Essential Role of the Iron-Regulated Outer Membrane Receptor FauA in Alcaligin Siderophore-Mediated Iron Uptake in *Bordetella* Species

TIMOTHY J. BRICKMAN[1,2] AND SANDRA K. ARMSTRONG[1,2]*

Department of Microbiology and Immunology, East Carolina University School of Medicine, Greenville, North Carolina 27858-4354,[1] and Department of Microbiology, University of Minnesota, Minneapolis, Minnesota 55455-0312[2]

Phenotypic analysis using heterologous host systems localized putative *Bordetella pertussis* ferric alcaligin transport genes and Fur-binding sequences to a 3.8-kb genetic region downstream from the *alcR* regulator gene. Nucleotide sequencing identified a TonB-dependent receptor family homolog gene, *fauA*, predicted to encode a polypeptide with high amino acid sequence similarity with known bacterial ferric siderophore receptors. In *Escherichia coli*, the *fauA* genes of both *B. pertussis* and *Bordetella bronchiseptica* directed the production of a 79-kDa polypeptide, approximating the predicted size of the mature FauA protein. *B. bronchiseptica fauA* insertion mutant BRM17 was unable to utilize ferric alcaligin, and in complementation analyses ferric alcaligin utilization was restored to this mutant by supplying the wild-type *fauA* gene in *trans*. Mutant BRM18, carrying a nonpolar in-frame *fauA* deletion mutation, was defective in ferric alcaligin utilization and [55]Fe-ferric alcaligin uptake and no longer produced a 79-kDa iron-regulated outer membrane protein. In complementation analyses, BRM18 merodiploids bearing the wild-type *fauA* gene in *trans* regained ferric alcaligin siderophore transport and utilization functions and produced the 79-kDa protein. Analysis of a plasmid-borne *fauA-lacZ* operon fusion confirmed that *fauA* is subject to iron regulation at the transcriptional level and that *cis*-acting transcriptional control elements mediating *fauA* iron repressibility reside within the 3.8-kb *Pst*I *fauA* DNA region. Moreover, expression of the *fauA-lacZ* fusion gene under iron starvation conditions was shown to be *alcR* dependent. FauA is a 79-kDa iron-regulated outer membrane receptor protein required for transport and utilization of ferric alcaligin siderophore complexes by *Bordetella* species.

Under iron-limiting growth conditions, bacteria express high-affinity transport systems to scavenge and assimilate nutritional iron. These transport processes often involve the synthesis and excretion of soluble siderophores that chelate ferric iron and are subsequently conveyed into the bacterial cell by ligand-specific cell surface receptors and permeases (34, 39). To exploit the availability of diverse iron sources that may be present in their environment, some microbes produce transport proteins that enable them to utilize siderophores produced by other microbial species (18, 39). For all gram-negative bacteria examined to date, siderophore-mediated iron uptake is dependent on the activities of TonB, ExbB, and ExbD, which work in conjunction with the cognate outer membrane receptor protein to translocate iron across the bacterial outer membrane against a concentration gradient (37). Further translocation of iron to the cytoplasm is accomplished by a periplasmic binding protein and an ATP binding cassette (ABC)-dependent permease in the cytoplasmic membrane.

Bordetella pertussis and *Bordetella bronchiseptica* are gram-negative pathogens that inhabit the respiratory mucosae of humans and nonhuman mammals. When nutritional iron is limiting in availability, these organisms produce and utilize the macrocyclic dihydroxamate siderophore alcaligin (16, 38). *B. pertussis* and *B. bronchiseptica* are also capable of utilizing iron complexed with the heterologous siderophores enterobactin (8), ferrichrome, and desferrioxamine B (9). In addition to

siderophores, the mammalian host-derived molecules lactoferrin (1), transferrin (1, 26), hemin (9), and hemoglobin (40) have been reported as iron sources for these bacteria. The capacity of *Bordetella* cells to utilize these alternative iron sources implies that they are capable of producing the cognate transporters required for their utilization.

Biosynthesis of the alcaligin siderophore in *B. pertussis* and *B. bronchiseptica* requires an ornithine decarboxylase activity that yields the requisite alcaligin precursor putrescine from ornithine (14). Dedicated alcaligin biosynthesis activities are encoded within the iron-regulated *alcABCDER* operon (29, 30). AlcA, AlcB, and AlcC are required for alcaligin biosynthesis; mutations in *alcA*, *alcB*, or *alcC* render *Bordetella* cells unable to produce alcaligin (30). The *alcA* (25, 30), *alcB*, and *alcC* gene products have strong primary amino acid sequence similarity with the *Escherichia coli* aerobactin biosynthesis enzymes IucD, IucB, and IucC, respectively (19, 30). Based on these amino acid sequence similarities and the known structure of alcaligin, it is postulated that AlcA is an oxygenase that catalyzes the hydroxylation of putrescine, and AlcB functions in an acylation step involving succinate. AlcC is similar to IucC aerobactin synthetase and may function in one of the final reactions yielding alcaligin. AlcD was reported to have no significant amino acid sequence similarity with any known proteins, while AlcE has similarity with iron-sulfur-containing dioxygenases (43). Although their role in alcaligin biosynthesis has not been established, the location of these two genes within the *alc* biosynthesis operon and the prediction that AlcE may function as a dioxygenase suggest that *alcD* and *alcE* are also involved in the biosynthesis of alcaligin. The *alcR* gene is involved in the regulation of both alcaligin biosynthesis (11, 43)

* Corresponding author. Mailing address: Box 196 UMHC, Department of Microbiology, University of Minnesota, 420 Delaware St. S.E., Minneapolis, MN 55455-0312. Phone: (612) 625-6947. Fax: (612) 626-0623. E-mail: sandra@lenti.med.umn.edu.

TABLE 1. *Bordetella* strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Reference or source
B. bronchiseptica strains		
B013N	*fauA*$^+$, nalidixic acid-resistant derivative of swine isolate strain B	6
BRM17	*fauA1*::Kan^r mutant derivative of B013N, with an insertion of a 1.2-kb *Bam*HI kanamycin resistance cassette into the *Bgl*II restriction site in *fauA*	This study
BRM18	Δ*fauA2* mutant derivative of B013N, with a nonpolar, in-frame deletion of 255 bp of *fauA* coding sequences	This study
BRM11	Δ*alcR1* mutant derivative of B013N	11
Recombinant plasmids		
pCP1.11	*B. pertussis* strain UT25 *fauA*$^+$ cosmid, source of sequenced *fauA* DNA, RK2 origin, RK2-Mob$^+$, RK2-Tra$^-$, Tc^r	30
pBRM6	20-kb *fauA*$^+$ *Sal*I genomic DNA fragment of *B. bronchiseptica alcC* mutant BRM6 cloned in pGEM3Z, Ap^r, ColE1 origin	6
p3Z44	7-kb *B. bronchiseptica fauA*$^+$ *Eco*RI-*Sal*I DNA fragment subcloned from pBRM6 to pGEM3Z, Ap^r, ColE1 origin	This study
p3Z48	3.8-kb *B. bronchiseptica fauA*$^+$ *Pst*I DNA fragment subcloned from p3Z44 to pGEM3Z, Ap^r, ColE1 origin	This study
p3Z49	As p3Z48 except opposite orientation of the 3.8-kb *fauA*$^+$ *Pst*I DNA insert fragment	This study
pBB24	3.8-kb *B. bronchiseptica fauA*$^+$ *Pst*I DNA fragment subcloned from p3Z44 to pBBR1MCS, Cm^r	This study
pBB24Δ*fauA*	As pBB24 except carrying the *B. bronchiseptica* Δ*fauA2* mutant allele on the *Pst*I DNA insert fragment	This study
p3Z63	3.8-kb *B. pertussis* UT25 *fauA*$^+$ *Pst*I DNA fragment subcloned from pCP1.11 to pGEM3Z, Ap^r, ColE1 origin	This study
p3Z69	As p3Z48 except carrying the *B. bronchiseptica* Δ*fauA2* mutant allele on the *Pst*I DNA insert fragment	This study
pBB24/mini-Tn*5 lacZ1*	As pBB24 except carrying the *B. bronchiseptica* φ(*fauA′-lacZ*$^+$)*1* allele generated by mini-Tn*5 lacZ1* transposon mutagenesis, Cm^r, Kan^r	This study

and transport of the ferric alcaligin complex (11). AlcR is an AraC-like transcriptional regulator similar to the *Pseudomonas aeruginosa* PchR pyochelin siderophore biosynthesis and transport regulator protein (27) and the YbtA yersiniabactin siderophore receptor gene regulator of *Yersinia pestis* (23). The *alcR* gene is transcribed primarily from the Fur-regulated promoter upstream from *alcA* as part of the *alcABCDER* operon and is also transcribed from a secondary Fur-regulated promoter located immediately upstream from *alcR* (11, 29). Adjacent to *alcR* is an open reading frame predicted to encode a multidrug efflux pump protein homolog (11, 43). This gene was named *bcr* in a previous study (43) on the basis of amino acid sequence similarity of the predicted product with the *E. coli* bicyclomycin resistance efflux protein, Bcr. However, since efflux pumps are generally not substrate specific (42) and the function of the *Bordetella* protein remains unknown, we presently refer to this gene as *orfX*.

