A Phase Variant of *Bordetella pertussis* with a Mutation in a New Locus Involved in the Regulation of Pertussis Toxin and Adenylate Cyclase Toxin Expression

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A novel nonhemolytic phase variant of *Bordetella pertussis* was characterized. This strain is strongly impaired in the transcription of the pertussis and adenylate cyclase toxins, whereas other known virulence-related factors such as the filamentous hemagglutinin, the fimbriae, and the outer membrane protein pertactin are expressed and regulated normally. Complementation and allelic exchange experiments demonstrated that the mutation is localized neither in the *bvg* locus involved in virulence regulation nor in the genes responsible for synthesis and transport of the toxins pertussis and adenylate cyclase. Instead, the mutation impairing transcription of at least the two toxin genes is located in a new genetic locus, which acts together with the BvgA/S two-component regulatory system on the expression of a subset of virulence genes. Further analysis suggested that most presumably the mutation affects a sequence-specific DNA-binding protein which contributes to transcriptional activation. The mutant was nonlethal in a murine respiratory model, which corresponds well with the lack of expression of the toxins. However, the clearing rate of this mutant from the lungs of mice was much lower than that of a *bvg* mutant, suggesting that factors other than the toxins may play a role in the persistence of the bacteria in the respiratory tract of mice.

Bordetella pertussis, the etiological agent of whooping cough, produces many factors involved in its virulence; most of them are secreted or associated with the cell surface. Among them, there are factors believed to be involved in adhesion to epithelial cells such as the filamentous hemagglutinin (FHA), the fimbriae (FIM), and the outer membrane protein pertactin (PRN) (33, 45, 62). In addition, several toxins are produced, such as the ADP-ribosylating pertussis toxin (PTX) (35, 43) and the extracellular invasive adenylate cyclase toxin, which also has hemolytic properties (HLY-CYA) (17). These toxins seem to play a major role during the initiation of infection and the colonization of the host tissue as well as causing the systemic effects associated with the disease (41, 60).

Regulation of these factors is coordinately controlled and responsive to changes in the environment, a reversible phenomenon called phenotypic modulation (24, 31, 38, 59). The two-component BvgA/BvgS system is responsible for this regulation and in vitro perceives signals such as $MgSO_4$, nicotinic acid, and temperature (2, 39, 58). Many years ago, another regulatory phenomenon was described for this organism: upon in vitro cultivation, the *Bordetella* species spontaneously lost their virulence properties with a relatively high frequency. This phenomenon was called phase variation, and it is rarely reversible (34). So far, all characterized spontaneous phase variants have been shown to contain small deletions or frameshift mutations in the *bvgS* gene (3). Recently, a model for the virulence regulation in *B. pertussis* was proposed, in which the BvgS sensor protein perceives external stimuli and transmits these signals via phosphorylation by its histidine kinase-transmitter domain to the receiver domain of the BvgA response regulator. BvgA in its phosphorylated form can then activate

transcription of the various virulence loci (49, 52). However, the fine analysis of regulation of the various virulence genes revealed some interesting differences, such as different behaviors of the promoters in the heterologous host *Escherichia coli* or different binding properties of the transcriptional activator BvgA for the various promoters (48, 51). These results indicated that the original model was too simplistic and that additional factors may be involved in the expression of at least certain loci of the virulence regulon (22, 27).

We now have identified a *B. pertussis* phase variant with a mutation in a new genetic locus involved in the regulation of expression of the toxins PTX and HLY-CYA. We show that in this strain transcription of the *ptx* and *cya* loci is strongly reduced, whereas other virulence factors are not impaired. This mutant is not able to cause lethality in a murine model; nevertheless, it shows a very low clearing rate from the lungs of the mice, suggesting a role for factors in addition to the toxins in the persistence of the bacteria in the respiratory tract of the animals.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1. *B. pertussis* strains were grown on Bordet-Gengou (BG) plates supplemented with 15% sheep blood (6) or in Stainer-Scholte liquid medium (54). When necessary, antibiotics were added to the following final concentrations: streptomycin, 200 μg/ml; tetracycline, 12.5 μg/ml; kanamycin, 30 μg/ml; gentamicin, 10 μg/ml; and nalidixic acid, 30 μg/ml. Bacterial conjugations were carried out as already described (23), with *E. coli* SM10 or S17.1 as the donor (53). Modulating growth conditions were generated by adding 30 mM $MgSO_4$ to the media.

DNA manipulations. All DNA manipulations were carried

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and source or reference
E. coli DH5-α	F⁻ φ80dlacZ M15 (lacZYA-argF)U169 endA1 recA1 hsdR17 (r_K^-, m_K^-) deoR thi-1 susE44 gyrA96 relA1 lambda⁻ (GIBCO BRL)
B. pertussis	
Tohama I	B. pertussis wild type (28, 50)
TI ptx	Derivative of Tohama I; the entire ptx operon including the promoter is substituted by a kanamycin resistance gene (I.R.I.S., Siena, Italy)
BP347	Derivative of Tohama I; bvgS::Tn5 (59)
BP359	Derivative of Tohama I; bvgA::Tn5 (59)
BC75	Derivative of Tohama I; insertion of Tn5tac1 in an unknown locus (8)
TI-Tn5BC75	Derivative of Tohama I carrying the Tn5tac1 insertion of BC75 (this study)
BC7500	Derivative of BC75; elimination of the Tn5tac1 insertion by the respective wild-type sequence (this study)
TI-ptx75	Derivative of TI ptx; contains the ptx operon of BC75 on its chromosome (this study)
BC7500-bvgTI	Derivative of BC7500; contains the wild-type bvg locus on its chromosome (this study)
Plasmids	
pBluescript	Cloning vector (Stratagene)
pRTP1	Mobilizable suicide vector (55)
pSS1129	Like pRTP1, but containing a gentamicin resistance gene (57)
pLAFR2	Mobilizable cloning vector (15)
pLA-HLYS1	Derivative of pLAFR2 containing cyaCABDE operon (16)
pLAFR2-2	Derivative of pLAFR2 with complete ptx synthesis and transport operons (10)
pLA-5,7-vir	Derivative of pLAFR2 containing the bvgA and bvgS genes (3)
pBP2	Derivative of pLAFR2 containing a ptx::CAT fusion with intact ptx promoter (23)
pBP33	Derivative of pBP2 with a deletion in the ptx promoter (23)
pRK-CAT	pRK415 with a lac::cat fusion (21)
pRK-14-CAT	Like pRK-CAT but containing the upstream activating sequence of the ptx promoter between the lac promoter and the cat gene (21)
pSS1212	Derivative of pRTP1 with kanamycin resistance gene flanked by the noncoding flanking regions of the bvg operon (58)
pNMD50	Derivative of pSS1129 containing a 14-kb BamHI fragment containing the entire bvg locus (this study)

out by standard procedures (36). For the subcloning experiments, E. coli DH5-α was used as a host (GIBCO-BRL, Gaithersburg, Md.) and pBluescript (Stratagene, San Diego, Calif.) was used as the vector. For the cloning experiments in B. pertussis, the replicating cosmid vector pLAFR2, which is a derivative of the RP4 plasmid, was used (15). Allelic exchange experiments were carried out with the mobilizable suicide vector pRTP1 or pSS1129, either of which is unable to replicate in B. pertussis (55, 57).

