture methods. Several strains of *B. bronchiseptica* were used in this study (Table 1). Cultures were routinely grown on brucella agar (Difco Laboratories, Detroit, Mich.) at 37°C. *B. bronchiseptica* was also grown at 37°C in Stainer and Scholte medium agitated at 500 rpm with pH maintained at 7.4 and dissolved oxygen at 80% saturation (16). Stock cultures were preserved in brucella broth at −70°C, with 10% (vol/vol) dimethyl sulfoxide (Fisher, Pittsburgh, Pa.) or 20% (vol/vol) glycerol added as stabilizer.

Microscopy. Morphology of *B. bronchiseptica* colonies was observed at ×7 to ×20 magnification using a dissecting microscope illuminated by diffused transmitted light. Nomenclature defined by Bemis et al. (3) for colonial morphotypes of *B. bronchiseptica* was used (see footnotes, Table 1).

Dark-field microscopy was used to monitor precipitation of pili by polyethylene glycol (PEG) and gross contamination of purified preparation by cellular debris or vesicles. This was done at ×65 to ×400 magnification using a microscope equipped with an oil-immersion dark-field condenser.

Purified pili were prepared for electron microscopy by negative staining. A drop of pili was applied to a carbon film supported by collodion on a 300-mesh copper grid. The specimen was washed and stained for 10 to 30 s with 1% uranyl acetate. Excess liquid was removed with filter paper, and the grid was allowed to air dry. The specimen was examined in a Hitachi HS-8-1 electron microscope operating at 60 kV. A carbon grating replica was used to calibrate the microscope.

Purification of pili. Cells from 16- to 20-h agar culture were used to isolate pili. The pilus purification was previously described (11). Briefly, cells were harvested in 0.05 M Tris-buffered saline (pH 7.2) and blended in a Sorvall Omnimixer in an ice bath at a speed of 10,000 to 13,000 rpm for 15 min. The bacteria were removed by centrifugation at 12,000 × *g* for 30 min. The pilus proteins in the supernatant were precipitated with 3% (wt/vol) PEG (PEG-6000; Kodak, Rochester, N.Y.) and 0.5 M sodium chloride. After overnight incubation at 4°C, the precipitated proteins were collected by centrifugation (12,000 × *g* for 60 min) and dissolved in one-tenth of the original volume of Tris-buffered saline. Insoluble materials after resuspension were removed by centrifugation (27,000 × *g* for 60 min). Precipitation was repeated until a homogeneous suspension of dark-field paracrystals was obtained.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used for determination of purity and subunit molecular size. Gel and buffer conditions were as described by Laemmli (9). Approximately 2 to 10 μg of proteins was applied per lane. The samples were prepared by heating in a boiling water bath for 2 min and electrophoresed at 20 to 30 mA per 1.5-mm-thick slab. Gels were fixed and stained in methanol-acetic acid-water (5:1:5) containing 0.2% Coomassie blue R250.

Densitometric tracing was performed using a spectrophotometer equipped with a gel scanner (Gilford Instruments, Oberlin, Ohio). Absorbance at 595 nm was monitored with a 0.10-by-2.36-mm aperture insert.

Electrophoretic transfer of SDS-PAGE-separated proteins

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TABLE 1. Relative proportions of pilus protein subunits expressed by various *B. bronchiseptica* strains

Strain	Origin	Source[a]	Phase[b]	% Distribution			Pilus phenotype
				21	22K	24K	
5389	Swine	Hill	1	9	87	4	A
87	Canine	Bemis	I	88	12	0	B
5389	Swine	Hill	I	61	31	2	B
5944	Swine	Hill	I	67	24	0	B
5998	Swine	Hill	I	71	23	0	B
588880	Canine	Hill	I	74	18	8	B
D-1	Canine	ATCC	I	ND[c]	ND	ND	B
55	Swine	ATCC	1	ND	ND	ND	B
GP	Guinea pig	Osen	I	ND	ND	ND	B
B133	Swine	Bemis	I	62	14	24	C
B133	Swine	NVSL	I	86	12	20	C
2-9	Swine	Bemis	I	50	13	33	C
6126	Swine	Hill	I	69	0	29	D
110H	Canine	Bemis	1→I	51	1	47	D
7-8	Swine	Bemis	I	68	1	28	D
110NH	Canine	Bemis	R	0	0	0	F[d]
B133	Swine	NVSL	R	0	0	0	F

[a] ATCC, American Type Culture Collection, Rockville, Md.; Bemis, D. Bemis, University of Tennessee, Knoxville; Hill, H. Hill, Iowa State University, Ames; NVSL, National Veterinary Services Laboratory, Ames, Iowa, Osen, E. Osen, Syntex Research, Palo Alto, Calif.

