An iron-regulated outer-membrane protein specific to *Bordetella bronchiseptica* and homologous to ferric siderophore receptors

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The *bfrA* (*Bordetella bronchiseptica* ferric iron repressed outer-membrane protein) gene was cloned from *Bordetella bronchiseptica* by screening a library of TnphoA insertion mutants for iron-repressed fusions to *phoA*. The *bfrA* gene encoded an 80 kDa outer-membrane protein with a high level of amino acid sequence identity to several bacterial proteins belonging to the family of Ton B-dependent outer-membrane receptors. BfrA was especially homologous to Cir of *Escherichia coli*, IrgA of *Vibrio cholerae* and to three previously characterized ferric enterobactin receptors. DNA hybridization results indicated that *bfrA* was not present in other *Bordetella* species. Expression of the *bfrA* gene was induced by low iron availability from a promoter overlapped by a sequence resembling a consensus Fur-binding sequence, and *bfrA* expression was derepressed in a *B. bronchiseptica* *fur* mutant. Utilization of the *Bordetella* siderophore alcaligin and the exogenous siderophore enterobactin was unaffected in *bfrA* mutants. Upon attempting to find the specificity of BfrA, 2,3-dihydroxybenzoylserine (DHBS) was shown to be utilized in a *bfeA* (*Bordetella ferric enterobactin receptor gene*)-dependent manner by *B. bronchiseptica* and *B. pertussis*. In addition, the hydroxamate siderophores ferrichrome and desferrioxamine B, and the iron source haemin were shown to be utilized independently of *bfeA* and *bfrA* in *B. bronchiseptica* and *B. pertussis*.

**Keywords:** *Bordetella*, TonB-dependent receptors, iron sources

**INTRODUCTION**

In extracellular bacterial pathogens, uptake of the essential metal iron is often facilitated by the secretion of siderophores capable of binding ferric iron with high affinity. In many Gram-negative species these ferric siderophores are then internalized via specific outer-membrane receptors. *Bordetella* species are known to secrete the hydroxamate siderophore alcaligin to scavenge iron upon iron limiting conditions (Moore *et al.*, 1995; Brickman & Armstrong, 1996a). Some of the genes involved in alcaligin synthesis have been identified recently (Giardina *et al.*, 1996; Kang *et al.*, 1996; Brickman & Armstrong, 1996b).

In addition to utilizing endogenous siderophores, many bacteria are also capable of taking up ferric complexes containing siderophores secreted by other organisms. The important mammalian respiratory pathogens *B. bronchiseptica*, *B. pertussis* and *B. parapertussis* contain the *fepA* gene homologue, *bfeA*, and the former two species have been shown to utilize the exogenous siderophore enterobactin in a process requiring the receptor BfeA (Beall & Sanden, 1995b).

Genes studied so far that are involved in siderophore biosynthesis and iron uptake in *Bordetella* are repressed by iron availability (Beall & Sanden, 1995b; Giardina *et al.*, 1995; Kang *et al.*, 1996) and this repression is apparently mediated by the iron-binding repressor protein Fur (Beall & Sanden, 1995a; Brickman & Armstrong, 1995). To identify additional *Bordetella* genes encoding iron-regulated exported proteins,
TnphoA mutagenesis of B. bronchiseptica was used. We report the sequence and regulation of a gene specific to this Bordetella species that encodes a protein homologous to the bacterial ferric enterobactin receptors FepA (Lundrigan & Kadner, 1986), PfeA (Dean & Poole, 1993) and BfeA (Beall & Sanden, 1995b), the ferric-DHB (2,3-dihydroxybenzoate) and ferric-DHBS (2,3-dihydroxybenzyloxyserine) receptor Cir (Nau & Konisky, 1988) and the Vibrio cholerae virulence protein IrgA (Goldberg et al., 1992). During the course of this study, additional iron sources usable by B. bronchiseptica and B. pertussis were found.

