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Characterization of *vir*-Activated Tn*phoA* Gene Fusions in *Bordetella pertussis*

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The expression of many of the known virulence determinants of *Bordetella pertussis* is coordinately regulated by the *vir* regulatory locus and reduced in response to environmental signals called modulators. We have previously identified eight Tn*phoA* gene fusions in *B. pertussis* in which the expression of alkaline phosphatase was maximal in the absence of the modulators nicotinic acid and $MgSO_4$. We have termed the genes identified by these fusions *vir*-activated genes. Here we report the characterization of these Tn*phoA* mutant strains. Four fusion strains were defective in known virulence determinants. For one of these, fusion strain SK39, Southern blot hybridization demonstrated that Tn*phoA* was inserted in the S1 subunit gene of pertussis toxin. Hemagglutination assays, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblots identified three fusions strains, SK16, SK75, and SK91, that were defective in filamentous hemagglutinin. Whereas all three filamentous hemagglutinin-defective mutants showed either normal or enhanced colonization, the pertussis toxin-defective mutant showed a marked defect in pulmonary persistence. Of the four other fusion strains, two were deficient in outer membrane proteins. One of these, strain SK8, was defective in a major outer membrane protein of 95 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This strain colonized mouse lungs less well and did not induce lymphocytosis after aerosol challenge. The other strain, SK34, was defective in four outer membrane proteins, three of which were detectable only on a Western blot with polyclonal sera against *B. pertussis*. Two of our gene fusion strains did not show any defect in identifiable *vir*-regulated proteins.

Bordetella pertussis, the causative organism of whooping cough, produces a number of factors that aid in the establishment of disease. The production of these factors is controlled by the *vir* (also known as *bvg*) locus in response to environmental stimuli in a phenomenon known as antigenic modulation (11, 20, 32, 33). It has been shown that transcription of the genes encoding pertussis toxin (PTX), filamentous hemagglutinin (FHA), dermonecrotic toxin, and adenylate cyclase is positively controlled by this locus (19, 36). More recently, genes that are activated by the *vir* locus (*vir*-activated genes or *vag* genes) have been identified by gene fusion methods using Tn*phoA* and Tn*5 lac* (10, 39).

Current interest in the development of a new vaccine for pertussis has stimulated an interest in its pathogenesis. Although many putative virulence factors have been identified, establishing their role in pathogenesis has been hampered by the lack of an ideal animal model for the disease. Genetic studies have provided some insight. For example, Weiss et al. characterized several mutants that are defective in putative virulence factors and showed that Tn*5* insertion mutants defective in adenylate cyclase-hemolysin and PTX had a higher 50% lethal dose for newborn mice after intranasal challenge (36–38). In our studies we have used the aerosol challenge model developed by Sato et al. (29) to infect 17-day-old mice. Although this is not a model of human pertussis per se, certain parameters of pertussis in humans, such as specific attachment of the bacteria to the ciliated epithelium of the respiratory tract, leukocytosis, increased severity of the disease in neonates, and pneumonia, are observed (29).

In this paper we describe the characterization of *vag*::Tn*phoA* fusion strains. Whereas several of these mutants were found to be defective in production of known virulence determinants, others either showed no detectable defect or altered expression of certain outer membrane proteins. Mutants with known defects were also tested for their ability to induce leukocytosis and to persist in the lungs of infected mice after respiratory challenge.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *B. pertussis* strains used in this study are listed in Table 1. Strains were grown in Bordet-Gengou agar (BG, Difco Laboratories, Detroit, Mich.) supplemented with 10 ml of glycerol per liter and 250 ml of defibrinated sheep blood per liter. Liquid cultures were grown in modified Stainer Scholte (SS) broth (9). For modulation studies, BG agar or SS broth was supplemented with either $MgSO_4$ (20 mM) or 5 mM nicotinic acid. Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; streptomycin 100 μg/ml; and tetracycline, 12.5 μg/ml.

Chemicals. Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co., St. Louis, Mo.