[b] Nomenclature on colonial morphotypes as described by Bemis et al. (3). Phase 1 colonies are small (<1 mm), pulvinated, and smooth. Intermediate-phase colonies are larger (1 to 2 mm), convex, and smooth. Rough-phase colonies are large (>2 mm), umbonate, and rough. Other parameters such as hemolysis were not considered.

[c] ND, No densitometric quantitation, only visual estimation of stained protein band intensity.

[d] F, Presence of flagella and few pili observed.

to nitrocellulose paper was performed using a Tris-glycine-methanol buffer in an overnight run at 100 mA according to procedures which accompanied the Trans-Blot apparatus (Bio-Rad Laboratories, Richmond, Calif.).

Serological procedures. Antisera were prepared in New Zealand white rabbits by subcutaneous injections of solutions of purified pili emulsified in Freund incomplete adjuvant (Difco). Three injections of approximately 150 μg of protein were given at biweekly intervals. The rabbits were bled 7 to 10 days after the last injection.

Antisera were titrated by an antibody enzyme-linked immunosorbent assay (ELISA). The method was a modification of the procedure described by Voller et al. (18). Optimal antigen coating occurred at 2 μg/ml. The first antibody was rabbit antipilus serum. The second antibody was horseradish peroxidase conjugated to goat anti-rabbit immunoglobulin G (L and H chains; Cappel Labs, West Chester, Pa.). The substrate was *O*-dianisidine hydrochloride (Sigma, St. Louis, Mo.) and hydrogen peroxide. Serum titers were calculated from a standard curve which was based on the reaction (absorbance at 450 nm) of a serially diluted reference serum with its homologous antigen. The reference serum used in this study was strain 2-9 and was arbitrarily assigned a titer of 10,000 U. Absorbances between 0.1 and 1.2 U were used in all calculations. Titer of the test serum was the product of value interpolated from the standard curve and the dilution factor of the test serum in the assay.

Immunodetection of antigen by specific antisera was done by the method described in the Immun-Blot assay kit (Bio-Rad). Antigens were electrophoretically transferred to nitrocellulose paper, and nonspecific binding sites were blocked by incubation in 3% gelatin. The paper was then incubated successively in test antibody, goat anti-rabbit immunoglobulin G antibodies conjugated with peroxidase, and substrates (hydrogen peroxide and 4-chloro-1-naphthol).

Mouse protection test. CF-1 mice (35 days old) were immunized intraperitoneally with 200 μl of a vaccine containing various amounts of pili and 400 μg of aluminum hydroxide gel. A second dose was administered 14 days later. Mice in the control group were not vaccinated. Ten days after the second vaccination, mice were challenged by intraperitoneal injection of a culture suspension of 1×10^7 to 7×10^7 CFU of *B. bronchiseptica* 2-9. Survival was monitored for 10 days postchallenge.

