Interspecies Variations in *Bordetella* Catecholamine Receptor Gene Regulation and Function

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Bordetella bronchiseptica can use catecholamines to obtain iron from transferrin and lactoferrin via uptake pathways involving the BfrA, BfrD, and BfrE outer membrane receptor proteins, and although *Bordetella pertussis* has the *bfrD* and *bfrE* genes, the role of these genes in iron uptake has not been demonstrated. In this study, the *bfrD* and *bfrE* genes of *B. pertussis* were shown to be functional in *B. bronchiseptica*, but neither *B. bronchiseptica bfrD* nor *bfrE* imparted catecholamine utilization to *B. pertussis*. Gene fusion analyses found that expression of *B. bronchiseptica bfrA* was increased during iron starvation, as is common for iron receptor genes, but that expression of the *bfrD* and *bfrE* genes of both species was decreased during iron limitation. As shown previously for *B. pertussis*, *bfrD* expression in *B. bronchiseptica* was also dependent on the BvgAS virulence regulatory system; however, in contrast to the case in *B. pertussis*, the known modulators nicotinic acid and sulfate, which silence Bvg-activated genes, did not silence expression of *bfrD* in *B. bronchiseptica*. Further studies using a *B. bronchiseptica bvgAS* mutant expressing the *B. pertussis bvgAS* genes revealed that the interspecies differences in *bfrD* modulation are partly due to BvgAS differences. Mouse respiratory infection experiments determined that catecholamine utilization contributes to the *in vivo* fitness of *B. bronchiseptica* and *B. pertussis*. Additional evidence of the *in vivo* importance of the *B. pertussis* receptors was obtained from serologic studies demonstrating pertussis patient serum reactivity with the *B. pertussis* BfrD and BfrE proteins.

Bordetella pertussis, *Bordetella parapertussis*, and *Bordetella bronchiseptica* are Gram-negative bacterial respiratory pathogens that have been termed the classical species of *Bordetella*. *B. pertussis* is a strict human pathogen that causes whooping cough (pertussis), which has been increasing in frequency (1–4). *B. parapertussis* causes a pertussis-like infection in humans and can also infect sheep, whereas *B. bronchiseptica* infects a range of nonhuman mammalian hosts as well as immunocompromised humans. The classical species of *Bordetella* are obligate pathogens that colonize the ciliated respiratory epithelium of the host, where they replicate and produce multiple virulence factors that promote their successful *in vivo* growth and cause symptoms of disease. Many genes, including most of the known *Bordetella* virulence factor genes, are positively regulated at the transcriptional level by the BvgAS two-component regulatory system (4–6).

Like virtually all other microbial pathogens, *Bordetella* species require iron for growth (7–9). Gram-negative bacteria generally rely on the TonB system to supply the energy for receptor-mediated transport of various iron sources across the bacterial outer membrane to the periplasm (10). *B. bronchiseptica* has 19 genes encoding known or predicted TonB-dependent outer membrane receptors, *B. pertussis* has 15 such genes, and *B. parapertussis* has 14 of the receptor genes (11, 12). In response to iron starvation, the classical bordetellae, including *B. pertussis* and *B. bronchiseptica*, produce and use the alcaligin siderophore for iron retrieval (13, 14) and can also use xenosiderophores, including enterobactin, produced by members of the *Enterobacteriaceae* (15); the fungal siderophore ferrichrome; and desferrioxamine B, produced by *Streptomyces* spp. (16). *Bordetella* species also use heme iron sources (17–19). Recently, we found that *B. bronchiseptica* can use neuroendocrine catecholamine hormones, such as norepinephrine, to obtain iron from the host glycoproteins transferrin and lactoferrin (20). The TonB-dependent outer membrane receptor proteins BfrA, BfrD, and BfrE were each determined to be involved in catecholamine-mediated utilization of transferrin

iron. Each of these receptors also functioned in utilization of transferrin iron via both enterobactin and its breakdown product, 2,3-dihydroxybenzoylserine, in *B. bronchiseptica*.

Although the classical *Bordetella* species are highly genetically related, *B. pertussis* and *B. parapertussis* do not have the *bfrA* catecholamine receptor gene. *B. pertussis* has apparently intact *bfrD* and *bfrE* genes; however, its ability to use catecholamines to obtain iron from either transferrin or lactoferrin has not yet been demonstrated successfully (20). *B. parapertussis* has *bfrD* and an apparent *bfrE* pseudogene; the ability of this species to use norepinephrine to obtain iron from transferrin was modest, and it was unable to use norepinephrine to obtain iron from lactoferrin. The *bfrD* and *bfrE* genes lie adjacent to each other on the *Bordetella* chromosome, and *bfrD* appears to have arisen by duplication of *bfrE* (20). The *bfrD* and *bfrE* nucleotide sequences are 56.7% similar (21), and the deduced proteins have 57.5% pairwise identity. Although its involvement in iron acquisition was unknown at the time, BfrD was first identified in a study of outer membrane proteins that were produced in wild-type Bvg$^+$ virulent-phase *B. pertussis* but were not produced by a *bvgS* mutant strain (22). A *B. pertussis* mutational analysis later showed that *bfrD* was transcriptionally activated by BvgAS and that its expression was decreased under modulating *in vitro* growth conditions that used nicotinic

TABLE 1 *Bordetella* strains used in this study

Strain	Relevant genotype or description	Source or reference(s)
B. pertussis strains		
BP338	Tohama I derivative, clinical isolate, ca. 1954	5, 65
BP347	BP338; *bvgS* transposon mutant	5
UT25Sm1	UT25; streptomycin resistant; clinical isolate, 1975	66, 67
PM29	UT25Sm1 with integrated chromosomal *bfrD-lacZ* fusion	This study
PM30	UT25Sm1 with integrated chromosomal *bfrE-lacZ* fusion	This study
PM21	UT25Sm1 with in-frame deletion in *bfrD*	This study
PM22	UT25Sm1 with deletion of *bfrD* and *bfrE*	This study
B. bronchiseptica strains		
RB50	Rabbit isolate; streptomycin resistant	68
RB52	RB50 *bvgAS* deletion mutant derivative containing *bvgAS* from *B. pertussis* BP338	52
RB54	RB50 *bvgS* deletion mutant derivative	68
RBB20	RB50 *bfrA bfrDE* triple mutant; in-frame deletion in *bfrA* and deletion in *bfrD-bfrE*	This study
B013N	Swine isolate; nalidixic acid resistant	69, 70
BRM26	B013N derivative; Δ*alcA*; alcaligin deficient	71

acid or sulfate to silence expression of Bvg-activated genes (21). Since *B. pertussis* BfrD was recently proposed as a potential pertussis vaccine candidate (23), more information about its function in *B. pertussis* would be useful.

In this characterization of the *Bordetella* catecholamine receptors, our analyses revealed that in these organisms, *bfrA* and *bfrE* transcriptional activities were modest, and *bfrD* expression appeared to be elevated under iron-replete growth conditions. In *B. bronchiseptica*, *bfrD* transcription was activated by Bvg but was relatively insensitive to modulation compared to the case in *B. pertussis*. We investigated the functionality of the *B. pertussis* BfrD and BfrE receptor proteins and demonstrated the involvement of catecholamine receptors in *Bordetella* growth during host respiratory infection.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *Bordetella* strains used in this study are listed in Table 1. *Bordetella* strains were cultured on Bordet-Gengou (BG) agar (1), and *Escherichia coli* strains were grown using Luria-Bertani broth or agar. Stainer-Scholte (SS) defined medium (24, 25) was used for *Bordetella* liquid cultures, and *B. pertussis* SS cultures were supplemented with 0.1 or 0.5% Casamino Acids. Glassware was acid cleaned and rinsed in distilled deionized water prior to use. Iron-replete SS medium contained 36 μM ferrous sulfate, and iron-depleted SS medium lacked an iron supplement and was deferrated using Chelex 100 resin (Bio-Rad Laboratories, Hercules, CA). In some studies, modulation conditions were achieved by addition of nicotinic acid or magnesium sulfate to SS medium at the indicated concentrations. Antibiotics were used as appropriate, at the following concentrations: tetracycline, 15 μg/ml; ampicillin, 100 μg/ml; gentamicin, 10 μg/ml; kanamycin, 50 μg/ml; and streptomycin, 20 μg/ml. Growth was monitored spectrophotometrically at a wavelength of 600 nm.

General genetic procedures. Standard methods were used for construction of recombinant plasmids (26). Reaction mixtures for PCR contained 5% dimethyl sulfoxide. Oligonucleotide primers were obtained from Integrated DNA Technologies (Coralville, IA). Plasmids were transferred to *E. coli* strains by electroporation and to *Bordetella* strains by conjugation using *E. coli* DH5α as the donor strain, with plasmid pRK2013 providing mobilization functions (27). Plasmid pRK415 (28) was used as a cloning vector for *Bordetella* strains. pGEM3Z (Promega, Madison, WI) and pBlueScript II KS(+) (Agilent Technologies, Santa Clara, CA) were used as cloning vectors for *E. coli*.

The *Bordetella* genome sequences (http://www.ncbi.nlm.nih.gov /nucleotide) (*B. pertussis* Tohama I, accession no. NC_002929; *B. parapertussis*, accession no. NC_002928; and *B. bronchiseptica* RB50, accession no. NC_002927) were accessed at the GeneDB website (http://www .genedb.org/), developed and maintained by the Sanger Institute's Pathogen Sequencing Unit. DNA and protein analyses and PCR primer design used the Geneious, version 6.1.8, software package (Biomatters Ltd., Auckland, New Zealand).