DNA manipulations. Standard methods were used for plasmid and chromosomal DNA isolation, restriction enzyme digestions, agarose gel electrophoresis, and Southern blot analysis (15). DNA restriction enzymes were purchased from New England BioLabs, Inc., Beverly, Mass.

Antisera and antibodies. Antisera to virulent-phase *B. pertussis* were generated by injecting New Zealand White

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TABLE 1. *B. pertussis* strains used in this study

Strain	Genotype	Phenotype or description	Reference or strain
18323		Wild type	ATCC 9797
SK39	*vag-39*::Tn*phoA*	PTX$^-$	10
SK75	*vag-75*::Tn*phoA*	FHA$^-$	10
SK91	*vag-91*::Tn*phoA*	FHA$^-$	10
SK16	*vag-16*::Tn*phoA*	FHA$^-$	10
SK8	*vag-8*::Tn*phoA*	Lacks 95-kDa OMP	10
SK34	*vag-34*::Tn*phoA*	Lacks 93$^-$, 91$^-$, 62$^-$, and 30-kDa OMP	10
SK49	*vag-49*::Tn*phoA*	No designation	10
SK25	*vag-25*::Tn*phoA*	No designation	10

rabbits subcutaneously with *B. pertussis* 18323 harvested from a 2-day BG plate, washed once in saline, and resuspended in 0.5 ml saline, and 0.5 ml of Freund complete adjuvant. Animals were boosted 2 and 4 weeks later as described above but with Freunds incomplete adjuvant. Rabbits were bled 14 days later and serum stored at 4°C until use.

Monoclonal antibody BPE3 against the 69-kDa outer membrane protein was kindly provided by M. Brennan, Food and Drug Administration, Bethesda, Md. Monoclonal antibodies X3E against FHA and X2X5 against the S1 subunit of PTX were kindly provided by J. Kenimer and E. Leininger, Food and Drug Administration.

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10, 12, or 15% polyacrylamide gels was performed as described previously (12). After electrophoresis, gels were stained with Coomassie blue or proteins were transferred by electrophoresis to nitrocellulose (34). Incubation and development of Western immunoblots were performed as described previously (23). Western blots probed with anti-phase I sera were incubated with sera diluted 1/1,000. Western blots for FHA were performed on Tricine-urea extracts of bacteria grown on BG plates. Briefly, 10^9 bacteria were resuspended for 20 min in 4 M urea–0.1 M Tricine (pH 8.0) at room temperature and then placed at −70°C overnight. Samples were centrifuged at 12,000 × *g* for 10 min, and aliquots of the supernatant containing 10 μg of protein were loaded on an SDS-PAGE 4 to 20% polyacrylamide gradient gel (Integrated Separation Systems/Enprotech, Hyde Park, Mass.). Proteins were transferred to Immobilon membranes (Millipore, Bedford, Mass.) and incubated with monoclonal antibody X3E (diluted 1/1,000) and alkaline phosphatase-conjugated goat anti-mouse immunoglobulin as described previously (2). Filters were developed with the Protoblot substrate (Promega Biotec, Madison, Wis.).

PTX ELISA. PTX was assayed in supernatant fluids by using a fetuin enzyme-linked immunosorbent assay (ELISA, Spiro method; Gibco Laboratories, Grand Island, N.Y.). Plates were sensitized with 100 μg of fetuin per ml. Purified PTX (List Biological Laboratories, Inc., Campbell, Calif.) was used as a standard. Anti-PTX antiserum was provided by L. Winberry (Massachusetts Public Health Laboratories, Jamaica Plain, Mass.) and used at a 1/1,000 dilution.

