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Identification and Characterization of Iron-Regulated *Bordetella pertussis* Alcaligin Siderophore Biosynthesis Genes

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***Bordetella bronchiseptica* mutants BRM1, BRM6, and BRM9 fail to produce the native dihydroxamate siderophore alcaligin. A 4.5-kb *Bam*HI-*Sma*I *Bordetella pertussis* genomic DNA fragment carried multiple genes required to restore alcaligin production to these siderophore-deficient mutants. Phenotypic complementation analysis using subclones of the 4.5-kb genomic region demonstrated that the closely linked BRM1 and BRM9 mutations were genetically separable from the BRM6 mutation, and both insertions exerted strong polar effects on expression of the downstream gene defined by the BRM6 mutation, suggesting a polycistronic transcriptional organization of these alcaligin biosynthesis genes. Subcloning and complementation experiments localized the putative *Bordetella* promoter to a 0.7-kb *Bam*HI-*Sph*I subregion of the cloned genomic DNA fragment. Nucleotide sequencing, phenotypic analysis of mutants, and protein expression by the 4.5-kb DNA fragment in *Escherichia coli* suggested the presence of three alcaligin system genes, namely, *alcA*, *alcB*, and *alcC*. The deduced protein products of *alcA*, *alcB*, and *alcC* have significant primary amino acid sequence similarities with known microbial siderophore biosynthesis enzymes. Primer extension analysis mapped the transcriptional start site of the putative alcaligin biosynthesis operon containing *alcABC* to a promoter region overlapping a proposed Fur repressor-binding site and demonstrated iron regulation at the transcriptional level.**

Iron is a fundamental nutritional requirement for virtually all cells, and its assimilation is considered essential for invading pathogenic bacteria to establish infection in the iron-limiting environment of the host (13, 56). Additionally, iron serves as an environmental modulator of the production of certain virulence factors in a number of bacteria (14, 24, 31, 32, 46). Despite host iron sequestration, mediated primarily by the glycoprotein family of iron-binding transferrins, pathogens multiply successfully in vivo because they express efficient iron-scavenging systems in response to decreased iron availability. These iron retrieval systems utilize two general strategies: one involving high-affinity iron-chelating soluble siderophores (30, 40) and the other using siderophore-independent cell surface receptor mechanisms allowing iron uptake directly from host sources such as transferrin, lactoferrin, and heme compounds (7, 35, 38, 54).

Bordetella pertussis, the causative agent of human whooping cough or pertussis, and *Bordetella bronchiseptica*, the agent of swine atrophic rhinitis and kennel cough in dogs, are bacterial pathogens that infect the respiratory epithelial mucosae of their hosts. Early reports described the production of putative siderophores by both *B. pertussis* and *B. bronchiseptica* in response to iron deficiency (1, 23). Armstrong and Clements isolated and characterized *B. bronchiseptica* transposon-induced siderophore-deficient mutants; DNA hybridization studies using sequences flanking those transposon insertions confirmed the existence of homologs of *B. bronchiseptica* siderophore system genes in *B. pertussis* (6). The siderophores of *B. pertussis* and *B. bronchiseptica* have been characterized structurally, and each has been identified as the macrocyclic dihydroxamate siderophore known as alcaligin (11, 37), previously isolated from the taxonomically related bacterial species *Alcaligenes denitrificans* subsp. *xylosoxydans* (41, 42). Characterization of *B. bronchiseptica* manganese-resistant mutants exhibiting deregulated expression of alcaligin and iron-repressed proteins and restoration of iron repressibility by the *B. pertussis fur* gene confirmed the role of the Fur repressor in regulation of these traits in both *Bordetella* species (9).

B. bronchiseptica siderophore mutants generated in an earlier study (6) were placed into distinct siderophore phenotypic complementation groups on the basis of cross-feeding assays, suggesting that multiple genes are involved in alcaligin biosynthesis. The *B. pertussis* alcaligin biosynthesis gene *odc*, defined by the mutation of *B. bronchiseptica* group III siderophore mutant BRM3, was shown to encode an ornithine decarboxylase activity which catalyzes the conversion of ornithine to putrescine, a required precursor of alcaligin in these species (10).

In this study, we have isolated alcaligin biosynthesis genes of *B. pertussis* which functionally restored alcaligin production to *B. bronchiseptica* siderophore mutants belonging to phenotypic complementation group I. Our results identify an iron-regulated polycistronic alcaligin biosynthesis gene cluster in *Bordetella* spp. predicted to encode proteins with primary amino acid sequence similarity to biosynthetic enzymes of other siderophore systems.

MATERIALS AND METHODS

Bacterial strains and plasmids. Wild-type strain *B. bronchiseptica* B013N, *B. bronchiseptica* alcaligin biosynthetic mutants BRM1, BRM6, and BRM9 (6), and wild-type *B. pertussis* UT25 (19) have been described previously. *Escherichia coli* DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) and DH10B (Bethesda Research Laboratories) were used as hosts for general DNA manipulations. *E. coli* HB101 harboring pRK2013 (20) was used to provide mobilization functions in conjugations. *E. coli* K38(pGP1-2) (53) was used for T7 promoter-directed conditional protein expression experiments. Plasmid vectors pGEM3Z (Promega, Madison, Wis.), pBluescript SK$^+$ (Stratagene, La Jolla, Calif.), pBBR1MCS (29), and pRK415 (28) were used for the construction of recombinant plasmids. The cosmid pCP13-based gene library of *B. pertussis* UT25 has been described (12). Plasmids pBRM1, pBRM6, and pBRM9 contain the cloned mini-Tn*5 lacZ*1 chromosomal insertions of mutants BRM1, BRM6, and BRM9,

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respectively, along with flanking genomic sequences (6). Plasmid pBRM1 was used as the source of *B. bronchiseptica* chromosomal DNA for probes in DNA hybridization experiments.