METHODS

Media, growth conditions, and antibiotics. L broth was used for growth of Escherichia coli and B. bronchiseptica, and Stainer–Sholte minimal medium (von Koenig et al., 1988) containing 0.1% Casamino acids (SC) was used for growth of Bordetella strains. L agar containing 100 μM 2,2-diprydyl (DP) and 150 μg 5-bromo-4-chloro-3-indolyl phosphate (XP) ml⁻¹ (DP-XP agar) was used to screen iron-repressed phoA fusions in B. bronchiseptica. The iron-limiting medium for Bordetella strain bioassays was SC agar containing 45 μg ethylenediaminedi(o-hydroxyphenylacetic acid) (EDDHA) ml⁻¹ (SC-EDDHA). For iron source utilization assays, SC-EDDHA was overlaid with 3 ml soft agar containing 10⁶ cells ml⁻¹. Disks containing 10 μl of different iron sources were laid upon the surface and growth halo diameters for B. bronchiseptica and B. pertussis strains were measured after incubation at 35 °C for 16 h and 48 h, respectively. SC broth supplemented with either 100 μM DP or 50 mM FeCl₃ was used for preparation of RNA from B. bronchiseptica strains. Antibiotics were used at the following concentrations (μg ml⁻¹): kanamycin, 35; ampicillin and nalidixic acid, 100; gentamycin, 10.

Iron sources. Enterobactin and DHBS extracts were prepared as described by Porra et al. (1972) and bioassay stocks were 50 μM and 200 μM, respectively. The source of alcaligin used for this study was SC broth cultures supplemented by 100 μM alc alcaligin (Brickman & Armstrong, 1995) grown in the presence of 50 μM putrescine (Brickman & Armstrong, 1996b). The following were obtained from Sigma and the indicated stock concentrations were used in bioassays: haemin, 125 μM; ferrichrome (from Ustilago sphaerogena), 50 μM; desferoxamine B (desferoxamine mesylate), 50 μM; DHB, 1–30 mM; salicylic acid, 1–30 mM; sodium citrate, 0.1–1 M; rhodotorulic acid, 1–3 mM.

Alkaline phosphatase (AP) assay. AP activities of B. bronchiseptica strains containing translational bfrA-phoA fusions were measured as described by Brickman & Beckwith (1975).

Transformation and conjugation. Plasmids were transformed into E. coli by standard methods. TnphoA mutagenesis (Manoil & Beckwith, 1985) was performed as described by Taylor et al. (1989) by introducing the suicide plasmid pRT733 into strain 19385 by conjugation with the donor strain SM10 (pir) (pRT733). Transconjugants were selected on DP-XP agar containing nalidixic acid and kanamycin. Plasmid pSS2141 derivatives (see below) were introduced into B. bronchiseptica strains by conjugation with the donor strain SM10 as described by Stibitz (1994).

Plasmids and strains. The strains and plasmids used for this study are described in Table 1. Plasmid pSS2141, derived from pSS1894 (Merkel & Stibitz, 1995), was used for construction of plasmid pSBG. Plasmid pSBG contains a bfrA::TnphoA insertion shown in pS233 (Fig. 1a). Plasmid pSPH1 was derived from pSBG in a chromosome walking step described below and in Results. Plasmids pS233 and pS1394 are pUC19 derivatives containing constructs cloned from the bfrA::TnphoA mutant strains 19233 and 191394, respectively, and the inserts in these plasmids are depicted in Fig. 1(a). Plasmid pSBFE', which was used for insertional inactivation of the bfeA gene in strains 19233 and 191394, is a pSS2141 derivative which contains a 736 bp structural gene fragment of bfeA (Beall & Sanden, 1995b).

