JOURNAL OF BACTERIOLOGY, Feb. 1998, p. 871–880 Vol. 180, No. 4
0021-9193/98/$04.00+0
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Identification of AlcR, an AraC-Type Regulator of Alcaligin Siderophore Synthesis in *Bordetella bronchiseptica* and *Bordetella pertussis*

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Received 5 September 1997/Accepted 6 December 1997

A Fur titration assay was used to isolate DNA fragments bearing putative Fur binding sites (FBS) from a partial *Bordetella bronchiseptica* genomic DNA library. A recombinant plasmid bearing a 3.5-kb DNA insert was further studied. Successive deletions in the cloned fragment enabled us to map a putative FBS at about 2 kb from one end. Sequence analysis revealed the presence of an FBS upstream from a new gene encoding an AraC-type transcriptional regulator. The deduced protein displays similarity to PchR, an activator of pyochelin siderophore and ferripyochelin receptor synthesis in *Pseudomonas aeruginosa*. Homologous genes in *Bordetella pertussis* and *Bordetella parapertussis* were PCR amplified, and sequence comparisons indicated a very high conservation in the three species. The *B. pertussis* and *B. bronchiseptica* chromosomal genes were inactivated by allelic exchange. Under low-iron growth conditions, the mutants did not secrete the alcaligin siderophore and lacked AlcC, an alcaligin biosynthetic enzyme. Alcaligin production was restored after transformation with a plasmid bearing the wild-type gene. On the basis of its role in regulation of alcaligin biosynthesis, the new gene was designated *alcR*. Additional sequence determination showed that *alcR* is located about 2 kb downstream from the *alcABC* operon and is transcribed in the same orientation. Two tightly linked open reading frames, *alcD* and *alcE*, were identified between *alcC* and *alcR*. AlcE is a putative iron-sulfur protein; AlcD shows no homology with the proteins in the database. The production of major virulence factors and colonization in the mouse respiratory infection model are AlcR independent.

To succeed in colonization of the host and subsequently cause disease, bacterial pathogens must first adhere to target tissues and concomitantly obtain nutrients which are essential for their growth. Iron is usually one such essential nutrient, and the ability of a pathogen to scavenge iron is an important virulence trait (55). In animals, the iron is not freely available to microorganisms, as it is bound to proteins such as transferrin (TF) and lactoferrin (LF) in the serum and other secretory fluids. Therefore, in order to survive, bacteria have evolved various iron uptake mechanisms. Some species, e.g., *Escherichia coli* and *Pseudomonas* spp., secrete low-molecular-weight siderophores which display a high affinity for ferric ions (36). These molecules can remove Fe(III) from TF or LF, and iron-loaded siderophores can bind to specific receptors on the bacterial surface to finally deliver the iron into the cell. Other bacteria, e.g., *Neisseria* spp. and *Haemophilus influenzae*, do not synthesize siderophores but produce receptors for the TF- and LF-iron complexes allowing iron uptake through direct contact between these host iron-binding proteins and the bacterial cell surface (9, 23, 46, 47). *Bordetella pertussis*, the etiologic agent of whooping cough in humans, and *Bordetella bronchiseptica*, the causative agent of swine atrophic rhinitis and kennel cough, may possess both iron uptake systems, since they synthesize alcaligin, a hydroxamate-type siderophore (1, 21, 34), as well as an outer membrane LF-binding protein (31, 43).

Iron uptake systems are usually expressed only under iron-limited growth conditions. In several species, this regulation mechanism involves the Fur (ferric ion uptake regulation) protein (24). In iron-rich growth conditions, the Fur repressor chelates Fe(II), binds to operator sequences in the promoter region of its target genes, and blocks transcription. These operators are called Fur-binding sites (FBS) or iron boxes. Under low-iron conditions, Fur is unable to bind to the FBS (14). Fur and iron may also modulate the expression of genes encoding virulence factors unrelated to iron metabolism, such as exotoxin A in *Pseudomonas aeruginosa* (40, 41), Shiga-like toxin in *E. coli* (13), or pH-regulated proteins in *Salmonella typhimurium* (17). Thus, the iron status of the environment appears to be used as a signal to trigger the expression of virulence genes in many pathogens.

Little is known about iron regulation in the bordetellae. The *fur* genes of *B. pertussis*, *B. bronchiseptica*, and *Bordetella parapertussis* have been cloned and sequenced recently (4, 12, 39). Several iron-repressed or iron-induced proteins have been detected (1, 3, 31), but only a few Fur target genes have been identified so far. Among them is the *alcABC* operon, coding for the first three enzymes of the alcaligin siderophore biosynthesis pathway (20, 28). Other cloned Fur-repressed genes encode outer membrane proteins BfeA, BfrB, and BfrC, receptors for ferric enterobactin and other hydroxamate siderophores in *B. pertussis* and *B. bronchiseptica* (5, 7), and BfrA, an unidentified exogenous siderophore receptor specific to *B. bronchiseptica* (6). The alcaligin receptor and its structural gene have not been characterized yet. To further elucidate the iron regulatory network in bordetellae and to study its involvement in virulence expression, we used the Fur titration assay (FURTA) of Stojiljkovic et al. to isolate Fur target genes (54). The same genetic approach has led to the recent identification of the *B. pertussis sodA* gene, encoding an Mn-containing superoxide dismutase (22). We present here the cloning and sequencing of a new Fur-repressed gene, *alcR*, encoding an

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant features[a]	Source or reference
Strains		
E. coli		
H1717	*aroB fhuF*::λ*plac*Mu; Ap^r	54
XL1-Blue	High-efficiency transformation; Tc^r	Stratagene
SM10	Mobilizing strain; Km^r	50
S17.1	Mobilizing strain; Sm^r	50
B. avium 103004	Sm^r	Institut Pasteur, Paris, France
B. bronchiseptica		
BB1015	Sm^r derivative of NL1015 but not *rpsL*	This study
BBEP205	Derivative of BB1015 but *alcR*::Km^r	This study
B. parapertussis PEP		37
B. pertussis		
BPSM	Tohama I *rpsL*; Sm^r Nal^r	32
BPEP184	Derivative of BPSM but *alcR*::Km^r	This study
BP953	Tohama I *rpsL fhaB*::*lacZ ptx*::*phoA*; Sm^r Nal^r	53
BPEP214	Derivative of BP953 but *alcR*::Km^r	This study
Plasmids		
pBBR1MCS	Broad-host-range vector; Cm^r	29
pBCSK$^+$	High-copy-number cloning vector; Cm^r	Stratagene
pEP279	pBCSK$^+$ containing a 3.5-kb *Pst*I fragment carrying the *B. bronchiseptica alcR* gene; Cm^r	This study
pEP300	Derivative of pEP279 with a Km^r cassette inserted in the *alcR* gene; Cm^r Km^r	This study
pEP301	pBBR1MCS carrying a 1.6-kb *Kpn*I-*Pst*I fragment derived from pEP279; Cm^r	This study
pEP308	Derivative of pJQ200KS$^+$ carrying a Km^r cassette flanked by *alcR*-derived sequences; Gn^r Km^r	This study
pEP319	Derivative of pSS1129 carrying a Km^r cassette flanked by *alcR*-derived sequences; Gn^r Ap^r Km^r	This study
pJQ200KS$^+$	*Bordetella* suicide vector; Gn^r	42
pSS1129	*Bordetella* suicide vector; Gn^r Ap^r	52
pUC4K	Source of Km^r cassette	Pharmacia

[a] Ap^r, Cm^r, Gn^r, Km^r, Nal^r, Sm^r, and Tc^r, resistance to ampicillin, chloramphenicol, gentamicin, kanamycin, nalidixic acid, streptomycin, and tetracycline, respectively.

