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Surface Proteins of *Bordetella pertussis*: Comparison of Virulent and Avirulent Strains and Effects of Phenotypic Modulation

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The surface proteins of several *Bordetella* strains and their modulated derivatives were examined by surface radioiodination, cell fractionation, and Western blotting. A surface protein with a high M_r, missing in a mutant lacking the filamentous hemagglutinin, was identified in virulent *Bordetella pertussis* and *Bordetella parapertussis* cells and was absent in avirulent *B. pertussis* strains. The electrophoretic profiles of lipopolysaccharide and the 40,000-M_r anion-selective porin were not determinants which correlated with phase variation or phenotypic modulation. At least three envelope proteins (91,000, 32,000, and 30,000 molecular weight) were found only in virulent *B. pertussis* strains and were absent or diminished in the avirulent phase and most phenotypically modulated strains. Two transposon-induced mutants unable to produce hemolysin, dermonecrotic toxin, pertussis toxin, and filamentous hemagglutinin also lacked these three envelope proteins, confirming that virulence-associated envelope proteins were genetically regulated with other virulence-associated traits.

Virulent *Bordetella pertussis* produces an array of substances which may be involved in the pathogenesis of whooping cough. These substances include pertussis toxin (19), adenylate cyclase (44), hemolysin (40), dermonecrotic toxin (4), filamentous hemagglutinin (5), and agglutinogens (7). The virulent phenotype of *B. pertussis* is unstable (6, 13, 16, 17, 22, 25, 28, 38, 41), giving rise to phase variants at a frequency of about 10^{-6} (13, 28, 41). Such a phase change results in the simultaneous absence of the aforementioned virulence-associated factors, as well as loss of cytochrome *d*-629 (8) and changes in the cell envelope protein profile (9, 27, 36). Other reported properties of avirulent-phase variants include acquisition of the ability to grow on bloodless solid media (13, 25, 30, 38), increased resistance to certain antibiotics and fatty acids (6, 25, 30, 41), and differences in colonial morphology (16, 28, 38). Spontaneous mutations may occur in any of the individual virulence-associated genes. Phase variation, however, is thought to be regulated by the *vir* locus, which coordinates the expression of multiple virulence-associated genes (41). Tn*5* transposon-induced mutants lacking individual virulence factors have been isolated, as well as two avirulent Tn*5* mutants in which the genetic lesion occurs within the *vir* region (41, 42). These latter two mutants lack pertussis toxin, hemolysin, and filamentous hemagglutinin; one *vir* mutant was shown to be avirulent in infant mice and to lack adenylate cyclase and dermonecrotic toxin (42, 43).

A second type of variation, phenotypic modulation, results in the reversible loss of the ability of virulent organisms to produce pertussis toxin (15, 35), adenylate cyclase (23), hemolysin (21), filamentous hemagglutinin (21), agglutinogens (21, 23, 31, 37), dermonecrotic toxin (15, 37), certain cell envelope proteins (9, 15, 27, 37), and cytochrome *d*-629 (8). Thus, the phenotype of modulated cells closely resembles that of avirulent-phase organisms. Modulation can be induced by culturing cells at 25°C or in the presence of high concentrations of magnesium ions (21) or pyridines such as nicotinic acid (31, 37). The mechanism causing phenotypic modulation is not known, but the effects are reversible upon return of the cells to the usual culture conditions.

Avirulent-phase strains (9, 27, 28, 36) as well as phenotypically modulated cells (9, 23, 27, 37) fail to produce certain cell envelope proteins in the 30,000- and the 90,000- to 200,000-molecular-weight regions. Several of these envelope proteins have been identified as being exposed on the surface of the cell, as evidenced by their accessibility to radioiodination (1, 28, 32) and reactivity with cell surface-specific antibodies (1, 26). In this study, we used surface radiolabeling to examine the cell surface proteins of modulated and normally grown *Bordetella* strains and Tn*5* mutants. Our results indicate that at least three surface-exposed, virulence-associated envelope proteins are coordinately regulated with other virulence-associated factors during modulation or as a result of mutation. Additionally, the ability of strains to grow on bloodless media appears to be a characteristic acquired independently of mutation to the avirulent phase.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in this study are listed in Table 1. All strains were stored as stock cultures frozen in sheep blood at −70°C and were grown on Bordet-Gengou plates (10) or in modified Stainer-Scholte medium (37). To induce phenotypic modulation, we passaged cells twice in modified Stainer-Scholte broth containing 500 μg of nicotinic acid (Sigma Chemical Co., St. Louis, Mo.) per ml (37). Strains modulated with nicotinic acid are designated in the text by addition of the suffix NA to the strain name. Trypticase soy agar (TSA) (BBL Microbiology Systems, Cockeysville, Md.) and nutrient agar plates were prepared according to the directions of the manufacturer, and TSA was supplemented with nicotinic acid (to 500 μg/ml) as specified in the text.

