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Differential Bvg Phase-Dependent Regulation and Combinatorial Role in Pathogenesis of Two *Bordetella* Paralogs, BipA and BcfA[∇]

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To successfully colonize their mammalian hosts, many bacteria produce multiple virulence factors that play essential roles in disease processes and pathogenesis. Some of these molecules are adhesins that allow efficient attachment to host cells, a prerequisite for successful host colonization. *Bordetella* spp. express a number of proteins which either play a direct role in attachment to the respiratory epithelia or exhibit similarity to known bacterial adhesins. One such recently identified protein is BipA. Despite the similarity of BipA to intimins and invasins, deletion of this protein from *B. bronchiseptica* did not result in any significant defect in respiratory tract colonization. In this study, we identified an open reading frame in *B. bronchiseptica*, designated *bcfA* (encoding BcfA [*b*ordetella *c*olonization *f*actor *A*]), that is similar to *bipA*. In contrast to the maximal expression of *bipA* in the Bvg intermediate (Bvg^i) phase, *bcfA* is expressed at high levels in both the Bvg$^+$ and Bvg^i phases. We show here that BvgA and phosphorylated BvgA bind differentially to the *bcfA* promoter region. Utilizing immunoblot assays, we found that BcfA is localized to the outer membrane and that it is expressed during animal infection. While deletion of either *bipA* or *bcfA* did not significantly affect respiratory tract colonization, concomitant deletion of both genes resulted in a defect in colonization of the rat trachea. Our results indicate that the two paralogous proteins have a combinatorial role in mediating efficient respiratory tract colonization.

Bordetellae are small, aerobic, gram-negative coccobacilli that colonize the respiratory tracts of humans and animals (25). One of the three classical *Bordetella* species, *Bordetella pertussis*, infects only humans and causes the acute respiratory disease whooping cough (37). *Bordetella parapertussis* strains can be divided into two genetically distinct types, those which infect humans and cause a pertussis-like illness and those which cause respiratory infections in sheep (25, 35). In contrast to the other two species, *Bordetella bronchiseptica* has a broad host range and infects a variety of nonhuman animals (14, 25); it typically establishes asymptomatic infections but can cause atrophic rhinitis in pigs, kennel cough in dogs, snuffles in rabbits, and bronchopneumonia in guinea pigs (14).

Efficient and productive colonization of the respiratory tract by *Bordetella* requires interactions of multiple factors that allow bacterial adherence to the respiratory epithelium, leading to the eventual development of disease. The majority of these virulence determinants are controlled by a two-component regulatory system known as BvgAS. BvgA is a DNA-binding response regulator, and BvgS is a transmembrane sensor protein kinase (25, 36). When a signal is perceived, BvgS is autophosphorylated at a histidine residue (33). Phosphorylation of BvgA at a conserved aspartate residue then occurs through a series of phosphotransfer reactions from BvgS (4, 34). Phosphorylated BvgA (BvgA-P) can bind to the cognate promoter regions of the Bvg-regulated genes and mediates activation and/or repression of transcription (5, 29). A striking feature of the BvgAS regulatory circuitry is its ability to control the transition among multiple phenotypic states, of which at least three, the Bvg$^+$, Bvg$^-$, and Bvg intermediate (Bvg^i), are known. Each of these phases is characterized by differential expression of known Bvg-regulated gene products (9, 11). For example, during the Bvg$^+$ phase a variety of Bvg-activated factors, including adhesins and toxins, are maximally expressed, and the Bvg-repressed genes are minimally expressed (9, 11). For both *B. pertussis* and *B. bronchiseptica*, it has been demonstrated that the Bvg$^+$ phase is necessary and sufficient for respiratory tract colonization (1, 23).

The transition to the Bvg$^-$ phase occurs as a result of either mutational inactivation of BvgAS or growth in the presence of modulating signals (sulfate anion, nicotinic acid, or low temperature). This phase is characterized by expression of the Bvg-repressed factors (e.g., flagella in *B. bronchiseptica* and outer membrane proteins having unknown functions in *B. pertussis*) and repression of Bvg-activated genes (25). It has been suggested that this phase may be responsible for survival of *B. bronchiseptica* in the environment (9). The Bvg^i phase is expressed either as a result of specific genetic mutations in BvgS or as a result of growth of wild-type (wt) *Bordetella* strains in the presence of semimodulating concentrations of chemical signals (9). The Bvg^i phase is distinguished principally by maximal expression of a set of antigens, and BipA (*Bordetella* intermediate phase protein A) is the first of these antigens to be identified at the molecular level (11, 32). The role of the

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
RB50	*B. bronchiseptica* wt strain	9
RB53	Bvg^+ phase-locked derivative	9
RB53i	Bvg^i phase-locked derivative	9
RB54	Bvg^- phase locked, Δ*bvgS*	9
SM10λpir	*thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km^r (λpir)	30
pSTBlue-1	Blunt end cloning vector	Novagen
pRKD22	pSTBlue-1 derivative containing the 640-bp promoter region of *bcfA* cloned into the EcoRV site of pSTBlue1	This study
pEGZ	*lacZ* transcriptional fusion vector	24
pRKD23	pEGZ derivative; EcoRI fragment from pRKD22 cloned into EcoRI site of pEGZ upstream of promoterless *lacZ*	This study
pRE112	Allelic exchange vector, Cm^r	12
pRKD40	pRE112 derivative, *bcfA* deletion plasmid	This study
pET24(a)	T7-based expression plasmid	Novagen
pNS101	pET24(a) derivative, BcfA overexpression plasmid	This study
BL21(DE3)/pLysE	Overexpression strain	Novagen
RKD101 (Δ*bcfA*)	RB50 derivative with in-frame chromosomal deletion of *bcfA*	This study
RB25 (Δ*bipA*)	RB50 derivative with in-frame chromosomal deletion of *bipA*	32
MM101 (Δ*bipA* Δ*bcfA*)	Δ*bipA* derivative with in-frame chromosomal deletion of *bcfA*	This study

Bvg^i phase in the *Bordetella* infectious cycle is presently unclear.

At its amino terminus, BipA exhibits similarity with intimin proteins of enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) and with invasins of *Yersinia* species, which has led to suggestions that BipA plays a role in the *Bordetella* infectious cycle (32). So far, a significant role for BipA in *Bordetella* pathogenesis has not been identified.

In this study, we identified a *B. bronchiseptica* open reading frame (ORF) designated *bcfA*, which is a paralog of *bipA*. We found that the Bvg phase-dependent expression profile of *bcfA* is strikingly different from that of *bipA*. In contrast to the maximal expression of *bipA* in the Bvg^i phase, *bcfA* is expressed at high levels in both the Bvg^+ and Bvg^i phases. We have identified DNA sequences similar to the consensus BvgA binding site in the region upstream of the *bcfA* ORF. Utilizing an electrophoretic mobility shift assay (EMSA), we observed higher-order BvgA-DNA complexes in the presence of acetyl phosphate. Our results also show that BcfA is localized to the outer membrane and that it is expressed during *Bordetella* infection of rats. By comparing strains having single- and double-deletion mutations in *bipA* and *bcfA* in intranasally infected rats, we found that BipA and BcfA have overlapping functions in mediating efficient colonization of the trachea.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Bordetella* strains were maintained on Bordet-Gengou (BG) agar (Becton Dickinson Microbiology Systems) supplemented with 7.5% defibrinated sheep blood. For RNA extraction and β-galactosidase assays, cells were grown in Stainer-Scholte (SS) broth at 37°C with shaking (31). *E. coli* strains were grown in Luria-Bertani medium at 37°C with shaking. The growth media were supplemented with antibiotics at the following concentrations when necessary: ampicillin, 100 μg/ml; chloramphenicol, 50 μg/ml; gentamicin sulfate, 25 μg/ml; streptomycin, 50 μg/ml; and kanamycin, 25 μg/ml.