Purification of PTX. PTX was partially purified from culture supernatants by using Affi-Gel Blue (Bio-Rad, Richmond, Calif.) as described by Sekura et al. (30). A 1/100 vol. of Affi-Gel Blue was added to culture supernatants. Tubes were rotated overnight and spun in a microfuge, and the Affi-Gel Blue was washed with 0.25 M Na_3PO_4 (pH 6.0) and then 0.05 M Tris-HCl (pH 7.4). PTX was eluted from the Affi-Gel Blue into 0.05 M Tris–0.75 M $MgCl_2$ (pH 7.4) at 4°C. Samples were spun to remove the Affi-Gel Blue, heated to 100°C with 2 volumes of 2× sample buffer containing 2-mercaptoethanol, and then loaded on a 4 to 20% polyacrylamide gradient gel. Western blots were performed as described above with the anti-S1 monoclonal antibody X2X5 diluted 1/500.

CHO cell clustering assay. The CHO cell clustering assay described by Hewlett et al. was used to screen for PTX (7). Two-day SS culture supernatants were filtered through 0.2-μm-pore-size polysulfone filters (Acrodisc, Fisher Scientific, Medford, Mass.). After centrifugation, the cell pellets were resuspended in 1/10 volume of 50 mM Tris–50 mM EDTA (pH 7.5) containing 2 mg of lysozyme per ml. After incubation for 1 h at 27°C, the pellets were vortex mixed and filtered through 0.2-μm-pore-size polysulfone filters. A 1/10 dilution of supernatants and cell pellets was made in Ham nutrient mixture F12 (Hazelton Research Products, Inc., Lenexa, Kans.) supplemented with 2% newborn calf serum, 50 U of penicillin, and 50 μg of streptomycin per ml. Confluent 24- to 48-h cultures of CHO cells in 60- by 15-mm plates were trypsinized and resuspended to 2.5 × 10^4 cells per ml in F12 plus 2% newborn calf serum. Then 200 μl of CHO cells was added to doubling dilutions of *B. pertussis* cells or supernatant preparations in 96-well microtiter plates. PTX (List Laboratories) was used as a control. Plates were incubated at 37°C in 7% CO_2 for 48 h before the results were read.

Assays for FHA. Supernatants from 2-day SS cultures of *B. pertussis* were assayed for hemagglutination of goose blood erythrocytes as previously described (28). In addition, Western blots of cell lysates were probed with the anti-FHA monoclonal antibody as described above.

Assay for dermonecrotic toxin. Dermonecrotic toxin was assayed by using the infant mouse nuchal injection model of Cowell et al. (3). *B. pertussis* cells were isolated from 2-day SS cultures that had been spun down and resuspended in saline to an optical density at 600 nm of 1.0; 50-μl samples of undiluted cells or cells diluted 1/10 were injected subcutaneously into the nuchal areas of 4-day-old CD-1 mice. After 20 h at 30°C, this area was observed for darkening.

Assay for adenylate cyclase. Cell-associated adenylate cyclase was measured by using a competitive binding assay kit (Amersham Corp., Arlington Heights, Ill.). *B. pertussis* cells were isolated from 2-day SS cultures, spun in a microfuge, and resuspended in an equal volume of 60 mM Tris-HCl (pH 7.8)–10 mM $MgCl_2$; dilutions were then made in this buffer. The assay was performed in the presence and absence of 60 mM calmodulin.

OMP preparations. Triton X-100-insoluble outer membrane proteins (OMPs) were prepared from 20-ml SS cultures by the procedure of Hantke (5). Samples were resuspended in 500 μl of sample buffer containing 2-mercaptoethanol. Gel samples were heated to 100°C for 5 min before samples were loaded.

Aerosol challenge assay. Specific-pathogen-free BALB/cAnNcR mice were obtained on day 17 postpartum from the Animal Production Program, Division of Cancer Treatment, National Cancer Institute, Frederick, Md. The animals were housed in filtered microisolators with free access to food and water. A 21-h culture of bacteria was harvested from a BG agar slant and resuspended in sterile saline to approximately 10^9 CFU/ml. The challenge inoculum was administered to the mice as an aerosol for 30 min as previously described

