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Expression of the Putative Siderophore Receptor Gene *bfrZ* Is Controlled by the Extracytoplasmic-Function Sigma Factor BupI in *Bordetella bronchiseptica*

ELIZABETH PRADEL† AND CAMILLE LOCHT*

INSERM U447, Institut Pasteur de Lille, 59019 Lille Cedex, France

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A new gene from *Bordetella bronchiseptica*, *bfrZ* encoding a putative siderophore receptor, was identified in a Fur-repressor titration assay. A *bfrZ* null mutant was constructed by allelic exchange. The protein profile of this mutant is similar to that of the wild-type parent strain. The BfrZ$^-$-BfrZ$^+$ isogenic pair was tested for utilization of 132 different siderophores as iron sources. None of these iron sources acted as a ligand for BfrZ. Translational *bfrZ*::*phoA* and transcriptional *bfrZ*::*lacZ* fusions were introduced into the *B. bronchiseptica bfrZ* locus. No alkaline phosphatase or β-galactosidase activity was detected. Sequence analysis of the *bfrZ* upstream region revealed the presence of two tightly linked genes, *bupI* and *bupR*. Both of these genes are located downstream from a Fur-binding sequence. BupI is homologous to *Escherichia coli* FecI and *Pseudomonas putida* PupI and belongs to the family of extracytoplasmic-function sigma factors involved in transcription of genes with extracytoplasmic functions. BupR is homologous to the FecR and PupR antisigma factors and is predicted to be localized in the inner membrane. Similar to the surface signaling receptors FecA and PupB, BfrZ bears an N-terminal extension. We found that *bfrZ* is not transcribed when *bupI* and *bupR* are expressed at the same level. However, overexpression of *bupI* from a multicopy plasmid triggers *bfrZ* transcription, and under these conditions BfrZ was detected in membrane fractions. By analogy with the FecI-FecR-FecA and PupI-PupR-PupB systems, our data suggest that *bfrZ* expression is inducible by binding of the cognate ligand to BfrZ and transduction of a signal through the envelope.

Iron is essential for the growth of most microorganisms but is usually not readily accessible. In an oxic environment at neutral pH, the concentration of free Fe^{3+} in solution is less than 10^{-18} M, and in the host iron is sequestered by proteins such as transferrin and lactoferrin (for a recent review see reference 9). To fulfill their iron requirement, bacteria have developed very efficient iron uptake systems. Most aerobic and facultatively aerobic bacteria secrete one or two small Fe^{3+}-complexing molecules, named siderophores, and produce specific siderophore receptors at their surfaces. Bacteria usually synthesize multiple receptors to scavenge exogenous iron chelates secreted by other microbial species. However, some pathogens (for instance, *Neisseria* spp. and *Haemophilus influenzae*) use heme and the ferriproteins of their hosts as iron sources without producing siderophores (20, 27, 42). *Serratia marcescens* and *Pseudomonas aeruginosa* secrete a small protein which can release heme from hemoglobin and then bind to a receptor at the cell surface (28).

Ferrisiderophore transport systems and their regulation have been well characterized in *Escherichia coli* and several other gram-negative bacteria. The TonB-ExbB-ExbD envelope complex (Ton system) enables transfer of all iron-loaded siderophores to the periplasm, and then siderophore-specific ABC-type systems transport the iron chelates across the inner membrane (9, 32). Genes encoding iron uptake systems are repressed by the Fur protein under high-iron growth conditions (see reference 13 for a recent review). The Fur-Fe^{2+} complex binds to promoters containing target sequences named Fur-binding sequences (FBS) and thus blocks transcription. The FBS consensus sequence was recently reexamined and is thought to result from combination of at least three adjacent 6-bp NATA-TAT motifs (18) instead of a previously proposed 19-bp palindromic AT-rich sequence (13). In the absence of Fe^{2+}, Fur does not bind to the FBS, and promoters are derepressed. Recently, positive Fur-Fe^{2+} regulation of the *E. coli* iron superoxide dismutase promoter was reported. The mechanism of this activation has not been elucidated yet, but it does not involve an FBS (16).

Several iron transport genes are also positively regulated by their cognate ligands via a surface signaling mechanism (8, 13). The ferric dicitrate uptake system is the only ligand-inducible iron uptake system known in *E. coli*. It has been extensively studied, and the following regulation model has been proposed and refined (8, 17, 22). Binding of ferric dicitrate to the outer membrane FecA receptor generates transmission of a signal from the periplasmic N-terminal extension of FecA to the C-terminal periplasmic domain of inner membrane protein FecR via the Ton system. FecR then transduces the signal across the cytoplasmic membrane and activates the sigma factor FecI. FecI binds to the RNA polymerase core enzyme, and the complex initiates transcription of the *fecABCDE* operon. The *fecIR* and *fecABCDE* operons are linked on the chromosome, and both are Fur repressed (1). A similar surface signaling mechanism has been identified in *Pseudomonas putida* WCS358 for *pupB* regulation (24). PupB is a receptor for pseudobactins BN7 and BN8. Upon binding to PupB, these

* Corresponding author. Mailing address: INSERM U447, Institut Pasteur de Lille, 59019 Lille Cedex, France. Phone: (33) 3 20 87 11 51. Fax: (33) 3 20 87 11 58. E-mail: camille.locht@pasteur-lille.fr.