Cloning the bfrA promoter region and structural gene. Transconjugants containing iron-repressed TnphoA fusions were found by replica streaking blue colonies from DP-XP agar onto DP-XP agar and onto XP agar containing 50 μM FeCl₃. To clone a bfrA::phoA translational fusion, chromosomal DNA from the TnphoA insertion mutant 19233, containing an iron-repressed phoA fusion, was cleaved with SalI and ligated with pUC19. This ligation was used to transform E. coli strain DH10B to kanamycin resistance, resulting in plasmid pS233. Plasmid pS1394 was obtained by the same procedure with partially SalI-digested chromosomal DNA from the iron-regulated TnphoA fusion strain 191394. The BgIII fragment from pS233 was cloned into the BamHI site of the conjugative vector pSS2141, resulting in the integrative plasmid pSBG. Plasmid pSBG was introduced onto the chromosome of strain 19383 by conjugation followed by selection for gentamycin and nalidixic acid resistance. Plasmid pSBG contained an Sphi site appropriately located such that the vector origin of replication, the bla gene, and downstream bfeA sequence would be cloned in the following chromosome walking step utilizing Sphi digestion. For this step, chromosomal DNA from one transconjugant containing a Campbell-type insertion of pSBG was digested with Sphi, followed by ligating the DNA and transforming E. coli strain DH10B to ampicillin resistance, resulting in plasmid pSPH1 (Fig. 1).

DNA sequencing. Plasmids pS233, pS1394, pSPH1 and appropriate plasmid subclones were sequenced with Dye-Deoxy Terminator Kits (Applied Biosystems) as described by the manufacturer using oligonucleotides annealing to bfrA region DNA or the M13 multiple cloning site. Reactions were loaded onto 6% polyacrylamide gels and electrophoresed on an Applied Biosystems 373 Sequencer.

Southern analysis. Southern analysis was performed using Bordetella chromosomal DNA prepared as described previously (Beall & Sanden, 1995a) with the Genius Kit (Boehringer Mannheim). A 498 bp SalI bfrA structural gene fragment was labelled and used as a probe with hybridization and high stringency washes at 65 °C as described by the manufacturer. Low stringency Southern analysis was performed by the same protocol, except that hybridization and washes were at 53 °C.

Total- and outer-membrane preparations. Extracts enriched in either total membrane or outer membrane were prepared by sonication followed by high speed centrifugation and sarcosyl extraction as described by Nikaido (1994).

Immunoblotting. Proteins electrophoresed on 10% SDS-polyacrylamide gels were transferred and immunostained as described by Blake et al. (1984) with antiserum against purified E. coli AP obtained from S Prime-3 Prime Inc.

RNA isolation and primer extension. Total RNA was prepared with kits (5 Prime-3 Prime Inc.) as described by the manufacturer followed by phenol/chloroform extraction and ethanol precipitation. The primer 5’GGCGTCTGGATGGC-
## Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or characteristics*</th>
<th>Source or reference</th>
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<tr>
<td><strong>E. coli</strong></td>
<td></td>
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</tr>
<tr>
<td>SM10</td>
<td>RP4-2 Tc::Mu conjugation strain</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>SM10( spir)</td>
<td>As SM10, but spir6K</td>
<td>Taylor et al. (1989)</td>
</tr>
<tr>
<td>DH10B</td>
<td>merA ΔmcrBC ΔhsdR ΔhsdM deoR recA1 endA1 lacZAM15</td>
<td>BRL</td>
</tr>
<tr>
<td><strong>B. bronchiseptica</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19385</td>
<td>ATCC 19385 NalR (wild-type dog isolate)</td>
<td>Laboratory collection</td>
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<tr>
<td>19233</td>
<td>19385(bfrA::TnphoA), TnphoA insertion at base 233 of bfrA</td>
<td>This study</td>
</tr>
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<td>191394</td>
<td>19385(bfrA::TnphoA), TnphoA insertion at base 1394 of bfrA</td>
<td>This study</td>
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<td>19233B</td>
<td>192330pSBFE' (bfeA insertional mutant)</td>
<td>This study</td>
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<td>B013NW</td>
<td>B013NpSBG</td>
<td>This study</td>
</tr>
<tr>
<td>B013NF</td>
<td>B013NMnR4pSBG</td>
<td>This study</td>
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<td><strong>B. pertussis</strong></td>
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<td>82</td>
<td>Wild-type clinical isolate</td>
<td>Beall &amp; Sanden (1995b)</td>
</tr>
<tr>
<td>84</td>
<td>820pKS3 (bfeA insertional mutant)</td>
<td>Beall &amp; Sanden (1995b)</td>
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<td><strong>B. parapertussis</strong></td>
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<td>A168</td>
<td>Wild-type clinical isolate</td>
<td>Beall &amp; Sanden (1995b)</td>
</tr>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pSBFE'</td>
<td>pSS2141 containing a 736 bp structural gene fragment of bfeA, for integrational inactivation of bfeA</td>
<td>This study</td>
</tr>
<tr>
<td>pS233</td>
<td>pUC19 containing a SalI fragment, encompassing 77 codons of bfrA fused to phoA and the KmR gene cloned from TnphoA mutant strain 19233</td>
<td>This study</td>
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<tr>
<td>pSBG</td>
<td>pSS2141 containing a BglII fragment from pSS233, encompassing the bfrA-phoA fusion</td>
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<tr>
<td>pSPH1</td>
<td>Contains entire bfrA gene and flanking sequence, obtained by homologous integration of pSBG followed by excision with SphI, ligation and transformation of E. coli to ApR</td>
<td>This study</td>
</tr>
<tr>
<td>pS1394</td>
<td>pUC19 containing a SalI fragment, encompassing 464 codons of bfrA fused to phoA and the KmR gene cloned from TnphoA mutant strain 191394</td>
<td>This study</td>
</tr>
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</table>