Dermonecrotic toxin assay. BP359 cells lysed by sonication were shown to lack dermonecrotic toxin when assayed by the method of Cowell et al. (4).

Radioiodination of intact bacteria. Intact bacterial cells were harvested from Bordet-Gengou plates and washed with phosphate-buffered saline. The cells were radiolabeled as described previously (1, 34) with $Na^{125}I$ (Amersham Corp.,

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TABLE 1. Properties of bacterial strains used in this study

Strain	Phase (mutation)[a]	Agglutinogens[b]	Origin (reference)
Bordetella pertussis			
UT25	Virulent	1, 3, 6	Clinical isolate, this laboratory (10)
UT25D	Avirulent	NT[c]	Laboratory-passaged derivative of UT25 (10)
114	Virulent	1, 3, 6	Vaccine strain, C. R. Manclark, Bureau of Biologics, Bethesda, Md.
165	Virulent	1, 2, 3, 4, 6	Vaccine strain, E. Hewlett, University of Virginia, Charlottesville
BP338	Virulent (Nal^r)	1, 2, 3, 4	Spontaneous Nal^r mutant of Tohama I, A. A. Weiss (42)
BP347	*vir-1*::Tn*5*; Nal^r	NT	Tn*5*-induced mutant of BP338 (42)
BP348	*hly-1*::Tn*5*; Nal^r	NT	Tn*5*-induced mutant of BP338 (42)
BP349	*hly-2*::Tn*5*; Nal^r	NT	Tn*5*-induced mutant of BP338 (42)
BP353	*fha-1*::Tn*5*; Nal^r	1, 3	Tn*5*-induced mutant of BP338 (42)
BP357	*ptx-2*::Tn*5*; Nal^r	1, 3	Tn*5*-induced mutant of BP338 (42)
BP359	*vir-2*::Tn*5*; Nal^r	NT	Tn*5*-induced mutant of BP338 (41)
MU8	Virulent	NT	Clinical isolate, this laboratory
MU41	Virulent	NT	Clinical isolate, this laboratory
Bordetella bronchiseptica SC89-21-1		NT	Stock laboratory strain, M. J. Pickett, University of California, Los Angeles
Bordetella parapertussis ONT1	Virulent	NT	Clinical isolate, J. Regan (33)

[a] Definitions of mutations: Nal^r, nalidixic acid resistant; *hly*, hemolysin; *fha*, filamentous hemagglutinin; *ptx*, pertussis toxin; *vir*, virulent-phase locus. Mutants in *vir* were deficient in multiple virulence-related properties including pertussis toxin, hemolysin, and filamentous hemagglutinin.
[b] Serotypes for all strains except UT25 kindly provided by J. Cowell, Bethesda, Md.
[c] NT, Not tested.

Arlington Heights, Ill.) and Iodo-beads (Pierce Chemical Co., Rockford, Ill.) as the catalyst.

Cell envelope preparation. Total cell envelopes were prepared as described in a previous study (2). Briefly, cells grown in modified Stainer-Scholte broth were harvested and suspended in a solution of 0.75 M sucrose and 10 mM Tris hydrochloride (pH 7.8) and were treated with 1.5 mM EDTA and lysed by sonication. The lysate was cleared of unbroken cells by centrifugation at 6,000 × *g* for 15 min, and the resulting supernatant was centrifuged at 100,000 × *g* for 1 h to obtain total cell envelopes.

Analysis by SDS-PAGE. A 7.5 to 20% acrylamide gradient gel system containing 0.5 M urea was used to examine radioiodinated proteins or cell envelopes (37). Each gel lane was loaded with 20 μg of protein or 50,000 cpm. Autoradiograms were produced by incubating dried gels against Kodak X-Omat XAR-2 film (Eastman Kodak Co., Rochester, N.Y.) for 24 h at room temperature. Protein gels were stained with Coomassie blue.