† Present address: INSERM CJF 9606, Faculté de Médecine, 13385 Marseille Cedex 05, France.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid(s)	Relevant features[a]	Source or reference
E. coli strains		
CC102	CC118 with F42 *lacI3 zzf-2*::Tn*phoA*; Km^r	30
CC118	*recA1* Δ*phoA20*	30
GM2163	*dam-13*::Tn*9 dcm-6*; Cm^r	This laboratory
H1717	*aroB fhuF*::λ*plac*Mu; Km^r	46
SM10	Mobilizing strain; Km^r	45
XL1-Blue	High-efficiency transformation; Tc^r	Stratagene
B. bronchiseptica strains		
BB1015	Sm^r but not *rpsL*	39
BBEP173	BB1015 *fur173*	This laboratory
BBEP185	BB1015 *bfrZ*::*lacZ* (pEP589 integration); Gn^r	This study
BBEP186	BBEP173 *bfrZ*::*lacZ* (pEP589 integration); Gn^r	This study
BBEP187	BBEP205 *bfrZ*::*lacZ* (pEP589 integration); Gn^r	This study
BBEP205	BB1015 *alcR*::Km^r	39
BBEP231	BB1015 *bfrZ*::Km^r	This study
BBEP250	BB1015 *bfrZ*::*phoA* (pEP515 integration); $BfrZ^+$ Km^r Gn^r	This study
BBEP251	BBEP173 *bfrZ*::*phoA* (pEP515 integration); $BfrZ^+$ Km^r Gn^r	This study
B. pertussis strain	BPSM TohamaI *rpsL*; Sm^r	31
Plasmids		
$pBCSK^+$	High-copy-number vector; Cm^r	Stratagene
pUC18, pUC19	High-copy-number vector; Ap^r	Roche
pUC4K	Source of Km^r cassette	Pharmacia
pBBR1MCS	Broad-host-range vector; Cm^r	25
pFus2	*Bordetella* suicide vector to generate *lacZ* transcriptional fusions; Gn^r	2
pJK200SK	*Bordetella* suicide vector; Gn^r	41
pEP278	$pBCSK^+$ bearing *bfrZ′* on a 2.6-kb *Pst*I fragment	This study
pEP410	pUC19 bearing *bfrZ*::Km^r	This study
pEP416	pJQ200SK bearing *bfrZ*::Km^r	This study
pEP434	pUC18 bearing *bfrZ*::Km^r and *bfrZ* downstream region	This study
pEP453	pBBR1MCS bearing *bfrZ* on a *Pst*I-*Sac*I fragment	This study
pEP482	pEP453 bearing *bfrZ*::Tn*phoA*	This study
pEP484	pEP482 deleted for the transposase gene (Δ*Bam*HI)	This study
pEP515	pJQ200SK bearing *bfrZ*::*phoA*	This study
pEP589	pFus2 bearing *′bfrZ′* to generate a *bfrZ-lacZ* fusion	This study
pEP596	$pBCSK^+$ bearing *bfrZ* upstream region	This study
pEP624	pBBR1MCS bearing *bupI bupR bfrZ′* on an *Nsi*I-*Sca*I fragment	This study
pEP625	pBBR1MCS bearing *bupI bupR′* on an *Nsi*I-*Sal*I fragment	This study

[a] Ap^r, Cm^r, Gn^r, Km^r, Sm^r, and Tc^r, resistance to ampicillin, chloramphenicol, gentamicin, kanamycin, streptomycin, and tetracycline, respectively.

siderophores induce transduction of a signal from the receptor to the cytoplasmic PupI sigma factor via the Ton system and the PupR antisigma factor localized in the envelope. The *pupIR* operon contains an FBS and is located upstream from *pupB* (24).

Iron uptake systems and their regulation in *Bordetella* have not been completely characterized yet. *Bordetella bronchiseptica*, the etiologic agent of swine atrophic rhinitis and kennel cough, and *Bordetella pertussis*, the agent of whooping cough, both secrete the siderophore alcaligin (34). The *Bordetella fur* gene has been identified and has been shown to mediate iron regulation in these species (6, 10; Pradel and Locht, Abstr. 95th Gen. Meet. Am. Soc. Microbiol. 1995, abstr. B-357, 1995). The *alcABCDE* operon encodes enzymes of the alcaligin biosynthesis pathway (19, 21, 39). AlcR is an AraC type of activator of the *alc* operon and of the alcaligin receptor gene *fauA* (7, 11, 39). The *alcABCDE, alcR*, and *fauA* promoters contain FBS.

In addition to alcaligin, *B. bronchiseptica* and *B. pertussis* have been shown to use enterobactin, heme, hemoglobin, ferrichrome, and desferal as iron sources (4). Four exogenous siderophore receptors have been identified in *B. bronchiseptica*: BfeA, BfrA, BfrB, and BfrC (3–5). *B. pertussis* produces only three of these, and BfrA is specific to *B. bronchiseptica* (4). BfeA binds enterobactin, but the ligands of the Bfr receptors have not been identified yet. The Ton system has been shown to be required for utilization of siderophores, heme, and hemoglobin (36, 40).

To gain more insight into the iron regulatory network in *Bordetella*, we analyzed a *B. bronchiseptica* Fur-repressed gene that we had previously isolated in a genetic screen analysis by a Fur titration assay (FURTA) (39, 46). This gene, named *bfrZ*, encodes a new TonB-dependent receptor for an unidentified ligand, probably an exogenous siderophore. We show here that *bfrZ* expression is controlled by BupI and BupR, a pair of sigma-antisigma transcription factors. We suggest that upon binding of the cognate siderophore to BfrZ, a signal is transduced through the envelope to BupI present in the cytoplasm to induce *bfrZ* transcription.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this work are listed in Table 1. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) medium (33) or on solid media obtained by adding 1.5%

(wt/vol) Bacto Agar. In the FURTA (46), the Lac phenotype of *E. coli* H1717 transformants was tested on MacConkey lactose agar plates containing 50 μM $FeCl_3$. *Bordetella* strains were grown at 37°C on Bordet-Gengou agar plates supplemented with 1% glycerol and 15% sheep blood. PhoA and β-galactosidase activities of *E. coli* or *B. bronchiseptica* strains were assayed on LB medium plates containing 40 μg of bromo-4-chloro-3-indolyl-phosphate (XP) (Sigma, St. Quentin Fallavier, France) per ml and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Sigma), respectively. Liquid cultures of *Bordetella* spp. were grown in modified Stainer-Scholte (SS) medium containing 10 mg of $FeSO_4 \cdot 7H_2O$ per liter (40). The low-iron medium used was SS medium without added $FeSO_4 \cdot 7H_2O$ (SS-Fe). To ensure that the conditions were iron limiting for *B. bronchiseptica*, the Chrome Azurol S assay was used to assess alcaligin production by *Bordetella* cells grown in SS-Fe as described previously (40). Modulation conditions were obtained by adding 50 mM $MgSO_4$ to SS medium or to LB medium plates. When necessary, antibiotics were included in the growth media at the following final concentrations: ampicillin, 150 μg/ml; chloramphenicol, 30 μg/ml; gentamicin, 10 μg/ml; kanamycin, 30 or 300 μg/ml for pEP453 Tn*phoA* mutagenesis; and streptomycin, 100 μg/ml.