RESULTS

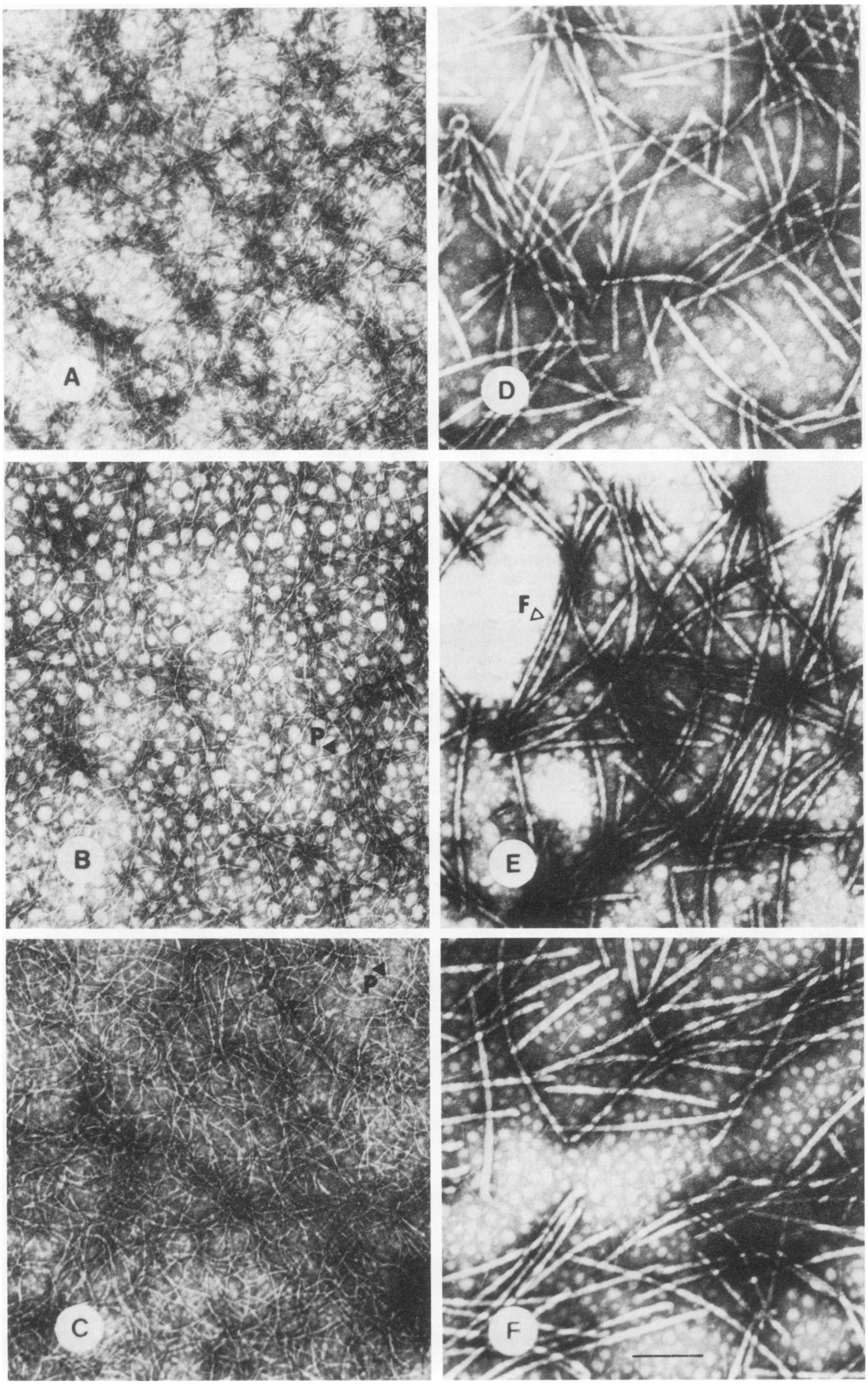
Variations in colonial morphology. Nomenclature defined by Bemis et al. (3) for the various colonial morphotypes of *B. bronchiseptica* on brucella agar was followed in this study (see footnotes, Table 1). With the exception of strains 110H, 110NH, 55, and B133 (NVSL), all *B. bronchiseptica* isolates exhibited intermediate phase, medium size, and smooth colonies typical of morphology when grown on brucella agar (Table 1). Upon serial laboratory passage, a phase 1 variant was selected from strain 5389. Strain 110H, previously described to be a phase 1 culture (3), appeared to have characteristics intermediate between strain 5389 phase 1 and the other *B. bronchiseptica* intermediate-phase strains and was therefore designated as 1→I. Strain B133 (NVSL) is the pig challenge strain used for producing experimental atrophic rhinitis at the National Veterinary Services Laboratory, Ames, Iowa. This colony differed in size as well as vertical contour from that of strain B133 (obtained from D. Bemis, University of Tennessee, Knoxville). Strain B133 resembled the rough phase of *B. bronchiseptica*.

The frequency of colonial phase variation was low, and colonial morphology was quite stable on agar cultures. All *B. bronchiseptica* strains were characterized in terms of pilus production and pilus subunit profile.

Purification of pili. Pili were extracted and purified from *B. bronchiseptica* cultures grown on brucella agar. Dark-field pilus aggregates were immediately evident after the addition of PEG to the crude culture extracts. Needlelike paracrystalline structures were observed. The precipitation process was repeated several times to obtain a homogeneous pilus suspension. The final products were examined under electron microscopy and analyzed by SDS-gel electrophoresis.

Pilus morphology. Electron microscopy showed the presence of pili in PEG precipitates (Fig. 1). While most of the pilus preparations contained flagella as a minor contaminant, preparations from strains B133 (NVSL) and 110NH contained flagella as the major protein (Fig. 1D and E). The two B133 strains studied here were apparently not similar as they differed in colonial morphology, piliation, and flagellation. The apparent size of a pilus rod was about 3 to 4 nm in diameter, and the length varied. One preparation from strain 5389 phase 1 culture (Fig. 1G and H) showed the presence of regular arrays of pilus aggregates in a pattern similar to the angle-layered crystals of *E. coli* type 1 pili (5). This type of pilus aggregation was also observed in *B. pertussis* (4). Occasionally, membraneous vesicles were also observed.