Detection of lipopolysaccharide (LPS) in whole-cell lysates in electrophoretic gels was accomplished by using a method modified from Peppler (29). Whole cells (approximately 30 to 40 μg of protein) were incubated for 2 h at 56°C in 20 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) digestion buffer (final concentrations, 0.625% SDS, 0.625% 2-mercaptoethanol, 6.25% glycerol, 0.125 M Tris hydrochloride [pH 6.8], and 0.0025% bromphenol blue) containing 0.5 mg of proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml. The samples were then treated at 100°C for 10 min and subjected to SDS-PAGE. LPS was visualized by the silver staining technique of Hitchcock and Brown (14).

Western blotting analysis. A monoclonal antibody which recognizes a cell surface protein found only in virulent *B. pertussis* was used to probe several strains and their modulated derivatives cultured in Stainer-Scholte broth. Whole-cell proteins from SDS-PAGE gels were transferred to a nitrocellulose sheet, which was blocked with bovine serum albumin, and incubated with hybridoma culture supernatant P8E7 (11) and processed as described previously (12).

RESULTS

Growth of phase variants on bloodless media. The growth properties of the strains used in this study are presented in Table 2. UT25D, an avirulent phase IV variant of virulent UT25, was able to grow well and produce isolated colonies on TSA or nutrient agar within 3 days. Phase I strains UT25, 114, 165, and BP338 were unable to grow on TSA or nutrient agar plates even after heavy inoculation. No Tn*5* mutants derived from BP338 were capable of growth on TSA or nutrient agar. The *Bordetella bronchiseptica* and *Bordetella parapertussis* strains examined demonstrated good growth on the bloodless media after 1 to 2 days of incubation.

UT25NA, phenotypically modulated by culture in Stainer-Scholte broth with added nicotinic acid, was heavily streaked onto TSA plates containing 500 μg of nicotinic acid per ml. No growth of the cells was detected even after incubation for 1 week at 35°C in a humidified chamber.

Analysis of radioiodinated cell surface proteins. (i) Phase I *Bordetella* species. As shown in the autoradiogram in Fig. 1, all the *B. pertussis* strains demonstrated similar protein profiles, which included the presence of the 40-kilodalton anion-selective porin (2). *B. bronchiseptica* and *B. parapertussis* also possessed a similarly sized surface protein. Strains MU8 and MU41 were recent clinical isolates passaged twice on Bordet-Gengou medium before radioiodination. These two isolates appeared to possess lower levels of the porin when compared with the other *B. pertussis* strains. MU8 and MU41 were not examined for production of virulence-associated factors such as pertussis toxin, filamentous hemagglutinin, or dermonecrotic toxin, but were isolated from symptomatic patients with culture-proven and fluorescent antibody-positive pertussis.

A high-molecular-weight doublet band, indicated by the asterisk in Fig. 1, can be seen in all the *B. pertussis* strains as

TABLE 2. Summary of the phase characteristics of various *Bordetella* strains[a]

Strain	Phase (mutation)[b]	Growth on TSA[c]	High-M_r surface protein	Envelope proteins		LPS type
				91K	30K doublet	
B. pertussis						
UT25	Virulent	−	+	+	+	a
UT25D	Avirulent	+	−	−	−	ab
UT25NA[d]	Modulated	−	−[e]	−	−	ab
114	Virulent	−	+	+	+	a
114NA	Modulated	NT	NT	−	−	ab
165	Virulent	−	+	+	+	a(b)[f]
165NA	Modulated	NT	NT	−	−	ab
BP338	Virulent (Nal^r)	−	+	+	+	a(b)
BP338NA		NT	NT	+	+	a
BP347	*vir-1*::Tn*5*; Nal^r	−	−	−	−	ab
BP347NA		NT	NT	−	−	ab
BP348	*hly-1*::Tn*5*; Nal^r	−	+	+	+	a(b)
BP348NA		NT	NT	+	+	ab
BP349	*hly-2*::Tn*5*; Nal^r	−	+	+	?[g]	NT
BP353	*fha-1*::Tn*5*; Nal^r	−	−	+	?	NT
BP357	*ptx-2*::Tn*5*; Nal^r	−	+	+	?	NT
BP359	*vir-2*::Tn*5*; Nal^r	−	−	−	−	ab
BP359NA		NT	NT	−	−	ab
MU8	Virulent	NT	+	+	?	NT
MU41	Virulent	NT	+	+	?	NT
B. bronchiseptica SC89-21-1		+	−	−	?	NT
B. parapertussis ONT1	Virulent	+	+	−	?	NT