Cloning of *Bordetella* receptor genes. The *Bordetella* *bfrD* and *bfrE* genes, along with their native upstream sequences, were cloned into pRK415. *bfrD* was PCR amplified from the *B. pertussis* UT25 genomic DNA template by using primers bfrD-up (5′-CCCCAAGCTTAGCGCC AATCTGCCGTTTCTCA-3′) and bfrD-low (5′-CCCCTGCAGTATCCA GCAAGGGCGCCGTCATCT-3′). The 3.2-kb product was cloned into pBlueScript II KS(+) as a HindIII-PstI fragment, yielding plasmid pBKS+20, and then subcloned into pRK415, producing plasmid pRK71. Similarly, *B. pertussis* *bfrE* was PCR amplified using primers bfrE-up (5′-CCCCAAGCTTCAACGCAGATGCCGGCTACAACAA-3′) and bfrE-low (5′-CCCCTCTAGACCCGGGTGGCTCGATGGCAAACA-3′), cloned as a 2.8-kb HindIII-XbaI fragment into pBlueScript II KS(+), and subcloned into pRK415 (pRK72). *B. bronchiseptica* *bfrD* was PCR amplified using primers bfrD-up and bfrD-low, and the 3.2-kb product was cloned into pGEM3Z as a HindIII-PstI fragment and then subcloned into pRK415, resulting in plasmid pRK66. Construction of plasmid pRK67, containing *B. bronchiseptica* *bfrE*, was achieved by PCR amplification using primers bfrE-up and bfrE-low, cloning of the 2.8-kb product into pGEM3Z as a HindIII-XbaI fragment, and subcloning into pRK415.

Construction and analysis of transcriptional *lacZ* fusions. Low-copy-number plasmid-borne *B. bronchiseptica* *bfrA*, *bfrD*, and *bfrE* promoter fusions to *lacZ* were constructed using the gene fusion plasmid vector pMP220 (29). A 3.3-kb DNA fragment encompassing the predicted promoter region of *bfrA* was amplified by PCR, using primers bfrA-A (5′-GGCCAAGCTTGTGCGGCACGACCGGTTTTGACG-3′) and bfrA-B (5′-GGCCAAGCTTGCAGCCCGCGGTGGTGGAGAAG-3′), and cloned into pBlueScript II KS(+) by using the primer HindIII adapter sites. A 1.1-kb BamHI-PstI DNA subfragment was cloned into a pGEM3Z intermediate and then excised with KpnI-PstI and subcloned into pMP220 to produce the *bfrA-lacZ* fusion plasmid pMP6. Primers bfrD-up and bfrD-low were used to PCR amplify a 3.2-kb product containing the *B. bronchiseptica* RB50 *bfrD* region. Digestion of the 3.2-kb product with HindIII and EcoRI yielded a 930-bp DNA fragment that was cloned (pBKS+15) and subsequently subcloned into pMP220 as a KpnI-PstI fragment to produce pMP7. The *B. bronchiseptica* *bfrE* promoter region was cloned into pMP220 as a 238-bp

PCR product (amplified with primers PbfrE3 [5′-CCCGAATTCATGCT GACGTTCAAGCTGAG-3′] and PbfrE4 [5′-CCCCTGCAGAAAAATCT GAATAACAACGATTC-3′]), using EcoRI and PstI primer adapters, to yield plasmid pMP8. A *cyaA-lacZ* gene fusion plasmid was constructed by PCR amplification of the *B. pertussis* UT25 *cyaA* promoter region (30), using primers cyaP3 (5′-GGCCGAATTCGAGTTCGGTGTCGGCGTCC ATTAG-3′) and cyaP4 (5′-GGCCCTGCAGTGTGTAGCGCTCAGAAC CTCATCC-3′), cloning of the 624-bp PstI DNA subfragment into pGEM3Z, and excision of the insert DNA fragment with KpnI and SphI for subcloning into pMP220 to produce pMP2. Fusion plasmids were transferred to *B. bronchiseptica* and *B. pertussis* strains by triparental mating.

Single-copy chromosomal *lacZ* fusion construction used the integrative fusion plasmid pFUS2 (21). A 351-bp PCR product consisting of the *bfrD* promoter region of *B. pertussis* strain UT25 was cloned into pFUS2 by using BamHI and KpnI adapters (5′-CCGGTACCGCCACCGTCAAA TGGGA-3′ and 5′-CCGGATCCCAGGGCGGGATTGTTGCCGT-3′); similarly, a 402-bp *B. pertussis bfrE* fragment was cloned by using BamHI and KpnI adapters (5′-CCGGTACCCGGAGAAAAGCCGCACCGT-3′ and 5′-CCGGATCCATTGCGACCGTTGTCCGCGT-3′). Each pFUS2 construct was conjugally transferred to *B. pertussis* strain UT25Sm1, and plasmid integrants at *bfrD* and *bfrE* were selected on BG agar containing gentamicin, streptomycin, and colicin B (31).

Transcriptional activities of *lacZ* fusion genes were monitored using β-galactosidase assays (32). *Bordetella* strains carrying gene fusion plasmids or chromosomal gene fusions were grown in iron-replete SS medium with antibiotic selection and then subcultured in iron-replete SS medium, iron-depleted SS medium, or iron-replete SS medium containing supplements, as indicated. After 24 and 48 h of growth, LacZ activities were measured. Values are reported as mean LacZ activities (in Miller units; $n = 3$) ± 1 standard deviation (SD). The results presented are representative of at least two experimental trials. Mean LacZ activities were compared using Student's paired *t* test (two-tailed distribution, hypothesized difference = 0) in StatView, version 4.51, software (Abacus Concepts, Inc.). The *P* value is the probability that the mean LacZ activity is the same between strains or under different growth conditions, and *P* values of ≤0.05 were considered to be significant.

Construction of *Bordetella* mutant strains. A *bfrA bfrD bfrE* triple mutant was constructed in the *B. bronchiseptica* RB50 strain background. Briefly, a *bfrD bfrE* deletion mutant was first constructed as described previously (20). *bfrD* and *bfrE* are adjacent to each other on the chromosome, and the mutation resulted in the removal of a 4,393-bp DNA segment spanning the genes. The *bfrDE* deletion was transferred to strain RB50 by allelic exchange to produce strain RBB19, and the deletion was confirmed by PCR. RBB19 was then made *bfrA* by using the allelic exchange plasmid pEG7.31, carrying a *bfrA* deletion mutation (20). The 810-bp in-frame *bfrA* deletion mutation was confirmed by PCR and yielded the Δ*bfrA* Δ*bfrDE* triple mutant RBB20. For construction of a *B. pertussis bfrD* mutant, pBKS+20 was cut with AatII and religated, resulting in a 1,392-bp in-frame deletion in *bfrD*. The Δ*bfrD* allele was subcloned into the suicide plasmid pSS1129 (33) as a BamHI-HindIII fragment, yielding pSS39. The mutation was transferred to *B. pertussis* UT25Sm1 by allelic exchange and confirmed by PCR.

Growth assays. Norepinephrine-mediated growth stimulation by transferrin was measured by determining the optical density at 600 nm (OD_{600}) of liquid SS cultures as described previously (20). *B. bronchiseptica* strains were grown on BG agar for 24 h, subcultured into iron-replete SS medium, and grown for 18 h. The cells were washed with SS basal medium lacking iron and used as inocula for the test cultures, at an initial OD_{600} of 0.01. Test media included iron-replete SS medium or iron-depleted SS medium containing 10 mM sodium bicarbonate and 200 μg/ml transferrin (approximately 2.5 μM; 30% iron saturated), with or without 50 μM norepinephrine. For *B. pertussis* strains, growth from BG agar was used to inoculate iron-replete SS medium. After growth for 24 h, the bacteria were harvested, washed, and subcultured into test media as

described for *B. bronchiseptica* growth assays, but at an initial OD_{600} of 0.02. Growth yield results are reported as the means of results from triplicate cultures, and each experiment was performed at least twice independently. Mean growth yields were compared using Student's paired *t* test (two-tailed distribution, hypothesized difference = 0) in StatView, version 4.51, software (Abacus Concepts, Inc.). The *P* value is the probability that the mean growth yield is the same between strains or growth conditions, and *P* values of ≤0.05 were considered to be significant.

Mouse infections. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Minnesota, and all procedures conformed to the guidelines of the *Guide for the Care and Use of Laboratory Animals* (34). *Bordetella* inocula for mouse competition infection experiments were prepared as described previously (35, 36). Wild-type *B. bronchiseptica* strain RB50 and the isogenic Δ*bfrA* Δ*bfrDE* triple mutant strain RBB20 were cultivated on BG agar and then subcultured onto iron-replete SS medium and grown for 24 h. The bacteria were washed and resuspended in 0.9% NaCl, and the two strain suspensions were combined at an estimated 1:1 strain ratio to prepare a mixed-strain suspension determined by CFU counting to have 9.3×10^5 total CFU/ml. Wild-type *B. pertussis* strain UT25Sm1 and the isogenic Δ*bfrD* mutant strain PM21 were grown on BG agar, subcultured onto SS medium containing 0.5% Casamino Acids, and then combined to produce a 1:1 strain ratio inoculum containing 2.5×10^7 total CFU/ml. BALB/cAnNHsd mice (10 to 20 g) (Harlan Sprague Dawley, Inc.) were briefly sedated by isoflurane inhalation and then infected intranasally with a 20-μl volume containing ~5 × 10^5 total CFU of a 1:1 mixture of *B. bronchiseptica* strains RB50 and RBB20 or, for *B. pertussis*, 1×10^6 total CFU of UT25Sm1 and PM21.

At the indicated time points postinfection, 5 mice were euthanized, and lungs and tracheal homogenates from individual mice were plated on BG agar for total CFU determination. The nasal tissue homogenates from each mouse group of 5 were pooled and plated. Differential enumeration of the bacterial strains was accomplished by picking 100 colonies, patching them onto BG agar, and performing *in situ* DNA hybridizations at high stringency, as described previously (26). The 0.9-kb *bfrD*-specific DNA hybridization probe was PCR amplified using primers bfrD1 and bfrD2 and *Bordetella* genomic DNA templates and then radiolabeled with [α-^{32}P]dCTP (MP Biomedicals, Santa Ana, CA) by using the Random Primers DNA labeling system (Invitrogen). The competitive index (CI) was calculated as the mutant/wild-type CFU ratio in the output recovered at each time point divided by the mutant/wild-type CFU ratio in the input inoculum. Three experimental trials were conducted. Student's *t* test was used to determine whether the mean CI at each time point differed significantly from the hypothesized mean value of 1.00 (the predicted mean CI if there were no difference in fitness between the two strains) and from the mean CI at the preceding time point. *P* values of ≤0.05 were considered to be significant.