In the present study, we localized a *Bordetella* genetic region downstream from *orfX* that is involved in ferric alcaligin transport and identified the *fauA* gene encoding the ferric alcaligin outer membrane receptor protein. FauA was localized in the outer membrane fraction prepared from iron-starved *B. bronchiseptica* cells and was produced in *E. coli* by using an inducible protein expression system. *B. bronchiseptica fauA* mutants were unable to transport and utilize ferric alcaligin, and these functions were restored to the mutants by genetic complementation using the cloned *B. bronchiseptica* or *B. pertussis fauA* gene. Analysis of a *fauA-lacZ* operon fusion confirmed that expression of *fauA* is regulated at the transcriptional level by iron and by the AlcR regulator protein.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *B. bronchiseptica* strains used in this study are listed in Table 1. *E. coli* DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) was used as the host for routine plasmid propagation and

DNA cloning procedures and as the donor strain in triparental matings. *E. coli* SURE (Stratagene, La Jolla, Calif.) was used for propagation of M13 mp18 bacteriophages (41), and *E. coli* host strain CJ236 (28) was used to produce uracil-containing single-stranded M13 bacteriophage DNA for in vitro site-directed mutagenesis procedures. *E. coli* S17-1(λ*pir*) (36, 48) was the donor strain for pUT mini-Tn*5 lacZ1* suicide plasmids in transposon mutagenesis, and *E. coli* K38(pGP1-2) was the host strain in bacteriophage T7 RNA polymerase/promoter system protein expression studies (51). *E. coli* H1717 (*fhuE-lacZ aroB*) served as the indicator host strain in Fur repressor titration assays (50) used to identify potential *Bordetella* Fur-binding DNA sequences borne on multicopy plasmids. Pyochelin- and pyoverdin-deficient *P. aeruginosa* mutant strain IA1 (5) was used as a heterologous host for identification of genes encoding putative *Bordetella* ferric alcaligin transport and utilization functions.

B. bronchiseptica and *E. coli* strains were grown in Luria-Bertani (LB) broth or on LB agar plates (44). *E. coli* strains for bacteriophage T7 RNA polymerase/promoter protein expression experiments were cultured in M9 medium (44) supplemented with 0.2% glucose and 0.01% L-amino acids (minus cysteine and methionine). Defined broth culture medium for *Bordetella* strains was Stainer-Scholte medium (SS) (49) modified as described elsewhere (46); iron-replete SS contained 36 μM FeSO$_4$, and iron-depleted SS culture medium was prepared by treatment of the medium with Chelex 100 (Bio-Rad, Hercules, Calif.) as described by Armstrong and Clements (6). Growth was monitored as optical density, using a spectrophotometer or a Klett-Summerson colorimeter equipped with a no. 54 filter (Klett Mfg. Co., Long Island City, N.Y.). Antibiotics were used at the indicated concentrations: ampicillin, 100 μg/ml; chloramphenicol, 30 μg/ml; gentamicin, 10 μg/ml; kanamycin, 50 μg/ml; and nalidixic acid, 35 μg/ml.

Plasmids and genetic methods. Plasmid cloning vectors pGEM3Z (Promega, Madison, Wis.), pBBR1MCS (31), and pBluescript II KS$^+$ (Stratagene) were used in the construction of recombinant plasmids. Recombinant *Bordetella* plasmids used in this study are listed in Table 1. Plasmid pRK2013 (24) was used to provide transfer functions in triparental matings. Plasmid pBSL15 (3) was the source of the kanamycin resistance gene used in the construction of *B. bronchiseptica* insertion mutant BRM17. Suicide plasmid vectors pEG7 and pEG18.3 (2) were used in allelic exchange procedures in the construction of *B. bronchiseptica* mutant BRM18, and suicide plasmid pUT mini-Tn*5 lacZ1* (20) was used to generate *fauA-lacZ* transcriptional fusions.

General genetic techniques were performed essentially as described previously (44). Verification of allelic exchange in *Bordetella* mutants was by Southern hybridization analysis or PCR analysis of genomic DNA samples. DNA probes used in nucleic acid hybridizations were radiolabeled by the random priming method (22) using the Random Primers DNA labeling system (Gibco BRL, Gaithersburg, Md.) and [α-^{32}P]dCTP (ICN Radiochemicals, Irvine, Calif.). The nucleotide sequence of *fauA* was determined for both DNA strands by using the

dideoxy-chain termination method (45) and double-stranded plasmid DNA templates, with nucleotide sequencing services provided by the University of Tennessee Molecular Biology Resource Facility and by the University of Minnesota Microchemical Facility. Management and analysis of nucleotide sequence data used the Lasergene sequence analysis software system for the Macintosh PowerPC computer (DNASTAR, Inc., Madison, Wis.). Database searches and data retrievals were performed by using the BLAST (4) server provided by the National Center for Biotechnology Information at the National Library of Medicine. Multiple amino acid sequence alignments were performed by the CLUSTAL V method, using the MegAlign module of the Lasergene sequence analysis software system (DNASTAR). Putative *Bordetella* Fur-binding sequences were identified by using the MegAlign software to locate DNA regions of at least 50% identity over a 30-nucleotide search window with the dyad sequence, 5′-GATAATGATAATCATTATC-3′, representing the proposed consensus *E. coli* Fur-binding site (17, 21). Translated *fauA* DNA sequences were scanned for the occurrence of PROSITE protein patterns by using the ScanProsite tool of the ExPASy molecular biology server of the Swiss Institute of Bioinformatics. Stained protein gels and protein gel autoradiographs were imaged by using a Hewlett Packard ScanJet 4c color flatbed scanner and Adobe Photoshop LE software for the Power Macintosh computer.

Conjugal transfer of plasmids to *Bordetella* strains was accomplished as described previously (14). Transformation of *Bordetella* or *E. coli* strains by electroporation was performed by using standard methods (Bio-Rad).

The Fur repressor titration assay used to screen *B. pertussis* cosmid pCP1.11 *fauA* region subclones for functional Fur repressor-binding sequences was performed as described by Stojiljkovic and coworkers (50), using lactose MacConkey agar supplemented with 30 μM ferrous ammonium sulfate and appropriate antibiotics to select for plasmid maintenance.

Construction of a *B. bronchiseptica fauA* insertion mutant. A *fauA* insertion mutation was constructed by ligation of the 1.2-kb *Bam*HI kanamycin resistance cassette of plasmid pBSL15 into the unique *Bgl*II site of the *B. bronchiseptica* DNA insert fragment of p3Z48. Since this plasmid is a ColE1 replicon and unable to replicate in *Bordetella* strains, it was used as a suicide plasmid to deliver the mutation to the *B. bronchiseptica* chromosome by homologous recombination. The plasmid was introduced into *B. bronchiseptica* wild-type strain B013N by electroporation, and kanamycin-resistant clonal isolates were subsequently screened for loss of the ampicillin resistance plasmid marker by replicate plating on LB agar and LB agar containing ampicillin. Allelic exchange in *B. bronchiseptica* mutant BRM17 was confirmed by Southern hybridization using *fauA*-specific and plasmid vector DNA probes.

