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 (Gorrige 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; Stojilkovic 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 (Netlands, 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 charge, the residual 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-dipiridyl (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 & Beckwith, 1975). 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 3164 pg 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 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 BamHI sites of these plasmids to obtain pPH1, pPH2 and pPH3. Plasmid pSS1129 was a gift from Dr Scot 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 *phoA* 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

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant features</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pPHO1, 2, 3</td>
<td>pBR322-derived phoA fusion vector, Ap'</td>
<td>T. Kenney</td>
</tr>
<tr>
<td>pPH1, 2, 3</td>
<td>pPHO1, 2, 3 with Kan' cassette</td>
<td>This work</td>
</tr>
<tr>
<td>pKP5</td>
<td>pPH1 derivative containing translational bfe-phoA fusion preceded by 1-5 kb of chromosomal sequence, Ap' Kan'</td>
<td>This work</td>
</tr>
<tr>
<td>pKP3</td>
<td>pKP5 deletion derivative containing bfe-phoA preceded by 370 bp of chromosomal sequence</td>
<td>This work</td>
</tr>
<tr>
<td>pDG102</td>
<td>Kan' cassette source</td>
<td>Driks et al. (1994)</td>
</tr>
<tr>
<td>pKS3</td>
<td>pUK19 containing 736 bp structural gene fragment of bfeA</td>
<td>This work</td>
</tr>
<tr>
<td>pKP1</td>
<td>pUC19 derivative containing the bfeA gene, 1-5 kb of upstream and 400 bp of downstream flanking sequence</td>
<td>This work</td>
</tr>
<tr>
<td>pSS1129</td>
<td>Broad-host-range integrational vector, Gm' Ap' and putative promoter region</td>
<td>Stibitz (1994)</td>
</tr>
<tr>
<td>pVK11, 12</td>
<td>pSS1129 derivatives containing 5' end of bfeA</td>
<td>This work</td>
</tr>
<tr>
<td>pGN11, 12</td>
<td>pSS1129 derivatives containing 3' end of bfeA</td>
<td>This work</td>
</tr>
<tr>
<td>pRK415</td>
<td>Broad-host-range replicative vector, Tet' and putative promoter region</td>
<td>Keen et al. (1988)</td>
</tr>
<tr>
<td>p415B1, 2</td>
<td>pRK415 derivatives containing bfeA and putative promoter region</td>
<td>This work</td>
</tr>
<tr>
<td>pCI</td>
<td>T7 expression vector</td>
<td>Promega</td>
</tr>
<tr>
<td>pCI7</td>
<td>pCI derivative containing bfeA under control of the T7 promoter</td>
<td>This work</td>
</tr>
</tbody>
</table>

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., 1995). KS330 also contains the lpp-5308 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 pH1, pH2 and pH3 that had been digested with a restriction enzyme producing compatible ends to the chromosomal fragments. For the work described here, only the ligation of SauI-digested chromosomal DNA into a mixture of EcoRV-cleaved pH1, pH2 and pH3 is described. This ligation mixture was transformed into strain KS330 and plated on L-agar containing ampicillin, 150 μg XGal/ml and 200 μM DP. Individual blue colonies were streaked in duplicate onto L-agar containing both XP and DP, and onto L-agar containing XGal 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 SauI 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 SauI 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 bfeA 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td>KS330</td>
<td>Δlac-proAB F′(traD36 proAB lacI51 zafZM15)</td>
<td>Messing (1983)</td>
</tr>
<tr>
<td>JM101</td>
<td>Δlac-proAB lacI51 zafZM15</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>LE392</td>
<td>Δlac-proAB lacI51 zafZM15</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>SM10</td>
<td>RP4-2 Tc::Mu, conjugation strain</td>
<td>Stibitz (1994)</td>
</tr>
<tr>
<td>MT912</td>
<td>thi trpT proE proC leuB lacY mtl ysl rpsL avj fhuA tix supF58 lacY1 or Δ(lacI51)6 gal1k2 gat22 melB trpR35</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>MC4100</td>
<td>araD139 argF lacI51 zafZM15 deoC1 pti E25 rbsR</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>H5058</td>
<td>tsx araB cir fu fepA</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>H1443</td>
<td>as MC4100 but areB</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>H1876</td>
<td>as H1443 but fepA::Mud1X cir fepA::Tn10</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>H306</td>
<td>tonB</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>BL21(ΔDE3)</td>
<td>F′ ampT rTn10</td>
<td>Tabor &amp; Richardson (1985)</td>
</tr>
</tbody>
</table>

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

[Sall-Stal 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).

[S3] Methionine labelling of plasmid-encoded proteins. The labelling procedure has been described previously (Taber & Richardson, 1985). The DNA fragment cloned into pC17 immediately downstream of the T7 promoter as well as the promoter-distal vector sequence was transcribed by phage T7 RNA polymerase encoded on phage ΔDE3. On phage ΔDE3, 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). Four 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 XA 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,
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.*, 1970) 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-
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.
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 Fasta 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) and 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 & McCleare, 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 bfeA promoter in 14 of 19 positions (sources of the references for the consensus and bfeA sequences given in Braun et al., 1990).

