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Comparison of *bipA* Alleles within and across *Bordetella* Species

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The *Bordetella* BvgAS signal transduction system controls the expression of at least three phenotypic phases, the Bvg$^+$ or virulent phase, the Bvg$^-$ or avirulent phase, and the Bvg^i or Bvg intermediate phase, which has been hypothesized to be important for transmission. *bipA*, the first identified Bvg^i-phase gene, encodes a protein with similarity to the well-characterized bacterial adhesins intimin and invasin. Proteins encoded by the *bipA* genes present in *Bordetella pertussis* Tohama I and *Bordetella bronchiseptica* RB50 differ in the number of 90-amino-acid repeats which they possess and in the sequence of the C-terminal domain. To investigate the possibility that *bipA* alleles segregate according to host specificity and to gain insight into the role of BipA and the Bvg^i phase in the *Bordetella* infectious cycle, we compared *bipA* alleles across members of the *B. bronchiseptica* cluster, which includes both human-infective (*B. pertussis* and *B. parapertussis*$_{hu}$) and non-human-infective (*B. bronchiseptica* and *B. parapertussis*$_{ov}$) strains. *bipA* genes were present in most, but not all, strains. All *bipA* genes present in *B. bronchiseptica* strains were identical to *bipA* of RB50 (at least with regard to the DNA sequence of the 3′ C-terminal-domain-encoding region, the number of 90-amino-acid repeats encoded, and expression patterns). Although all *bipA* genes present in the other *Bordetella* strains were identical in the 3′ C-terminal-domain-encoding region to *bipA* of *B. pertussis* Tohama I, they varied in the number of 90-amino-acid repeats that they encoded and in expression level. Notably, the genes present in *B. parapertussis*$_{hu}$ strains were pseudogenes, and the genes present in *B. parapertussis*$_{ov}$ strains were expressed at significantly reduced levels compared with the levels in *B. pertussis* and *B. bronchiseptica* strains. Our results indicate that there is a correlation between specific *bipA* alleles and specific hosts. They also support the hypothesis that both horizontal gene transfer and fine-tuning of gene expression patterns contribute to the evolution of host adaptation in lineages of the *B. bronchiseptica* cluster.

Bordetellae are gram-negative coccobacilli that cause respiratory infections in mammals, birds, and reptiles. The classical bordetellae (19), *Bordetella pertussis*, *Bordetella parapertussis*, and *Bordetella bronchiseptica*, are so closely related that they are now considered subspecies or strains of a single species (5, 27, 37, 39, 40, 57). Because the progenitor organism of this group appears to have been *B. bronchiseptica*, with *B. pertussis* and *B. parapertussis* strains diverging at different times and from different branches of the phylogenetic tree, these bacteria have been referred to as the *B. bronchiseptica* cluster (19). The organisms identified as *B. bronchiseptica* have a broad host range, infecting nearly all four-legged mammals, and cause infections that range from asymptomatic colonization to tracheobronchitis and pneumonia (22). In contrast, *B. pertussis* strains uniformly cause the acute and severe childhood illness known as whooping cough or pertussis and are adapted exclusively to humans (8). *B. parapertussis* strains comprise two separate lineages. *B. parapertussis*$_{hu}$ strains have been isolated only from humans and cause a respiratory illness that is very similar to that caused by *B. pertussis* (24, 60), while *B. parapertussis*$_{ov}$ strains were isolated from the respiratory tracts of healthy sheep, as well as sheep with chronic nonprogressive pneumonia (13, 43).

Members of the *B. bronchiseptica* cluster express overlapping sets of highly related virulence factors. These include putative adhesins, such as filamentous hemagglutinin (45), fimbriae (Fim) (36), pertactin (Prn) (46), tracheal colonization factor (TcfA) (17), and BrkA (*Bordetella* resistance to killing) (16), and toxins, such as adenylate cyclase toxin (CyaA) (25), dermonecrotic toxin (Dnt) (33), tracheal cytotoxin (20, 21), pertussis toxin (Ptx) (38, 42), and proteins secreted via a type III secretion system encoded by the *bsc* locus (62, 63). With the exception of tracheal cytotoxin, all of these factors are positively regulated by BvgAS sensory transduction systems that are nearly identical and functionally interchangeable (34, 51, 56, 59); hence, expression of these molecules characterizes a phenotypic state designated the Bvg$^+$ phase. Experiments with phase-locked and ectopic expression mutants have shown that the Bvg$^+$ phase is necessary and sufficient for the development of respiratory infection (1, 11, 35).

Comparisons of Bvg$^+$-phase factors and their expression patterns across the *Bordetella* species have provided useful information for understanding phylogenetic and evolutionary relationships among these bacteria (5–7, 31, 48). Such information has also proven to be valuable for formulating hypotheses regarding the roles of the factors in the *Bordetella* infectious cycle. For example, since Ptx is expressed only by *B. pertussis* strains (5), it can be concluded that Ptx is not absolutely required for respiratory infection. However, since only *B. pertussis* strains induce leukocytosis (24, 58), it is likely that Ptx plays a significant role in causing this specific parameter of disease. Reciprocally, as type III secretion systems appear to be functional only in *B. bronchiseptica* and *B. parapertussis*$_{ov}$ strains, which commonly cause chronic subclinical infections, it has been hypothesized that type III secreted proteins may

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function to down-regulate the host immune response and thereby contribute to long-term persistence (62).

Comparative analyses have similarly provided insight into Bvg-repressed phenotypes and the role of the Bvg^- or avirulent phase. Bvg-repressed phenotypes in *B. bronchiseptica* include the expression of flagella, motility, chemotaxis, and the ability to grow under nutrient-limiting conditions, and it has been hypothesized that the role of the Bvg^- phase is to allow the bacteria to survive for extended periods of time in the environment while they are between mammalian hosts (1, 9, 10). *B. pertussis*, *B. parapertussis*$_{hu}$, and *B. parapertussis*$_{ov}$ strains, which do not express these phenotypes, are thought to be restricted to transmission via direct contact or aerosol droplets (9, 10). The Bvg-repressed genes expressed exclusively by *B. pertussis* include *vrg* and *vra* loci (28, 50). Although the functions of the products of these genes are unknown, determination of these functions should allow formulation of hypotheses regarding the role of the Bvg^- phase in this species.

A third phenotypic phase, induced by growth in the presence of semimodulating concentrations of nicotinic acid or $MgSO_4$ (chemicals that down-regulate BvgAS activity) or by a specific mutation in *bvgS*, has been described for *B. bronchiseptica* strain RB50 (12). This Bvg intermediate (Bvg^i) phase, characterized by expression of a subset of Bvg^+-phase factors, lack of expression of Bvg^--phase factors, and expression of phenotypes that are maximally if not exclusively expressed in this phase, is hypothesized to be important for aerosol transmission (12). The Bvg^i-specific phenotypes identified so far include autoaggregation (12) and expression of the recently identified outer membrane protein BipA (14, 54). The function of BipA is unknown; however, the predicted similarity of this protein to intimin of enteropathogenic and enterohemorrhagic *Escherichia coli* and invasin of *Yersinia* spp., including a similar C-terminal exposed topology, suggests that it may play a role in adherence (54). Initial studies revealed the presence of a BipA cross-reactive protein in whole-cell lysates of *B. pertussis* strain GMT1 (54), and DNA sequence data from the Sanger Centre's *B. pertussis* genome project (http://www.sanger.ac.uk/Projects/B_pertussis/) indicate that there is a *bipA* homolog in *B. pertussis* Tohama I. The majority of the predicted amino acid differences between the BipA proteins produced by *B. bronchiseptica* RB50 and *B. pertussis* Tohama I are located at the extreme C terminus. This observation suggests the intriguing possibility that if BipA functions in adherence like intimin and invasin, it could be involved in host specificity since the C-terminal domains of intimin and invasin are directly involved in binding to receptor proteins on host cells (26, 30, 32, 61) and variability in this region among intimin family members has been hypothesized to play a role in tissue tropism (18, 41, 44). The conservation of *bipA* and the Bvg^i phase in bordetellae is unknown. To study the role of BipA and the Bvg^i phase in the *Bordetella* infectious cycle, we conducted a comparative analysis of *bipA* homologs and their expression patterns within and across strains of the *B. bronchiseptica* cluster.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in Table 1. *Bordetella* strains were grown on Bordet-Gengou agar supplemented with 15% defibrinated sheep blood or in Stainer-Scholte (SS) broth (49). *E. coli* strains were grown in L broth or on L agar. When appropriate, media were supplemented with ampicillin (100 μg ml^{-1}), gentamicin (30 μg ml^{-1}), streptomycin (70 μg ml^{-1}), rifampin (20 μg ml^{-1}), kanamycin (50 μg ml^{-1}), or sucrose (10%). *B. parapertussis*$_{ov}$ strain Fr107i and *B. parapertussis*$_{hu}$ strain 12822i were constructed by transferring the *bvgS*-I1 mutation to the chromosomes of Fr107 and 12822, respectively, by allelic exchange with plasmid pEG129, as described previously (12).

Recombinant DNA techniques. Standard methods were used for preparation of plasmid DNA, restriction endonuclease digestion, agarose gel electrophoresis, DNA ligation, and other DNA manipulations (47). Nucleotide sequences were determined by Laragen, Inc. (Los Angeles, Calif.).