[a] +, Positive; −, negative or markedly reduced; NT, not tested.
[b] Terminology as in Table 1.
[c] All strains were additionally tested for growth on nutrient agar; results were the same as for TSA.
[d] NA indicates that cells were modulated with nicotinic acid.
[e] Data not shown.
[f] Parentheses indicate faintly staining band.
[g] ? indicates that this parameter could not be determined from autoradiograms of surface-radioiodinated cells.

well as *B. parapertussis* ONT1. This doublet was only faintly visible in *B. pertussis* UT25 and *B. parapertussis* but became readily apparent if the autoradiogram was allowed to incubate longer before developing. A surface protein with an apparent molecular weight of 91,000 was observed in all the *B. pertussis* strains but not in *B. bronchiseptica* or *B. parapertussis*. All the *B. pertussis* strains except BP338 possessed a protein with an apparent molecular weight of 34,000, which could represent a strain-specific molecule or agglutinogen. The protein profile of *B. parapertussis* more closely resembled that of *B. pertussis* than did *B. bronchiseptica*. *B. bronchiseptica* appeared to lack many higher-molecular-weight (>60,000) surface proteins.

(ii) Phase variants and Tn*5* mutants. When phase I UT25, phase IV UT25D, and virulent-phase BP338 and its Tn*5* mutant derivatives were radioiodinated, their cell surface protein profiles demonstrated distinct differences (Fig. 2). The high-molecular-weight doublet observed in the *B. pertussis* and *B. parapertussis* samples of Fig. 1 is evident in Fig. 2 (asterisk) only in virulent-phase UT25, BP338, BP348, BP349, and BP357. The doublet was not observed in UT25D, BP347 (*vir-1*), BP353 (*fha*), or BP359 (*vir-2*). A major band with an apparent molecular weight of 91,000 was decreased or lacking in avirulent UT25D, BP347, and BP359. BP357 demonstrated a unique band at approximately 100,000 molecular weight which was not detectable in the other samples. This particular strain is unable to produce pertussis toxin owing to transpositional inactivation, but polar effects may be responsible for the presence of the unique high-molecular-weight surface protein. The major band representing the anion-selective porin at 40,000 molecular weight was apparent in all the strains examined.

Other investigators have noted virulence-associated changes in *B. pertussis* envelope protein profiles in the 30,000-molecular-weight range (9, 27, 28, 36). In this study such changes were difficult to discern in autoradiograms of surface-radioiodinated cells owing to the abundance of labeled polypeptides in that molecular weight range. Among the Tn*5* mutants, BP347 and BP359 displayed similar but distinctive patterns of radiolabeling in the 14,000-molecular-weight zone of electrophoretic gels. The radiolabeled band(s) seemed more intense and migrated at a slightly higher M_r than those proteins of the other Tn*5* derivatives, the parental strain BP338, UT25, or UT25D. Again, this could be due to polar effects which can occur with transposon mutagenesis, possibly resulting in the insertion of aberrant molecules into the outer membrane.

Analysis of cell envelopes. (i) Envelope proteins. Selected virulent strains and Tn*5* mutants were grown in Stainer-Scholte broth containing nicotinic acid to evoke phenotypic modulation, and cell envelopes derived from these samples were examined by SDS-PAGE (Fig. 3). The 91,000-molecular-weight virulence-associated envelope protein (91K protein) was easily detected in strains UT25, 114, and 165, but was absent or markedly decreased in the nicotinic acid-modulated derivatives of these strains. This 91K protein was present to a lesser extent in the envelope protein profiles of BP338 and BP348 and was visible to the same extent in their nicotinic acid-modulated counterparts. Two envelope proteins with apparent molecular weights of ap-