Construction of a *B. bronchiseptica fauA* deletion mutant. A nonpolar *fauA* mutation was constructed by in vitro site-directed mutagenesis using the Kunkel method (32), and the mutated *fauA* gene was introduced into wild-type *B. bronchiseptica* strain B013N by allelic exchange. The purified 3.8-kb *fauA Pst*I DNA fragment of plasmid p3Z49 was ligated with *Pst*I-digested bacteriophage M13 mp18 replicative-form DNA, and the ligation mixture was used to transfect *E. coli* host strain SURE. Recombinant bacteriophages bearing the 3.8-kb *Pst*I *B. bronchiseptica* DNA insert fragment were plaque purified and used to infect *E. coli* host strain CJ236. Bacteriophages were recovered from infected CJ236 culture supernatants by polyethylene glycol-NaCl precipitation, and single-stranded uracil-containing bacteriophage DNA was purified by using standard procedures. The mutagenic 42-mer oligonucleotide fau1 (5′-TTGCCCGAGCC TGTCATCAGGGATTGTGGTGTTTCGCGTGGC-3′) is complementary to two noncontiguous regions of the *fauA* sense strand, spanning the site of the desired deletion mutation. This primer was phosphorylated using T4 polynucleotide kinase and ATP, annealed to the single-stranded uracil-containing *fauA* template DNA, and extended by using T7 DNA polymerase and deoxyribonucleotides in the presence of T4 DNA ligase. A portion of the resulting reaction mixture was used to transfect *E. coli* SURE, and replicative-form DNA samples isolated from plaque-purified bacteriophage progeny were mapped by using the unique *Eco*RV and *Bgl*II restriction endonuclease sites in *fauA* that flank the desired mutation. Bacteriophage DNA samples from phage clones produced from two independent mutagenesis reactions were sequenced to confirm the desired deletion of *fauA* coding sequences.

The *sacBR* positive selection allelic exchange system of Akerley and coworkers (2) was used to cross the *fauA* deletion mutation carried on the 3.8-kb *fauA Pst*I DNA fragment into the chromosome of wild-type *B. bronchiseptica* strain B013N. Deletion mutations were verified by PCR analysis of genomic DNA samples prepared from putative Δ*fauA* mutants, using oligonucleotide primers 5′-GAC ACTCTCGCCACGCGAAAC-3′ (upstream primer) and 5′-CGTGATGGGCA CGGAGATGTC-3′ (downstream primer) flanking the chromosomal deletion mutation. Based on the known *fauA* nucleotide sequence, these primers were predicted to direct the synthesis of a 430-bp PCR product from the wild-type *fauA* region and a 175-bp PCR product from Δ*fauA* mutants. Agarose gel electrophoresis was used to determine the sizes of PCR reaction products corresponding to the presence of wild-type or Δ*fauA* mutant alleles in the bacterial chromosome.

Measurement of *Bordetella* siderophore activity. The chrome azurol S universal siderophore detection assay (47) was used to quantitate alcaligin production by *Bordetella* cells grown in iron-depleted SS as described previously (6, 16) to monitor iron nutritional status of bacteria. Siderophore assays were performed in triplicate.

Ferric alcaligin utilization bioassays. Growth stimulation of *B. bronchiseptica* strains and siderophore-deficient *P. aeruginosa* mutant strain IA1 by ferric alcaligin siderophore complexes was measured by quantitative ferric alcaligin utilization bioassays using iron-restricted agar plates containing ethylenediaminedi-[(o-hydroxyphenyl)acetic acid] (EDDA) at a concentration of 100 μg/ml, performed as described previously (11, 16). Alcaligin siderophore was purified from spent *Bordetella* culture supernatants as described by Brickman and coworkers (16) and used at aqueous concentrations of 250, 125, and 63 μg/ml.

Ferric alcaligin transport assays. *B. bronchiseptica* cells were assayed for the ability to bind and transport ferric alcaligin by measuring ^{55}Fe(III) accumulation by the method of Langman et al. (33) as modified by Brickman and coworkers (16), using ^{55}Fe(III) supplied as 5 μM alcaligin–3.3 μM ^{55}Fe(III) complexes (3:2 molar ratio, pH 8.0). Cell-associated ^{55}Fe counts per minute were measured by scintillation counting.

Production of plasmid-encoded FauA proteins in *E. coli*. Plasmid-encoded FauA proteins were conditionally produced in *E. coli* K38(pGP1-2) by using the bacteriophage T7 polymerase/promoter system of Tabor and Richardson (51). After induction of *fauA* expression at mid-logarithmic growth phase by incubation at 42°C, approximately 5×10^7 cells (0.1 units of optical density at 600 nm) were harvested by centrifugation, solubilized in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer at 100°C for 7 min and analyzed by SDS-PAGE on 10% polyacrylamide gels. Proteins were visualized by intrinsic radiolabeling using Tran^{35}S-label (ICN Biochemicals, Inc.), SDS-PAGE, and autoradiography of dried electrophoretic gels.

Cell fractionation and SDS-PAGE. *Bordetella* cells from iron-replete and iron-depleted SS cultures were harvested by centrifugation at late-logarithmic growth phase, resuspended in 50 mM HEPES buffer (pH 7.4), and lysed by passage through a French pressure cell (American Instrument Company, Silver Spring, Md.). The insoluble cell fraction sediment obtained by ultracentrifugation was extracted with Triton X-100 detergent as described previously (46), and the outer membrane protein-enriched detergent-insoluble fraction was resuspended in 50 mM HEPES buffer (pH 7.4) and solubilized in SDS-PAGE sample buffer at 100°C for 7 min. Proteins were resolved by SDS-PAGE on 7.5 or 10% polyacrylamide gels containing 0.5 M urea as described previously (46), with approximately 20 μg of protein applied to each lane. Protein bands were visualized by staining with Coomassie blue dye.

Construction and analysis of a *fauA-lacZ* transcriptional fusion plasmid. Transposon mini-Tn5 *lacZ1* (20) was delivered to *B. bronchiseptica* carrying *fauA* plasmid pBB24 by conjugal transfer of suicide plasmid pUT mini-Tn5 *lacZ1* from donor strain *E. coli* S17-1(λ*pir*). After 5 h of mating on an LB agar plate surface, bacterial growth was harvested and total plasmid DNA was prepared by the alkaline lysis method (12). Plasmid DNA pools were used to transform *E. coli* DH5α to kanamycin and chloramphenicol resistance, and mutated pBB24 plasmids subsequently isolated from transformants were screened by restriction endonuclease mapping for insertions in *fauA*. Plasmids carrying *fauA* insertions in the *fauA* sense orientation were transferred to wild-type *B. bronchiseptica* strain B013N and *alcR* mutant strain BRM11 by conjugation. Transconjugants were assayed for β-galactosidase activity by the method of Miller (35) as modified by Brickman et al. (15) after culture in iron-replete or in iron-depleted SS medium.

Nucleotide sequence accession number. The GenBank accession number assigned to the *B. pertussis* UT25 *fauA* gene is AF135154.