Plasmid rescue. The 5′ end of *bipA* from *B. parapertussis*$_{hu}$ strain 12822 was cloned by plasmid rescue. Briefly, a suicide plasmid derivative of plasmid pEG7 carrying a 621-bp PCR product corresponding to the C-terminal 307 amino acids of BipA from *B. parapertussis*$_{hu}$ strain 12822 was introduced into 12822 by conjugation, and cointegrates were selected on Bordet-Gengou–gentamicin agar. Chromosomal DNA was then prepared, digested with *Fse*I (which does not cut within the pEG7 derivative), self-ligated, and transformed into *E. coli* DH5α. Plasmid DNA recovered from the transformants carried chromosomal DNA from the *bipA* locus flanking the 621-bp region on the plasmid that provided homology for recombination. This DNA was characterized by restriction endonuclease digestion and nucleotide sequence analysis.

β-Galactosidase assays. To measure *bipA* expression, plasmid pTEN34, which contains a 321-bp fragment of *bipA* corresponding to nucleotides encoding amino acids 70 to 177 of BipA fused to the promoterless *lacZ* gene on plasmid pEGZ (34), was constructed. Integration of this suicide plasmid into the chromosomes of the various strains at the *bipA* locus resulted in *bipA-lacZ* fusions. PCR was used to confirm that plasmids had integrated at the *bipA* locus as intended. β-Galactosidase assays were performed as previously described (34).

PCR and Southern blotting. Primers used for PCR and Southern blotting are listed in Table 2. PCR was performed by using total cellular DNA templates. The cycling parameters were 94°C for 4 min and 30 cycles of 94°C for 30 s, 55 or 60°C for 45 s, and 72°C for 1 min, with a final extension step of 72°C for 5 min. Southern blotting was performed as previously described (2).

Western immunoblotting. Immunoblotting was performed as previously described (11, 12). Polyclonal anti-BipA antibody was used at a 1:5,000 dilution, and horseradish peroxidase-conjugated anti-rabbit immunoglobulin (secondary antibody) was used at a dilution of 1:5,000. Antigen-antibody complexes were detected by enhanced chemiluminescence (Amersham).

RESULTS

Prevalence of *bipA* homologs among *Bordetella* strains that cause respiratory infections in mammals. We used PCR to investigate the prevalence of the *bipA* gene within and across the *Bordetella* species that cause respiratory infections in mammals. The collection of strains tested (Table 1) included members of all of the main subdivisions of the *B. pertussis*, *B. bronchiseptica*, *B. parapertussis*$_{hu}$, and *B. parapertussis*$_{ov}$ species as determined by van der Zee et al. (57) except the *B. bronchiseptica* lineage represented by ET21, which was unavailable. A few recent clinical isolates of *B. pertussis* (strains CS, 6235, and 6068) and *B. bronchiseptica* (strains Cb2 and JC100) were also included. The primers used, QCPCRBipF and QCPCRBipR (Table 2 and primers 1 and 2 in Fig. 1), amplified a 727-bp fragment from *B. bronchiseptica* and *B. pertussis* genomic DNA corresponding to the region encoding amino acids 115 to 355 of BipA. Products that were approximately 727 bp long, and no other products, were amplified from all strains tested except *B. bronchiseptica* strains 548, 590, and 675 (data not shown), suggesting that most, but not all, bordetellae that infect mammals contain *bipA* homologs.

The number of 90-amino-acid repeats varies across, but not within (except for *B. pertussis* strain 18323), *Bordetella species*. The *bipA* gene of *B. pertussis* Tohama I is predicted to encode a 1,308-amino-acid protein containing five highly conserved 90-amino-acid repeats extending from amino acid 568 to amino acid 1017, while the *bipA* gene of *B. bronchiseptica*

TABLE 1. Strains used in this study

Species	Strain	Electrophoretic type	Host	Geographic origin	Reference or source
B. bronchiseptica	RB50		Rabbit	United States	11
	548	6	Pig	The Netherlands	57
	590	6	Dog	United States	57
	595	4	Dog	United States	57
	625	16	Rat	United States	57
	675	14	Human	Germany	57
	676	3	Pig	Australia	57
	704	1	Rabbit	United States	58
	Cb2		Dog	United States	This study
	JC100		Human	United States	This study
	RB53				11
	RB53i				11
B. pertussis	Tohama I	37	Human	Japan	27
	18323	38	Human	United States	ATCC 9797
	CS		Human	United States	This study
	6235		Human	United States	This study
	6068		Human	United States	This study
	GMT1		Human	United States	34
	GMT1i				12
B. parapertussis$_{hu}$	12822	28[a]	Human	Germany	24
	Ccug38844	28[a]	Human	Germany	24
	No7	28[a]	Human	France	24
	840994	28[a]	Human	Finland	24
	803	28[a]	Human	Italy	24
	H789	28[a]	Human	The Netherlands	24
B. parapertussis$_{ov}$	Fr107		Ovine	New Zealand	13
	J1		Ovine	Scotland	43
	C		Ovine	Scotland	43
	H1		Ovine	Scotland	43
	Fr107i				This study
E. coli	DH5α(pEG129)				12
	DH5α(pTEN34)				This study

[a] The electrophoretic type was not determined, but it was almost definitely electrophoretic type 28 as all *B. parapertussis*$_{hu}$ strains tested by van der Zee et al. were electrophoretic type 28 strains.

RB50 is predicted to encode a 1,578-amino-acid protein containing eight of these highly conserved 90-amino-acid repeats (54) (Fig. 1). To estimate the number of 90-amino-acid repeat domains encoded by the *bipA* genes in the various strains included in this study, we performed Southern blot analyses. Genomic DNA digested with *Bsi*HKI was probed with an 870-bp *Kpn*I-*Bam*HI fragment corresponding to the region encoding amino acids 1018 to 1310 of BipA of *B. bronchiseptica* (Fig. 1 and 2A). This probe hybridized to ~2.75-kb fragments in all *B. bronchiseptica* strains tested except strains 548, 590, and 675. These data confirm the PCR results, indicating that all *B. bronchiseptica* strains tested except 548, 590, and 675 contain *bipA* homologs. They further suggest that all *B. bronchiseptica* strains containing *bipA* genes have the potential to encode BipA proteins containing eight 90-amino-acid repeats. With the exception of strain 18323, the probe hybridized to a ~1.95-kb fragment in all *B. pertussis* strains tested (Fig. 2A and data not shown), suggesting that the *bipA* genes in these strains encode proteins containing five 90-amino-acid repeats. The probe hybridized to a ~870-bp fragment in strain 18323, suggesting that this strain encodes a protein containing only one of the 90-amino-acid domains. The probe also recognized a ~870-bp fragment in all *B. parapertussis*$_{hu}$ strains, suggesting that these strains also encode only one 90-amino-acid domain. A ~2.2-kb fragment was recognized by the probe in all *B. parapertussis*$_{ov}$ strains tested, suggesting that these strains encode proteins containing six 90-amino-acid repeats. These results were confirmed by PCR by using primers bBipRepF1 and bBipRepR1 (primers 3 and 4 in Fig. 1), which anneal to regions flanking the DNA region encoding the 90-amino-acid repeat (Fig. 2B). These primers amplified 2.2-kb fragments in

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	DNA sequence
QCPCRbipF	5′AATTGCTTGGACAGCGGTTC3′
QCPCRbipR	5′GCGACTATCTCAAGCGTGAA3′
bBipRepF1	5′GTCGCGGATTATCGCCG3′
bBipRepR1	5′GGCACGGCGGATCCGGACAAC3′
BipCtFwd2	5′CACGGCGGATCCGGACAACGAT3′
pBipCtRvs3	5′CGCCTGCTCGCCAGACAGTG3′
BipCtFwd3	5′CGGACAACGATGTGACGGT3′
pBipCtRvs1	5′GTCTTACGGCGCTTAGTA3′
KB1For	5′CGCGGTACCCCGGCCTGTTCGACGTGCCG3′
LMBipNtc	5′GGGGATCCGGTTCCGAGAGATTGATGTTCAGG3′
LM7BipNt	5′CCTATGCTGCATGTATTGGGTTCC3′

