A *Bordetella pertussis* *fepA* homologue required for utilization of exogenous ferric enterobactin

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The *bfeA* (*Bordetella* ferric enterobactin) receptor gene was cloned from a *Bordetella pertussis* chromosomal library by using a screen in *Escherichia coli* to detect iron-repressed genes encoding exported proteins translationally fused to the *E. coli* *phoA* gene. The *bfeA* gene encoded a protein with a molecular mass of approximately 80 kDa and about 50% amino acid sequence identity to both the *fepA*- and *pfeA*-encoded enterobactin receptors of *E. coli* and *Pseudomonas aeruginosa*, respectively. Enterobactin prepared from iron-starved *E. coli* cultures supported growth of *B. pertussis* and *Bordetella bronchiseptica* in the presence of the iron chelator ethylenediamine-di-(*o*-hydroxyphenylacetic acid) (EDDA). Expression of the *bfeA* gene was induced by low iron availability, and iron-regulated expression appeared to be dependent upon the presence of the sequence contained within 370 bp upstream of the *bfeA* structural gene. An internal fragment of the *bfeA* structural gene and flanking regions were shown by Southern analysis to be highly conserved among *Bordetella* species. Insertional inactivation of *bfeA* in both *B. pertussis* and *B. bronchiseptica* greatly impaired their ability to grow in the presence of enterobactin and EDDA. These findings suggest that enterobactin produced by other respiratory flora could aid in the colonization of the respiratory tract by *Bordetella* species.

Keywords: *Bordetella*, enterobactin receptor, heterologous siderophore.

INTRODUCTION

Bacterial pathogens must obtain iron for growth, and for extracellular pathogens this requires the scavenging of iron from extracellular iron-binding glycoproteins in the host. This is often facilitated by the secretion of siderophores capable of binding ferric iron with high affinity. In many Gram-negative organisms, specific outer membrane receptors then bind iron-siderophore complexes which are internalized in a process requiring the integral cytoplasmic membrane protein TonB (Wang & Newton, 1971; Hantke & Braun, 1975; Braun & Hantke, 1991).

Enterobactin, a cyclic triester of 2,3-dihydroxy-*N*-benzoyl-L-serine, is a siderophore produced upon iron

depletion by many members of the family *Enterobacteriaceae*, including *Escherichia coli* (O'Brien & Gibson, 1970) and *Salmonella typhimurium* (Pollack & Neilands, 1970). In *E. coli* transport of ferric-enterobactin across the inner and outer membranes requires several proteins, including TonB and the receptor FepA (Wayne & Neilands, 1976). Recently the *pfeA* gene of *Pseudomonas aeruginosa*, which encodes a ferric enterobactin receptor very similar structurally to FepA, was characterized and was found to be required for efficient utilization of exogenously supplied enterobactin (Dean & Poole, 1993a). There is also strong evidence of a *fepA* homologue in *Haemophilus* species that allows the usage of ferric enterobactin as an iron source (Williams *et al.*, 1990). The work presented here demonstrates *fepA*-homologue-dependent utilization of exogenous enterobactin by *Bordetella* species.

The genes encoding several other iron-regulated outer membrane receptors involved in iron uptake from various Gram-negative pathogens have been identified. For

Abbreviations: AP, alkaline phosphatase; DP, 2,2-dipyridyl; EDDA, ethylenediamine-di-(*o*-hydroxyphenylacetic acid); SS-Fe, Stainer-Sholte lacking added iron; XP, 5-bromo-4-chloro-3-indolyl phosphate.

The GenBank accession number for the sequence reported in this paper is U13950.

example, in *P. aeruginosa*, besides a ferric enterobactin receptor, outer membrane receptors for ferric pyoverdinin (Meyer *et al.*, 1990) and ferric pyochelin (Heinrichs *et al.*, 1991) have been identified. In contrast, no siderophore receptors of *Bordetella* species have been identified, even though *Bordetella* species are known to acquire iron from transferrin and lactoferrin by a hydroxamate-siderophore-dependent mechanism (Gorringe *et al.*, 1990; Agiato & Dyer, 1992; Armstrong & Clements, 1993; Moore *et al.*, 1995). In addition, *Bordetella* species apparently have at least one outer membrane protein that binds to transferrins directly (Redhead *et al.*, 1987; Menozzi *et al.*, 1991; Redhead & Hill, 1991).

Iron transport systems in many Gram-negative bacterial species are repressed at high intracellular iron concentrations by the iron-binding repressor Fur (Calderwood & Mekalanos, 1987; Braun *et al.*, 1990; Stojiljkovic *et al.*, 1994). As with the Fur proteins of several other Gram-negative bacteria, the Fur protein of *Bordetella pertussis* is functional in *E. coli* and apparently binds to a DNA sequence similar to that recognized by the *E. coli* Fur protein (Beall & Sanden, 1995; Brickman & Armstrong, 1995). On the basis of this observation we screened for *B. pertussis* genes encoding transported proteins which were iron-repressed in *E. coli*. Here we report the characterization of *bfeA* (*Bordetella* ferric enterobactin receptor), which was cloned by this approach.

METHODS

Preparation of enterobactin. Enterobactin extracts were prepared from cultures of *E. coli* strain MT912 in 500 ml iron-starved M9 minimal medium incubated for 12 h at 35 °C. Ethyl acetate extraction was used as previously described (Langman *et al.*, 1972) with some modifications (Neilands, 1981). Culture supernatants were not acidified and enterobactin was not iron saturated during extraction. Ethyl acetate extracts were washed with 1/40th volume of 0.1 M sodium citrate buffer to remove charged species, and following evaporation of the ethyl acetate the residue was dissolved in methanol and stored at -20 °C. These preparations were used either immediately or within 2 weeks of preparation. During this time there was no significant decrease in the growth enhancement conferred by these extracts upon the *E. coli aroB* strain H1443 in media containing 2,2-dipyridyl (DP) (data not shown). The concentration of enterobactin was measured in ethyl acetate extracts from its absorbance at 316 nm as described by Langman *et al.* (1972).

Assays for enterobactin utilization. Growth of *B. pertussis* and *B. bronchiseptica* cultures was monitored in modified Stainer-Sholte minimal medium (von Koenig *et al.*, 1988) lacking added iron (SS-Fe) containing 20–45 µg EDDA ml⁻¹ and 5 µM enterobactin. To assess growth on solid medium *B. pertussis* strains were grown on Regan Lowe agar, washed in SS-Fe, and streaked onto SS-Fe agar containing 15–30 µg EDDA ml⁻¹ and 5 µM enterobactin. The presence or absence of growth was determined after 4–5 d incubation at 37 °C. Overnight Luria (L) broth cultures of *B. bronchiseptica* were diluted to an OD₆₀₀ of 0.3 and 100 µl was spread on L-agar plates containing 100 µg EDDA ml⁻¹ or SS-Fe agar plates lacking added iron and containing 45 µg EDDA ml⁻¹. Disks were laid on the surface of the agar, to which 10 µl threefold-concentrated overnight L-broth culture of enterobactin-producing *E. coli* strains or 5 µl of 50 µM enterobactin was added. The diameters of growth zones

around the disks were measured after 24 h at 35 °C. Culture from *aroB* mutant strains (strains H5058, H1443 or H1876; see Table 2) was added to the disks as negative controls.

Alkaline phosphatase (AP) assay. AP activities of *B. pertussis*, *B. bronchiseptica* and *E. coli* strains containing a translational *bfeA-phoA* fusion were measured as previously described (Brickman & Beckwith, 1975).