Protein profiles of *B. bronchiseptica* pili. PEG-precipitated pili were analyzed by SDS-gel electrophoresis. These prep-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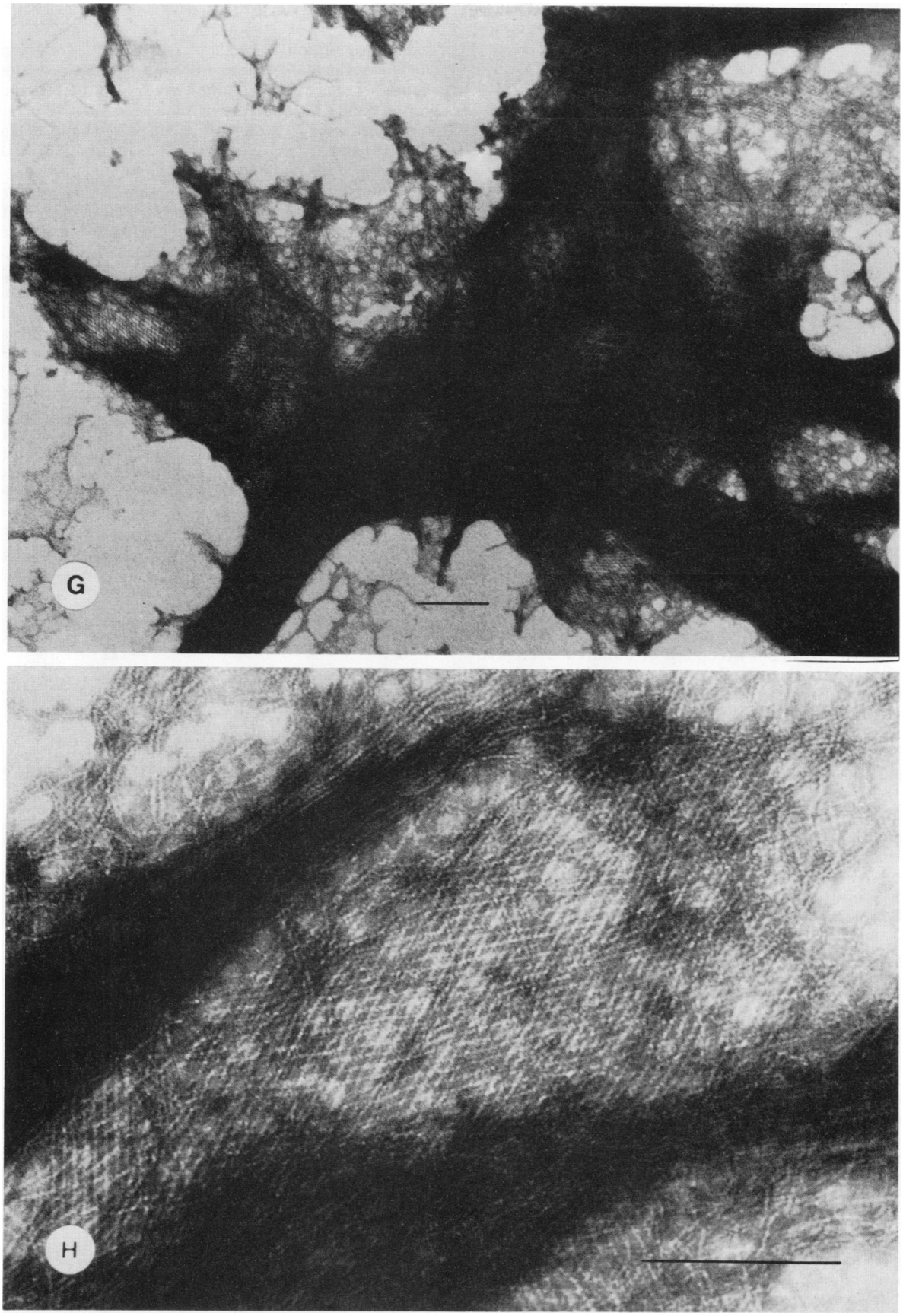

FIG. 1. Electron micrographs of PEG precipitates of culture extract of *B. bronchiseptica*. (A) Strain 2-9; (B) strain B133 (Bemis); (C) strain GP; (D) strain B133 (NVSL); (E) strain 110NH; (F) strain 87; (G and H) strain 5389 (phase 1). Specimens were prepared by adhesion to carbon-shadowed collodion membranes and negative staining. P, Pilus; F, flagellum. Bar, 0.2 μm.

