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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 Tn*phoA* 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,

Abbreviations: AP, alkaline phosphatase; DP, 2,2-dipyridyl; DHB, 2,3-dihydroxybenzoate; DHBS, 2,3-dihydroxybenzoylserine; EDDHA, ethylenediaminedi(*o*-hydroxyphenylacetic acid); OMP, outer-membrane protein; SC, Stainer–Sholte minimal medium with Casamino acids; XP, 5-bromo-4-chloro-3-indolyl phosphate.

The GenBank accession number for the nucleotide sequence reported in this paper is U56084.

Tn*phoA* mutagenesis of *B. bronchiseptica* was used. Here 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-dihydroxybenzoylserine) 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-dipyridyl (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 10^6 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 $FeCl_3$ 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 culture supernatant obtained from the *B. pertussis fur* mutant, B013NMn^{R}4 (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; desferrioxamine B (deferoxamine 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. Tn*phoA* 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(*λpir*)(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*::Tn*phoA* 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*::Tn*phoA* 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 Tn*phoA* fusions were found by replica streaking blue colonies from DP-XP agar onto DP-XP agar and onto XP agar containing 50 μM $FeCl_3$. To clone a *bfrA–phoA* translational fusion, chromosomal DNA from the Tn*phoA* insertion mutant 19233, containing an iron-repressed *phoA* fusion, was cleaved with *Sal*I 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 *Sal*I-digested chromosomal DNA from the iron-regulated Tn*phoA* fusion strain 191394. The *Bgl*II fragment from pS233 was cloned into the *Bam*HI site of the conjugative vector pSS2141, resulting in the integrative plasmid pSBG. Plasmid pSBG was introduced onto the chromosome of strain 19385 by conjugation followed by selection for gentamycin and nalidixic acid resistance. Plasmid pSBG contained an *Sph*I 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 *Sph*I digestion. For this step, chromosomal DNA from one transconjugant containing a Campbell-type insertion of pSBG was digested with *Sph*I, 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 *Sal*I *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 5 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′ GGCGTCTGGATGCG-

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or characteristics*	Source or reference
E. coli		
SM10	RP4-2 Tc::Mu conjugation strain	Simon *et al.* (1983)
SM10(*λpir*)	As SM10, but *λpir6K*	Taylor *et al.* (1989)
DH10B	*mcrA* Δ*mcrBC* Δ*hsdR* Δ*hsdM deoR recA1 endA1 lacZ*ΔM15	BRL
B. bronchiseptica		
19385	ATCC 19385 Nal^R (wild-type dog isolate)	Laboratory collection
19233	19385(*bfrA*::Tn*phoA*), Tn*phoA* insertion at base 233 of *bfrA*	This study
191394	19385(*bfrA*::Tn*phoA*), Tn*phoA* insertion at base 1394 of *bfrA*	This study
19233B	19233ΩpSBFE′ (*bfeA* insertional mutant)	This study
B013N	Wild-type pig isolate	Brickman & Armstrong (1995)
B013NMn^{R}4	B013N(*fur*)	Brickman & Armstrong (1995)
B013NW	B013NΩpSBG	This study
B013NF	B013NMn^{R}4ΩpSBG	This study
B. pertussis		
82	Wild-type clinical isolate	Beall & Sanden (1995b)
84	82ΩpKS3 (*bfeA* insertional mutant)	Beall & Sanden (1995b)
B. parapertussis		
A168	Wild-type clinical isolate	Beall & Sanden (1995b)
Plasmids		
pSS2141	Broad host-range integrational vector, Gm^R Ap^R	Merkel & Stibitz (1995)
pSBFE′	pSS2141 containing a 736 bp structural gene fragment of *bfeA*, for integrational inactivation of *bfeA*	This study
pS233	pUC19 containing a *Sal*I fragment, encompassing 77 codons of *bfrA* fused to *phoA* and the Km^R gene cloned from Tn*phoA* mutant strain 19233	This study
pSBG	pSS2141 containing a *Bgl*II fragment from pS233, encompassing the *bfrA*–*phoA* fusion	This study
pSPH1	Contains entire *bfrA* gene and flanking sequence, obtained by homologous integration of pSBG followed by excision with *Sph*I, ligation and transformation of *E. coli* to Ap^R	This study
pS1394	pUC19 containing a *Sal*I fragment, encompassing 464 codons of *bfrA* fused to *phoA* and the Km^R gene cloned from Tn*phoA* mutant strain 191394	This study

*Ω indicates the strain contains a single homologous inserted copy of the indicated plasmid. Ap^R, ampicillin resistance; Gm^R, gentamycin resistance; Km^R, kanamycin resistance; Nal^R, nalidixic acid resistance.