Media, growth conditions and antibiotics. Regan Lowe agar (Regan & Lowe, 1977) was used for the routine growth of *B. pertussis* strains. L-broth was used for growth of *E. coli* and *B. bronchiseptica*. L-broth or L-agar containing 50–450 µg EDDA ml⁻¹ or 50–200 µM DP was used as iron-deficient medium for *E. coli* and *B. bronchiseptica*. SS-Fe supplemented with 0–45 µg EDDA ml⁻¹ was used as iron-limiting media for *B. pertussis* and *B. bronchiseptica*. SS-Fe containing 50 µM FeCl₃ (SS+Fe) was used for some experiments. Antibiotics were used at the following concentrations for the growth of *E. coli*, *B. bronchiseptica* and *B. pertussis*: kanamycin at 35 µg ml⁻¹; ampicillin and nalidixic acid at 100 µg ml⁻¹; gentamicin at 10 µg ml⁻¹; tetracycline at 15 µg ml⁻¹. Human holo-transferrin was obtained from Sigma and used in some experiments to supplement media at the concentration of 200 µg ml⁻¹.

Transformation and conjugation. Plasmids were transformed into *E. coli* by standard methods. Chromosomal integration of plasmids into *B. pertussis* and *B. bronchiseptica* was facilitated by electroporation with a Bio-Rad Gene Pulser at a voltage of 2500 V and at a capacitance of 25 µF, producing time constants of 4.0–4.6 ms. After electroporation of *B. pertussis*, cells were spread onto the surface of Regan Lowe agar and incubated at 37 °C for 3 h. At this time kanamycin was spread over the plates to a concentration of 35 µg ml⁻¹. Transformant colonies typically appeared after 4–6 d at 37 °C. Following electroporation of *B. bronchiseptica*, cells were incubated in L-broth for 2 h before plating on kanamycin L-agar. The integrational plasmids pVK11, pVK12, pGN11 and pGN12 were introduced into *B. bronchiseptica* and *B. pertussis* by conjugation with the donor strain SM10 as previously described for other pSS1129 derivatives (Stibitz, 1994). Transformants and conjugants were subjected to Southern analysis for verification of the expected chromosomal constructs. Chromosomal constructs were always maintained in *Bordetella* strains by the presence of kanamycin or gentamicin, which had no detectable influence on growth rate in any of the media used.

Plasmids and strains. Plasmids used for this work are described in Table 1. Plasmids pPHO1, pPHO2, and pPHO3 were a gift from Dr Terri Kenney (Emory University). These plasmids are pBR322 derivatives and contain several unique restriction sites preceding a promoterless *phoA* gene lacking its 5' sequence including the first 11 codons encoding the mature AP. The plasmids differ from each other only by their translational reading frames relative to *phoA* in the multiple cloning site. The kanamycin resistance cassette from pDG102 (Driks *et al.*, 1994) was inserted into the *Bam*HI sites of these plasmids to obtain pPH1, pPH2 and pPH3. Plasmid pSS1129 was a gift from Dr Scott Stibitz (Stibitz, 1994) and was used for construction of plasmids pVK11, pVK12, pGN11 and pGN12 used for integrational analysis of the *bfeA* region in *B. pertussis* and *B. bronchiseptica*. The DNA fragments within integrational plasmids used for determining the functional boundaries of the *bfeA* gene are shown in Fig. 7. Plasmid pPK5 is a pPH1 derivative, while pKS3 is a derivative of pUK19 which contains a kanamycin resistance gene selectable in a wide range of bacterial species (Driks *et al.*, 1994). Plasmids p415B1 and p415B2, used for complementation of *Bordetella bfeA* mutants, were derivatives of the broad-host-range replicative plasmid

Table 1. Plasmids used in this study

Plasmid	Relevant features	Reference or source
pPHO1, 2, 3	pBR322-derived <i>phoA</i> fusion vector, Ap ^r	T. Kenney
pPH1, 2, 3	pPHO1, 2, 3 with Kan ^r cassette	This work
pPK5	pPH1 derivative containing translational <i>bfe-phoA</i> fusion preceded by 1.5 kb of chromosomal sequence, Ap ^r Kan ^r	This work
pKP3	pPK5 deletion derivative containing <i>bfe-phoA</i> preceded by 370 bp of chromosomal sequence	This work
pDG102	Kan ^r cassette source	Driks <i>et al.</i> (1994)
pUK19	pUC19 with Kan ^r cassette, Ap ^r Kan ^r	Driks <i>et al.</i> (1994)
pKS3	pUK19 containing 736 bp structural gene fragment of <i>bfeA</i>	This work
pKP1	pUC19 derivative containing the <i>bfeA</i> gene, 1.5 kb of upstream and 400 bp of downstream flanking sequence	This work
pSS1129	Broad-host-range integrational vector, Gm ^r Ap ^r	Stibitz (1994)
pVK11, 12	pSS1129 derivatives containing 5' end of <i>bfeA</i> and putative promoter region	This work
pGN11, 12	pSS1129 derivatives containing 3' end of <i>bfeA</i>	This work
pRK415	Broad-host-range replicative vector, Tet ^r	Keen <i>et al.</i> (1988)
p415B1, 2	pRK415 derivatives containing <i>bfeA</i> and putative promoter region	This work
pCI	T7 expression vector	Promega
pCI7	pCI derivative containing <i>bfeA</i> under control of the T7 promoter	This work

pRK415 (Keen *et al.*, 1988). Plasmid pCI7, which is a derivative of pCI (Promega), contains the *bfeA* structural gene on a 2.3 kb *XmnI*-*NcoI* fragment under the control of the phage T7 promoter.

The strains used for this study are described in Table 2. *E. coli* strain KS330 contains the *degP4* allele, which decreases degradation of various periplasmic fusion proteins (Strauch & Beckwith, 1988; Strauch *et al.*, 1989). KS330 also contains the *lpp-5508* mutation, which results in a leaky outer membrane through which periplasmic protein or outer membrane protein fusions to *E. coli* AP can diffuse (Strauch & Beckwith, 1988; Giladi *et al.*, 1993). On agar plates containing XP (5-bromo-4-chloro-3-indolyl phosphate), fusions of outer membrane proteins to AP in strain KS330 often result in a blue halo on the agar, while enzymic activity of AP fusions to periplasmic domains of inner membrane proteins remains cell-associated (Strauch & Beckwith, 1988; Giladi *et al.*, 1993).

Cloning the *bfeA* promoter region and structural gene.

Random *B. pertussis* chromosomal fusions to *phoA* were made by digesting chromosomal DNA to completion by any one of a number of restriction enzymes, and ligating the digests with a mixture of phosphatase-treated pPH1, pPH2 and pPH3 that had been digested with a restriction enzyme producing compatible ends to the chromosomal fragments. For the work described here, only the ligation of *StuI*-digested chromosomal DNA into a mixture of *EcoRV*-cleaved pPH1, pPH2 and pPH3 is described. This ligation mixture was transformed into strain KS330 and plated on L-agar containing ampicillin, 150 µg XP ml⁻¹, and 200 µM DP. Individual blue colonies were streaked in duplicate onto L-agar containing both XP and DP, and onto L-agar containing XP and 20 µM added FeCl₃. Colonies exhibiting

more activity with iron-deficient conditions were stored for future analysis. Plasmid minipreps were prepared for sequence analysis as described by Bron (1990) from colonies exhibiting both blue haloes and increased AP activity upon iron limitation. The plasmid that was analysed for this work contained a 2.5 kb *StuI* fragment cloned in the *EcoRV* site of pPH3 and was designated pPK5. A derivative of pPK5 was made by deletion of an *EcoRV* fragment, resulting in the removal of all but 373 bp of chromosomal sequence upstream of *bfeA*. This chromosomal fragment encompassing the chromosomal region from the *EcoRV* to the *StuI* site within *bfeA*, together with the vector kanamycin resistance determinant, was subcloned in both orientations as an *EcoRI* fragment into the conjugative vector pSS1129. These plasmids were designated pVK11 and pVK12.