Analysis of *Bordetella* proteins. Recombinant BfrD and BfrE proteins were produced in *E. coli*. The *bfrD* coding sequence minus the predicted signal sequence was PCR amplified from *B. pertussis* strain UT25 by using primers His-bfrD up (5′-GGGCTCGAGGCGTCCACGGCGGTCCAG-3′) and His-bfrD low (5′-GGGGAAGCTTGGGACGCTTCGCCCTCTA-3′). The 2.5-kb PCR product was cloned into the expression vector pBAD/His-A (Invitrogen) as an XhoI-HindIII DNA fragment (pBAD/*bfrD*). Similarly, the *bfrE* sequence minus the predicted signal sequence was amplified from strain UT25 by using primers His-bfrE up (5′-GGGCTCGA GACCAGCGCGGGCGTTACC-3′) and His-bfrE low (5′-GGGGAAGCT TGCAATGCGCCGGTTTCC-3′). The 2.2-kb PCR product was cloned into pBAD/His-A as an XhoI-HindIII fragment (pBAD/*bfrE*). Plasmids were transformed into *E. coli* strain TOP10 (Invitrogen) via electroporation. A 25-ml Luria-Bertani broth culture containing 200 μg/ml ampicillin and 0.02% arabinose inducer was inoculated to an initial OD_{600} of 0.04 with TOP10(pBAD/*bfrD*) or TOP10(pBAD/*bfrE*) cells taken directly from the primary transformation plates. The cultures were incubated at 35°C

for 4 h, and the cells were harvested and suspended to 20 OD_{600} units/ml in 50 mM HEPES, pH 7.4.

Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5% polyacrylamide gels, and proteins were visualized by staining with Coomassie blue dye. For immunoblot studies, bacterial sample suspensions were normalized by using the OD_{600} and heated to 100°C in solubilization buffer, and the cleared lysate was loaded onto 7.5% polyacrylamide gels for electrophoresis. Purified BfrD and BfrE proteins were obtained by cutting relevant protein bands from electrophoretic gels. BfrD and BfrE protein identities were confirmed by tandem mass spectrometry (MS/MS), and proteins in the remainder of the gel slices were subjected to electrophoresis as indicated.

Research involving materials from human subjects was performed in accordance with federal guidelines and institutional policies. Immunoblot analyses used sera from *B. pertussis* culture-positive patient donors (aged 6 to 17 years) who had previously been vaccinated and sera from a control group of healthy adult donors not known to have had previous *B. pertussis* infection. Serum samples were provided by Alison Weiss, University of Cincinnati (37), or were from our laboratory collection. The sera were analyzed for antibody reactivity with the recombinant *B. pertussis* BfrD and BfrE proteins by using standard immunoblotting methods, as described previously (38). Blots with control and patient sera were performed in parallel on replicate gels, using the same serum dilution. The blots were developed using the same developer solution and were imaged together on a flatbed scanner, using identical image settings.

Reverse-phase LC-MS/MS analysis. The putative BfrD and BfrE protein bands were excised from Coomassie blue-stained SDS-PAGE gels and treated with trypsin. Trypsin treatment and liquid chromatography-MS/MS (LC-MS/MS) were performed at the University of Minnesota Center for Mass Spectrometry and Proteomics facility. Peptide products were separated on a C_{18} Nanotrap column (Michrom BioResources, Auburn, CA) and subjected to tandem mass spectrometry using a ThermoFinnigan (ABI, Inc., Foster City, CA) LTQ ion-trap mass spectrometer (MS). Mass spectrometry data were analyzed using Sequest (version 27, rev. 12; Thermo Fisher Scientific, San Jose, CA). Sequest was set up to search the NCBI protein sequence database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=protein), including *E. coli* proteins and the *B. pertussis* Tohama I BfrD (accession no. NP_879666.1) and BfrE (accession no. NP_879667.1) proteins, assuming trypsin cleavage. Sequest was searched with a fragment ion mass tolerance of 1.00 Da. An iodoacetamide derivative of cysteine was specified as a fixed modification, and oxidation of methionine was set as a variable modification. Scaffold (version Scaffold_4.4.1; Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at >95.0% probability as specified by the Peptide Prophet algorithm (39). Protein identifications were accepted if they could be established at >99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (40). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

RESULTS

The *B. pertussis* BfrD and BfrE receptors are functional. In *B. bronchiseptica*, the BfrA, BfrD, and BfrE receptors each promote catecholamine-mediated uptake of transferrin iron. *B. pertussis* has the *bfrD* and *bfrE* genes (99.9% and 99.8% identical to those of *B. bronchiseptica*, respectively), but we have not yet been able to demonstrate the utilization of catecholamines for iron acquisition by *B. pertussis* (20). Since it is possible that the *B. pertussis* BfrD and BfrE proteins are not functional, the *B. pertussis* genes were cloned and transferred to *B. bronchiseptica* strain RBB21, a *bfrA bfrD bfrE* mutant that is unable to use catecholamines to obtain transferrin iron and also lacks alcaligin production (*alcA*). The
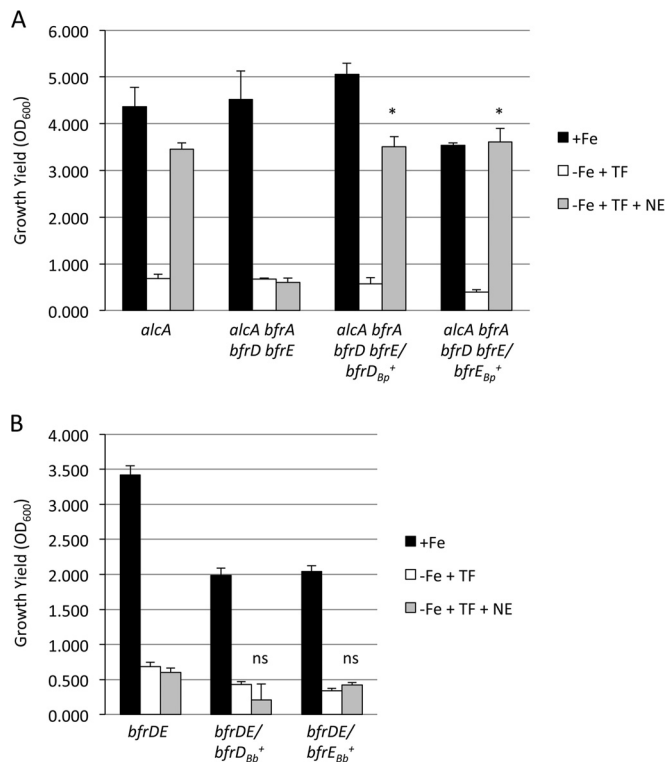

FIG 1 The *B. pertussis* *bfrD* and *bfrE* genes confer a norepinephrine-mediated transferrin utilization phenotype to *B. bronchiseptica*, but the *B. bronchiseptica* *bfrD* and *bfrE* genes do not promote norepinephrine-mediated transferrin utilization in *B. pertussis*. (A) *B. bronchiseptica* strains RBB22 (*alcA*) and RBB21 (*alcA bfrA bfrD bfrE*), each carrying the pRK415 plasmid vector, and RBB21, carrying the *B. pertussis* *bfrD*⁺ plasmid pRK71 or *bfrE*⁺ plasmid pRK72, were cultured in iron-depleted SS medium with transferrin, either without norepinephrine (−Fe + TF) or with 50 μM norepinephrine (−Fe + TF + NE). Iron-replete control cultures contained 36 μM iron (+Fe). Growth yields ($n = 3$; values are means and SD) were determined after 48 h. (B) *B. pertussis* strain PM22 (*bfrD bfrE*) carrying the pRK415 plasmid vector and PM22 carrying the *B. bronchiseptica* *bfrD*⁺ (pRK66) or *bfrE*⁺ (pRK67) plasmid were grown in SS media as described for panel A. Growth yields ($n = 3$; means and SD) were determined after 48 h. Statistically significant differences in mean growth yields in −Fe + TF + NE cultures compared with control strains were calculated using Student's paired *t* test as described in Materials and Methods. *, $P \leq 0.05$; ns, not significant.

plasmid-bearing strains were examined for the ability to grow in iron-replete SS defined medium and iron-depleted SS medium supplemented with 30% iron-saturated transferrin as the sole iron source, either lacking or containing 50 μM norepinephrine (Fig. 1A). As expected, compared with the RBB22 *alcA* parent strain, the *bfrA bfrD bfrE* mutant derivative RBB21 grew poorly with norepinephrine as the only means to obtain transferrin iron. However, RBB21 carrying the *B. pertussis* *bfrD* or *bfrE* gene grew as well as the RBB22 parent strain in norepinephrine-supplemented medium. These results indicate that *B. pertussis* *bfrD* and *bfrE* encode receptors that function in norepinephrine-mediated iron retrieval in *B. bronchiseptica*.