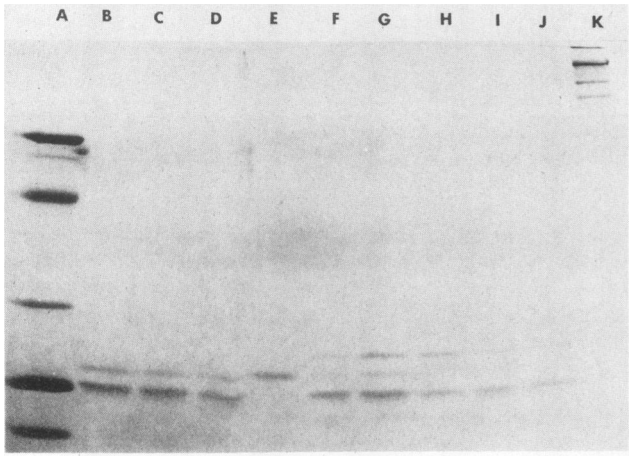

FIG. 2. SDS-PAGE protein profile of PEG-precipitated pilus preparations of *B. bronchiseptica*. Lanes: A, marker proteins (bovine serum albumin, 66K; ovalbumin, 45K; *E. coli* K88, 26K; 987p pilus, 21K; K99 pilus, 18.5K); B, strain 5998; C, strain 5944; D, strain 5389 (intermediate phase); E, strain 5389 (phase 1); F, strain B133; G, strain 2-9; H, strain 110H; I, strain 6126; J, strain 7-8; K, filamentous hemagglutinins of *B. pertussis*.

arations were composed of proteins with molecular size around 22,000 daltons (22K) (Fig. 2), which were distinct from *B. pertussis* filamentous hemagglutinins purified and provided (14) by J. Cowell, Pertussis Branch, Food and Drug Administration. With the exception of strains 87, 110NH, and B133 (NVSL), flagella were not present in any significant quantities. However, flagella proteins were detected if an increased load of PEG-purified preparations was applied to the gel (data not shown). Flagella were not seen in strains 110H and 5389 (phase 1).

The pilus-producing strains can be classified into four groups or phenotypes based on SDS-PAGE protein profile of the pili produced. Group A strain produced a major pilus protein at 22K. Group B strains produced two protein bands at 21K and 22K. Group C strains produced three protein bands at 21K, 24K, and 22K in decreasing quantities. Group D strains produced approximately equal quantities of 21K and 24K proteins and a very small amount of 22K protein. Table 1 summarizes the relative proportions of these protein subunits. The only instance in which *B. bronchiseptica* pili were purified close to single-band homogeneity in SDS-gel electrophoresis was with a preparation from an agar culture of strain 5389 phase 1 variant. An intermediate-phase variant of strain 5389 produced a mixture of 21K and 22K proteins. Strains B133 (NVSL; Fig. 1D) and 110NH (Fig. 1E) produced large amounts of flagella with subunit molecular weight at about 45K. An intermediate-phase variant selected from strain B133 (NVSL), however, produced a highly enriched pilus preparation with very little flagellar contamination (data not shown). Pili were also purified from a liquid culture of *B. bronchiseptica* (strain B133) grown in Stainer-Scholte medium. When this preparation was compared with pili isolated from the same strain grown on brucella agar, a drastic shift of protein profiles was found (Fig. 3).

Since PEG precipitation is a general procedure applicable to the purification of many pilus types (11; S. W. Lee, Ph.D. thesis, University of Pittsburgh, Pittsburgh, Pa., 1980), an investigation was undertaken to determine the structural and antigenic relationship of the 21K, 22K, and 24K proteins. Preparative SDS-gel electrophoresis of pili purified from agar-grown strain B133 cells was performed, and the protein bands were isolated. These proteins migrated very closely to each other. Particular attention was given to cutting out the protein bands. The polyacrylamide gel was lightly stained in Coomassie blue dye and destained immediately. The blue protein bands were carefully excised and pulverized in Freund incomplete adjuvant for immunization of rabbits. This procedure yielded pure pilus subunits as confirmed by rerunning the isolated proteins in SDS-PAGE. Antisera prepared against each band were compared serologically by ELISA test and immunoblot.

Serology of pili. Antisera prepared against PEG-precipitated pili were found to exhibit high antibody titers against all *B. bronchiseptica* pili as assayed by ELISA (Table 2). Immunoblot techniques, using antisera prepared against each protein species of PEG-precipitated pili, also detected cross-reactions among the three pilus subunits (Fig. 4). Anti-24K serum was most reactive, reacting with both 21K and 22K subunits. Anti-22K serum reacted with 24K subunit but not with 21K subunit. Anti-21K serum reacted with 22K subunit and slightly with 24K subunit. Thus, each antiserum had unique specificity against the three pilus subunits.