Electrophoresis and immunoblotting methods. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in vertical slab gel instruments (Hoefer Scientific Instruments, San Francisco, Calif.), and proteins were blotted in a semidry transfer chamber onto nitrocellulose filters [Schleicher & Schüll, Dassel, Germany]), or ready-to-use 8 to 25% polyacrylamide gels and the PhastSystem (Pharmacia, Uppsala, Sweden) were applied, and the proteins were transferred to Hybond C-Super membranes (Amersham, Little Chalfont, United Kingdom). After blocking, the membranes were incubated at a 10^{-3} dilution with the respective antisera at 4°C overnight. Generally, polyclonal antisera against the purified factors were used, with the exception of the serotype 2 FIM (FIM2) and the S1 subunit of PTX, for which monoclonal sera were used (10, 59). Either the immunochemical detection was performed with horseradish peroxidase-labeled sheep anti-mouse immunoglobulins and the enhanced chemiluminescence system (ECL, Amersham), or the filters were transferred to a solution containing 6 ml of 0.3% 4-chloro-1-naphthol (Sigma, Deisenhofen, Germany), 41 µl of 30% H_2O_2 (Merck, Darmstadt, Germany), and 94 ml of Tris-buffered saline for the detection of the peroxidase activity.

Assays for biological activities of Bordetella virulence factors. HLY-CYA activities were measured as previously described (17). One unit of HLY-CYA activity corresponds to 1

nmol of cyclic AMP formed per min at 30°C at pH 8.0. The CHO toxicity assay and the estimation of the hemagglutinating activity in bacterial supernatants were carried out as described elsewhere (42, 44).

RNA procedures. RNA was prepared from bacteria grown in liquid culture as already described (24). The same amount of total RNA of each strain was blotted onto nitrocellulose filters (Schleicher & Schüll) with a slot blot apparatus (Bio-Rad, Richmond, Calif.). Radioactive labeling of specific DNA restriction fragments for the hybridization experiments was carried out with a random priming kit (Pharmacia). The specific DNA probes used were an 892-bp KpnI-XbaI fragment containing most of the PTX S1 gene (43), a 499-bp EcoRI-PstI fragment containing the N-terminal part of the fhaB gene (45), a 513-bp BamHI-SalI fragment internal to the cyaA gene (17), and a 19-bp oligonucleotide complementary to nucleotides 270 to 289 of the published sequence of the recA gene (14). Hybridization of the DNA fragments to the RNA blots was carried out under stringent conditions according to standard procedures (36). At least three independent RNA preparations were used for each strain, and for the determination of background hybridization due to contaminating, DNA, samples treated with RNase were included in control experiments.

CAT assay. The chloramphenicol acetyltransferase (CAT) assays were performed as already described (23). For each strain, at least three independent cultures were used for the CAT assay. The protein concentrations of the various samples used in the various assays were standardized according to the method of Bradford (Bio-Rad). Quantitation was carried out by scanning of the autoradiographs on an Elscript 400 densitometer (Hirschmann Inc., Unterhaching, Germany). In the case of plasmid-borne cat gene fusions, the relative copy numbers of the various constructs were determined as described earlier (24).
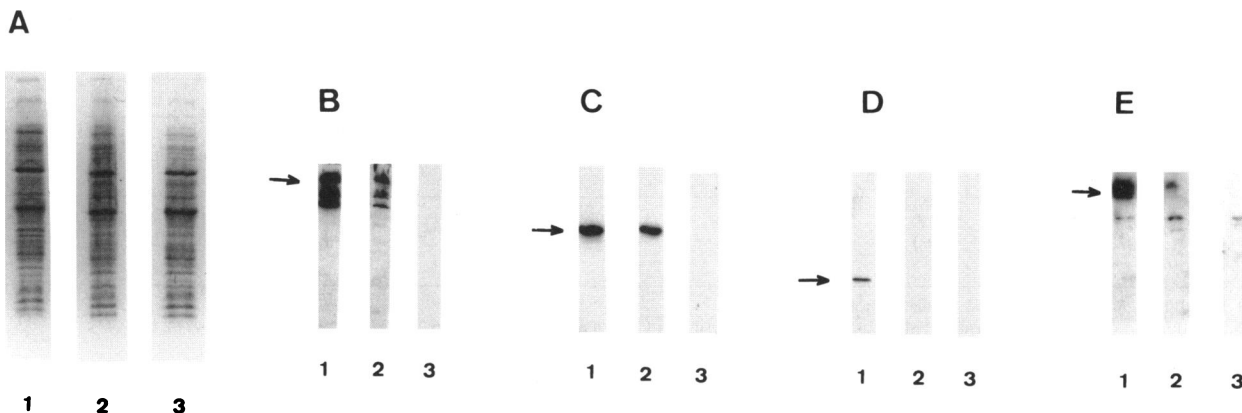
FIG. 1. Production of FHA, HLY-CYA, PTX, and PRN by *B. pertussis* Tohama I (lanes 1), BC75 (lanes 2), and BP359 (lanes 3). Bacterial suspensions were loaded onto 8 to 25% polyacrylamide gels (A). After electrophoresis, proteins were blotted and immunological detection was carried out with specific antisera against purified FHA (B), PRN (C), PTX (D), and HLY-CYA (E) as described in Materials and Methods.