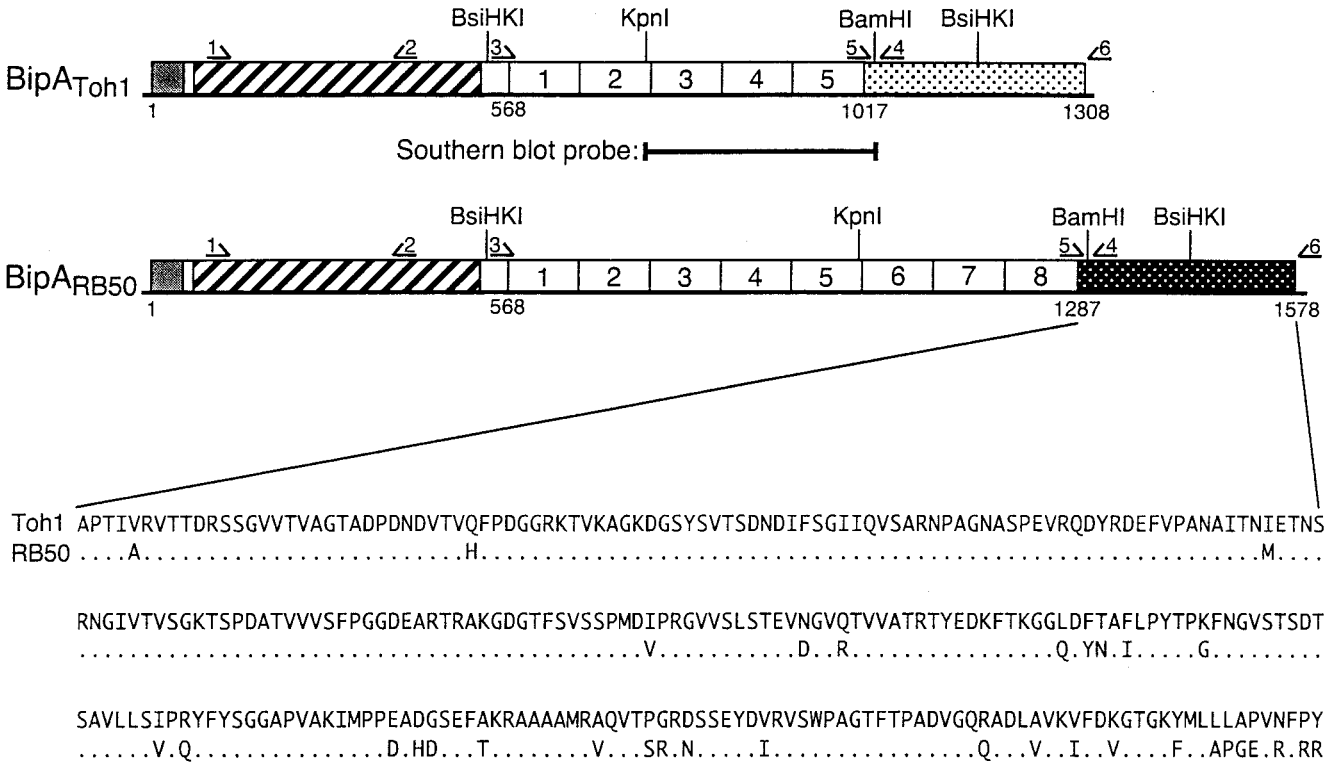

FIG. 1. Schematic diagram of the *bipA* loci of *B. bronchiseptica* RB50 and *B. pertussis* Tohama I. The open reading frames of *B. bronchiseptica* RB50 and *B. pertussis* Tohama I code for N-terminal signal sequences (shaded boxes), followed by ~410-amino-acid regions with similarity to intimin and invasin (cross-hatched boxes) and then by five (*B. pertussis*) or eight (*B. bronchiseptica*) 90-amino-acid repeated domains (indicated by the numbers 1 to 5 and 1 to 8, respectively) and finally by 291-amino-acid C-terminal domains. The amino acid sequences of the C-terminal domains, as predicted from their nucleotide sequences, are shown. Restriction endonuclease sites and the locations of primers (arrows 1 to 6) used in this study are indicated. The fragment used as a probe for Southern blotting is also indicated.

B. bronchiseptica strains, 1.35-kb fragments in *B. pertussis* strains, 1.6-kb fragments in *B. parapertussis*$_{ov}$ strains, and 270-bp fragments in *B. parapertussis*$_{hu}$ strains and *B. pertussis* 18323.

The nucleotide sequences encoding the BipA C-terminal domains of all *B. bronchiseptica* strains are identical to those of RB50, and the nucleotide sequences encoding the BipA C-terminal domains of all other strains are identical to those of Tohama I. Nearly all (34 of 39) of the predicted amino acid differences encoded by the *bipA* genes of *B. pertussis* Tohama I and *B. bronchiseptica* RB50 (exclusive of the additional 90-amino-acid repeats in BipA of RB50) are located in the C-terminal domain, and most are located at the extreme C terminus (Fig. 1) (54). To investigate the variability in this region within and across the *Bordetella* species, we used primers BipCtFwd2 and pBipCtRvs3 (primers 5 and 6 in Fig. 1) to PCR amplify 875-bp DNA fragments corresponding to the C-terminal domains of *bipA* and had their nucleotide sequences determined. The nucleotide sequences of these fragments from all *B. bronchiseptica* strains listed in Table 1 were identical to the nucleotide sequence of RB50. The nucleotide sequences of all other *Bordetella* strains listed in Table 1 were identical to the nucleotide sequence of Tohama I. These data suggest that all *B. bronchiseptica* strains that contain *bipA* encode BipA proteins with the same amino acid sequence in their C-terminal domains and that all non-*B. bronchiseptica* strains encode BipA proteins with C-terminal domains identical to the C-terminal domain of *B. pertussis* Tohama I BipA.

BipA protein can be detected in *B. bronchiseptica* and *B. pertussis* strains but not in *B. parapertussis*$_{hu}$ or *B. parapertussis*$_{ov}$ strains. To investigate BipA protein expression in the various strains, we performed Western blot analyses with whole-cell lysates prepared from cultures grown under Bvg$^+$-, Bvg^i-, and Bvg$^-$-phase conditions (i.e., in medium containing various concentrations of nicotinic acid or $MgSO_4$ or both). Polyclonal antibodies raised against the C terminus of BipA (54) recognized polypeptides of the expected sizes in lysates of all *B. bronchiseptica* and *B. pertussis* strains tested (except *B. bronchiseptica* strains 548, 590, and 675) but failed to recognize polypeptides in lysates made from *B. parapertussis*$_{hu}$ or *B. parapertussis*$_{ov}$ strains (data not shown). Because the conditions under which the Bvg^i phase is expressed can vary within and across species (34), we constructed *B. parapertussis*$_{hu}$ and *B. parapertussis*$_{ov}$ strains containing the *bvgS*-I1 mutation (12). In both *B. bronchiseptica* RB50 and *B. pertussis* GMT1, this mutation causes the bacteria to express Bvg^i-phase phenotypes when they are grown under Bvg$^+$-phase conditions (12, 54). Western blot analysis of whole-cell lysates of these strains confirmed that the BipA protein could be detected in *B. pertussis* and *B. bronchiseptica* but not in *B. parapertussis*$_{hu}$ or *B. parapertussis*$_{ov}$ (Fig. 3). Our inability to detect BipA in lysates

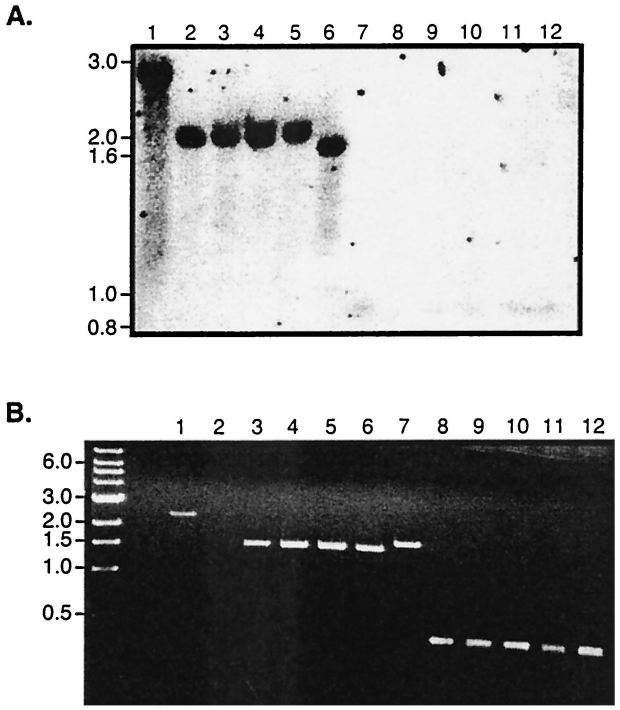

FIG. 2. Comparison of the 90-amino-acid repeat domain-containing regions of *bipA* within and across *Bordetella* species. (A) Southern blot. *Bsi*HKI-digested genomic DNA was probed with the 870-bp *Kpn*I-*Bam*HI fragment shown in Fig. 1. Lane 1, *B. bronchiseptica* RB50; lane 2, *B. parapertussis*$_{ov}$ Fr107; lane 3, *B. parapertussis*$_{ov}$ JI; lane 4, *B. parapertussis*$_{ov}$ C; lane 5, *B. parapertussis*$_{ov}$ HI; lane 6, *B. pertussis* Tohama I; lane 7, *B. parapertussis*$_{hu}$ 12822; lane 8, *B. parapertussis*$_{hu}$ No7; lane 9, *B. parapertussis*$_{hu}$ 840994; lane 10, *B. parapertussis*$_{hu}$ 803; lane 11, *B. parapertussis*$_{hu}$ 789; lane 12, *B. pertussis* 18323. The positions of molecular weight markers are indicated on the left. (B) PCR. DNA fragments were amplified from genomic DNA by using primers bBipRepF1 and bBipRepR1 (primers 3 and 4 in Fig. 1). Lane 1, *B. bronchiseptica* strain RB50; lane 2, *B. bronchiseptica* strain 590; lanes 3 to 6, *B. pertussis* strains Tohama I, GMT1, 6068, and CS, respectively; lane 7, *B. parapertussis*$_{ov}$ Fr107; lane 8, *B. pertussis* strain 18323; lanes 9 to 12, *B. parapertussis*$_{hu}$ strains 12822, No7, 840994, and 803, respectively. The sizes of the 1-kb-marker fragments (in kilobase pairs) are indicated on the left.

of *B. parapertussis*$_{hu}$ strain 12822 contradicts our previous report (54). We repeated this experiment with all *B. parapertussis*$_{hu}$ isolates included in this study grown with various concentrations of the chemical modulators nicotinic acid and $MgSO_4$ and also with selected strains in which the *bvgS*-I1 mutation had been introduced, and we still failed to detect the BipA protein (data not shown). Together with the results shown below, these data indicate conclusively that *B. parapertussis*$_{hu}$ strains do not express the BipA protein, indicating that our previously reported result was in error.