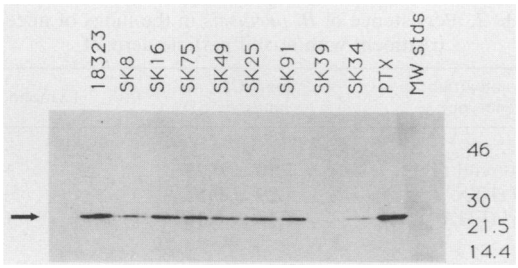

FIG. 1. Affi-Gel-purified supernatants from strain 18323 and Tn*phoA* fusion strains were separated on a 4 to 20% polyacrylamide gradient SDS-PAGE gel, electroblotted onto nitrocellulose, and probed with monoclonal antibody X2X5 against the S1 subunit of PTX. All lanes except SK39 show a band with an apparent molecular mass of 28 kDa, indicated by the arrow, corresponding to the S1 subunit of the PTX. Lane 10 contains 1 μg purified PTX. Molecular weight markers (MW stds) are indicated.

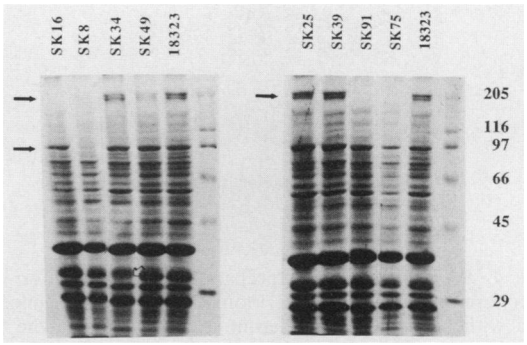

FIG. 2. *B. pertussis* OMP preparation separated on a 10% polyacrylamide SDS-PAGE gel and stained with Coomassie blue. SK16, SK75, and SK91 no longer produce the 220-kDa FHA species, indicated by the top arrows. SK8 is defective for a 95-kDa OMP, shown by an arrow. An open arrow identifies the 30-kDa OMP missing in SK34. Samples run in each lane are indicated on the gel. Molecular weight markers are shown to the right.

(29). Upon removal from the aerosol chamber, a group of mice were sacrificed; their lungs, including the bronchial tree, were removed and homogenized in sterile phosphate-buffered saline, and the homogenate was plated to determine the numbers of viable *B. pertussis* in the lungs. All mice tested had approximately 10^4 CFU in their lungs 1 h after aerosol infection. To determine bacterial persistence, mice were sacrificed after 14 days and the number of CFU in the lungs was determined as described above. Mice were bled from the orbital sinus periodically after infection, and leukocyte counts were determined in a model ZM Coulter counter (Coulter Electronics, Hialeah, Fla.).

RESULTS

Characterization of *vir*-activated Tn*phoA* fusions for loss of known putative virulence determinants. Strains containing positively regulated Tn*phoA* fusions were characterized for loss of PTX, FHA, pertactin, adenylate cyclase, and dermonecrotic toxin activity (Table 1).

Strain SK39 was defective for the production of PTX based on a fetuin ELISA. Culture supernatants and whole-cell lysates prepared from this strain were also negative for PTX in a CHO cell assay. Southern blot hybridization with a *ptx*-derived probe, a 1.7-kb *Bgl*II-*Xba*I fragment internal to the *ptx* operon, demonstrated that the Tn*phoA* is inserted into the gene encoding the S1 subunit of PTX between the *Apa*I site at 609 bp and the *Xba*I site at 1,313 bp (data not shown). When Affi-Gel-purified supernatants were analyzed on Western blots with a monoclonal antibody against the S1 subunit, they showed loss of the 28-kDa S1 subunit (Fig. 1). A negative fetuin ELISA would indicate that the S2, S3, S4, and S5 subunits are not present, since the subunits of the B oligomer are required for binding to fetuin (41). The loss of these subunits presumably results from transcriptional polarity on the genes for these subunits, which are known to be downstream of the S1 subunit (14, 21).