Growth conditions. *B. bronchiseptica* B013N and siderophore biosynthesis mutants were grown in parallel in iron-replete or iron-depleted Stainer Scholte (SS) liquid medium (47, 49) as described previously (6). Optical densities of SS cultures were monitored with a Klett-Summerson colorimeter fitted with a no. 54 filter (Klett Mfg. Co., Long Island City, N.Y.). *B. bronchiseptica* and *B. pertussis* were cultured on blood agar and Bordet-Gengou (8, 19) agar, respectively; *E. coli* was grown on Luria-Bertani medium (36). When required, the following antibiotics were added to culture media at the indicated concentrations: ampicillin (100 μg/ml), chloramphenicol (30 μg/ml), gentamicin (10 μg/ml), kanamycin (50 μg/ml), nalidixic acid (35 μg/ml), streptomycin (50 μg/ml), and tetracycline (15 μg/ml).

Siderophore detection. Chrome azurol S (CAS) agar for assessment of *B. bronchiseptica* alcaligin production was prepared as described earlier (6). The CAS universal siderophore detection assay (48) was used to monitor siderophore production by *Bordetella* cells grown in iron-depleted SS by measuring the decrease in A_{630} of the CAS dye reaction as reported previously (6).

General DNA techniques. Conjugal transfer of plasmids from *E. coli* to *Bordetella* spp. was by triparental mating as described previously (10). General genetic techniques were performed as described in the procedures of Sambrook et al. (43). Nucleotide sequencing using double-stranded plasmid templates was performed by the dideoxy chain termination method (44) as modified by DeShazer et al. (18) with [^{32}P]dATP (ICN Radiochemicals, Irvine, Calif.) and a Sequenase version 2.0 kit (United States Biochemical Corp., Cleveland, Ohio). The nucleotide sequence of the *alcABC* promoter-operator region was determined for both DNA strands, while analyses of the predicted products of *alcB* and *alcC* were based on the nucleotide sequence from one strand. Nucleotide sequence data management and analysis used DNA Strider 1.2 software (Service de Biochimie et de Génétique Moléculaire, Gif-Sur-Yvette, France) for the Macintosh computer. Database searches and data retrievals were done with the BLAST (3) and RETRIEVE electronic mail servers developed by the National Center for Biotechnology Information at the National Library of Medicine. For protein database BLASTP searches, *Bordetella* DNA sequences were translated in all six possible reading frames, and the resulting amino acid sequences were transmitted to the National Center for Biotechnology Information for analysis with the nonredundant database mandatory DATALIB search parameter. All other search parameters were BLAST default values. Multiple amino acid sequence alignments were performed by the J. Hein method (26) with the MegAlign module of a demonstration version of the Lasergene sequence analysis software system for the Macintosh PowerPC computer (DNASTAR, Inc., Madison, Wis.). DotPlot comparisons used to identify putative Fur-binding sequences were also performed with the MegAlign module of the Lasergene sequence analysis software system for the Macintosh PowerPC computer, with search parameters programmed to identify *Bordetella* DNA regions of at least 50% identity over a 30-nucleotide search window with the dyad sequence 5′-GATA ATGATAATCATTATC-3′.

Southern and in situ DNA hybridizations were performed at high stringency as described previously (43). Hybridization probes derived from a 2-kb *Not*I-*Eco*RI *B. bronchiseptica* DNA fragment of pBRM1 were labelled with [^{32}P]dCTP (ICN Radiochemicals) by the random priming method with the Prime-a-Gene labeling system (Promega).

RNA isolation. RNA was isolated by a modification of the acid guanidinium thiocyanate-phenol-chloroform extraction method of Chomczynski and Sacchi (15). Solutions used in RNA isolation were treated with 0.1% diethylpyrocarbonate whenever possible. *B. pertussis* UT25 was streaked from frozen stocks onto Bordet-Gengou agar plates and cultured at 37°C for 30 to 48 h, at which time plate growth was used to inoculate iron-replete SS broth seed cultures. Seed cultures were grown for 30 to 48 h at 37°C with shaking, harvested, washed twice with iron-depleted SS, and used to inoculate 50 ml of iron-replete or iron-depleted SS to an initial density of 30 Klett units. Cultures were grown at 37°C with shaking until the mid-logarithmic growth phase, reaching densities of 175 to 225 Klett units after 16 to 24 h.

To prepare approximately 50 ml of guanidine thiocyanate (GdSCN) lysis solution for *Bordetella* RNA isolation, 23.6 g of solid GdSCN was dissolved in 28 ml of H_2O in a sterile bottle, to which was added 2.5 ml of 0.5 M sodium citrate (pH 7.0), 1.25 ml of 20% *N*-lauroylsarcosine, and 0.36 ml of 2-mercaptoethanol. Cells were pelleted by centrifugation at 4°C, immediately resuspended in 5 ml of GdSCN lysis solution, and lysed by vortexing. One milliliter of 2 M sodium acetate (pH 4.0) was added to acidify the mixture, 5 ml of unbuffered H_2O-saturated phenol was added, and the mixture was vortexed vigorously. One milliliter of $CHCl_3$-isoamyl alcohol (49:1) was added and mixed well, and the tubes were placed on ice for 15 min. The mixtures were centrifuged at 10,000 × *g* at 4°C for 10 min to separate the phases, the upper aqueous phase was transferred to a fresh tube, and RNA was precipitated by the addition of an equal volume of isopropanol and held overnight at −20°C. RNA was recovered by centrifugation at 10,000 × *g* at 4°C for 15 min. The RNA pellets were drained, briefly air dried, and redissolved in 0.7 ml of GdSCN lysis solution prior to transfer to microcentrifuge tubes and the addition of an equal volume of isopropanol. After incubation at −20°C for at least 1 h, the RNA precipitate was sedimented in a microcentrifuge at maximum speed for 15 min at 4°C, and the pellet was drained well and rinsed twice with 1-ml volumes of cold 70% ethanol. The RNA was air dried, dissolved in H_2O, and quantitated spectrophotometrically. After quantitation, the RNA solution was divided among microcentrifuge tubes to provide 50 μg of RNA per tube, which was precipitated by the addition of 0.1 volume of 3 M sodium acetate (pH 5.2) plus 2.5 volumes of ethanol. RNA was stored as a precipitate under ethanol at −70°C.