RESULTS

Preliminary identification of putative ferric alcaligin transport and utilization genes and Fur-binding sequences by phenotypic analysis using heterologous host systems. Since microbial siderophore system genes are usually clustered on the chromosome, it was hypothesized that alcaligin transport genes may reside near known alcaligin biosynthesis genes on *B. pertussis* cosmid pCP1.11 or on the adjacent overlapping cosmids described previously (30). Prior alcaligin biosynthesis studies using a heterologous host system established that pyochelin- and pyoverdin-deficient *P. aeruginosa* mutant strain IA1 (5) carrying pCP1.11 produced authentic alcaligin (10). In parallel studies using mutant strain IA1 as a host system for expression of potential *Bordetella* alcaligin transport and utilization genes, analysis of subcloned segments of pCP1.11 identified an approximately 4-kb *Pst*I genetic region downstream from *alcR* (Fig. 1) that conferred a positive growth stimulation phenotype to IA1 in bioassays using purified alcaligin as an iron source (data not shown). When cloned to high-copy-number plasmids, the same *B. pertussis* genetic region had strong in vivo Fur repressor-binding activity by the Fur repressor titration assay in *E. coli* (data not shown), consistent with the presence of
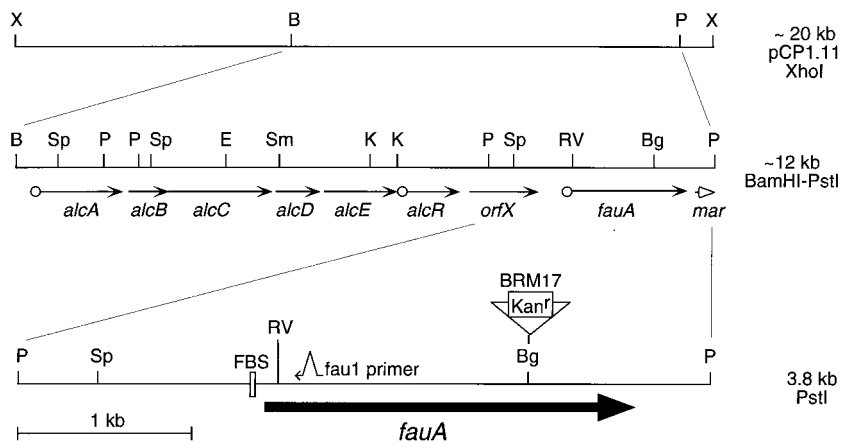
FIG. 1. Spatial organization of known *Bordetella* alcaligin siderophore system genes. The 20-kb *Xho*I insert DNA fragment of *B. pertussis* cosmid pCP1.11 is depicted at the top. The enlarged map in the center represents an approximately 12-kb *Bam*HI-*Pst*I subregion of pCP1.11; arrows indicate the direction of transcription and genetic limits of all known genes within the *Bordetella* alcaligin gene cluster, and open circles represent known or hypothetical Fur-regulated promoter-operator regions. The detailed lowermost map is enlarged to represent the 3.8-kb *Pst*I DNA subregion encoding *fauA*, and a 1-kb scale bar is shown. FBS indicates the position of the computer-predicted Fur-binding site immediately upstream from the start of the *fauA* coding sequences. The approximate position of sequences complementary to mutagenic primer fau1 used in the construction of Δ*fauA* mutant BRM18 is indicated by the small arrow. The triangle indicates the position of the *Bgl*II site of insertion of the kanamycin resistance cassette (Kan^r) in *fauA* mutant BRM17. Abbreviations: X, *Xho*I; B, *Bam*HI; E, *Eco*RI; Sp, *Sph*I; P, *Pst*I; Sm, *Sma*I; K, *Kpn*I; RV, *Eco*RV; Bg, *Bgl*II.

previously unidentified functional Fur repressor-binding sequences. Based on these preliminary data suggesting the existence of previously unknown ferric alcaligin transport and utilization genes and functional Fur-binding sequences downstream from *alcR*, the corresponding DNA region was further analyzed by nucleotide sequencing.

Nucleotide sequencing of putative ferric alcaligin transport and utilization genes. Complete nucleotide sequence analysis of an approximately 3-kb genetic region downstream from *alcR* identified a large open reading frame encoding a putative *B. pertussis* TonB-dependent receptor family homolog. In BLASTP searches, the GenBank database sequences producing the six highest-scoring alignments with the deduced *Bordetella* receptor homolog (GenBank accession no. AF135154) were ferric siderophore receptor proteins. These included the ferripyoverdin receptor FpvA of *P. aeruginosa* (GenBank accession no. U07359), the *Pseudomonas putida* ferric pseudobactin 358 receptor PupA (GenBank accession no. P25184), the *E. coli* FhuE outer membrane receptor for ferric coprogen, ferric ferrioxamine B, and ferric rhodotorulic acid (GenBank accession no. P16869), the *P. putida* PupB ferric pseudobactin BN7/BN8 receptor (GenBank accession no. P38047), *P. aeruginosa* ferric pyochelin receptor FptA (GenBank accession no. P42512), and PbuA, the ferric pseudobactin M114 receptor of *Pseudomonas* sp. strain M114 (GenBank accession no. Q08017). Overall percent similarity between the *Bordetella* ferric siderophore receptor homolog and these highest-scoring database retrievals ranged from 33.1% for FpvA to 27.0% for PbuA at the primary amino acid sequence level.

The *Bordetella* ferric siderophore receptor homolog gene was tentatively named *fauA*, for ferric alcaligin uptake gene. As deduced from the nucleotide sequence, the FauA precursor protein is a 734-amino-acid polypeptide with a calculated molecular mass of approximately 81.6 kDa. A PROSITE database search revealed that at C-terminal amino acid positions 717 to 734, FauA exhibits the TonB-dependent receptor protein signature 2 (PROSITE PDOC00354) that is characteristic of most other members of the TonB-dependent receptor family (Fig. 2). Consistent with the earlier identification of functional

Fur-binding DNA sequences in the *fauA* genetic region by Fur repressor titration assay, a computer-predicted Fur repressor-binding site, 5′-AAAAAAGATAATTCCTATT-3′, was identified at nucleotide positions 35 to 50 upstream from the start of the *fauA* coding sequence (Fig. 1).

Beginning approximately 300 bp downstream from the end of the *fauA* coding region and extending to the *Pst*I terminus of the sequenced DNA region (Fig. 1) is a partial open reading frame encoding a polypeptide with primary amino acid sequence similarity to multiple antibiotic resistance protein MarC of *E. coli* (GenBank accession no. P31123) and *Salmonella typhimurium* (GenBank accession no. Q56068). MarC proteins are members of SwissProt uncharacterized protein family UPF0056 of integral membrane proteins. This *Bordetella marC*-like gene, tentatively named *mar*, is transcribed in the same polarity as *fauA*, but it is not yet known whether it is
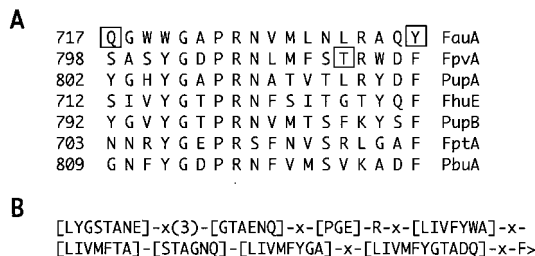

FIG. 2. Partial amino acid sequence alignments of FauA with TonB-dependent bacterial siderophore receptor proteins of high similarity. (A) Multiple sequence alignments of the FauA C terminus with C termini of bacterial siderophore receptor proteins producing the six highest-scoring alignments with FauA in GenBank database searches (GenBank accession numbers: *P. aeruginosa* FpvA, U07359; *P. putida* PupA, P25184; *E. coli* FhuE, P16869; *P. putida* PupB, P38047; *P. aeruginosa* FptA, P42512; *Pseudomonas* sp. strain M114 PbuA, Q08017). With the exceptions of residues Q717 and Y734 of FauA and T812 of FpvA (boxed), all amino acid residues shown are consistent with the PROSITE database TonB-dependent receptor protein signature 2 (PROSITE PDOC00354) consensus pattern (B) shared by most members of the TonB-dependent receptor family.

TABLE 2. Utilization of ferric alcaligin by wild-type and mutant *B. bronchiseptica* strains[a]

Indicator strain	Growth zone (mm) surrounding wells containing purified alcaligin at concn of:		
	63 µg/ml	125 µg/ml	250 µg/ml
B013N (wild type)	16	20	24
BRM17	—	—	—
BRM17(pBBR1MCS[b])	—	—	—
BRM17(pBB24Δ*fauA*)	—	—	—
BRM17(pBB24)	17	22	27
BRM18	—	—	—
BRM18(pBBR1MCS)	—	—	—
BRM18(pBB24)	18	23	28

[a] Aqueous alcaligin solution (50 µl/well) at the indicated concentration was added to 6-mm-diameter wells in EDDA-containing iron-restricted agar medium seeded with indicator strains, and diameters of growth zones were measured after 18 h incubation at 37°C. No growth occurred in the absence of added alcaligin siderophore. —, no detectable growth stimulation.

[b] Plasmid vector control.

operonic with *fauA* or whether it encodes any function related to iron metabolism.