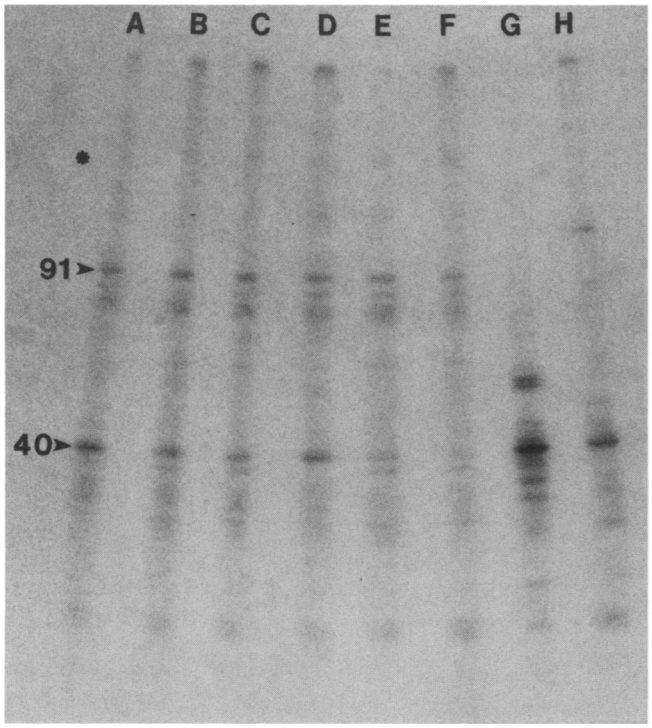

FIG. 1. Autoradiogram of surface-radioiodinated *Bordetella* species. Washed whole cells were radiolabeled with ^{125}I and subjected to SDS-PAGE and autoradiography. Lanes: A, *B. pertussis* UT25; B, *B. pertussis* 114; C, *B. pertussis* 165; D, *B. pertussis* BP338; E, *B. pertussis* MU8; F, *B. pertussis* MU41; G, *B. bronchiseptica* SC89-21-1; H, *B. parapertussis* ONT1. The asterisk indicates a high-molecular-weight doublet. Arrows denote major bands with apparent molecular weights of 91,000 and 40,000, as determined by reference to the following molecular weight standards: phosphorylase *b*, 92,500; bovine serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; and lysozyme, 14,400.

proximately 30,000 and 32,000 (the 30K doublet) were distinctly observed in strains UT25, 114, and 165, but were found in decreased amounts in those same strains which were modulated. BP338 and BP348 also possessed the 30K and 32K proteins even when grown in the presence of nicotinic acid. These data indicated that in the strains examined at least three virulence-associated envelope proteins existed and that growth in medium containing high levels of nicotinic acid caused phenotypic modulation and concomitant reduction in the synthesis of those polypeptides for some *B. pertussis* strains. BP338 and its Tn*5* derivatives appeared to be more refractory to the effects of nicotinic acid. Two Tn*5* mutants, BP347 (*vir-1*) and BP359 (*vir-2*), lacked or showed a dramatic decrease in the amounts of the three virulence-associated proteins regardless of exposure to nicotinic acid.

(ii) LPS. The same strains used for the analysis of envelope proteins were examined with regard to their LPS staining profile (Fig. 3). When whole cells were pretreated with proteinase K and subjected to SDS-PAGE and silver staining, all the strains demonstrated the major band a characteristic of *Bordetella* LPS (29). The intensity of the faster-migrating band b varied with the strain and exposure to nicotinic acid, but no definite correlation could be demonstrated between LPS bands and a virulence-associated or modulated phenotype. For example, UT25 lacked LPS band b, whereas both UT25D and UT25NA clearly displayed the faster-migrating band b. The LPS profiles of the two Tn*5* *vir* mutants showed distinct bands a and b even when exposed to nicotinic acid. The parent strain, BP338, demonstrated a faint band b and an absence of this band when grown in the presence of nicotinic acid.

Western blot analysis. Previous studies have described the specificity and characteristics of monoclonal antibody P8E7 (1, 11, 26). This antibody has been shown to specifically react with a surface-exposed envelope protein associated with the virulent phenotype and having an apparent molecular weight of 91,000. Western blots of whole-cell proteins probed with monoclonal antibody P8E7 showed a direct correlation with the presence of the 91K surface-exposed envelope protein observed in virulent strains (Fig. 4). P8E7 reacted strongly with virulent-phase UT25, 114, and 165, but reacted weakly with the modulated derivatives of these strains. The antibody recognized the 91K protein relatively equally in BP338, BP338NA, BP348, and BP348NA, although the bands of reactivity were not as intense as those of the other *B. pertussis* strains shown in this figure. The Tn*5* *vir* mutants BP347, BP347NA, BP359, and BP359NA did not demonstrate any reactivity with P8E7.