To clone the entire *bfeA* gene and additional downstream DNA, the integrational plasmid pPK5 was used to transform *B. pertussis* strain 82 to kanamycin resistance, and the chromosomal DNA was then digested with *KpnI*. The DNA was ligated with *KpnI*-digested pUC19 and used to transform *E. coli* LE392 to kanamycin resistance, resulting in plasmid pKP1.

DNA sequencing. Appropriate M13mp18 and M13mp19 (Messing, 1983) subclones were sequenced according to the Sequenase protocol (US Biochemical Corp.) using the universal primer and various oligonucleotides corresponding to the *bfeA* region. pPK5 DNA was sequenced according to the Sequenase protocol for double-stranded DNA with a primer to the 5' end of the *phoA* gene. Both strands of the *bfeA* region were sequenced to obtain the data in Fig. 2.

Southern analysis. This was performed using *Bordetella* chromosomal DNA prepared as previously described (Beall & Sanden, 1995) with the Genius Kit (Boehringer Mannheim). A 736 bp

Table 2. Bacterial strains

Strain	Genotype	Source or reference
<i>E. coli</i>		
KS330	<i>lpp-5508 degP4 ΔphoA</i>	Strauch & Beckwith (1988)
JM101	$\Delta(lac-proAB)$ F'(traD36 proAB lacI ^q lacZΔM15)	Messing (1983)
LE392	<i>hsdR514</i> ($r_K^- m_K^+$) <i>supE44</i> <i>supF58 lacY1</i> or $\Delta(lacIZY)$ 6 <i>galK2 galT22 melB1 trpR55</i>	Laboratory stock
SM10	RP4-2 Tc::Mu, conjugation strain	Stibitz (1994)
MT912	<i>thi trpT purE proC leuB lacY mtl xyl rpsL azi fhuA tsx supA fepA Kan^r</i>	M. McIntosh
MC4100	<i>araD139 Δ(argF-lac) U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR</i>	K. Hantke
H5058	<i>tsx aroB cir fhu fepA</i>	K. Hantke
H1443	as MC4100 but <i>aroB</i>	K. Hantke
H1876	as H1443 but <i>fhu::Mud1X cir fepA::Tn10</i>	K. Hantke
H306	<i>tonB</i>	K. Hantke
BL21(ΔDE3)	F' <i>ompT</i> $r_B^- m^- r_B^-$	Tabor & Richardson (1985)
<i>B. pertussis</i>		
82	Wild-type Nal ^R clinical isolate	Laboratory collection
83	82ΩpPK5	This work
84	82ΩpKS3	This work
85	82ΩpGN11	This work
86	82ΩpVK11	This work
<i>B. bronchiseptica</i>		
19385	ATCC 19385 Nal ^R (wild-type)	Laboratory collection
19386	19385ΩpPK5	This work
19387	19385ΩpKS3	This work
19388	19385ΩpGN11	This work
19389	19385ΩpVK11	This work
<i>B. paraptentis</i>		
A168	Wild-type clinical isolate	Laboratory collection
<i>B. avium</i>	Wild-type	Laboratory collection

* The symbol Ω indicates the strain contains a single homologously inserted copy of the indicated plasmid.

SalI-StuI bfeA structural gene fragment was labelled and used as a probe as described by the manufacturers.

Outer membrane preparations. Protein extracts enriched in outer membrane proteins were prepared by Triton X-100 extraction as described by Schneider & Parker (1982).

[³⁵S]Methionine labelling of plasmid-encoded proteins. The labelling procedure has been described previously (Tabor & Richardson, 1985). The DNA fragment cloned into pCI7 immediately downstream of the T7 promoter as well as the promoter-distal vector sequence was transcribed by phage T7 RNA polymerase encoded on phage λDE3. On phage λDE3, expression of T7 RNA polymerase was induced by the addition of 0.5 mM IPTG since it is under control of the *lac* promoter. Precursors of transported proteins were enriched by the addition of 5 mM sodium azide before labelling (Fortin *et al.*, 1990; Oliver *et al.*, 1990). Protein extracts were subjected to SDS-PAGE and autoradiography.

RESULTS

Cloning *bfeA*

To identify iron-regulated genes from *B. pertussis* encoding exported proteins we screened for iron-repressed AP activity in *E. coli* KS330 transformed with plasmid-borne libraries of random *B. pertussis* DNA fragments fused to a truncated *phoA* gene. Plasmids from these transformants were likely to contain promoter regions normally regulated by the *B. pertussis* Fur protein, since the Fur proteins of *B. pertussis* and *E. coli* appear to be functionally interchangeable to a significant degree (Beall & Sanden, 1995; Brickman & Armstrong, 1995). Since the missing sequence in the truncated *phoA* carries the signal sequence and first 11 codons encoding the mature enzyme, the AP activity of resulting translational fusions depends upon a chromosomal-fragment-encoded membrane export sequence that situates the PhoA portion of the fusion extracytoplasmically (Manoil & Beckwith, 1985). Five of 400 transformants with AP activity resulting from transformation with a *StuI* fragment-*phoA* library showed enhanced activity in the presence of DP. Two of these showed blue haloes on XP agar containing DP and were subjected to further analysis. These transformants contained identical plasmids, designated pPK5, with the truncated *phoA* gene fused to the 5' portion of a gene designated *bfeA* (Fig. 1a). The characterization of *bfeA* is described below.

The *bfeA-phoA* fusion that was isolated in pPK5 contained a 2.5 kb *StuI* chromosomal fragment from *B. pertussis* with a 352 codon open reading frame which was fused in-frame to the truncated *phoA* at the *EcoRV* site of pPH1 (Fig. 1a). pPK5 was used to transform *B. pertussis* strain 82 to kanamycin resistance, which resulted in the Campbell-type insertion of the entire plasmid into the chromosome (Fig. 1a, b). DNA from one transformant was cleaved with *KpnI*, ligated with *KpnI*-digested pUC19 and subsequently used to transform *E. coli* LE392 to kanamycin resistance (Fig. 1c). The plasmid contained in these transformants, designated pKP1, contained the rest of the *bfeA* gene and about 400 bp of additional downstream sequence (Fig. 1c).

Nucleotide sequence of the *bfeA* gene

The DNA sequence of appropriate restriction fragments from pKP1 and pPK5 revealed an open reading frame preceded by a potential ribosome-binding site (Fig. 2,

Fig. 1. Construction of *B. pertussis* and *B. bronchiseptica* strains with a chromosomal *bfeA-phoA* fusion and cloning of the entire *bfeA* gene. Dashed lines indicate vector sequence; solid lines indicate *B. pertussis* chromosomal DNA. (a) Plasmid pPK5 containing a translational *bfeA-phoA* fusion was (b) transformed into *B. pertussis* and *B. bronchiseptica*, resulting in homologous integration into the chromosome. (c) Chromosomal DNA from a *B. pertussis* strain containing the integrated pPK5 was cleaved with *KpnI*, ligated into pUC19, and used to transform *E. coli* to kanamycin resistance, resulting in pKP1, which contains the entire *bfeA* gene.

positions 398–401); the open reading frame extends 352 codons to the junction of the *phoA* fusion at a *StuI* site (Fig. 2, position 1450). The sequence obtained with subclones of pKP1, which overlapped and extended downstream of the *StuI* library junction site, extended this open reading frame to 735 codons.