TGCGTGGAATG 3′, complementary to bases 524–548 of the *bfrA* sequence represented in Fig. 2 was end-labelled with ^{32}P 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 Tn*phoA* 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 Tn*phoA* 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 Tn*phoA* insertion strain 19233 with *Sal*I, which cleaved within Tn*phoA* 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

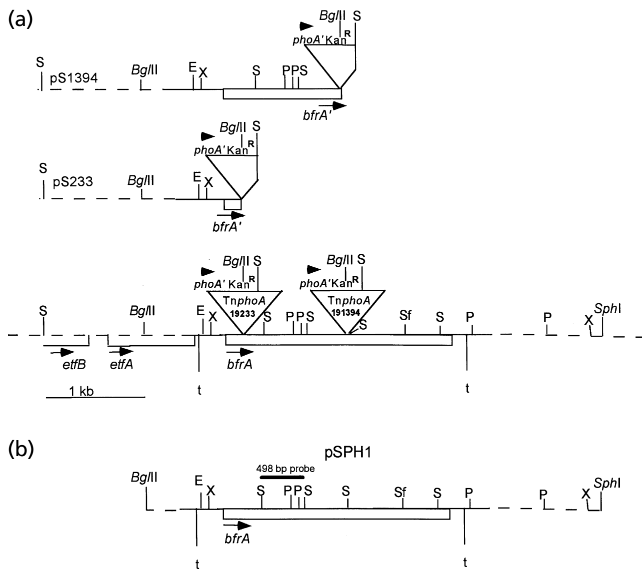

Fig. 1. (a) The *B. bronchiseptica bfrA* chromosomal region, Tn*phoA* fusions isolated during this study and relevant plasmid inserts. The *bfrA* region is shown with the positions of two independently isolated Tn*phoA* insertions (bases 233 and 1394 of the *bfrA* structural gene within strains 19233 and 191394, respectively). The solid line indicates sequenced DNA shown in Fig. 2. t designates sequences that may function as transcriptional terminators. Plasmids pS233 and pS1394 are pUC19 derivatives containing the indicated *bfrA–phoA* fusions. These plasmids were obtained by digestion of chromosomal DNA of the respective Tn*phoA* mutants with *Sal*I, ligation and transformation of *E. coli* to kanamycin resistance. Just upstream of *bfrA* lie homologues of electron transfer flavoprotein subunit genes *etfA* and *etfB* located by partial sequence analysis. E, *Eco*RI; P, *Pst*I; S, *Sal*I; Sf, *Sfu*I; X, *Xmn*I. (b) Diagram of insert in 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 *Bgl*II fragment from pS233 was integrated into the *B. bronchiseptica* chromosome by selecting for gentamycin resistance, followed by digestion of the chromosomal DNA with *Sph*I. *Sph*I cleaved downstream of *bfrA* (shown), and also in an appropriate location within the integrated pSS2141 vector sequence (not shown). The ligated *Sph*I-digested chromosomal DNA was used to transform *E. coli* to ampicillin resistance, resulting in pSPH1. The 498 bp *bfrA* structural gene fragment used as a probe for Southern analysis in Fig. 6 is indicated.

contain the 5′ end of a gene later designated *bfrA*. Next, a *Bgl*II fragment from pS233, consisting of a 1·2 kb fragment of *B. bronchiseptica* DNA fused to a 3·4 kb portion of Tn*phoA*, was subcloned into the *Bam*HI site of the conjugative integrable vector pSS2141. The resulting plasmid, pSBG, was used in a chromosome walking step that resulted in plasmid pSPH1. Plasmid pSPH1 contained an additional 3·4 kb of DNA downstream of the initial *pho*A fusion in strain 19233 (Fig. 1b).

The DNA sequence of pS233 revealed an ORF extending 77 codons to the junction of the *phoA* fusion in strain 19233 (Fig. 1a; Fig. 2, position 712). The DNA sequence of pS1394, containing a chromosomal fragment from *bfrA*::Tn*phoA* mutant strain 191394, overlapped and extended this ORF, designated *bfrA*, 464 codons to the junction of this *phoA* fusion (Fig. 1; Fig. 2, position 1775). Finally, plasmid pSPH1 was found to include the entire *bfrA* structural gene consisting of 734 codons (Fig. 1b; Fig. 2, positions 482–2743). Just downstream of the *bfrA* stop codon lies an inverted repeat which possibly functions as a transcriptional terminator (Fig. 2, positions 2770–2802).

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 α and β subunits of electron transport flavoproteins (Fig. 2). Upstream of *etfA* lies *etfB* (β 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 GCACCCCAC. 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*::Tn*phoA* 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 B013NMn^{R}4 (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-

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CGTGGCCATCAACAAGGATCCGGAAGCGCCGATGTTCGGCGTGGCCGACTACGGCCTGGTGGGCGACCTGTTCCAGGTCGTGCCCGAACTGACCGGCGCGCTCTGAGCGGCGCCTGTCCC  120
 V  A  I  N  K  D  P  E  A  P  M  F  G  V  A  D  Y  G  L  V  G  D  L  F  Q  V  V  P  E  L  T  G  A  L  * etfA  STOP

                                                                            1          2          3          4          5          6          7
CGCGGCGGCAACGAGGTGCCTGAGCAACGAGGTGCCTGTCCCCGCGGGGACAGGCACCCCACGCACCCCACGCACCCCCGCACCCCACGCACCCCACGCACCCCACGCACCCCACGCAC  240
8          9
CCCACGCCCCCCCGCACCCGGCGTTCCTTCCCGCATCAACCGCCGAATTCCCGCCAATTGCCGGGATTCGGACGCCTTCGTGTTGTATTCAAGCTGCAATAAATCCCCTCCAGGCCCCCA  360