Three fusion strains, SK16, SK75, and SK91, were defective for FHA on the basis of hemagglutination data (not shown) and the loss of the 200-kDa bands on an SDS-PAGE gel (Fig. 2). Immunoblots probed with the monoclonal antibody X3E indicated that both SK16 and SK75 produced no reactive material and that SK91 produced some lower-molecular-weight bands but lacked the 200-kDa species (Fig. 3). None of the mutants carried inserts in the structural gene for FHA based on Southern blot analysis with *B. pertussis* DNA adjacent to the fusion junction of each mutant to probe four individual clones, each containing the FHA structural gene (13a). This suggests that the FHA^- phenotype of these Tn*phoA* mutants may be associated with the loss of accessory gene products involved in FHA biogenesis or assembly. Western blot analysis with monoclonal antibodies against the type 2 and type 3 fimbriae detected subunits in all eight fusion strains (20a).

No fusions defective in DNT were found, as measured with the neonatal mouse model. This was not entirely unexpected, since this protein has been localized to the cytoplasm (3). Only strains harboring active Tn*phoA* fusions were selected for study, and alkaline phosphatase activity

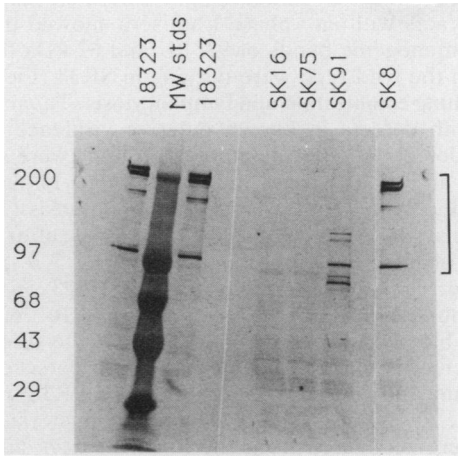

FIG. 3. Tricine-urea extracts of 18323, SK8, and three FHA-defective strains (SK16, SK75, SK91) were separated on a 4 to 20% polyacrylamide gradient SDS-PAGE gel and transferred to an Immobilon transfer membrane. The blot was incubated with monoclonal antibody X3E against FHA. The lanes marked 18323 and SK8 show a doublet with apparent molecular masses of 220 and 208 kDa and smaller bands of 173 and 104 kDa, corresponding to breakdown products of FHA; these are indicated by the bracket. SK16 and SK75 show no material that was cross-reactive with this monoclonal antibody. SK91 shows a number of bands that are cross-reactive with the X3E monoclonal antibody, one which may be identical to the 104-kDa band seen on the 18323 and SK8 lanes. Molecular weight markers (MW stds) are indicated.

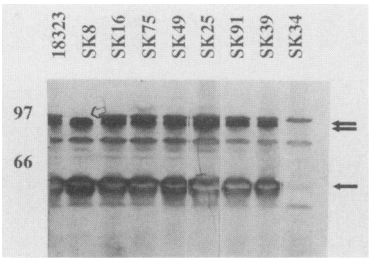

FIG. 4. *B. pertussis* OMP preparations separated on a 10% polyacrylamide SDS-PAGE gel, transferred to nitrocellulose, and probed with anti-phase I antiserum. Arrows indicate the 95-kDa band, missing in SK8, and three bands of 93, 91, and 62 kDa missing in strain SK34. The samples run in each lane are as indicated on the gel. Molecular weight markers are indicated.

will not be expressed unless the target gene encodes a secreted protein. No fusion strains were defective in adenylate cyclase enzymatic activity.

A potent antigen in *B. pertussis* vaccine preparations is the 69-kDa protein pertactin, which is thought to be an important adherence factor (2, 13, 22). None of our *vir*-activated Tn*phoA* fusions was defective in this protein, based on Western blots probed with the monoclonal antibody to pertactin, BPE3.