*Ω indicates the strain contains a single homologous inserted copy of the indicated plasmid. ApR, ampicillin resistance; GmR, gentamycin resistance; KmR, kanamycin resistance; NalR, nalidixic acid resistance.

TGCGTGGAAATG 3', complementary to bases 524–548 of the bfrA sequence represented in Fig. 2 was end-labelled with 32P using T4 polynucleotide kinase. Approximately 1 pmol of primer was annealed to 40 μg total RNA at 60 °C and extended at 52 °C with avian myeloblastosis virus reverse transcriptase (Promega) as described by Moran (1993).

**RESULTS**

**Cloning and nucleotide sequence of the bfrA gene**

Following TnphoA mutagenesis of *B. bronchiseptica* strain 19385, iron-repressed translational fusions to phoA were identified by screening kanamycin-resistant blue colonies on DP-XP plates for those requiring DP for full expression of AP. Of a total of approximately 3000 initial blue colonies, 12 independent colonies meeting this criterion were picked for further analysis. Three of these were found later by sequence and Southern analysis (data not shown for one of these) to contain independent insertions within the same gene, bfrA, which is the focus of this study. Studies with the remaining nine TnphoA mutants are in progress. The putative bfrA iron-repressed promoter and chromosomal DNA downstream of the bfrA promoter were cloned using the strategy described in Methods. This involved digesting chromosomal DNA from the TnphoA insertion strain 19233 with SalI, which cleaved within TnphoA adjacent to the phoA and kanamycin resistance gene sequences (Fig. 1a). Ligation of this digest with plasmid pUC19 followed by transforming strain DH10B to kanamycin resistance resulted in the isolation of plasmid pS233 (Fig. 1a), which was found to
B. bronchiseptica bfrA fusions isolated during this study and relevant plasmid insertions.

Fig. 1. (a) The B. bronchiseptica bfrA chromosomal region, TnphoA fusions isolated during this study and relevant plasmid insertions. The bfrA region is shown with the positions of two independently isolated TnphoA insertions (bases 233 and 1394 of the bfrA structural gene within strains 19233 and 191394, respectively). The solid line indicates sequenced DNA shown in Fig. 2. (b) Diagram of plasmid pSPH1 containing the entire bfrA gene. This plasmid was obtained in a chromosome walking step. A conjugative plasmid, pSBG (a derivative of pSS2141) containing the indicated bfrA-phoA fusions. These plasmids were obtained by digestion of chromosomal DNA of the respective TnphoA mutants with SalI, ligation and transformation of E. coli to kanamycin resistance. Just upstream of bfrA ile homologues of electron transfer flavoprotein subunit genes etfA and etfB located by partial sequence analysis. E. coli; P, PstI; S, SalI; Si, SfiI; X, XmnI. (b) Diagram of plasmid pSPH1 containing the entire bfrA gene. This plasmid was obtained in a chromosome walking step. A conjugative plasmid, pSBG (a derivative of pSS2141) containing the indicated bfrA-phoA fusions. These plasmids were obtained by digestion of chromosomal DNA of the respective TnphoA mutants with SalI, ligation and transformation of E. coli to kanamycin resistance. Just upstream of bfrA ile homologues of electron transfer flavoprotein subunit genes etfA and etfB located by partial sequence analysis. E. coli; P, PstI; S, SalI; Si, SfiI; X, XmnI.