                                   Fur1           -35                   Fur2   -10        +1
AAACTGAACTTTTCCTGAATTAATTCTGACGTTTAGTTGAATAGAAGAATAGGAATGAATAGCATTGTCATTCCTTGCAAACCGTTACCGCGGCAGCCGCGCGGTCTTTCCTTCGATTCC  480
                    ****  * *   ***                ***** * **** **

bfrA start
CATGTCAGAACAGAAAAGCAATCAACGCTCCGGGGGAGCGCAGCATTCCACGCACGCATCCAGACGCCGGCCGATCCTGCGCCACGCGCCGCTGGCCCTGGCGATCGCCGCGCTCGGCGC  600
 M  S  E  Q  K  S  N  Q  R  S  G  G  A  Q  H  S  T  H  A  S  R  R  R  P  I  L  R  H  A  P  L  A  L  A  I  A  A  L  G  A
                                                                                                                 √233
CGCCCAGGCCCAGACCGCCAGCCCGCAGGAAGACACCCTGGGCATCACGCAGATGGACACCGTGGTCGTGACCGCCTCCGGGTTCGAGCAGGAAATCAAGAACGCGCCGGCCTCGATCTC  720
 A  Q  A  Q  T  A  S  P  Q  E  D  T  L  G  I  T  Q  M  D  T  V  V  V  T  A  S  G  F  E  Q  E  I  K  N  A  P  A  S  I  S

CGTCATCACCCGCGAGCAGCTCGAAAGCAAGCCCTTCCACAACCTGGCCGACGCGGTCGCCGACGTCGAAGGCGTCAGCGTCGAACGCGGCGGCAAGGCGGGCGGCATGAACATCAGCAT  840
 V  I  T  R  E  Q  L  E  S  K  P  F  H  N  L  A  D  A  V  A  D  V  E  G  V  S  V  E  R  G  G  K  A  G  G  M  N  I  S  I

CCGCGGCCTGCCCAGCGACTACACCCTGGTGCTGGTCGACGGCAAGCGCCTGAGCCAGAACAGCTCGGGCGCCCGCCCCAACGGCTTCGGCGACGTGGACACCAACTTCATCCCGCCCAT  960
 R  G  L  P  S  D  Y  T  L  V  L  V  D  G  K  R  L  S  Q  N  S  S  G  A  R  P  N  G  F  G  D  V  D  T  N  F  I  P  P  M

GTCCGCCATCGACCGCATCGAAGTCGTGCGCGGCCCTATGTCCACCCTGTATGGCTCGGACGCCATGGGCGGCGTGATCAACATCATCACCCGCAAGGTCGCCAGGGAATGGACCGGCCA 1080
 S  A  I  D  R  I  E  V  V  R  G  P  M  S  T  L  Y  G  S  D  A  M  G  G  V  I  N  I  I  T  R  K  V  A  R  E  W  T  G  Q

GGTCACGCTGGACGGCACCGCCCAGGGCGACAACCGCTACGGCAACAACTACGGCTCGTCGTTCTACCTGAGCGGCCCGCTGCAGACCGACAAGCTGGGCCTGTCGCTGCGCGGAGGCCT 1200
 V  T  L  D  G  T  A  Q  G  D  N  R  Y  G  N  N  Y  G  S  S  F  Y  L  S  G  P  L  Q  T  D  K  L  G  L  S  L  R  G  G  L

GTACCGTCGCCTGAGCGCGCACGGCAGCTACCCGGCCAACCAGGCCGAATACGACAGCGGCGACTACTCCGGCGACATCGCCAGCTTCAGCGGCCTGGGCGACAGCCTGCAGCGCAACGT 1320
 Y  R  R  L  S  A  H  G  S  Y  P  A  N  Q  A  E  Y  D  S  G  D  Y  S  G  D  I  A  S  F  S  G  L  G  D  S  L  Q  R  N  V

CGGCCTGCGCCTGGCGCTCACGCCCAACCGCAACCACGACATCCTGTTCGACGTCGACGCCAACTGGCAGACCTTCGACAACGCCAACGGCGAACTCGGCACCCTCAACGCCGACGTGGC 1440
 G  L  R  L  A  L  T  P  N  R  N  H  D  I  L  F  D  V  D  A  N  W  Q  T  F  D  N  A  N  G  E  L  G  T  L  N  A  D  V  A

GCCCAACCGCCAGGGCGGCGGCTATGAGCCCGAGATGAAGTTCAATCGCCAGCGCTACGCCCTGACCCACCTGGGCCGCTACGACGGCGCGATCAGCTCCGACACCAGCCTGCTGTACGA 1560
 P  N  R  Q  G  G  G  Y  E  P  E  M  K  F  N  R  Q  R  Y  A  L  T  H  L  G  R  Y  D  G  A  I  S  S  D  T  S  L  L  Y  D

CACCACCGAGACCATCGGCCGCACCAACCCCATGAGCACGCCGCGCCAGCCGAGCGATGGGGAAAAGCGCGAACTGGAATACGAGAACTGGGTGTTCGACACCAAGTGGACCATGCCCCT 1680
 T  T  E  T  I  G  R  T  N  P  M  S  T  P  R  Q  P  S  D  G  E  K  R  E  L  E  Y  E  N  W  V  F  D  T  K  W  T  M  P  L
                                                                                                          √1394
GTTCAATGACCGCCACAACCTCACGATGGGCGGCCAGTGGCGCGAGCAGAAGTTCAAGGACACCCTGGTGAGCGCGCCCCTGAACCTGCGCCAGTACCAGTGGGCCCTGTTCGCCGAAGA 1800
 F  N  D  R  H  N  L  T  M  G  G  Q  W  R  E  Q  K  F  K  D  T  L  V  S  A  P  L  N  L  R  Q  Y  Q  W  A  L  F  A  E  D