Invasion assay. The invasion assay was carried out essentially as described by Ewanowich et al. (13). Briefly, in tissue culture trays (12-well) about 10^5 HeLa cells per well were seeded and incubated for about 18 h at 37°C in Eagle's minimal salt medium (GIBCO) supplemented with 5% fetal calf serum (GIBCO) in an atmosphere containing 5% CO_2. The *B. pertussis* strains were grown for 2 days on BG plates and resuspended in Eagle's medium. The bacteria were added at a multiplicity of infection of 100 to the HeLa cells and incubated for 5 h. Then, the monolayers were washed intensively, and gentamicin (GIBCO) was added to a final concentration of 100 µg/ml. After 2 h of incubation, required for the killing of the extracellular bacteria, the monolayers were washed several times and the cells were lysed in distilled water. Under these conditions, extracellular bacteria were eliminated to 99.9% (data not shown). Then, the surviving intracellular bacteria were counted on BG plates. Four independent assays were carried out for the comparative quantitation of the experiment.

Intranasal infection of mice. The *B. pertussis* strains were grown on BG plates. Bacteria were then resuspended and diluted in 1% Casamino Acids and then serially diluted to provide challenge inoculum dilutions for the evaluation of the 50% lethal dose. For the respiratory infection, 50 µl of bacterial suspension was injected intranasally into groups of 10 3- to 4-week-old female Swiss mice (CERJ, St. Berthevin, France). The 50% lethal doses for the challenge inocula were determined over a time course of 30 days by recording daily the number of surviving mice.

Sublethal challenges were performed by intranasal injection as described above. Infected mice were sacrificed by cervical dislocation 1 h after exposure (at time designated day 0) and at various days thereafter (three mice per time point). The lungs were removed and homogenized in saline with tissue grinders. Dilutions of lung homogenates were sampled on BG plates, and CFU were counted after 3 to 4 days of incubation at 36°C.

Mice were bled at days 35 and 95 after sublethal infection in order to assess the presence of circulating antibodies.

RESULTS

Properties of the BC75 mutant. BC75 is a derivative of *B. pertussis* Tohama I, obtained after mutagenesis with Tn5tac1 (8). It was chosen for this analysis because of its interesting phenotype. The mutant is not hemolytic on blood agar plates, and several changes in its growth properties and in the protein

pattern of whole-cell lysates compared with those of the wild-type strain demonstrate a pleiotropic effect of the mutation (16).

The expression of virulence-related factors in this strain was investigated by immunoblots with specific antibodies against the various factors (Fig. 1 and 2). In suspensions of Tohama I, the antibodies raised against purified FHA detected the 220-kDa FHA protein and its major degradation products, whereas the anti-PRN antibodies reacted with a single protein corresponding to the 69-kDa PRN. The anti-HLY-CYA antibodies detected the 200-kDa HLY-CYA protein and a smaller polypeptide which is unrelated to HLY-CYA since it is also present in a mutant carrying a deletion of the *cyaA* structural gene (data not shown). The monoclonal antibodies directed against the PTX S1 subunit and FIM2 detected the 28-kDa S1 protein and the 23-kDa major subunit of FIM2, respectively. Suspensions of the BC75 strain only showed trace amounts of HLY-CYA, which is in agreement with its nonhemolytic phenotype. In contrast, nearly normal amounts of FHA, PRN, and the serotype 2 fimbrial proteins were detected (Fig. 1 and 2). Surprisingly, no PTX could be found in the immunoblots. None of these factors noted above could be detected in the *bvg* mutant strain BP359.

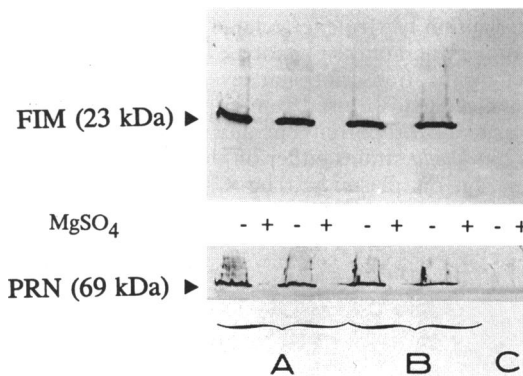To confirm the results of these immunoblots, we performed

FIG. 2. Immunological detection of PRN and FIM in suspensions of various *B. pertussis* strains grown in the absence (−) or the presence (+) of $MgSO_4$ with specific antisera. A, Tohama I strain; B, BC75 strain; C, BP347 strain.

TABLE 2. Some properties of *B. pertussis* Tohama I, BC75, BP359, and BP347

Bordetella strain	HLY-CYA			PTX		FHA		LD_{50} in mice[c]
	Hemolysis[a]	Immunoblot[a]	Enzymatic activity (U)	Immunoblot[a]	CHO assay[b]	Immunoblot[a]	Hemagglutination[b]	
Tohama I	+++	+++	30	+++	1:10,000	+++	1:256	8×10^7
BC75	−	(+)	3–5	−	1:250	+++	1:128	$>10^9$
BP347-BP359	−	−	0	−	1:10	−	1:1	$>10^9$

[a] +++, strong signal; (+), very weak signal; −, not detectable.
[b] Maximal dilution of bacterial supernatant still able to cause toxicity in the CHO assay or hemagglutination with erythrocytes.
[c] Bacterial challenge (in CFU) sufficient to cause 50% lethality in mice.

assays for biochemical or biological activities of some of these proteins (Table 2). In agreement with the results of the immunoblots, the assay for HLY-CYA activity in the BC75 supernatant revealed activity 10 times lower than that in the supernatant of the wild type. Similarly, the very sensitive CHO assay for PTX showed at least a 40-fold reduced toxic activity in BC75 supernatants (Table 2). In agreement with the presence of FHA on the immunoblots, the hemagglutinating activity of BC75 supernatants with erythrocytes was only slightly reduced compared with that of the wild-type supernatants. However, these factors could not be detected by immunoblotting or the other assays in the *bvg* mutant strain BP347 or BP359. Therefore, the BC75 mutant has a very particular phenotype which is strongly impaired in the expression of the two toxins PTX and HLY-CYA, but not in that of such factors as FHA, PRN, and FIM, which are produced in about normal amounts.