Characterization of Tn*phoA* mutants for loss of OMPs. Tn*phoA* creates fusion proteins between target genes and *phoA*, and only if the target gene encodes a secreted or membrane-spanning protein will these hybrid proteins will show alkaline phosphatase activity. We therefore screened for loss of OMPs on SDS-PAGE gels stained with Coomassie blue. This demonstrated that strain SK8 was defective in the production of a major OMP of 95 kDa (Fig. 2). Another strain, SK34, was missing a 30-kDa protein from the SDS-PAGE profile (Fig. 2). In addition, Western blot analysis of SK34 extracts with anti-phase I antisera showed that three highly immunogenic bands of 93, 91, and 62 kDa that were present in the wild type were missing in SK34 (Fig. 4).

Mouse lung colonization and lymphocytosis. Tn*phoA* fusion strains with defects in known putative virulence determinants or defects in outer membrane proteins were screened for their ability to persist in mouse lungs 14 days after aerosol inoculation and to induce lymphocytosis (Table 2). Wild-type strain 18323 colonized mouse lungs; after 14 days, the peak of infection, approximately $\log_{10}$ 7.5 *B. pertussis* cells were recovered. In addition strain 18323 was able to induce lymphocytosis of greater than 75×10^3 leukocytes per μl of blood 14 days after infection. Of the three fusion strains defective in FHA, SK16 and SK75 persisted in the lungs significantly better than did 18323 (for SK16, $P < 0.01$; for SK75, $P < 0.05$); $\log_{10}$ 8.49 and 8.5 bacteria, respectively, were recovered from the lungs 14 days after challenge. SK91, which had more FHA-cross-reactive material than SK16 and SK75 based on a Western blot probed with anti-FHA monoclonal antibody X3E (Fig. 3), was recovered at the same level as 18323. All three FHA-defective strains induced lymphocytosis in mice 16 days after infection.

Fusion strain SK34, which is defective in four OMPs, was able to colonize significantly better than was 18323 ($P < 0.01$). Animals infected with this strain showed an increase in peripheral blood leukocyte count within 16 days of challenge.

Two Tn*phoA* fusion strains were defective in the mouse aerosol challenge model. SK39, carrying a Tn*phoA* inserted in the PTX S1 subunit gene, did not induce lymphocytosis and colonized about 100-fold less than did 18323. Strain SK8, which no longer produces a major OMP of 95kDa, was reduced about 10-fold in pulmonary colonization. In addition, the animals infected with SK8 did not exhibit an increase in peripheral blood leukocyte count. SK8 did not appear to be defective in the CHO cell assay. After 2 days of growth in SS medium, 4.8 μg of PTX per ml per unit of optical density at 600 nm were produced, equivalent to the amount produced by the parental strain 18323 (4.9 μg/ml per unit of optical density at 600 nm).

TABLE 2. Persistence of *B. pertussis* in the lungs of mice after treatment with mixed particle aerosol

Expt and strain (phenotype)	$\log_{10}$ CFU in lungs[a] ± SD	Lymphocytosis[b]
Expt 1		
18323 (wild type)	7.10 ± 0.23	+
SK39 (PTX^-)	5.29 ± 0.53[c]	−
SK16[d] (FHA^-)	8.49 ± 0.16[c]	+
Expt 2		
18323	7.62 ± 0.48	+
SK75 (FHA^-)	8.50 ± 0.47[c]	+
SK91 (FHA^-)	7.72 ± 0.71	+
Expt 3		
18323	7.59 ± 0.37	+
SK8 (OMP^-)	6.46 ± 0.21	−
SK34 (OMP^-)	8.82 ± 0.26[c]	+

[a] Mean CFU in the lungs of mice 14 days after challenge with approximately $\log_{10}$ 4.5 organisms (five to eight mice per group).

[b] +, $>75 \times 10^3$ leukocytes per μl of blood 14 days postinfection. Normal levels are $<25 \times 10^3$ leukocytes per μl. These results were determined on a second group of mice.

[c] Statistically significant compared with results from strain 18323 with the Student *t* test ($P < 0.01$, except for SK75 [$P < 0.05$]).