The *B. bronchiseptica* *bfrD* and *bfrE* genes were tested for the ability to confer norepinephrine utilization on *B. pertussis*. The growth of the *B. pertussis* *bfrD bfrE* mutant PM22 carrying the plasmid-borne *B. bronchiseptica* *bfrD* or *bfrE* gene was not stimulated by norepinephrine in the presence of transferrin (Fig. 1B).
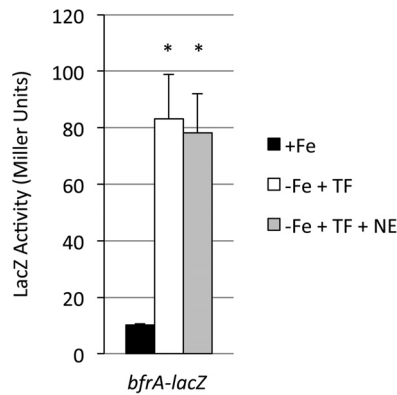
FIG 2 Transcriptional regulation of *B. bronchiseptica bfrA*. RB50(pMP6) (*bfrA-lacZ*) was grown in iron-replete (+Fe) SS medium or in iron-depleted medium with transferrin in the absence (−Fe + TF) or presence (−Fe + TF + NE) of norepinephrine. LacZ activity ($n = 3$; means and SD) was measured after 24 h of growth. *, $P \leq 0.05$ for comparison to +Fe cultures. There was no significant difference in *bfrA-lacZ* expression in cells grown in −Fe + TF medium and those grown in −Fe + TF + NE medium.
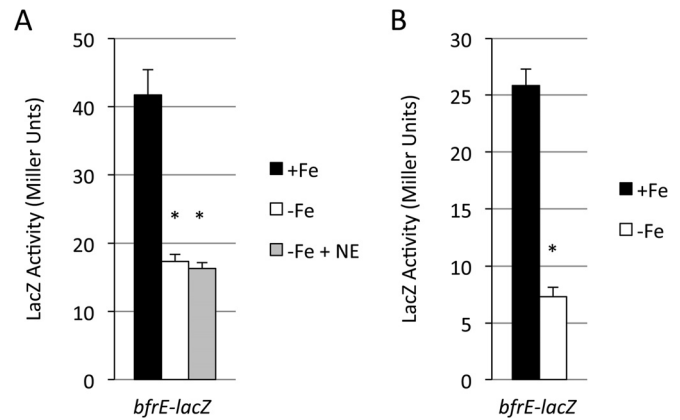
FIG 3 Transcriptional regulation of *B. bronchiseptica* and *B. pertussis bfrE*. (A) *B. bronchiseptica* strain RB50(pMP8) (*bfrE-lacZ*) was cultured in iron-replete (+Fe) SS medium or in iron-depleted medium in the absence (−Fe) or presence (−Fe + NE) of norepinephrine. LacZ activity ($n = 3$; means and SD) was measured after 24 h of growth. (B) *B. pertussis* strain PM30, with the *lacZ* fusion plasmid pFUS2 integrated at the chromosomal *bfrE* locus, was grown under iron-replete (+Fe) or iron-depleted (−Fe) conditions, and LacZ activity ($n = 3$; means and SD) was measured after 48 h of growth. *, $P \leq 0.05$ for comparison to +Fe cultures. For *B. bronchiseptica* RB50 in panel A, there was no significant difference in *bfrE-lacZ* expression between cells grown in −Fe versus −Fe + NE medium.

The identities of the presumptive *B. bronchiseptica* cytoplasmic membrane transporter genes required for catecholamine utilization are not yet known. The lack of observed *in vitro B. pertussis* catecholamine utilization is not likely due to nonfunctional BfrD or BfrE receptor proteins but may be due to an absent or defective cytoplasmic membrane transport apparatus or other catecholamine utilization function, or it may be a technical limitation of the *in vitro* growth assay system used for *B. pertussis*.

Analyses of *bfrA, bfrD,* and *bfrE* transcription. Bacterial iron transport genes are typically repressed by Fur (or functionally similar proteins) with its bound iron corepressor under iron-replete growth conditions. When internal iron stores are depleted, Fur is inactive and the iron assimilation genes are derepressed (41, 42). In addition, some iron system genes are positively regulated by mechanisms involving the cognate iron source acting as the inducer (9, 43). In a microarray study of the transcriptional responses of *B. pertussis* and *B. bronchiseptica* to iron starvation stress, the *bfrA, bfrD,* and *bfrE* genes were not identified as being significantly iron regulated (44). Using the consensus Fur binding sequence 5′-GATAATGATAATCATTATC-3′ (45, 46) as the query, *in silico* analysis of the *bfrA, bfrD,* and *bfrE* upstream DNA regions identified potential Fur binding sites. Three overlapping sites, with 7 or 8 mismatches, are positioned 46, 52, and 58 nucleotides (nt) upstream of *bfrA*. As reported by Passerini de Rossi et al. (47), one potential 19-nt Fur binding site (8 mismatches) is located 121 nt upstream of *bfrD. bfrD* and *bfrE* are adjacent on the chromosome but are separated by 211 bp and are transcribed independently (20). A possible Fur binding site with 8 mismatches is positioned 12 bp upstream of the *bfrE* coding sequence. The ability of the upstream regions of these genes to bind Fur *in vivo* was examined using the Fur titration assay (48). In this *E. coli* assay, a functional Fur binding site introduced on a multicopy plasmid can titrate Fur, thus relieving the repression of a chromosomal *fhuF-lacZ* fusion that is normally repressed under iron-replete growth conditions, resulting in a LacZ$^+$ phenotype. Compared with plasmid vector controls and a plasmid bearing the consensus Fur binding sequence which demonstrated strong Fur binding (LacZ) activity, the upstream DNA regions of *bfrA, bfrD,* and *bfrE*

exhibited variable, weak Fur binding (data not shown). To evaluate the transcriptional regulation of the catecholamine utilization genes, gene fusions were analyzed in *Bordetella* cells.

bfrA. Of the three classical *Bordetella* species, only *B. bronchiseptica* has the *bfrA* receptor gene. *B. bronchiseptica* wild-type strain RB50 carrying a low-copy-number *bfrA-lacZ* transcriptional fusion plasmid (pMP6) was grown in iron-replete SS defined medium or iron-restricted SS medium containing transferrin (30% iron saturated) as the sole iron source (Fig. 2). *Bordetella bfrA* transcription levels were low in iron-replete medium but were elevated upon iron restriction in the presence of transferrin; the presence or absence of norepinephrine did not influence *bfrA* expression. Compared with our previous siderophore and heme receptor gene-*lacZ* fusion analyses of iron-replete or iron-starved *Bordetella* cells (49–51), the overall *bfrA* expression levels were quite low. Similar results were obtained using other *B. bronchiseptica* host strains (data not shown). LacZ transcriptional fusions provide an established, reliable measure of *Bordetella* promoter activity, and the growth media and conditions that were used do not produce artifactual LacZ assay results. Similarly low *bfrA* expression levels were observed in our transcriptome studies of iron-replete versus iron-starved *B. bronchiseptica* strain RB50 (44). Experiments using iron-replete or iron-restricted SS medium that lacked transferrin showed comparable *B. bronchiseptica bfrA* transcription levels, indicating that it was the lack of iron, not the presence of transferrin, that promoted *bfrA* gene expression (data not shown).

bfrE. In *B. bronchiseptica* strain RB50, *bfrE-lacZ* expression (pMP8) was low under all SS medium growth conditions (Fig. 3A). There was no effect of norepinephrine on *bfrE-lacZ* expression. The presence of iron in the medium was associated with elevated *bfrE* transcriptional activity compared with that under iron-restricted conditions. As observed for *bfrA*, addition of transferrin did not alter transcription of *bfrE* (data not shown). A dif-
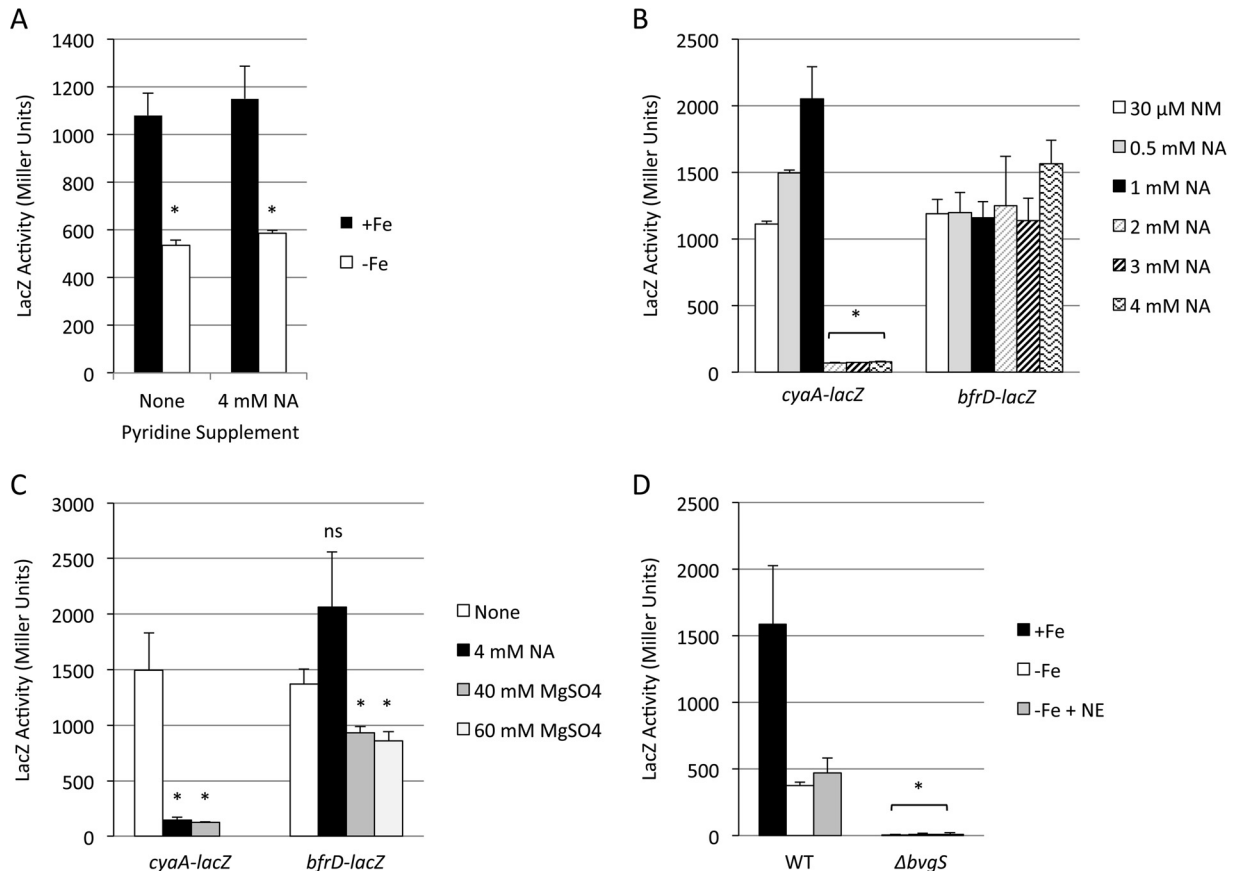
FIG 4 Effects of iron starvation and the BvgAS system on transcriptional regulation of *B. bronchiseptica bfrD*. (A) RB50(pMP7) was grown in iron-replete (+Fe) SS medium or iron-depleted medium (−Fe) in the absence or presence of the Bvg modulator nicotinic acid (NA; 4 mM), and LacZ activity was measured after 24 h of growth (*n* = 3; means and SD). *, *P* ≤ 0.05 for comparison to +Fe cultures. There was no statistically significant difference in *bfrD-lacZ* expression between cells grown with 4 mM NA and those grown in its absence. (B) RB50 carrying either pMP7 (*bfrD-lacZ*) or the known Bvg-responsive *cyaA-lacZ* control plasmid pMP2 was grown in iron-replete SS medium supplemented with either the standard pyridine supplement 30 µM nicotinamide (NM) or nicotinic acid (NA) at the indicated concentrations, and LacZ activity was measured after 24 h of growth (*n* = 3; means and SD). For *cyaA-lacZ* data, statistically significant differences for comparing cells grown in NM to those grown in 2 to 4 mM NA are indicated as follows: *, *P* ≤ 0.05. There were no statistically significant differences in *bfrD-lacZ* expression between cells grown in NM and those grown in NA at any concentration. (C) RB50(pMP2) (*cyaA-lacZ*) and RB50(pMP7) (*bfrD-lacZ*) were grown in standard SS medium (none) or in the same medium supplemented with nicotinic acid (NA) or magnesium sulfate at the indicated concentrations. LacZ activity was measured after 24 h of growth (*n* = 3; means and SD). Statistically significant differences in cells grown with the modulator NA or MgSO₄ and those grown in the absence of modulators are indicated as follows: *, *P* ≤ 0.05 (ns, not significant). (D) RB50 (WT) and its Δ*bvgS* derivative RB54, each carrying the *bfrD-lacZ* fusion plasmid pMP7, were grown in iron-replete (+Fe) SS medium or in iron-depleted medium in the absence (−Fe) or presence (−Fe + NE) of norepinephrine. LacZ activity was measured after 24 h of growth (*n* = 3; means and SD). *, *P* ≤ 0.05 for comparing *bfrD* expression in strains RB54 and RB50 under each of the three growth conditions. There were no statistically significant differences between cells grown in −Fe and −Fe + NE media.