The 5′ ends of the *bipA* loci of *B. parapertussis*$_{hu}$ and *B. parapertussis*$_{ov}$ strains differ from those of *B. pertussis* and *B. bronchiseptica*. Because we did not detect BipA expression in the *B. parapertussis*$_{hu}$ and *B. parapertussis*$_{ov}$ strains by Western blotting (Fig. 3) despite the presence of *bipA* hybridizing sequences in the genomes (Fig. 2), we investigated the 5′ ends of the *bipA* loci in these strains by PCR. Primers designed to amplify 1.36-kb fragments from the 5′ ends of the *bipA* loci of *B. bronchiseptica* and *B. pertussis* (KB1For and LMBipNtc [Table 2]) amplified fragments of the expected sizes from *B. bronchiseptica*, *B. pertussis*, and *B. parapertussis*$_{ov}$ strains but failed to amplify fragments from *B. parapertussis*$_{hu}$ strains (Fig. 4A). Other primers designed to amplify DNA fragments within the same region also failed to yield products from *B. parapertussis*$_{hu}$ strains (data not shown). These results suggested that the nucleotide sequence at the 5′ end of *bipA* in *B. parapertussis*$_{hu}$ strains differed from the nucleotide sequences of the other bordetellae. We therefore cloned the *bipA* 5′ region from the chromosome of *B. parapertussis*$_{hu}$ strain 12822 by plasmid rescue (see Materials and Methods) and determined the nucleotide sequence of the resulting clone. The sequence obtained matched exactly DNA sequence data newly released by the Sanger Centre (http://www.sanger.ac.uk/Projects/B_parapertussis/) and indicated that the homology between *bipA* of *B. parapertussis*$_{hu}$ strain 12822 and the *bipA* genes of *B. bronchiseptica* RB50 and *B. pertussis* Tohama I begins with nucleotides encoding amino acid 67 (a glycine residue) of the BipA protein. Strain 12822 sequences immediately 5′ to this position exhibited no similarity to the 5′ ends of the *bipA* loci of RB50 and Tohama I and lacked discernible translation initiation elements. Promoter elements and putative BvgA binding sites were also not recognizable. Also, 793 bp 5′ to the beginning of the truncated *′bipA* gene, an IS*1001* element was found with its TnpA-encoding gene oriented in the opposite direction relative to the orientation of *′bipA*. By searching the Sanger Centre's *Bordetella* genome databases, we found sequences identical to the 793-bp sequence that intervenes between IS*1001* and *′bipA* in 12822 in the RB50 genome (at a site unlinked to *bipA*) but not in the Tohama I genome. Sequences immediately 3′ to *′bipA* in 12822 matched the sequences 3′ to *bipA* in Tohama I (99.1% identity) and RB50 (94.9% identity) for 700 bp, at which point there was a 450-bp gap in the 12822 sequence. Beyond that gap, the 12822, RB50, and Tohama I sequences were nearly identical for 690 bp, and then the Tohama I se-

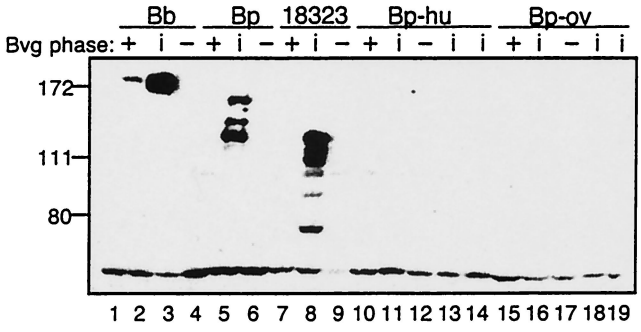

FIG. 3. BipA protein expression as determined by Western blotting. Whole-cell lysates of *B. bronchiseptica* RB50 (lanes 1 to 3), *B. pertussis* Tohama I (lanes 4 to 6), *B. pertussis* 18323 (18323) (lanes 7 to 9), *B. parapertussis*$_{hu}$ 12822 (lanes 10 to 12), *B. parapertussis*$_{hu}$ No7 (Bvg^i phase only) (lane 13), *B. parapertussis*$_{hu}$ 803 (Bvg^i phase only) (lane 14), *B. parapertussis*$_{ov}$ Fr107 (Fr107) (lanes 15 to 17), *B. parapertussis*$_{ov}$ JI (Bvg^i phase only) (lane 18), and *B. parapertussis*$_{ov}$ HI (Bvg^i phase only) (lane 19) were analyzed by Western blotting with anti-BipA (CT4) antibody. The positions of molecular size markers (in kilodaltons) are indicated on the left. Bb, *B. bronchiseptica* RB50; Bp, *B. pertussis* Tohama I; Bp-hu, *B. parapertussis*$_{hu}$; Bp-ov, *B. parapertussis*$_{ov}$.

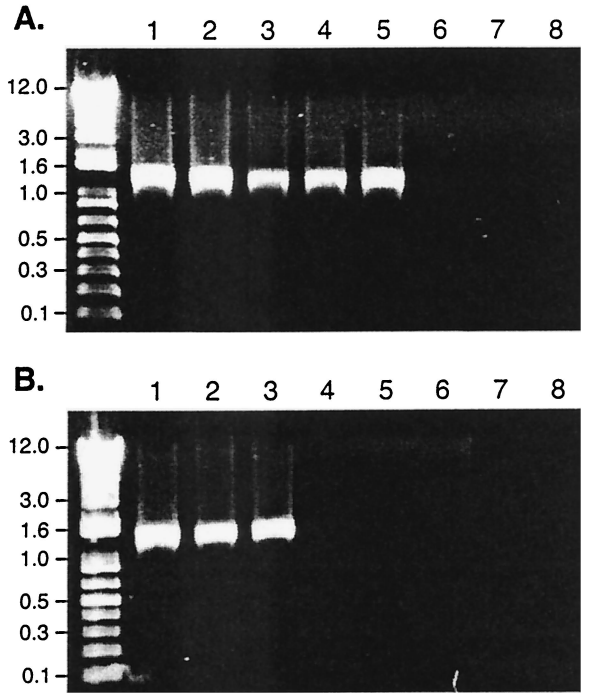

FIG. 4. Comparison of the 5′ regions of *bipA* homologs by PCR. (A) Genomic DNA amplified by PCR by using primers KB1For and LMBipNtc. Lane 1, *B. bronchiseptica* RB50; lane 2, *B. pertussis* Tohama I; lane 3, *B. parapertussis*$_{ov}$ Fr107; lane 4, *B. parapertussis*$_{ov}$ JI; lane 5, *B. parapertussis*$_{ov}$ HI; lane 6, *B. parapertussis*$_{hu}$ 12822; lane 7, *B. parapertussis*$_{hu}$ No7; lane 8, *B. parapertussis*$_{hu}$ 803. (B) Genomic DNA amplified by PCR by using primers LM7BipNt and LMBipNtc. Lane 1, *B. parapertussis*$_{hu}$ 12822; lane 2, *B. parapertussis*$_{hu}$ No7; lane 3, *B. parapertussis*$_{hu}$ 803; lane 4, *B. bronchiseptica* RB50; lane 5, *B. pertussis* Tohama I; lane 6, *B. parapertussis*$_{ov}$ Fr107; lane 7, *B. parapertussis*$_{ov}$ JI; lane 8, *B. parapertussis*$_{ov}$ HI.

quences diverged significantly while the RB50 and 12822 sequences remained nearly identical for at least another 3 kb. The region of DNA containing *′bipA* and ~700 bp 3′ to *′bipA* in 12822, therefore, appears to be of *B. pertussis* origin, while the flanking sequences are more similar to *B. bronchiseptica* sequences, as expected for *B. parapertussis*$_{hu}$ DNA in general due to its closer phylogenetic relationship with *B. bronchiseptica* DNA than with *B. pertussis* DNA. To determine if the genetic organization of the *bipA* region in 12822 was conserved in other *B. parapertussis*$_{hu}$ isolates, we designed primers to amplify a 1.58-kb fragment from the 5′ end of *bipA* from *B. parapertussis*$_{hu}$ 12822 (LM7BipNt and LMBipNtc [Table 2]). LM7BipNt annealed immediately 3′ to the IS*1001* element, within the 793-bp intervening sequences. These primers amplified 1.58-kb fragments from all *B. parapertussis*$_{hu}$ strains tested and did not yield PCR products from *B. bronchiseptica*, *B. pertussis*, or *B. parapertussis*$_{ov}$ strains (Fig. 4B). The *bipA* homolog in all *B. parapertussis*$_{hu}$ strains tested, therefore, appears to be a pseudogene.