RNA transcript mapping by primer extension. Primer extension mapping was performed as described previously (43), with 50 μg of total RNA and 100 fmol of ^{32}P-end-labelled oligonucleotide primer per reaction. RNA was denatured at 85°C for 10 min and then hybridized with radiolabelled antisense primer overnight at 42°C. Extension reactions used 400 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) at 37°C. The 35-mer oligonucleotide primer used to map the 5′ terminus of the *alc* transcript (5′-G TGTCTGTCCCGTATGCCGCCTTGCCGAAGCACTG-3′) was the antisense of nucleotides 529 to 563 in Fig. 2 and was also used to generate the accompanying DNA sequencing ladder from an *alc* plasmid template.

Cell fractionation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). *B. bronchiseptica* cells harvested from both iron-replete and iron-depleted SS cultures were resuspended in 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.4) and disrupted with a French pressure cell (American Instrument Company, Silver Spring, Md.). The pressates were centrifuged at 2,316 × *g* for 5 min to sediment unbroken cells, and the resulting supernatants were centrifuged at 132,300 × *g* at 4°C for 1 h to obtain soluble and insoluble cell fractions.

For analysis of total cellular proteins, *B. pertussis* cells were harvested from iron-replete and iron-depleted SS cultures and solubilized, and the proteins were resolved by SDS-PAGE on 10% polyacrylamide gels containing 0.5 M urea (47). Approximately 40 μg of protein was applied per lane. Proteins were visualized by Coomassie blue staining.

Expression of plasmid-encoded proteins. Plasmid-encoded polypeptides were radiolabelled with Tran^{35}S-label (ICN Biochemicals, Inc., Irvine, Calif.) by use of *E. coli* host strain K38(pGP1-2) and the T7 polymerase-promoter conditional expression technique of Tabor and Richardson (53). The proteins were visualized by autoradiography after fractionation by SDS-PAGE on 12% polyacrylamide gels.

Construction of *B. pertussis alcB* and *alcC* mutants. A kanamycin resistance gene cassette isolated from plasmid pBSL86 (2) as a *Sph*I fragment was ligated into the *Sph*I site internal to *alcB* on the 4.5-kb *Bam*HI-*Sma*I *B. pertussis* DNA fragment. The *Bam*HI-*Eco*RI subfragment encompassing the mutated *alcB* region was then subcloned to the allelic exchange plasmid vector pSS1129 (50). Two plasmids, carrying *alcB* interrupted by the kanamycin cassette in both possible orientations with respect to *alc* transcription, were each conjugally transferred to the streptomycin-resistant derivative of *B. pertussis* UT25, UT25Sm1 (5, 10), and the mutations were transferred to the chromosome by homologous recombination as described by Stibitz (50). Allelic exchange in each mutant was verified by Southern hybridization analysis.

The internal 0.6-kb *Cla*I fragment of the *B. pertussis alcC* gene was deleted from the 4.6-kb *B. pertussis alcABC Sma*I fragment by digestion with *Cla*I followed by religation, and the remaining 4.0-kb region containing the mutation was subcloned to the allelic exchange plasmid vector pRTP1 (50). The *alcC* deletion mutation was transferred to the chromosome of *B. pertussis* UT25Sm1 by homologous recombination and confirmed by Southern hybridization analysis as described for the *alcB* mutants.

Nucleotide sequence accession number. The GenBank accession number assigned to the nucleotide sequence of the *B. pertussis* UT25 *alcABC* control region is U34894.

RESULTS

Identification of *B. pertussis* alcaligin biosynthesis genes by restoration of siderophore production to *B. bronchiseptica* mutants. A previous study identified and characterized *B. bronchiseptica* alcaligin biosynthesis mutants BRM1, BRM6, and BRM9 assigned to phenotypic complementation group I on the basis of cross-feeding assays (6). The genomic regions containing the insertions of these mutants were cloned and mapped; nucleotide sequencing primed from the transposon ends of mini-Tn*5 lacZ*1 revealed that the sites of insertion mutations defining BRM1 and BRM9 are separated by approximately 200 bp (Fig. 1). Restriction mapping placed the BRM6 mutation approximately 1.4 kb from that of BRM9. To isolate *B. pertussis* genes involved in alcaligin biosynthesis, a 2-kb *Not*I-*Eco*RI *B. bronchiseptica* DNA fragment flanking the transposon in mutant BRM1 was used as a source of hybridization probe DNA, resulting in the identification of 14 homologous recombinant cosmids representing five distinct overlapping clones from our *B. pertussis* UT25 genomic library.