AraC-type transcriptional regulator in *B. bronchiseptica*, *B. pertussis*, and *B. parapertussis*. This gene is located 2 kb downstream from the alcaligin biosynthesis operon. Sequence analysis of the *alcABC-alcR* intergenic region suggests that the *alc* operon may contain two additional open reading frames (ORFs). Construction and characterization of *B. pertussis* and *B. bronchiseptica alcR* mutants showed that AlcR is necessary for expression of the *alcABC* operon and thus required for alcaligin production but that it is not involved in the expression of the major *B. pertussis* virulence factors, filamentous hemagglutinin (FHA), pertussis toxin (PTX), pertactin (PRN), and adenylate cyclase hemolysin (AC-Hly). In vivo studies revealed that AlcR is not required for colonization in the mouse respiratory infection model.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this work are listed in Table 1. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) medium (33) or on solid media obtained by addition of Bacto-Agar (1.5% [wt/vol]; Difco). *Bordetella* strains were grown at 37°C on Bordet-Gengou (BG) (10) agar base plates (Difco) supplemented with 1% glycerol and 15% sheep blood. Liquid cultures were grown in Stainer-Scholte (SS) medium (51) containing 10 μg of $FeSO_4 \cdot 7H_2O$ per ml (iron-rich SS medium) or in SS medium without addition of $FeSO_4 \cdot 7H_2O$ (iron-limited SS medium). *Bordetella avium* was grown in SS medium supplemented with 2 mg of 2-ketoglutarate per ml, 2 mg of pyruvate per ml, 10 μg of pantothenate per ml, 20 μg of L-phenylalanine per ml, and 0.5 mg of nicotinamide per ml. When necessary, antibiotics were included in the growth media at the following concentrations (in micrograms per milliliter): ampicillin, 150; chloramphenicol, 30; gentamicin, 10; kanamycin, 30; nalidixic acid, 30; streptomycin, 100; and tetracycline, 20.

DNA techniques. Plasmid DNA was routinely isolated by the alkaline lysis method (45) or purified by using a Nucleobond AX kit (Macherey-Nagel, Hoerdt, France) for sequencing. Restriction endonucleases and T4 DNA ligase were obtained from Boehringer Mannheim and used according to standard procedures (45). The *Pst*I DNA fragment inserted into pEP279 and appropriate subclones was sequenced by using a T7 polymerase kit from Pharmacia, α-^{35}S-dCTP, and a combination of universal, reverse, and custom-synthesized primers (Pharmacia). The sequence was later confirmed by using an ABI PRISM Dye Terminator Cycle Sequencing kit and an ABI PRISM 377 sequencer (Perkin-Elmer). The *B. pertussis* and *B. parapertussis alcR* chromosomal locus was amplified by PCR with Vent DNA polymerase (New England Biolabs Inc., Beverly, Mass.) and oligonucleotides B10 (5′-GACGATGAAATCGGTGAGCGC-3′) and B3′ (5′-GCGCCGAAGGCTGGCAGGTAG-3′), which start at positions 1826 and 3306 (complementary strand) in the deposited *B. bronchiseptica* sequence, respectively. PCR products were cloned into the *Eco*RV site of pBCSK$^+$. For each *Bordetella* species, sequence analysis was carried out on two independently isolated recombinant plasmids bearing inserts in the opposite orientation.

FURTA. The FURTA was essentially performed as described by Stojiljkovic et al. (54). A partial *B. bronchiseptica* BB1015 genomic library had been constructed previously to isolate the *fur* gene in this organism (38). This strain is an Sm^r derivative of NL1015 (31). Chromosomal *Pst*I DNA fragments with sizes ranging from 2 to 4 kb had been cloned into the high-copy-number plasmid pBCSK$^+$ (Stratagene, San Diego, Calif.). *E. coli* H1717 carrying the chromosomal Fur-repressible *fhuF*::*lacZ* fusion was transformed with this partial library, and Cm^r transformants were screened for the Lac$^+$ phenotype on MacConkey lactose agar plates (Difco) containing 50 μM $FeCl_3$. Four red colonies (Lac$^+$) were isolated. Restriction mapping of the four recombinant plasmids (pEP276 to pEP279) showed that each one contained a distinct DNA insert. The recombinant plasmid conferring the strongest Lac$^+$ phenotype in the assay, pEP279, was further studied.

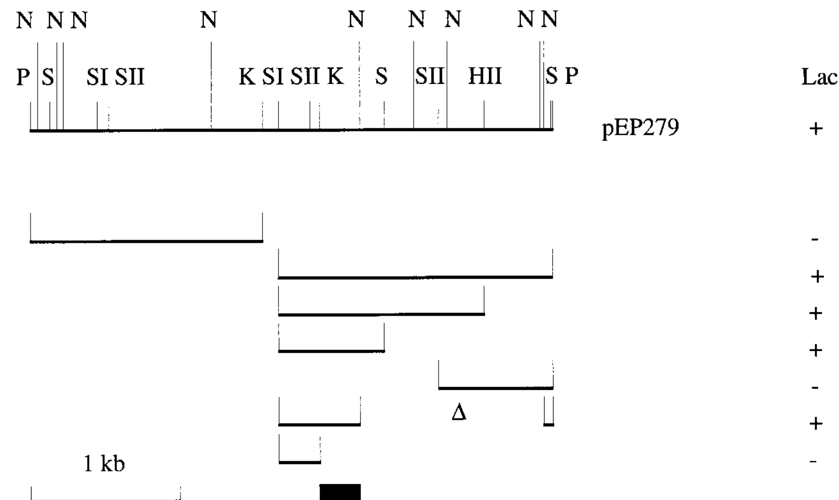

FIG. 1. Mapping of the region of pEP279 conferring a Lac$^+$ phenotype in the FURTA. Subclones of the 3.5-kb *Pst*I insert were constructed by deletions in pEP279 using the following restriction enzymes: HII, *Hin*cII; K, *Kpn*I; N, *Nar*I; P, *Pst*I; S, *Sma*I; SI, *Sac*I; and SII, *Sac*II. The recombinant plasmid-associated Lac phenotypes are indicated on the right. The deduced localization of the FBS is also shown (black box).

Computer analysis of sequences. The nucleotide and protein sequences were analyzed by using DNA Strider 1.2 software (Service de Biochimie et de Génétique Moléculaire du CEA, Saclay, France). Sequence homology was identified with the help of the BLASTN and BLASTP programs (2).

Construction of *alcR* mutants. A *Hin*cII DNA restriction fragment of 1.3 kb containing the Km^r cassette from pUC4K (Pharmacia) was inserted into the unique *Nru*I site of pEP279. The resulting plasmid, pEP300, was digested with *Bam*HI and *Sal*I, and the 4.8-kb DNA fragment bearing *alcR*::Km^r was subcloned into Gn^r suicide vector pJQ200KS$^+$ (42). The obtained plasmid, pEP308, was transformed into *E. coli* S17.1 (50) and then transferred to *B. bronchiseptica* BB1015 by conjugation as previously described (52). Exconjugants bearing the pEP308 insertion in the chromosome were selected on BG agar-streptomycin-gentamicin plates. The *Bacillus subtilis sacB* gene present on pJQ200KS$^+$ had been shown to confer sucrose sensitivity to several gram-negative bacteria (42). We tried to select for double recombinants by plating exconjugants on plates of BG agar plus kanamycin or LB plus kanamycin supplemented with 5 or 10% sucrose, but all exconjugants were sucrose resistant. This suggests that, in contrast to the case for *E. coli*, the *sacB* gene was not expressed from its own promoter in *B. bronchiseptica*. To isolate *alcR* mutants without the help of counterselection, one exconjugant was grown to stationary phase in iron-rich SS medium plus streptomycin, and culture dilutions were plated onto BG agar plus streptomycin plus kanamycin. Of 333 clones tested, 15 spontaneous Km^r Sm^r Gn^s mutants were isolated. One of the *alcR*::Km^r mutants was designated BBEP205.

For the allelic exchange in *B. pertussis*, we constructed pEP319, a derivative of suicide vector pSS1129 (52). For this purpose, pEP300 was digested with *Eco*RI, and the resulting 3.4-kb *Eco*RI DNA fragment carrying *alcR*::Km^r was cloned into pSS1129 to give pEP319. *E. coli* SM10 (50) was transformed with pEP319 and used as a donor in conjugation with *B. pertussis* BPSM. Exconjugants were selected on BG agar-nalidixic acid-kanamycin plates. One such exconjugant was grown to stationary phase, and culture dilutions were plated onto BG agar plus kanamycin. Of 116 isolated colonies tested, only one, designated BPEP184, showed a Sm^r Km^r Gn^s double-recombination phenotype. Correct allelic exchange in BBEP205 and BPEP184 was confirmed by Southern blot hybridization with the *Kpn*I-*Pst*I fragment of pEP279 carrying *alcR* (data not shown). For complementation studies, replicative plasmids pBBR1MCS and pEP301 were conjugated into *alcR* mutants, with SM10 used as a donor.

BPEP214 was obtained by conjugating BP953 (53) and SM10(pEP319) and selecting for the *alcR*::Km^r allelic exchange as described above.