A database (GenBank) search revealed roughly 50% identity of the deduced *bfeA* product with the *fepA* and *pfeA* gene products of *E. coli* and *P. aeruginosa* respectively (Fig. 3). A putative signal peptide of 25 residues was predicted for BfeA, with a cleavage site after the sequence AMA (Fig. 3), which is in agreement with the motif recognized by signal peptidase I (Perlman & Halvorson, 1983). The amino acid sequences of BfeA, FepA and PfeA, as with many other outer membrane proteins, contain hydrophobic residues at positions 3, 5, 7 and 9 from the C-terminal phenylalanine and also share a conserved arginine residue at position 11 from the C-terminus (Struyve *et al.*, 1991) (Fig. 3). The region corresponding to the TonB box of *E. coli* (residues 34–40

of FepA; boxed in Fig. 3) (Lundrigan & Kadner, 1986) does not appear to be particularly well conserved between BfeA and FepA. Two other regions in BfeA corresponding to TonB-dependent receptors (695–704 and 142–170 of FepA) are very similar to FepA and identical to both FepA and PfeA at residues highly conserved in TonB-dependent receptors (specific residues not shown; Baumler *et al.*, 1992) (Fig. 3). Significantly, two regions corresponding to ligand-binding sites of FepA (residues also boxed in Fig. 3) (Murphy *et al.*, 1990) are well conserved between the three proteins.

Based on the deduced similarity of BfeA to the FepA and PfeA proteins, and the blue halo results obtained in *E. coli* KS330(pPK5) it is likely that the BfeA-PhoA hybrid protein encoded by pPK5 is an outer membrane protein. This protein presumably contains 327 mature BfeA residues, with the fusion junction corresponding to residue 342 of FepA (Fig. 3). This residue lies within a 49-residue region of FepA that is well conserved with BfeA and contains a surface-exposed epitope previously impli-

EcoRV

1 AGTTCGGCGCGCACGGCGGCCCTGCAGATCGCGGATATCGACCCGGTGGCCACCACGCGGGCGCCACCTCGTGGTATCAAACACGCCCTGGAAAA

101 CCGGCGCGTCTCTTGCCTGGGACCGACAGGTAGTAGCGCGCGCGCTGTGGCGCAGACATCGATCAGCGTCTGGCCCGCCACCAGGCGCGCGCGCGCC

201 GCGCGCGGTGCGCGTGGAGGAGTCTGGGTCAATGAATGGCGATAGTGCAAAAGGCTTATGGCGCGTGGCGAAACCTTTGCCGGGATCTTCTCTGACA ⁻³⁵

301 GAATCATGAACGCCAAATCTTATTAGAAATCATTCCCAATCGCAAACAGGCCGATACATGGCTGGCCGCCATGTGGACGGGCGCGCACGCACACGGA ⁻¹⁰ **FUR1** **FUR2**

401 GAAGCCGCATGTCCACCCCGGATTCGCGTGCATTACGCCAGCGCGTCACTCTGTGGCGCATCCGGCTGGCGCATGGCGCAGACGCCACCCAGAT **FUR3** **NcoI** **SD**

