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

Bernard Beall and Gary N. Sanden

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
example, in *P. aeruginosa*, besides a ferric enterobactin receptor, outer membrane receptors for ferric pyoverdin (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 1995). In addition, the Fur protein of *B. bronchiseptica* is iron-repressed 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 precipitated. 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* stains 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 40–46 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 pPH01, pPH02, and pPH03 are 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 BamHI 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 phP derivative, while pPK3 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

<table>
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<tr>
<th>Plasmid</th>
<th>Relevant features</th>
<th>Reference or source</th>
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<tr>
<td>pPHO1, 2, 3</td>
<td>pBR322-derived <em>phoA</em> fusion vector, Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>T. Kenney</td>
</tr>
<tr>
<td>pPH1, 2, 3</td>
<td>pPHO1, 2, 3 with Kan&lt;sup&gt;+&lt;/sup&gt; cassette</td>
<td>This work</td>
</tr>
<tr>
<td>pKP5</td>
<td>pPH1 derivative containing translational <em>bfeA</em> fusion preceded by 1.5 kb of chromosomal sequence, Ap&lt;sup&gt;+&lt;/sup&gt; Kan&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pKP3</td>
<td>pKP5 deletion derivative containing <em>bfeA</em> fusion proceeded by 370 bp of chromosomal sequence</td>
<td>This work</td>
</tr>
<tr>
<td>pDG102</td>
<td>Kan&lt;sup&gt;+&lt;/sup&gt; cassette source</td>
<td>Driks et al. (1994)</td>
</tr>
<tr>
<td>pUK19</td>
<td>pUC19 with Kan&lt;sup&gt;+&lt;/sup&gt; cassette, Ap&lt;sup&gt;+&lt;/sup&gt; Kan&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Driks et al. (1994)</td>
</tr>
<tr>
<td>pKS3</td>
<td>pUK19 containing 736 bp structural gene fragment of <em>bfeA</em></td>
<td>This work</td>
</tr>
<tr>
<td>pKP1</td>
<td>pUC19 derivative containing the <em>bfeA</em> gene, 1.5 kb of upstream and 400 bp of downstream flanking sequence</td>
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<td>pSS1129</td>
<td>Broad-host-range integrationald vector, Gm&lt;sup&gt;+&lt;/sup&gt; Ap&lt;sup&gt;+&lt;/sup&gt; and putative promoter region</td>
<td>Stibitz (1994)</td>
</tr>
<tr>
<td>pVK11, 12</td>
<td>pSS1129 derivatives containing 5' end of <em>bfeA</em> and putative promoter region</td>
<td>This work</td>
</tr>
<tr>
<td>pGN11, 12</td>
<td>pSS1129 derivatives containing 3' end of <em>bfeA</em></td>
<td>This work</td>
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<tr>
<td>pRK415</td>
<td>Broad-host-range replicative vector, Tet&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Keen et al. (1988)</td>
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<td>p415B1, 2</td>
<td>pRK415 derivatives containing <em>bfeA</em> and putative promoter region</td>
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<tr>
<td>pCI</td>
<td>T7 expression vector</td>
<td>Promega</td>
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<tr>
<td>pCI7</td>
<td>pCI derivative containing <em>bfeA</em> under control of the T7 promoter</td>
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pRK415 (Keen et al., 1988). Plasmid pCI7, which is a derivative of pCI (Promega), contains the *bfeA* structural gene on a 2.3 kb XmaI-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-508 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 ligation of 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 *Stul*-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 X 1<sup>−</sup>, 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 *Stul* 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 *Stul* 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. The DNA was ligated with *KpnI*-digested pUC19 and used to transform *E. coli* LE392 to kanamycin resistance, resulting in plasmid pKP1.

**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**

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<th>Genotype</th>
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<td>JM101</td>
<td>Δ(lac-pro-AB) F'(traD36 pro-AB lacI9 lacZAM15)</td>
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<td>LE392</td>
<td>bsdR514 (rB mB) supE44 supF58 lacY1 or Δ(lacI9Z) 6 galK2 galT22 melB1 trpR55</td>
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<td>SM10</td>
<td>RP4-2 Te::Mu, conjugation strain</td>
<td>Sibitz (1994)</td>
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<tr>
<td>MT912</td>
<td>thi trpT purE proC leuB lacY</td>
<td>M. McIntosh</td>
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<tr>
<td></td>
<td>mil xyl rpsL avj fbiA tix</td>
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<tr>
<td></td>
<td>supA fepA Kan'</td>
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<td>araD139 (argF-lac) U169</td>
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<td>rpsL150 relA1 fkb5301</td>
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<td>as MC4100 but arB</td>
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<td>H306</td>
<td>tonB</td>
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<td><strong>B. avium</strong></td>
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*The symbol Ω indicates the strain contains a single homologously inserted copy of the indicated plasmid.

**SaII-StuI bfeA structural gene fragment was labelled and used as a probe 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).

**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 pKPl, 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 pKPl and pPK5 revealed an open reading frame preceded by a potential ribosome-binding site (Fig. 2,
positions 398–401); the open reading frame extends 352
codons to the junction of the phoA fusion at a S\text{stul} site
(Fig. 2, position 1450). The sequence obtained with
subclones of pKP1, which overlapped and extended
downstream of the S\text{stul} library junction site, extended this
open reading frame to 735 codons.