Construction and analysis of *B. bronchiseptica* fauA insertion mutant BRM17. To preliminarily address the hypothesized role of *fauA* in transport and utilization of ferric alcaligin, *fauA* insertion mutant BRM17 was constructed by insertion of a kanamycin resistance cassette at the unique *Bgl*II site of *fauA* (Fig. 1). In growth stimulation bioassays, BRM17 was fully defective in utilization of ferric alcaligin as an iron source (Table 2). Genetic complementation of BRM17 by the wild-type 3.8-kb *B. bronchiseptica Pst*I *fauA* DNA region as plasmid pBB24 resulted in restoration of ferric alcaligin utilization. In these complementation analyses, BRM17 carrying multicopy *fauA* plasmids reproducibly displayed larger growth haloes than the wild-type indicator strain, presumably because of FauA overproduction and enhanced ferric alcaligin utilization. Complementation of the BRM17 ferric alcaligin utilization defect by *B. bronchiseptica* plasmids carrying the 3.8-kb *Pst*I *fauA* DNA region was independent of insert DNA orientation with respect to the plasmid cloning vector (data not shown), suggesting that *fauA* was expressed from a natural *Bordetella* promoter borne on that plasmid construct. In addition, a *B. pertussis fauA* plasmid carrying the 3.8-kb *Pst*I *fauA* DNA region (Fig. 1) was equally capable of restoring ferric alcaligin utilization to BRM17 (data not shown), indicating that the *B. pertussis fauA* gene was fully functional in *B. bronchiseptica* by this growth stimulation bioassay method.

Construction of a *B. bronchiseptica* fauA deletion mutant. A specific *fauA* deletion mutation was constructed in vitro, and the mutated *B. bronchiseptica fauA* gene was crossed into wild-type *B. bronchiseptica* strain B013N by allelic exchange as described in Materials and Methods. The mutation was designed to generate an in-frame deletion removing 255 bp (85 codons) of *fauA* coding sequences that specify a FauA segment (amino acid residues 89 to 173 in the FauA precursor protein) that is highly conserved among known TonB-dependent outer membrane siderophore complex receptors; thus, the deleted sequences were hypothesized to encode a region of functional importance in the FauA protein. Further, an in-frame deletion was desired in order to avoid potential polarity effects of a mutation on unknown, potentially cotranscribed downstream genes that may also be involved in ferric alcaligin transport or utilization.

After in vitro mutagenesis of cloned *fauA* sequences, bacte-

riophage DNA from clones produced from two independent mutagenesis reactions was sequenced, and both were found to carry the desired deletion mutation. To preliminarily assess the phenotypic effect of the mutation, the mutated *fauA Pst*I fragment was subcloned to the broad-host-range plasmid vector pBBR1MCS to produce pBB24Δ*fauA*, and this plasmid was introduced into *B. bronchiseptica fauA* insertion mutant BRM17, which is defective in ferric alcaligin utilization. Unlike the wild-type *fauA* plasmid equivalent pBB24, mutated plasmid pBB24Δ*fauA* was unable to complement the growth defect of BRM17 when supplied with ferric alcaligin in growth stimulation bioassays (Table 2). These data were consistent with the prediction that the *fauA* deletion mutation would ultimately result in a functionally defective FauA protein once transferred to the *B. bronchiseptica* chromosome by allelic exchange.

A positive selection allelic exchange system (2) was used to cross the *fauA* deletion mutation into the chromosome of wild-type *B. bronchiseptica* strain B013N, and the allelic exchange was confirmed by genomic PCR analysis. Electrophoretic analysis of PCR products generated from genomic DNA samples prepared from four putative B013N Δ*fauA* mutants by using oligonucleotide primers flanking the deletion junction revealed that three of the four putative mutants carried only the Δ*fauA* allele (data not shown). The fourth putative B013N Δ*fauA* mutant carried only a wild-type *fauA* allele, yielding a single PCR product indistinguishable from that produced from a wild-type control genomic DNA template.

FauA requirement for growth stimulation by ferric alcaligin siderophore. The three B013N Δ*fauA* mutants identified by genomic PCR analysis were assayed for growth stimulation by ferric alcaligin under iron starvation culture conditions using bioassays conducted in parallel with the wild-type parent strain B013N. Consistent with the results of physical mapping of the chromosomal *fauA* regions by genomic PCR analysis, the deletion mutants examined in growth stimulation bioassays were fully defective in ferric alcaligin transport or utilization compared with the wild-type control strain. One B013N Δ*fauA* mutant chosen for further study was designated BRM18 (Table 1). In complementation analyses, growth stimulation by ferric alcaligin in bioassays was restored to BRM18 by the wild-type *B. bronchiseptica fauA* gene supplied as plasmid pBB24 (Table 2). As in BRM17 complementation analyses, growth stimulation zones for the complemented BRM18 Δ*fauA* mutant were consistently larger than those produced with wild-type indicator cells. BRM18 growth stimulation by ferric alcaligin was also restored by plasmids carrying the analogous wild-type 3.8-kb *Pst*I *fauA* genetic region of *B. pertussis* strain UT25 (Fig. 1), indicating that the *fauA* alleles of the two different *Bordetella* species are interchangeable in this functional assay (data not shown). These growth stimulation bioassays confirmed the predicted correlation between the deletion mutation detected by genomic PCR mapping and a defective ferric alcaligin transport or utilization phenotype. Genetic complementation results confirmed that ferric alcaligin utilization could be fully restored to BRM18 by the wild-type *fauA* gene of either *B. pertussis* or *B. bronchiseptica* in *trans*.

FauA requirement for ferric alcaligin-mediated iron transport. *B. bronchiseptica* Δ*fauA* mutant BRM18 and the wild-type parent strain B013N were assayed for the ability to transport iron supplied as ^{55}Fe(III)-alcaligin complexes. Iron transport by iron-starved *Bordetella* cells was monitored as the accumulation of cell-associated ^{55}Fe (Fig. 3). Compared with wild-type B013N iron transport activity, Δ*fauA* mutant strain BRM18 carrying the pBBR1MCS control plasmid vector was grossly defective in ^{55}Fe uptake. Consistent with the results of
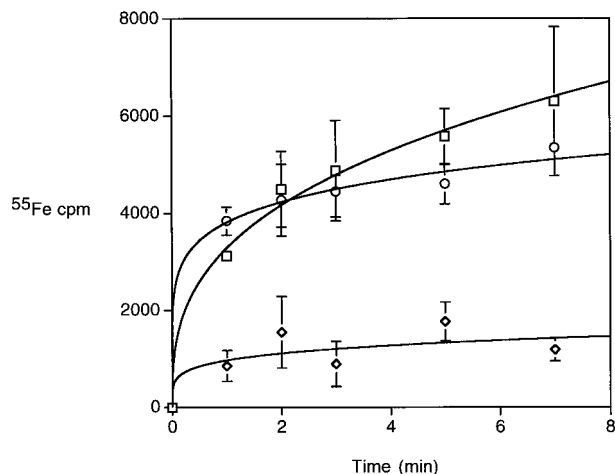
FIG. 3. *Bordetella* ^{55}Fe-ferric alcaligin transport assays. The rate of uptake of the ^{55}Fe label of ^{55}Fe-ferric alcaligin by iron-starved *B. bronchiseptica* wild-type strain B013N (squares), Δ*fauA* mutant BRM18 (diamonds), and Δ*fauA* mutant BRM18 carrying wild-type *fauA* plasmid pBB24 (circles), using an alcaligin concentration of 5 μM, together with 3.3 μM ^{55}Fe(III) is shown over a 7-min period of time. Mean ^{55}Fe counts per minute ± standard deviations (*n* = 3) are plotted as a function of time.
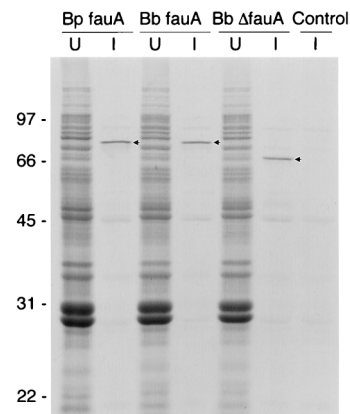