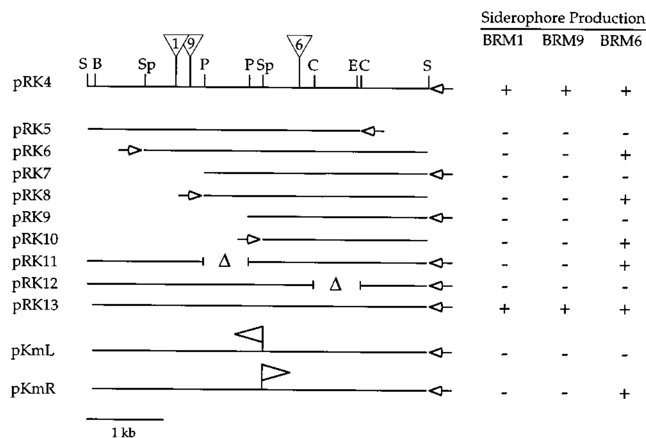

FIG. 1. Genetic complementation of group I *B. bronchiseptica* alcaligin biosynthesis mutants by cloned *B. pertussis* genomic DNA. The relative positions of the insertional mutation sites of BRM1, BRM6, and BRM9 are shown as numbered triangles. Open arrowheads indicate the positions and orientations of the vector *lac* promoter of the recombinant plasmids; delta symbols indicate deleted sequences. Plasmids pKmL and pKmR each contain a kanamycin resistance gene insertion at a *Sph*I site; the direction of transcription of the kanamycin resistance gene is indicated by the open flags. *B. bronchiseptica* alcaligin biosynthetic mutants carrying the plasmids were evaluated for the ability (+) or inability (−) to produce siderophore on CAS agar. Abbreviations: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; P, *Pvu*II; S, *Sma*I; Sp, *Sph*I.

Initially, these cosmids were conjugally transferred to the group I mutant BRM9, and all were found to complement its alcaligin biosynthetic defect. A 4.6-kb *B. pertussis Sma*I DNA fragment shared by all of the recombinant cosmids was subcloned from representative cosmid pCP1.11 and found to restore siderophore biosynthetic capability to all three group I mutants (Fig. 1, plasmid pRK4).

Further subcloning, deletion, and complementation analysis revealed that the minimal DNA region required to restore alcaligin siderophore biosynthesis to mutants BRM1, BRM6, and BRM9 was a 4.5-kb *Bam*HI-*Sma*I subfragment (plasmid pRK13). A smaller DNA fragment lacking the *Cla*I-*Sma*I region (pRK5) did not restore alcaligin production to any of the group I mutants. Subclones lacking 5′ DNA sequences (plasmids pRK6 through pRK10) complemented mutant BRM6 in an orientation-dependent manner, suggesting that expression of the complementing biosynthetic activity was driven from the vector *lac* promoter; none of these plasmids conferred alcaligin production to mutants BRM1 and BRM9. Furthermore, since pRK6 and pRK8, but not pRK7, restored siderophore production capability to BRM6, we hypothesized that the native *Bordetella* promoter likely resides in the 5′ *Bam*HI-*Sph*I DNA region, directing transcription of a distal gene encoding an activity lacking in mutant BRM6. Plasmids pRK5, pRK11, and pRK12 failed to restore alcaligin end product biosynthesis in *trans* to BRM1 and BRM9, consistent with strong polarity effects of the mini-Tn*5 lacZ*1 mutations on the downstream gene defined by BRM6. Deletion derivative pRK11 complemented the alcaligin defect of mutant BRM6 but not BRM1 or BRM9, localizing DNA sequences predicted to encode the biosynthetic functions affected in those two mutants. While plasmid pRK10 restored alcaligin synthesis to BRM6, pRK12, with an internal *Cla*I deletion, did not complement the siderophore production defect of any group I mutant, suggesting that the gene defining the BRM6 mutation resides on the 2.2-kb *Sph*I-*Sma*I DNA subregion.

Insertion of a transcriptionally active kanamycin resistance gene in either orientation into the right-most *Sph*I site of the 4.5-kb *Bam*HI-*Sma*I fragment resulted in the failure of that cloned fragment to restore siderophore production to either BRM1 or BRM9 (Fig. 1, plasmids pKmL and pKmR). However, alcaligin production by BRM6 carrying either pKmL or pKmR was dependent on the transcriptional orientation of the resistance cassette. That is, the insertion at *Sph*I in pKmL was strongly polar, whereas that in pKmR (transcribed in the same proposed orientation as that of the *alc* genes) was not polar; thus, pKmR could restore alcaligin biosynthesis to BRM6.

These data show that the BRM6 mutation is genetically separable from those of BRM1 and BRM9 and are consistent with the presence on pRK13 of at least two distinct alcaligin biosynthesis genes cotranscribed from a *Bordetella* promoter located within the 0.7-kb *Bam*HI-*Sph*I DNA region.

***B. pertussis* alcaligin-deficient mutants.** Allelic exchange of mutated *alc* DNA fragments from plasmids pKmL and pKmR (Fig. 1) with wild-type *alc* alleles of *B. pertussis* UT25 resulted in two *B. pertussis* mutants (PM-2 and PM-3, respectively), both of which were unable to produce detectable levels of the siderophore alcaligin. These results indicate that the genetic region spanning that *Sph*I restriction site is involved in alcaligin synthesis.

Deletion of the internal *Cla*I DNA fragment from the alcaligin genetic region (as shown for pRK12 in Fig. 1) followed by allelic exchange in *B. pertussis* UT25 resulted in mutant PM6-1, which also failed to produce detectable alcaligin.

Nucleotide sequence analysis of the 0.7-kb *Bam*HI-*Sph*I DNA fragment required for expression of *alc* activities. Genetic complementation analysis of BRM1, BRM6, and BRM9 with subclones of the 4.6-kb *Sma*I DNA fragment of *B. pertussis* had determined that a terminal 0.7-kb *Bam*HI-*Sph*I subregion was required for expression of alcaligin biosynthesis activities affected in group I mutants. Nucleotide sequence analysis of the *Bam*HI-*Sph*I DNA fragment (Fig. 2) revealed the presence of sequences predicted to encode the N-terminal 47 amino acids of a *B. pertussis* homolog of oxygenase enzymes involved in siderophore biosynthesis in other bacterial species. The open reading frame beginning at nucleotide position 582 and extending through the *Sph*I site exhibits 47% amino acid sequence identity with *E. coli* L-lysine N^6-hydroxylase IucD involved in aerobactin siderophore biosynthesis (16, 27), 32% identity with *Pseudomonas aeruginosa* L-ornithine N^5-oxygenase PvdA required for pyoverdin siderophore biosynthesis (55), and 40% identity with *Ustilago maydis* L-ornithine N^5-oxygenase Sid1 involved in ferrichrome siderophore biosynthesis (34). A study published while this manuscript was in review reported the nucleotide sequence of the *B. bronchiseptica alcA* gene (GenBank accession number U32117) and also noted the similarity of the deduced protein product to these oxygenase enzymes (22). Comparison of the nucleotide sequences from the two *Bordetella* species revealed 99.4% identity over a shared 349-bp region.