CAS assay. The chrome azurol S (CAS) assay (48) was used to assess alcaligin siderophore production by *Bordetella* cells as described previously (3). Briefly, cells were grown to stationary phase in iron-limited SS medium. A 0.5-ml volume of culture supernatant was added to 0.5 ml of CAS solution, and the A_{630} of the CAS dye was measured after incubation for 4 h at room temperature.

Cell fractionation and protein analysis. Cells from 20-ml *B. bronchiseptica* or *B. pertussis* cultures were resuspended in 4 ml of HEPES (50 mM; pH 7.4) and disrupted with a French pressure cell (SLM-Aminco, Rochester, N.Y.). The pressates were centrifuged at 2,065 × *g* for 5 min at 4°C to sediment cellular debris and unbroken cells. Whole-cell lysates (WCLs) were saved or centrifuged at 111,000 × *g* for 1 h at 4°C to separate soluble and insoluble cell fractions.

Proteins (30 μg loaded per lane) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 5% stacking gel and a 12% separating gel (45). Following electrophoresis, proteins were visualized by Coomassie blue staining.

β-gal and AP assays. β-Galactosidase (β-gal) and alkaline phosphatase (AP) specific activities generated by *fhaB*::*lacZ* and *ptx*::*phoA* fusions in strains BP953 and BPEP214 were measured on WCLs as previously described (11, 33), except that samples were incubated at 37 instead of 28°C to facilitate detection of the low-level AP activity in these strains.

Colonization assay in the murine model. BPSM or BPEP184 cells were grown for 24 h on BG agar, and then 3- to 4-week-old mice were intranasally challenged with 5 × 10^6 cells from one of the strains. Infected mice were sacrificed by cervical dislocation 1 h after exposure and at 5, 8, 12, and 19 days thereafter (four mice per time point). The lungs were removed and homogenized in saline with tissue grinders. Enumeration of bacteria was performed on BG agar. To assess the stability of the *alcR* mutation, bacteria reisolated from the lungs of BPEP184-infected mice were tested for their resistance to kanamycin and absence of siderophore production. Both phenotypes had been retained.

Nucleotide sequence accession number. The nucleotide sequence of the *B. bronchiseptica* 3,526-bp *Pst*I DNA fragment in pEP279 has been assigned EMBL accession no. AJ000061.

RESULTS

Isolation of a Fur-repressed gene. We used the FURTA system (54) to isolate potential Fur-binding fragments in a partial *B. bronchiseptica* genomic DNA library that we had previously constructed (38). *E. coli* H1717 bearing the Fur-repressible *fhuF*::*lacZ* fusion was transformed with the pool of recombinant plasmids, and four red colonies (Lac$^+$ phenotype) were isolated on iron-rich MacConkey agar plates, suggesting that the cloned *B. bronchiseptica* sequences were interfering with Fur repression of the chromosomal *lac* fusion. One such plasmid, pEP279, was studied further since it gave the strongest Lac$^+$ phenotype in the assay. Restriction mapping showed that pEP279 contained a 3.5-kb *Pst*I DNA fragment. Successive deletions in the insert enabled the region conferring the Lac$^+$ phenotype to be localized to a 0.6-kb *Sac*I-*Nar*I fragment (Fig. 1). The *Sac*I-*Kpn*I portion derived from this fragment did not confer a Lac$^+$ phenotype to H1717; thus, the potential FBS was mapped to the other 0.3-kb half, between the *Kpn*I and *Nar*I sites, as shown in Fig. 1.

In order to characterize the putative Fur-repressed gene in pEP279, we determined the nucleotide sequence of the 1.5-kb *Kpn*I-*Pst*I fragment bearing the potential FBS (Fig. 2). A sequence homologous to the *E. coli* Fur-binding consensus GAT AATGATAATCATTATC (13 of 19 matches) was identified

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   1  ggtacctgagcaacatgggagagcagcttggtgacagcgccgcgccgcatgtcgcatcga
       KpnI                                          FBS
  61  cccaacggcccatgccatttcgctcatgaaataaaagcgaatgaattgcattatcattaa
 121  cagctcATGCGGACCAGGCCTCCTCCGGCAACGCCAATGACACAACCGACGCTCCCCCCA
            M  R  T  R  P  P  P  A  T  P  M  T  Q  P  T  L  P  P
 181  CCGCCCCGCACCCGATTCACGGTCTCGGACTTCAACCGCATGGGCGGGTCGGTCGGCTTC
      P  P  R  T  R  F  T  V  S  D  F  N  R  M  G  G  S  V  G  F
                                         NarI
 241  AATTACCGCTTGCCCGGCCAGGAAGGCGGGGGCGCCGGCATTGAGAACCTTTGCATCGCC
      N  Y  R  L  P  G  Q  E  G  G  G  A  G  I  E  N  L  C  I  A
 301  GAGGGCCGTGTGGAGGAATGTGAGATCCGCCCCGGCATCACCCTGATCGTCTCCGACGTG
      E  G  R  V  E  E  C  E  I  R  P  G  I  T  L  I  V  S  D  V
 361  CATGTGTATCATCATTACGAATCGACGTCCGTCATGACGCCGCGCTTTTCAGCCATCGTT
      H  V  Y  H  H  Y  E  S  T  S  V  M  T  P  R  F  S  A  I  V
 421  ATGCTCCAGGGCCAGGCCCGGGCTCGGCTGGACAAGCAGGACGACGTGCGCCTGGCCGCT
      M  L  Q  G  Q  A  R  A  R  L  D  K  Q  D  D  V  R  L  A  A
                                                   ⇩↓
 481  CAGAGCGGCCTCAATGCGCTATATGGCGATACCGTCGCCATGACGAGCGTTCATCCGGCC
      Q  S  G  L  N  A  L  Y  G  D  T  V  A  M  T  S  V  H  P  A
 541  GGCCAGCGTCTGCGCAGCGTCAATCTCTCCGTGACGGCCCCTGAAGCGGCCGACGATGAG
      G  Q  R  L  R  S  V  N  L  S  V  T  A  P  E  A  A  D  D  E
 601  TACACCAGCGAAATCATATGGAAACTCATGCAGTCCTCGGCGCCCAGGCTGCGCCGCTGG
      Y  T  S  E  I  I  W  K  L  M  Q  S  S  A  P  R  L  R  R  W
 661  CCGGTGCCGCACCATTTGCTGCTATCCATTGAGCATCTGCTGGAATGCGACTGGGATCAA
      P  V  P  H  H  L  L  L  S  I  E  H  L  L  E  C  D  W  D  Q
                                ↓
 721  CCTCTACGCAATATGGTGCGTGAAGGCGTGGGCACGCAACTGCTGGCCCACGCCTTGGCT
      P  L  R  N  M  V  R  E  G  V  G  T  Q  L  L  A  H  A  L  A
 781  GCGCTGCAACACGCCCCTGTGACCCACCGCGGCCTGACCCAGCGGGATCGCCAGCTTCTG
      A  L  Q  H  A  P  V  T  H  R  G  L  T  Q  R  D  R  Q  L  L
 841  GAGAGGGTGCGCGAGCGCCTGCACGAGGCGCCCGGCGAAGACCATACTCTGGATGACCTG
      E  R  V  R  E  R  L  H  E  A  P  G  E  D  H  T  L  D  D  L
 901  GCGCGACTGGCCTGCATGAGTCCAAGCACGCTGCGTGCAAAGTTCCATGCCGTGTATCAC
      A  R  L  A  C  M  S  P  S  T  L  R  A  K  F  H  A  V  Y  H
 961  CGTTCGGTATTCAGCTGGCTGCGCGAACGCCGCCTGGAGGTTGCCCGCGAACAATTGGCT
      R  S  V  F  S  W  L  R  E  R  R  L  E  V  A  R  E  Q  L  A
1021  CGGGGTTGGAGCGTACAGCAGGCGGCGCACTTCGTCGGCTACCGCCACGCGACCAATTTC
      R  G  W  S  V  Q  Q  A  A  H  F  V  G  Y  R  H  A  T  N  F
                  NruI
1081  GCCACGGCGTTTCGCGAACGCTACGGCGTCGCGCCCAGTCAACTCGGTtagcggctggtg
      A  T  A  F  R  E  R  Y  G  V  A  P  S  Q  L  G  *  ⇩
1141  ccttgcggcggcaggcattgacattgcggcattgaccatcccattcctgttgccagctgg
1201  atcatcatggaaggccgctgcccgctccgggcggccttgtccatggagccgccatgagcc
1261  gcgccgcgtcggccccctcatattcgctcatgatggtaatgctggggctgctgtcctgcg
1321  tcgcacccgccagcatcgacgcctacctgccagccttcggcgcgctgctacgcgagttcg
1381  gcgtaccccaggattccgagcaactcacgctcggcgtatacatgttctgctatgcggcga
1441  tgctcttgctgcacggaacactgtctgactctctgggcaggcgtcgcgttgtgctgggcg
1501  ccctgggcttctatgtttggggcgccctgcttgccacggccgcacccggttttggctggc
1561  tgctggcggcccgggccctgcag
                         PstI
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FIG. 2. Nucleotide sequence of *alcR* of *B. bronchiseptica* and deduced amino acid sequence of AlcR. Selected restriction enzyme sites, the putative FBS, and possible initiation codons are indicated (underlined). Differences detected in the *B. pertussis* and *B. parapertussis* sequences are also shown (underlined and labelled ⇩ and ↓, respectively).