ferent *B. bronchiseptica* host strain showed similar *bfrE-lacZ* expression patterns (data not shown). To assess *bfrE* expression in *B. pertussis*, the promoter of the *B. pertussis bfrE* gene was fused to *lacZ* and integrated into the chromosome of strain UT25Sm1 at the *bfrE* locus. In this human-adapted species, transcription of *bfrE* was quite modest, and as with *B. bronchiseptica*, it was elevated under iron-replete conditions (Fig. 3B); expression was not affected by the presence of norepinephrine (data not shown). These results indicate that in *B. bronchiseptica* and *B. pertussis* grown under these culture conditions, expression levels of *bfrE* are generally low but are elevated under iron-replete conditions.

bfrD. Transcription of *bfrD* in *B. bronchiseptica* was monitored in strain RB50 carrying the low-copy-number *bfrD-lacZ* transcriptional fusion plasmid pMP7. Similar to the pattern observed with *bfrE*, *bfrD* expression levels in RB50 were high under iron-replete conditions and decreased when bacteria were grown under

iron-depleted conditions (Fig. 4A), but overall *bfrD* expression levels were much higher than those of *bfrE*. Iron titration experiments showed optimal *bfrD* expression when cells were cultured at iron concentrations ranging from 4.5 µM to 36 µM; growth with iron concentrations of 1 to 2 µM resulted in significantly lower *bfrD-lacZ* expression levels (data not shown).

In *B. pertussis*, *bfrD* was reported to be a highly expressed Bvg-activated gene, exhibiting no significant expression in *bvg* mutant strains and decreased expression in the presence of modulator compounds, such as nicotinic acid or magnesium sulfate (21). As a first step toward determining whether *bfrD* expression is regulated by Bvg in *B. bronchiseptica*, wild-type strain RB50(pMP7) was grown in the presence or absence of modulating compounds, and *bfrD* expression was measured (Fig. 4A and B). As predicted, expression of the Bvg-activated, adenylate cyclase-encoding *cyaA-lacZ* fusion control was dramatically decreased when RB50
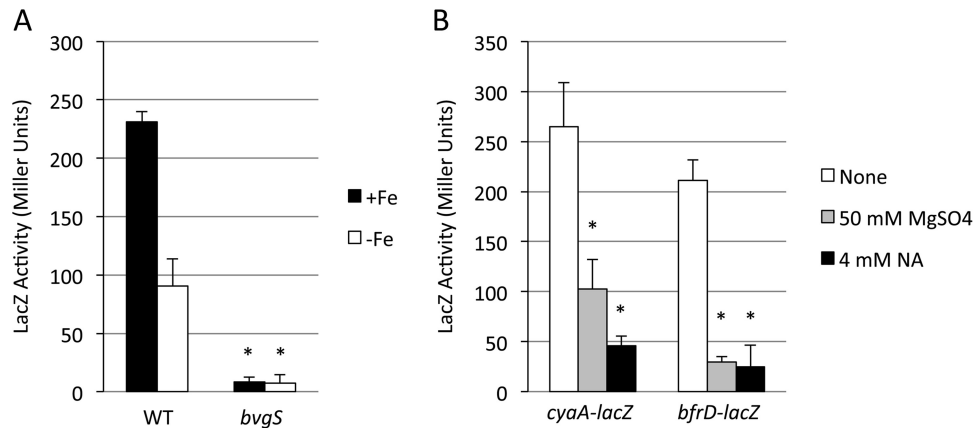
FIG 5 Transcription of *B. bronchiseptica bfrD* in *B. pertussis* is regulated by Bvg and modulated by nicotinic acid and sulfate. (A) Wild-type (WT) *B. pertussis* strain BP338 and the isogenic *bvgS* mutant strain BP347, both carrying pMP7 (*bfrD-lacZ*), were grown in iron-replete (+Fe) SS medium or iron-depleted medium (−Fe), and LacZ activity was measured after 48 h ($n = 3$; means and SD). *, $P \leq 0.05$ for differences in *bfrD-lacZ* expression between BP338 and BP347 under each growth condition. For comparison of the LacZ activity of BP338(pMP7) in −Fe compared with +Fe medium, the *P* value was ≤0.05. (B) BP338 carrying either the *cyaA-lacZ* (pMP2) or *bfrD-lacZ* (pMP7) plasmid was grown in standard SS medium with no additions (none) or supplemented with the indicated concentrations of magnesium sulfate or nicotinic acid (NA) as a modulator. LacZ activity was measured after 24 h of growth ($n = 3$; means and SD). *, $P \leq 0.05$ for comparing fusion gene expression in strain BP338 under nonmodulating (none) versus modulating growth conditions.

(pMP2) was cultured in the presence of 2 to 4 mM nicotinic acid compared with expression levels when the strain was cultured in the absence of nicotinic acid (Fig. 4B). In contrast, nicotinic acid had no modulating effect on *bfrD-lacZ* expression in *B. bronchiseptica* (Fig. 4A and B). Compared with expression of *cyaA*, expression of *bfrD* was also significantly less sensitive to the modulating influence of sulfate (Fig. 4C); thus, *bfrD* expression is relatively insensitive to these modulators in *B. bronchiseptica*. As with *bfrA* and *bfrE*, norepinephrine supplementation of cultures had no effect on expression of *bfrD* (Fig. 4D). To determine whether *bfrD* transcription is Bvg dependent in *B. bronchiseptica*, as it is in *B. pertussis*, *bfrD-lacZ* expression was measured in wild-type RB50 compared with its isogenic Δ*bvgS* mutant derivative RB54 (Fig. 4D). Expression of *bfrD* in strain RB54 was negligible under all growth conditions. These results demonstrate that in *B. bronchiseptica*, transcription of *bfrD* is strictly Bvg dependent but is insensitive to nicotinic acid and sulfate modulation.

The same pMP7 plasmid, containing the *B. bronchiseptica bfrD* promoter region fused with *lacZ*, was transferred to *B. pertussis* wild-type strain BP338 and its *bvgS* mutant derivative BP347. Similar to the *B. bronchiseptica* results, transcription of *bfrD* in *B. pertussis* was greater under iron-replete than iron-restricted growth conditions, and expression was severely reduced in the *bvgS* mutant strain BP347 (Fig. 5A). However, in contrast to the case in *B. bronchiseptica*, *bfrD* expression in *B. pertussis* was modulated by sulfate and nicotinic acid, as was that of the *cyaA-lacZ* control (Fig. 5B). A single-copy *B. pertussis bfrD-lacZ* fusion was integrated into the chromosome of a different *B. pertussis* strain (UT25Sm1). As with the pMP7-borne *B. bronchiseptica*-derived *bfrD-lacZ* fusion, expression of the *B. pertussis* chromosomal *bfrD-lacZ* fusion was higher under iron-replete conditions (data not shown) and was subject to sulfate- and nicotinic acid-mediated modulation (Fig. 6).