Seven nucleotides upstream from the predicted AUG start codon is the Shine-Dalgarno-like sequence 5′-AGGCGA-3′, which may direct initiation of AlcA translation. Further upstream, within an extensive region of unusually low G+C nucleotide composition (34% versus the typical 66 to 70% for *Bordetella* spp.) spanning nucleotide positions 380 to 490, are two extensively overlapping 19-nucleotide sequences, 5′-GAG AATAGAAGTCATAATT-3′ (positions 418 to 436) and 5′-A ATAGAAGTCATAATTATT-3′ (positions 421 to 439), which share 12 of 19 and 11 of 19 residues, respectively, with the reported *E. coli* consensus Fur-binding site 5′-GATAATGAT AATCATTATC-3′ (17, 52). Since these 19-nucleotide sequences overlap by 16 nucleotides, they likely represent a sin-

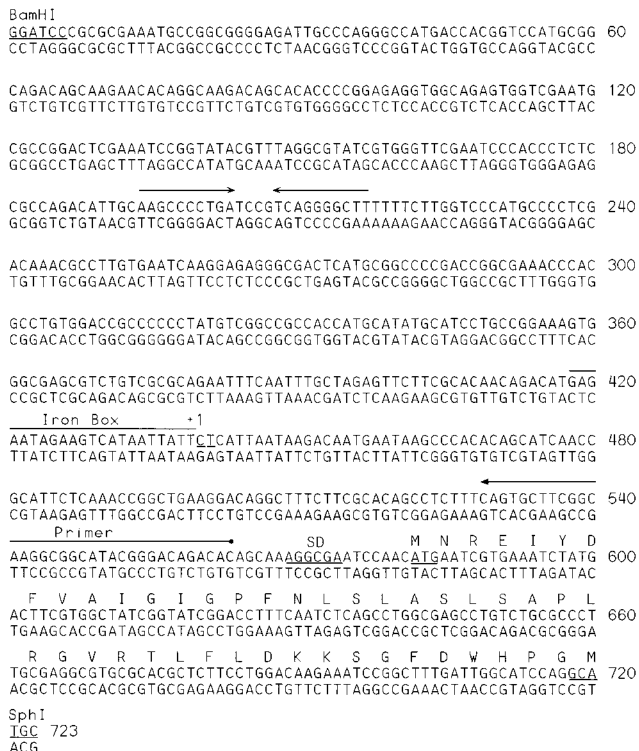

FIG. 2. Nucleotide sequence analysis of the 723-bp *B. pertussis Bam*HI-*Sph*I fragment. The nucleotide sequence including the *alcABC* operon control region and the N-terminal coding region of *alcA* is shown. The transcription initiation sites (+1) determined by primer extension analysis with the designated antisense primer are underlined. Iron Box, sequences resembling an *E. coli* Fur-binding site. The position of a Shine-Dalgarno-like sequence (SD) upstream from an open reading frame (*alcA*) is indicated. The upstream position of a putative transcription terminator is indicated by converging arrows.

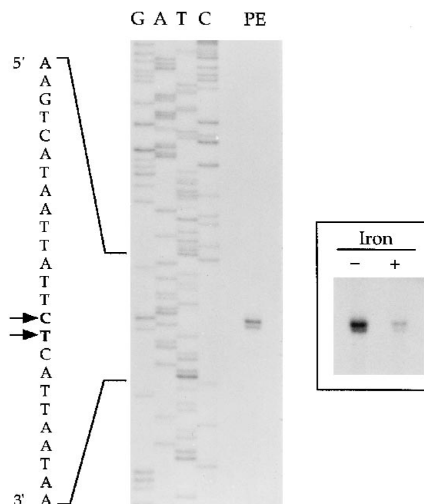

FIG. 3. Promoter mapping by primer extension (PE). (Left) Extension products resulting from the use of the antisense primer shown in Fig. 2, identifying a major transcription initiation site corresponding to the C residue at position 440, and a less-abundant product corresponding to a start site at the adjacent T residue at position 441. (Right) Overexposed autoradiograph demonstrating that iron-depleted growth conditions (−) for *B. pertussis* results in an 18-fold increase in steady-state levels of *alcA*-specific mRNA compared with that resulting from iron-replete (+) growth conditions.

gle repressor-binding site. Because the most common mechanism of transcription repression is interference with RNA polymerase binding, we hypothesized that the *B. pertussis* promoter directing *alcA* transcription was in the vicinity of these predicted Fur-binding sequences. The identification of a predicted rho-independent transcriptional terminator occupying nucleotide positions 193 to 221 further suggested that *alcA* transcription originated between that terminator and the start of the putative oxygenase open reading frame at nucleotide position 582.