between the *Kpn*I and *Nar*I restriction sites, in agreement with the FURTA system genetic data (Fig. 2). Sequence analysis revealed an ORF starting 120 bp downstream from the *Kpn*I site and stretching approximately 1 kb towards the *Pst*I site. In Fur-repressed promoters, FBS usually overlap the AT-rich −10 promoter region. A close examination of the proposed FBS sequence GCGAATGAATTGCATTATC revealed several putative −10 boxes; among them, the TATCAT sequence

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AlcR  EYTSEIIWKLMQSSAPRLRRWPVPHHLLLSIEHLLECDWDQPLRNMVREGVGTQLLAHAL
          ++I +      PR+   P P  +      +  C    P R++   G   +L A +
PchR  WLPEQLIRR--PGGDPRIMSCPAPRAMQALASQIATCQMLGPTRDLYLGGKALELAALSA

                                               Helix-Turn-Helix
AlcR  AAL--QHAPVTHRGLTQRDRQLLERVRERLHEAPGEDHTLDDLARLACMSPSTLRAKFHA
        L  +  PV    +T  + + +   R+ L  A  E  +LD LA    M+P  L A F
PchR  QFLSGEGRPVEEPRITCSEVERIHAARDLLVGALQEPPSLDTLASRVGMNPRKLTAGFRK

                                   AraC signature
AlcR  VYHRSVFSWLRERRLEVAREQLA-RGWSVQQAAHFVGYRHATNFATAFRERYGVAPSQLG
      V+  SVF +L+E RL  A   L     +V   A+ VGY  A +F+ AFR+RYG++PS+
PchR  VFGASVFGYLQEYRLREAHRMLCDEEANVSTVAYRVGYSPA-HFSIAFRKRYGISPSEIR
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FIG. 3. Alignment of the deduced C-terminal amino acid sequences of AlcR of *B. bronchiseptica* and PchR of *P. aeruginosa*. The final 180 residues of each protein are shown. The putative helix-turn-helix and AraC signature motifs (underlined), exact matches, and conserved changes (+) are indicated.

was the closest to the *E. coli* TATAAT −10 consensus (25). Twenty nucleotides upstream from this hexamer lies the sequence ATGAAA, sharing four nucleotides with the *E. coli* −35 consensus TTGACA. These determinants may constitute the promoter region. Six in-frame codons (three ATGs and three GTGs [Fig. 2]) could initiate translation, but four of them are not preceded by a sequence close to the canonical AAGGAGG *E. coli* ribosome binding site (RBS). Thus, translation most probably initiates either at the third ATG, located 10 bp downstream from a putative GGA RBS, or further down at the first GTG, situated 5 bp downstream from another GAGG potential RBS. However, the use of other triplets as start codons cannot be ruled out. The calculated molecular mass of the predicted translation product would then be 34 or 31 kDa, respectively.

Scanning of the Swiss Protein Data Base revealed that the C-terminal amino acid sequence deduced from the ORF is homologous to that of PchR, an AraC family activator of pyochelin siderophore and ferripyochelin receptor synthesis in *P. aeruginosa* (26, 27). An alignment of the conserved region is shown in Fig. 3. To a lesser extent, the C-terminal domain of the ORF is also homologous to that of YbtA, another AraC-type regulator controlling pesticin siderophore and yersiniabactin receptor synthesis (16). A second ORF, starting 126 bp downstream from the first one and running to the *Pst*I site, was detected. The deduced 110-residue sequence shows 56% similarity to the N-terminal region of the 337-residue *E. coli* Bcr protein conferring bicyclomycin and sulfonamide resistance (8). Thus, this second gene could encode an inner membrane drug translocase of the Bcr family.

Presence of the Fur-repressed gene in other *Bordetella* genomes. *B. pertussis* and *B. parapertussis* are human pathogens closely related to *B. bronchiseptica*, while *B. avium*, a poultry pathogen, is more distant, according to phylogenetic analysis (35). PCR experiments were performed on genomic DNA from *B. pertussis* BPSM, *B. parapertussis* PEP, and *B. avium* 103004, with oligonucleotides hybridizing to the flanking sequences of the *B. bronchiseptica* Fur-repressed gene. PCR amplification products of the expected size were obtained with chromosomal DNA from *B. pertussis* and *B. parapertussis*, but under the same conditions, no amplification product was generated with *B. avium* DNA (data not shown). Furthermore, *B. avium* genomic DNA did not hybridize to the *Kpn*I-*Pst*I fragment of pEP279 used as a probe in Southern blot experiments (data not shown). Thus, *B. avium* 103004 may lack this gene, or the sequence may be too divergent to be detected under the hybridization conditions tested.

Sequence comparison of the *B. bronchiseptica* DNA fragment with the cloned *B. pertussis* and *B. parapertussis* 1.48-kb PCR products revealed only two differences in each fragment (Fig. 2). In the *B. pertussis* sequence, the first distinction was an A-to-G alteration in the ORF (Ser-to-Gly change in the deduced protein), and the second was a G-to-A switch, 61 bp downstream from the stop codon. In the *B. parapertussis* sequence, both differences mapped in the ORF; the first one was identical to that in the *B. pertussis* gene, and the second change, a G-to-A switch, leads to a substitution of Ser for Gly in the deduced product. Ser-Gly substitutions are considered conservative changes; thus, the predicted proteins probably have the same function in *B. bronchiseptica*, *B. pertussis*, and *B. parapertussis.*

Characterization of *B. pertussis* and *B. bronchiseptica alcR* mutants. Under low-iron growth conditions, *B. pertussis* and *B. bronchiseptica* have been shown to secrete the same alcaligin siderophore (Sid$^+$ phenotype) (34). To our knowledge, *B. parapertussis* and *B. avium* siderophores have not been isolated. We found that *B. parapertussis* grown under iron-restricted conditions produced siderophore, but, interestingly, no siderophore was detected in the culture supernatant of *B. avium* 103004 (data not shown). In order to determine whether the cloned gene is involved in siderophore production, *B. pertussis* and *B. bronchiseptica* mutants were generated by exchange with an interrupted allele. In this construct, a kanamycin resistance cassette was inserted at the *Nru*I site located in the 3′ region of the ORF (Fig. 2). This insertion disrupted the AraC signature motif of the putative regulatory protein (Fig. 3).

Isogenic *B. pertussis* and *B. bronchiseptica* wild-type and mutant strains were grown to stationary phase in low-iron SS medium. No difference in growth rate or in final yields between members of isogenic pairs could be detected, even after growth for several generations in such an iron-restricted medium (data not shown). Bacterial cells and culture supernatants were separated by centrifugation and saved. WCLs were subjected to SDS-PAGE analysis, and culture supernatants were tested for siderophore activity in the CAS assay (Fig. 4A, lanes 1, 2, 5,