***lacZ* transcriptional fusions and β-galactosidase assays.** To construct *lacZ* transcriptional fusions, a 640-bp DNA fragment consisting of 445 bp upstream and 195 bp downstream of the *bcfA* translational site was amplified from RB50 using primers BcfA5 and BcfA6. The PCR fragment was cloned as a blunt-ended fragment into the EcoRV site of the linearized pSTBlue-1 vector (Novagen), resulting in plasmid pRKD22. This plasmid was subsequently digested with EcoRI, and the resultant fragment was then cloned into the EcoRI site of suicide plasmid pEGZ (24), leading to creation of the fusion plasmid pRKD23. This placed the *bcfA* promoter fragment upstream of a promoterless *lacZ* gene. pRKD23 was then integrated into the genomes of the different *Bordetella* strains by a single crossover at the *bcfA* locus, as described previously (11). β-Galactosidase assays were performed as previously described (10, 11). To detect β-galactosidase activity under conditions in which the BvgAS system was modulated, the wt strain was grown in the presence of 40 mM $MgSO_4$.

RNA isolation and real-time RT-PCR. Total RNA was isolated using an RNeasy kit from QIAGEN and was treated with RNase-free DNase I (Invitrogen) to degrade contaminating DNA. Then 2 to 3 μg of RNA was primed with random hexamers (Invitrogen), and cDNA was prepared utilizing the Superscript III (Invitrogen) reverse transcriptase (RT) according to the manufacturer's protocol.

Specific primers (Table 2) for various genes were designed using the ABI PRISM Primer Express software (PE Applied Biosystems) in order to obtain amplicons that were similar sizes. Diluted (1:25) reverse transcription products obtained as described above and a blank control without cDNA were used as templates and were amplified using the TaqMan universal PCR Master Mix (PE Applied Biosystems). The Master Mix contained deoxynucleoside triphosphates, including dUTP, Ampli*Taq* Gold DNA polymerase, Amperase UNG, optimized buffer, and a passive reference dye. For each PCR, a mixture (20 μl) containing template cDNA, 1× Master Mix, 250 nM sense primer, 250 nM antisense primer, and the TaqMan probe at a concentration of 500 nM was placed in 96-well optically clear PCR plates (Greiner Bio-one). Amplification and detection of the specific products were performed with an ABI PRISM 7000 sequence detection system (PE Applied Biosystems) using the following protocol: one cycle of 50°C for 2 min and one cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The threshold value was set manually and was kept constant for all comparison groups. To control for variations in RNA quantity and quality, the constitutively expressed *Bordetella* gene *recA* was used as the endogenous reference control. Relative levels of gene expression were determined using the comparative threshold cycle (C_T) method according to the manufacturer's instructions (user bulletin 2, ABI PRISM sequence detection system). The critical C_T was defined as the cycle at which the fluorescence became detectable above the background fluorescence, and it was inversely proportional to the logarithm of the initial number of template molecules. The ΔC_T values were determined by subtracting the *recA* C_T value from the gene-specific C_T values. The $\Delta\Delta C_T$ value was calculated by subtracting the ΔC_T value obtained with the ΔC_T calibrator value. For measurement of the relative levels of the Bvg-activated genes *bcfA*, *fhaB* (encoding filamentous hemagglutinin), *bipA*, and *cyaA* (encoding adenylate cyclase toxin), the ΔC_T value obtained from the Bvg^- phase-locked cells was designated the calibrator, since this strain exhibits the lowest expression of these target genes. Similarly, for measurement of the relative levels of the Bvg-re-

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TABLE 2. Oligonucleotide primers used in this study

Primer	Sequence or reference[a]
BcfA1	5′-CTAG**TCTAGA**CCTACATATCCGTAGGATTG-3′
BcfA2	5′-TT**GGCGCGCC**GTCTGCGCGACCCGCAGCAT-3′
BcfA3	5′-TT**GGCGCGCC**TGATCGGTGGCGAGGGCGGC-3′
BcfA4	5′-CGG**GGTACC**CTTGAACAGCGGCAGCACGTCG-3′
BcfA5	5′-CGCATGGGATTCTCCCGGGTA-3′
BcfA6	5′-GCTGTCGGCGGCATCCTGGCGATC-3′
BcfA7	5′-GGCGAGCGCGACCGCGTCTTGCT-3′
BcfA8	5′-GGCTGAGATTGACGCCAAGCTGCA-3′
BcfA9	5′-CGCCATGCCTTGAA-3′
BcfA10	5′-(**DFAM**)-GGGCGCCAACGCATT-(**DTAM**)-3′
BcfA11	5′-TGGTTCTTGCCGAA-3′
BcfA12	5′-CGC**GGATCC**GTGAAGCAAGCCATCCACG-3′
BcfA13	5′-CCC**AAGCTT**CCCAGCAGGCCGCCCTC-3
FhaF	5′-ATCCGACCTACACCGAATGG-3′
FhaR	5′-GAGTGTGCGCCGATTTTCAG-3′
BipAF	5′-GGGTCTGCCCTTTCTGCGCAATCTG-3′
BipAR	5′-GCCGATCACCTTGGTCTGCTCCAG-3′
PrnF	5′-GCCGCTGCAGCCGGAAGACCTTC-3′
PrnR	5′-GCAGCTCCGTCGCGACGATGTCG-3′
RecF	24
RecR	24
RecA9	5′-ACGTGCAATACGCCTCCAA-3′
RecA10	5′-(**DFAM**)-TGGGCGTCAACCTGACCGACCT-(**DTAM**)-3′
RecA11	5′-TGTCCGGCTGGGAGATCA-3′
Fha9	5′-TGTCCGCCATGGAGTATTTCA-3′
Fha10	5′-(**DFAM**)-CCCGGTGAGCCTGACAGCCCT-(**DTAM**)-3′
Fha11	5′-CCAGCAGATAATCCAGGAGTTCAT-3′
Fla9	5′-GATCCAGCAGGAAGTCAACCA-3′
Fla10	5′-(**DFAM**)-AAATCAACCGCATCGCCGAGCA-(**DTAM**)-3′
Fla11	5′-GACCTGATGCCGTTGAAGTC-3′
BipA109	5′-GGCCCAGGTCAATGATGTCTT-3′
BipA110	5′-(**DFAM**)-AACCTGGCTCGGGAATCGGGTC-(**DTAM**)-3′
BipA111	5′-CCTTGCAGATTGCGCAGA-3′

[a] Bold type indicates restriction enzyme sites. DFAM, 6-carboxyfluorescein (reporter fluorochrome); DTAM, 6-carboxytetramethylrhodamine (quencher fluorochrome).

pressed gene *flaA* (coding for flagellin), the ΔC_T value obtained from the Bvg$^+$ phase-locked cells was designated the calibrator, since this strain exhibits the lowest expression of *flaA*. We also performed a validation experiment to ensure that the efficiencies of target amplification and the efficiencies of reference amplification were similar by determining the ΔC_T values with template dilution. Data from at least three measurements, each performed with at least two different batches of RNA, were plotted, and standard deviations for independent values were determined.