[d] At day 14, only two mice were alive from the group of eight; in all other cases the results reflect the entire group.

DISCUSSION

We have used Tn*phoA* transposon mutagenesis to isolate fusion strains that are defective in *vir*-regulated genes. Once these mutants were characterized we used them to study the pathogenesis of infection and the control of gene expression during the infectious process.

The first stage of infection is thought to be adherence to the ciliated epithelium, which extends from the nasopharynx and trachea to the respiratory bronchioles. Filamentous structures are thought to be candidates for mediating adherence of *B. pertussis*, since many other organisms use fimbrial structures to adhere to host cells (4, 6, 16, 27). *B. pertussis* possesses both FHA and fimbriae (1); although FHA has been implicated in in vitro studies as an important mediator of adherence to both ciliated cells and macrophages, the role of the fimbriae is less clear (25, 26, 35). We isolated three FHA-deficient mutants, SK16, SK75, and SK91, none of which showed any defect in respiratory infection in the mouse aerosol model. Indeed, two of them, SK16 and SK75, appeared more able than 18323 to colonize and persist in the lungs. These results are consistent with reports from other workers that FHA-defective strains are not impaired in their ability to colonize the lower respiratory tract of mice (8, 38, 40). However, using a strain that synthesizes a truncated FHA, Kimura et al. have shown that FHA is an important

factor for the initial colonization of the trachea. Of note is the observation that this mutant recolonized the trachea 5 to 10 days after challenge, when ciliostasis had presumably occurred (8). Thus, the colonization proficiency of our FHA$^-$ mutants is consistent with these and other in vitro studies which suggest that adherence is multifactorial and may involve a 69-kDa protein, FHA, and PTX (13, 25, 26). It is possible that FHA-defective mutants are able to utilize OMPs and possibly other adherence mechanisms in vivo. Consistent with this conclusion, our FHA-defective Tn*phoA* insertion mutants were not defective in production of pertactin, PTX, or type 2 and type 3 fimbrial subunits.

Strain SK39 carries a Tn*phoA* insertion in the gene encoding the S1 subunit of PTX. This strain produced no toxin detectable by the CHO cell assay and did not induce lymphocytosis in infected animals. Although SK39 was able to persist in the lungs of mice, it colonized at a level about 100-fold less than its parent, 18323. What might be the nature of the colonization defect associated with loss of PTX in SK39?

PTX has been implicated in the adherence of *B. pertussis* to ciliated epithelial cells inasmuch as a *ptx* deletion mutant shows reduced adherence in vitro (26). This adherence effect presumably is associated with the receptor binding action of the PTX B promoter. Thus, SK39 may be reduced in toxin-mediated bronchial adherence even though it does produce other putative adherence factors, like FHA and fimbriae. Alternatively, the colonization defect of SK39 may reflect enhanced clearance of the strain by a mechanism that does not involve differences in adherence per se. For example, it is possible that PTX, through its disruption of phagocyte and lymphocyte function, interferes with host defense mechanisms and thus allows toxigenic organisms to attain a higher level of colonization (17, 31).

In this regard, it is interesting that mutants of *Vibrio cholerae* that have deletions in genes encoding cholera toxin are also reduced in their capacity to colonize the intestinal mucosa of rabbits (24). Because the degree of this defect is about the same as we see here for our *B. pertussis ptx* mutant SK39 (i.e., most *ctx* mutants colonize 10- to 100-fold less than do their parental strains), it is tempting to speculate that both toxins may act to compromise a similar mucosal defense mechanism that may involve lymphocytes, macrophages, or some other type of effector cell. Like the PTX B protomer, the cholera toxin B protomer has receptor binding activity. However, *V. cholerae ctxA* mutants, which still secrete a functional B promoter, are just as defective in colonization as are mutants that lack both A and B subunits, suggesting that it is the activity of the toxin rather than binding that stimulates colonization. Given that both cholera toxin and PTX can elevate cyclic AMP levels in host tissues, it is reasonable to suggest that this common pharmacological effect may be involved mechanistically in the toxin-mediated enhancement of colonization seen in both cases (18, 24). Clearly, the adenylate cyclase toxin of *B. pertussis* might also contribute to colonization by a similar cyclic AMP-dependent mechanism inasmuch as adenylate cyclase mutants have also been found to be highly defective in the pulmonary colonization of mice (38, 40). Support for this hypothesis will require studying the colonization properties of *B. pertussis* mutants that produce PTX molecules with normal binding and adherence properties but without the capacity to activate the adenylate cyclase.