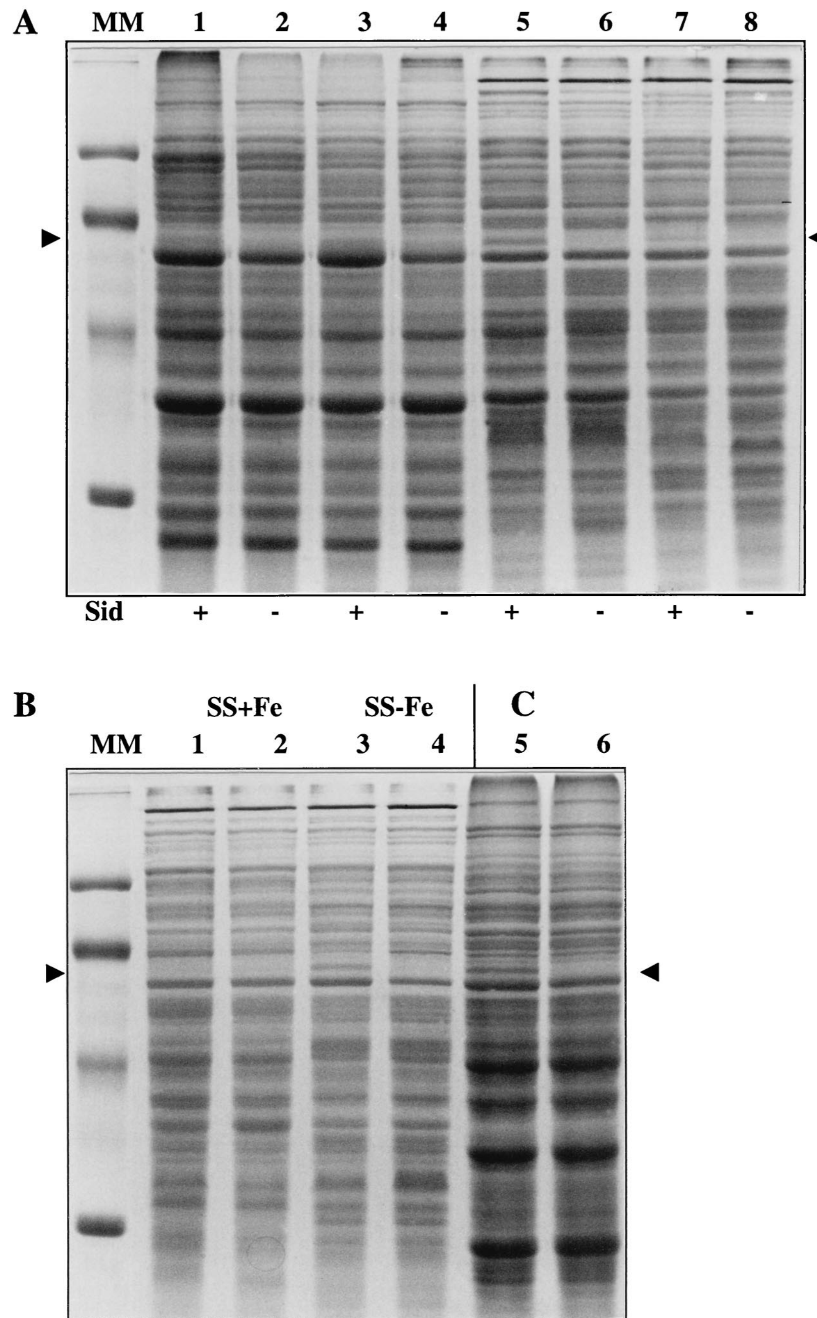

FIG. 4. Effect of *alcR* inactivation on protein and siderophore production in *B. pertussis* and *B. bronchiseptica* as determined by SDS-PAGE analysis. (A) WCLs of cultures grown in iron-limited SS medium. Lanes: 1, BPSM (wild type); 2, BPEP184 *alcR*::Km^r; 3, BPEP184(pEP301); 4, BPEP184(pBBR1MCS); 5, BB1015 (wild type); 6, BBEP205 *alcR*::Km^r; 7, BBEP205(pEP301); 8, BBEP205(pBBR1MCS). Siderophore (Sid) production in matching culture supernatants tested by the CAS assay is indicated below the gel (+, high level of activity; −, no siderophore activity detected). (B) WCLs of BB1015 (lanes 1 and 3) and BBEP205 *alcR*::Km^r (lanes 2 and 4) grown in iron-rich SS medium (SS+Fe) (lanes 1 and 2) or iron-limited SS medium (SS-Fe) (lanes 3 and 4). (C) Soluble proteins prepared from BPSM (lane 5) and BPEP184 *alcR*::Km^r (lane 6) grown in iron-limited SS medium. For both gels, the molecular masses of markers in lane MM are 97.4, 66.2, 45, and 31 kDa, from top to bottom. The 60-kDa iron-repressed protein is indicated (arrowheads).

and 6). No siderophore activity was detected in the culture supernatant of either mutant (Sid$^-$ phenotype; Fig. 4A, lanes 2 and 6). The isogenic pairs presented similar protein profiles except for a 60-kDa polypeptide synthesized by wild-type *B. pertussis* and *B. bronchiseptica* strains (Fig. 4A, lanes 1 and 5) which was clearly absent in BPEP184 and BBEP205 (lanes 2 and 6).

Each mutant was then transformed with pBBR1MCS or with pEP301, a pBBR1MCS derivative bearing an intact copy of the *B. bronchiseptica* Fur-repressed gene. The transformants were grown in iron-limited SS medium, and WCLs were analyzed by SDS-PAGE, while culture supernatants were tested in the CAS assay (Fig. 4A, lanes 3, 4, 7, and 8). BPEP184(pEP301) and BBEP205(pEP301) produced siderophores and synthe-

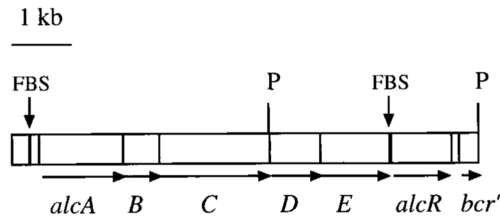

FIG. 5. Physical map of the *alc* locus of *B. bronchiseptica*. Blocks representing ORFs are drawn to scale from the 4.3-kb *alcABC* sequence determined by Giardina et al. (20) and from the sequence data for the 3.5-kb *Pst*I DNA fragment presented in this study. *Pst*I restriction sites (P), FBS upstream from *alcA* and *alcR* (black bars), and *bcr′*, the 5′ extremity of a putative bicyclomycin resistance gene downstream from *alcR*, are indicated.

sized the 60-kDa polypeptide (Fig. 4A, lanes 3 and 7), whereas no siderophore and no 60-kDa polypeptide were synthesized by BPEP184 and BBEP205 bearing pBBR1MCS (lanes 4 and 8). This complementation experiment indicated that neither the Sid$^-$ phenotype nor the absence of the 60-kDa protein resulted from a polar effect of the disruption on downstream genes. Both phenotypes were directly linked to the absence of a functional Fur-repressed gene. This new gene was designated *alcR* owing to its involvement in alcaligin siderophore production.

The AlcR-dependent 60-kDa polypeptide is a soluble iron-repressed protein. To investigate whether the production of the 60-kDa protein was iron regulated, *B. bronchiseptica* BB1015 and BBEP205 were grown in iron-rich and iron-limited SS media. SDS-PAGE analysis of WCLs revealed that the AlcR-dependent 60-kDa protein was synthesized by BB1015 only under low-iron growth conditions (Fig. 4B, compare lanes 1 and 3). Thus, this iron-repressed protein was designated IRP60. In agreement with our previous observations, BBEP205 AlcR$^-$ grown in iron-limited or iron-rich SS medium did not produce IRP60 (Fig. 4B, lanes 2 and 4). To determine the IRP60 cell location, soluble and membrane protein fractions were prepared from lysates of BPSM and BPEP184 cells grown in iron-limited SS medium. The protein samples were analyzed by SDS-PAGE. The 60-kDa protein was identified in the soluble protein fraction from BPSM, while it was absent in the BPEP184 extract (Fig. 4C, compare lanes 5 and 6). IRP60 was not detected in membrane preparations (data not shown). These observations suggest that IRP60 is a cytoplasmic or periplasmic protein.