Antisera generated against the pilus subunits were also active in the ELISA against many pilus preparations (Table 3). Since in most cases the antigen used in this ELISA contains a mixture of the various subunit proteins, it is difficult to determine whether the activities originated from

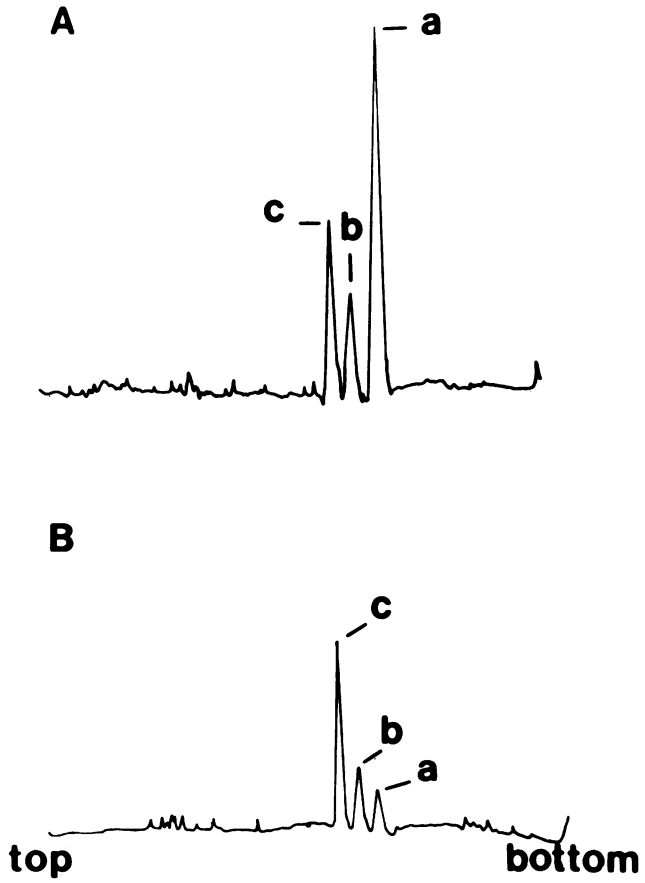

FIG. 3. SDS-PAGE protein profile of pili purified from strain B133. (A) Brucella agar culture; (B) Stainer and Scholte liquid medium. Pilus subunits: a, 21K; b, 22K; c, 24K.

TABLE 2. Serological cross-reactivities of antisera prepared against PEG-precipitated pili

Antigen phenotype	Strain	Activity[a] in pilus antiserum from following phenotype and strain:									
		A; 5389	B				C		D		
			5998	5944	5389	588880	B133	2-9	110H	6126	7-8
A	5389	100	38	14	52	22	41	48	40	31	33
B	5998	152	100	103	140	80	90	152	100	113	93
	5944	135	94	100	115	88	100	143	99	107	102
	5389	100	70	68	100	74	76	98	81	88	71
	588880	151	65	76	109	100	79	168	87	85	103
C	B133	59	65	98	109	77	100	135	90	112	93
	2-9	59	35	38	82	38	97	100	59	76	60
D	110H	52	55	74	104	55	88	120	100	86	79
	6126	77	64	75	108	80	97	116	80	100	103
	7-8	93	78	98	113	68	88	145	100	112	100

[a] Activity expressed as percent homologous titer. Standardized ELISA titers were used in the calculation.

the homologous or heterologous reactions. It is also difficult to explain the inactivity of the 22K antiserum against some of the phenotype B pilus preparations, e.g., strain 5998. The ELISA measures the activity of antiserum to native pilus proteins, whereas the immunoblot measures the activity of antiserum to SDS-denatured proteins. Thus, the serological relationship of the 21K, 22K, and 24K proteins appears complex and open to speculation. Normal rabbit serum had very low titers against pili.

Recently, pili were discovered on *B. pertussis* (1, 4). A preparation of purified *B. pertussis* serotype 2 pili (20) was obtained through the courtesy of J. Cowell. This preparation migrated very closely to the 22K subunit of *B. bronchiseptica* pili but was slightly larger (Fig. 5, lanes b). *B. pertussis* pili were also purified from ATCC strain 9797 by PEG precipitation (data not shown). Antiserum against this antigen was found to react very well with serotype 2 pili of *B. pertussis* pili (Table 4). Strain 5389 phase 1 pilus antiserum was only weakly active against pili of *B. pertussis*, and the antiserum to strain 2-9 pili was not at all reactive with the *B. pertussis* pili. However, antisera to 21K, 22K, or 24K were active in both ELISA (Table 4) and immunoblots (Fig. 5).