To analyze whether the BvgA/S system is functional in BC75, we investigated whether this strain is still able to modulate its virulence properties. The immunoblot in Fig. 2 shows that PRN and FIM are still subject to the phenotypic modulation by $MgSO_4$. Also, the expression of FHA is abolished under modulative conditions (data not shown). This demonstrates that the environmental regulation mediated by the Bvg system is still functional in BC75. Indeed, immunoblots with specific antisera raised against BvgA and BvgS demonstrated that both proteins are produced by BC75, although in a slightly reduced amount compared with that produced by the wild-type strain (Fig. 3). In contrast, the *bvgS* mutant BP347 did not show any detectable amount of BvgS, but it still produced some BvgA protein, presumably because of a weak constitutive promoter in front of the *bvgA* gene (52).

Transcription of virulence-related genes in BC75. In order to determine whether the phenotype of the mutant is caused by a defect at the transcriptional level, we performed several experiments. First, we used transcriptional fusions of the *ptx* promoter to a *cat* reporter gene and introduced them into the various *Bordetella* strains either on the chromosome via allelic exchange or on plasmids. The CAT activities were then

measured under nonmodulating and modulating growth conditions (Fig. 4). The plasmid-borne *ptx* fusion on the construct pBP2 present in the wild-type strain showed the highest level of CAT activity, about three times higher than that of the equivalent fusion on the chromosome (TI ptx::cat). This corresponds well with the copy number of the vector pLAFR2, which was estimated to be about two to three copies per cell (15). The plasmid-borne *ptx* fusion expressed in BC75 (BC75 pBP2) had approximately a 10-fold reduction in activity compared with that of the equivalent fusion in the wild-type strain (TI pBP2). This was, however, a level of activity four times higher than that of the same fusion present in the *bvg* mutant BP347. Interestingly, the activity of the plasmid-borne fusion in BC75 was still subject to modulation. Background activity was defined by the construct pBP33, in which the *ptx* promoter is inactivated because of a deletion in its upstream activating sequence (23), and by the activity of the fusions in the *bvg* mutant BP347 (Fig. 4).

To confirm the CAT fusion results and to extend this analysis to other factors, we extracted total RNA from the various strains and performed an RNA slot blot analysis with specific DNA probes. As shown in Fig. 5, this analysis confirmed that the gene for *fha* is transcribed at wild-type levels in BC75, whereas transcription of *fha* in the *bvg* mutant BP347 is severely reduced. The transcription of the *ptx* and *cya* operons was strongly reduced in BC75 compared with that in Tohama I (6- to 10-fold as determined by densitometric scanning) but was still significantly higher than in the *bvg* mutant. The detection of *recA* message, which is not affected in these mutants, was used to standardize RNA between the samples. Therefore, we can conclude that the impairment of toxin expression in BC75 is due to a defect at the transcriptional level.

Localization of the mutation in BC75. BC75 was a strain obtained after a transposon mutagenesis, and we naturally assumed that the insertion of the transposon had caused the mutant phenotype. The locus containing the Tn5tac1 insertion was cloned on a 14-kb SalI fragment, and the DNA flanking the transposon insertion site was sequenced. This analysis showed that the transposon was located in an unknown gene locus with no similarity to known *Bordetella* genes (9). Consequently, it was hypothesized that a yet-unknown gene involved in the expression of CYA and PTX had been inactivated by the transposon.

To prove this hypothesis, we introduced the cloned locus containing the transposon into the corresponding region of the wild-type *Bordetella* strain via allelic exchange. Surprisingly, no detectable change in the phenotype of the resulting strain TI-Tn5BC75 was observed. To confirm this finding, we eliminated the transposon from the chromosome of BC75 by
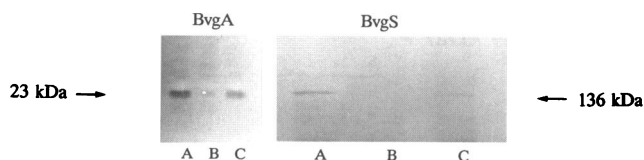

FIG. 3. Immunological detection of BvgA and BvgS proteins in *B. pertussis* Tohama I (lanes A), BP347 (lanes B), and BC75 (lanes C) with specific polyclonal antisera.
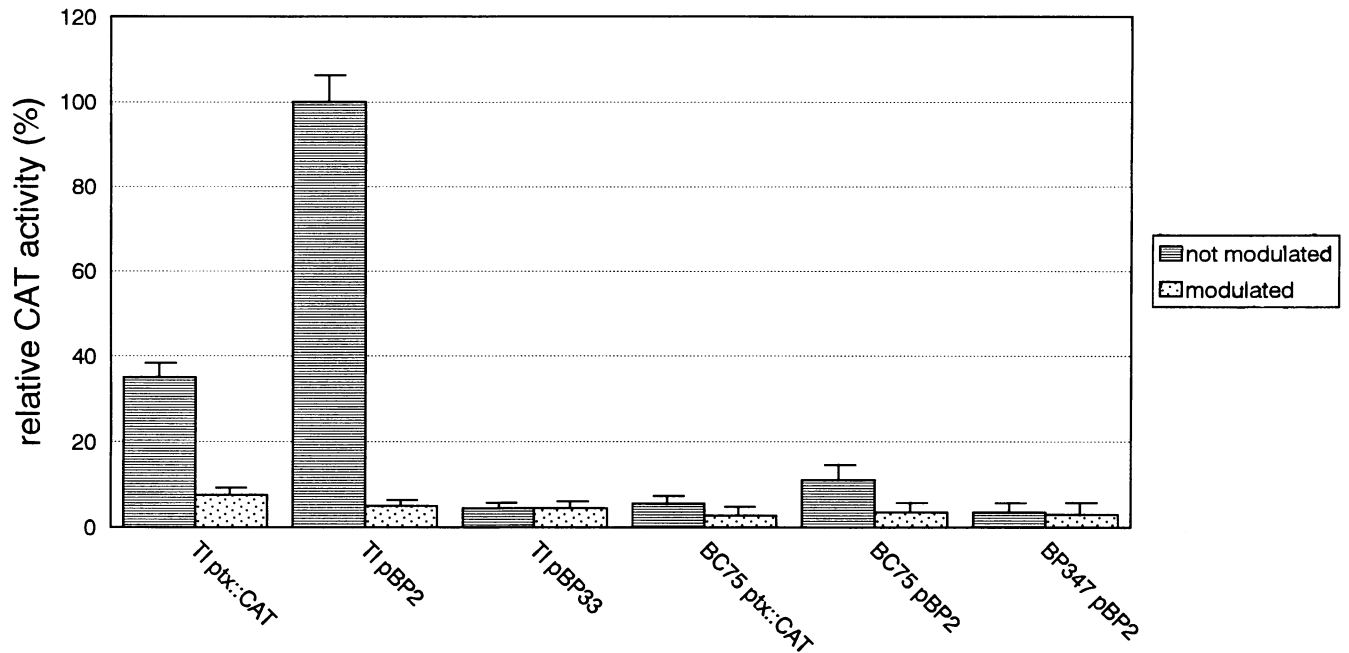
FIG. 4. Analysis of transcription from the *ptx* promoter in *B. pertussis* Tohama I (TI), BC75, and BP347 in the absence (not modulated) or the presence of MgSO₄ (modulated). The activity of pBP2 in strain Tohama I was taken as 100%, and the activities of the other strains were calculated relative to it. The vertical bars on the top of the columns represent the standard deviations.