The *alcR* gene is located downstream from the *alcABC* operon. Structural genes of regulatory proteins sometimes lie in the vicinity of their target genes. To identify putative AlcR-regulated genes, the nucleotide sequence upstream from *alcR* in pEP279 was determined up to the first *Pst*I cloning site shown in Fig. 1. A BLASTN search (2) in the nonredundant GenBank/EMBL/DDBJ/PDB library revealed a 300-bp sequence overlap with the 3′ end of the *alcABC* operon encoding alcaligin biosynthesis enzymes (20, 28). Thus, *alcR* was mapped about 2 kb downstream from the *alcC* gene on the chromosome and in the same orientation as the *alcABC* operon (Fig. 5). We noticed a few discrepancies between the *B. bronchiseptica alcC* downstream sequence deposited in the databank by Giardina et al. (20) and our sequence determination. The differences corresponded to three base substitutions, two base insertions, and one base inversion. Sequence analysis of the *alcR* upstream region revealed two tightly linked ORFs oriented in the same direction as *alcC* (Fig. 5). The first ORF contains an ATG codon overlapping the *alcC* stop codon (Fig. 6A) and could encode a 29-kDa polypeptide. The second ORF, 14 bp downstream from the stop codon of the preceding ORF and 6 bp downstream from a putative TAAGGAG RBS (Fig. 6A), could specify a 45-kDa protein. Such a tight organization suggests that these genes are part of the *alcABC* operon. Thus, they were designated *alcD* and *alcE*. Amino acid sequences deduced from *alcD* (AlcD) and *alcE* (AlcE) were subjected to a BLASTP search (2) in the library cited above. Homology between AlcE and TdnA1, the large subunit of *Pseudomonas putida* terminal dioxygenase, was detected. TdnA is an iron-sulfur protein involved in aniline degradation (18). A high degree of similarity was observed around the putative iron-sulfur center of TdnA1, as shown in Fig. 6B, suggesting that AlcE might also be an iron-sulfur protein. A scan with AlcD failed to reveal any homology with sequences in the bank.

AlcR is not involved in FHA, AC-Hly, PRN, or PTX production or in colonization. Since in other pathogens the production of important virulence factors may be iron regulated, we investigated whether AlcR is involved in the production of FHA, AC-Hly, PRN, or PTX in *B. pertussis*. The WCLs and culture supernatants of BPSM and BPEP184 were compared by SDS-PAGE and immunoblot analyses. The two strains produced similar amounts of FHA, AC-Hly, PRN, and PTX, indicating that AlcR is not required for the synthesis of these four virulence factors (data not shown). The results for FHA and PTX were confirmed by using transcriptional reporter gene fusions to the *fhaB* and *ptx* chromosomal genes. For this purpose, the *alcR*::Km^r mutation was introduced into BP953 (*fhaB*::*lacZ ptx*::*phoA*) (53) by allelic exchange to generate BPEP214. The β-gal and AP activities of the isogenic AlcR$^+$-AlcR$^-$ strains were then compared (data not shown). The *alcR* disruption had no significant effect on β-gal and AP activities, indicating that AlcR is not involved in *fhaB* or *ptx* expression.

FHA, PRN, PTX, and AC-Hly are the major adhesins and toxins in *B. pertussis* and as such play an important role in the initiation, amplification, and persistence of the bacterial infection in the mouse respiratory infection model. As none of these virulence factors proved to be AlcR dependent, we tested whether AlcR, as an activator of siderophore synthesis, was required for efficient colonization of *B. pertussis* in this animal model. Mice were infected with either BPSM or BPEP184. The two strains presented similar colonization profiles, with a 3-log increase in bacterial counts during the first week after exposure followed by a slow decline over the subsequent 2 weeks (data not shown). Similar results were obtained with the *B. bronchiseptica* AlcR$^+$-AlcR$^-$ isogenic pair (data not shown). Therefore, at least in the mouse model, AlcR plays no important role in colonization.

DISCUSSION

As a first step to elucidate the iron regulatory network in *Bordetella* spp., we chose to identify target genes of the global iron regulator Fur. We have isolated a clone bearing an FBS from a *B. bronchiseptica* partial library by using the *E. coli* FURTA (54). The FBS in the plasmid was mapped about 2 kb downstream from one end of the cloned 3.5-kb fragment. Sequence analysis of the 1.5-kb region downstream of the FBS revealed an ORF, *alcR*, which could encode an AraC-like regulatory protein homologous to PchR, a regulator of pyochelin and ferripyochelin receptor synthesis in *P. aeruginosa* (26, 27, 49), and to YbtA, a regulator of pesticin and yersiniabactin receptor synthesis in *Yersinia pestis* (16). No significant ORF running in the opposite direction in the 2-kb flanking region was detected, strongly suggesting that the target of Fur repression is the downstream *alcR* gene. In the same orientation, and about 120 bp downstream from *alcR*, an ORF (*bcr*)

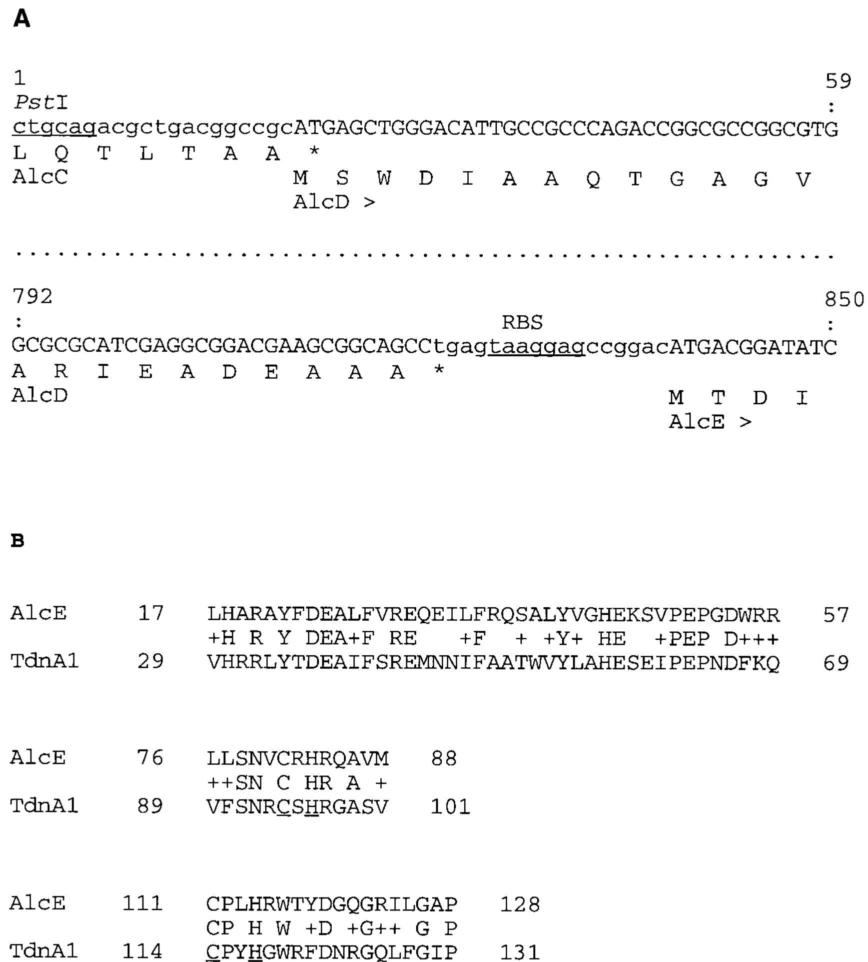

FIG. 6. (A) Flanking sequences of the *alcD* gene and deduced amino acid sequences. The tight genetic organization of the *alcC*, *alcD*, and *alcE* genes is shown. The *Pst*I restriction site (underlined), the 3′-terminal sequence of *alcC* (lowercase), and the deduced C-terminal sequence of AlcC and the *alcD* and *alcE* putative coding sequences and predicted translation products (uppercase) are shown. A potential RBS upstream from *alcE* is also indicated (underlined). (B) Partial amino acid sequence alignments of the putative AlcE protein with *P. putida* TdnA1. C and H residues forming the predicted iron-sulfur reaction center in TdnA1 (underlined) and conservative changes (+) are indicated.

encoding a putative inner membrane drug resistance translocase was detected. The large spacing between *alcR* and *bcr* suggests that *bcr* is transcribed from its own promoter, but since no obvious terminator was found in the *alcR-bcr* intergenic sequence, the possibility that *alcR* and *bcr* are cotranscribed and form an operon cannot be ruled out. The *alcR* upstream sequence in the cloned 3.5-kb fragment was shown to contain the very end (7 C-terminal amino acid residues) of the previously identified *alcC* gene (20, 28), followed by two tightly linked ORFs running in the same direction. Such a spatial arrangement suggests that these ORFs are part of the alcaligin biosynthesis *alcABC* operon (28). We designated them *alcD* and *alcE*. A homology search indicated that *alcE* could encode an iron-sulfur protein. No homology was detected for the putative AlcD protein. In their paper reporting the identification of the *alcABC* operon, Kang et al. suggested that in addition to AlcA, AlcB, and AlcC, at least two other enzymes are required to complete the siderophore biosynthesis pathway from succinyl-hydroxy-putrescine to alcaligin (28). The *alcD* and *alcE* genes could code for these enzymes. Experiments are in progress to determine whether the *alcD* and *alcE* ORFs are involved in alcaligin biosynthesis.