A search of the SWISS-PROT protein database revealed significant homology of the deduced bfrA product with several proteins in the family of TonB-dependent receptors (not shown). BfrA had the most amino acid sequence identity with Cir (34%), IrgA (33%), BfeA (26%), PfeA (25%) and FepA (22%) (Fig. 3). Regions of the BfrA protein with the most pronounced homology to these outer-membrane proteins (OMPs) included a 'Ton B box' (region I in Fig. 3) and two other portions highly similar to sequences conserved in Ton B-dependent receptors (regions II and III, Fig. 3). There are two regions of FepA implicated in other studies as putative ligand-binding sites (Murphy et al., 1990) (bold sequences in Fig. 3). While well conserved between the ferric enterobactin receptors FepA, PfeA and BfeA, these regions are not well conserved or absent in BfrA, IrgA and Cir.

A signal peptide of 43 residues was predicted for BfrA (von Heijne, 1985), similar to the predicted PupA (Pseudomonas putida ferric pseudobactin receptor) leader peptide in that it is unusually long and has an extended basic N-terminal region containing 6 basic residues (Fig. 3; Bitter et al., 1991). Among the features conserved between BfrA and many other OMPs are a C-terminal phenylalanine and an arginine residue at position 11 relative to the C terminus (Fig. 3).

Although not the focus of this study, other features of the sequence shown in Fig. 2 include the 3' end of an apparent homologue of etfA (Finocchiaro et al., 1988; Bedzyk et al., 1993) which together with etfB, encode the \( \alpha \) and \( \beta \) subunits of electron transport flavoproteins (Fig. 2). Upstream of etfA lies etfB (\( \beta \) subunit flavoprotein gene), which was found by partial sequence of the region upstream of etfA (Fig. 1a, data not shown). Downstream of etfA, and just upstream of a possible transcriptional terminator, lie eight perfect repeats of the 9-base sequence GCACCGCCAC. The significance of this unusual repeated sequence is unknown.

Expression of the bfrA gene

In the presence of DP, both of the B. bronchiseptica bfrA::TnphoA mutant strains 19233 and 191394 showed iron-repressed AP activity (Table 2). To determine if bfrA gene expression was Fur-regulated, plasmid pSBG, which contains a bfrA-phoA translational fusion, was introduced as a Campbell-type insertion into the fur mutant B013NMnR4 (Brickman & Armstrong, 1995) and its wild-type parental strain B013N. Activity of this fusion was constitutive in the fur mutant strain in iron-replete and iron-limiting con-
**Bordetella bronchiseptica** iron-regulated gene

Fig. 2. Nucleotide sequence of the bfrA gene and the amino acid sequence of the ORF BfrA. Inverted repeats possibly functioning in transcription termination are overlined (positions 284-313 and 2770-2802). A minimal potential ribosome binding site is indicated at positions 474-475 (underlined). -35 and -10 hexamers homologous to corresponding regions of $\sigma^{70}$-recognized promoters are overlined upstream of the transcriptional start site (+1), a T residue at position 432 (in bold type). Two 19-base sequences with homology to the consensus iron box recognized by the *E. coli* Fur protein are underlined with asterisks below bases conserved with the consensus. The positions of the TnphoA insertions in strains 19233 and 191394 are indicated by 4233 and 41394, respectively. Upstream of the potential terminator preceding bfrA are eight perfect repeats of the sequence GCACCCCAC, which is situated downstream of an etfA (electron transport flavoprotein subunit $\alpha$) homologue.