CGAATGGCGCATCGTCGACGACCTGGCCCTGACCATGGGCGCGCGCTATGACCGCAACGAGCAGTTCGGCGGCAAGTGGAGCCCGCGCGGCTACCTGGTCTGGAACGCCACGCCGGCCTG 1920
 E  W  R  I  V  D  D  L  A  L  T  M  G  A  R  Y  D  R  N  E  Q  F  G  G  K  W  S  P  R  G  Y  L  V  W  N  A  T  P  A  W

GACCGTCAAGGGCGGCGTCAGCAAGGGCTACAAGACGCCGGACATCAACCTCATGACCGACGGCATCATCGGCCTGGGCGCGCAAGGCACCATGCCGCTGCTGGGCAACTCGCAGCTCAA 2040
 T  V  K  G  G  V  S  K  G  Y  K  T  P  D  I  N  L  M  T  D  G  I  I  G  L  G  A  Q  G  T  M  P  L  L  G  N  S  Q  L  K

GCCGGAATCCAGCACCTCGTCCGAACTCGGCGTGCTGTTCGACGACGGCGAAGGACTGACGGGCAACCTGACCGGCTTTCACACCAAGTTCAAGGACAAGATCGACACCCAGAACGTGCC 2160
 P  E  S  S  T  S  S  E  L  G  V  L  F  D  D  G  E  G  L  T  G  N  L  T  G  F  H  T  K  F  K  D  K  I  D  T  Q  N  V  P

CAACTGCCTGGCCGCCGGCGGCCCCGTGCCGGGGTGCCTGGACCTGGGCGTGTGGGAGCGCAACGGCGTGCCGGTGGCCAACTTCTCGCAACGCGTGAACGTGGATACCGCCACCATCCA 2280
 N  C  L  A  A  G  G  P  V  P  G  C  L  D  L  G  V  W  E  R  N  G  V  P  V  A  N  F  S  Q  R  V  N  V  D  T  A  T  I  Q

GGGCTTCGAGCTGGGCGGGCGCATCCCGCTGTTCGAAGGCTGGTCCTTCAGCGGCAACTACACCCTGACCGCCAGCGAAATCACCTCCGGCGCCAAGCAGGGCCAGCCGCTGGGCTCGCA 2400
 G  F  E  L  G  G  R  I  P  L  F  E  G  W  S  F  S  G  N  Y  T  L  T  A  S  E  I  T  S  G  A  K  Q  G  Q  P  L  G  S  Q

GCCGCGCCACAGCCTGAACCTGGGCCTGAACTGGCGCGTCAACGAACGCTTCAACGCCTGGGTGCGCGGCGAGTACCGGGCCAAGCAGTTCAACGACATGAACTGGGAAAAGGAGCAGGT 2520
 P  R  H  S  L  N  L  G  L  N  W  R  V  N  E  R  F  N  A  W  V  R  G  E  Y  R  A  K  Q  F  N  D  M  N  W  E  K  E  Q  V

GTTCTACAGCCCGTACTGGCTGGCCAGCCTGGGCGGCTCGTACGTGCTGAACAAGAACGTGACGCTGTCGGCCTCGGTGTACAACCTGTTCGACAAGAACTTCGTCGACTACGGCCCCAC 2640
 F  Y  S  P  Y  W  L  A  S  L  G  G  S  Y  V  L  N  K  N  V  T  L  S  A  S  V  Y  N  L  F  D  K  N  F  V  D  Y  G  P  T

CAAGGTCGGCACCAGCGCCCCCACGGCCGCCACCTCGTGGAGCAACTCGTACCGCCAGGTGCTGGAAGGCCGCCGCCTGTGGGTGTCGGCGAACATTACGTTCTGATCCCGCGAAAGCGG 2760
 K  V  G  T  S  A  P  T  A  A  T  S  W  S  N  S  Y  R  Q  V  L  E  G  R  R  L  W  V  S  A  N  I  T  F  * bfrA STOP

GCAACCGCAAAGGAAAAGCCGGCGCAAGCCGGCTTTTCCTTGTCGGCGCCGGCGCGCCCTACTGCGGTTGCAGGCCGGCGTCCTTGACGATGGCGCCCCAGCGCGGCAGCTCCTCACGCA 2880

GCATGGCCTCGAACCCTTGCGGCGTGGTCGTATGCGCCACCACCCCGGAGGTTCTCCAGCTTCTCGGTCACTTCCTTTTCCTGCAGCACGGCGTTGAACACGCTGTTCAGCTTCTCCACC 3000

ACCGCGGGCTGCGCGCCCTTGGGCAGCAGGAAGCCGATCCAGACGTGCGCCGGGACGGTGCCAAAGCCGCTTTCCTTCATGGTGGGCACGTCCGGGAACAGCTTGAGGCGGTCCTCGGAG 3120

CCGATGGCCAGCGCCTTCAGCTTGCCCGAACTGACGAACGCCTGCACGG 3169
```

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 σ^{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 Tn*phoA* insertions in strains 19233 and 191394 are indicated by √233 and √1394, 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 α) homologue.

ditions, 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 (TTGAAT-17 bp-TAGCAT) with high similarity to the consensus sequence of *E. coli* σ^{70}-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