FIG. 4. Production of *Bordetella* FauA polypeptides, using a bacteriophage T7 RNA polymerase/promoter expression system in *E. coli*. Intrinsically labeled plasmid-encoded FauA proteins were resolved by SDS-PAGE and visualized by autoradiography. Whole-cell lysates of *E. coli* K38(pGP1-2) containing p3Z63 (*B. pertussis* 3.8-kb *Pst*I *fauA* fragment; Bp fauA), p3Z48 (*B. bronchiseptica* 3.8-kb *Pst*I *fauA* fragment; Bb fauA), p3Z69 (*B. bronchiseptica* 3.8-kb *Pst*I Δ*fauA* fragment deleted of the 255-bp internal *fauA* region; Bb Δ*fauA*), and pGEM3Z (plasmid vector control; Control) are shown. U, uninduced; I, induced for expression of T7 promoter-directed genes by temperature shift and in the presence of rifampin. Positions of marker proteins are given along with their apparent molecular masses in kilodaltons. The radiolabeled FauA products are indicated by arrows.

the growth stimulation bioassays, *B. bronchiseptica fauA* plasmid pBB24 complemented the BRM18 ferric alcaligin transport defect, restoring accumulation of cell-associated ^{55}Fe to wild-type levels. Although cell-associated ^{55}Fe levels for the complemented mutant are not significantly different from the wild-type level, mathematical curve fitting indicated that the initial ^{55}Fe uptake rate for the complemented mutant was greater than the wild-type rate, possibly reflecting a higher receptor density at the cell surface.

Bacteriophage T7 promoter-directed FauA protein production. Protein production directed by the 3.8-kb *Bordetella Pst*I *fauA* DNA region was examined by using a bacteriophage T7 RNA polymerase/promoter expression system in *E. coli* (Fig. 4). In the *fauA* sense orientation with respect to the plasmid vector T7 *gene 10* promoter, both *B. pertussis* and *B. bronchiseptica* 3.8-kb *Pst*I DNA regions directed the production of a single strongly radiolabeled polypeptide migrating at an apparent molecular mass of approximately 79 kDa. These products approximate the predicted size (ca. 77.7 kDa) of the mature FauA protein, based on amino acid sequence comparisons of FauA with processed forms of similar ferric siderophore receptor proteins. The cloned *B. bronchiseptica* Δ*fauA* region directed the production of a smaller polypeptide with an apparent molecular mass of approximately 69 kDa, consistent with the loss of an 85-amino-acid segment resulting from the Δ*fauA* in-frame deletion.

FauA protein production in *B. bronchiseptica*. In this and previous SDS-PAGE analyses (11, 13), a polypeptide with an apparent molecular mass of approximately 79 kDa was detected in whole-cell lysates and outer membrane-enriched fractions of B013N cultured under iron-depleted conditions. The 79-kDa iron-regulated outer membrane protein was not present at detectable levels in samples prepared from *B. bronchiseptica* Δ*fauA* mutant BRM18 but was very strongly produced under iron-depleted culture conditions by BRM18 carrying wild-type *fauA* plasmid pBB24 (Fig. 5). Production of this 79-kDa iron-regulated outer membrane protein correlates with the ability of *fauA* plasmid pBB24 to restore ferric alcaligin growth stimulation and ^{55}Fe-alcaligin uptake to mutant

BRM18. The protein has an apparent molecular mass consistent with predictions based on the *fauA* nucleotide sequence and appears indistinguishable from wild-type FauA proteins produced in T7 protein expression experiments in *E. coli*. Elevated levels of FauA detected in outer membrane preparations of BRM18 carrying wild-type *fauA* plasmid pBB24 correlate with enhanced ferric alcaligin growth stimulation observed in bioassays for *fauA* mutants BRM17 and BRM18 complemented with this plasmid.

Iron regulation and *alcR*-dependent expression of a *fauA-lacZ* transcriptional fusion gene. Fifteen mini-Tn*5 lacZ1* transposon insertions were mapped to *fauA* sequences in plasmid pBB24 by restriction endonuclease mapping. One insertion chosen for further analysis mapped near the center of *fauA*, approximately 900 bp downstream from the start of the *fauA* coding sequence. The orientation of this transposon insertion in the *fauA* sense placed *lacZ* reporter gene transcription under the control of any putative *fauA* regulatory sequences
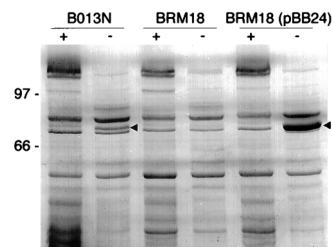

FIG. 5. SDS-PAGE analysis of iron-regulated FauA outer membrane protein production in *B. bronchiseptica*. FauA outer membrane protein production by wild-type *B. bronchiseptica* B013N, Δ*fauA* mutant BRM18, and Δ*fauA* mutant BRM18 carrying wild-type *fauA* plasmid pBB24 was analyzed by SDS-PAGE. Outer membrane proteins were visualized by staining gels with Coomassie blue dye. +, outer membrane proteins from iron-replete cells; −, outer membrane proteins from iron-depleted cells. Arrowheads indicate positions of the 79-kDa FauA protein in the wild-type B013N and BRM18(pBB24) outer membrane samples. Positions of marker proteins are given along with their apparent molecular masses in kilodaltons.
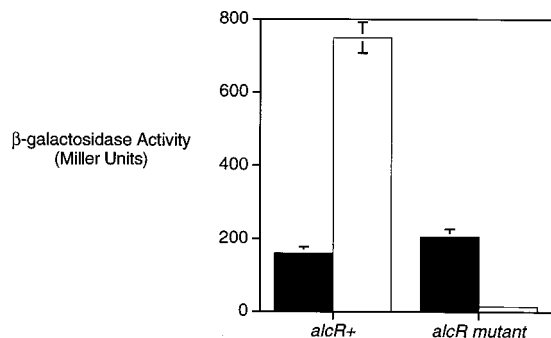
FIG. 6. Iron regulation and *alcR*-dependent expression of a *fauA-lacZ* transcriptional fusion gene. Transcriptional activity of a *fauA-lacZ* fusion gene carried as plasmid pBB24/mini-Tn5 *lacZ1* in wild-type *B. bronchiseptica* B013N (*alcR*+) or isogenic *alcR* mutant strain BRM11 (*alcR mutant*) was assessed by measuring β-galactosidase activity of bacteria cultured under iron-replete (■) and iron-depleted (□) conditions. Mean β-galactosidase activities ± standard deviations (*n* = 3) are shown in Miller units.

present on the 3.8-kb *Pst*I *fauA* insert DNA fragment of pBB24. The fusion plasmid, pBB24/mini-Tn5 *lacZ1*, was transferred to wild-type *B. bronchiseptica* strain B013N and isogenic *alcR* mutant strain BRM11 by conjugation, and *fauA* transcriptional activity was monitored as β-galactosidase reporter gene activity of cells cultured under iron-replete versus iron-depleted conditions in SS defined medium. In this plasmid context in wild-type *B. bronchiseptica* strain B013N, *fauA* transcriptional activity was elevated nearly fivefold by iron starvation (Fig. 6). Analysis of the *fauA-lacZ* fusion gene in *alcR* mutant strain BRM11 revealed that *fauA* transcription was dependent on the regulatory gene *alcR*; expression of the *fauA* fusion gene in the *alcR* mutant was drastically reduced. The apparent requirement of *alcR* for *fauA* expression is consistent with the previously observed loss of production of an iron-repressible 79-kDa membrane protein by *B. bronchiseptica alcR* mutants BRM10 and BRM11 (11). The *alcR* mutant BRM10, which is also defective in alcaligin production, was identified in a screening for ferric alcaligin transport mutants on the basis of a ferric alcaligin utilization-defective phenotype. These data indicate that *fauA* transcription is iron regulated and *alcR* dependent and that *cis*-acting transcriptional control elements are active within the pBB24 plasmid-borne *fauA* DNA region.