Mapping of transcriptional initiation sites for the *alcA* gene. Expression of the *alc* operon is directed from a limited genetic region bounded by the upstream *Bam*HI restriction site and the *alcA* coding sequences (Fig. 2). Sites of in vivo transcription initiation from the *alcA* upstream region were localized by primer extension analysis with total cellular RNA isolated from *B. pertussis* UT25 grown under iron-replete or iron-depleted conditions. Reverse transcription of mRNA primed by a synthetic oligonucleotide representing the antisense of nucleotide positions 529 to 563 in Fig. 2 resulted in two products (Fig. 3) corresponding to a primary transcription initiation site (+1) at the C residue at position 440 in Fig. 2, located 142 nucleotides upstream from the putative start of the *alcA* coding region, and a less frequently utilized initiation site at the adjacent T residue at position 441. Densitometric analysis of autoradiographs indicated that approximately 60% of *alc* transcripts originate at the primary site of initiation, whereas 40% of *alc* transcripts start at the minor site. These sites are positioned near the 3′ terminus of the *B. pertussis* sequences strongly resembling the consensus *E. coli* Fur-binding site spanning residue positions 418 to 439. The spatial organization of the *alc* promoter region and the putative Fur-binding sequences suggest a promoter occlusion mechanism of transcription repression. Densitometric analysis of extension product signal intensities confirmed that steady-state *alcA* mRNA levels are approximately 18-fold higher in the iron-depleted RNA population than in the iron-replete RNA, suggesting that *alcA* promoter activity is responsive to iron levels in the culture medium.

Examination of sequences immediately upstream from the +1 sites revealed the presence of sequences with similarity to *E. coli* σ^{70} promoter determinants. The major *alcA* initiation site is optimally spaced 7 nucleotides from the hexameric sequence AGTCAT, which shares 3 of the 6 most highly conserved nucleotides with the *E. coli* σ^{70} −10 promoter consensus sequence TATAAT (25), including the so-called invariant T at the last position. Seventeen nucleotides upstream from this Pribnow-like sequence is the sequence CGCACA, which shares 3 of the 6 most highly favored nucleotides with the *E. coli* σ^{70} −35 consensus sequence TTGACA (25). Other sequences resembling *E. coli* σ^{70} promoter determinants exist, although at suboptimal spacing intervals with respect to the start sites. The major transcription initiation site is spaced only 4 nucleotides from the hexameric sequence CATAAT, which more closely resembles the *E. coli* σ^{70} −10 consensus sequence TATAAT, and 22 nucleotides upstream from this sequence is the hexameric sequence TTCGCA, which contains 4 of the 6 most highly favored nucleotides shared among *E. coli* σ^{70} −35 promoter determinants. However, in the absence of genetic evidence, the actual promoter sequences directing *alcA* transcription are not known with certainty. The existing database of *Bordetella* promoters is relatively small compared with that of *E. coli*, and the most well-characterized *Bordetella* promoters are dependent on the transcriptional activator BvgA (4, 51).

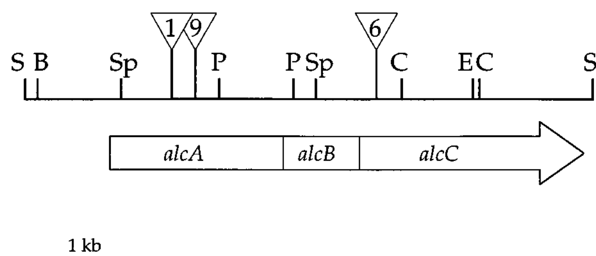

FIG. 4. Genetic organization of *alc* system genes. The positions and orientations of the *alc* genes and restriction enzyme cleavage sites are based on nucleotide sequence data as described in the text. The mutation sites of BRM1, BRM6, and BRM9 are shown as numbered triangles. Abbreviations: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; P, *Pvu*II; S, *Sma*I; Sp, *Sph*I.

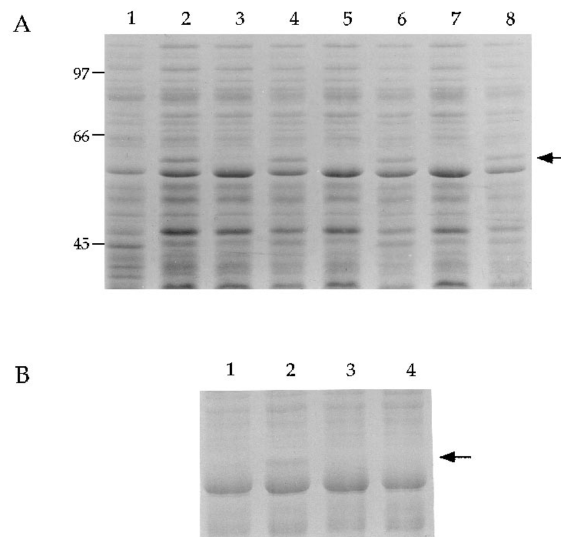

FIG. 5. SDS-PAGE analysis of *B. bronchiseptica* and *B. pertussis* alcaligin mutants. (A) Soluble fractions of *B. bronchiseptica* cells were prepared and subjected to electrophoresis as described in Materials and Methods. Lanes: 1, wild-type B013N grown under iron-replete conditions; 2, B013N grown under iron-depleted conditions; 3 to 8, soluble fractions of group I mutants carrying plasmids and grown under iron-depleted conditions (3, BRM1/pRK415 [vector]; 4, BRM1/pRK4; 5, BRM6/pRK415; 6, BRM6/pRK4; 7, BRM9/pRK415; 8, BRM9/pRK4). Positions of molecular mass standards are shown in kilodaltons on the left. (B) Total cellular proteins of *B. pertussis* wild-type strain UT25 and *alcC* mutant PM6-1. Lanes: 1, UT25 grown under iron-replete conditions; 2, UT25 grown under iron-depleted conditions; 3, PM6-1 grown under iron-replete conditions; 4, PM6-1 grown under iron-depleted conditions. The position of the 59-kDa soluble protein is denoted by an arrow on the right in both panels.