DNA techniques. Plasmid DNA was isolated by the alkaline lysis method (43). Restriction enzymes and T4 DNA ligase were obtained from Roche (Meylan, France) and were used according to standard procedures (43). Cloned DNA fragments were sequenced by using an ABI PRISM dye terminator cycle sequencing kit and an ABI PRISM 377 sequencer (PE Applied Biosystems, Warrington, United Kingdom) along with a combination of universal, reverse, and custom-synthesized primers. PCRs were carried out with Vent DNA polymerase (New England Biolabs, Inc., Beverly, Mass.).

Computer analysis of sequences. The nucleotide and protein sequences were analyzed by using the DNA Strider 1.2 software (Service de Biochimie et de Génétique Moléculaire du CEA, Saclay, France). Sequence similarities were identified with the BLASTN, BLASTX, and BLASTP programs (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence alignment was performed with the multalin 5.3.3 software (12). Oligonucleotides were designed with the Oligo 5.0 software (NBI, Plymouth, Minn.).

Construction of pEP278 and pEP416 and cloning of the *bfrZ* 3′ extremity. Plasmid pEP278 bearing *bfrZ′* on a 2.6-kb *Pst*I fragment was isolated with the FURTA from a partial *B. bronchiseptica* BB1015 genomic library as described previously (39). The 2.4-kb *Sph*I-*Pst*I fragment bearing *′bfrZ′* was isolated from pEP278 and cloned into pUC19. The resulting plasmid was introduced into *E. coli* GM2163 *dcm* (Cm^r) and then reisolated from this strain to enable digestion with *Stu*I. The *Hin*cII Km^r cassette was isolated from pUC4K and inserted into the unique *Stu*I site of *bfrZ* (Fig. 1). The resulting plasmid, pEP410, was digested with *Sph*I and *Xba*I, and the 3.7-kb fragment bearing *bfrZ*::Km^r was cloned into the *Bordetella* suicide vector pJQ200SK, digested with the same restriction enzymes, to obtain pEP416. *E. coli* SM10 was transformed with pEP416 and used as a donor in conjugations with *B. bronchiseptica* BB1015. *B. bronchiseptica* BBEP231 was isolated as an Sm^r Km^r Gn^s exconjugant, and the correct allelic exchange in this strain was confirmed by Southern blot hybridization (data not shown). To isolate the *bfrZ* downstream region, genomic DNA of BBEP231 was digested with *Sph*I, and 4- to 5-kb restriction fragments were cloned into pUC18. Recombinant pEP431 Ap^r Km^r was selected, and the nucleotide sequence of the *bfrZ* 3′ extremity was determined.

Siderophore utilization plate assay. *B. bronchiseptica* cells and plates were prepared as described previously (40). Filter paper disks impregnated with 10 μl of a siderophore solution (5 mM) were applied to the surfaces. Growth stimulation around the disks was evaluated after 12 h of incubation at 37°C.

Construction of pEP453 and Tn*phoA* mutagenesis. The complete *bfrZ* gene was reconstituted in *Bordetella* replicative plasmid pBBR1MCS as a 3-kb *Pst*I-*Sac*I fragment to obtain pEP453. Tn*phoA* (30) was introduced into *E. coli* CC118(pEP453) by mating with *E. coli* CC102. Transpositions of Tn*phoA* onto pEP453 were selected on LB medium containing XP and 300 μg of kanamycin per ml. Plasmids were prepared from pools of bacteria and transformed into *E. coli* CC118. None of the resulting Cm^r Km^r colonies showed a strong $PhoA^+$ phenotype (no dark blue colonies). One clone which exhibited rather low yet detectable PhoA activity (pale blue colonies) was named CC118(pEP482) and was studied further. Sequencing of the fusion joint indicated that Tn*phoA* was inserted in frame into *bfrZ*, 65 bp downstream from the *Stu*I site (Fig. 1). Plasmid pEP482 was digested with *Bam*HI to delete the transposase gene and was religated to generate pEP484.

Construction of *B. bronchiseptica bfrZ*::*phoA* chromosomal fusions. A 6.6-kb *Bam*HI-*Eco*RV fragment containing *bfrZ*::*phoA*Km^r from pEP484 was inserted into the *Bordetella* Gn^r suicide vector pJQ200SK opened with *Bam*HI and *Sma*I to generate pEP515. As this construct bore the *bfrZ* promoter region up to the *Pst*I site, pEP515 integration into the chromosome conserved an intact *bfrZ* copy.

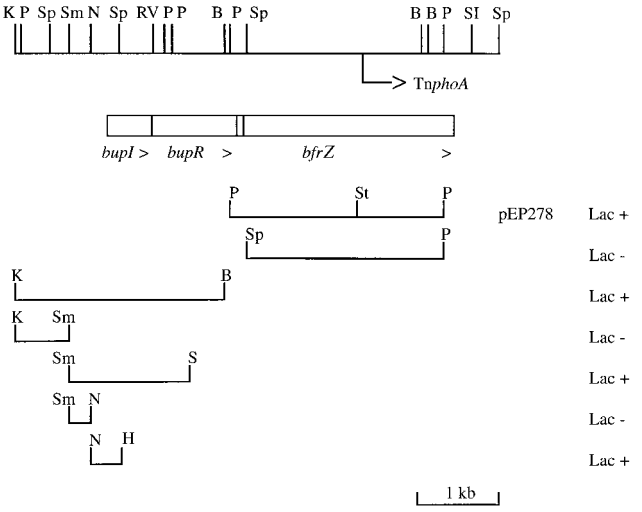

FIG. 1. Physical map of the *B. bronchiseptica bupI bupR bfrZ* locus and relevant constructions described in this study. The recombinant plasmid-associated Lac phenotypes in the FURTA are indicated on the right. In this genetic test, a Lac^+ phenotype indicates the presence of an FBS on a multicopy plasmid. The boxes representing ORFs are drawn to scale; the arrowheads indicate the direction of transcription. The Tn*phoA* insertion site is indicated by an arrow. Certain restriction sites are indicated as follows: B, *Bam*HI; H, *Hin*cII; K, *Kpn*I; N, *Nsi*I; P, *Pst*I; RV, *Eco*RV; S, *Sal*I; SI, *Sac*I; Sc, *Sca*I; Sm, *Sma*I; Sp, *Sph*I; St, *Stu*I.