M S T P R F A L H Y A S A S V L L A A S G L A M A Q T A T Q I

501 CCACGATCCGTCGAGGTGCAGCAGATGGCGACGGTGCAGGTGCTGGGACGGCGGAAGAGGAAATCAAGGAGTCTGGGCGTCTCGGTTCATCACCGCC

601 GAGGAGATCGCCCGCGCGCCACCAATGACCTGTCCGACCTGATCCGCGCGAACCAGCGCTCAACCTGACCGGCAACAGCGCCAGCGCGCGCGGG

701 GCAACAGCCCGCAGGTTCGACATCCGCGCATGGGCCCCGAGAACCCCTCATCTGATCGACGGAAGCCCGTCACTCGCGCAATGCGGTGCGCTATGG **SalI**

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

801 CTGGAACGGCGACCGGACACGCGCGGGGACACCAACTGGGTGCCCGCGAGGAAGTCGAGCGCATCGAAGTGATCCGCGGCCCGCGCGCCCGCTAC

901 GGTTCGGGGCCATGGCGCGTGGTCAACATCATCACAAGCGCCCCCGATCGCGCCACCGGCTCCATCACCTACTACAGAACAGCGCGGAAGACA **NcoI**

G S G A M G G V V N I I T K R P A D R A T G S I T Y Y T N Q P E D S

1001 GCCGCGAGGGCAACACCAACCGCTCAATGCGCGCATCAGCGCGCGATCAGCGACCGTGAAGCGGCTGTACGCAACTACAACAGACCAATCC **SphI**

R E G N T N R V N A R I S A P I S D T L S M R L Y G N Y N K T N P

1101 GGATGCCCGGACATCAACGCCGCCACGCGAACCAGCGCAACCGGCAACCCCTCGACCGCGGACGCGAGGGCGTCAACACGAGGACCTGAGCGCG

1201 CTGTTCTCGTGGAAGCCGACAGCCACAACCCGTGGACCTGGACCTGGGCTTCAGCGCGCAGGGCAACCTGTTTCGCGCGGACACCATGAACAACGCCA

1301 ACAGCGACTTCTCGGACAGCTGTACGGCAAGGAAACCAATCGATGTACCGGAGAACTATGCGCTGACGCACCGCGCGTATACGATCGGGGACCTC

S D F S D S L Y G K E T N A M Y R E N Y A L T H R G V Y D W G T S

1401 GCGCGCCAGCGTGGCTATGACTACAGCGCAACGCGCGCCAGCGCAAGGCTTGGCGCGCGCCCGAGGGCGCGCCACCGCGGGCGGCTACGACACC **StuI**

R A S V G Y D Y T R N A R Q R E G L A G G P E G A P T A G G Y D T

1501 GCGCGCTGAAGAACTGGCGCGCGCGCGGAGGCGAGCGTCCGTTTCATCTCGGTTTCGAGCAGGTGCGCCAGGTGCGCGTGAATGGCTGCGCGAAT

1601 CGCTGGAAGACCCCGCGCGCAGCGCCAGACCTATACCGCGCGGCCATCGGCGGCGACGCGCCCGGCGGACCGGACCGGAAATCGCGCGAGACAGCTA

L E D P A G G T R Q T Y T G G A I G G T A P A D R D P K S R Q T S Y

1701 TGCGCTGTTCCGCGGACCAACATCGAGATCGAGCGCACCATGCTCACGCCGCGGTGCGCCTGGACCACAACGCGAATTCGGCAGCACTGGAGT **EcoRI**

A L F A E D N I E I D E R T L P G V R L D H N S E F G S T N W S

1801 CCCAGCTGAACGCTCGTACGCGCTACCGACGCGCTCAAGGGTGGCATCGCGCGCGCTACAAGGCGCCCAACCTCTACCAATCAACCCCACTACC

P S L N A S Y A V T D A L K G G I A R A Y K A P N L Y Q S N P N Y L

1901 TGCTGTACAGCGCGCAATGGCTGCGCTGGCCTCGCAGACCAACCAACGGCTGCTATCTGGTTCGGAACGAGGACCTCTCGCGGAAACAGCGTCAA

L Y S R G N G C L A S Q T N T N G C Y L V G N E D L S P E T S V N

2001 CAAGGAAATCGGCTTCGAGTACGACCCGGGACGTTGGCGCACCAGCATGGCCTATTTCGCAACGACTACCGCAACAGATCGTCGCGGCACCGACGTC

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

2101 CAGTACCGCTGGCAATGGCGCCGGGTGCTGCAATGGACCAACAGCGGCAAGGCGGTGGTGAAGGGCTGGAAGGCAACCTGTTTCATTCGCTGGCCA

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

2201 GCAATCTCGACTGGAACCAACTTCACCTACATGATCCAGTCCAAGGAAAGGCCACCGGCGAACCCTTGAGCGTGATCCCGAATACACCATCAACAG

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

2301 CACGTGGACTGGTTCTACACGCGCAGCTGTGCTTCCAGGCAATCTCACCTATTACGCAAGCAGGAAGGCCGTCACCAATGTACGCACCGCGTC

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

2401 GAACTGAACGGCGACGGCGCCAGACCATCAGTCCGTATGCCCTGGCGGGCCTGAGCATGGGTACGAAGTCAACCGGAACCTGAAGTTCGCGCTGGCG

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

2501 TGAGCAACCTGTTGCAAGCAGCTGTACCGCAAGGCAATGCCAGCAGCGCGGGCGCGGCCACTACAACGAACCGGGCGCGCTATTACGCCACGGC

S N L F D K Q L Y R E G N A S S A G A A T Y N E P G R A Y Y A T A

2601 GACGGTGTCTGTTCTGACCATGGCGACGGCTCTCTGCGCGCTCGCGCGCCCCCTGGCTGCTGGCGCGCGCCCTGCGCGCGCGCGCGGCGGCGGCGG **NcoI**

T V S F -

2701 CGGCGCGGTGCCGAGCAACCGCGCGCGCGCGCGCGCTGGGCGTGTGGGCTGTTCCAGCAGACCGTGTGGCGCGCGCGGGGGCTTGGCG

2801 AACGTGCGCTCACCTGGCCGTGCCGCGGG

Fig. 2. Nucleotide sequence of the *bfeA* gene and the predicted amino acid sequence of the BfeA protein. The *StuI* site at position 1452 was the site of the *bfeA* fusion to *phoA*. An inverted repeat possibly functioning in transcription termination is underlined (positions 176–203). A potential ribosome-binding site is indicated at positions 398–401. Potential –35 and –10 hexamers are overlined. Three 19-base sequences with homology to the consensus iron box recognized by the *E. coli* Fur protein are in bold.

PfeA	MSSRALPAVFFLLSSCLLANAVHAAGQGDGSVIELGRCNTHAETKQAPGVS	56
BfeA	MSTPRPALHYASASVLLAASGLAMAQTATQIHDPQVQMATVQLCTNEEIKESLGS	60
FepA	MNKKIHLALLVNLGIYGVAAQAEPTDTPVSHDITIVTNAEQNLQAPGVS	51
PfeA	IITAEDIAKPPSNDLSQIIRTPMGVNLGTSSSGQRGNRQIDIRMGPPENTLILVDGK	116
BfeA	VITAEIARRPPTNDLSLIRREPGLVNLGTSSSGQRGNRQIDIRMGPPENTLILVDGK	120
FepA	TITADEIRKPVARDVSKIIRTPMGVNLGTSSSGQRGNRQIDIRMGPPENTLILVDGK	111
PfeA	PVSSRSVRYGWRGERDSRGDTNWVPADQVERIEVIRGPAARYGNGAAGGVNIIITKA	176
BfeA	PVTSRNAVRYGWRGERDRDTRGDTNWVPADQVERIEVIRGPAARYGSGAMGVNIIITKP	181
FepA	PVSSRSVRYGWRGERDRDTRGDTNWVPADQVERIEVIRGPAARYGNGAAGGVNIIITKG	171
PfeA	GAETHGNLSVSNFPQHKAGSERSFGLNGPLTENLSYRVYGNIAKTDSDWDINAGH	236
BfeA	ADRATGSITYTTPQEDSRGNTNRVNARISAPISDTLSMRLYGNYNKTPDARDINAGH	241
FepA	SGEWHGSDWAFNAPEHKEGATKRTNFSLTGPGDEFSPRLYGNLKDQADWDINQGH	231
PfeA	ESNRTGKQAGTLPAAGREGVRNKIDIGLLSWRLTPEQTLFEAGFSRQGNIIYTDQTNTS	296
BfeA	ANTSNDGNPST--AGREGVINQDLSALFSWKADSHNTVDLDMGFSRQGNLFAGDTMNAN	299
FepA	QSAAGTYATTLPAGREGVINKDINGVVRWDFAPLQSLLEAGYSRQGNLYAGDTQNTS	291
PfeA	NNYVKQMLGHETNMYRETSVTHRGWDGFG--SSLAYLQYEKTRNSRINEGLAGGTGTF	356
BfeA	SDFSLSLYGKETNAMYRENYALTHRGVYDWG--TSRASVGYDYTNARQREGLAGGTGTF	356
FepA	DSYTRSKYQDETNRLYKQNYALTTHRGWDGFG--SSLAYLQYEKTRNSRINEGLAGGTGTF	351
PfeA	--PNNAGFYTTATLRDLTAHGEVNLPLHLGYEQLTLGSEWTEQKLDPPSSN--TREG	411
BfeA	--APTAGGYTTATLRDLTAHGEVNLPLHLGYEQLTLGSEWTEQKLDPPSSN--TREG	411
FepA	NEKATQGFVDLDDVLMHSEVNLPLDPLVNLTLGTEWNLQRMKDLSSNALTGTNTG	412
PfeA	GSIPGLAGKNSSSSARIFSLPAEDNIELMPGTMPLTGLRMDHHDIVGDNWSPSLNLSH	471
BfeA	GAIGGTAPADNDPKSRQTSYALPAEDNIELDERMTLPGLRMDHHDIVGDNWSPSLNLSH	472
FepA	GAIDGVSTDRSPYSKAEISLPAEDNIELDERMTLPGLRMDHHDIVGDNWSPSLNLSH	471
PfeA	ALTERVTLKAGIARAYKAPNLYQLNPDLVLYSRGQGCY--QSTSCYLRGNDGLKAETS	528
BfeA	AVTD--ALKGGIARAYKAPNLYQSNPNLYLYSRGQGCY--QSTSCYLRGNDGLKAETS	530
FepA	GLGDDFTLKMGIARAYKAPNLYQSNPNLYLYSRGQGCY--QSTSCYLRGNDGLKAETS	528
PfeA	VNKGLEIYSHDGLVAGLTIFYRDNKYKIESGLSPVDHASGGKDYANAAIYQWENVPKA	588
BfeA	VNKGLEIYSHDGLVAGLTIFYRDNKYKIESGLSPVDHASGGKDYANAAIYQWENVPKA	584
FepA	VNKGLEIYSHDGLVAGLTIFYRDNKYKIESGLSPVDHASGGKDYANAAIYQWENVPKA	582
PfeA	VVEGLEGLTLPLADGLKWSNNLTMYLQSKNKETGDLVSVTPRYTLNMLDQATDLSL	648
BfeA	VVEGLEGLTLPLADGLKWSNNLTMYLQSKNKETGDLVSVTPRYTLNMLDQATDLSL	644
FepA	VVEGLEGLTLPLADGLKWSNNLTMYLQSKNKETGDLVSVTPRYTLNMLDQATDLSL	642
PfeA	QATVTWYGKQK--PKKYDYGDRVTGSANDOLSPYATAGLGTYRLSKNLSLQAGVDNLPD	707
BfeA	QANLTYGKQKQPSNTNVRTGVELNGDGRQITSPYATAGLGTYRLSKNLSLQAGVDNLPD	704
FepA	QATVTWYGKQK--PKKYDYGDRVTGSANDOLSPYATAGLGTYRLSKNLSLQAGVDNLPD	701
PfeA	KRLFRAGNAQGVVGD-----GAGATYNEPGRTFYTSLTASF	745
BfeA	KQLYREGNAS-----SAGATYNEPGRATATATVSF	735
FepA	KRLFRAGNAQTTGDLGAGANYIAGAGATYNEPGRATATATVSF	745

Fig. 3. Comparison of BfeA with the enterobactin receptors FepA and PfeA of *E. coli* and *P. aeruginosa*, respectively. The comparisons were done with the FASTP algorithm of Pearson & Lipman (1988). Sequences corresponding to the TonB box of *E. coli* receptors (residues 34–40 of FepA) and putative ligand-binding sites of FepA are boxed (residues 323–358 and 404–422) (Murphy & Klebba, 1990). The predicted first residues of the mature proteins are underlined.

cated in ligand binding (Murphy *et al.*, 1990). The 'blue halo effect' (Giladi *et al.*, 1993) that we observed with strain KS330(pPK5) was also consistent with previous studies in which FepA–PhoA fusion proteins containing 227–710 residues of the mature FepA N-terminus were localized to the outer membrane (Murphy & Klebba, 1989).