Sequence comparison of the *B. bronchiseptica*, *B. pertussis*, and *B. parapertussis alcR* genes showed that the predicted product is highly conserved in these species. Both *B. bronchiseptica* and *B. pertussis* produce alcaligin (34), and in this paper we show that *B. parapertussis* also synthesizes siderophores. It is highly probable that this siderophore is alcaligin. In contrast, no siderophore production was observed in the culture supernatant of *B. avium* 103004 grown in iron-restricted conditions. Concomitantly, no sequence hybridizing to an *alcR* probe could be detected in Southern blot experiments with *B. avium* genomic DNA. Another *B. avium* strain in our collection presented also a Sid$^-$ phenotype (38). In neither of these *B. avium* strains could the production of siderophore be induced by a plasmid-borne copy of the *B. bronchiseptica alcR* gene (38), suggesting that they were not simply *alcR* mutants. To our knowledge, *B. avium* iron uptake systems have not been studied yet, and we do not know whether the absence of siderophore production is a physiological trait of the *B. avium* species. An LF-binding protein has been identified in the *B. avium* outer membrane (30), but the role of this protein in iron uptake remains to be elucidated.

B. bronchiseptica and *B. pertussis alcR* mutants, obtained by

in vitro mutagenesis and gene replacement, were deficient in alcaligin production. They were also deficient in the synthesis of a soluble 60-kDa iron-repressed protein (IRP60). Both phenotypes were complemented by an intact plasmid-borne copy of *alcR*, showing that neither of them resulted from a polar effect of the *alcR* disruption. Thus, AlcR is required for alcaligin and IRP60 synthesis. Kang et al. demonstrated that AlcC is a soluble iron-repressed protein and that, although predicted to have a size of 70 kDa, AlcC migrates with an apparent molecular mass of 59 kDa during SDS-PAGE (28). It is therefore possible that IRP60 is in fact AlcC, suggesting that AlcR is an activator of the *alcC* gene. Complementation studies have shown that *alcC* is transcribed from the *alcA* promoter and that the *alcABC* locus constitutes an operon (28). Kang et al. also compared the protein profiles for two *B. bronchiseptica* mutants bearing polar mutations in the *alcA* gene with that for a wild-type strain. They reported that the only observed difference was the absence of the AlcC polypeptide in the *alcA* polar mutants (28). Since the *B. pertussis* and *B. bronchiseptica alcR* mutants described in this paper present the same protein profile phenotype as *alcA* mutants, it is very likely that AlcR is an activator of the whole *alc* operon in both species, including the putative new *alcD* and *alcE* genes located downstream from *alcC*. Regulatory genes are often autoregulated; however, a comparison of the *alcA* and *alcR* promoter regions did not reveal any potential AlcR binding sites. Overexpression and purification of AlcR will enable us to determine the N-terminal sequence of the protein as well as characterize its DNA binding sequence. Alternatively, AlcR may be an intermediate regulator and may activate the promoter of another, as-yet-unidentified gene, the latter being the final activator of the *alc* operon. The identification of AlcR target genes will help establish its position in the iron regulatory network.

A putative FBS overlaps the −10 box of the *alcABC* promoter, and transcription of the *alcABC* genes is iron repressed (28). In addition, *alcABC* transcription appears to be AlcR dependent, suggesting the following model for very tight Fur repression of the *alc* operon. Under iron-rich growth conditions, the Fur-Fe(II) complex binds to the *alcR* promoter, generating an AlcR depletion which in turn shuts off *alcABC* operon transcription. Concomitantly, the Fur-Fe(II) complex binds directly to the *alcABC* promoter, ensuring a tight double-level repression. Under iron-restricted growth conditions, both promoters are derepressed and AlcR activates *alcABC* transcription, either directly or via another regulatory protein.

The iron regulatory network strongly influences the production of virulence factors in a number of gram-positive and gram-negative pathogenic bacteria, such as *Corynebacterium diphtheria*, *E. coli*, *P. aeruginosa*, and many others (for a review, see reference 15). The production of the major virulence factors FHA, PTX, PRN, and AC-Hly in *B. pertussis* was not affected by the inactivation of the *alcR* gene. The behaviors of the AlcR-deficient mutant and the parental strain in the murine respiratory model were similar probably because the expression of adhesins and toxins was neither enhanced nor repressed in the absence of AlcR. Siderophore production has been shown to contribute significantly to virulence in a number of bacterial pathogens (55), and, like most bacteria, *Bordetella* spp. also require iron for growth (31). The *alcR* mutation did not affect colonization in the mouse model, although the mutant strain was unable to secrete siderophore in vitro under iron-limited growth conditions. Our observations therefore suggest that, in addition to alcaligin production, *B. pertussis* has evolved a second iron uptake mechanism, unless low-level siderophore synthesis, below the detection limit of the CAS assay, occurs in the absence of the regulator. Beall and coworkers recently identified three *B. pertussis* iron-regulated genes encoding BfeA, BfrB, and BfrC, three outer membrane proteins homologous to receptors of enterobactin, ferrichrome, and another hydroxamate siderophore, respectively (5, 7). They also showed that, in addition to these proteins, *B. bronchiseptica* synthesizes BfrA, an unidentified exogenous siderophore receptor (6). Thus, via these specific receptors, *Bordetella* spp. may perhaps scavenge various heterologous ferrisiderophores secreted by the commensal flora of the airways. If the synthesis of these receptors is AlcR independent, the multiplicity of heterologous siderophore-mediated iron uptake systems could compensate for the absence of alcaligin in *alcR* mutants. Alternatively, Redhead and Hill have suggested that *B. pertussis* has recourse to a TF-LF receptor to scavenge iron from the host (43). Menozzi et al. have isolated LF-binding outer membrane proteins from both *B. pertussis* and *B. bronchiseptica* (31). It is therefore possible that *Bordetella* spp. use both siderophore-mediated and siderophore-independent iron uptake systems. The testing of mutants bearing deletions of the alcaligin biosynthesis genes in the mouse colonization model could help determine if the alcaligin system is essential.

Interestingly, in certain *B. bronchiseptica* strains, siderophore production is repressed by BvgA, a global activator of most *Bordetella* virulence factors (19). This implies that in these strains siderophores are produced only in the avirulent phase, suggesting that siderophores may interfere with colonization in certain circumstances. Consistent with this assumption, Register et al. observed that a *B. bronchiseptica* mutant deficient in siderophore synthesis in fact expressed enhanced virulence in neonatal pigs (44). The relationship between siderophore production and virulence thus appears to be quite complex in *Bordetella* spp. and deserves further study.

ACKNOWLEDGMENTS

We thank Klaus Hantke for providing the FURTA system, Scott Stibitz for the gift of strain BP953, and Michael Kovach for the gift of pBBR1MCS. We are grateful to Eve Willery and Nathalie Reveneau for technical assistance with automatic sequencing, to Sabine Thiberge for technical assistance with mouse experiments, and to Carine Capiau for the gift of antipertactin serum. We thank Emmanuelle Fort for photographic work and Franco Menozzi for critically reading the manuscript.

This work was supported by the INSERM, the Institute Pasteur de Lille, the Institut Pasteur de Paris, the Région Nord-Pas-de-Calais, and the Ministère de l'Enseignement Supérieur et de la Recherche.