E. coli SM10(pEP515) was mated with *B. bronchiseptica* BB1015 and BBEP173 *fur173* to isolate the *bfrZ*::*phoA* Sm^r Km^r exconjugants BBEP250 and BBEP251, respectively.

Construction of *B. bronchiseptica bfrZ*::*lacZ* mutants. An internal *bfrZ* fragment was amplified from pEP278 by using oligonucleotides *Hin*dIII-*bfrZ* (5′-AAGCTTCGTTGTCGGGCAGCAATCTC-3′) and *bfrZ*-*Bam*HI (5′-GGATCCGCTCTTGGGCTCCTGGAAG-3′), which hybridized 264 bp downstream from the *Sph*I site and 136 bp upstream from the *Sca*I site (complementary strand), respectively. The 680-bp PCR product was cloned into the *Hin*cII site of $pBCSK^+$. The resulting plasmid was digested with *Hin*dIII and *Bam*HI, and the 680-bp fragment obtained was ligated into pFus2 (2) digested with the same enzymes to generate pEP589. This *Bordetella* Gn^r suicide plasmid was introduced into *B. bronchiseptica* BB1015, BBEP173 *fur173*, and BBEP205 *alcR*::Km^r by conjugation with *E. coli* SM10(pEP589). For each strain, one Sm^r Gn^r exconjugant bearing pEP589 integrated into the chromosome was studied further; these exconjugants were designated BBEP185, BBEP186, and BBEP187. In contrast to the *bfrZ*::*phoA* translational mutants, the *bfrZ*::*lacZ* transcriptional mutants were defective for BfrZ production, as pEP589 did not contain the *bfrZ* promoter.

Cloning of the *bfrZ* upstream region and construction of pEP624 and pEP625. *B. bronchiseptica* BBEP185 genomic DNA was digested with *Not*I, which did not cut pEP589, and ligated. The ligation mixture was used to transform *E. coli* XL1-Blue to Gn^r. A recombinant plasmid resulting from intramolecular ligation of a chromosomal *Not*I fragment containing pEP589 was isolated. Restriction mapping of this plasmid enabled us to localize the *bfrZ* upstream region in a 6-kb *Eco*RI fragment. This fragment was cloned into $pBCSK^+$ to obtain pEP596. The 3-kb region upstream from *bfrZ* was sequenced up to the *Kpn*I site shown in Fig. 1. Plasmids pEP624 and pEP625 were derivatives of pBBR1MCS containing a 3-kb *Nsi*I-*Sca*I fragment bearing *bupI bupR bfrZ′* cloned into the *Nsi*I and *Sma*I sites and a 1.2-kb *Nsi*I-*Sal*I *bupI bupR′* fragment inserted into the *Nsi*I and *Sal*I sites, respectively.

Cell fractionation and protein analysis. Cells from 100-ml *B. bronchiseptica* cultures grown in SS-Fe to an optical density at 600 nm of 3 were harvested by centrifugation, resuspended in 10 ml of 10 mM HEPES (pH 7.4) containing DNase (10 μg/ml) and disrupted with a French pressure cell (SLM-Aminco, Rochester, N.Y.). The pressates were centrifuged at 2,065 × *g* for 10 min at 4°C to sediment cellular debris and unbroken cells. Whole-cell lysates were then centrifuged at 111,000 × *g* for 1 h at 4°C to separate soluble and insoluble cell fractions. Whole-membrane pellets were resuspended in 3 ml of 1% Sarkosyl in 10 mM HEPES buffer (pH 7.4) and incubated for 30 min at room temperature.

The suspensions were centrifuged at 111,000 × *g* for 1 h at 4°C to pellet outer-membrane-enriched fractions. The pellets were resuspended in 300 μl of 10 mM HEPES buffer (pH 7.4). After solubilization at 95°C for 5 min in Laemmli buffer (26), proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by using a 5% stacking gel and a 10% separating gel.

Nucleotide sequence accession number. The nucleotide sequence of the *B. bronchiseptica* 5,909-bp *Kpn*I-*Sph*I DNA fragment has been assigned EMBL accession no. AJ251793.

RESULTS

Identification of *bfrZ*. The FURTA (46) was used to detect potential Fur-binding fragments in a *B. bronchiseptica* genomic DNA library as described previously (39). In this genetic screen analysis, recombinant high-copy-number plasmids were introduced into an *E. coli* Δ*lac* strain bearing a Fur-repressible *fhuF*::*lacZ* fusion (Lac$^-$ phenotype in high-iron growth conditions) and transformants were selected on iron-rich MacConkey agar plates containing antibiotics. Cloned sequences containing FBS titrated out the Fur repressor, resulting in derepression of the chromosomal *lacZ* fusion. Such transformants formed red colonies on MacConkey agar plates (Lac$^+$ phenotype). Plasmid pEP278 was isolated and studied further since it gave a strong Lac$^+$ phenotype in the assay. Restriction mapping showed that pEP278 contained a 2.6-kb *Pst*I DNA fragment (Fig. 1). Deletion of a 200-bp *Pst*I-*Sph*I fragment at one end of the insert conferred a Lac$^-$ phenotype in the FURTA, suggesting that this region contained an FBS (Fig. 1). The nucleotide sequence of the *Pst*I fragment was determined. Consistent with the genetic data, a sequence homologous to the recently reexamined *E. coli* Fur-binding consensus sequence (18) was identified 123 bp upstream from the *Sph*I site (aATAAcGAatcTCAaTAT; 12 of 18 matches). Sequence analysis revealed a large open reading frame (ORF) starting 80 bp downstream from the putative FBS and extending to the opposite *Pst*I site. Of three potential in-frame initiation codons, only the first ATG was preceded by a sequence resembling the canonical AAGAGG *E. coli* ribosome binding site. Thus, translation most probably starts at this ATG, which is located 4 bp downstream from a putative AAGGGAGAA ribosome binding site.