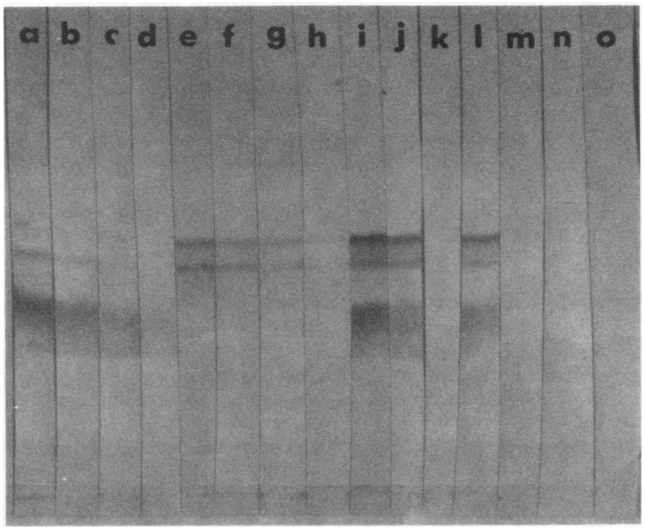

FIG. 4. Immunoblots of *B. bronchiseptica* B133 pili using specific antisera. Lanes: a through d, 21K antiserum at 1/100, 1/200, 1/400, and 1/800; e through h, 22K antiserum at 1/100, 1/200, 1/400, and 1/800; i and j, 24K antiserum at 1/100 and 1/200; k, blank; l, 24K antiserum at 1/800; m through o, normal rabbit serum at 1/100, 1/200, and 1/400.

Mouse protection. A preparation of strain 2-9 pili was tested in the mouse protection test. Aluminum hydroxide was used as the adjuvant. Mice were protected against an intraperitoneal challenge of virulent *B. bronchiseptica* by vaccination with purified *B. bronchiseptica* pili (Table 5). Immunization using pili purified from another strain was also effective. The 50% protective dose was determined to be 0.8 ng of pilus antigen.

DISCUSSION

Most isolates of *B. bronchiseptica* exhibited intermediate colonial morphology (Table 1). We did not see a consistent subunit variation of pili purified from canine, swine, or guinea pig origin. Based on electron microscopy and protein profiles of purified pili prepared from the various colonial variants reported in this study, there appeared to be a correlation of piliation and flagellation with colonial morphology. Phase 1 colonies were usually highly piliated and lacked flagella. Intermediate-phase colonies were also highly piliated, but flagella were sometimes found in significant quantities. Rough-phase colonies produced largely flagella and little pili.

We found that pili isolated and purified from *B. bronchi-*

TABLE 3. ELISA activities of 21K, 22K, and 24K pilus antisera

PEG precipitate from strain:	Pilus phenotype	Pilus antiserum activity[a]		
		21K	22K	24K
5389	A	2,778	487	2,070
87[b]	B	1,597	3	193
5998	B	2,427	24	704
2-9	C	2,623	445	2,746
B133	C	2,671	377	2,242
6126	D	2,339	298	2,270
7-8	D	2,210	298	2,768
110NH	Flagella	19	3	15

[a] Standardized titers as described in the text.
[b] Strain 87 contained a significant contamination of flagella.

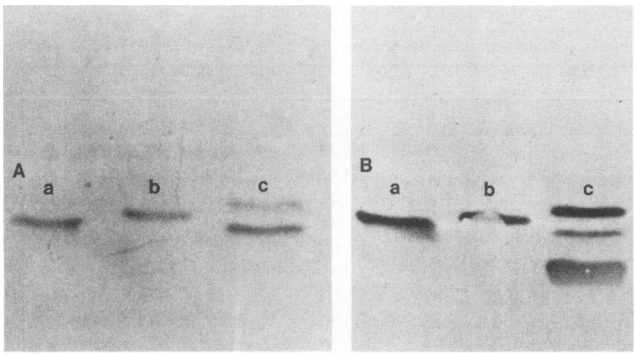

FIG. 5. Immunoblots of *B. bronchiseptica* and *B. pertussis* pili using specific *B. bronchiseptica* pilus antisera. (A) Reaction with 22K pilus antiserum; (B) reaction with 24K pilus antiserum. Lanes: a, *B. bronchiseptica* 5389 (phase 1); b, *B. pertussis* Tohama 325; c, *B. bronchiseptica* 2-9.