substitution with the corresponding wild-type sequence. For this purpose, we screened a chromosomal library of Tohama I in pBluescript with the labeled flanking region of the cloned Tn5tac1 insertion from BC75. After cloning in the vector pSS1129, this wild-type sequence was then used to eliminate the Tn5 insertion in BC75 via allelic exchange. However, the phenotype of the resulting strain, BC7500, did not show differences in the expression of the toxins compared with BC75. In conclusion, these results demonstrated that the phenotype of BC75 has not been caused by the insertion of the transposon, but by a spontaneous mutation present in BC75 in addition to the mutation caused by Tn5tac1.

To analyze whether the relevant mutation in BC75 is linked to known virulence genes, the following experiments were performed. So far, all mutations affecting the expression of more than one virulence factor in *Bordetella* species in *trans* were mapped to the *bvg* locus. In *B. pertussis*, all known *bvg* mutations could be complemented in *trans* by the *bvg* locus.
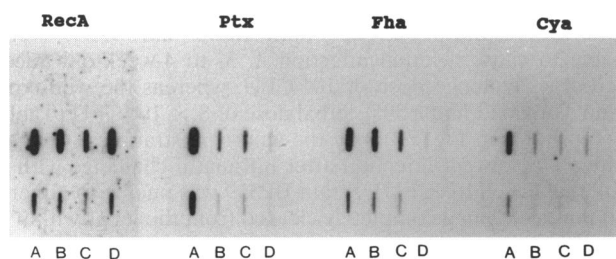

FIG. 5. RNA slot blot analysis of *B. pertussis* Tohama I (lanes A), BC75 (two different samples) (lanes B and C), and BP347 (lanes D) with specific probes for the *recA*, *fha*, *ptx*, and *cya* messages. The top spot corresponds to 5 μg of RNA; the bottom spot corresponds to 2 μg of RNA.

However, the transfer of the plasmid pLA-5,7-vir containing the *bvg* locus into BC75 did not result in complementation of the mutant phenotype, whereas the *bvg* mutant BP347 was complemented to the wild-type phenotype by this plasmid. Some spontaneous phase variants of *Bordetella bronchiseptica* carrying mutations in the *bvg* locus were described. These phase variants could not be complemented in *trans* by the *bvg* genes present on the vector pLAFR2, possibly because of a gene dosage effect (3). In these cases, complementation could only be obtained after exchanging parts of the mutant *bvg* locus with the wild-type locus because of homologous recombination. To exclude a similar situation in the case of BC75, we first deleted the *bvg* locus on the BC7500 chromosome and replaced it with a kanamycin resistance gene by allelic replacement with the plasmid pSS1212 (57). Then, we introduced the wild-type *bvg* locus on the chromosome by allelic exchange with the plasmid pNMD50 (Table 1). Introduction of the wild-type *bvg* genes did not restore the wild-type phenotype in BC75. These experiments clearly demonstrate that the mutation in BC75 is not linked to the *bvg* locus.

To exclude the remote possibility that the *ptx* and *cya* operons themselves carry mutations leading to the phenotype, we tried to complement BC75 in *trans* with cosmids containing the PTX structural gene and the recently described transport genes (pLAFR2-2) (10, 61) and the entire HLY-CYA locus (pLA-HLYS1) (16). However, the introduction of these constructs into BC75 did not restore the wild-type phenotype. In addition, we deleted either the *ptx* genes, including their promoter region, or a portion of the *cya* genes containing the *cya* promoter region in BC7500, replacing them with a kanamycin resistance gene by allelic exchange, and then introduced the respective wild-type operons cloned from Tohama I. The phenotype of these strains showed no difference from that of BC75 (data not shown). Finally, we cloned the *ptx* operon of BC75 on an *Eco*RI fragment containing the PTX structural
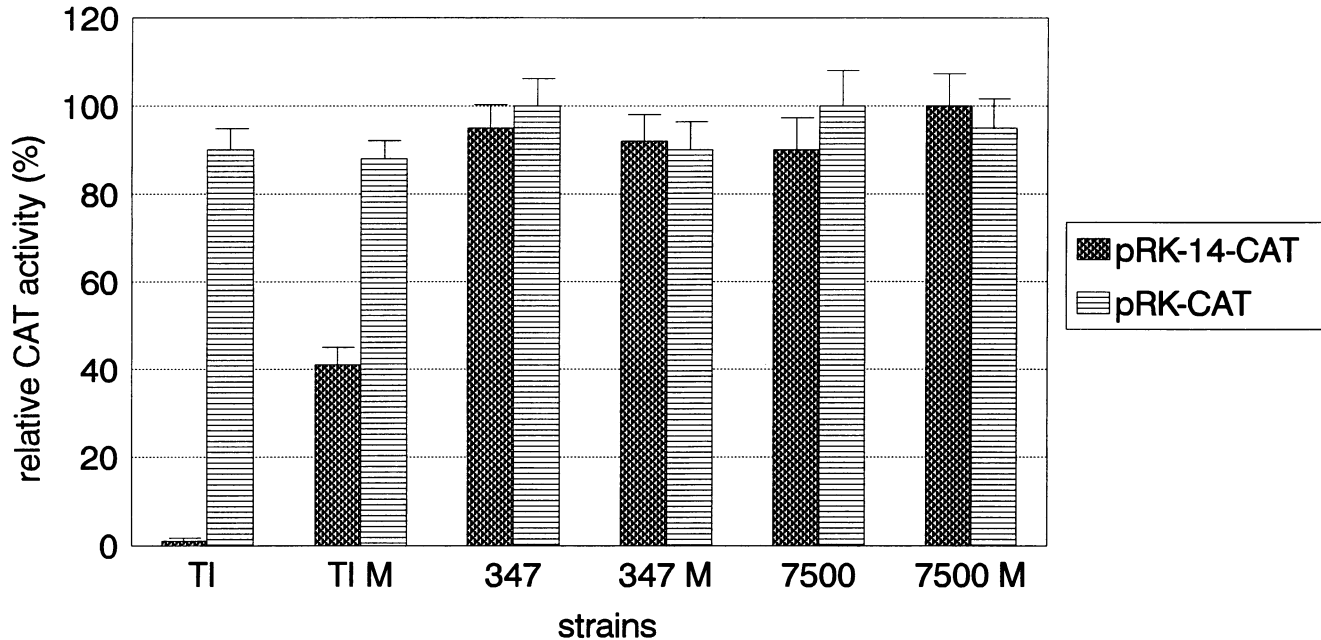
FIG. 6. Transcription interference assay with *B. pertussis* Tohama I (TI), BP347 (347), and BC7500 (7500) in the absence or presence of $MgSO_4$ (M). The CAT activity of BC7500 (M) pRK-14-CAT was taken as 100%, and the other activities were calculated relative to it. The vertical bars on the top of the columns represent the standard deviations.