Scanning of the Swiss Protein Data Base revealed that the deduced amino acid sequence of the 821-residue ORF is homologous to that of TonB-dependent receptors. The closest homologue whose ligand has been identified is the ferripyoverdin receptor FpvA of *P. aeruginosa* PAO1 (GenBank accession no. P48632), with 22% identity, 38% similarity, and 11% gaps in a 699-amino-acid (aa) overlap. The ORF was named *bfrZ* by analogy with the previously characterized putative *B. bronchiseptica* siderophore receptor genes *bfrA, bfrB*, and *bfrC* (3, 4). The N-terminal region of BfrZ was predicted to contain a 41-residue cleavable signal peptide. BLASTP searches in the GenBank database indicated that the N-terminal sequence of the proposed mature BfrZ protein is similar to that of a class of *Pseudomonas* sp. siderophore receptors whose production is induced in the presence of their cognate siderophores. A sequence alignment of this region with the corresponding regions of the pseudobactin M114 receptor PbuA of *Pseudomonas* sp. strain M114 (GenBank accession no. Q08017), the ferripyoverdin receptor FpvA of *P. aeruginosa* (GenBank accession no. P48632), and the pseudobactin BN7-BN8 receptor PupB of *P. putida* (GenBank accession no. P38047) is presented in Fig. 2. The N-terminal periplasmic extension of PupB has been shown to be involved in signal transduction and transcriptional control of *pupB* and other iron transport genes (24). A similar role is suspected for the N terminus of FpvA (44).

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BfrE    1 QPAQALEQPL SFDMAPQPLG QALIELNRRT ALSIAFDPRQ VEHFQAPAIR
PbuA    1        QEW TLNIPSQPLA QALQTLGQQT SLQIIYSPES LQGLRSTALN
FpvA    1        QEV EFDIPPQALG SALQEFGRQA DIQVLYRPEE VRNKRSSAIK
PupB    1     AAQAQA DFDIPAGPLA PALAHFGQSA HILLSYPTAL TEGRSTSGLA

BfrE   51 GVMTSEQALR QALSGSNLAY SLRGQVATVR QQEVA....Q LMPVTVS 93
PbuA   44 GRYQDDESLK AMLNGTGIRY QRDGNTVTV. LGPATGSAME LAPTNVN 90
FpvA   44 GKLEPNQAIT ELLRGTGASV DFQGNAITIS VAEAADSSVD LGATMIT 90
PupB   47 GRFDIDQGLA ILLAGTGLEA SRGANASYSL QASASTGALE LSAVSIS 93
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FIG. 2. Sequence alignment for the deduced mature N terminus of BfrZ and the N-terminal periplasmic extensions of three inducible *Pseudomonas* siderophore receptors. PbuA, pseudobactin M114 receptor of *Pseudomonas* sp. strain M114; FpvA, ferripyoverdin receptor of *P. aeruginosa*; PupB, pseudobactin BN7-BN8 receptor of *P. putida*. Conserved residues are indicated by boldface type.

The presence of *bfrZ* in other *Bordetella* genomes was tested by Southern blot hybridization using the 2.6-kb *Pst*I fragment of pEP278 as a probe. No hybridization signal was detected with *B. pertussis, Bordetella parapertussis*, or *Bordetella avium* genomic DNA (data not shown). Thus, similar to *bfrA, bfrZ* is probably specific for *B. bronchiseptica*.

Characterization of a *bfrZ* mutant and cloning of the *bfrZ* 3′ terminus. *B. bronchiseptica* BBEP231, a *bfrZ*::Km^r mutant, was constructed by allelic exchange as described in Materials and Methods. Whole-membrane and outer-membrane-enriched fractions of BBEP231 and the BB1015 parent strain grown in low-iron medium were analyzed by SDS-PAGE. No difference in the protein profiles was observed, suggesting that BfrZ is not abundantly present in the outer membrane (data not shown). BBEP231 exogenous siderophore utilization and BB1015 exogenous siderophore utilization were compared in a plate bioassay. More than 110 pyoverdins and 22 other siderophores from the Jean-Marie Meyer collection (Strasbourg, France) were tested. The two strains had identical iron source utilization profiles (data not shown). Thus, none of the siderophores tested proved to be the BfrZ ligand.

To isolate the *bfrZ* 3′ extremity, BBEP231 genomic DNA was digested with *Sph*I and cloned into pUC18 to obtain pEP431. Sequencing of the 900-bp *Bam*HI-*Sph*I fragment indicated that the *bfrZ* stop codon is located 123 bp downstream from the *Pst*I site (Fig. 1). The deduced mature BfrZ protein is a 819-residue molecule with a calculated molecular mass (MM) of 91.3 kDa. Its C-terminal sequence contains a TonB-dependent receptor signature, TIVWGNERRAMLNAQLSF (PROSITE accession no. PDOC00354). A truncated ORF starting about 200 bp downstream from *bfrZ* and having the same orientation was detected. In a 112-residue overlap, the translation product exhibited 59% similarity with a 127-aa putative inner membrane protein encoded by a *Sphingomonas aromaticivorans* catabolic plasmid (GenBank accession no. O58848).

Expression of *bfrZ*::*phoA* and *bfrZ*::*lacZ* fusions. A translational *bfrZ*::*phoA* fusion was isolated by Tn*phoA* mutagenesis of pEP453, an *E. coli-Bordetella* shuttle vector containing the whole *bfrZ* gene on a *Pst*I-*Sph*I fragment. The fusion junction of the Tn*phoA* insertion is indicated in Fig. 1. The mutagenized

plasmid, pEP482, and its derivative deleted for the transposase gene, pEP484, conferred low but detectable levels of PhoA activity to *E. coli* Δ*phoA* cells (data not shown). However, *B. bronchiseptica* BB1015(pEP484) grown in low-iron conditions expressed no detectable PhoA activity, although these conditions were sufficient to induce alcaligin production. In addition, no PhoA activity was detected in *B. bronchiseptica* BBEP205 *alcR*::Km^r bearing pEP484, suggesting that *bfrZ* expression is not repressed by AlcR, the regulator of alcaligin biosynthesis and alcaligin receptor genes (data not shown). *B. pertussis* BPSM(pEP484) did not produce any detectable PhoA activity either (data not shown). The same *bfrZ*::*phoA* fusion was introduced into the *bfrZ* locus of *B. bronchiseptica* BB1015 and its *fur173* derivative, BBEP173. During growth in low-iron medium the resulting strains, BBEP250 and BBEP251, expressed no PhoA activity. Taken together, these observations suggest that either the hybrid BfrZ-PhoA protein is highly unstable in *Bordetella* or additional sequences not present in pEP484 are required for *bfrZ* expression in *Bordetella*.