Our inability to detect BipA in *B. parapertussis*$_{ov}$ strains by Western blotting was not due to gross differences in the 5′ regions of the *bipA* loci of these strains, as primers designed to amplify the promoter regions of *bipA* in RB50 and Tohama I amplified fragments of the same size in *B. parapertussis*$_{ov}$ strains (Fig. 4A). We therefore determined the nucleotide sequences of the 5′ ends of the *bipA* genes of all of the *B. parapertussis*$_{ov}$ strains used in this study. All of the sequences obtained were identical to each other, and although they were nearly identical to the sequences of *B. pertussis* and *B. bronchiseptica*, there were notable differences in the region between the BvgA binding sites designated IR1 and HS2 and at position −11 relative to the transcriptional start site that were identified in *B. bronchiseptica* (Fig. 5).

***bipA* transcription is maximal under Bvg^i-phase conditions in *B. pertussis*, *B. bronchiseptica*, and *B. parapertussis*$_{ov}$, but the levels are different in different species.** To compare *bipA* transcription levels in the various species, we created *bipA-lacZ* fusions in representative strains (*B. bronchiseptica* strain RB50, *B. pertussis* strain GMT1, and *B. parapertussis*$_{ov}$ strain Fr107) using plasmid pTEN34, which contained a 321-bp fragment of *bipA* corresponding to nucleotides encoding amino acids 70 to 177 of BipA fused to a promoterless *lacZ* gene. Integration of

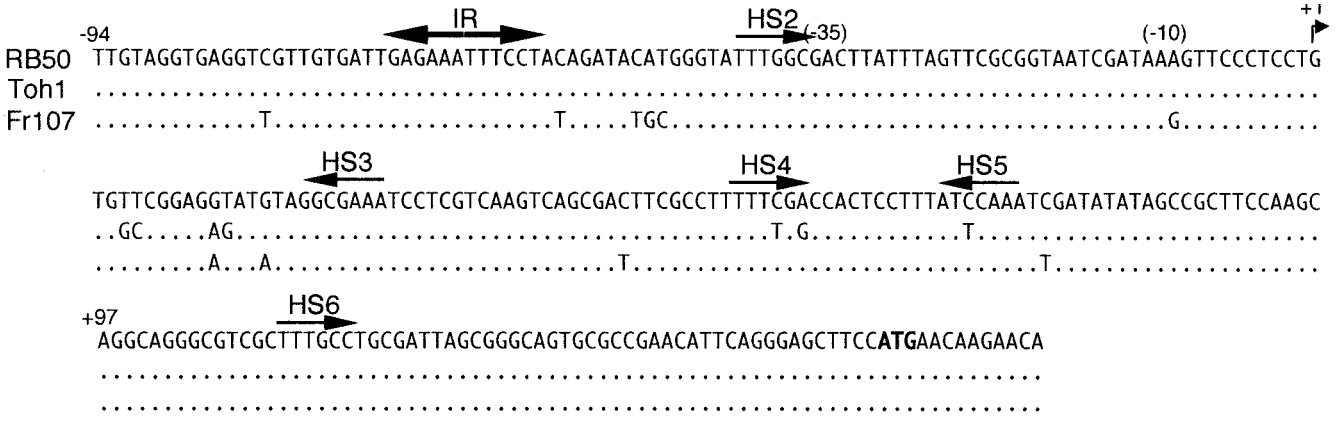

FIG. 5. Comparison of *bipA* promoter regions. The nucleotide sequences of the promoter regions of *B. bronchiseptica* RB50, *B. pertussis* Tohama I, and *B. parapertussis*$_{ov}$ Fr107 are shown. BvgA binding sites identified for RB50 and hypothesized to be required for transcriptional activation of *bipA* are indicated by arrows and are labeled IR1 and HS2 (14). BvgA binding sites hypothesized to be required for repression of *bipA* under Bvg$^+$-phase conditions are also indicated by arrows and are labeled HS3, HS4, HS5, and HS6. The translation initiation codon is indicated by boldface type.

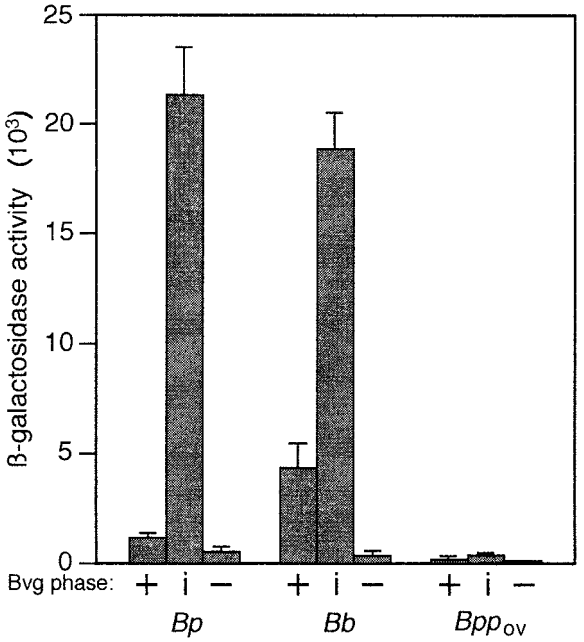

FIG. 6. *bipA* expression patterns: β-galactosidase activity in *B. bronchiseptica* RB50 (Bb), *B. pertussis* GMT1 (Bp), and *B. parapertussis*$_{ov}$ Fr107 (Bpp$_{ov}$) in the Bvg$^+$, Bvg^i, and Bvg$^-$ phases. The units are nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein. The error bars indicate one standard deviation.

this suicide plasmid into the chromosome resulted in the formation of a *bipA'-'lacZ* fusion. To determine expression levels under Bvg$^+$- and Bvg$^-$-phase conditions, we used wild-type strains (RB50::pTEN34, GMT1::pTEN34, and Fr107::pTEN34) grown in SS medium and in SS medium containing 20 mM $MgSO_4$, respectively. To determine Bvg^i-phase expression levels, we used strains containing the *bvgS*-I1 mutation (RB50i::pTEN34, GMT1i::pTEN34, and Fr107i::pTEN34) grown in SS medium. Consistent with previous reports (14, 54), the levels of *bipA* expression in *B. bronchiseptica* were maximal in the Bvg^i phase (Fig. 6). The *bipA-lacZ* expression levels in *B. pertussis* were nearly the same as those in *B. bronchiseptica* in the Bvg^i and Bvg$^-$ phases. The level of *bipA-lacZ* expression in *B. pertussis* grown under Bvg$^+$-phase conditions, however, was only about 15% of that of *B. bronchiseptica* (~500 β-galactosidase units, compared with ~4,200 β-galactosidase units) (Fig. 6). This result is consistent with the fact that the BipA protein can be detected by Western blot analysis in lysates of Bvg$^+$-phase *B. bronchiseptica* but not in lysates of Bvg$^+$-phase *B. pertussis* (Fig. 3). *bipA* expression in *B. parapertussis*$_{ov}$ strain Fr107 was significantly decreased under all conditions compared with *bipA* expression in *B. bronchiseptica* and *B. pertussis*. The overall expression pattern (maximal in the Bvg^i phase), however, was the same as that in the other species (Fig. 6). Our inability to detect the BipA protein in lysates of *B. parapertussis*$_{ov}$ strains by Western blotting was therefore most likely due to a decreased level of transcription compared with that in *B. bronchiseptica* and *B. pertussis*.

DISCUSSION

We have been studying *bipA* and its product, BipA, to determine its role and the role of the Bvg^i phase in the *Bordetella* infectious cycle. Initial DNA sequence analyses revealed significant similarity between the predicted amino acid sequence of BipA and the amino acid sequences of intimin and invasin, suggesting that BipA represents a new member of this class of bacterial adhesins (54). The fact that most of the amino acid differences between the BipA homologs of *B. bronchiseptica* RB50 and *B. pertussis* Tohama I are located within the C-terminal domain, the region analogous to the regions of intimin and invasin that participate in direct contact with receptors on host cells, suggested further that if BipA does function as an adhesin, it might play a role in determining host specificity. To explore this possibility by using a genomic approach, we determined the prevalence, genetic organization, and expression pattern of *bipA* alleles in strains representing various clades of the *B. bronchiseptica* cluster. Our results are summarized in Fig. 7.