DISCUSSION

We (26, 37) as well as others (9, 27, 28, 36) have noted differences in the cell envelope profiles of modulated and phase IV (degraded) derivatives of *B. pertussis* when compared with virulent-phase strains. *B. bronchiseptica* and *B. parapertussis*, although proposed to be phylogenetically very closely related to *B. pertussis* (20, 24), displayed

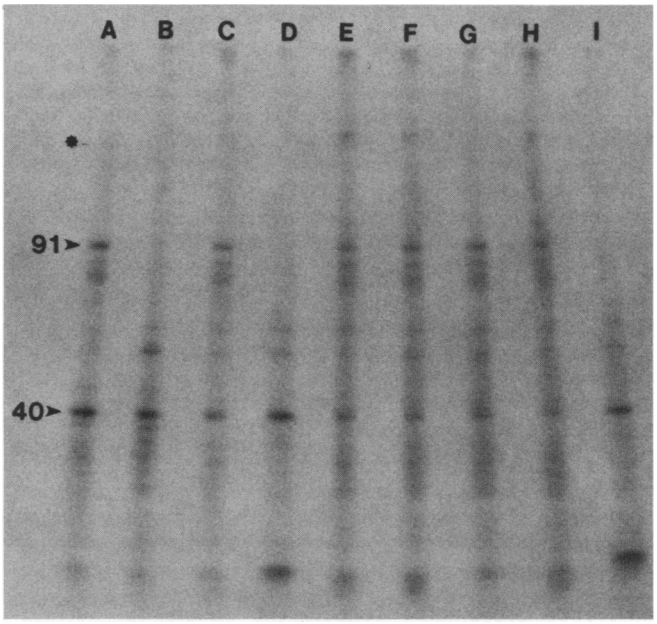

FIG. 2. Autoradiogram of surface-radioiodinated *B. pertussis*. Intact cells were radiolabeled as described in the text and analyzed by SDS-PAGE and autoradiography. Lanes: A, virulent-phase UT25; B, avirulent-phase UT25D; C, virulent-phase BP338; D, BP347; E, BP348; F, BP349; G, BP353; H, BP357; I, BP359. The asterisk indicates the region in which a high-molecular-weight doublet occurred. The arrows indicate the positions of proteins with apparent molecular weights of 91,000 and 40,000 as determined by reference to the molecular weight standards listed in the legend to Fig. 1.