To bypass potential hybrid protein instability, a transcriptional *bfrZ*::*lacZ* fusion was constructed and introduced into *B. bronchiseptica* BB1015, BBEP173 *fur173*, and BBEP205 *alcR*::Km^r. None of the strains obtained expressed β-galactosidase activity in low-iron growth conditions. These data suggest that *bfrZ* transcription is tightly regulated, as has been shown for several siderophore receptor genes in *Pseudomonas* spp. (13).

Identification of the *bupI* and *bupR* genes upstream from *bfrZ*. The *bfrZ* upstream region was subcloned from pEP595, a large recombinant plasmid generated by digestion of BBEP185 *bfrZ*::*lacZ* genomic DNA with *Not*I followed by intramolecular ligation. Sequence analysis of the 2.6-kb *Kpn*I-*Pst*I fragment upstream from *bfrZ* revealed the presence of two tightly linked ORFs separated from *bfrZ* by 100 bp and having the same orientation (Fig. 1). BLASTP searches performed with sequences in the GenBank database indicated that the first ORF translates into a product homologous to *P. putida* PupI and *E. coli* FecI ECF sigma factors (GenBank accession no. S46355 and JV0111, respectively). The second ORF encodes a protein similar to the *P. putida* PupR and *E. coli* FecR antisigma factors (GenBank accession no. S46356 and B37804, respectively). By analogy with the *P. putida* system, the ORFs were designated *bupI* and *bupR*.

The deduced BupI protein is a 177-aa molecule with a calculated MM of 18.8 kDa. It exhibits 29% identity and 49% similarity with PupI in a 130-aa overlap and 26% identity and 50% similarity with FecI in a 129-aa overlap. An alignment of the sequences of these proteins is shown in Fig. 3A. Similar to FecI and PupI, BupI is predicted to possess a C-terminal helix-turn-helix motif. The deduced BupR protein is a 344-aa molecule with a calculated MM of 38.2 kDa. It exhibits 24% identity and 42% similarity with FecR in a 306-aa overlap and 22% identity and 37% similarity with PupR in a 310-aa overlap (Fig. 3B). FecR contains a unique hydrophobic transmembrane segment (aa 85 to 105) which anchors it in the inner membrane, while its N- and C-terminal regions are cytosolic and periplasmic, respectively (37, 48). Protein structure prediction programs suggested that PupR and BupR both have this topology and that they possess a transmembrane region

A

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BupI   1 MGGELETPGP GQGWTSLKEC LSAVQRAVAA RVGGQHSAPD LVQEAYARML
PupI   1 M.LPSSDPLL CDVALLYRQQ HSWLTRWLRQ RLNCSQSAAD LAQDTFIRLL
FecI   1 MSDRATTTAS LTFESLYGTH HGWLKSWLTR KLQSAFDADD IAQDTFLRVM

BupI  51 DRPAAASLVN LPGYLYRAAL NIAYNGSARN SVENRVHGQL ALSGDEASAA
PupI  50 NKEQVPQLHA PRTFLAKVAQ SVLCNHYRRQ KLERAYLEAL AMLPEPVVPS
FecI  51 VSETLSTIRD PRSFLCTIAK RVMVDLFRRN ALEKAYLEML ALMPEGGAPS

BupI 101 LDPARICADR QALGRVMAAI DALPPRCREV FVLYRFEGLA QAETAARLGI
PupI 100 LETQAILLE. .TLIALDAAL DGLERPVREA FLLSQVDGLG HTELAQRLAV
FecI 101 PEERESQLE. .TLQLLDSML DGLNGKTREA FLLSQLDGLT YSETAHKLGV

BupI 151 SRNMVEKHVI RAMGACRAAL NEERPVA 177
PupI 148 SVTTVKKYLI KAGALCIMLD HSLDLP  173
FecI 149 SLSSVKKYVA KAVEHCLLER LEYGL  173
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B

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BupR   1 MHCRRQISRP FDVRTGPPSP PELRDPAIDD EAMRWYLALR ETGADDPDHA
PupR   1                MNGQGA TSIPGEVAE. QAMHWHLELQ EPAVS....A
FecR   1                MNPL.L TDSRRQALR. SASHWYAVLS GERVS....P

BupR  51 PLQARFRNWL EQDPRHRPAY DACARDWARL APLQTHYLAT RPGTRRHRGA
PupR  32 ATLAACMSWR QAHPLHEHAW QRTQVFAQRL REMRSPGQRP LAHAALRPQQ
FecR  31 QQEARWQQWY EQDQDNQWAW QQVENLRNQL GGV..PGD.V ASRALHDTRL

BupR 101 WRAACALALV LVVTTIGGLA WQAHTYADFS LLGARQFDTP LEQTAALRMR
PupR  82 SRRTAIKQLS LLMAAGAGLA WYLKDAALVQ DWRA.DYHSR IGEQRRLTLA
FecR  78 TRRHVMKGLL LLLGAGGG.. WQLWQSETGE GLRA.DYRTA KGTVSRQQLE

BupR 151 DGTRVDMDVG TTLTVAYDDA RREVVLESGA AFFDVARDS. QRPFLIRTPA
PupR 130 DGTQVQLNTD SALNVAFDQQ ARRLRLVRGE MLITRPALAD SRPLWVDTEH
FecR 125 DGSLLTLNTQ SAADVRFDAH QRTVRLWYGE IAITTAKDAL QRPFRVLTRQ

BupR 200 GEVHVLGTAF EVQRLADGLL VNVARGHVRI AAPESPAGVE LL.TGQSVRL
PupR 180 GRLESTLAQF NVRLHGQHTQ ATVYQGSVAL QPALHAYPPI LLGAGEQASF
FecR 175 GQLTALGTEF TVRQQDNFTQ LDVQQHAVEV LLASAPAQKR IVNAGESLQF

BupR 249 RQSRFDAVRP IGVNQVAAWR HNRLVFDNRT LGEVAQAITR RGDWTVHVDP
PupR 230 NQQGLLARQA VA.AVAPAWS QGMLVAQGQP LAAFIEDLAR YRRGHLACDP
FecR 225 SASEFGAVKP LD.DESTSWT KDILSFSDKP LGEVIATLTR YRNGVLRCDP

BupR 299 AAARLPITLA VQLNDVANSL QALPEILAVD VVRTGRT.LT ISARSRP344
PupR 279 ALAGLRVSGT FPLENTDKII AAVAETLQLE VQHFTRYWVT LKPRMA 324
FecR 274 AVAGLRLSGT FPLKNTDAIL NVIAQTLPVK IQSITRYWIN ISPL   317
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FIG. 3. Alignment of the deduced sequences of the *B. bronchiseptica* BupI (A) and BupR (B) proteins with the sequences of their *E. coli* FecI and FecR and *P. putida* PupI and PupR homologues. Conserved residues are indicated by boldface type. The highlighted motifs are helix-turn-helix C-terminal regions (A) and predicted transmembrane segments (B).

formed by aa 85 to 105 and aa 103 to 123, respectively (http://www.bmm.icnet.uk/~prof).