Potential –35 and –10 hexamer sequences homologous to the consensus sequence recognized by the major form

Fig. 4. Identification of the *bfeA* gene product. *E. coli* BL21(ΔDE3) harbouring the vector (pCI, lane 3) or *bfeA* cloned into pCI (pCI7, lanes 1 and 2) untreated (lanes 2 and 3) or treated with sodium azide (lane 1) were labelled with [³⁵S]methionine for 5 min as previously described (Tabor & Richardson, 1985; Baumber & Hantke, 1992). Bands were detected by autoradiography. β-Lac, β-lactamase. Results are representative of repeated experiments using several independent transformants.

of *E. coli* RNA polymerase lie at positions 285–323 (Fig. 2) (Hawley & McClure, 1983). These putative –35 and –10 sequences are both overlapped by sequences similar to Fur-binding sites. The sequence from 318 to 336 overlapping the –10 hexamer is identical to the consensus Fur-binding site in 11 of 19 positions (Calderwood & Mekalanos, 1987) and identical to the Fur-binding site in the *E. coli fhuA* promoter in 14 of 19 positions (sources of the references for the consensus and *fhuA* sequences given in Braun *et al.*, 1990).

Identification of the *bfeA* gene product

Plasmid pCI7, containing the *bfeA* gene on a 2.3 kb *XmnI*–*NcoI* fragment (position 333–2620; Fig. 2) under the control of the phage T7 promoter, was used to selectively label the *bfeA* gene product with [³⁵S]methionine (Fig. 4). The *bla* gene encoding β-lactamase is in the same orientation as *bfeA* on this plasmid, situated downstream of *bfeA*, and therefore is also under T7 promoter control. Upon IPTG induction of T7 RNA polymerase a protein of approximately 78 kDa was radiolabelled in cells containing pCI7; this probably corresponds to the mature form of BfeA (Fig. 4, lanes 1 and 2). To prevent cleavage of the BfeA precursor, sodium azide, an inhibitor of SecA-mediated protein export (Fortin *et al.*, 1990; Oliver *et al.*, 1990) was added to half of the sample before labelling. While the precursor of the plasmid-encoded exported protein β-lactamase was

Fig. 5. Conservation of the *bfeA* gene and flanking region in *Bordetella* species. Chromosomal DNAs from *B. pertussis* (lanes 1, 5 and 8), *B. paraptentussis* (lanes 2, 6 and 9), *B. bronchiseptica* (lanes 3 and 7), and *B. avium* (lane 4) were subjected to high-stringency Southern analysis. The 736 bp *SalI*-*StuI* *bfeA* structural gene fragment (bases 717–1451 in Fig. 2) was used as the probe.

enriched by azide treatment, a band corresponding to the BfeA precursor was not evident and the band putatively corresponding to the mature BfeA protein was significantly reduced compared to the untreated sample (Fig. 4; compare lanes 1 and 2). The reduction of mature BfeA in azide-treated cells would be consistent with the expected inhibition of SecA-mediated protein transport. It is possible that the accumulation of BfeA precursor resulting from azide treatment was not observed due to degradation in the cytoplasm.

We were unable to detect the BfeA protein in stained 7.5–12% gels after SDS-PAGE of outer membrane protein extracts of *B. pertussis* or *B. bronchiseptica* (with isogenic *bfeA* mutants for comparison). This may have been due to the relatively low abundance of BfeA causing it to be obscured by other outer membrane proteins of the same mobility.

Conservation of the *bfeA* gene among *Bordetella* species

As seen in Fig. 5, *bfeA* and flanking regions appear to be conserved among *Bordetella* species. The region is highly conserved between *B. pertussis*, *B. bronchiseptica* and *B. paraptentussis*, since DNA from each of these three species gave restriction fragments indistinguishable in size that hybridized strongly to a *bfeA* structural gene fragment probe. This result was seen with three different restriction digests (Fig. 5) (results of *SalI* for *B. bronchiseptica* not shown). *EcoRV* digests of *B. avium* DNA produced a single, less strongly hybridizing fragment of a different

Table 3. Growth zones of *B. bronchiseptica* strains on L-agar containing 100 µg EDDA ml⁻¹ around disks impregnated with enterobactin extract or enterobactin-producing *E. coli* strains

The diameters of growth zones were measured in mm after 24 h at 37 °C. To maintain chromosomal insertions, antibiotic selection was used as needed and *E. coli* strains were transformed with appropriate resistance vectors. The results are the mean for at least five separate experiments; the standard deviation in each case was less than 4 mm.

<i>B. bronchiseptica</i> strain*	<i>E. coli</i> strain†			Enterobactin extract
	MC4100	MT912	H306	
19385 (wild-type)	10	21	15	30
19386	11	20	14	32
(= 19385ΩpPK5)				
19389	10	20	13	30
(= 19385ΩpVK11)†				
19388	11	19	14	32
(= 19385ΩpGN11)†				
19387	0	0	0	0
(= 19385ΩpKS3)				
19387(p415B1)‡	12	21	16	29
19387(pRK415)	0	0	0	0

* The symbol Ω indicates Campbell-type integration of the indicated plasmid containing the chromosomal restriction fragments depicted in Fig. 7.

† All these strains are *aroB*⁺. All the *B. bronchiseptica* strains listed were also tested with the *aroB* *E. coli* strains H5058, H1443 and H1876; no growth was obtained with any of these strain combinations.

‡ An identical plasmid, except containing the opposite orientation of the chromosomal fragment, was used with identical results.

size from the respective hybridizing *EcoRV* fragments generated from the other three *Bordetella* species (Fig. 5, lane 4).

Utilization of enterobactin by *B. pertussis* and *B. bronchiseptica*

To determine if *Bordetella* species were capable of utilizing ferric enterobactin, a disk method was employed as described in Methods. Only *aroB*⁺ enterobactin-producing *E. coli* strains were capable of stimulating growth of *B. bronchiseptica* on SS-Fe containing 45 µg EDDA ml⁻¹ or L-agar plates containing 100 µg EDDA ml⁻¹ (Table 3). It was not possible to demonstrate stimulation of *B. pertussis* growth in this manner since evidently the *E. coli* strains secreted substance(s) inhibitory or lytic to *B. pertussis*. This was shown by control experiments in the absence of EDDA, which resulted in wide zones devoid of growth around the *E. coli*-containing disks (data not shown). Enterobactin-enhanced growth by *B. pertussis* and *B. bronchiseptica* was apparent in SS-Fe broth cultures

Fig. 6. BfeA-dependent enterobactin-enhanced growth of *B. pertussis*. The *bfeA* mutant strain 84 (squares), strain 84(p415B1) (triangles), and the parental wild-type strain 82 (circles) were grown to mid-exponential phase in iron-replete medium, washed, and inoculated into SS-Fe containing 25 μ g EDDA ml⁻¹ (open symbols) or the same medium additionally containing 5 μ M enterobactin (filled symbols). Strains 84 and 84(p415B1) cultures also contained 15 μ g kanamycin ml⁻¹. These results show the mean and standard deviation of three identical experiments.