B. pertussis and *B. bronchiseptica* have been reported to vary in their sensitivity to modulator compounds, and differences in their BvgS proteins were implicated (52). In those studies, a derivative of *B. bronchiseptica* RB50, RB52, was constructed by replacing its

bvgAS genes with those of *B. pertussis* strain BP338. In the present study, the *bfrD-lacZ* plasmid pMP7 was examined in strain RB52 for transcriptional responses to modulator compounds. Compared with the expression levels in the wild-type *B. bronchiseptica* RB50 host, in *B. bronchiseptica* RB52 the *B. pertussis bvgAS* alleles conferred increased sensitivity of *bfrD-lacZ* expression to modulation, with markedly decreased transcription at 60 mM MgSO₄ (Fig. 7A). Conversely, in RB52, *cyaA-lacZ* expression showed a decreased sensitivity to sulfate modulation compared to the expression in RB50. Modulation using 4 mM and 8 mM nicotinic acid yielded *bfrD* and *cyaA* expression patterns similar to those seen with MgSO₄ modulation in the two *B. bronchiseptica* strains (Fig. 7B). For unknown reasons, growth of the wild-type strain in 8 mM nicotinic acid consistently appeared to stimulate *bfrD* transcription. Overall, these results indicate that the relative resistance
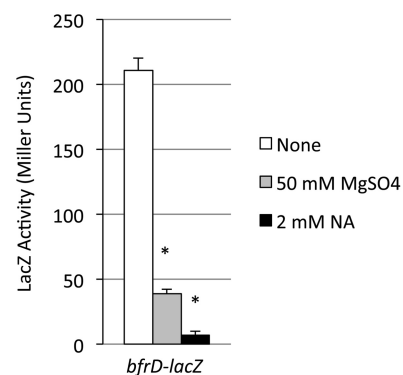

FIG 6 Transcription of the *B. pertussis bfrD* gene in *B. pertussis* is modulated by nicotinic acid and sulfate. *B. pertussis* strain PM29, with the *lacZ* fusion plasmid pFUS2 integrated at the chromosomal *bfrD* locus, was grown in standard SS medium with no additions (none) or with the indicated concentrations of magnesium sulfate or nicotinic acid (NA) as a modulator. LacZ activity was measured after 24 h of growth ($n = 3$; means and SD). *, $P \leq 0.05$ for comparing *bfrD* expression in cells grown under modulating versus nonmodulating conditions.
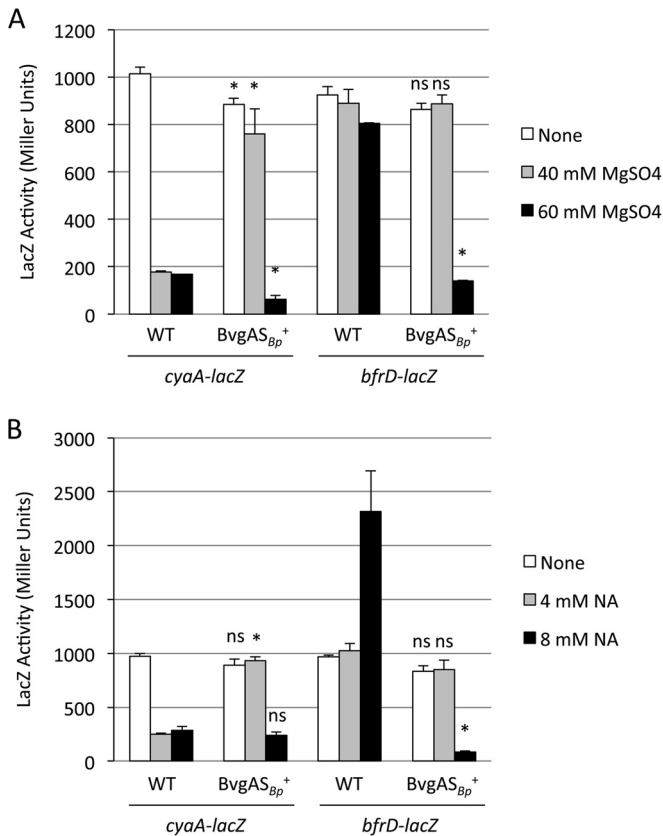
FIG 7 Transcription of *bfrD* in *B. bronchiseptica* RB52, which has the *B. bronchiseptica bvgAS* genes replaced by the orthologous *bvgAS* genes of *B. pertussis*, is modulated by nicotinic acid and sulfate. Wild-type strain RB50 (WT) and its isogenic derivative RB52 (BvgAS$_{Bp}^+$), carrying pMP2 (*cyaA-lacZ*) or pMP7 (*bfrD-lacZ*), were grown in standard SS medium with no additions (none) or supplemented with the indicated concentrations of magnesium sulfate (A) or nicotinic acid (NA) (B) as a modulator. LacZ activity was measured after 24 h of growth (*n* = 3; means and SD). *, $P \leq 0.05$ for comparing fusion gene expression in strains RB52 and RB50 grown under each condition; ns, not significant.

of *bfrD* transcription to modulation in *B. bronchiseptica* compared with that in *B. pertussis* is due in part to species-specific differences in BvgAS. However, since *bfrD* transcription in *B. pertussis* BP338 (the source of *bvgAS* in RB52) was effectively modulated by 4 mM nicotinic acid and 50 mM MgSO$_4$ (Fig. 5B), other cellular differences are also likely to influence expression.

In aggregate, these results indicate that in *B. pertussis* and *B. bronchiseptica*, the transcriptional activity of *bfrD* is higher under iron-replete than iron-restricted growth conditions. Furthermore, in *B. bronchiseptica*, *bfrD* is under strict transcriptional control of the BvgAS system, as it is in *B. pertussis*. *B. bronchiseptica* transcription of *bfrD* was not modulated by compounds given at concentrations known to decrease expression of other Bvg-activated genes, as in *B. pertussis*, despite strong interspecies conservation of the putative *bfrD* upstream control regions.

Contributions of BfrA, BfrD, and BfrE to *Bordetella* growth *in vivo*. To determine if the BfrA, BfrD, and BfrE catecholamine receptors were important for growth in a host, mixed-infection competition experiments were performed. Mice were infected intranasally with a 1:1 mixture of *B. bronchiseptica* RB50 (wild-type strain) and RBB20 (isogenic *bfrA bfrD bfrE* mutant). At 3, 7, 14, 21,

28, and 35 days postinfection, infected mouse respiratory tract tissues were obtained, total and differential CFU counts were determined, and competitive index values were calculated (Fig. 8). Bacterial counts in the lungs and trachea (Fig. 8A) peaked at day 7 postinfection, gradually declining to 1.7×10^4 CFU per mouse by day 35. In these tissues, the *B. bronchiseptica bfrA bfrD bfrE* triple mutant exhibited little fitness defect until after day 14, in the later stages of infection (Fig. 8C). In the nasal cavity, bacterial counts hovered at $\sim 1.6 \times 10^6$ per animal until late infection, declining by approximately 1 log by day 35 (Fig. 8B). Interestingly, by midinfection (day 21), the mutant had an apparent fitness advantage in the nasal cavity (Fig. 8D).

Since *bfrD* is activated by Bvg and expressed by *B. pertussis in vitro* and production of the BfrD protein has been reported for *B. pertussis* (22, 23, 47), mixed-infection competition experiments were performed to determine whether BfrD is important for growth of this *Bordetella* species in a host. Bacterial counts from the lungs and trachea peaked at day 7 and declined significantly by day 28 (Fig. 9A), which is characteristic of *B. pertussis* mouse infections. The *B. pertussis bfrD* mutant showed a modest but significant fitness defect in the lungs and trachea of mice early in infection (Fig. 9C). In the nasal cavity, CFU values similarly decreased over the infection period (Fig. 9B), and the mutant exhibited an apparently reduced fitness at early and late infection time points (Fig. 9D). Together, these results suggest a moderate yet significant role for the *B. bronchiseptica* and *B. pertussis* catecholamine receptors in this mouse model of infection.

In a previous study from our laboratory, immunoblot screening of human sera from culture-positive patients diagnosed with pertussis demonstrated that, compared with control sera, the majority of infected patient sera demonstrated strong antibody reactivity to iron-repressible membrane proteins, including the *B. pertussis* outer membrane receptors for alcaligin, enterobactin, and heme (38). These human sera were used in similar analyses of *B. pertussis* BfrD and BfrE (Fig. 10). Evaluation of BfrD and BfrE produced in *B. pertussis* and *B. bronchiseptica* was complicated by the naturally low levels of receptor protein production. Therefore, *B. pertussis bfrD* and *bfrE* were expressed in *E. coli*, and the overproduced proteins were used as antigens for immunoblot analysis. Compared with sera from uninfected donors, sera from pertussis patients exhibited stronger reactivity (and consistently showed more background reactivity, similar to the results of our previous study [38]) with the recombinant BfrD and BfrE proteins. Multiple uninfected- and convalescent-phase-serum paired sample sets were analyzed in parallel, yielding similar results. The serum reactivity could also be due to antibodies that cross-react with other bacterial proteins, and there is likely cross-reactivity between the highly similar BfrD and BfrE proteins. However, overall, these results suggest that in the context of human infection, *B. pertussis* produces the BfrD and BfrE receptor proteins, which elicit an antibody response.