Sequence analysis of the DNA region upstream from *bupI* did not reveal any homology with sequences in the database. The 2.5-kb *Kpn*I-*Bam*HI fragment was tested with the FURTA and found to be positive (Fig. 1). Several subclones were constructed and tested with the FURTA in order to localize the FBS more precisely. As shown in Fig. 1, the 380-bp *Nsi*I-*Hin*cII fragment conferred a Lac$^+$ phenotype in the assay. Examination of the corresponding nucleotide sequence indicated that a putative FBS was present 21 bp upstream from the predicted *bupI* initiation codon (atTAATGAgAtTtgTTAT; 12 of 18 matches). A GC-rich 10-bp inverted repeat, CCGCCAGGAC AGGCCGTCCTGGCGG, located 245 bp upstream from *bupI*, could form a transcriptional termination signal. Along with the fact that only 1 bp separates *bupI* and *bupR*, these observations suggest that *bupI bupR* is a Fur-repressed operon.

***bupI* overexpression induces *bfrZ* expression.** To test whether BupI and BupR are involved in *bfrZ* expression, we constructed pEP624 and pEP625, which were derivatives of *Bordetella* replicative plasmid pBBR1MCS bearing the intact *bupI bupR* operon and *bupI* and a truncated *bupR* gene, respectively. These plasmids were introduced into *B. bronchiseptica* BBEP250 *bfrZ*::*phoA*. BBEP250(pBBR1MCS) and BBEP250

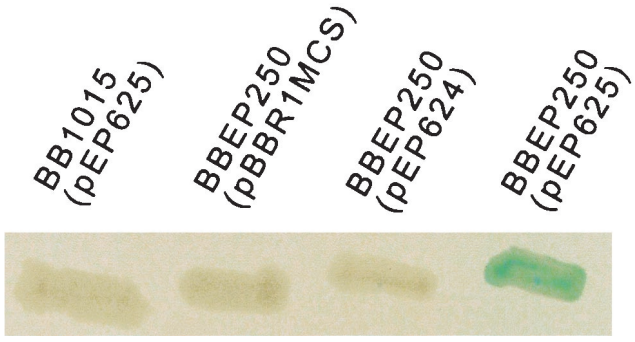

FIG. 4. Alkaline phosphatase activities of *B. bronchiseptica* BB1015 (pEP625), BBEP250(pBBR1MCS), BBEP250(pEP624), and BBEP250 (pEP625) on LB medium plates containing XP and chloramphonicol. pEP624 and pEP625 are pBBR1MCS derivatives bearing *bupI bupR* and *bupI*, respectively. White colonies are $PhoA^-$, and blue colonies are $PhoA^+$.

(pEP624) were found to form white patches on plates containing XP ($PhoA^-$ phenotype), while the BBEP250(pEP625) patches were blue ($PhoA^+$) (Fig. 4). The BB1015(pEP625) control strain was $PhoA^-$, which showed that pEP625 did not induce expression of a resident *B. bronchiseptica* phosphatase activity. In addition, transfer of pEP625 into BBEP185 induced *bfrZ*::*lacZ* expression (data not shown). These results indicated that an excess of BupI over BupR induces *bfrZ* transcription. BBEP250(pBBR1MCS), BBEP250(pEP624), BBEP250 (pEP625), and BBEP185(pEP625) were grown in the presence of 50 mM $MgSO_4$ to modulate virulence gene expression. Expression of the *bfrZ* fusions was not affected (data not shown), suggesting that *bfrZ* is not controlled by the BvgA-BvgS virulence factor regulatory system (29).

Outer membrane protein-enriched fractions were prepared from BB1015(pBBR1MCS), BB1015(pEP625), BBEP250 (pBBR1MCS), BBEP250(pEP625), BBEP231(pEP625), and BBEP185(pEP625) grown in low-iron medium and were analyzed by SDS-PAGE (Fig. 5). As shown in Fig. 5, a protein with an apparent MM of approximately 90 kDa was detected in the extracts from cells bearing an intact *bfrZ* chromosomal gene and *bupI* on a multicopy plasmid (lanes 2 and 4). As the calculated MM of BfrZ is 91 kDa, the protein present in Fig. 5, lanes 2 and 4, was most probably BfrZ. This protein was not visible in extracts from cells carrying an intact *bfrZ* gene but no multicopy *bupI* gene (Fig. 5, lanes 1 and 3) or in extracts from cells harboring *bupI* on a plasmid but having an interrupted *bfrZ* gene on the chromosome (lanes 5 and 6). However, in strains bearing an inactivated *bfrZ* gene (lanes 5 and 6), *bupI* overexpression seemed to induce production of a protein that migrated slightly faster than would be predicted from the MM of BfrZ. This membrane protein may be another siderophore receptor whose synthesis requires BupI. Alternatively, BupI overproduction could generate cross-talk and activate expression of a gene normally transcribed via another extracytoplasmic-function (ECF) sigma factor.