A role for PTX as a colonization factor makes the interpretation of some mutant phenotypes more problematic. For example, strain SK8 produces normal amounts of PTX and adherence factors in vitro yet was defective in vivo in colonization and in induction of lymphocytosis. Since lymphocytosis is a good indicator of effective delivery of PTX to host tissues, and since PTX is also a colonization factor, it is difficult to discern the exact nature of the virulence defect of SK8. The reduced pulmonary persistence of SK8 might suggest that the 95-kDa OMP may itself influence colonization of the lung by, for example, acting as an adhesin. The resultant reduced cell density of SK8 in the lung would then fail to produce sufficient PTX to induce lymphocytosis. Alternatively, SK8 may simply be defective in the delivery of PTX to target tissues by, for example, lacking a critical protease or glycosidase that affects toxin receptor availability. As a consequence of the reduced delivery of toxin to target cells, this strain would be reduced in colonization and induction of lymphocytosis. Both interpretations suggest that the *vir*-regulated 95-kDa OMP probably plays an important role in establishing or maintaining infection. However, differentiating the direct versus indirect effects of this protein is important, since it greatly influences the interpretation of mutant phenotypes, 50% lethal dose data, and even strategies for immune intervention. Similar complications have been previously noted in the characterization of FHA$^-$ mutants (40).

We have determined that SK34 is also deficient in OMPs. A Coomassie blue-stained SDS-PAGE gel shows loss of a 30-kDa protein. In addition, a Western blot of OMPs probed with anti-phase I antisera shows the loss of three immunogenic bands that are present in the wild type. The loss of four proteins may be due to a polar effect of Tn*phoA* on downstream genes, or these proteins could be the breakdown products of a larger protein, such as is seen with FHA on an SDS-PAGE gel. A third possibility is that the *vag-34* gene product is a regulatory protein and affects the expression of these proteins. This strain is not defective in the mouse aerosol challenge model. However, three of the proteins appear to be highly immunogenic and thus the antibody response to the *vag-34* gene product(s) might be important in protection.

Two of our fusion strains, SK49 and SK25, showed no defect in known virulence determinants, nor did they differ from the wild type on SDS-PAGE gels or Western blots. However, measurement of alkaline phosphatase activities from these strains showed that they are regulated. The products of the *vag-25* and *vag-49* genes are therefore produced with the other known virulence determinants and may be important in the infectious process.

In summary, we have used Tn*phoA* insertional mutagenesis to assess the role of *B. pertussis* virulence factors in colonization with a mouse model and to identify new genes positively regulated by the *bvg* locus. We have identified four new *vir*-activated genes, one of which (*vag-8*) appears to be important for persistence in a mouse aerosol model. Before this, only strains defective in adenylate cyclase and hemolysin or in PTX have shown any defects in virulence or persistence in animal models (37, 39). Using our SK39 mutant, we have confirmed that PTX is important in the colonization and persistence of *B. pertussis*. Our three strains that were defective in FHA showed no defect in persistence. Although the role of invasion in the pathogenesis of *B. pertussis* is unclear, it has been suggested that it may enable the bacterium to establish a carrier state isolated from the immune system (36). It is possible that the *vag* gene products that show no defect in current in vivo and in vitro model systems aid in the pathogenesis of the human host or in the establishment of a carrier state. Thus, the Tn*phoA*

insertion mutants described in this paper should enable us to understand how the *vag* gene products contribute to the pathogenesis of *B. pertussis*.

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