Time point analysis. To modulate *bvg* activity, wt *B. bronchiseptica* strain RB50 was grown in 50 ml of SS medium in the presence of 40 mM $MgSO_4$ for 18 h. At time zero, the culture was spun down and resuspended in 100 ml of SS medium lacking $MgSO_4$. At 5 and 30 min and 1 and 4 h, 5 ml of the bacterial culture was utilized to prepare total RNA as described above.

After reverse transcription of the RNA, an aliquot of cDNA (5%) was used as the template in an RT-PCR. In order to eliminate possible interference by genomic DNA, mock reactions without RT were also performed. The RT-PCR was carried out as previously described (10, 11) with gene-specific primers. The primer pairs used are listed in Table 2. Genomic DNA prepared from wt strain RB50 was used as the positive control for the PCR. Aliquots of the amplified products obtained were electrophoresed on 1% agarose gels. Images of the ethidium bromide-stained gels were captured by using the Alpha Innotech Gel Doc system (Alpha Innotech Corporation).

EMSAs. The 640-bp DNA fragment used for construction of *lacZ* fusions (see above) was end labeled by using T4 polynucleotide kinase (New England Biolabs, Massachusetts) with [γ-^{32}P]ATP (Amersham Biosciences, New Jersey). Unincorporated radioactivity was removed by passage through G-50 quick spin columns (Amersham Biosciences, New Jersey). Each reaction mixture (20 μl) contained purified BvgA or BvgA-P and the radiolabeled promoter DNA in 1× binding buffer [10 mM Tris-HCl (pH 7.8), 2 mM $MgCl_2$ 50 mM NaCl, 1 mM dithiothreitol [DTT], 0.5 μg of poly(dI-dC), 0.01% NP-40, 100 ng of bovine serum albumin, 10% glycerol]. BvgA was phosphorylated as described previously by incubation of the protein at room temperature for 15 min in 1× binding buffer containing 20 mM acetyl phosphate (10, 29). The reaction mixtures were incubated at 37°C for 15 min to allow binding of BvgA/BvgA-P to radiolabeled promoter. The samples were electrophoresed and visualized by autoradiography as previously described (10, 29).

For nonspecific competition, a DNA fragment corresponding to the internal region (positions 453 to 978) of the *bcfA* ORF was amplified using primers BcfA7 and BcfA8. Various concentrations of the fragment were included in the binding reaction mixtures. For specific competition, the *bcfA* promoter fragment was amplified using primers BcfA5 and BcfA6, and various concentrations of the unlabeled DNA fragment were included in the reaction mixtures.

Overexpression and purification of BcfA and antibody production. The entire *bcfA* ORF except the stop codon was cloned as a BamHI-HindIII fragment in similarly digested plasmid pET24(a) (Novagen), resulting in the BcfA overexpression plasmid pNS101. Amplification was carried out using a mixture of the *Pfu* (0.6 U) and *Taq* (0.5 U) DNA polymerases. *E. coli* BL21(DE3)/pLysE cells containing pNS101 were grown in 2× TY (16 g of Bacto tryptone [Difco], 10 g of yeast extract, 5 g/liter NaCl, 0.4% glucose) in the presence of kanamycin and chloramphenicol at 37°C. The cells were grown to an optical density at 600 nm of 0.8 to 1.0 and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h. The cells were harvested by centrifugation, resuspended in 30 ml of TGED buffer (10 mM Tris-HCl [pH 7.9], 0.1 mM EDTA [pH 8.0], 0.2 mM DTT, 0.05% sodium deoxycholate, 5% glycerol, 2 mM phenylmethylsulfonyl fluoride), incubated on ice for 20 min, and lysed by passage through a French pressure cell three times at 14,000 to 16,000 lb/in^2. The lysate was centrifuged, and the soluble BcfA was purified from the clarified lysate using a T7.Tag affinity purification kit (Novagen) according to the manufacturer's instructions.

For antibody production, after induction the cells were resuspended in TGED buffer, lysed by passage through a French pressure cell, and centrifuged. The pellet was washed once with TGED buffer containing 0.5 M NaCl, 10 ml of 1× Bugbuster protein extraction reagent (Novagen), 10 μl of Benzonase nuclease, and 1 mg/ml of lysozyme. After centrifugation, the cell pellet was resuspended in 5 ml of TGED buffer containing 6 M guanidine hydrochloride and incubated on ice for 10 min. Another 5 ml of cold TGED buffer was added, and the suspension was incubated on ice for an additional 10 min. The lysate was spun down, and the supernatant was dialyzed in 2 liters of cold TGED buffer at 4°C for 18 h. The dialysate was centrifuged, and the pellet containing overproduced BcfA was resuspended in protein loading buffer (50 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% glycerol, 5 mM DTT) and electrophoresed on a standard SDS-polyacrylamide protein gel. The band corresponding to the BcfA protein was excised and utilized for production of anti-BcfA antibodies in rats. Antibody was produced on a fee-for-service basis by Covance. The polyclonal serum was enriched further for BcfA-specific antibodies by incubating (for 3 to 4 h) a 1:50 dilution of the serum with overnight cultures of the RKD110 (Δ*bcfA*) strain at 4°C. After adsorption, bacteria were centrifuged at 12,000 rpm at 4°C. The enrichment procedure was performed at least four times by utilizing the supernatant from each preceding step for serum depletion.

Preparation of outer membrane fractions, SDS-polyacrylamide gel electrophoresis, and immunoblot analysis. Stationary-phase cultures of the different strains were centrifuged at 17,000 rpm, and the cell pellets were resuspended in cell disruption buffer (10 mM Tris-HCl [pH 8.0], 20% sucrose, 1 mM EDTA, 0.1 mg/ml lysozyme). After incubation on ice for 10 min, the samples were frozen in dry ice and then thawed in cold water. The bacterial cells were sonicated on ice and centrifuged initially at 3,700 rpm for 10 min to pellet unlysed cells. The clarified suspension obtained was spun at 17,000 rpm for 1 h, and the pellet was resuspended in an appropriate volume of 1× phosphate-buffered saline (PBS). For separation of inner and outer membrane proteins, the membrane fractions were incubated with 2% Triton X-100 for 30 min on ice and then centrifuged at 17,000 rpm for 1 h. The pellet (Triton X-100 insoluble) consisting of the outer membrane proteins or purified recombinant BcfA were separated on SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with depleted anti-BcfA serum (1:5,000), anti-BipA antibody (1:5,000 dilution) (32), or rat serum (1:1,000 dilution) harvested from rats infected with the different strains 30 days postinoculation. The secondary antibody (1:2,000) used was either goat anti-rat immunoglobulin G or goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase. For detection of proteins, the Amersham ECL Western blotting system was utilized.

Construction of deletion strains. Based on the preannotated sequence information for *B. bronchiseptica* wt strain RB50 present in the Sanger Center database, we designed primers to delete a region encoding amino acids 10 to 882 of the 903 amino acids encoded by the *bcfA* ORF. After construction of the deletion strains and testing of these strains in animal models, we found that in the updated database the *bcfA* ORF was extended to include an upstream in-frame stretch of nucleotides encoding 66 amino acids. Thus, based on the current annotation, the in-frame deletion of *bcfA* encompassed a region corresponding to the sequence encoding amino acids 76 to 958 of the 969 amino acids encoded by the ORF.