DISCUSSION

We isolated and characterized the *bupI bupR bfrZ* locus, the first example of a putative inducible exogenous siderophore uptake system in *B. bronchiseptica*. The *bupI bupR* genes seem to be transcribed as a single unit from a Fur-repressed promoter, while *bfrZ* expression requires an excess of BupI over BupR in the absence of the cognate ligand. By analogy with the well-characterized *E. coli* FecI-FecR-FecA (1, 8) and *P. putida* PupI-PupR-PupB (24) systems, our data suggest that BupI is an ECF sigma factor localized in the cytoplasm, BupR is an antisigma factor anchored in the inner membrane, and BfrZ is an outer membrane siderophore receptor. However, in the absence of the phenotype of a *bfrZ* mutant with respect to siderophore uptake, we cannot rule out the possibility that BfrZ is involved in transport of a noniron ligand. Nicholson and Beall previously isolated another Fur-repressed *B. bronchiseptica* putative ECF sigma-antisigma pair, BtfI-BtfR, but its target gene(s) has not been identified yet (M. L. Nicholson and B. Beall, Abstr. 98th Gen. Meet. Am. Soc. Microbiol., abstr. B-60, 1998). Other observations have suggested that expression of the *bfeA* enterobactin receptor gene is inducible, as uptake experiments have indicated that the ability of *B. bronchiseptica* to bind enterobactin is increased when cells are precultured with this exogenous siderophore (47). However, the regulation mechanism remains to be elucidated. In *P. aeruginosa*, induction of the enterobactin uptake system involves phosphorylation of a two-component regulatory system (14, 15).

The G+C content of the *bupI bupR bfrZ* locus is 67%, which is similar to those of other *Bordetella* genes. Southern blot experiments indicated that *bfrZ* is not present in the strictly human pathogen *B. pertussis*. We recently used the *Bordetella* BLAST server of the Sanger Centre to scan the available *B. pertussis* and *B. bronchiseptica* genomic DNA sequences for homology with *bfrZ* (http://www.sanger.ac.uk). No *bfrZ* sequence was detected in the 105 assembled contigs, which covered most of the *B. pertussis* genome. A unique *bfrZ* gene was identified in the 1,777 contigs, which covered part of the *B. bronchiseptica* genome. The *B. pertussis* genome is about 1 Mb smaller than that of *B. bronchiseptica* (http://www.sanger.ac.uk). The latter species has a much wider ecological niche as it can survive and grow even in lakewater (38). Another siderophore receptor, BfrA, has also been shown to be specific to *B. bronchiseptica* (4). Considering the importance of iron to sustaining life, it is not surprising that *B. bronchiseptica* has evolved or conserved a significant iron-scavenging potential in addition to other survival strategies.

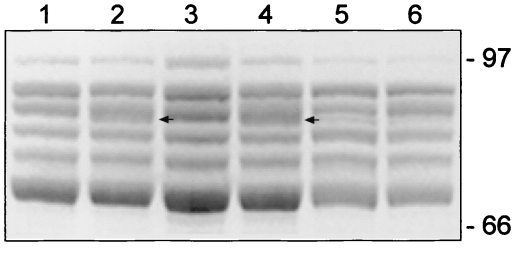

FIG. 5. Effect of *bupI* overexpression on outer membrane protein composition as determined by SDS-PAGE analysis. Cells were grown in low-iron medium. Lane 1, *B. bronchiseptica* BB1015 $bfrZ^+$ (pBBR1MCS); lane 2, BB1015 $bfrZ^+$ (pEP625); lane 3, BBEP250 $bfrZ^+$ *bfrZ*::*phoA* (pBBR1MCS); lane 4, BBEP250 $bfrZ^+$ *bfrZ*::*phoA* (pEP625); lane 5, BBEP231 *bfrZ*::Km^r (pEP625); lane 6, BBEP185 *bfrZ*::*lacZ* (pEP625). The arrows indicate the position of BfrZ. The positions of the MM standards are indicated on the right.

So far, about 200 siderophores secreted by bacteria or fungi have been identified, but many more remain to be isolated (35). We were able to test a wide range of pyoverdins and other siderophores for their ability to stimulate the growth of *B. bronchiseptica* in iron-depleted conditions. In addition to the previously identified siderophores ferrichrome and desferal, we observed that coprogen, schizokinen, ferricrocin, vicibactin, ferrichrysin, ferrirubin, aerobactin, protochelin, and several pyoverdins are iron sources for *B. bronchiseptica* (data not shown). However, we were unable to identify the BfrZ ligand, either because it is absent from the siderophore library or because it is transported via a second receptor in a *B. bronchiseptica bfrZ* null mutant. Koster et al. reported that a *P. putida pupI*::Tn*5* mutant still uses pseudobactin BN8 as an iron source although it does not produce the cognate receptor PupB, suggesting that an additional receptor for the BN8 siderophore is produced in this strain (23). Pseudobactins BN7, BN8, and M114 are not included in the collection that we tested; thus, we cannot comment on their utilization by *B. bronchiseptica*. We used the *B. bronchiseptica bfrZ*$^+$ *bfrZ*::*phoA* mutant as a reporter strain to assay *bfrZ* induction in the presence of siderophores from the collection. No increase in PhoA activity was detected in a plate test, suggesting that the BfrZ ligand is missing from the siderophore library (data not shown).

In the *E. coli* ferric dicitrate transport system, the N-terminal cytoplasmic region of FecR is required and is sufficient for *fecA* expression (48). An *E. coli* strain bearing a nonsense mutation in codon 19 of *fecR* does not express *fecA*, but the production of a 56-residue FecR is sufficient to activate the FecI sigma factor to transcribe *fecA* in the absence of the citrate inducer. In *P. putida*, disruption of *pupR* by deletion of its 5′ region and insertion of an Sm^r interposon triggers constitutive *pupB* expression (24). Thus, PupR is not necessary for *pupB* expression. Overproduction of PupI from a plasmid in conjunction with chromosomal expression of *pupR* leads to *pupB* expression. PupR appears to be more like a stoichiometric repressor of PupI than an enzymatic activator (24). However, intact PupR is required for optimal *pupB* transcription in the presence of the pseudobactin BN8 inducer (24). The Fec and Pup systems are related but may differ with respect to the role of the antisigma factor. In *B. bronchiseptica*, construction of *bupR* mutants and additional *bfrZ* expression studies are required to evaluate the function of BupR. As the cognate siderophore of BfrZ has not been identified yet, construction of a chimeric receptor can also be envisioned. By analogy with the PupB-PupA chimera constructed by Koster et al. (24), a hybrid protein formed by the N-terminal extension of BfrZ and the mature FauA alcaligin receptor could be tested for its ability to transduce a signal to the BupI-BupR system and initiate *bfrZ* transcription upon binding of alcaligin to the FauA moiety.

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