PCR and Southern blot analyses showed that most, but not all, strains contain *bipA* genes. In *B. parapertussis*$_{hu}$ strains, these genes appear to be pseudogenes. Although *B. parapertussis*$_{ov}$ strains contain intact *bipA* loci, the genes were expressed at very low levels compared with the levels of expression in *B. bronchiseptica* and *B. pertussis* strains. These low levels of expression were probably due to nucleotide differences at positions −11, −51 to −53, and −59 relative to the predicted transcriptional start site as these positions are predicted to be involved in BvgA-mediated transcriptional activation in *B. bronchiseptica* (14). The fact that *bipA* is absent from some strains and either is not expressed or is expressed at only a very low level in other strains suggests that if BipA plays a role in the *Bordetella* infectious cycle, the role is either not essential or can be compensated for by another gene(s) in some strains. Alternatively, BipA could play an important role in a behavior that is unique to those strains in which it is expressed. We and other workers have shown that the Bvg$^+$ phase is necessary and sufficient for respiratory colonization and that failure to repress Bvg$^-$-phase phenotypes is detrimental to the development of infection (1, 11, 12, 35). Based on these and other data, we hypothesized that the Bvg^i phase is important for transmission (9, 10, 12). As transmission routes may include survival in one or more in vivo niches or ex vivo environments and as the various species, or even lineages within species, may be differentially restricted in their modes of transmission, it is conceivable that factors required for specific routes could be absolutely required in some strains and dispensable in others. BipA's limited distribution, therefore, may reflect a specialized role in this process.

In our analyses we identified five *bipA* alleles, and in most cases strains of a single species contained the same allele; the only exception was *B. pertussis* strain 18323, which contained its own unique allele. With regard to the C-terminus-encoding region, however, only two types were identified, those identical to the RB50 region (present in all *B. bronchiseptica* strains containing *bipA*) and those identical to the Tohama I region (present in all non-*B. bronchiseptica* strains). If only strains expressing high levels of BipA are considered, the two types segregate according to host specificity; all human-infective

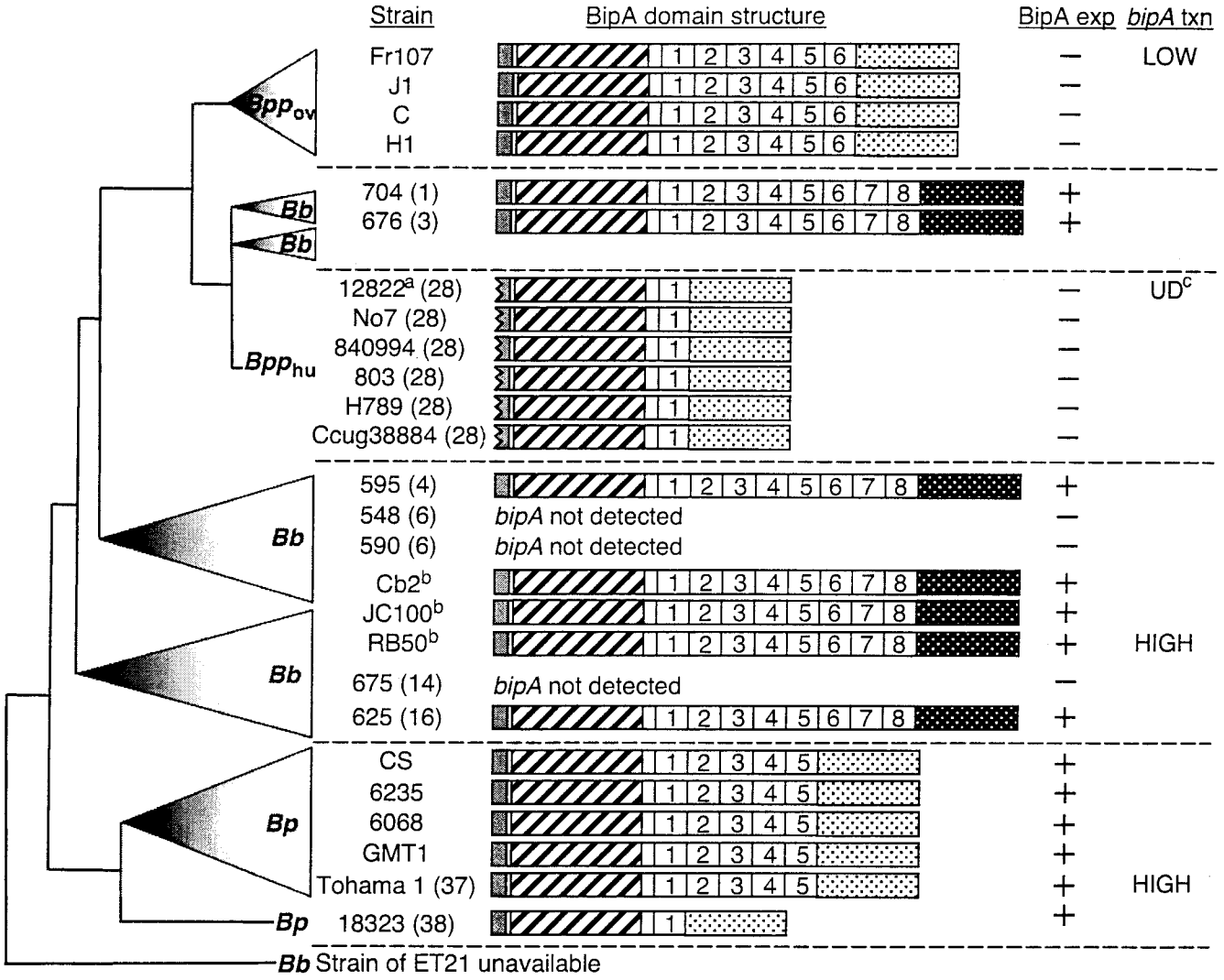

FIG. 7. Schematic diagram of *bipA* alleles in strains of the *B. bronchiseptica* cluster. The phylogenetic relationships shown are based on the work of van der Zee et al. (58), and the dendrogram was adapted from the study of Gerlach et al. (19). The strains used in this study are included, and where known, electrophoretic types (ET) are indicated in parentheses after the strain designations. The cross-hatched area represents the region of BipA that exhibits amino acid sequence similarity with intimin and invasin, the numbered boxes represent the 90-amino-acid repeats, and the distribution of the two different C-terminal sequences are indicated (black dots on a white background, Tohama I-like; white dots on a black background, RB50-like). Although the electrophoretic types of the *B. parapertussis*$_{hu}$ strains used in this study were not determined, they are almost definitely electrophoretic type 28 as all *B. parapertussis*$_{hu}$ strains tested by van der Zee et al. were electrophoretic type 28 strains. The electrophoretic types of strains RB50, Cb2, and JC100 are unknown so these strains have been placed between the two large clusters of *B. bronchiseptica* strains. For RB50, this is consistent with its lack of IS elements. The ability to detect BipA by Western blotting (BipA exp) and the relative level of *bipA* expression under Bvg^i-phase conditions (*bipA* txn) are indicated. Bpp$_{ov}$, *B. parapertussis*$_{ov}$; Bb, *B. bronchiseptica*; Bpp$_{hu}$, *B. parapertussis*$_{hu}$; Bp, *B. pertussis*; UD, undetectable.

strains contain alleles encoding Tohama I-like C termini, while all non-human-infective strains contain alleles encoding RB50-like C termini. Host specificity is likely, although not necessarily, controlled by specific interactions that occur when the bacterium first encounters a potential host (i.e., at the level of adherence). Although similarity to intimin and invasin suggests that BipA plays a role in adherence, we were unable to detect BipA-dependent adherence in any of multiple cell lines, including those of respiratory, endothelial, and macrophage origin (54; unpublished observations). It is still possible, however, that BipA mediates adherence to specific respiratory cells that are either unavailable as cell lines or are available but lack a specific receptor(s) when they are cultured in vitro or that BipA mediates adherence to noncellular components of the respiratory tract, such as mucus. We are currently performing experiments to test these possibilities.

In addition to nucleotide sequence differences at their 3′ ends, *bipA* alleles were distinguished by differences in the number of 90-amino-acid repeats which they encoded. It is possible that these differences reflect variability in surface characteristics displayed by the different species. It is known that the lipopolysaccharide structures of *B. pertussis*, *B. parapertussis*$_{hu}$, and *B. bronchiseptica* differ; *B. pertussis* strains express only lipid A and a branched-chain core oligosaccharide, while *B.*

bronchiseptica and *B. parapertussis*$_{hu}$ strains add O-antigen-like homopolymers to their core structures under Bvg$^-$-phase conditions (3, 4, 15, 23, 29, 55). Although predominance of O antigens or other large surface molecules in the Bvg^i phase is unknown, our results suggest that the surfaces of *B. bronchiseptica* and *B. pertussis* differ such that eight 90-amino-acid domains are required for exposure of the C terminus of BipA in *B. bronchiseptica*, while only five 90-amino-acid domains may be required for exposure of the C terminus of BipA in *B. pertussis*. It is curious that *bipA* of *B. pertussis* 18323 encodes only one 90-amino-acid domain. In many respects, 18323 appears to be more similar to *B. parapertussis*$_{hu}$ and *B. bronchiseptica* strains than to *B. pertussis* strains (5, 19, 40, 52). The presence of only one 90-amino-acid domain in BipA of 18323, like the data obtained for the *B. parapertussis*$_{hu}$ strains, provides more evidence for the unique position of this *B. pertussis* strain on the *Bordetella* phylogenetic tree. The fact that the *bipA* allele in 18323 is expressed in a pattern similar to the pattern in *B. bronchiseptica* and other *B. pertussis* strains, however, provides support for the hypothesis that BipA confers a selective advantage to members of these species. If BipA does provide some selective advantage for 18323, however, the surface characteristics of 18323 must be such that the C terminus of a BipA protein with only one 90-amino-acid domain is exposed.