Fig. 7. Integrational analysis of the *bfeA* gene region. The region from 38 bp upstream of the *EcoRV* site to approximately 50 bp upstream of the *KpnI* site represents the sequenced DNA shown in Fig. 2. Plasmid pKS3 is a derivative of plasmid pUK19 while pVK11, pVK12, pGN11 and pGN12 are pSS1129 derivatives. Plasmid pPK5 is a pPK3 derivative (see Methods). The resultant plasmids were integrated into the chromosome by conjugation (pVK11, pVK12, pGN11 and pGN12) or by transformation (pPK5 and pKS3) of wild-type *B. pertussis* and *B. bronchiseptica*. Ability to utilize enterobactin was assessed.

containing EDDA (Fig. 6, shown for *B. pertussis* only). Similar results were obtained by inoculating *B. pertussis* strain 82 onto minimal agar medium containing EDDA and 5 μ M enterobactin (not shown).

Enterobactin utilization is *bfeA*-dependent in *B. pertussis* and *B. bronchiseptica*

To facilitate the insertional inactivation of *bfeA* in *B. pertussis* and *B. bronchiseptica*, a fragment of the *B. pertussis* *bfeA* structural gene was subcloned into the vector pUK19 (Driks *et al.*, 1994) to make pKS3 (Fig. 7). pKS3

cannot replicate in *Bordetella* species but contains a kanamycin resistance gene that is selectable in these hosts. As predicted, integration of pKS3 into the chromosomes of *B. bronchiseptica* and *B. pertussis* resulted in each containing a *bfeA* allele truncated at the *StuI* site (identical hybridization data for each strain not shown) and coincided with drastically reduced enterobactin utilization in strains 84 (Fig. 6) and 19387 (Table 3). The wild-type *B. pertussis* and *B. bronchiseptica* strains were unable to grow on solid medium containing EDDA, but unlike the *bfeA* mutants 84 and 19387, the addition of enterobactin stimulated their growth (data not shown). *aroB*⁺ strains of *E. coli* capable of enterobactin synthesis stimulated the growth of *B. bronchiseptica* on L-agar containing 100 μ g EDDA ml⁻¹, while *aroB* strains did not allow growth.

Previous work has demonstrated the ability of *B. pertussis* and *B. bronchiseptica* to utilize human holo-lactoferrin and holo-transferrin (Agiato & Dyer, 1992; Agiato-Foster & Dyer, 1993; Menozzi *et al.*, 1991). Insertional inactivation of *bfeA* in *B. pertussis* and *B. bronchiseptica* by Campbell insertion of pKS3 had no effect on the ability of these strains to utilize human holo-transferrin in similar experiments (data not shown). This observation was expected, since catechol siderophores are not detectable in iron-starved *Bordetella* cultures (Agiato & Dyer, 1992; Armstrong & Clements, 1993; Gorringe *et al.*, 1990). These *bfeA* mutants were also identical to the wild-type parental strains in their growth rates and cell density accumulation in SS + Fe, SS-Fe without added iron, and in the presence of low EDDA and DP concentrations. These results indicated that the normal growth of these strains and their use of endogenous siderophores was not affected (data not shown).

Functional boundaries of *bfeA*

The 3 kb *EcoRV*–*KpnI* fragment from an *EcoRV* deletion derivative of pKP1 was cloned as a *Bam*HI fragment in both orientations into the replicative plasmid pRK415, resulting in plasmids p415B1 and p415B2. These plasmids, which contain the *bfeA* gene and 370 bp of upstream sequence, were introduced by conjugation into the *bfeA* mutant strains 84 and 19387. Enterobactin utilization experiments indicated that both p415B1 and p415B2 fully complemented these *bfeA* mutants (Table 3, Fig. 6).

Chromosomal integration of the pSS1129 derivatives pVK11 and pVK12, which contain opposite orientations of a fragment consisting of about 1 kb of 5' *bfeA* sequence preceded by 370 bp of upstream DNA (Fig. 7), had no discernible effect on enterobactin utilization in either *B. pertussis* (not shown) or *B. bronchiseptica* (Table 3). Integration of pVK11 and pVK12 by a single crossover event into the chromosome resulted in one complete chromosomal copy of the *bfeA* structural gene with 370 bp of upstream chromosomal sequence (hybridization data not shown). Integration of pGN11 and pGN12 (Fig. 7), containing a sequence (subcloned into the pSS1129 *EcoRI* site in opposite orientations) extending from the *EcoRI* site within *bfeA* to the *NcoI* site that barely encompasses the 3' end of *bfeA*, had no effect on

enterobactin utilization in *B. pertussis* (data not shown) or *B. bronchiseptica* (Table 3). These results, together with the ability of plasmids p415B1 and p415B2 to fully complement the *bfeA* mutants (Table 3, Fig. 6), indicated that the expression of *bfeA* does not require further upstream sequence. These results also suggested that the defective phenotype conferred by Campbell insertion of pKS3 is not likely to be due to polarity effects of the insertion on downstream genes.

The *bfeA* gene on a multicopy plasmid does not complement *E. coli fepA* mutants

Plasmid pKP1 was transformed into the three *E. coli fepA* mutants described in Table 2, but the capability to utilize enterobactin was not conferred upon these strains. Strain MT912(pKP1) was grown in L-broth containing different inhibitory concentrations (50–450 µg ml⁻¹) of EDDA and actually grew somewhat more slowly than strain MT912(pUK19) under the same conditions (data not shown). Similarly, the *aroB* strains H5058(pKP1), H1876(pKP1) and H1443(pKP1) were spread onto kanamycin L-agar plates containing different inhibitory concentrations (50–200 µM) of DP and tested for the ability to grow around disks impregnated with H306(pUK19), MT912(pUK19), or enterobactin extract. Only the control strain H1443(pKP1), which, unlike the other *aroB* strains, is *fepA*⁺, displayed a zone of growth around the disks showing enterobactin utilization (not shown). The *bfeA-phoA* fusion was well expressed from pPK5 in strain KS330(pPK5) (Table 4). Since pPK5 contains the same region upstream of *bfeA* that is upstream of *bfeA* in pKP1, it is likely that *bfeA* was expressed in the *fepA* mutants tested. Sequence analysis of the putative *bfeA* promoter region and structural gene sequences shared between pPK5 and pKP1 showed no differences. Additionally, the *XmnI-NcoI* fragment subcloned from pKP1 into a T7 expression vector was demonstrated to encode a protein of the predicted size of BfeA (Fig. 4).

Expression of the *bfeA* gene

The presence of potential Fur-binding sites overlapping the putative *bfeA* promoter and the observation that *bfeA* expression is iron-regulated in *E. coli* (Table 4) suggests that expression of *bfeA* is repressed by Fur, with iron as its co-repressor. The *bfeA* gene was cloned as a consequence of its iron-regulated expression in *E. coli* KS330(pPK5). Higher AP activity resulted from expression of the *bfeA-phoA* translational fusion in the presence of the iron chelator DP than during growth under iron-sufficient conditions (Table 4). The relatively high level of AP activity in strain KS330(pPK5) during growth with non-limiting iron concentrations was possibly caused by inefficient regulation of *bfeA* expression. This could be due to low-affinity binding to the promoter region by the *E. coli* Fur and/or a multi-copy effect, since multiple copies of Fur-regulated genes are not as tightly regulated as when they are in a single copy (Stojiljkovic *et al.*, 1994). In contrast, the same *bfeA-phoA* translational fusion

present as a single chromosomal copy in *B. pertussis* and *B. bronchiseptica* was tightly iron-regulated (Table 4).

AP activity was induced equally in *B. bronchiseptica* 19386 by growth in SS-Fe or SS-Fe + 50 µM DP (data not shown), indicating that the iron levels in SS-Fe were low enough for complete derepression of *bfeA*. This observation correlated with a slightly slower growth rate and decreased final cell density of *B. pertussis* and *B. bronchiseptica* strains in SS-Fe compared to growth in SS+Fe (data not shown) and the apparently complete deregulation of iron-regulated proteins in SS-Fe in *B. bronchiseptica* and *B. pertussis* seen by SDS-PAGE (data not shown). In contrast to *pfeA* expression in *P. aeruginosa*, which requires the presence of enterobactin in addition to iron limitation for its induction (Poole *et al.*, 1990; Dean & Poole, 1993a, b), *bfeA* expression was induced solely by iron limitation in both *Bordetella* species (Table 4). As described previously for iron-mediated repression of siderophore production in *B. pertussis* (Agiato & Dyer, 1992), the presence of 200 µg ml⁻¹ of human holotransferrin in addition to growth-inhibiting levels of EDDA in SS-Fe restored growth and eliminated *bfeA* expression in *B. pertussis* and in *B. bronchiseptica* (data not shown).