```
                                                                 I
FepA ..................MNKKIHS...LALLVNLGIYGVA...QAQEPTDTP.VSH..DDTIVVTAA..EQNLQ..APGVSTITADEIRKNPVARDVSKII 71
PfeA ..................MSSRALP...AVPFLLLSSCLLANAVHAAGQGDGS.VIELGEQTVVATAQ..EETKQ..APGVSIITAEDIAKRPPSNDLSQII  76
BfeA ..................MSTPRFALHYASASVLLAASGLAMAQTATQIHDPSQVQQMATVQVLGTAE..EEIKE..SLGVSVITAEEIARRPPTNDLSDLI  80
BfrA MSEQKSNQRSGGAQHSTHASRRRPILRHAPLALAIAALGAAQAQTASPQEDTLGITQM..DTVVVTASGFEQEIKNAPASISVITREQLESKPF.HNLADAV  99
IrgA ..................MSRFNPSPVSLSVTLGLMFSASAFAQDATKTDET.......ETMVVTAAGYAQVIQNAPASISVISREDLESRYY.RDVTDAL  73
Cir  ..................MFRLNPFVR...VGLCLSAISCAWPVLAVDDDG........ETMVVTASSVEQNLKDAPASISVITQEDLQRKPV.QNLKDVL  71
                                        A    A                      TA                S I

                                                                                               II
FepA  RTMPGVNLTGNSTSGQRGNNRQIDIRGMGPENTLILIDGKPVSSRNSVRQGWRGERDTRGDTSWVPP.EMIERIEVLRGPARARYGNGAAGGVVNIITKK  170
PfeA  RTMPGVNLTGNSSSGQRGNNRQIDIRGMGPENTLILVDGKPVSSRNSVRYGWRGERDSRGDTNWVPA.DQVERIEVIRGPAAARYGNGAAGGVVNIITKQ  175
BfeA  RREPGVNLTGNSASGARGNSRQVDIRGMGPENTLILIDGKPVTSRNAVRYGWNGDRDTRGDTNWVPA.EEVERIEVIRGPAAARYGSGAMGGVVNIITKR  179
BfrA  ADVEGVSVER....GGKAGGMNISIRGLPSDYTLVLVDGKRLSQNSSG..ARPNGFG.DVDTNFIPPMSAIDRIEVVRGPMSTLYGSDAMGGVINIITRK  192
IrgA  KSVPGVTVTG....GG..DTTDISIRGMGSNYTLILVDGKR..QTSRQ..TRPNSDGPGIEQGWLPPLQAIERIEVIRGPMSTLYGSDAIGGVINIITRK  163
Cir   KEVPGVQLTN....EG.DNRKGVSIRGLDSSYTLILVDGKRVNSRNAV..FRHN....DFDLNWI.PVDSIERIEVVRGPMSSLYGSDALGGVVNIITKK  169
         GV                   IRG     TL L DGK                                      RIEV RGP    YG  A GGV NIIT

FepA  GSGEWHGSWDAYFNAPEHKEEGATKRTNFSLTGPL.GDEFSFRLYGNLDKTQADAWDINQGHQSARAGTYATTLP..AGREGVINKDINGVVRWDFAPLQ  267
PfeA  AGAETHGNLSVYSNFPQHKAEGASERMSFGLNGPL.TENLSYRVYGNIAKTDSDDWDINAGHESNRTGKQAGTLP..AGREGVRNKDIDGLLSWRLTPEQ  272
BfeA  PADRATGSITYYTNQPEDSREGNTNRVNARISAPI.SDTLSMRLYGNYNKTNPDARDINAGHANTSDNGNPST....AGREGVINQDLSALFSWKADSHN  274
BfrA  VAREWTGQVTLDGTAQGDNRYGNNYGSSFYLSGPLQTDKLGLSLRGGLYRRLSAHGSYPANQAEYDSGDYSGDIASFSGLGDSLQRNVGLRLALTPNRNH  292
IrgA  DQQQWSGNVQLSTVVQENRASGDEQSANFFVTGPL.SDALSLQVYGQTTQR........DEDEIE..........HGYGDKSLRSLTSKLNYQLNPDH  242
Cir   IGQKWSGTVTVDTTIQEHRDRGDTYNGQFFTSGPLIDGVLGMKAYGSLAKR......EKDDPQNSTTTDTGETPRIEGFSS...RDGNVEFAWTPNQNH  249
          G         G        P        G                                       G

FepA  SLELEAGYSRQG...NLYAGDTQNTNSDSYTRSKYGDETNRLYRQNYALTWNGGWDNGVTTSNWVQYEHTRNSRIPEGLAGGTEGKFNEKATQDFVDIDL  364
PfeA  TLEFEAGFSRQG...NIYTGDTQNTNSNNYVKQMLGHETNRMYRETYSVTHRGEWDFGSSLA.YLQYEKTRNSRINEGLAGGTEGIFDPNNA.GFYTATL  367
BfeA  TVDLDMGFSRQG...NLFAGDTMNNANSDFSDSLYGKETNAMYRENYALTHRGVYDWGTSRAS.VGYDYTRNARQREGLAGGPEG...APTAGGYDTARL  367
BfrA  DILFDVDANWQTFDNANGELGTLNADVAPNRQGGGYEPEMKFNRQRYALTHLGRYDGAISSDTSLLYDTTETIGRTNPMSTPRQ.....PSDGEKRELEY  387
IrgA  QLQLEAGVSAQDRENNVG.....KSAQSSGCRGTCSNTDNQYRRNHVAVSHQGDWQGVGQSDTYLQYEE....................NTNKSREMSI  316
Cir   DFTAGYGFDRQDRDSD..................SLDKNRLERQNYSVSHNGRWDYGTSELKYY............GEKVEN.....KNPGNSSPITS  312
                Q                                            G