septica were structurally heterogeneous in SDS-PAGE (Fig. 2). Structural heterogeneity in subunits of gonococcal pili has been reported (7, 10, 13, 17). An alternative interpretation of the data is that the various bands detected by SDS-PAGE were impurities. However, dark-field microscopy of PEG precipitates showed homogeneous suspension of needlelike pilus aggregates and the absence of cellular contaminants. Electron microscopy also indicated large amounts of pili in all PEG precipitates. Phenotype A pili showed predominately 22K proteins, and phenotype D pili showed essentially an absence of 22K proteins and the presence of 21K and 24K proteins. A conclusion may be drawn that the 21K, 22K, and 24K proteins are pilus proteins. A corollary that follows is that *B. bronchiseptica* pili are comprised of a single subunit polypeptide, and the various pilus variants may be present on the cell surfaces. Piliation phase variation is influenced by the growth environment (5, 6). This is further illustrated by the contrasting protein profiles of purified pili isolated from a single strain grown under different culture conditions (Fig. 3).

B. bronchiseptica appeared to be structurally heterogeneous but serologically cross-reactive. All antipilus sera contained high antibody titers (Table 2). Since some pili used in the immunization of rabbits were not homogeneous, the heterologous titers may be due not to antigenic cross-reactivity of the pilus subunits but rather to activity resulting from the presence of antigen used in immunization. While this is possible, an argument against this interpretation could be data shown with phenotype A and D pili (Table 1). The 22K protein of phenotype A pili was present only in a very small quantity (<1%) in phenotype D pili, and yet significantly high antibody titers were detected in the heterologous reaction (Table 2). Antigenic cross-reactivity of *B. bronchiseptica* pili is further supported by the data in Tables 3 and 4 and by immunoblots (Fig. 5).

TABLE 4. ELISA activities of rabbit antisera against purified *B. bronchiseptica* and *B. pertussis* pili

Pilus antiserum	ELISA activity[a] against pilus prepn		
	B. bronchiseptica pili		*B. pertussis* pili, serotype 2
	Strain 2-9	Strain 5389	
B. bronchiseptica			
Strain 2-9	9,434	4,420	14
21K protein	2,623	2,778	1,013
22K protein	445	481	1,078
24K protein	2,746	2,070	4,443
Strain 5389	7,728	13,028	865
B. pertussis 9797	462	1,648	>1,571[b]
Normal rabbit serum	12	ND[c]	27

[a] Standardized ELISA titers as described in the text.
[b] Endpoint of titration not determined.
[c] ND, Not done.

TABLE 5. Protection of mice against *B. bronchiseptica* intraperitoneal infection

Expt	Vaccine received	Survivors/total (%)
1	None	0/10 (0)
	B. bronchiseptica B133 pilus, 40.0 μg	10/10 (100)
	B. bronchiseptica 2-9 pilus, 29.0 μg	12/12 (100)
2	None	1/10 (10)
	B. bronchiseptica 2-9 pilus	
	4.0 ng	11/16 (69)
	0.4 ng	6/16 (38)
	0.04 ng	3/16 (19)

Asworth et al. (1) reported that the subunit size of *B. pertussis* pili was 22K. *B. pertussis* pili from Tohama strain 325 (20) as well as from strain ATCC 9797 migrated very close to but slightly larger than the 22K subunit of *B. bronchiseptica* pili (Fig. 5). Antigenic reactivity of intact *B. bronchiseptica* pili with *B. pertussis* pili was generally slight (Table 4). The strongest reaction was detected with phenotype A *B. bronchiseptica* pili (865/13028 or 6.6%; Table 4). However, ELISA titers of antisera against the SDS-PAGE-separated pilus subunits were high. This was also reflected in the immunoblots (Fig. 4). One interpretation is that *B. bronchiseptica* and *B. pertussis* pili share similar amino acid sequences which are not located on the surface of pili but are exposed when pili are denatured and reduced by SDS and mercaptoethanol. Amino acid sequence homology of pili isolated from bacteria of different genera and species has been reported (8).

Our data also show that pili are immunogens in *B. bronchiseptica* bacterins (Table 5). Recently, a bacterin containing purified *B. bronchiseptica* antigen was described for control of canine bordetellosis (15). The nature of this antigen has not been defined, and the relationship of this antigen to pili is not clear.

The isolation of pili and understanding of their antigenicity should facilitate the study of the role of pili in the pathogenicity of *B. bronchiseptica*.

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