DISCUSSION

In our previous report of the purification and spectroscopic analysis of alcaligin produced by *B. pertussis* and *B. bronchiseptica*, evidence for biological activity of the purified alcaligin siderophore was obtained by using growth stimulation bioassays and quantitative ferric alcaligin transport assays with *Bordetella* indicator strains (16). Uptake rates and saturability of iron uptake observed in quantitative ferric alcaligin transport assays were consistent with the involvement of a high-affinity receptor-mediated ferric alcaligin transport system and provided the first direct evidence of iron delivery to *Bordetella* cells mediated by the native alcaligin siderophore. Despite the demonstration of rapid and saturable transport of iron supplied as ferric alcaligin, the specific outer membrane receptor for ferric alcaligin or other potential alcaligin transport proteins remained unidentified.

The first *Bordetella* ferric siderophore receptor identified was the *B. pertussis* BfeA receptor for the heterologous siderophore ferric enterobactin (8), found in a screen of *E. coli* to detect iron-repressible genes encoding exported proteins. BfeA was found to share a high degree of similarity with the ferric enterobactin receptors FepA of *E. coli* and PfeA of *P. aeruginosa*, and growth stimulation by ferric enterobactin was shown to be defective in *B. pertussis* and *B. bronchiseptica bfeA* mutants. Later, using the same *E. coli* screening approach, the putative ferric siderophore receptor gene *bfrA* was identified in *B. bronchiseptica* (9). The *bfrA* gene was repressible by Fur and iron and was predicted to encode an 80-kDa outer membrane protein with strong similarity with members of the TonB-dependent outer membrane receptor family, especially with Cir of *E. coli* and IrgA of *Vibrio cholerae*, and with three known ferric enterobactin receptors. In phenotypic analyses, *Bordetella bfrA* mutants were found to be unaffected in their ability to utilize ferric alcaligin, ferric enterobactin, and 2,3-dihydroxybenzoylserine. 2,3-Dihydroxybenzylserine utilization was shown to be dependent on the *Bordetella* ferric enterobactin receptor BfeA. Ferrichrome, desferrioxamine B, and hemin were shown to stimulate *Bordetella* growth, albeit by mechanisms functioning independently of both the BfeA and BfrA proteins in *B. bronchiseptica* and *B. pertussis*. The specificity of *bfrA* and its potential role in iron transport remain unknown. Two additional Fur-regulated ferric siderophore receptor homologs of unknown specificity, *bfrB* and *bfrC*, have since been identified in *B. bronchiseptica* (7). *B. bronchiseptica* and *B. pertussis bfrB* and *bfrC* mutants and a *B. bronchiseptica bfrB bfrC* double mutant had no phenotypic defects related to growth stimulation by any known *Bordetella* iron sources. Since phenotypic characterization of ferric siderophore receptor homolog mutants determined that BfrA, BfrB, and BfrC are not involved in the utilization of any known *Bordetella* iron sources, these data suggest that the iron-scavenging potential of *Bordetella* species may include additional, unidentified iron sources. With the exception of BfeA, it has proven difficult to predict the specificity of the putative *Bordetella* siderophore receptors based on nucleotide sequence information. Recent analysis of *B. pertussis* and *B. bronchiseptica tonB* mutants confirmed that as with other characterized gram-negative bacteria, TonB function is required for specific utilization of all known iron sources by these species (40).

In this study, the hypothesis that previously unknown *Bordetella* ferric alcaligin siderophore transport genes might be clustered near known alcaligin biosynthesis genes was tested by screening for *B. pertussis* genes encoding ferric alcaligin utilization functions in a heterologous host system. We identified a 3.8-kb *Pst*I genetic region mapping immediately downstream from the known alcaligin system gene *alcR* that conferred a strong positive ferric alcaligin growth stimulation phenotype to siderophore-deficient *P. aeruginosa* IA1, an indicator strain that is normally incapable of ferric alcaligin utilization. The ability of the *fauA* gene alone to confer a ferric alcaligin utilization phenotype to IA1 implies that IA1 is capable of supplying any additional transport and utilization activities required for growth stimulation by ferric alcaligin. Nucleotide sequencing of the region identified a putative ferric siderophore receptor gene, *fauA*, with deduced primary amino acid sequence similarity with known members of the TonB-dependent receptor family. The *fauA* DNA regions of *B. pertussis* UT25 and *B. bronchiseptica* B013N each directed the production of a 79-kDa protein in *E. coli*, approximating the predicted size of the mature FauA protein.

Functional Fur repressor-binding sequences were detected on the 3.8-kb *Pst*I DNA fragment by the Fur repressor titration assay in *E. coli*. Analysis of a plasmid-borne *fauA-lacZ* operon fusion in wild-type *B. bronchiseptica* provided evidence of iron regulation of *fauA* at the transcriptional level, mediated by

cis-acting control elements residing within the 3.8-kb *Pst*I *fauA* DNA region. By using an *alcR* mutant host strain, transcription of the *fauA-lacZ* fusion gene was also shown to be *alcR* dependent, an effect that is consistent with the previous observation of *alcR*-dependent production of an unidentified 79-kDa membrane protein, presumably FauA, in phenotypic analysis of *B. bronchiseptica alcR* mutants (11). A previous report also described a 79-kDa protein that was normally iron regulated but constitutively produced by a *B. bronchiseptica fur* mutant (13). Complete genetic characterization of *fauA* regulation will require further analysis.

Presumptive evidence for the requisite role of *fauA* in ferric alcaligin utilization was obtained by phenotypic analysis of *B. bronchiseptica fauA* insertion mutant BRM17. BRM17 was incapable of utilizing ferric alcaligin in growth stimulation bioassays, and in complementation analyses ferric alcaligin utilization was restored to BRM17 by the wild-type *fauA* gene of either *B. pertussis* or *B. bronchiseptica* supplied in *trans*. The ability of the *fauA* gene alone to complement the ferric alcaligin utilization defect of BRM17 when supplied in *trans* suggested that the BRM17 mutant phenotype was not due to polarity effects of the insertion mutation. However, to test the requirement for FauA function in ferric alcaligin transport and utilization in the absence of potential polarity effects of insertion mutations on any downstream genes, a nonpolar in-frame *fauA* deletion mutation was crossed into *B. bronchiseptica* B013N to produce Δ*fauA* mutant BRM18. BRM18 was defective in ferric alcaligin utilization in growth stimulation bioassays and in iron transport activity using quantitative ^{55}Fe-ferric alcaligin uptake assays. BRM18 also failed to produce detectable quantities of a 79-kDa iron-regulated outer membrane protein produced by the wild-type parent strain B013N. In complementation studies, BRM18 merodiploids carrying a wild-type *fauA* gene in *trans* regained ferric alcaligin transport and utilization capacity, and production of the 79-kDa iron-regulated outer membrane protein was restored.

Genetic and biochemical evidence presented in this report corroborates and extends our previous observations of production of a 79-kDa outer membrane protein regulated by Fur and AlcR (11, 13), the ferric alcaligin utilization defect associated with *alcR* mutants (11), and the rapid and saturable transport of ferric alcaligin siderophore complexes by *B. bronchiseptica* (16). The *fauA* gene encodes the iron-regulated outer membrane receptor protein required for ferric alcaligin transport and utilization. Further studies will be necessary to identify additional gene products involved in transport and utilization of ferric alcaligin by *Bordetella* species.