genes and the promoter region and introduced this operon by allelic exchange into Tohama I containing a deletion of the entire *ptx* operon. The resulting strain, TI-ptx75, produced normal amounts of PTX as detected in immunoblots.

In conclusion, these experiments demonstrated that the lack of expression of the toxins in BC75 is due neither to mutations in the *bvg* regulatory system nor to mutations in the toxin operons themselves. Therefore, the mutation defines an unknown locus involved in transcription of the toxin genes.

Transcription interference assay. In an attempt to detect regulatory proteins binding to the *ptx* promoter in vivo, a transcription interference assay was performed as described earlier (21). The construct pRK-14-CAT, containing the *ptx* promoter region from positions -62 to -181 inserted between the constitutive *lac* promoter and the promoterless *cat* reporter gene on the replicating pRK415 vector, and the control construct pRK-CAT with no such intervening sequence were introduced into the various *Bordetella* strains. With these constructs, the regulatory region of the *ptx* promoter was recently shown to impair transcription from the *lac* promoter in *B. bronchiseptica* under nonmodulative conditions, providing indirect evidence for the binding of regulatory protein(s) to this promoter region (21).

The two constructs were introduced by conjugation into the various *B. pertussis* strains, and the CAT activities were measured from bacteria grown under modulating or nonmodulating growth conditions. As shown in Fig. 6, strong CAT activity is observed in the *bvg* mutant strain BP347. In contrast, a very low level of activity is detected in the wild-type strain Tohama I under nonmodulating conditions. A strong effect of modulation was observed only in the wild-type strain in which the CAT activity increased to about 40% of the activity of BP347. This result is in agreement with the recently published experiment carried out with *B. bronchiseptica* (21). The *bvg* mutant BP347 showed no significant variations under the two different growth conditions. Interestingly, strain BC7500 behaved essentially

like the *bvg* mutant (about 100% CAT activity). Therefore, it appears that a factor binding to this part of the *ptx* promoter region in Tohama I is not able to bind or is missing in BC7500 and BP347, which may account for the defect in transcription from the *ptx* promoter.

Invasion by BC75 of HeLa cells. The BC75 mutant is strongly impaired in the expression of the two toxins PTX and HLY-CYA, whereas the other known virulence factors are produced in nearly normal amounts. Therefore, we analyzed some of its virulence properties in vivo with cell culture and murine respiratory systems. Recent evidence suggests that *Bordetella* species are able to invade eukaryotic cells, although with a relatively low efficiency compared with other facultative intracellular bacteria (13). The in vivo significance of this phenomenon and the role of the various virulence-related factors in this process are still unclear. Therefore, we analyzed the invasive properties of BC75. Invasion assays with HeLa cells showed no significant difference in the invasive properties of BC75 compared with the wild-type strain Tohama I, whereas the avirulent *bvg* mutant strain BP359 showed about 50-fold less invasiveness compared with Tohama I (Fig. 7A).

Characterization of BC75 in a murine respiratory model. In a murine respiratory model, both BC75 and BP359 were unable to cause a lethal infection in 3- to 4-week-old mice, even at a challenge dose of 10^9 CFU, whereas the wild-type strain Tohama I had a 50% lethal dose of 8×10^7 CFU (Table 2). As shown in Fig. 7B, only the wild-type strain was able to induce a persistent infection after intranasal challenge with a sublethal dose (10^5 CFU). Strain BP359 was unable to adhere and multiply, being completely cleared from the lungs within 3 days. BC75 was strongly impaired in the ability to multiply and colonize in the first 5 days of infection and showed a much higher clearance rate compared with the wild-type strain, but a much lower rate than BP359. Indeed, BC75 was recovered from the animals after 20 days postinfection.