- Conditions, while the parental strain showed iron-repressed AP activity (Table 2).

Primer extension of total RNA from iron-starved wild-type *B. bronchiseptica* with an end-labelled primer revealed a transcript with a 5' terminus mapping to position 432 (Fig. 2; Fig. 4, lane 1). This 5' transcript terminus maps just downstream of a sequence (TTGAAAT-17 bp-TAGCAT) with high similarity to the consensus sequence of *E. coli* $\alpha^{54}$-directed promoters (TTGaca-17 bp-TAtaAT, the upper case positions are the most conserved) (Hawley & McClure, 1983). Overlapping the -10 hexamer just upstream of the transcript 5' terminus lies a sequence identical in 12 of 19 positions
to the consensus Fur binding site (Calderwood & Mekalanos, 1987) (positions 411–429 in Fig. 2). This sequence was the only iron box found with a homology search of the entire 1369 bp of shown in Fig. 2. Another sequence with some similarity to the consensus Fur binding site overlaps the -35 hexamer (9 of 18 identical positions, bases 379–397 in Fig. 2).
**Table 2.** bfrA–phoA expression experiments

Cells were grown to late exponential phase in SC broth containing 50 μM FeCl₃ (+Fe) or in SC broth containing 100 μM DP (−Fe).

<table>
<thead>
<tr>
<th>B. bronchiseptica strain and relevant features</th>
<th>AP units (mean ± SD, n = 3)</th>
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<tbody>
<tr>
<td>191394 (bfrA::TnphoA at base 1394 of bfrA)</td>
<td>+Fe  7 ± 3, 47 ± 9</td>
</tr>
<tr>
<td>19233 (bfrA::TnphoA at base 233 of bfrA)</td>
<td>−Fe  9 ± 3, 60 ± 12</td>
</tr>
<tr>
<td>B013W (wild-type, bfrA::TnphoA at base 233 of bfrA in integrated plasmid)</td>
<td>+Fe  8 ± 4, 70 ± 8</td>
</tr>
<tr>
<td>B013F (as B013W, except fur mutant)</td>
<td>−Fe  81 ± 7, 74 ± 13</td>
</tr>
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</table>

**Fig. 4.** Mapping the transcription start of the bfrA gene (arrow). A 5'-labelled oligonucleotide complementary to the 5' sequence of the non-transcribed strand of the bfrA structural gene was used to prime cDNA synthesis from total RNA prepared from late-exponential phase cultures of strain 19385 (lanes 1 and 2) during iron-limiting (lane 1) and iron-replete (lane 2) conditions. The DNA sequence (A, C, G, T) was generated on the non-transcribed strand with the same labelled oligonucleotide used for the primer extensions.

**Identification of a BfrA–PhoA fusion protein**

Western analysis of whole-cell, total-membrane-enriched and outer-membrane-enriched protein extracts of iron-starved strain 191394 with antibody against the E. coli PhoA protein revealed a protein with an electrophoretic mobility corresponding to a protein of 97 kDa (Fig. 5, lanes 1, 5 and 7). This is close to the predicted size of the BfrA–PhoA fusion protein which should contain the first 421 residues of the mature BfrA protein fused to 461 residues of PhoA. Smaller potential degradation products that reacted with anti-PhoA (Fig. 5, lanes 1, 5 and 7) were also seen upon Western analysis of strain 191394 protein extracts.

We could not identify proteins corresponding to BfrA or BfrA–PhoA fusion proteins in stained 7.5–12% gels after SDS-PAGE of protein extracts (using comparisons of strains 19385, 19233 and 191394) subjected to SDS-PAGE on 7.5% and 10% gels. This was most likely due to the low abundance of these proteins (data not shown).