ACKNOWLEDGMENTS

REFERENCES

1. **Agiato, L.-A., and D. W. Dyer.** 1992. Siderophore production and membrane alterations by *Bordetella pertussis* in response to iron starvation. Infect. Immun. **60:**117–123.
2. **Akerley, B. J., P. A. Cotter, and J. F. Miller.** 1995. Ectopic expression of the flagellar regulon alters development of the *Bordetella*-host interaction. Cell **80:**611–620.
3. **Alexeyev, M. F.** 1995. Three kanamycin resistance gene cassettes with different polylinkers. BioTechniques **18:**52–56.
4. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
5. **Ankenbauer, R., S. Sriyosachati, and C. D. Cox.** 1985. Effects of siderophores on the growth of *Pseudomonas aeruginosa* in human serum and transferrin. Infect. Immun. **49:**132–140.
6. **Armstrong, S. K., and M. O. Clements.** 1993. Isolation and characterization of *Bordetella bronchiseptica* mutants deficient in siderophore activity. J. Bacteriol. **175:**1144–1152.
7. **Beall, B.** 1988. Two iron-regulated putative ferric siderophore receptor genes in *Bordetella bronchiseptica* and *Bordetella pertussis*. Res. Microbiol. **149:**189–201.
8. **Beall, B., and G. N. Sanden.** 1995. A *Bordetella pertussis fepA* homologue required for utilization of exogenous ferric enterobactin. Microbiology **141:**3193–3205.
9. **Beall, B., and T. Hoenes.** 1997. An iron-regulated outer-membrane protein specific to *Bordetella bronchiseptica* and homologous to ferric siderophore receptors. Microbiology **143:**135–145.
10. **Beaumont, F. C., and S. K. Armstrong.** 1995. A heterologous expression system for the genetic analysis of *Bordetella* siderophore synthesis, abstr. B-361, p. 228. *In* Abstracts of the 95th General Meeting of the American Society for Microbiology 1995. American Society for Microbiology, Washington, D.C.
11. **Beaumont, F. C., H. Y. Kang, T. J. Brickman, and S. K. Armstrong.** 1998. Identification and characterization of *alcR*, a gene encoding an AraC-like regulator of alcaligin siderophore biosynthesis and transport genes in *Bordetella pertussis* and *Bordetella bronchiseptica*. J. Bacteriol. **180:**862–870.
12. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
13. **Brickman, T. J., and S. K. Armstrong.** 1995. *Bordetella pertussis fur* gene restores iron-repressibility of siderophore and protein expression to deregulated *Bordetella bronchiseptica* mutants. J. Bacteriol. **177:**268–270.
14. **Brickman, T. J., and S. K. Armstrong.** 1996. The ornithine decarboxylase gene *odc* is required for alcaligin siderophore biosynthesis in *Bordetella* spp.: putrescine is a precursor of alcaligin. J. Bacteriol. **178:**54–60.
15. **Brickman, T. J., B. A. Ozenberger, and M. A. McIntosh.** 1990. Regulation of divergent transcription from the iron-responsive *fepB-entC* promoter-operator regions in *Escherichia coli*. J. Mol. Biol. **212:**669–682.
16. **Brickman, T. J., J.-G. Hansel, M. J. Miller, and S. K. Armstrong.** 1996. Purification, spectroscopic analysis, and biological activity of the macrocyclic dihydroxamate siderophore alcaligin produced by *Bordetella pertussis* and *Bordetella bronchiseptica*. BioMetals **9:**191–203.
17. **Calderwood, S. B., and J. J. Mekalanos.** 1987. Iron regulation of Shiga-like toxin expression in *Escherichia coli* is mediated by the *fur* locus. J. Bacteriol. **169:**4759–4764.
18. **Crosa, J. H.** 1997. Signal transduction and transcriptional and posttranscriptional control of iron-regulated genes in bacteria. Microbiol. Mol. Biol. Rev. **61:**319–336.
19. **de Lorenzo, V., A. Bindereif, B. H. Paw, and J. B. Neilands.** 1986. Aerobactin biosynthesis and transport genes of plasmid ColV-K30 in *Escherichia coli* K-12. J. Bacteriol. **165:**570–578.
20. **de Lorenzo, V., M. Herrero, U. Jakubzik, and K. N. Timmis.** 1990. Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J. Bacteriol. **172:**6568–6572.
21. **de Lorenzo, V., S. Wee, M. Herrero, and J. B. Neilands.** 1987. Operator sequences of the aerobactin operon of plasmid ColV-K30 binding the ferric uptake regulation (*fur*) repressor. J. Bacteriol. **169:**2624–2630.
22. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
23. **Fetherston, J. D., S. W. Bearden, and R. D. Perry.** 1996. YbtA, an AraC-type regulator of the *Yersinia pestis* pesticin/yersiniabactin receptor. Mol. Microbiol. **22:**315–325.
24. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. USA **76:**1648–1652.
25. **Giardina, P. C., L.-A. Foster, S. I. Toth, B. A. Roe, and D. W. Dyer.** 1995. Identification of *alcA*, a *Bordetella bronchiseptica* gene necessary for alcaligin production. Gene **167:**133–136.
26. **Gorringe, A. R., G. Woods, and A. Robinson.** 1990. Growth and siderophore production by *Bordetella pertussis* under iron-restricted conditions. FEMS Microbiol. Lett. **66:**101–106.
27. **Heinrichs, D. E., and K. Poole.** 1993. Cloning and sequence analysis of a gene (*pchR*) encoding an AraC family activator of pyochelin and ferripyochelin receptor synthesis in *Pseudomonas aeruginosa*. J. Bacteriol. **175:**5882–5889.
28. **Joyce, C. M., and N. D. F. Grindley.** 1984. Method for determining whether a gene of *Escherichia coli* is essential: application to the *polA* gene. J. Bacteriol. **158:**636–643.
29. **Kang, H. Y., and S. K. Armstrong.** 1998. Transcriptional analysis of the *Bordetella* alcaligin siderophore biosynthesis operon. J. Bacteriol. **180:**855–861.

30. **Kang, H. Y., F. C. Beaumont, T. J. Brickman, and S. K. Armstrong.** 1996. Identification and characterization of iron-regulated *Bordetella pertussis* alcaligin siderophore biosynthesis genes. J. Bacteriol. **178:**4877–4883.

31. **Kovach, M. E., R. W. Phillips, P. H. Elzer, R. M. Roop II, and K. M. Peterson.** 1994. pBBR1MCS: a broad-host-range cloning vector. BioTechniques **16:**800–802.

32. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82:**488–492.

33. **Langman, L., I. G. Young, G. E. Frost, H. Rosenberg, and F. Gibson.** 1972. Enterochelin system of iron transport in *Escherichia coli*: mutations affecting ferric-enterochelin esterase. J. Bacteriol. **112:**1142–1149.

34. **Lankford, C. E.** 1973. Bacterial assimilation of iron. Crit. Rev. Microbiol. **2:**273–331.

35. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

36. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170:**2575–2583.

37. **Moeck, G. S., and J. W. Coulton.** 1998. TonB-dependent iron acquisition: mechanisms of siderophore-mediated active transport. Mol. Microbiol. **28:** 675–681.

38. **Moore, C. H., L.-A. Foster, D. G. Gerbig, D. W. Dyer, and B. W. Gibson.** 1995. Identification of alcaligin as the siderophore produced by *Bordetella pertussis* and *Bordetella bronchiseptica*. J. Bacteriol. **177:**1116–1118.

39. **Neilands, J. B.** 1981. Microbial iron compounds. Annu. Rev. Biochem. **50:** 715–731.

40. **Nicholson, M. L., and B. Beall.** 1999. Disruption of *tonB* in *Bordetella bronchiseptica* and *Bordetella pertussis* prevents utilization of ferric siderophores, haemin and haemoglobin as iron sources. Microbiology **145:**2453–2462.

41. **Norrander, J., T. Kempe, and J. Messing.** 1983. Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene **26:**101–106.

42. **Paulsen, I. T., M. H. Brown, and R. A. Skurray.** 1996. Proton-dependent multidrug efflux systems. Microbiol. Rev. **60:**575–608.

43. **Pradel, E., N. Guiso, and C. Locht.** 1998. Identification of AlcR, an AraC-type regulator of alcaligin siderophore synthesis in *Bordetella bronchiseptica* and *Bordetella pertussis*. J. Bacteriol. **180:**871–880.

44. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

45. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.

46. **Schneider, D. R., and C. D. Parker.** 1982. Effect of pyridines on phenotypic properties of *Bordetella pertussis*. Infect. Immun. **38:**548–553.

47. **Schwyn, B., and J. B. Neilands.** 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. **160:**47–56.

48. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. Bio/Technology **1:**784–791.

49. **Stainer, D. W., and M. J. Scholte.** 1970. A simple chemically defined medium for the production of phase I *Bordetella pertussis*. J. Gen. Microbiol. **63:**211–220.

50. **Stojiljkovic, I., A. J. Baumler, and K. Hantke.** 1994. Fur regulon in gram-negative bacteria. Identification and characterization of new iron-regulated *Escherichia coli* genes by a Fur titration assay. J. Mol. Biol. **236:**531–545.

51. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82:**1074–1078.