A database (GenBank) search revealed roughly 50% identity of the deduced \text{bfeA} product with the \text{fepA} and
\text{pfeA} gene products of \text{E. coli} and \text{P. aetribactena}
respectively (Fig. 3). A putative signal peptide of 25 residues was
predicted for \text{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 \text{BfeA}, \text{FepA} and
\text{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 \text{et al.}, 1991) (Fig. 3). The region
corresponding to the TonB box of \text{E. coli} (residues 34–40
of \text{FepA}; boxed in Fig. 3) (Lundrigan & Kadner, 1986)
does not appear to be particularly well conserved between
\text{BfeA} and \text{FepA}. Two other regions in \text{BfeA corre-
sponding to TonB-dependent receptors (695–704 and
142–170 of \text{FepA}) are very similar to \text{FepA and identical
to both \text{FepA and PfeA at residues highly conserved in
TonB-dependent receptors (specific residues not shown;
Baumler \text{et al.}, 1992) (Fig. 3). Significantly, two regions
corresponding to ligand-binding sites of \text{FepA (residues
also boxed in Fig. 3) (Murphy \text{et al.}, 1990) are well
conserved between the three proteins.

Based on the deduced similarity of \text{BfeA} to the \text{FepA} and
\text{PfeA proteins, and the blue halo results obtained in \text{E. coli}
KS330(pPK5) it is likely that the \text{BfeA-PhoA hybrid
protein encoded by pPK5 is an outer membrane protein.}
This protein presumably contains 327 mature \text{BfeA
residues, with the fusion junction corresponding to
residue 342 of \text{FepA (Fig. 3). This residue lies within a 49-
residue region of \text{FepA that is well conserved with \text{BfeA
and contains a surface-exposed epitope previously impli-}
**Fig. 2.** Nucleotide sequence of the *bfeA* gene and the predicted amino acid sequence of the BfeA protein. The *Stul* 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.
227–710 residues of the mature FepA N-terminus were localized to the outer membrane (Murphy & Klebba, 1990). The predicted first residues of the mature proteins are underlined.

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.

Potential –35 and –10 hexamer sequences homologous to the consensus sequence recognized by the major form 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 (sources of the references for the consensus and PfeA 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 for 5 min as previously described (Tabor & Richardson, 1985; Baumber & Hanke, 1992). Bands were detected by autoradiography. β-Lac, β-lactamase. Results are representative of repeated experiments using several independent transformants.

Fig. 4. Identification of the bfeA gene product. E. coli BL21(DE3) 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, 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 not detected by autoradiography, bands corresponding to the mature form of BfeA (Fig. 4, lanes 1 and 2) were labelled with [35S]methionine for 5 min as previously described (Tabor & Richardson, 1985; Baumber & Hanke, 1992). Bands were detected by autoradiography. β-Lac, β-lactamase. Results are representative of repeated experiments using several independent transformants.

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 radio-labelled 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 not detected by autoradiography, bands corresponding to the mature form of BfeA (Fig. 4, lanes 1 and 2) were labelled with [35S]methionine for 5 min as previously described (Tabor & Richardson, 1985; Baumber & Hanke, 1992). Bands were detected by autoradiography. β-Lac, β-lactamase. Results are representative of repeated experiments using several independent transformants.
Fig. 5. Conservation of the bfeA gene and flanking region in Bordetella species. Chromosomal DNAs from B. pertussis (lanes 1, 5 and 8), B. parapertussis (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-Stul bfeA structural gene fragment (bases 717-1451 in Fig. 2) was used as the probe.

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. parapertussis, 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 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 araB+ 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.

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

<table>
<thead>
<tr>
<th>B. bronchiseptica strain*</th>
<th>E. coli strain†</th>
<th>Enterobactin extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>19385 (wild-type)</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>19386</td>
<td>11</td>
<td>32</td>
</tr>
<tr>
<td>(= 19385pPK5)</td>
<td>20</td>
<td>13</td>
</tr>
<tr>
<td>19389</td>
<td>11</td>
<td>14</td>
</tr>
<tr>
<td>(= 19385pVK11)†</td>
<td>11</td>
<td>14</td>
</tr>
<tr>
<td>19388</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(= 19385pGN11)†</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>19387</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>19387 (pRK415)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* The symbol † indicates Campbell-type integration of the indicated plasmid containing the chromosomal restriction fragments depicted in Fig. 7.
† All these strains are araB+. All the B. bronchiseptica strains listed were also tested with the araB+ 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.

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.

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.

Enterobactin-enhanced growth by B. pertussis on SS-Fe containing 100 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.
**Bordetella ferric enterobactin receptor gene**

The 3 kb EcoRV–Kpnl fragment from an EcoRV deletion derivative of pPK1 was cloned as a BamHI 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

Figure 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 µg EDDA ml⁻¹ (open symbols) or the same medium additionally containing 5 µM enterobactin (filled symbols). Strains 84 and 84(p415B1) cultures also contained 15 µg kanamycin ml⁻¹. These results show the mean and standard deviation of three identical experiments.

Figure 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 Kpnl site represents the sequenced DNA shown in Fig. 2. Plasmid pPK3 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 µM 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
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 kanamyacin 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–pboA 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 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–pboA 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 nonlimiting 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 (Stojilkovic et al., 1994). In contrast, the same bfeA–pboA 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 deregression 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).

**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; Hanke & 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 FepA, 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...
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 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 14 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 OD_{500} 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 OD_{500} of approximately 0.6 in SS–Fe containing 25 μM EDDA plus 5 μM enterobactin after more than 2 d at 37 °C (Fig. 6), while B. bronchiseptica could reach an OD_{500} of greater than 2.0 in SS–Fe containing 45 μM EDDA plus 5 μM 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 paraprophilus 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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