FepA  DDVMLHSEVNLPIDFLVNQTLTLGTEWNQQRMKDLSSNQALTGTNTGGAIDGVSTTDRSPYSKAEIFSLFAENNMELTDSTIVTPGLRFDHHSIVGNNWS  464
PfeA  RDLTAHGEVNLPLHLGYEQTLTLGSEWTEQKLDDPSSNTQ..NTEEGGSIPGLAGKNRSSSSSARIFSLFAEDNIELMPGTMLTPGLRWDHHDIVGDNWS  465
BfeA  KNWRAAAEASVPFHLGFEQVATVGVEWLRESLEDPAGTRQ...TYTGGAIGGTAPADRDPKSRQTSYALFAEDNIEIDERTMLTPGVRLDHNSEFGSNWS  464
BfrA  ENWVFDTKWTMPL.FNDRHNLTMGGQWREQKFKDTL...VSAPLNL................RQYQWALFAEDEWRIVDDLALTMGARYDRNEQFGGKWS  467
IrgA  DNTVFKSTLVAPI...GEHMLSFGVEGKHESLEDKTSNKISSRTHI................SNTQWAGFIEDEWALAEQFRLTFGGRLDHDKNYGSHFS  396
Cir   ESNTVDGKYTLPL.TAINQFLTVGGEWRHDKLSDAVNLTGGTSSKT................SASQYALFVEDEWRIFEPLALTTGVRMDDHETYGEHWS  395
                P       G      D                                      F ED      T G R D   G  S

FepA  PALNISQGLGDDFTLKMGIARAYKAPSLYQTNPNYILYSKGQGCYASAG...GCYLQGNDDLKAETSINKEIGLEFKRD.....GWLAGVTWFRNDYRNK  556
PfeA  PSLNLSHALTERVTLKAGIARAYKAPNLYQLNPDYLLYSRGQGCYGQST...SCYLRGNDGLKAETSVNKELGIEYSHD.....GLVAGLTYFRNDYKNK  557
BfeA  PSLNASYAVTD..ALKGGIARAYKAPNLYQSNPNYLLYSRGNGCLASQTNTNGCYLVGNEDLSPETSVNKEIGFEYDPG.....TWRTSMAYFRNDYRNK  557
BfrA  PRGYLVWNATPAWTVKGGVSKGYKTPDINLMTDGI..........IGLGAQGTMPLLGNSQLKPESSTSSELGVLFDDG....EGLTGNLTGFHTKFKDK  553
IrgA  PRVYGVWNLDPLWTVKGGVSTGFRAPQLREVTPDW..........GQVSGGGN..IYGNPDLQPETSINKELSLMYSTG....SGLAASLTAFHNDFKDK  481
CirA  PRAYLVYNATDTVTVKGGWATAFKAPSLLQLSPDW..........TSNSCRGACKIVGSPDLKPETSESWELGLYYMGEEGWLEGVESSVTVFRNDVKDR  485
      P          K G     P                                G  L  E S   E                      F

FepA  IEAGYVAVGQNAVG.............TD......LYQWDNVPKAVVEGLEGSLNVPVSETVMWTNNITYML..KSENKTTGDRLSIIPEYTLNSTLSWQ  645
PfeA  IESGLSPVDHASGG.............KGDYANAAIYQWENVPKAVVEGLEGTLTLPLADGLKWSNNLTYML..QSKNKETGDVLSVTPRYTLNSMLDWQ  642
BfeA  IVAGTDVQYRLANG..................ARVLQWTNSGKAVVEGLEGNLFIPLASNLDWNTNFTYMI..QSKEKATGEPLSVIPEYTINSTLDWF  636
BfrA  IDTQNVPNCLAAGG.PVPGCLDLGVWERNGVPVANFSQRVNVDTATIQGFELGGRIPLFEGWSFSGNYTLTASEITSGAKQGQPLGSQPRHSLNLGLNWR  652
IrgA  ITRVACPANICTAG.P.........NQWGATPTY....RVNIDEAETYGAEATLSLPITESVELSSSYTYTHSEQKSGNFAGRPLLQLPKHLFNANLSWQ  567
Cir   ISISRTSDVNAAPGYQNFVGFETGANGRR.IPVFSY...YNVNKARNQGVETELKIPFNDEWKLSINYTYNDGRDVSNG.ENKPLSDLPFHTANGTLDWK  580
      I       G                                       G E    P        T              L   P   N L W

                                                                                   III
FepA  ..AREDLSMQTTFTWYGKQQPKKYNYKGQPAVGPETKE.ISPYSIVGLSATWDVTKNVSLTGGVDNLFDKRLWRAGNAQTTGDLAGANYIAG.AGAYTYN  731
PfeA  ..ATDDLSLQATVTWYGKQKPKKYDYHGDRVTGSANDQ.LSPYAIAGLGGTYRLSKNLSLGAGVDNLFDKRLFRAGNAQGVVG......IDG.AGAATYN  732
BfeA  ..YTPQLSFQANLTYYGKQEGPSTNVRTGVELNGDGRQTISPYALAGLSMGYEVNRNLKFRVGVSNLFDKQLYREGNA............SS.AGAATYN  731
BfrA  ..VNERFNAWVRGEYRAKQFNDMNWEKEQVFYSP........YWLASLGGSYVLNKNVTLSASVYNLFDK..NFVDYGPTKVGTSAPTAATSWSNSYRQV  740
IrgA  ..TTDRLNSWANLNYRGKEMQPEGGASNDDFIAPS.......YTFIDTGVTYALTDTATIKAAVYNLFDQEVNYAEYG....................YV  638
Cir   PLALEDWSFYVSGHYTGQKRADSATAKT....PGG.......YTIWNTGAAWQVTKDVKLRAGVLNLGDKDLSRDDYS....................YN  649
                                           Y                          V NL D