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. **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.
3. **Armstrong, S. K., and M. O. Clements.** 1993. Isolation and characterization of *Bordetella bronchiseptica* mutants deficient in siderophore activity. J. Bacteriol. **175:**1144–1152.
4. **Beall, B., and G. N. Sanden.** 1995. Cloning and initial characterization of the *Bordetella pertussis fur* gene. Curr. Microbiol. **30:**1–4.
5. **Beall, B., and G. N. Sanden.** 1995. A *Bordetella pertussis fepA* homologue required for utilization of exogenous ferric enterobactin. Microbiology **141:** 3193–3205.
6. **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.
7. **Beall, B., and T. Hoenes.** 1997. Three unlinked genes in *Bordetella* encoding putative ferric siderophore receptors, abstr. B-239, p. 69. *In* Abstracts of the 97th General Meeting of the American Society for Microbiology 1997. American Society for Microbiology, Washington, D.C.
8. **Bentley, J., L. S. Hyatt, K. Ainley, J. H. Parish, R. B. Herbert, and G. R. White.** 1993. Cloning and sequence analysis of an *Escherichia coli* gene conferring bicyclomycin resistance. Gene **127:**117–120.
9. **Bonnah, R. A., R.-H. Yu, and A. B. Schryvers.** 1995. Biochemical analysis of

lactoferrin receptors in the Neisseriaceae: identification of a second bacterial lactoferrin receptor protein. Microb. Pathog. **19:**285–297.
10. **Bordet, J., and O. Gengou.** 1906. Le microbe de la coqueluche. Ann. Inst. Pasteur (Paris) **20:**731–741.
11. **Brickman, E., and J. Beckwith.** 1975. Analysis of the regulation of *Escherichia coli* alkaline phosphatase synthesis using deletions and φ80 transducing phages. J. Mol. Biol. **96:**307–316.
12. **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.
13. **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.
14. **Coy, M., and J. B. Neilands.** 1991. Structural dynamics and functional domains of the Fur protein. Biochemistry **30:**8201–8210.
15. **Crosa, J. H.** 1987. Bacterial iron metabolism, plasmids and other virulence factors, p. 139–170. *In* J. J. Bullen and E. Griffiths (ed.), Iron and infection. John Wiley & Sons, Chichester, United Kingdom.
16. **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.
17. **Foster, J. W., and H. K. Hall.** 1992. Effect of *Salmonella typhimurium* ferric uptake regulator (*fur*) mutations on iron- and pH-regulated protein synthesis. J. Bacteriol. **174:**4317–4323.
18. **Fukumori, F., and C. P. Saint.** 1997. Nucleotide sequences and regulational analysis of genes involved in conversion of aniline to catechol in *Pseudomonas putida* UCC22(pTDN1). J. Bacteriol. **179:**399–408.
19. **Giardina, P. C., L.-A. Foster, J. M. Musser, B. J. Akerley, J. F. Miller, and D. W. Dyer.** 1995. *bvg* repression of alcaligin synthesis in *Bordetella bronchiseptica* is associated with phylogenetic lineage. J. Bacteriol. **177:**6058–6063.
20. **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.
21. **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.
22. **Graeff-Wohlleben, H., S. Killat, A. Banemann, N. Guiso, and R. Gross.** 1997. Cloning and characterization of an Mn-containing superoxide dismutase (SodA) of *Bordetella pertussis*. J. Bacteriol. **179:**2194–2201.
23. **Gray-Owen, S. D., S. Loosmore, and A. B. Schryvers.** 1995. Identification and characterization of genes encoding the human transferrin-binding proteins from *Haemophilus influenzae*. Infect. Immun. **63:**1201–1210.
24. **Hantke, K.** 1987. Selection procedure for deregulated iron transport mutants (*fur*) in *Escherichia coli* K12: *fur* not only affects iron metabolism. Mol. Gen. Genet. **210:**135–139.
25. **Hawley, D. K., and W. R. McClure.** 1983. Compilation and analysis of *Escherichia coli* promoter sequences. Nucleic Acids Res. **11:**2237–2255.
26. **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.
27. **Heinrichs, D. E., and K. Poole.** 1996. PchR, a regulator of ferripyochelin receptor gene (*fptA*) expression in *Pseudomonas aeruginosa*, functions both as an activator and as a repressor. J. Bacteriol. **178:**2586–2592.
28. **Kang, H. Y., T. J. Brickman, F. C. Beaumont, and S. K. Armstrong.** 1996. Identification and characterization of iron-regulated *Bordetella pertussis* alcaligin siderophore biosynthesis genes. J. Bacteriol. **178:**4877–4884.
29. **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.
30. **Menozzi, F.** Unpublished data.
31. **Menozzi, F. D., C. Gantiez, and C. Locht.** 1991. Identification and purification of transferrin- and lactoferrin-binding proteins of *Bordetella pertussis* and *Bordetella bronchiseptica*. Infect. Immun. **59:**3982–3988.
32. **Menozzi, F. D., R. Mutombo, G. Renauld, C. Gantiez, J. H. Hannah, E. Leininger, M. J. Brennan, and C. Locht.** 1994. Heparin-inhibitable lectin activity of the filamentous hemagglutinin adhesin of *Bordetella pertussis*. Infect. Immun. **62:**769–778.
33. **Miller, J. H.** 1992. A short course in bacterial genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
34. **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 *B. bronchiseptica*. J. Bacteriol. **177:**1116–1118.
35. **Müller, M., and A. Hildebrandt.** 1993. Nucleotide sequence of the 23S rRNA genes from *Bordetella pertussis*, *B. parapertussis*, *B. bronchiseptica*, and *B. avium*, and their implications for phylogenic analysis. Nucleic Acids Res. **21:**3320.
36. **Neilands, J. B., and K. Nakamura.** 1991. Detection, determination, isolation, characterization and regulation of microbial iron chelates, p. 1–15. *In* G. Winkelmann (ed.), Handbook of microbial iron chelates. CRC Press, Boca Raton, Fla.
37. **Nordmann, P., B. François, F. D. Menozzi, M. C. Commare, and A. Barois.** 1992. Whooping cough associated with *Bordetella parapertussis* in an HIV-infected child. Pediatr. Infect. Dis. J. **11:**248.
38. **Pradel, E.** Unpublished observation.
39. **Pradel, E., and C. Locht.** 1995. Cloning and sequencing of the *fur* gene from *Bordetella pertussis*, abstr. B-357, p. 227. *In* Abstracts of the 95th General Meeting of the American Society for Microbiology 1995. American Society for Microbiology, Washington, D.C.
40. **Prince, R. W., C. D. Cox, and M. L. Vasil.** 1991. Regulation of *toxA* and *regA* by *Escherichia coli fur* gene and identification of a Fur homolog in *Pseudomonas aeruginosa* PA103 and PAO1. Mol. Microbiol. **5:**2823–2831.
41. **Prince, R. W., D. G. Storey, and M. L. Vasil.** 1993. Coordinate regulation of siderophore and exotoxin A production: molecular cloning and sequencing of the *Pseudomonas aeruginosa fur* gene. J. Bacteriol. **175:**2589–2593.
42. **Quandt, J., and M. F. Hynes.** 1993. Versatile suicide vectors which allow direct selection for gene replacement in Gram-negative bacteria. Gene **127:**15–21.
43. **Redhead, K., and T. Hill.** 1991. Acquisition of iron from transferrin by *Bordetella pertussis*. FEMS Microbiol. Lett. **77:**303–308.
44. **Register, K. B., M. R. Ackermann, and D. W. Dyer.** 1994. Loss of siderophore production in *Bordetella bronchiseptica* affects virulence in neonatal pigs, abstr. B-173, p. 59. *In* Abstracts of the 94th General Meeting of the American Society for Microbiology 1994. American Society for Microbiology, Washington, D.C.
45. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
46. **Schryvers, A., and L. J. Morris.** 1988. Identification and characterization of the transferrin receptor from *Neisseria meningitidis*. Mol. Microbiol. **2:**281–288.
47. **Schryvers, A. B.** 1989. Identification of the transferrin- and lactoferrin-binding proteins in *Haemophilus influenzae*. J. Med. Microbiol. **29:**121–130.
48. **Schwyn, B., and J. B. Neilands.** 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. **160:**47–56.
49. **Serino, L., C. Reimmann, P. Visca, M. Beyeler, V. Della Chiesa, and D. Haas.** 1997. Biosynthesis of pyochelin and dihydroaeruginoic acid requires the iron-regulated *pchDCBA* operon in *Pseudomonas aeruginosa*. J. Bacteriol. **179:**248–257.
50. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. BioTechnology **1:**784–790.
51. **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.
52. **Stibitz, S.** 1994. Use of conditionally counterselectable suicide vectors for allelic exchange. Methods Enzymol. **235:**458–465.
53. **Stibitz, S., and N. H. Carbonetti.** 1994. Hfr mapping of mutations in *Bordetella pertussis* that define a genetic locus involved in virulence gene regulation. J. Bacteriol. **176:**7260–7266.
54. **Stojiljkovic, I., A. J. Baumler, and K. Hantke.** 1994. Fur regulon in gram-negative bacteria. Identification and characterization of new *Escherichia coli* iron-regulated genes by a Fur titration assay. J. Mol. Biol. **236:**531–545.
55. **Weinberg, E. D.** 1995. Acquisition of iron and other nutrients in vivo, p. 81–95. *In* J. A. Roth, C. A. Bolin, K. A. Brogden, F. C. Minion, and M. J. Wannemuehler (ed.), Virulence mechanisms of bacterial pathogens, 2nd ed. American Society for Microbiology, Washington, D.C.