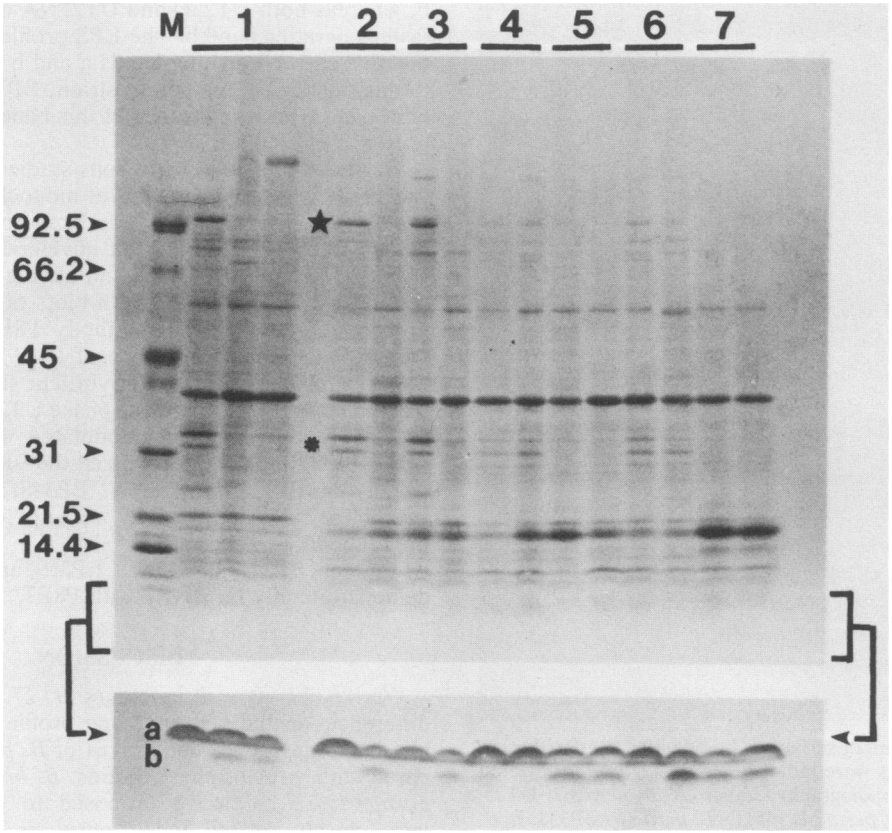

FIG. 3. Analysis of cell envelopes of *B. pertussis* strains and their modulated derivatives. Total cell envelopes were prepared and analyzed by SDS-PAGE and protein staining with Coomassie blue (top panel). Whole cells were pretreated with proteinase K and subjected to SDS-PAGE and LPS-specific silver staining (lower panel). Top panel: The first lane in sample sets 2 to 7 is the original strain, while the sample in the next lane is the nicotinic acid (NA)-modulated derivative of the strain. Set 1, UT25, avirulent UT25D, and UT25NA; set 2, 114 and 114NA; set 3, 165 and 165NA; set 4, BP338 and BP338NA; set 5, BP347 and BP347NA; set 6, BP348 and BP348NA; set 7, BP359 and BP359NA. Lane M, Molecular weight standards as described in the legend to Fig. 1. The star denotes the position of the virulence-associated 91K protein, while the asterisk indicates a region (ca. 30,000 molecular weight) in which the virulence-associated doublet migrated. Lower panel: Bacterial strains were identical to those listed for the top panel. The two major LPS bands are designated a and b. The brackets indicate the area of the gel in which the *Bordetella* LPS migrated.

surface-labeled protein profiles distinct from those of *B. pertussis*. The virulent *B. pertussis* strains examined in this investigation showed similar envelope protein profiles despite their differences in agglutinogen content.

Virulent strains cultured on Bordet-Gengou medium showed a surface radioiodinated protein doublet of high molecular weight which was absent in similarly cultured avirulent *B. pertussis*, BP353 (*fha*), BP347 (*vir-1*), and BP359 (*vir-2*), suggesting that this moiety was the filamentous hemagglutinin. Purified filamentous hemagglutinin migrates as a high-molecular-weight doublet or triplet (45). In total cell envelopes prepared by ultracentrifugation of lysates of cells grown in Stainer-Scholte broth, the high-molecular-weight surface-exposed doublet was not observed. This difference presumably reflects the paucity of surface-associated filamentous hemagglutinin on the cells grown in liquid medium.

A surface-exposed protein (91K) was observed in surface-radioiodinated virulent strains as well as unlabeled total envelopes of virulent strains. In a previous study (1), it was demonstrated that the virulent-phase-specific 30K and 32K proteins observed in total envelope samples were exposed at the cell surface, and their presence correlated directly with the presence of the 91K surface protein. UT25D, a degraded avirulent-phase strain, demonstrated a lack of the 91K, 32K, and 30K virulence-associated envelope proteins, as did the two Tn*5* *vir* mutants BP347 and BP359. Thus, the results of this investigation confirm that these three envelope proteins are coordinately regulated with the other virulence-associated factors (such as pertussis toxin, filamentous hemagglutinin, and hemolysin) of virulent *B. pertussis*.

We could not distinguish the 30K doublet for strains BP349, BP353, BP357, MU8, and MU41 in autoradiograms; however, the 91K protein was clearly evident in surface-radioiodinated preparations of these cells. Neither *B. bronchiseptica* nor *B. parapertussis* demonstrated a 91K envelope protein.

Nicotinic acid-modulated UT25NA, 114NA, and 165NA showed a decrease in the three virulence-associated envelope proteins, but BP338 and its Tn*5* derivatives were affected by nicotinic acid to a much lesser extent. We do not know whether other virulence-associated traits were affected by the nicotinic acid treatment of BP338 and its derivatives. Relative resistance to nicotinic acid modulation has been observed in other strains of *B. pertussis* (31).

Monoclonal antibody P8E7, reactive with the 91K protein, proved useful in screening strains for this surface antigen; antibodies specific for the 30K and 32K virulence-associated