FepA  EPGRTWYMSVNTHF 746
PfeA  EPGRTFYTSLTASF 746
BfeA  EPGRAYYATATVSF 735
BfrA  LEGRRLWVSANITF 754
IrgA  EDGRRYWLGLDIAF 652
Cir   EDGRRYFMAVDYRF 663
        GR        F
```

Fig. 3. Alignment of BfrA with enterobactin receptors (FepA, PfeA and BfeA of *E. coli*, *P. aeruginosa* and *B. pertussis*, respectively), IrgA (*V. cholerae* outer-membrane virulence protein of unknown function) and Cir (colicin I, ferric-DHB and ferric-DHBS receptor). The comparisons were done with the PILEUP program of the Genetics Computer Group version 8 software. The region corresponding to the TonB box of *E. coli* receptors (residues 34–40 of FepA, region I) and two other regions (II and III) conserved with TonB-dependent receptors are indicated (overlined). Putative ligand binding sites of FepA are in bold type (residues 323–358 and 404–422; Murphy *et al.*, 1990).

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 3169 bp of the sequence 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 $FeCl_3$ (+Fe) or in SC broth containing 100 μM DP (−Fe).

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

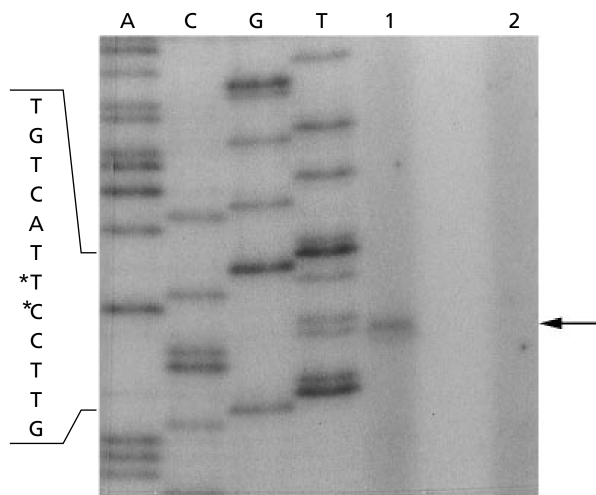

Fig. 4. Mapping the transcription start of the *bfrA* gene (arrow). A 5′-labelled oligonucleotide complementary to the 5′ sequence of the non-transcribed strand of the *bfrA* structural gene was used to prime cDNA synthesis from total RNA prepared from late-exponential phase cultures of strain 19385 (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 *Pst*I and *Sal*I 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 *Pst*I 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 *Sal*I 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

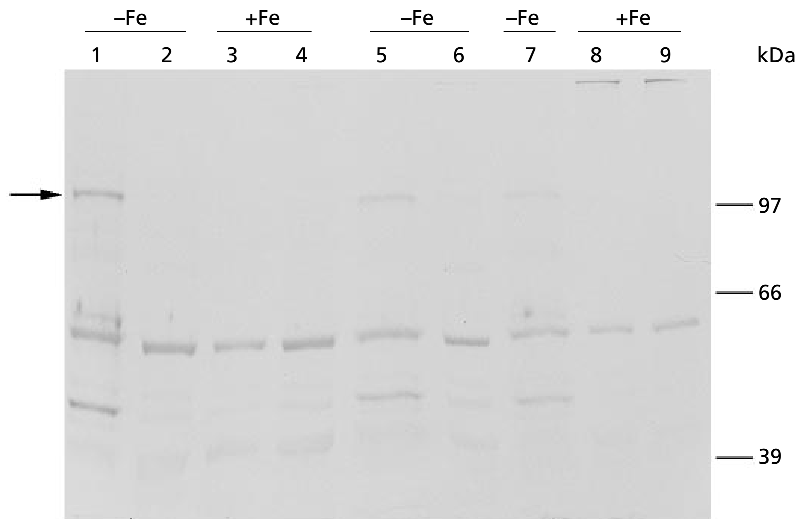

Fig. 5. Identification of a BfrA–PhoA fusion protein (arrow). Whole-cell extracts (lanes 1–4), total-membrane-enriched extracts (lanes 5–6) and outer-membrane-enriched extracts (lanes 7–9) from strain 191394 (*bfrA*::Tn*phoA*) (lanes 1, 3, 5, 7 and 8) and wild-type strain 19385 (lanes 2, 4, 6 and 9). Proteins electrophoresed on SDS-polyacrylamide gels were transferred and immunostained with antiserum against the *E. coli* PhoA (AP) protein.

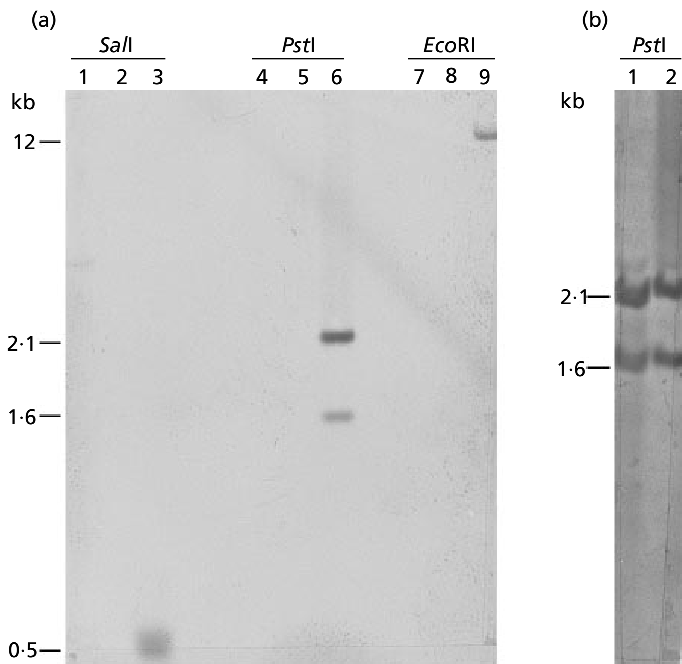

Fig. 6. (a) Lack of conservation of the *bfrA* gene among *Bordetella* species. Chromosomal DNA digests from *B. pertussis* (lanes 1, 4 and 7), *B. parapertussis* (lanes 2, 5 and 8) and *B. bronchiseptica* (lanes 3, 6 and 9) were subjected to high stringency Southern analysis. A 498 bp *bfrA* gene fragment (the *Sal*I fragment encompassing bases 875–1373 in Fig. 2) was used as probe. (b) Conservation of the *bfrA* gene and flanking region between a pig and a dog isolate of *B. bronchiseptica*. Chromosomal DNA from dog isolate strain 19385 (lane 1) and pig isolate strain B013N (lane 2) was subjected to high stringency Southern analysis as shown in (a).

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).

Table 3. Siderophore and haemin utilization by wild-type, *bfeA* mutant, *bfrA* mutant and *bfeA–bfrA* double mutant *Bordetella* strains on SC-EDDHA agar