**The bfrA gene is not present in B. pertussis and B. parapertussis**

A 498 bp structural bfrA gene fragment hybridized to identically sized restriction fragments from PstI and SalI digests of 19385 and B013N, B. bronchiseptica strains originally isolated from a dog and a pig, respectively (Fig. 6b, lanes 1 and 2; only the PstI digests are shown). Subsequently the 5' bfrA region of strain B013N was amplified by PCR and sequenced. It was found that the sequence of the first 50 codons of bfrA and the upstream region between etfA and bfrA was identical between strains 19385 and B013N (data not shown).

Unexpectedly, the 498 bp internal bfrA gene fragment did not hybridize to digests of three different B. pertussis isolates and two B. parapertussis clinical isolates (Fig. 6a, lanes 1, 2, 4, 5, 7 and 8; only one isolate of each is shown). This 498 bp DNA fragment (the SalI fragment encompassing bases 875–1373 in Fig. 2) is homologous to the bfeA enterobactin receptor gene (about 60% identical) and under conditions of low hybridization stringency, identically sized bfeA structural gene fragments from all three species could be seen to hybridize with the bfrA gene probe (data not shown, Beall & Sanden, 1995b). Additional evidence suggesting that bfrA is not present in B. pertussis and B. parapertussis came from the inability to amplify different regions of the bfrA gene from chromosomal DNA of these two species using bfrA-specific oligonucleotides (data not shown).

**bfrA mutants are not defective in utilization of alcaligin and enterobactin**

bfrA mutants were first assessed for their ability to use the Bordetella siderophore alcaligin (Moore et al., 1995; Brickman & Armstrong, 1996a). As shown in Table 3, bfrA (and bfeA) mutants utilized alcaligin as efficiently...
as the wild-type parental strain. Consistent with previous results (Beall & Sanden, 1995b), enterobactin utilization was abolished in bfeA and bfeA–bfrA double mutants, but was unaffected in bfrA single mutants. Enterobactin appeared to be utilized more efficiently by B. bronchiseptica strains than by B. pertussis strains, which was also consistent with previous findings (Table 3, Beall & Sanden, 1995b).

Other iron sources utilized by B. bronchiseptica and B. pertussis

During the course of attempting to find the specificity of the putative siderophore receptor BfrA, it was subsequently found that B. bronchiseptica and B. pertussis could utilize the enterobactin degradation product DHBS, ferrichrome, desferrioxamine B and haemin (Table 3). B. bronchiseptica bfrA mutants were unaffected in the use of these iron sources; however, DHBS-enhanced growth on SC-EDDHA was bfeA-dependent in B. bronchiseptica and B. pertussis. The pattern of iron source usage by B. bronchiseptica bfeA–bfrA double mutants was identical to the pattern seen in B. bronchiseptica bfeA mutants (Table 3).

DHB (an enterobactin precursor), salicylic acid (2-hydroxybenzoic acid), rhodotorulic acid and citrate did not promote growth of B. pertussis and B. bronchiseptica (data not shown).
growth zone diameters measured around haemin disks. Pathogenic bacteria are capable of utilizing haemin as an iron source. For example, haemin uptake in *Bordetella* strains requires a longer incubation period for these bioassays (Table 3). However, in spite of the ability of the *B. pertussis* bfrA gene expressed from a multicopy plasmid to complement *B. bronchiseptica* bfeA mutants (Beall & Sanden, 1995b), there was a significant difference between the two subspecies in enterobactin-enhanced growth on SC-EDDHA agar (Table 3). It is possible that this difference is due to the higher level of bfrA expression that is evident in *B. bronchiseptica* (Beall & Sanden, 1995b).

Two regions of FepA have been implicated in ligand binding by the use of monoclonal antibodies to block binding of ferric enterobactin and colicins to FepA (Murphy et al., 1990; Fig. 3). These experiments are consistent with the observation that these regions are fairly well conserved between FepA and two other enterobactin receptors, PfeA and BfeA (Fig. 3). The two putative ligand binding sites within FepA are largely not conserved in Cir, IrgA or BfrA, although significant homology is seen between all of these proteins throughout much of their respective lengths (Fig. 3). The apparent absence of the bfrA gene in *B. pertussis* and *B. parapertussis* (Fig. 6) was unexpected, since these two species, together with *B. bronchiseptica*, are considered subspecies (Kloos et al., 1981). This result was different from that seen with the *B. pertussis* bfeA gene, which on the basis of hybridization experiments, was highly conserved between the three subspecies (Beall & Sanden, 1995b).