An XbaI-AscI fragment (407 bp) containing sequences corresponding to the 5′ end, including the sequence encoding the first 75 amino acids, of the *bcfA* ORF

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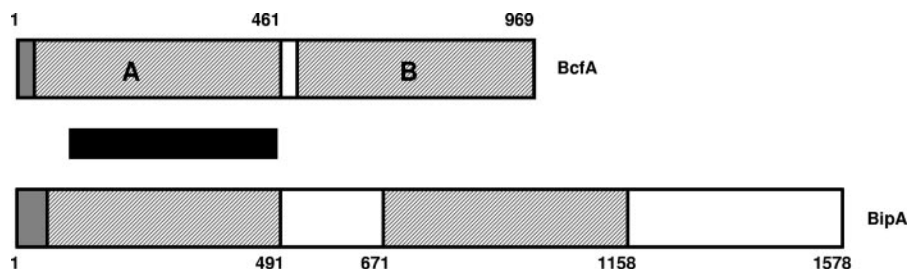

FIG. 1. Schematic diagrams showing the similarity of BcfA and BipA. The amino acid sequences of BipA and BcfA were aligned using the Needleman-Wunsch global alignment algorithm by utilizing EMBOSS Needle available at the European Bioinformatics Institute website. The homologous regions (regions A and B) in these two proteins are indicated by cross-hatching. Region A of BcfA (positions 17 to 461) exhibits 56% sequence identity to positions 35 to 491 of BipA, whereas region B (positions 473 to 969) exhibits 39% sequence identity to positions 671 to 1158 of BipA. The putative signal sequences are indicated by the shaded regions. The solid rectangle indicates the region of BipA and BcfA that exhibits similarity to intimins and invasins.

was amplified from the chromosome of RB50 using primers BcfA1 and BcfA2. A second AscI-KpnI fragment (525 bp) containing sequences corresponding to the 3′ end, including the sequence encoding the last 11 amino acids, of the *bcfA* ORF was also amplified using primers BcfA3 and BcfA4. These fragments were digested with the appropriate restriction enzymes and were used for three-way ligation with XbaI- and KpnI-digested suicide vector pRE112 (Cm^r) (12), resulting in plasmid pRKD40. This plasmid was transformed into SM10λpir and mobilized from this strain into RB50. After conjugation, cointegrants were selected on BG agar containing chloramphenicol and streptomycin. Colonies resulting from second recombination events were selected on Luria-Bertani agar containing 7.5% sucrose as described previously (10, 12). The genotype of deletion strain RKD110 (Δ*bcfA*) was confirmed by PCR and subsequent DNA sequencing. MM101, a Δ*bipA* Δ*bcfA* strain, was constructed essentially as described above for RKD110 (Δ*bcfA*), except that the parental strain used was the previously described Δ*bipA* strain RB25 (32).

Rat colonization experiments. Female Wistar rats (Charles River Laboratories) that were 4 to 5 weeks old were lightly anesthetized with halothane and inoculated intranasally with 5 μl of sterile PBS or with 40 to 100 CFU of either the wt strain or a mutant strain. The number of CFU delivered was confirmed by plating on BG agar containing streptomycin. The levels of colonization in the respiratory tract were determined by sacrificing rats 12 or 30 days postinoculation and removing the entire nasal septum and 1 cm of trachea. These tissues were then homogenized in 200 μl of sterile PBS, and various dilutions of the homogenates were plated on BG agar containing streptomycin to determine the number of CFU.

RESULTS

Identification of BcfA, a parolog of BipA, in *B. bronchiseptica*. It was reported previously that a BipA-deficient strain of *B. bronchiseptica* colonized the rabbit respiratory tract as efficiently as the wt strain, suggesting that this protein is not essential for colonization (32). Since bordetellae express multiple proteins that have either been demonstrated or been predicted to be involved in attachment to the respiratory epithelium (25), we hypothesized that the absence of BipA in *B. bronchiseptica* could be compensated for by either a known or a previously unidentified alternative factor, resulting in no apparent effect on colonization. In particular, we explored the possibility of a paralogous protein in *B. bronchiseptica*. We searched the recently sequenced *B. bronchiseptica* genome database for ORFs homologous to *bipA*. BLASTP (2) searches revealed the presence of an ORF (BB0110) whose product exhibited 49% identity in the N-terminal 809 amino acids to the BipA protein. In addition, these searches also revealed similarity of BB0110 to genes encoding intimins in EPEC and EHEC (8, 13), invasins in *Yersinia* species (17), and the putative *E. coli* adhesin EaeH (accession number AAZ57201). In the annotated *B. bronchiseptica* genome database, the protein encoded by BB0110 has been designated a putative adhesin, and based on our finding that this protein plays a role in respiratory tract colonization (see below), we designated this ORF *bcfA* (*b*ordetella *c*olonization *f*actor *A*).

***B. bronchiseptica* BcfA protein.** The *bcfA* ORF has the potential to code for a 969-amino-acid protein with a predicted molecular mass of 102 kDa (Fig. 1). The BipA protein of *B. bronchiseptica* is larger, having 1,578 amino acids and a predicted molecular mass of 164.5 kDa (Fig. 1). Analysis of the predicted BcfA protein sequence by the SignalP 3.0 method (3) revealed that it contains an unusually long putative signal sequence consisting of 44 amino acids (signal peptide probability, 0.942) (Fig. 1), including the characteristic positively charged N region, the hydrophobic core H region, and the C region with a consensus cleavage site for a Sec-dependent leader peptide. The predicted cleavage site of the signal sequence is located between the Ala44 and Gln45 residues (cleavage site probability, 0.928). The presence of a canonical signal sequence in BcfA suggests that this protein can traverse the inner membrane.

Expression of *bcfA* is regulated by BvgAS in *B. bronchiseptica*. The BvgAS locus regulates the expression of the majority of virulence genes in *Bordetella* (25). The similarity of *bcfA* to *bipA*, which is transcriptionally activated by BvgAS (11, 32), prompted us to determine whether BvgAS controls *bcfA* expression. We generated a *bcfA-lacZ* fusion and integrated it into different strains of *B. bronchiseptica* by homologous recombination (see Materials and Methods). Measurement of the β-galactosidase activities of log-phase cultures of these strains indicated that while *bcfA* was expressed at high levels in the wt strain, it was expressed at low levels in the Bvg^- phase-locked strain (Fig. 2). Growth of the wt strain in the presence of $MgSO_4$, a known modulator of BvgAS activity, resulted in very low levels of β-galactosidase activity (Fig. 2). Together, these results suggest that expression of *bcfA* is positively regulated by BvgAS.