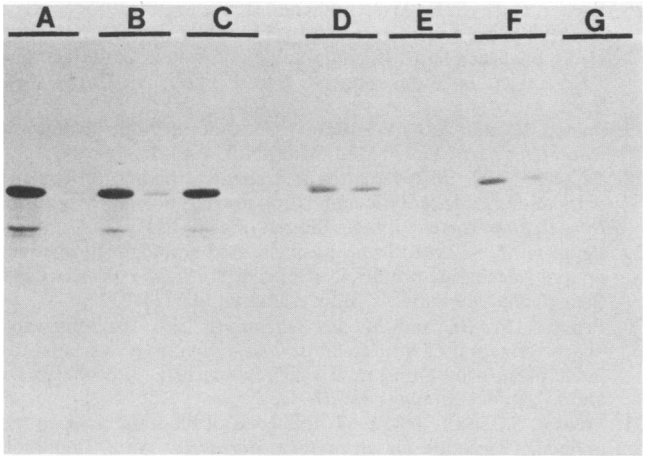

FIG. 4. Immunoblot analysis of strains of *B. pertussis* and their nicotinic acid (NA)-modulated derivatives. Whole-cell proteins of strains and their modulated counterparts were electrophoresed, transferred to nitrocellulose, and probed with the cell surface-specific monoclonal antibody P8E7 which recognizes the virulence-associated 91K envelope protein. Lanes A, UT25 and UT25NA; lanes B, 114 and 114NA; lanes C, 165 and 165NA; lanes D, BP338 and BP338NA; lanes E, BP347 and BP347NA; lanes F, BP348 and BP348NA; lanes G, BP359 and BP359NA.

envelope proteins would also be helpful in this regard. The functions of the three virulence-associated envelope proteins remain unclear. It is possible that they function as agglutinogens from an immunological standpoint, but their roles in bacterial metabolism or pathogenesis will remain poorly characterized until mutants lacking the individual proteins are isolated.

Phase III and IV (degraded) variants have been observed to be more resistant than their virulent counterparts to certain antibiotics (including erythromycin, penicillin, and tetracycline) and inhibitory compounds such as fatty acids (6, 10, 25, 30, 41). One explanation may be that the three virulence-associated envelope proteins function in the uptake of these inhibitors and antibiotics, and loss of or a decrease in these proteins will confer resistance. This theory may be plausible in the case of antibiotic resistance, but because modulated cells and the Tn*5 vir* mutants failed to grow on bloodless media, the three virulence-specific envelope proteins are not likely to be involved in the sensitivity of virulent strains to the inhibitory compounds found in agar. Bloodless media such as nutrient agar or Stainer-Scholte solidified with agar have been utilized to select for phase variants (13, 30). Goldman et al. (13) reported that variants selected on such media varied in their levels of virulence-associated factors and that phase variation was a nonrandom sequence of losses with the disappearance of hemolysin heralding the loss of adenylate cyclase, pertussis toxin, and then filamentous hemagglutinin. Peppler and Schrumpf (30) found two sets of phase variants: flat, nonhemolytic types which grew on nutrient agar, and flat, nonhemolytic types which could not grow on nutrient agar. These observations suggest that phase variation is separate and distinct from the acquisition of the ability to grow on bloodless media. This ability to grow on nutrient agar or TSA has been postulated to be the result of mutations independent of any which may confer other phase IV characteristics (25, 30). If this was true, then the Tn*5 vir* mutants would be amenable to serial passage on media containing decreasing amounts of blood to obtain strains with a fully degraded phase IV phenotype.

Two *Bordetella* variant LPS types have been described in the literature (29). It was thought that the two LPS types might reflect differences observed in phase variation, but there are no data to suggest such a relationship. Our LPS analyses also showed that there was no relationship between the absence of the LPS band b and the avirulent phase or modulated phenotype.

In nature, avirulent-phase variants can be cultured with significant frequency from patients with pertussis (18), whereas phenotypic modulation requires the in vitro exposure of the bacteria to unnatural and nonphysiological conditions. Under optimal conditions in synthetic and semisynthetic broth, the generation time of *B. pertussis* in our laboratory is about 6 to 8 h, in agreement with the observations of Idigbe et al. (15). One can begin to detect the effects of modulation within 10 h of culture in modulating medium (nicotinic acid or magnesium ions) (15, 35). In such media, bacteria appear to be maximally modulated after two passages or culture for 48 h (ca. eight generations) (15, 23, 35). When fully modulated cells are subcultured into normal broth (nonmodulating conditions), virulence-associated traits are rapidly reacquired, with full recovery within 20 h (35). Brownlie et al. (3) found that protein synthesis was required both for modulation and for its reversal.

The phenotypic effects of modulation resemble those of phase variation, suggesting that a common regulatory element is involved. The expression of virulence-associated genes is thought to be under the control of a *trans*-acting regulator (*vir*) during phase variation (41). It is possible that during phenotypic modulation, the modulating factors (low temperature, pyridines, magnesium ions) affect the *trans*-acting *vir* product or its synthesis. Another possibility is that modulation could be a general response of the cells to extreme environmental stress in vitro, similar to the SOS types of responses which occur when bacteria experience DNA damage, heat shock, or other metabolic insults (39).

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