Although the *E. coli* Cir receptor appears to function in ferric-DHB uptake (Hantke, 1990), the BfrA and IrgA putative siderophore receptors do not function detectably in ferric-DHB uptake since neither *V. cholerae* nor *Bordetella* species is capable of using this compound as an iron source (Goldberg et al., 1992; Table 3). The utilization of DHBS has not been reported.
in *V. cholerae*. DHBS functioned as a siderophore by a *bfeA*-dependent mechanism in *B. bronchiseptica* and also, less efficiently, in *B. pertussis*. We were unable to detect differences between wild-type and *bfrA* mutant *B. bronchiseptica* strains using DHBS in iron uptake bioassays (Table 3). Ferric-DHBS and ferric-DHB uptake in *E. coli* are apparently mediated by the receptors Fiu, Cir and FepA (Hantke, 1990). It should be noted that we were unable to detect DHB or DHBS utilization in *B. bronchiseptica* possibly a consequence of inefficient translation of *bfrA* in *E. coli* due to a poor ribosome binding site (Fig. 2).

In *E. coli*, ferric siderophore-receptor-mediated iron uptake across the outer membrane requires the cytoplasmic membrane TonB protein in an energy-dependent process (for review see Braun, 1995). Although a TonB protein has not been identified in *Bordetella* species, BfrA and BfeA are the two known OMPs from this genus that are homologous to the large family of TonB-dependent outer-membrane receptors. A region sharing high similarity to the seven residue ‘Ton B box’ is evident in BfrA, and differs from the Ton B box of FepA by only one conservative substitution (region I in Fig. 3). Two other regions in BfrA corresponding to TonB-dependent receptors (regions II and III of FepA in Fig. 3) are very similar to FepA and identical between all six of the OMPs at residues highly conserved in TonB-dependent receptors (Braun, 1995) (Fig. 3).

The *fur*-dependent iron regulation of *bfrA* (Fig. 4) and the putative Fur-binding sites preceding *bfeA* (Fig. 2) indicate that its expression is controlled by the transcriptional regulator Fur complexed to iron. It is possible that the expression of *bfrA* is affected by other transcription factors. The sequence AATAAATCCCA, present at positions 338–347 upstream of the *bfrA* promoter, is very similar to the consensus (G/C)CTAAATCCCC implicated as a site required for transcriptional activation in iron-regulated promoters of *Pseudomonas* species (Rombel et al., 1995) (the 2 non-matching bases at positions 338 and 339 were each seen in individual promoters used to generate the consensus). The significance of this sequence, if any, remains to be determined.

Recent work has shown that alcaligin production in *B. bronchiseptica* in certain strains is repressed not only by iron, but is also repressed by a *bug*-dependent mechanism (Giardina et al., 1995). We have not detected differences in alcaligin production or *bfeA* and *bfrA* expression by varying virulence factor modulating signals such as temperature and MgSO₄ concentrations in the two *B. bronchiseptica* backgrounds used for this study (data not shown).

Although IrgA, which shares extensive homology to BfrA, does not function in utilization of the *V. cholerae* siderophore vibriobactin and its function as an outer-membrane receptor is unknown (Goldberg et al., 1992), IrgA is an iron-regulated virulence factor in an animal model (Goldberg et al., 1990). Future studies may determine if BfrA has a role in *B. bronchiseptica* virulence. As with IrgA, the function of BfrA as an outer-membrane siderophore receptor also remains to be elucidated.

ACKNOWLEDGEMENTS

We are grateful to Tim Brickman, Klaus Hantke, Scott Stibitz and Ronald Taylor for strains, plasmids and advice. We thank Shambavi Subbarao for critically reading this manuscript. We appreciate the laboratory support provided by Gary Sanden.

REFERENCES