***bcfA* and *bipA* have distinct Bvg phase-dependent expression profiles.** Previously, we and others have shown that the various Bvg-regulated genes exhibit differences in their phase-dependent expression profiles (9, 11). In contrast to other known Bvg-regulated genes, *bipA* is expressed maximally in the Bvg^i phase and at low levels in the Bvg^+ phase (11). To compare the

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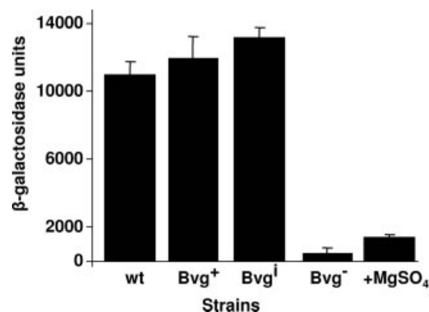

FIG. 2. Quantification of *bcfA* transcriptional activity in the wt, Bvg^+, Bvg^i, and Bvg^- phase-locked strains of *B. bronchiseptica* by β-galactosidase assays. The *bcfA-lacZ* fusion was integrated in the various strains, and the β-galactosidase activity was determined after growth to the mid-log phase in SS broth at 37°C. To modulate the BvgAS activity, the wt strain carrying the *bcfA-lacZ* fusion was grown in the presence of 40 mM $MgSO_4$. The error bars indicate standard deviations.

phase-dependent expression profile of *bcfA* directly with those of *bipA* and other Bvg-regulated genes, we performed real-time RT-PCR assays (Materials and Methods). As shown in Fig. 3A, *bcfA* was expressed at high levels in both the Bvg^+ and Bvg^i phases. *bcfA*-specific mRNA was barely detectable in the Bvg^- phase-locked strain, confirming the results of the β-galactosidase assays described above. Strikingly, these results demonstrate that the paralogs *bcfA* and *bipA* have distinct Bvg-dependent expression profiles, since the level of expression of *bipA* is maximal in the Bvg^i phase and low in the Bvg^+ phase (Fig. 3B) (10, 11). Our data also suggest that the expression profile of *bcfA* is similar to the phase-specific expression pattern of the previously described Bvg-activated gene *fhaB* (Fig. 3C) (5, 9). As expected based on previous results (9, 11), *cyaA* and *flaA* were expressed maximally only in the Bvg^+ and Bvg^- phases (Fig. 3D and E), respectively, further corroborating the validity of the real-time RT-PCR assays for accurately measuring the expression patterns of Bvg-regulated genes.

We independently confirmed the phase-dependent expression pattern of *bcfA* by measuring the β-galactosidase activities in log-phase cultures of the Bvg^+ and Bvg^i phase-locked strains. Our results (Fig. 2) indicate that the level of expression of *bcfA* is high in both the Bvg^+ and Bvg^i phases.

Kinetics of transcriptional activation of *bipA* and *bcfA*. Previous studies revealed the differential kinetics of expression of *bvg*-regulated genes upon induction of the BvgAS activity either by a temperature shift (25°C to 37°C) or by growth in the presence of chemical modulators followed by growth in the absence of these modulators (18, 20). We examined the transcriptional activation of *bcfA* in *B. bronchiseptica* over time following induction of the BvgAS system. RB50 cells were modulated by first growing them in the presence of 40 mM $MgSO_4$ and then growing them in medium without $MgSO_4$ for induction of the BvgAS system. After 0, 5, 30, 60, and 240 min of induction, total RNA was isolated from cells, and the kinetics of promoter activation were determined by RT-PCR using primers specific for *fha*, *prn*, *bipA*, and *bcfA*. We also utilized primers specific for a *bvg*-independent gene, *recA*, as a normalization standard. The RT-PCR assays revealed that *fhaB* and *bipA* were reproducibly activated as early as 5 min after induction and that *prn* was transcribed after 30 min of induction (Fig. 4). In accordance with our results, it has been shown previously that the expression of *fhaB* and *bipA* was activated almost immediately after a switch to inducing conditions, whereas there was a delay in the expression of *prn* (18, 20). Our results clearly demonstrate that transcriptional activation of *bcfA* was reproducibly observed within 5 min during growth under inducing conditions, suggesting that the activation kinetics of this gene parallel the activation kinetics of *fhaB* and *bipA*

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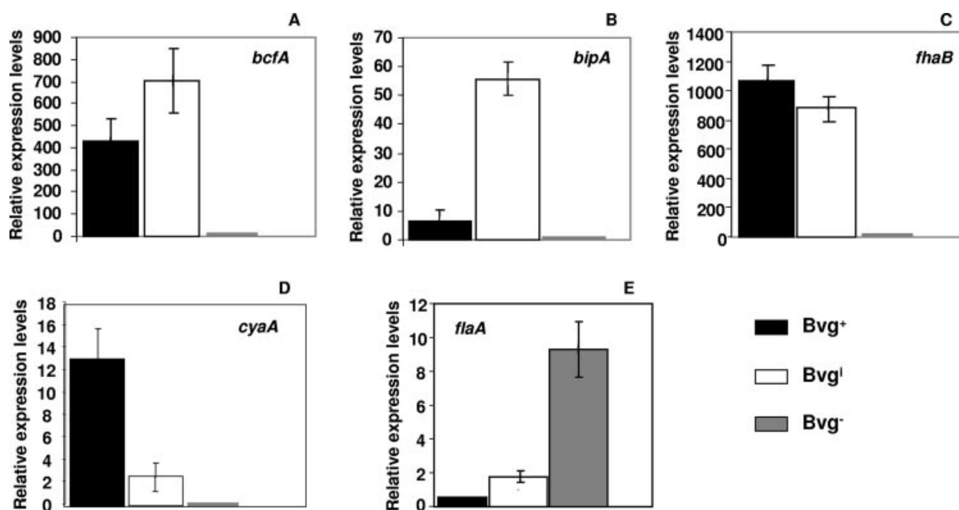

FIG. 3. Determination of the phase-dependent expression profiles of different Bvg-regulated genes in *B. bronchiseptica* by real-time RT-PCR analysis. cDNA prepared from various strains was used as a template for real-time RT-PCR as described in Materials and Methods. The ΔC_T values were determined by subtracting the *recA* C_T value from the gene-specific C_T values. The $\Delta\Delta C_T$ value for each gene was obtained by subtracting the ΔC_T value of either the Bvg^- phase-locked strain (for *bcfA*, *bipA*, *fhaB*, and *cyaA*) or the Bvg^+ phase-locked strain (for *flaA*) from the gene-specific ΔC_T value. The relative levels of expression of genes (*y* axes) were then calculated by using $2^{-\Delta\Delta C_T}$. Thus, for *bcfA*, *bipA*, *fhaB*, and *cyaA* the relative level of expression in the Bvg^- phase-locked strain was 2^0 or 1. Similarly, the level of expression of *flaA* in the Bvg^+ phase-locked strain was also 1. The bars indicate standard deviations for three independent values obtained from two different RNA batches.

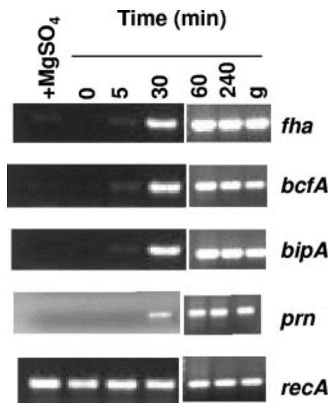

FIG. 4. Kinetics of transcriptional activation of different Bvg-activated genes and the Bvg-independent gene *recA*. The wt strain was grown in liquid cultures under modulating conditions (in the presence of 40 mM $MgSO_4$) for 18 h, spun, and resuspended in medium without $MgSO_4$. Total RNA was isolated at the indicated times, and cDNA was prepared as described in Materials and Methods. RT-PCR was used to detect the levels of transcripts of the genes indicated on the right. g, RB50 genomic DNA.

(Fig. 4). No detectable PCR products were obtained in the absence of RT, indicating that contaminating DNA was not present in the RNA preparation (data not shown).