DISCUSSION

Studies from this laboratory demonstrated *B. bronchiseptica* utilization of transferrin- and lactoferrin-bound iron via the catecholamines epinephrine, norepinephrine, dopamine, and L-3,4-dihydroxyphenylalanine (L-DOPA), as well as 2,5-dihydroxybenzoylserine (20). BfrA, BfrD, and BfrE were identified as outer membrane receptors involved in this iron acquisition mechanism, which operates in the absence of siderophores. Catecholamines
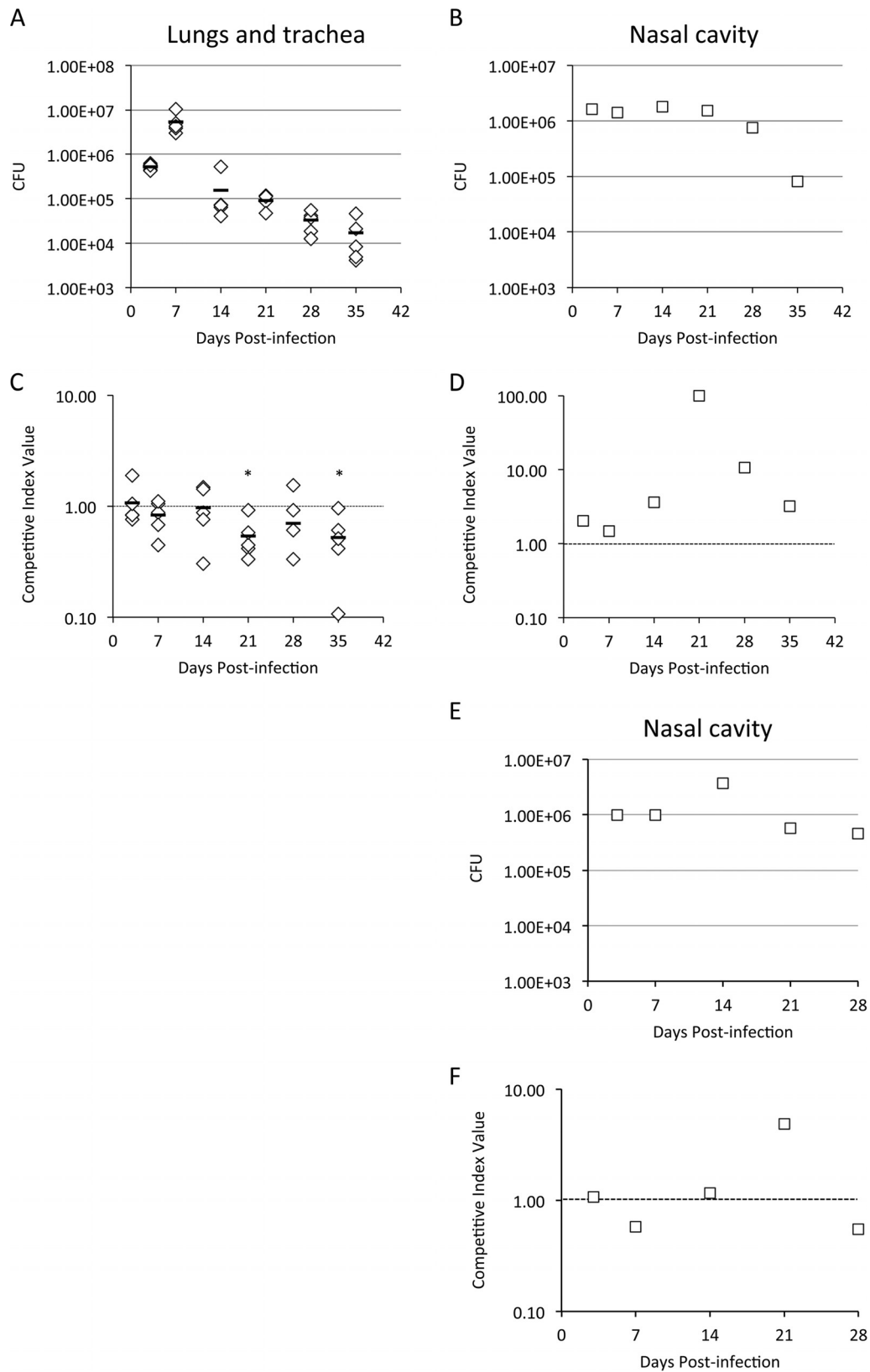
FIG 8 Relative *in vivo* fitness of a *B. bronchiseptica bfrA bfrD bfrE* triple receptor mutant during mouse respiratory infection. The graphs show the mean total CFU/mouse (*n* = 5) in the outputs recovered from lung and tracheal homogenates from individual mice (A) or from pooled nasal cavity homogenates (B and E) at various times postinfection. Nasal cavity data from two experiments are shown (experiment 1, panels B and D; and experiment 2, panels E and F). The lower limit of detection was 100 CFU per mouse. Competitive index (CI) values were calculated as the mutant/wild-type strain ratio in the outputs from lungs and tracheas of individual mice (C) or from pooled nasal cavities (D and F) recovered at each time point divided by the mutant/wild-type strain ratio in the input inoculum, as determined by differential colony hybridization. CI values are shown relative to the reference CI of 1.00 (dashed line). (C) For the lungs and trachea, the horizontal bars represent mean values, and each symbol represents the CI value determined for an individual mouse. *P* values of ≤0.05 (indicated with asterisks) were considered to be significant.
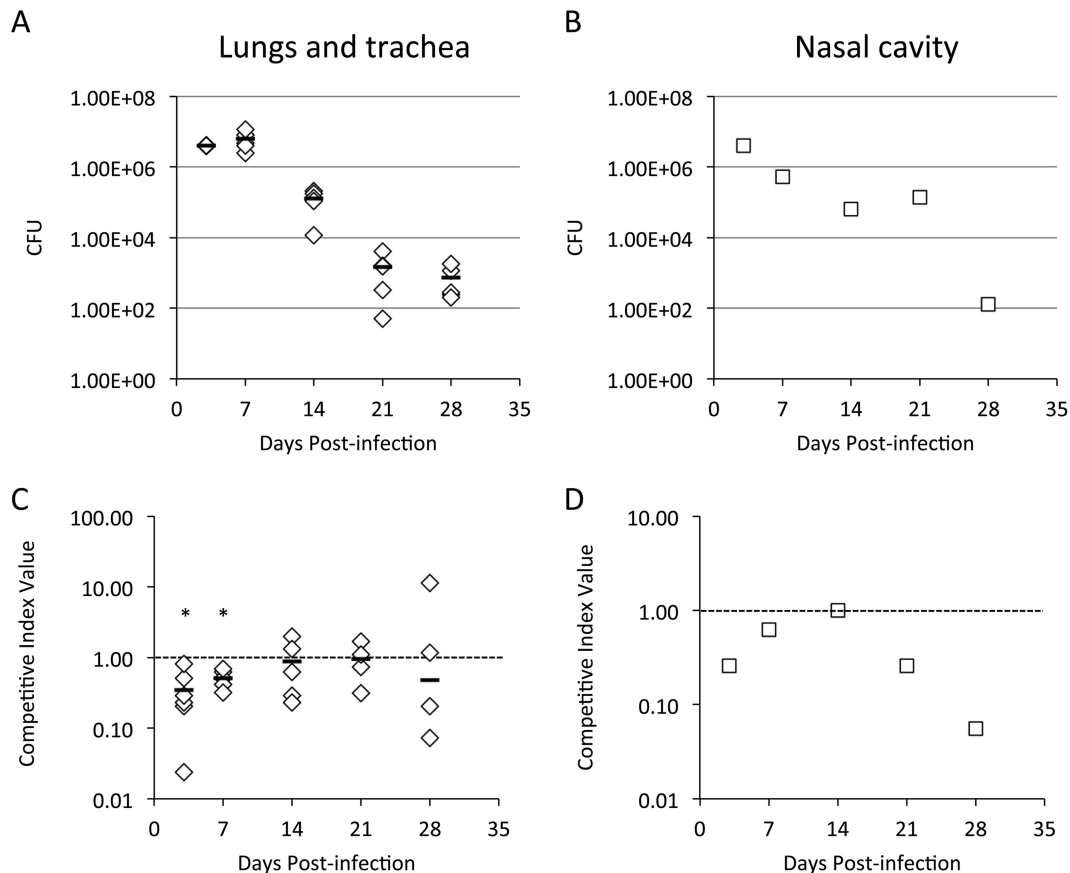
FIG 9 Relative *in vivo* fitness of a *B. pertussis* bfrD receptor mutant during mouse respiratory infection. The graphs show the mean total CFU/mouse (*n* = 5) in the outputs recovered from lung and tracheal homogenates from individual mice (A) or from pooled nasal cavity homogenates (B) at various times postinfection. The lower limit of detection was 100 CFU per mouse. CI values were calculated as described in the legend to Fig. 8, based on strain ratios determined by differential colony hybridization. CI values are shown relative to the reference CI of 1.00 (dashed line). (C) For the lungs and trachea, the horizontal bars represent mean values, and each symbol represents the CI value determined for an individual mouse.

act as chelators that effectively remove the iron from transferrin or lactoferrin (53). Catecholamine-iron complexes are predicted to diffuse to the *Bordetella* cell surface and interact with BfrA, BfrD, and/or BfrE for uptake to the periplasm. The machinery that transports the iron across the cytoplasmic membrane to the cytoplasm is not known, and there are no obvious candidate transporter genes colocalized on the chromosome near *bfrA*, *bfrD*, or *bfrE*.

Taxonomically related *Bordetella petrii* and *Achromobacter* species that live in the external environment have a single gene encoding a protein that is significantly more similar to BfrE (80% identity) than to BfrD (56 to 57% identity). This suggests that *bfrE* was duplicated in the classical pathogenic *Bordetella* species, giving rise to the adjacent *bfrD* gene, which evolved to be coregulated with virulence factor genes via BvgAS. *B. pertussis* has intact *bfrD* and *bfrE* genes, but demonstration of catecholamine-mediated growth on transferrin or lactoferrin iron by *B. pertussis* has not been successful with our *in vitro* culture system. However, the present study showed that the *B. pertussis* BfrD and BfrE proteins are functional for catecholamine utilization when the genes are expressed in *B. bronchiseptica*, illustrating differences between the human-adapted species and *B. bronchiseptica*. In our studies of *Bordetella* iron acquisition, we observed no significant differences