The *alcABC* gene cluster. Nucleotide sequencing of a 2.6-kb *B. pertussis* DNA region ranging from a position 170 bp upstream of the right-most *Pvu*II site to the *Sma*I site of the 4.5-kb *Bam*HI-*Sma*I fragment (data not shown) revealed the presence of two open reading frames, designated *alcB* and *alcC*. Figure 4 shows a genetic map of the proposed *alcABC* gene cluster based also, in part, on the *alcA* nucleotide sequence of *B. bronchiseptica* (22). The deduced 53-kDa AlcA protein of *B. bronchiseptica* has been reported to have 25% identity and 61% similarity with *E. coli* IucD (22), a result consistent with our analysis of the N-terminal coding region of the predicted *B. pertussis* AlcA protein. Analyses of the *B. pertussis alcBC* open reading frames, AlcB (23 kDa) and AlcC (70 kDa), revealed strong primary amino acid sequence similarities with enzymes IucB (GenBank accession number X76100 [33]) and IucC (GenBank accession number X76100 [33]) involved in the biosynthesis of the *E. coli* siderophore aerobactin. The *B. pertussis* deduced proteins AlcB and AlcC demonstrated 23% identity (over a 167-amino-acid region) with IucB and 28% identity (over a 563-amino-acid length) with IucC, respectively. The compact genetic organization and lack of canonical promoter sequences in *alc* intergenic regions, along with observed polarity effects of insertion mutations, are consistent with the existence of an operon transcribed from an iron-responsive promoter-operator region upstream of *alcA*.

Analysis of proteins of *Bordetella* alcaligin biosynthesis mutants. Initial SDS-PAGE experiments demonstrated that all three group I *B. bronchiseptica* mutants lacked the iron-repressed protein of 59 kDa, IR59 (9) (data not shown). Electrophoretic analysis showed that in the wild-type, this protein was in the soluble cell fraction, suggesting a cytoplasmic or periplasmic cellular location (Fig. 5A). Expression of the 59-kDa iron-repressed protein was restored to BRM1, BRM9, and BRM6 by supplying alcaligin biosynthetic genes in *trans* as plasmid pRK4. No other protein profile differences were observed between wild-type strain B013N and mutants BRM1, BRM9, and BRM6 in SDS-PAGE gels stained with Coomassie blue. These data suggest that the mutation in BRM6 disrupts the gene encoding IR59; BRM1 and BRM9 lack the protein as a result of the polar influence of the transposon mutations on this downstream gene. *B. pertussis* alcaligin mutant PM6-1, with a 0.6-kb *Cla*I deletion in *alcC*, also appeared to lack protein IR59 (Fig. 5B).

T7 promoter-directed protein expression. Protein expression directed by the 4.5-kb *Bam*HI-*Sma*I *B. pertussis* DNA fragment was examined by use of a bacteriophage T7 promoter expression system in *E. coli* (Fig. 6). No significant levels of any protein products were detected when the fragment was oriented with the vector T7 promoter adjacent to the *Sma*I site. However, when cloned in the opposite orientation, with the T7 promoter directing transcription in the same orientation as the proposed native *Bordetella* promoter, the plasmid directed the expression of multiple radiolabelled polypeptides ranging from 31 to 100 kDa. T7 plasmid 3 bears the same internal *Cla*I deletion as *B. pertussis* alcaligin biosynthesis mutant PM6-1 and also failed to express a 59-kDa radiolabelled protein; plasmid 4, encompassing the DNA region complementing BRM6, programmed the expression of the 59-kDa protein. Other experiments confirmed that the 59-kDa protein observed in the T7 expression experiments comigrated in electrophoretic gels with authentic IR59 of *B. bronchiseptica* B013N (data not shown). The T7 expression experiments produced a 51-kDa labelled protein which may be the product of the *alcA* gene, predicted to encode a protein of 53 kDa in *B. bronchiseptica* (22). In multiple experiments, expression of other radiolabelled species was consistent, although their presence did not correlate with any specific *alcABC* subregions examined, suggesting that the other observed products may represent artifacts of the *E. coli* expression system. The ca. 100-kDa doublet was sporadically observed in other experiments with variety of subclones (data not shown), suggesting that this polypeptide may also be an artifactual translational product or perhaps T7 RNA polymerase itself (53).

The results of the protein expression analyses together with the subcloning and complementation studies and nucleotide sequencing indicate the presence of three alcaligin biosynthesis genes (*alcA*, *alcB*, and *alcC*) on the 4.5-kb *Bam*HI-*Sma*I DNA fragment carried by pRK13, with the iron-repressed protein IR59 encoded by the promoter-distal gene *alcC*.

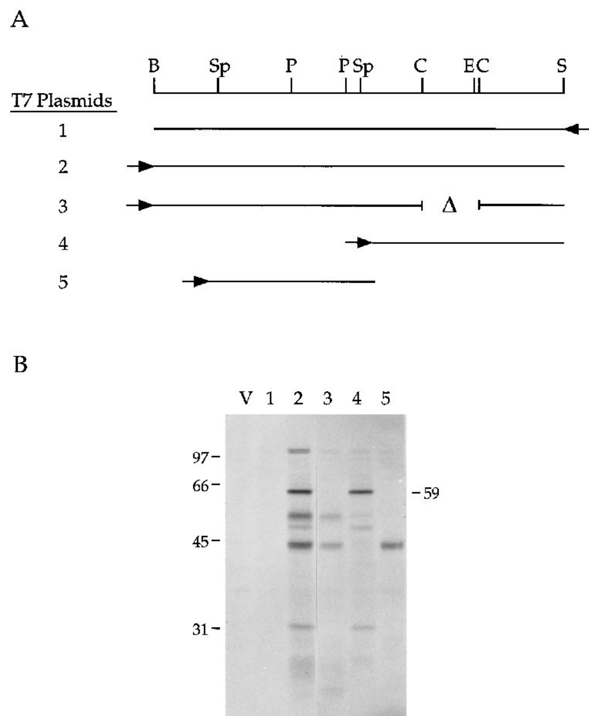

FIG. 6. Proteins expressed from the 4.5-kb *Bam*HI-*Sma*I *B. pertussis* DNA fragment by use of a T7 RNA polymerase-promoter system in *E. coli*. (A) Genetic limits of the cloned DNA fragments. Filled arrowheads represent the orientation of the vector T7 promoter; the delta symbol denotes deletion of the *Cla*I fragment. The T7 expression plasmid number is indicated on the left. Restriction site abbreviations are identical to those described in the legend to Fig. 1. (B) Autoradiogram of ^{35}S-labelled translational products resolved by SDS-PAGE. Lanes: V, vector pGEM3Z; 1 to 5, numbers correspond to the T7 plasmid numbers in panel A. Molecular mass standards (in kilodaltons) are shown on the left. The strongly radiolabelled polypeptide of 59 kDa expressed from the *alcC* region of the 4.5-kb *Bam*HI-*Sma*I fragment is indicated on the right.