Identification of BvgA box-like sequences upstream of the *bcfA* ORFs. Scanning of the sequences upstream of the predicted translational start site of the *bcfA* ORF revealed the presence of two directly joined inverted repeats (IR2 and IR3) (Fig. 5) that were similar to previously identified BvgA binding sites (5, 7, 11, 28). In addition to the conjoined inverted repeats, we also detected overlapping inverted repeat sequences (IR1) in which the two halves were separated by multiple nucleotides (Fig. 5). For some Bvg-regulated promoters it has been shown that BvgA binding repeats are not conjoined but are separated by multiple nucleotides (2 nucleotides for *cyaA*, 10 nucleotides for *ptx* encoding pertussis toxin, and 27 and 37 nucleotides for *bipA* [6, 10, 11, 19]). The presence of predicted BvgA binding sites upstream of the ORF, combined with our finding that *bcfA* is positively regulated by BvgAS, suggests that the BvgAS control of *bcfA* expression is direct.

BvgA and BvgA-P bind to the promoter region of *bcfA*. The presence of putative BvgA binding elements upstream of the *bcfA* ORF led us to speculate that BvgA binds the *bcfA* promoter. To demonstrate BvgA occupancy of the *bcfA* promoter, we performed an EMSA by utilizing a PCR fragment that was similar to the fragment used for β-galactosidase assays (see above and Materials and Methods). Purified recombinant BvgA and BvgA phosphorylated in vitro with acetyl phosphate (BvgA-P) were used for this analysis. The reaction mixtures also contained poly(dI-dC) as a nonspecific competitor DNA. The results of the EMSA are shown in Fig. 6A. Both BvgA and BvgA-P reduced the mobility of the radiolabeled DNA fragment. As a result of phosphorylation, BvgA occupancy of the radiolabeled DNA fragment resulted in higher-order protein-DNA complexes, which suggested that there were multiple binding sites or multimerization of BvgA as a result of phosphorylation (Fig. 6A, compare lanes 2 to 6 with lanes 8 to 12). In order to confirm the specificity of BvgA binding, we also performed competition EMSAs with different concentrations of specific and nonspecific competitors (see Materials and Methods). There was a gradual loss of BvgA binding activity as the concentration of the specific competitor increased (Fig. 6B). In contrast, in the presence of the nonspecific competitor, there was no significant loss of binding affinity of BvgA-P (Fig. 6C). Therefore, these results suggest that the interaction of BvgA-P with the *bcfA* promoter is DNA sequence specific.

BcfA is an outer membrane protein. Due to the homology of BcfA to BipA, particularly in the N-terminal region (Fig. 1), we hypothesized that BcfA is also localized to the outer membrane. We constructed two isogenic strains, RKD110 (Δ*bcfA*) and the double-deletion strain MM101 (Δ*bipA* Δ*bcfA*). These strains have in-frame deletions of the genes that are nonpolar. Notably, these strains are comparable to the wt strain with respect to growth in laboratory cultures (data not shown). We purified the outer membranes from RB50 (wt), RKD110 (Δ*bcfA*), RB25 (Δ*bipA*), and MM101 (Δ*bipA* Δ*bcfA*) as Triton X-100-insoluble fractions (see Materials and Methods). To facilitate detection of BcfA, polyclonal sera were raised against a BcfA-T7-tagged fusion protein purified from *E. coli* (see Materials and Methods). Using Western blotting with anti-BcfA antibody, we detected a polypeptide that migrated at

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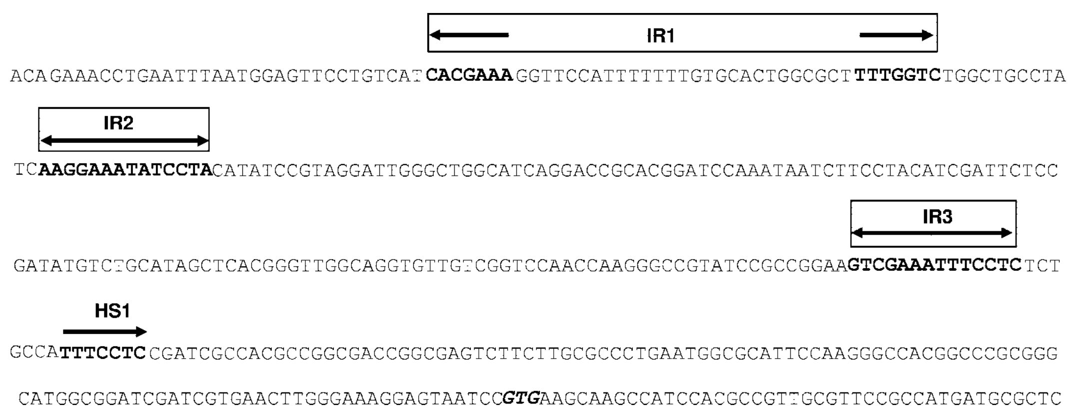

FIG. 5. Putative promoter region of *bcfA*. The arrows above the DNA sequences indicate sequence elements (bold type) that are similar to the consensus BvgA binding site. The predicted translational start codon is italicized. IR, inverted repeat. The boxes indicate regions corresponding to different inverted repeats. HS1 is the half-site.

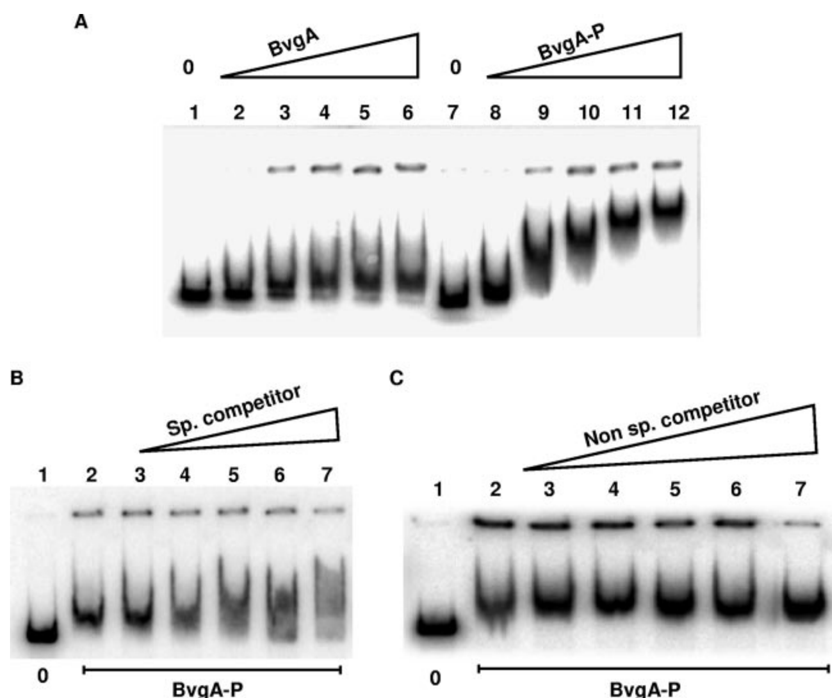

FIG. 6. EMSA. (A) A^{32}P-end-labeled *bcfA* promoter fragment was incubated with various concentrations of either BvgA (lanes 2 to 6) or BvgA-P (lanes 8 to 12). The DNA-protein complexes were separated by electrophoresis on a 4% nondenaturing polyacrylamide gel and visualized by autoradiography. Lanes 1 and 7, DNA alone; lanes 2 to 6, 0.12, 0.6, 1.2, 1.8, and 2.4 μg of BvgA, respectively; lanes 8 to 12, same as lanes 2 to 6 except that 20 mM acetyl phosphate was added to the reaction mixtures. (B) Specific competition (lanes 3 to 7) was carried out using a 10-, 50-, 100-, 300-, and 600-fold excess of unlabeled *bcfA* promoter fragment, respectively. Lane 1, DNA alone; lanes 2 to 7, 1.8 μg of BvgA-P. The reactions were carried out in the presence of 20 mM acetyl phosphate. Sp. competitor, specific competitor. (C) Nonspecific competition (lanes 3 to 7) was performed with a 10-, 50-, 100-, 200-, and 800-fold excess, respectively, of a 525-bp fragment encompassing a region corresponding to the *bcfA* ORF. Lane 1 contained DNA alone; lanes 2 to 7 contained 1.8 μg of BvgA-P. The reactions were carried out in the presence of 20 mM acetyl phosphate. Non sp. competitor, nonspecific competitor. Note that for panels B and C, the gels were run for a shorter time.