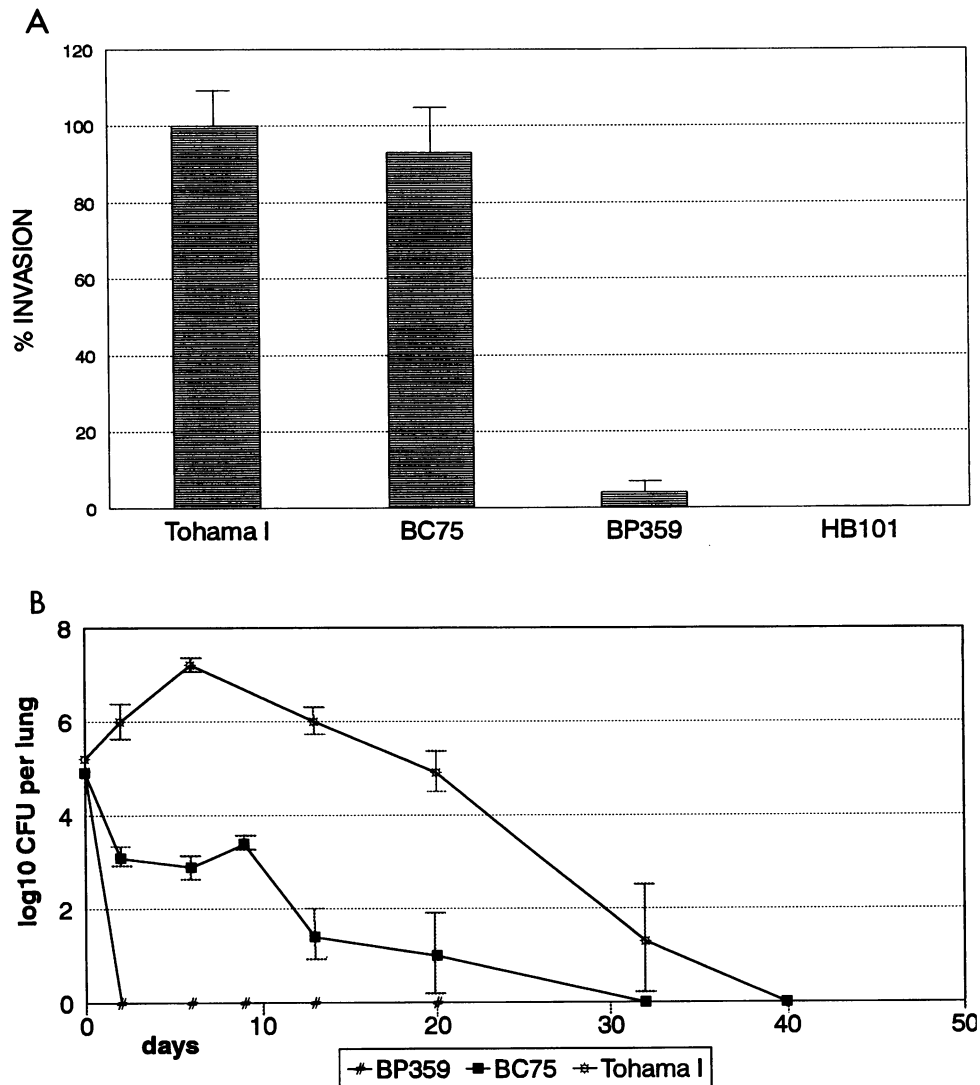Furthermore, we analyzed the specific antibody response

A

B

FIG. 7. (A) Invasion by *B. pertussis* Tohama I, BC75, and BP359 and *E. coli* HB101 of HeLa cells. The invasion rate observed with Tohama I was set at 100%. The vertical bars on the top of the columns represent the standard deviations. (B) Persistence of *B. pertussis* Tohama I, BC75, and BP359 in the lungs of sublethally infected mice. Mice (3 to 4 weeks old) were challenged intranasally with 10^5 CFU of Tohama I, BC75, and BP359. The plots show the geometric means ± standard deviations (bars) for three mice per time point.

after infection with the various live *B. pertussis* strains. As shown in Fig. 8, 35 days after intranasal infection of the mice with 5 × 10^5 CFU of the wild-type strain, antibodies against HLY-CYA, FHA, and PTX were detected, and they persisted for more than 3 months. Anti-PRN antibodies were also detected, but only about 150 days after infection (data not shown). In contrast, strain BP359 caused no antibody response, even after 3 months postinfection. This confirms the inability of the mutant to colonize the respiratory tract of the mice. In agreement with the in vitro phenotype of BC75, anti-FHA antibodies could be detected 35 days after infection. A very low level of antibodies recognizing HLY-CYA was detected only at 3 months postinfection with the BC75 mutant, but no anti-PTX antibodies were produced during the same time span.

DISCUSSION

The results in this paper describe the phenotype of a mutant strain of *B. pertussis*, BC75, and shed further light on the

mechanisms of regulation of expression of virulence factors in this organism.

Expression of PTX and HLY-CYA was shown to be impaired in this mutant because of a significant decrease in transcription of the *ptx* and *cya* genes, while expression of other virulence-related factors was apparently unaffected. Through various complementation experiments, we showed that the mutation causing the decrease in transcription of the toxin promoters is linked neither to the *bvg* locus nor to the known structural and/or transport genes of the toxins, but lies in a new genetic locus. Recently, this conclusion was confirmed by data from a new conjugative gene mapping system in *B. pertussis* (56). This technique, along with Southern hybridization analysis, was also used to map the location of the Tn5tac1 insertion in BC75, providing final confirmation that the mutation described in this paper and the transposon insertion are not linked (56).

However, the identity of the mutated factor in BC75 remains unknown. One possibility is that this factor is a DNA sequence-
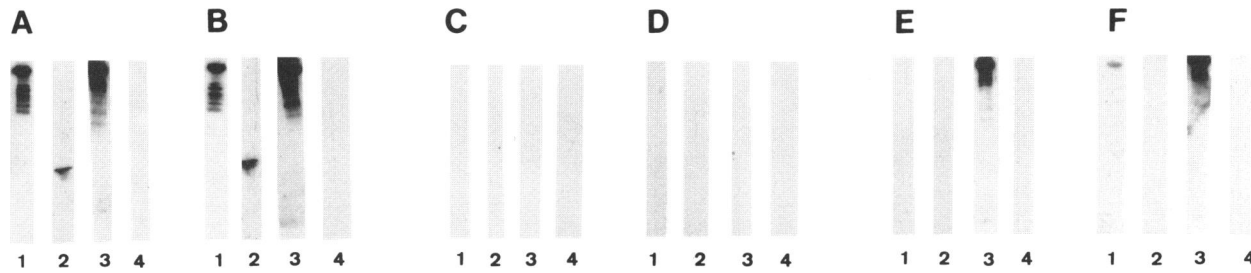
FIG. 8. Antibody response against FHA, PRN, HLY-CYA, and PTX of mice infected with *B. pertussis* Tohama I, BC75, and BP359. Groups of 10 3- to 4-week-old mice were infected intranasally with 5×10^5 CFU of *B. pertussis* Tohama I (A and B), BP359 (C and D), and BC75 (E and F). Mice were bled at day 35 (A, C, and E) or at day 95 (B, D, and F) postinfection, and antibodies against HLY-CYA (lanes 1), PTX (lanes 2), FHA (lanes 3), and PRN (lanes 4) were detected by Western blotting (immunoblotting).