in iron requirements or sensitivity to iron chelators between *B. bronchiseptica* and *B. pertussis* that may account for the differences in catecholamine receptor function. It is possible that our *in vitro* culture system using catecholamines and ferric transferrin does not recapitulate conditions allowing BfrD/BfrE function in *B. pertussis*. It is also possible that *B. pertussis* never acquired, or has lost, the other factors for catecholamine utilization, such as a cytoplasmic membrane transporter. However, previous studies showed that the *B. bronchiseptica* bfrA gene could promote norepinephrine-mediated utilization of transferrin iron by *B. pertussis*, which lacks a *bfrA* ortholog (20), suggesting that a catecholamine transporter system that can function with BfrA is active in *B. pertussis*. Database searches indicate that all sequenced *B. pertussis* strains have intact *bfrD* and *bfrE* genes, and BfrD was proposed as a potential vaccine antigen (23). Perhaps, in contrast to the case in *B. bronchiseptica*, *B. pertussis* BfrD/BfrE function continued to evolve in humans such that noncatecholamine host substrates were bound and transported. Our infection experiments indicate that BfrD does have an apparent function in *B. pertussis*, since a *bfrD* mutant is attenuated in mice, albeit modestly. Our studies also indicate that sera from humans convalescing from pertussis recognize the BfrD and BfrE proteins, suggesting the *in vivo* expression of *bfrD* and *bfrE* in *B. pertussis* in the natural human host.
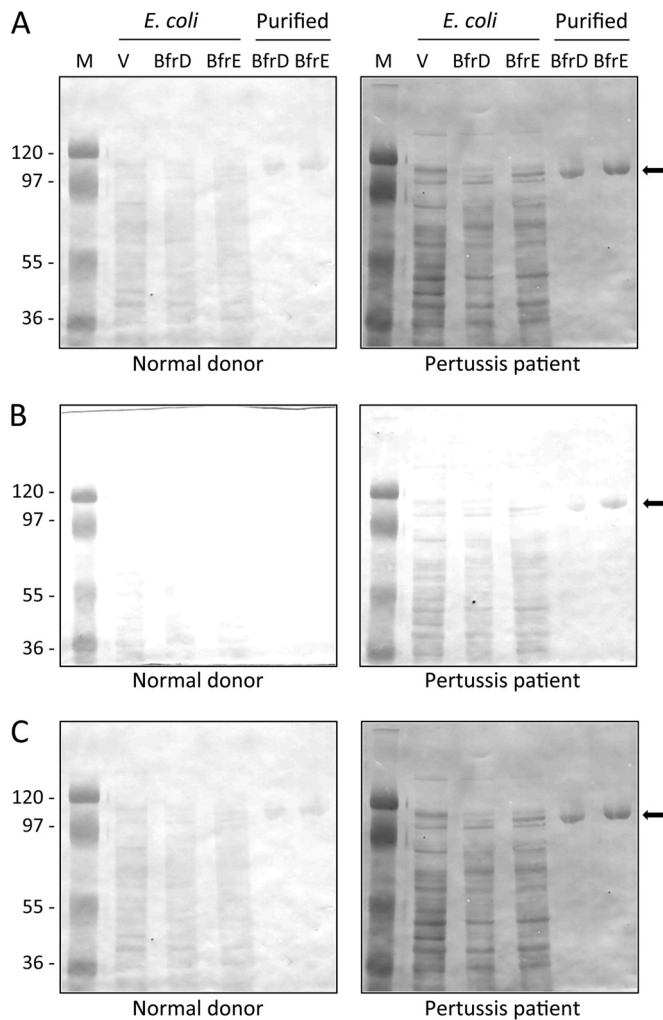
FIG 10 Immunoblot reactivity of human sera with *B. pertussis* BfrD and BfrE receptor proteins. (A) Protein samples were analyzed in replicate immunoblots by using sera from an uninfected human donor (normal donor) and a *B. pertussis* culture-positive human donor (pertussis patient). Lanes: *E. coli*, total cell proteins prepared from *E. coli* producing recombinant BfrD and BfrE proteins; Purified, gel-purified authentic BfrD and BfrE proteins; M, molecular mass markers; V, cell proteins from *E. coli* carrying the plasmid vector control. (B) The antigens analyzed in panel A were probed using sera from donors different from those shown in panel A. (C) The antigens analyzed in panels A and B were probed using sera from donors different from those shown in panels A and B. Positions of BfrD and BfrE are indicated by arrows.

Previously, we demonstrated convalescent-phase human serum reactivity to a variety of membrane proteins from iron-starved (versus iron-replete) *Bordetella* cells, including the TonB-dependent receptors for alcaligin, heme, and enterobactin (38).

Previous studies from this laboratory showed that *B. pertussis* mutants lacking the receptors for alcaligin (FauA), heme (BhuR), and enterobactin (BfeA) were attenuated for growth in the mouse respiratory tract (35, 36, 38). In the present study, our results demonstrated that a *B. pertussis bfrD* mutant was less fit than the wild-type parent strain in the lungs and trachea, especially during the first week of infection. Comparison of competitive index values from these experiments indicates that the *bfrD* mutant had a smaller fitness defect than those of the *fauA* and *bhuR* mutants but a larger fitness defect than that of the *bfeA* mutant. In the lungs and

trachea, a *B. bronchiseptica bfrA bfrD bfrE* triple receptor mutant exhibited decreased fitness in later stages of infection. Unexpectedly, the *B. bronchiseptica* competitive index in the nasal cavity suggested that the triple mutant had a fitness advantage over the wild-type strain, especially at day 21. Although significance cannot be determined because the nasal samples were pooled, similar nasal fitness results were obtained in another experiment. The *B. pertussis bfrD* mutant showed a fitness defect in the nasal cavity at both early and later infection time points, but unlike *B. bronchiseptica*, it did not exhibit any fitness advantage over the coinfecting wild-type strain. During our *in vitro* analyses of *B. bronchiseptica bfrA*, *bfrD*, and/or *bfrE* single- or multiple-receptor mutants, we did not observe overproduction of the alcaligin siderophore or any other effects on siderophore-mediated iron uptake that may explain the apparent nasal fitness advantage. A recent study examined mouse nasal colonization efficacies of *B. pertussis* and *B. bronchiseptica* and showed significant species differences that involved the respiratory microbiota (54). *B. bronchiseptica* nasal colonization was obtained using infecting doses as low as 5 CFU, whereas much higher doses of *B. pertussis* (10,000 CFU) or antibiotic pretreatment was required to overcome inhibition by the microflora. Our observed species differences in the mouse nasal cavity may reflect these known effects of the resident flora. The *Bordetella* species growth differences in the mouse respiratory tract as a whole also may reflect differences in catecholamine receptor functions. In addition, compared with *B. pertussis*, *B. bronchiseptica* generally causes chronic infection in its natural nonhuman mammalian hosts, such as mice (2, 4, 55), and one or more catecholamine receptors may play a role in persistence.

In previous transcriptome analyses of iron-starved versus iron-replete *B. pertussis* and *B. bronchiseptica*, differences in *bfrA*, *bfrD*, and *bfrE* expression levels did not meet the cutoff value to allow their assignment as either iron repressed or iron induced (44). In the present study, we found that *bfrA* and *bfrE* expression levels were low under all growth conditions. A previous study also reported very low expression of *bfrE-lacZ* in *B. pertussis* (21). BfrD was originally described as an outer membrane protein produced by Bvg$^+$ virulent-phase *B. pertussis* (22), and a subsequent mutagenesis study using *lacZ* gene fusions confirmed the Bvg regulation of *B. pertussis bfrD* (21). In those studies, the bacteria were cultured in iron-replete medium, since the functions of BfrD and BfrE were unknown at the time. In the present analysis, *bfrE* and *bfrD* exhibited higher transcription levels when bacteria were grown in iron-replete medium than in iron-depleted SS medium. Typically, iron acquisition genes are repressed by Fur and iron under iron-replete growth conditions, and thus show elevated expression during iron starvation. For *bfrE* in both *Bordetella* species, expression under both iron conditions was very low. In contrast, *bfrD* transcription was consistently and significantly higher in iron-replete medium than the expression in iron-starved *B. bronchiseptica* and *B. pertussis*. Iron starvation is an important cue for pathogenic bacteria, informing them that they are in the host environment, where free iron is scarce (56, 57). It is possible that having *bfrD* under BvgAS virulence control serves the same signaling purpose in *Bordetella* cells, supplanting the role of the iron starvation cue. However, since iron-replete *in vitro* growth conditions increased *bfrD* transcription, it may be that other signals feed into the regulatory pathway in the host environment.

Alternatively, other mechanisms, such as differential mRNA

stability or small regulatory RNA species, may control *bfrD* transcription in relationship to the organism's iron status. Although regulatory RNAs have been found in *B. pertussis*, those controlling TonB-dependent receptor gene expression have not been reported (58).

During modulation, *Bordetella* cells are in the Bvg$^-$ phenotypic phase; Bvg-activated virulence genes are minimally transcribed, and genes such as those involved in motility and chemotaxis are maximally expressed. The Bvg intermediate-phase genes are maximally expressed at intermediate modulator concentrations (2, 4). When *Bordetella* cells are switched from modulating conditions to nonmodulating conditions, the transit from the Bvg$^-$ to Bvg$^+$ phase is characterized by restoration of Bvg-activated gene expression, paralleling the increasing intracellular phosphorylated BvgA (BvgA$\sim$P) concentration. Expression of the class 2 genes (e.g., those encoding fimbriae, filamentous hemagglutinin, and BvgAS) resumes early after removal of modulators, and transcription of the class 1 genes (encoding pertussis toxin, adenylate cyclase, and the type III secretion system) resumes late in this process (59). A previous study noted that the *cyaA* adenylate cyclase gene, a class 1 late gene in *B. pertussis*, was transcribed significantly earlier in *B. bronchiseptica* (60). There is significant variability in the BvgS amino acid sequences of the classical *Bordetella* species (52, 61, 62). Using the *B. bronchiseptica* RB52 strain carrying the *B. pertussis bvgAS* genes, previous studies indicated that this difference in *cyaA* expression was not due to species differences in the *cyaA* promoter or *bvgAS* sequences (60). The authors postulated that the *cyaA* transcription profiles were likely due to other differences between the two *Bordetella* species. Our results demonstrated variations in *bfrD* expression between *B. pertussis* and *B. bronchiseptica*. Notably, *B. bronchiseptica bfrD* expression was resistant to modulation by nicotinic acid and sulfate, whereas in *B. pertussis*, the same gene was readily modulated, as was native *B. pertussis bfrD*. In strain RB52 carrying *bvgAS$_{Bp}$*, *bfrD* modulation was achieved only at the highest modulator concentrations, compared with no modulation in the wild-type parent strain RB50. These results indicate that the *Bordetella* species differences observed in *bfrD* transcription may be due to variations in BvgAS. It is clear that expression of *bfrD* in both *Bordetella* species requires BvgAS. However, in *B. bronchiseptica*, the relative resistance of *bfrD* to modulation indicates that very low concentrations of BvgA$\sim$P are sufficient for transcriptional activation and high-level expression of the gene.

As obligate mammalian pathogens, the classical *Bordetella* species evolved to utilize host nutritional resources. In the host respiratory tract, it is unknown whether catecholamines are the authentic substrates for the BfrA, BfrD, and BfrE receptors. Serum is known to exude onto the respiratory mucosa (63), and metabolomic studies of serum indicate the presence of not only catecholamines but also catechols and other aromatic compounds (64), which may also be substrates for the receptors. Given the differences in *B. pertussis* and *B. bronchiseptica* host ranges and the acute versus chronic nature of the infections they cause, their catecholamine receptors may have evolved to carry out distinct functions.

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