about 100 kDa, which corresponded to the migration position of purified recombinant BcfA, in the outer membrane fractions isolated from the wt and RB25 (Δ*bipA*) strains (Fig. 7A). Note that this polypeptide was not detected in the outer membrane fractions isolated from RKD110 (Δ*bcfA*) and MM101 (Δ*bipA* Δ*bcfA*), confirming that the polyclonal serum specifically recognized BcfA. Western blot analysis of the purified recombinant BcfA protein consistently revealed two bands, one, around 100 kDa, corresponding approximately to the predicted size of the BcfA protein and one higher-molecular-mass polypeptide which migrated at more than 180 kDa, suggesting that it may be a dimeric form of BcfA. The higher-molecular-weight band was not observed with outer membrane fractions (Fig. 7A), suggesting that it might also have been an artifact of the purification procedure.

As a positive control for a known outer membrane protein from *Bordetella*, we probed for BipA. A previously described anti-BipA antibody (32) raised against the C terminus of BipA recognized a polypeptide present in the outer membrane fractions from the wt and RKD110 (Δ*bcfA*) strains but not in the outer membrane fractions from the RB25 (Δ*bipA*) and MM101 (Δ*bipA* Δ*bcfA*) strains (Fig. 7B).

BcfA is expressed during the *Bordetella* infectious cycle in rats. To evaluate whether BcfA is expressed during infection, we used sera from rats infected for 30 days with the wt, RKD110 (Δ*bcfA*), RB25 (Δ*bipA*), and MM101 (Δ*bipA* Δ*bcfA*) strains as probes to detect the purified BcfA protein in Western blot assays. While the sera from the wt and RB25 (Δ*bipA*) strain-infected rats recognized the purified BcfA protein, sera from the RKD110 (Δ*bcfA*) and MM101 (Δ*bipA* Δ*bcfA*) strain-infected rats (Fig. 8) showed no significant reactivity. Interest-

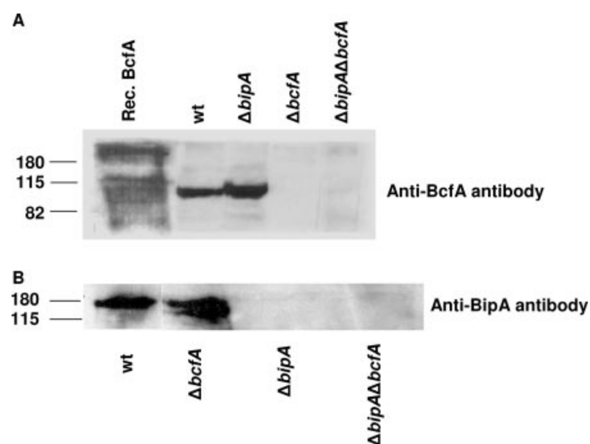

FIG. 7. BcfA is localized to the outer membrane. Outer membrane proteins were prepared by cellular fractionation of strains with the indicated genotype and were subjected to Western blot analysis using polyclonal antibodies raised against purified *B. bronchiseptica* BcfA (A) and BipA (B). The molecular masses of the protein markers (in kDa) are indicated on the left. The purified recombinant BcfA protein overexpressed in *E. coli* was loaded in lane Rec.BcfA.

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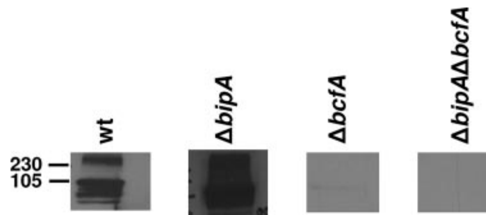

FIG. 8. BcfA is expressed during infection. The purified recombinant BcfA protein was subjected to SDS-polyacrylamide gel electrophoresis and Western blot analysis using serum collected 30 days postinoculation from rats infected with strains with the indicated genotype. The molecular masses of the protein markers (in kDa) are indicated on the left.

ingly, the higher-molecular-weight peptide that was observed in immunoblots with purified BcfA using anti-BcfA sera (Fig. 7A) was not recognized by sera from RKD110 (Δ*bcfA*)- and MM101 (Δ*bipA*Δ *bcfA*)-infected rats, suggesting that this polypeptide might be a higher-molecular-weight form (possibly a dimer) of BcfA (Fig. 8). In addition to sera from rats infected with the wt strain for 30 days, sera collected from rats infected with the wt strain for 12 days also recognized purified BcfA (data not shown).

Overlapping functions of BipA and BcfA in colonization of the lower respiratory tract. The similarity of BcfA to BipA and the failure to detect previously a function for *bipA* in colonization of the upper respiratory tract (32) led us to hypothesize that BcfA either alone or in combination with BipA plays a role in respiratory tract colonization. Groups of six female Wistar rats were separately inoculated intranasally with the wt, RB25 (Δ*bipA*), RKD110 (Δ*bcfA*), and MM101 (Δ*bipA* Δ*bcfA*) strains. For intranasal inoculation, we utilized the well-studied and frequently utilized low-volume, low-inoculum protocol (1, 15). At 12 and 30 days postinoculation, animals were sacrificed, and the levels of colonization were determined by removing the entire nasal septum and 1 cm of the trachea. Each tissue was homogenized in 200 μl of PBS, aliquots were diluted, and viable colonies were determined by plating homogenates on BG agar containing blood.

Consistent with previously described results (1, 15), high numbers of bacteria (range, 10^4 to 10^6 CFU) were recovered from the nasal septa and tracheas of the wt strain-inoculated animals at both times (Fig. 9). Although some animals (one animal each for the nasal septum at 12 days and 30 days and one animal for the trachea at 12 days) were not colonized by the RB25 (Δ*bipA*) strain, there was not a statistically significant difference in the mean colonization levels as a result of deletion of *bipA*. Note that the failure of the RB25 (Δ*bipA*) strain to elicit a significant colonization defect in rats is consistent with results reported previously for rabbits (32). Similarly, the difference in mean colonization levels for the wt strain and the RKD110 (Δ*bcfA*) strain was not statistically significant at any of the times tested. In contrast to the individual deletions of *bipA* and *bcfA*, combined deletion of these two genes resulted in a drastic defect in the colonization of the trachea at both the early and late times (Fig. 9). Notably, for five rats at 12 days and for four rats at 30 days, the number of CFU recovered from the trachea was either at or below the lower limit of detection (Fig. 9). At 12 days, three animals were not colonized in the nasal septum with the double-deletion strain, but this defect in nasal colonization was not apparent at 30 days, when all six animals were colonized, and the mean level of coloni-