E. coli KS330(pPK3) showed the same levels of AP activity as KS330(pPK5) (Table 4; pPK3 contains the same *bfeA-phoA* gene fusion as does pPK5 except that upstream chromosomal sequence has been deleted to the *EcoRV* site upstream of *bfeA*: Fig. 7) in both iron-limiting and iron-replete conditions. These results indicated that it is likely that the sequence(s) responsible for iron-regulated *bfeA* expression lies between the *bfeA* translational start and the upstream *EcoRV* site. This contention was reinforced by the wild-type phenotypes of *Bordetella* strains 86 (not shown), 19389, 84(p415B1,2) and 19387(p415B1,2) (Table 3, Fig. 7). In these strains the *bfeA* gene is preceded by chromosomal DNA only up to the *EcoRV* site 370 bp upstream, and three potential Fur-binding sites lie within this upstream sequence (Fig. 2).

DISCUSSION

The ability of Gram-negative bacteria such as *P. aeruginosa*, *E. coli* and *Haemophilus* species to utilize heterologous siderophores for iron acquisition has been established (Poole *et al.*, 1990; Hantke & Braun, 1975; Williams *et al.*, 1990). In this work we describe the cloning, nucleotide sequence, and phenotypic analysis of a gene highly homologous to the enterobactin receptor genes of *P. aeruginosa* and *E. coli* that is required for exogenous enterobactin utilization by *B. pertussis* and *B. bronchiseptica*.

As discussed earlier (Dean & Poole, 1993a), 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). These regions are indicated in Fig. 3 as residues 311–358 and 404–422 of FepA. As with PfeA, the central portion of BfeA corresponding to residues 311–358 of FepA is most similar to this region of FepA, with much less similarity at each end (18 identities to FepA over residues

Table 4. *bfeA*–*phoA* expression experiments

Strain	AP units (mean $\pm$ SD, $n = 3$)	
	+Fe	–Fe
<i>E. coli</i> KS330(pPK5)*	75 $\pm$ 20	300 $\pm$ 65
<i>E. coli</i> KS330(pPK3)*	97 $\pm$ 25	335 $\pm$ 53
<i>B. bronchiseptica</i> 19386†	5 $\pm$ 3	125 $\pm$ 24
<i>B. pertussis</i> 83†	2 $\pm$ 2	15 $\pm$ 4

* Mid-exponential-phase cells were incubated for 3 h in L-broth (+Fe) or in L-broth containing 200 μ M DP (–Fe).

† Cells were grown to late exponential phase in SS + Fe, containing 50 μ M FeCl₃ (+Fe), or in SS – Fe, lacking added iron supplement (–Fe).

330–358 with PfeA and 13 identities to FepA over this region with BfeA). The region of BfeA corresponding to residues 404–422 of FepA also has significant homology to FepA, with 9 identities and 6 conservative substitutions over 19 residues. The regions of BfeA similar to putative ligand-binding sites of FepA, the high homology of BfeA over its entire length with the FepA and PfeA enterobactin receptors, and the drastically decreased ability of *B. pertussis* and *B. bronchiseptica* *bfeA* mutants to utilize enterobactin implicate BfeA as a ferric enterobactin receptor.

The BfeA protein, unlike PfeA (Dean & Poole, 1993a), apparently does not function properly in *E. coli*, possibly due to its inability to interact with TonB or other proteins involved in transport and intracellular release of iron from ferric enterobactin. A region sharing some similarity with the seven-residue ‘Ton B box’ is evident in BfeA, but only two of seven residues are identical to FepA while four of the seven are identical between PfeA and FepA (Fig. 3, residues 34–40 of FepA). The two most conserved residues in the TonB box of the large group of TonB-dependent outer membrane receptors are the threonine and valine residues corresponding to positions of 35 and 37 of FepA (Baumler & Hantke, 1992) (Fig. 3), which are absent in BfeA. Further work is required to examine the conservation between *tonB* and the other genes required for ferric enterobactin utilization in *E. coli* with their counterparts in *Bordetella*.

The iron-regulated expression of *bfeA* (Table 4) and the putative Fur-binding sites preceding *bfeA* (Fig. 2) suggest that its expression may be controlled solely by the transcriptional regulator Fur complexed to iron. In *B. pertussis* and *B. bronchiseptica* expression of *bfeA* was stimulated by iron-limitation alone, while the addition of enterobactin during iron-limited growth had no effect on *bfeA* expression (data not shown). This differs from the expression of the *P. aeruginosa* *pfeA* gene, which requires the presence of enterobactin as well as low iron availability for its induction (Poole *et al.*, 1990). Immediately upstream of the *pfeA* gene lie the *pfeS* and *pfeR* genes, which constitute a ‘two-component’ transcriptional regulator

that mediates enterobactin-dependent transcription of *pfeA* (Dean & Poole, 1993b). Limited sequence analysis of a total of about 700 bp of the 1.4 kb of DNA upstream of *bfeA* in plasmid pPK5 revealed no homology to *pfeR* and *pfeS* (data not shown).

It should be noted here that *bfeA* expression studies and functional analysis of *bfeA* were much more straightforward in *B. bronchiseptica* than in *B. pertussis*. Both of these organisms can reach OD₆₀₀ values of at least 2.5 in SS–Fe, although *B. pertussis* has a much slower growth rate. Wild-type *B. pertussis* would only reach a maximal OD₆₀₀ of approximately 0.6 in SS–Fe containing 25 μ M EDDA plus 5 μ M enterobactin after more than 2 d at 37 °C (Fig. 6), while *B. bronchiseptica* could reach an OD₆₀₀ of greater than 2.0 in SS–Fe containing 45 μ M EDDA plus 5 μ M enterobactin within 24 h (data not shown). Similarly, the iron-regulated expression of *bfeA* was much more evident in *B. bronchiseptica* than in *B. pertussis* (Table 4).

The conservation of the *bfeA* region among *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* (Fig. 5) is consistent with them being considered subspecies (Kloos *et al.*, 1981). Similar studies concerning the more distant relative *B. avium* should prove interesting. DNA hybridization results indicate that *B. avium* has a DNA sequence homologous to the *bfeA* gene, but the homology is less than that observed between *bfeA* and the putative *bfeA* genes from *B. parapertussis* and *B. bronchiseptica* (Fig. 5).

Enterobactin-producing members of the family *Enterobacteriaceae* are occasionally encountered in clinical respiratory tract specimens, where they may be involved in transient colonization or infectious processes, including superinfection after infection by common respiratory pathogens such as *B. pertussis*. *Haemophilus parainfluenzae* and *Haemophilus paraphrophilus* are common commensals of the human upper respiratory tract that have been demonstrated to utilize exogenous enterobactin *in vitro*. These *Haemophilus* species are likely to internalize ferric enterobactin via a FepA homologue since they have a DNA sequence highly homologous to *fepA*, and *H. parainfluenzae* expresses an iron-repressible outer membrane protein that reacts with anti-FepA serum (Williams *et al.*, 1990). It seems likely that the expression of a functional enterobactin receptor in *Bordetella*, *Haemophilus* and *Pseudomonas* species may have served an evolutionary advantage. Further work will be necessary to determine the significance of the expression of these enterobactin receptors *in vivo*.

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