With regard to phylogenetic and evolutionary implications, our results are consistent with observations which indicate that there are significant genomic rearrangements within, and potentially across, *Bordetella* species (52, 53). As the progenitor organism for the *B. bronchiseptica* cluster is proposed to have been *B. bronchiseptica* (19, 57) and our analyses indicate that all *B. bronchiseptica* strains have the same *bipA* allele, vertical inheritance of *bipA* would require the occurrence and selection of the same set of mutations independently in each of three lineages in order for *B. pertussis*, *B. parapertussis*$_{hu}$, and *B. parapertussis*$_{ov}$ strains to encode the same BipA C-terminal domain, which is significantly different from that of *B. bronchiseptica*. Vertical inheritance of *bipA* is even less probable given the lack of *bipA* expression in *B. parapertussis*$_{hu}$ strains and the low level of expression of *bipA* in *B. parapertussis*$_{ov}$ strains. Moreover, a sequence comparison of *bipA* and flanking regions suggests strongly that the truncated *'bipA* gene present in *B. parapertussis*$_{hu}$ strain 12822 was acquired horizontally from *B. pertussis*. Although little is known about the genome of *B. parapertussis*$_{ov}$ strains, it seems likely that *bipA* was acquired horizontally in these strains as well, with subsequent mutations giving rise to variation in the number of regions encoding the 90-amino-acid repeat and nucleotide sequence differences at the promoter. We can only speculate about the selective advantages of these gene-swapping events. With regard to *B. parapertussis*$_{hu}$ strains, an interesting possibility is that acquisition of *'bipA* from *B. pertussis* resulted in replacement of a *B. bronchiseptica*-like *bipA* allele that conferred a selective disadvantage to *B. parapertussis*$_{hu}$ strains during their adaptation to human hosts. Similarly, one can imagine that expression of a *B. pertussis*-like *bipA* allele in *B. parapertussis*$_{ov}$ strains might be disadvantageous, leading to accumulation of promoter mutations that decrease transcription levels. The possibility that the wrong *bipA* allele could be detrimental at some point in the infectious cycle is an interesting one that we are investigating using chimeric strains.

Our results are consistent with the hypothesis that BipA plays a role in a specific mode of transmission that is used by a subset of *Bordetella* strains. They also contribute to the growing body of information indicating the highly fluid nature of the *Bordetella* genome and high degree of gene transfer and recombination within and among strains of the *B. bronchiseptica* cluster. The anticipated publication of the comparative genome sequences of *B. pertussis* Tohama I, *B. parapertussis*$_{hu}$ 12822, and *B. bronchiseptica* RB50 will undoubtedly provide considerably more insight into the complex phylogenetic and evolutionary relationships among these bacteria.

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REFERENCES

1. **Akerley, B. J., P. A. Cotter, and J. F. Miller.** 1995. Ectopic expression of the flagellar regulon alters development of the *Bordetella*-host interaction. Cell **80:**611–620.
2. **Akerley, B. J., and J. F. Miller.** 1993. Flagellin gene transcription in *Bordetella bronchiseptica* is regulated by the BvgAS virulence control system. J. Bacteriol. **175:**3468–3479.
3. **Allen, A., and D. Maskell.** 1996. The identification, cloning and mutagenesis of a genetic locus required for lipopolysaccharide biosynthesis in *Bordetella pertussis*. Mol. Microbiol. **19:**37–52.
4. **Allen, A. G., T. Isobe, and D. J. Maskell.** 1998. Identification and cloning of *waaF* (*rfaF*) from *Bordetella pertussis* and use to generate mutants of *Bordetella* spp. with deep rough lipopolysaccharide. J. Bacteriol. **180:**35–40.
5. **Arico, B., R. Gross, J. Smida, and R. Rappuoli.** 1987. Evolutionary relationships in the genus *Bordetella*. Mol. Microbiol. **1:**301–308.
6. **Arico, B., V. Scarlato, D. M. Monack, S. Falkow, and R. Rappuoli.** 1991. Structural and genetic analysis of the *bvg* locus in *Bordetella* species. Mol. Microbiol. **5:**2481–2491.
7. **Betsou, F., O. Sismeiro, A. Danchin, and N. Guiso.** 1995. Cloning and sequence of the *Bordetella bronchiseptica* adenylate cyclase-hemolysin-encoding gene: comparison with the *Bordetella pertussis* gene. Gene **162:**165–166.
8. **Cherry, J. D., and U. Heininger.** 1998. Pertussis, p. 1423–1440. *In* R. D. Feigin and J. D. Cherry (ed.), Textbook of pediatric infectious diseases, 4th ed. Saunders, Philadelphia, Pa.
9. **Cotter, P. A., and V. J. DiRita.** 2000. Bacterial virulence gene regulation: an evolutionary perspective. Annu. Rev. Microbiol. **54:**519–565.
10. **Cotter, P. A., and J. F. Miller.** 2001. *Bordetella*, p. 619–674. *In* E. A. Groisman (ed.), Principles of bacterial pathogenesis. Academic Press, San Diego, Calif.
11. **Cotter, P. A., and J. F. Miller.** 1994. BvgAS-mediated signal transduction: analysis of phase-locked regulatory mutants of *Bordetella bronchiseptica* in a rabbit model. Infect. Immun. **62:**3381–3390.
12. **Cotter, P. A., and J. F. Miller.** 1997. A mutation in the *Bordetella bronchiseptica bvgS* gene results in reduced virulence and increased resistance to starvation, and identifies a new class of Bvg-regulated antigens. Mol. Microbiol. **24:**671–685.
13. **Cullinane, L. C., M. R. Alley, R. B. Marshall, and B. W. Manktelow.** 1987. *B. parapertussis* from lambs. N. Z. Vet. J. **35:**175.
14. **Deora, R., H. J. Bootsma, J. F. Miller, and P. A. Cotter.** 2001. Diversity in the *Bordetella* virulence regulon: transcriptional control of a Bvg-intermediate phase gene. Mol. Microbiol. **40:**669–683.
15. **Di Fabio, J. L., M. Caroff, D. Karibian, J. C. Richards, and M. B. Perry.** 1992. Characterization of the common antigenic lipopolysaccharide O-chains produced by *Bordetella bronchiseptica* and *Bordetella parapertussis*. FEMS Microbiol. Lett. **76:**275–281.
16. **Fernandez, R. C., and A. A. Weiss.** 1994. Cloning and sequencing of a *Bordetella pertussis* serum resistance locus. Infect. Immun. **62:**4727–4738.
17. **Finn, T. M., and L. A. Stevens.** 1995. Tracheal colonization factor: a *Bordetella pertussis* secreted virulence determinant. Mol. Microbiol. **16:**625–634.
18. **Fitzhenry, R. J., D. J. Pickard, E. L. Hartland, S. Reece, G. Dougan, A. D. Phillips, and G. Frankel.** 2002. Intimin type influences the site of human intestinal mucosal colonisation by enterohaemorrhagic *Escherichia coli* O157:H7. Gut **50:**180–185.
19. **Gerlach, G., F. von Wintzingerode, B. Middendorf, and R. Gross.** 2001. Evolutionary trends in the genus *Bordetella*. Microbes Infect. **3:**61–72.