specific binding protein which activates transcription at the toxin promoters by RNA polymerase. Indeed, the result of the transcription interference assay suggests that a factor binding to the *ptx* promoter region between −62 and −181 under permissive conditions in the wild-type strain is missing or defective in both BC75 and the *bvg* mutant BP347. A tandemly repeated sequence between positions −117 and −157 was previously shown to be important for activation of the *ptx* promoter, and this sequence may represent the binding site of such an activator (23, 27). The position of this binding sequence cannot be changed without causing a dramatic decrease in the promoter activity (21). A similar sequence is located at the same position in the *cya* promoter and seems to be involved in its activation (18, 27). It is likely that a protein(s) binding to these sequences is too far from the RNA polymerase binding site to allow direct interaction of the activator with the RNA polymerase. Therefore, an additional factor which can influence the local structure of the DNA to bring the two proteins into contact may be required for the activation of this promoter (1). It may therefore be that BC75 contains a mutation in such a DNA bending factor which also binds in the region of the promoter included in the transcription interference assay. These findings recall the so-called histone-like proteins found in several other organisms (26, 37). Mutations in such proteins generally have pleiotropic effects on the expression of many genes, including virulence genes in several pathogenic organisms, because of an influence on the DNA topology (12). Indeed, the different growth properties of BC75 and several changes in its protein pattern observed in whole-cell lysates compared with the wild-type strain demonstrate some pleiotropy in its phenotype (16). Furthermore, several *Bordetella* virulence promoters, including the *ptx* and *cya* promoters, were recently shown to be very sensitive to drugs which can influence the DNA topology (20). A low-stringency Southern hybridization experiment with the gene of the histone-like factor OsmZ from *E. coli* revealed the presence of related sequences in the chromosome of *B. pertussis*. The introduction of this *osmZ* gene into BC75 did not influence the phenotype of the mutant (16). However, it is possible that the *E. coli* protein, although expressed in *B. pertussis*, may not be functional in the heterologous organism.

It is unclear how the *bvg* system is involved in the activation of the toxin promoters in *B. pertussis*. Expression of the factor identified by the mutation in BC75 may be *bvg*-dependent, involving transcriptional activation of its gene by BvgA, for example. This would be analogous to the cascade regulation of virulence factors of *Vibrio cholerae*, in which the sensor-regulator molecule ToxR in an unknown fashion activates the ToxT protein, which in turn activates expression of genes

encoding virulence factors (11). Alternatively, BvgA or BvgS may be part of a complex of factors, including the factor mutated in BC75, which interact at the *ptx* and *cya* promoters to allow activation of transcription. In any case, the phenotype of this mutant is consistent with recent data suggesting that factors in addition to the Bvg proteins are necessary for expression of the toxin loci (19, 27, 48).

Cloning of the gene mutated in BC75 will be a major step towards our understanding of the molecular mechanisms underlying the activation of the toxin promoters. Several groups have already tried to clone an activator of these promoters by various approaches, but without success. For example, an attempt was made to clone this factor in *E. coli* by screening for the activation of *ptx::lacZ* (40), *cya::lacZ* (19), or *ptx::cat* fusions (7). In addition, we have tried to obtain Tn5-induced *B. pertussis* mutants with a phenotype similar to that of BC75, but among 40,000 Tn5 mutants screened, none could be found (5). These negative results, while difficult to interpret, suggest that this factor could be toxic or nonfunctional in *E. coli* or that insertional inactivation of this gene may be lethal for *B. pertussis*.

Since BC75 has an unusual phenotype with regard to expression of virulence factors, we performed several experiments to assay for virulence characteristics. The ability of BC75 to enter eukaryotic cells was analyzed in an invasion assay, in which extracellular bacteria were killed by gentamicin treatment. Using this technique, several groups have already investigated invasion by wild-type and mutant strains of *Bordetella* species lacking various virulence factors. These groups showed that wild-type strains of *B. pertussis* can enter eukaryotic cells, albeit with a relatively low efficiency, whereas *bvg* mutants expressing none of the *bvg*-activated factors have strongly reduced invasiveness (13, 32). However, attempts to identify single factors specifically relevant for invasion did not result in clear-cut data, and some contradictory observations concerning the possible role for FHA in invasion were made. Recently, Lee et al. (32) observed that a *bvg* mutant with a phenotype somewhat similar to that of BC75 (i.e., strongly reduced expression of the toxins yet expression of a low level of FHA and PRN) was as invasive as the wild type. We were able to confirm this finding with BC75, which is strongly impaired only in the expression of the toxins and produces nearly normal amounts of FHA and PRN. Therefore, we can conclude that the two toxins do not play a role in bacterial entry into HeLa cells. Experiments to evaluate a function in intracellular survival are under way.

With a murine respiratory model, it has been shown that HLY-CYA and PTX are the two factors of *B. pertussis* which mediate a lethal infection (25, 30). As expected, the mutants

BP359 and BC75, which express these toxins at negligible levels at best, were unable to induce lethality in the adult mouse model (Table 2). In addition, these mutants were impaired in their ability to adhere and multiply in the mouse respiratory tract, which is in agreement with previous data indicating that HLY-CYA is required for initial growth in vivo and PTX is required for colonization. On the other hand, BC75 was able to persist in the host for at least 20 days, whereas the mutant strain BP359 was cleared in 3 days. Furthermore, after infection of mice with BP359, no circulating antibody was detected, whereas both the wild-type and the BC75 strains elicited antibodies that could be detected 35 days after infection. This result confirms the in vivo persistence of BC75 compared with BP359. A role of FHA and PRN in adhesion is well documented in vitro (33, 46), but their in vivo role is still unclear. In fact, it has been shown recently that mutants unable to produce either PRN, FHA, or both factors (29, 47) were able to adhere, multiply, or colonize the lungs of mice as efficiently as the parental strains. Therefore, the behavior of the BC75 mutant, which does not produce a significant amount of PTX and HLY-CYA in vivo, suggests a role for other factors in addition to the toxins in the persistence of *B. pertussis* in the respiratory tract of mice. The in vivo importance of other factors such as PRN, FHA, FIM, or the recently described *vrg* gene products (4) may become apparent only in the absence of the two toxins in the mouse model.

In conclusion, we have identified a new genetic locus, which participates in an unknown fashion in the expression of a subset of virulence genes in *B. pertussis*. This result suggests that there are differences in the fine regulation of expression of the various virulence factors, which could be of relevance for in vivo growth and for the development of disease by this pathogen. It is therefore possible that this organism has developed differential regulation of expression of factors involved in adhesion and of factors with mainly toxic properties, which may be important in other phases of the course of infection. Future experiments involving the exchange of control mechanisms for the various factors may provide some insight into the relevance of this differential regulation for *Bordetella* species in vivo.

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