Identification of the bfeA gene product

Plasmid pCI7, containing the bfeA gene on a 23 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 (lanes 1 and 2) or bfeA cloned into pHCI7 (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; Baumer & Hantke, 1992). Bands were detected by autoradiography. β-Lac, β-lactamase. Results are representative of repeated experiments using several independent transformants.
Conservation of the \textit{bfeA} gene among \textit{Bordetella} species

As seen in Fig. 5, \textit{bfeA} and flanking regions appear to be conserved among \textit{Bordetella} species. The region is highly conserved between \textit{B. pertussis}, \textit{B. bronchiseptica} and \textit{B. parapertussis}, since DNA from each of these three species gave restriction fragments indistinguishable in size that hybridized strongly to a \textit{bfeA} structural gene fragment probe. This result was seen with three different restriction digests (Fig. 5) (results of \textit{SalI} for \textit{B. bronchiseptica} not shown). \textit{EcoRV} digests of \textit{B. avium} DNA produced a single, less strongly hybridizing fragment of a different size from the respective hybridizing \textit{EcoRV} fragments generated from the other three \textit{Bordetella} species (Fig. 5, lane 4).

<table>
<thead>
<tr>
<th>\textit{PstI}</th>
<th>\textit{EcoRV}</th>
<th>\textit{SalI}</th>
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<tbody>
<tr>
<td>1</td>
<td>2</td>
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<tr>
<td>4</td>
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\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig5.png}
\caption{Conservation of the \textit{bfeA} gene and flanking region in \textit{Bordetella} species. Chromosomal DNAs from \textit{B. pertussis} (lanes 1, 5 and 8), \textit{B. parapertussis} (lanes 2, 6 and 9), \textit{B. bronchiseptica} (lanes 3 and 7), and \textit{B. avium} (lane 4) were subjected to high-stringency Southern analysis. The 736 bp \textit{SalI-StuI} \textit{bfeA} structural gene fragment (bases 717–1451 in Fig. 2) was used as the probe.}
\end{figure}

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\textit{B. bronchiseptica} & \textit{E. coli} strain & Enterobactin extract \\
strain* & & \\
\hline
19385 (wild-type) & 10 & 21 & 15 & 30 \\
19386 & 11 & 20 & 14 & 32 \\
( = 19385QpPK5) & 10 & 20 & 13 & 30 \\
19389 & 11 & 19 & 14 & 32 \\
( = 19385QpVK11)† & 0 & 0 & 0 & 0 \\
19388 & 12 & 21 & 16 & 29 \\
( = 19385QpGN11)† & 0 & 0 & 0 & 0 \\
19387 & 0 & 0 & 0 & 0 \\
( = 19385QpKS3) & 19387(p415Bl)‡ & 0 & 0 & 0 & 0 \\
19387(pRK415) & & & & \\
\hline
\end{tabular}
\caption{Growth zones of \textit{B. bronchiseptica} strains on L-agar containing 100 μg EDDA ml\(^{-1}\) around disks impregnated with enterobactin extract or enterobactin-producing \textit{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 \textit{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.

\begin{itemize}
\item * The symbol \textbf{Ω} indicates Campbell-type integration of the indicated plasmid containing the chromosomal restriction fragments depicted in Fig. 7.
\item † All these strains are \textit{aroB}*. All the \textit{B. bronchiseptica} strains listed were also tested with the \textit{aroB} \textit{E. coli} strains H5058, H1443 and H1876; no growth was obtained with any of these strain combinations.
\item ‡ An identical plasmid, except containing the opposite orientation of the chromosomal fragment, was used with identical results.
\end{itemize}

\section*{Utilization of enterobactin by \textit{B. pertussis} and \textit{B. bronchiseptica}}

To determine if \textit{Bordetella} species were capable of utilizing ferric enterobactin, a disk method was employed as described in Methods. Only \textit{aroB}+ enterobactin-producing \textit{E. coli} strains were capable of stimulating growth of \textit{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 \textit{B. pertussis} growth in this manner since evidently the \textit{E. coli} strains secreted substance(s) inhibitory or lytic to \textit{B. pertussis}. This was shown by control experiments in the absence of EDDA, which resulted in wide zones devoid of growth around the \textit{E. coli}-containing disks (data not shown). Enterobactin-enhanced growth by \textit{B. pertussis} and \textit{B. bronchiseptica} was apparent in SS–Fe broth cultures...
Bordetella ferric enterobactin receptor gene

**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 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 data 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 pPK5 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 assayed.

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 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 Stul 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 μg 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 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
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 pKPl 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(pKPl) 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(pKPl), H1876(pKPl) and H1443(pKPl) 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(pKPl), 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 pKPl, 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 pKPl showed no differences. Additionally, the XmnI–NcoI fragment subcloned from pKPl 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 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–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 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 *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–pboA 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; 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 FieA, 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>AP units (mean ± SD, n = 3)</th>
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<tr>
<td></td>
<td>+ Fe</td>
</tr>
<tr>
<td>E. coli KS330(pPK5)*</td>
<td>75 ± 20</td>
</tr>
<tr>
<td>E. coli KS330(pPK3)*</td>
<td>97 ± 25</td>
</tr>
<tr>
<td>B. bronchiseptica 19386†</td>
<td>5 ± 3</td>
</tr>
<tr>
<td>B. pertussis 83†</td>
<td>2 ± 2</td>
</tr>
</tbody>
</table>

*Mid-exponential-phase cells were incubated for 3 h in L-broth (+Fe) or in L-broth containing 200 μM DPD (−Fe).
† Cells were grown to late exponential phase in SS + Fe, containing 50 μM 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 OD₉₀₀ 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 OD₉₀₀ 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 OD₉₀₀ 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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REFERENCES


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