20. **Goldman, W. E., and B. T. Cookson.** 1988. Structure and functions of the *Bordetella* tracheal cytotoxin. Tokai J. Exp. Clin. Med. **13**(Suppl.)**:**187–191.
21. **Goldman, W. E., and L. A. Herwaldt.** 1985. *Bordetella pertussis* tracheal cytotoxin. Dev. Biol. Stand. **61:**103–111.
22. **Goodnow, R. A.** 1980. Biology of *Bordetella bronchiseptica.* Microbiol. Rev. **44:**722–738.
23. **Harvill, E. T., A. Preston, P. A. Cotter, A. G. Allen, D. J. Maskell, and J. F. Miller.** 2000. Multiple roles for *Bordetella* lipopolysaccharide molecules during respiratory tract infection. Infect. Immun. **68:**6720–6728.
24. **Heininger, U., K. Stehr, S. Schmitt-Grohe, C. Lorenz, R. Rost, P. D. Christenson, M. Uberall, and J. D. Cherry.** 1994. Clinical characteristics of illness caused by *Bordetella parapertussis* compared with illness caused by *Bordetella pertussis*. Pediatr. Infect. Dis. J. **13:**306–309.
25. **Hewlett, E. L., V. M. Gordon, J. D. McCaffery, W. M. Sutherland, and M. C. Gray.** 1989. Adenylate cyclase toxin from *Bordetella pertussis*. Identification and purification of the holotoxin molecule. J. Biol. Chem. **264:**19379–19384.
26. **Kenny, B., R. DeVinney, M. Stein, D. J. Reinscheid, E. A. Frey, and B. B. Finlay.** 1997. Enteropathogenic *E. coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells. Cell **91:**511–520.
27. **Kloos, W. E., N. Mohapatra, W. J. Dobrogosz, J. W. Ezzel, and C. R. Manclark.** 1981. Deoxyribonucleotide sequence relationships among *Bordetella* species. J. Int. Syst. Bacteriol. **31:**173–176.
28. **Knapp, S., and J. J. Mekalanos.** 1988. Two *trans*-acting regulatory genes (*vir* and *mod*) control antigenic modulation in *Bordetella pertussis*. J. Bacteriol. **170:**5059–5066.
29. **Le Blay, K., P. Gueirard, N. Guiso, and R. Chaby.** 1997. Antigenic polymorphism of the lipopolysaccharides from human and animal isolates of *Bordetella bronchiseptica*. Microbiology **143:**1433–1441.
30. **Leong, J. M., R. S. Fournier, and R. R. Isberg.** 1990. Identification of the integrin binding domain of the *Yersinia pseudotuberculosis* invasin protein. EMBO J **9:**1979–1989.
31. **Li, J., N. F. Fairweather, P. Novotny, G. Dougan, and I. G. Charles.** 1992. Cloning, nucleotide sequence and heterologous expression of the protective outer-membrane protein P.68 pertactin from *Bordetella bronchiseptica*. J. Gen. Microbiol. **138:**1697–1705.
32. **Liu, H., L. Magoun, S. Luperchio, D. B. Schauer, and J. M. Leong.** 1999. The Tir-binding region of enterohaemorrhagic *Escherichia coli* intimin is sufficient to trigger actin condensation after bacterial-induced host cell signalling. Mol. Microbiol. **34:**67–81.
33. **Livey, I., and A. Wardlaw.** 1984. Production and properties of *Bordetella pertussis* heat-labile toxin. J. Med. Microbiol. **17:**91–103.
34. **Martinez de Tejada, G., J. F. Miller, and P. A. Cotter.** 1996. Comparative analysis of the virulence control systems of *Bordetella pertussis* and *Bordetella bronchiseptica*. Mol. Microbiol. **22:**895–908.
35. **Merkel, T. J., S. Stibitz, J. M. Keith, M. Leef, and R. Shahin.** 1998. Contribution of regulation by the *bvg* locus to respiratory infection of mice by *Bordetella pertussis*. Infect. Immun. **66:**4367–4373.
36. **Mooi, F. R., W. H. Jansen, H. Brunings, H. Gielen, H. G. J. van der Heide, H. C. Walvoort, and P. A. M. Guinee.** 1992. Construction and analysis of *Bordetella pertussis* mutants defective in the production of fimbriae. Microbiol. Pathol. **12:**127–135.
37. **Muller, M., and A. Hildebrandt.** 1993. Nucleotide sequences of the 23S rRNA genes from *Bordetella pertussis*, *B. parapertussis*, *B. bronchiseptica* and *B. avium*, and their implications for phylogenetic analysis. Nucleic Acids Res. **21:**3320.
38. **Munoz, J. J.** 1988. Action of pertussigen (pertussis toxin) on the host immune system in pathogenesis and immunity in pertussis, p. 173–192. *In* A. C. Wardlaw and R. Parton (ed.), Pathogenesis and immunity in pertussis. Wiley and Sons, Chichester, United Kingdom.
39. **Musser, J. M., D. A. Bemis, H. Ishikawa, and R. K. Selander.** 1987. Clonal diversity and host distribution in *Bordetella bronchiseptica*. J. Bacteriol. **169:** 2793–2803.
40. **Musser, J. M., E. L. Hewlett, M. S. Peppler, and R. K. Selander.** 1986. Genetic diversity and relationships in populations of *Bordetella* spp. J. Bacteriol. **166:**230–237.
41. **Phillips, A. D., and G. Frankel.** 2000. Intimin-mediated tissue specificity in enteropathogenic *Escherichia coli* interaction with human intestinal organ cultures. J. Infect. Dis. **181:**1496–1500.
42. **Pittman, M.** 1979. Pertussis toxin: the cause of the harmful effects and prolonged immunity of whooping cough. A hypothesis. Rev. Infect. Dis. **1:**401–412.
43. **Porter, J. F., K. Connor, and W. Donachie.** 1994. Isolation and characterization of *Bordetella parapertussis*-like bacteria from ovine lungs. Microbiology **140:**255–261.
44. **Reece, S., C. P. Simmons, R. J. Fitzhenry, S. Matthews, A. D. Phillips, G. Dougan, and G. Frankel.** 2001. Site-directed mutagenesis of intimin alpha modulates intimin-mediated tissue tropism and host specificity. Mol. Microbiol. **40:**86–98.
45. **Relman, D. A., M. Domenighini, E. T. Tuomanen, R. Rappuoli, and S. Falkow.** 1989. Filamentous hemagglutinin of *Bordetella pertussis*: nucleotide sequence and crucial role in adherence. Proc. Natl. Acad. Sci. USA **86:**2634–2641.
46. **Roberts, M., N. F. Fairweather, E. Leininger, D. Pickard, E. L. Hewlett, A. Robinson, C. Hayward, G. Dougan, and I. G. Charles.** 1991. Construction and characterization of *Bordetella pertussis* mutants lacking the *vir*-regulated P.69 outer membrane protein. Mol. Microbiol. **5:**1393–1404.
47. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
48. **Savelkoul, P. H., D. P. de Kerf, R. J. Willems, F. R. Mooi, B. A. van der Zeijst, and W. Gaastra.** 1996. Characterization of the *fim2* and *fim3* fimbrial subunit genes of *Bordetella bronchiseptica*: roles of Fim2 and Fim3 fimbriae and flagella in adhesion. Infect. Immun. **64:**5098–5105.
49. **Stainer, D. W., and M. J. Scholte.** 1970. A simple chemically defined medium for the production of phase I *Bordetella pertussis*. J. Gen. Microbiol. **63:**211–220.
50. **Stenson, T. H., and M. S. Peppler.** 1995. Identification of two *bvg*-repressed surface proteins of *Bordetella pertussis*. Infect. Immun. **63:**3780–3789.
51. **Stibitz, S., and J. F. Miller.** 1994. Coordinate regulation of virulence in *Bordetella pertussis* mediated by the *vir* (*bvg*) locus, p. 407–422. *In* V. L. Miller, J. B. Kaper, D. A. Portnoy, and R. R. Isberg (ed.), The molecular biology of bacterial pathogenesis. ASM Press, Washington, D.C.
52. **Stibitz, S., and M. S. Yang.** 1997. Genomic fluidity of *Bordetella pertussis* assessed by a new method for chromosomal mapping. J. Bacteriol. **179:**5820–5826.
53. **Stibitz, S., and M. S. Yang.** 2001. Genomic plasticity in natural populations of *Bordetella pertussis*. J. Bacteriol. **181:**5512–5515.
54. **Stockbauer, K. E., B. Fuchslocher, J. F. Miller, and P. A. Cotter.** 2001. Identification and characterization of BipA, a *Bordetella* Bvg-intermediate phase protein. Mol. Microbiol. **39:**65–78.
55. **Turcotte, M. L., D. Martin, B. R. Brodeur, and M. S. Peppler.** 1997. Tn5-induced lipopolysaccharide mutations in *Bordetella pertussis* that affect outer membrane function. Microbiology **143:**2381–2394.
56. **Uhl, M. A., and J. F. Miller.** 1995. *Bordetella pertussis* BvgAS virulence control system, p. 333–349. *In* J. A. Hoch and T. Silhavy (ed.), Two-component signal transduction. ASM Press, Washington, D.C.
57. **van der Zee, A., F. Mooi, J. Van Embden, and J. Musser.** 1997. Molecular evolution and host adaptation of *Bordetella* spp.: phylogenetic analysis using multilocus enzyme electrophoresis and typing with three insertion sequences. J. Bacteriol. **179:**6609–6617.
58. **Verghese, M. W., C. D. Smith, and R. Snyderman.** 1985. Potential role for a guanine nucleotide regulatory protein in chemoattractant receptor mediated polyphosphoinositide metabolism, Calif.++ mobilization and cellular responses by leukocytes. Biochem. Biophys. Res. Commun. **127:**450–457.
59. **Weiss, A. A., and S. Falkow.** 1984. Genetic analysis of phase change in *Bordetella pertussis*. Infect. Immun. **43:**263–269.
60. **Wirsing von Konig, C. H., and H. Finger.** 1994. Role of pertussis toxin in causing symptoms of *Bordetella parapertussis* infection. Eur. J. Clin. Microbiol. Infect. Dis. **13:**455–458.
61. **Worley, M. J., I. Stojiljkovic, and F. Heffron.** 1998. The identification of exported proteins with gene fusions to invasin. Mol. Microbiol. **29:**1471–1480.
62. **Yuk, M. H., E. T. Harvill, P. A. Cotter, and J. F. Miller.** 2000. Modulation of host immune responses, induction of apoptosis and inhibition of NF-KB activation by the *Bordetella* type III secretion system. Mol. Microbiol. **35:** 991–1104.
63. **Yuk, M. H., E. T. Harvill, and J. F. Miller.** 1998. The bvgAS virulence control system regulates type III secretion in *Bordetella bronchiseptica*. Mol. Microbiol. **28:**945–959.

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