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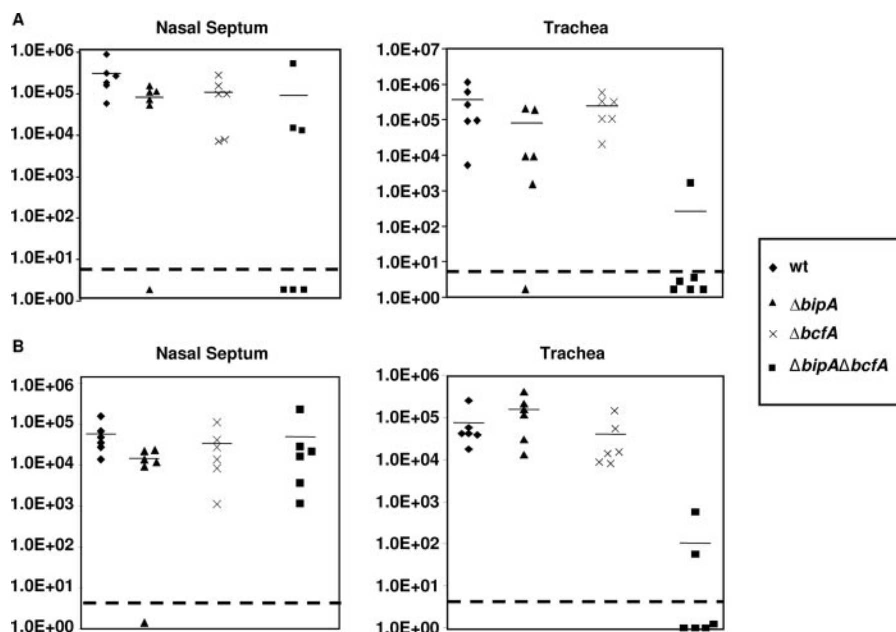

FIG. 9. Colonization of the rat respiratory tract by the wt strain and the isogenic mutant derivatives RB25 (Δ*bipA*), RKD110 (Δ*bcfA*), and MM101 (Δ*bipA* Δ*bcfA*) 12 days (A) and 30 days (B) postinoculation. Four- to five-week-old female Wistar rats were inoculated with the strains. The entire nasal septum and 1 cm of the trachea were harvested 12 days and 30 days postinoculation and homogenized, aliquots were plated, and CFU were enumerated as described in Materials and Methods. The horizontal bars indicate means for the groups. The dashed lines indicate the lower limit of detection.

zation at this time was not significantly different than that of the wt strain. These results suggest that BipA and BcfA have overlapping roles in colonization of the trachea.

DISCUSSION

Efficient colonization of the mammalian respiratory tract by *Bordetella* occurs due to production of a collection of virulence factors, including a wide variety of adhesins and toxins. Because of the presence of multiple potential adhesin molecules, it is easy to envision that there is redundancy in the adherence mechanisms of *Bordetella*. One or more adhesin molecules can mediate their activities by acting either in a concerted manner or synergistically. In this study, we identified a *Bordetella* gene, *bcfA*, which is a paralog of the previously identified *Bordetella* gene *bipA* (32). We were encouraged to search for a gene homologous to *bipA* because a previous study (32) failed to identify a role for BipA in respiratory tract colonization. With the aid of BLAST searches, we detected an ORF in *B. bronchiseptica* that exhibits significant similarity to BipA. Analysis of the BcfA amino acid sequence revealed that the most significant levels of sequence identity of the N-terminal region were the levels of sequence identity with the corresponding regions of BipA and intimins of EPEC and EHEC and invasins of *Yersinia* species. The N-terminal region in intimins and invasins is required for outer membrane localization and for export of the carboxyl termini of these proteins (21, 22). Western blot analysis of the outer membrane fractions demonstrated that BcfA, like BipA, is localized to the outer membrane.

The BvgAS signal transduction system is the principal regulator of virulence gene expression in the three classical *Bordetella* species. We investigated the Bvg-mediated control of *bcfA* expression in *B. bronchiseptica*. Our data show that BvgAS positively regulates the expression of *bcfA*. We and other workers have previously reported that *bipA* is expressed maximally in the Bvg^i phase (11, 32). Using real-time RT-PCR and β-galactosidase assays to quantify the expression of *bcfA* in different phenotypic phases, we found that in contrast to *bipA*, *bcfA* is expressed maximally in both the Bvg^+ and Bvg^i phases. In this respect, the expression profile of *bcfA* is similar to that of the gene encoding another well-studied *Bordetella* adhesin, FHA (Fig. 3) (9, 10). Our kinetic transcription activation results indicated that *bipA* and *bcfA* are activated concurrently after the initiation of an inducing signal. Although the results of these two experiments seem contradictory at first, it should be noted that they represent two different ways in which the transition between the different activation states of BvgA can occur. In the phase-locked mutant strains, the BvgAS system is locked in its different activation states (ranging from maximal activation [Bvg^+ phase] to submaximal activation [Bvg^i phase] to minimal activation [Bvg^- phase]). In contrast, when bordetellae are grown overnight in the presence of chemical modulators and then in the absence of the modulators, the activity of BvgA changes from minimal activation at early times to a maximal activation at late times. Thus, we hypothesized that genes that require a low concentration of BvgA-P to be activated are turned on first. While there are obvious differences in the arrangements and the affinities of various BvgA binding sites of *fhaB*, *bipA*, and *bcfA*, a common feature of the promoters of all these genes is the presence of a high-affinity BvgA binding site in the form of inverted heptanucleotide repeats centered at position −88.5 for *fha* (5), at IR1 for *bipA* (10, 11), and at IR2 for *bcfA* (Fig. 5) (unpublished results). Indeed we and other workers have demonstrated that under in vitro conditions using purified BvgA and RNA polymerase, activation of *fhaB* and *bipA* occurs at a relatively low concentration of BvgA-P (5, 18, 29). We hypothesize that similar to *fhaB* and *bipA*, *bcfA* requires a low concentration of BvgA-P to be activated in vitro, and we are currently testing this hypothesis.

Encouraged by the sequence identity of BcfA with BipA and other bacterial adhesins, we investigated whether BcfA singly or together with BipA plays a role in respiratory tract colonization. We utilized both single- and double-deletion mutants. Our results obtained with an intranasal rat model of colonization showed that no significant demonstrable phenotype for respiratory tract colonization resulted from deletion of *bipA* or *bcfA* individually. However, a deficiency of both BipA and BcfA resulted in a severe defect in tracheal colonization, suggesting that BipA and BcfA have collective and/or overlapping functions in the *Bordetella* life cycle in the host.

These findings raise an important question concerning the function of these two proteins in *Bordetella* infections. The simplest explanation is that these proteins act as adhesins, i.e., that they are directly involved in attachment to the respiratory epithelium. By utilizing epithelial cell lines and tracheal explants, we are currently testing whether BcfA is directly involved in attachment. It is also possible that the defect in colonization resulting from gene deletions is not dependent directly on adherence to respiratory epithelia and that BipA and BcfA are involved in modulating the components of the innate and/or adaptive immune systems. In this context, it is important to note that the major *Bordetella* adhesin FHA plays an accessory role in down-regulating the innate immune responses, resulting in increased bacterial persistence (16, 26, 27).

In conclusion, additional studies to determine the mechanistic basis of the role of BipA and BcfA in the *Bordetella* life cycle in the host organisms should provide important insights for understanding the multipartite nature of *Bordetella*-host interactions. Additionally, studies to determine the biochemical basis of the BvgAS-mediated control of the two genes could result in a clear and detailed understanding of how a single regulatory locus directs the observed variations in signal-dependent gene expression patterns.

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