The diameters of growth zones around disks containing the indicated iron source were measured in mm after 16 h (*B. bronchiseptica*) or 48 h (*B. pertussis*) at 35 °C. The results are the means for five separate experiments; the SD in each case was less than 3 mm. ENT, enterobactin; ALC, strain B013NMn^{R}4 supernatant which contains alcaligin; FC, ferrichrome; HEM, haemin; DF, desferrioxamine B.

Strain and features	ENT	DHBS	ALC	FC	HEM	DF
B. bronchiseptica						
19385 (wild-type)	14	7	8	22	9	16
191394 (*bfrA*::Tn*phoA*)	14	9	7	20	9	17
19233 (*bfrA*::Tn*phoA*)	13	8	8	20	10	16
19387 (*bfeA*)	0	0	8	22	9	16
19233B (*bfrA*::Tn*phoA bfeA*)	0	0	7	20	9	15
B. pertussis						
82	9	4	6	24	11	18
84 (*bfeA*)	0	0	6	25	11	18

DISCUSSION

In this study we describe the cloning, nucleotide sequence and iron-regulated expression of a gene, *bfrA*, that is homologous to siderophore receptors, especially to the ferric-DHB and ferric-DHBS receptor gene *cir* and ferric enterobactin receptor genes (*fepA*, *pfeA* and *bfeA*). Since *bfrA* mutants appeared fully functional in using the *Bordetella* siderophore alcaligin and appeared to have no readily apparent defect in iron uptake, BfrA may serve as a receptor for an exogenous siderophore synthesized by another organism.

bfrA mutants appeared fully functional in the use of enterobactin, the enterobactin degradation product DHBS, ferrichrome, desferrioxamine B and haemin. To our knowledge, utilization of the latter four iron sources has not been documented previously in *Bordetella*. *bfeA*-dependent utilization of DHBS was not unexpected, since the *E. coli* FepA receptor has been implicated in DHBS uptake (Hantke, 1990). It is likely that receptors other than BfeA, BfrA, and the as yet uncharacterized alcaligin receptor, function in the uptake of ferrichrome and desferrioxamine B. Studies of haemin uptake in *Bordetella*, which appeared to be an efficient process (Table 3), should prove interesting (the relatively small growth zone diameters measured around haemin disks do not reflect the observation that growth within these zones was more luxuriant than within the larger growth zones conferred by other iron sources (Table 3). Several pathogenic bacteria are capable of utilizing haemin as an iron source. For example, haemin uptake in *Yersinia enterocolitica* is TonB-dependent and requires an iron-regulated specific outer-membrane receptor (Stojiljkovic & Hantke, 1992). Haemin uptake in *Streptococcus pneumoniae* may be required for full virulence (Tai *et al*., 1993).

The levels of growth enhancement conferred by alcaligin, ferrichrome, desferrioxamine B and haemin appeared similar between *B. bronchiseptica* and *B. pertussis*, even though *B. pertussis* strains required a longer incubation period for these bioassays (Table 3). However, in spite of the ability of the *B. pertussis bfeA* 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 *bfeA* 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 a triple *fepA cir fiuA E. coli* mutant carrying *bfrA* on a multicopy plasmid (data not shown), but that is 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 AATAAATCCC, present at positions 338–347 upstream of the *bfrA* promoter, is very similar to the consensus (G/C)CTAAATCCC 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 *bvg*-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 $MgSO_4$ 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.

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