DISCUSSION

Both *Alcaligenes* and *Bordetella* spp., members of the *Alcaligenaceae*, produce the macrocyclic siderophore alcaligin. *B. pertussis* and *B. bronchiseptica* are considered genetically closely related on the basis of nucleic acid hybridization and multilocus enzyme electrophoresis analyses (39); correspondingly, the DNA sequences of *B. pertussis odc* and the known sequences for the *B. bronchiseptica odc* region revealed identity at the nucleotide sequence level (10). As it was in our studies of *Bordetella odc* (10) and *fur* (9), our strategy utilizing the more readily cultured *B. bronchiseptica* for mutant construction to identify and isolate *B. pertussis* genes has proven to be an effective approach to the study of siderophore-mediated iron acquisition in *B. pertussis*. A 4.5-kb *B. pertussis* genomic *Bam*HI-*Sma*I DNA fragment was identified; this fragment could restore alcaligin production to three *B. bronchiseptica* mutants originally assigned to the same siderophore phenotypic complementation group. *B. bronchiseptica* mutants BRM1 and BRM9 carry mini-Tn*5 lacZ*1 insertions which inactivate the alcaligin biosynthesis gene *alcA*, while the BRM6 mutation is located in the genetically distinct *alcC* locus. *B. pertussis* mutants constructed by allelic exchange, PM-2 and PM-3, defined the gene *alcB*, while mutant PM6-1 is defective in *alcC* function. Complementation analysis revealed strong polarity of the transposon insertions of BRM1 and BRM9 on downstream chromosomal gene expression, resulting in an alcaligin-deficient phenotype which could not be complemented by the *alcA* or *alcAB* region alone supplied in *trans*. Additional polarity effects were observed in BRM1, BRM6, and BRM9 in experiments using the kanamycin resistance gene insertions in the 4.5-kb *alc* DNA fragment. All three *B. bronchiseptica* group I mutants lacked a 59-kDa soluble iron-repressed protein which was restored along with alcaligin production by the 4.6-kb *Sma*I *alc* DNA fragment. In T7 bacteriophage promoter protein expression experiments, the *alcC* region directed the expression of an approximately 59-kDa polypeptide, consistent with the apparent molecular mass of the soluble protein lacking in the *B. bronchiseptica* mutants as well as in *B. pertussis alcC* mutant PM6-1.

Nucleotide sequencing of the 723-bp region encompassing the 5′ region of *alcA* revealed a putative rho-independent terminator structure and an intergenic region bearing one or two overlapping proposed Fur-binding sites and the start of an open reading frame predicted to encode a protein with similarity to oxygenases of other microbial hydroxamate siderophore biosynthesis systems. The location of *alcA* is in agreement with a recent report describing the *alcA* nucleotide sequence of the related species *B. bronchiseptica* (22). The nucleotide sequence of the *B. bronchiseptica alcA* gene is predicted to encode a 53-kDa protein with an N-terminal 47-amino-acid sequence identical to that of the deduced *B. pertussis* AlcA protein described in this study.

Primer extension analysis of mRNA from *B. pertussis* grown under iron-replete and iron-depleted conditions defined primary and secondary transcriptional start sites adjacent to the proposed Fur-binding site and demonstrated iron regulation at the transcriptional level. Studies in this laboratory are under way to confirm the involvement of the proposed Fur-binding sequences in the regulation of *alcABC* gene expression and to determine the 3′ limit of the transcript. A recent study described Bvg-regulated alcaligin production in certain biotypes of *B. bronchiseptica* (21). Although examination of the *B. pertussis alcA* upstream sequences reveals several potential BvgA-binding sites (45), our studies indicate that alcaligin production in the strains of *B. bronchiseptica* and *B. pertussis* used in this study is not under BvgAS control but is regulated by iron through the activity of the Fur repressor (9).

Nucleotide sequence analysis of the *alcBC* region of the 4.5-kb *alc* fragment revealed two additional open reading frames predicted to encode proteins with similarity to biosynthetic enzymes IucB and IucC of the *E. coli* aerobactin siderophore system. These preliminary data, along with results of genetic and phenotypic analyses of our *B. bronchiseptica* and *B. pertussis* mutants, are consistent with the presence of three alcaligin biosynthesis genes, *alcA*, *alcB*, and *alcC*, in a polycistronic transcriptional organization. The sizes of the predicted Alc proteins are as follows: AlcA, 53 kDa; AlcB, 23 kDa; AlcC, 70 kDa. The corresponding Iuc protein homologs, IucD (49 kDa), IucB (37 kDa), and IucC (67 kDa), possess the enzymatic activities L-lysine N^6-hydroxylase and N^6-hydroxylysine acetyl-coenzyme A N^6-transacetylase and an activity which participates in the condensation of two molecules of N^6-acetyl-N^6-hydroxylysine and citric acid, respectively (16). Despite apparent similarities between the Alc and Iuc proteins, Giardina et al. found that the *B. bronchiseptica alcA* gene did not complement an *E. coli iucD* mutant (22). This is not surprising, given the obvious structural differences between alcaligin and aerobactin. Our protein analyses of *B. bronchiseptica* and *B. pertussis* mutants and T7 protein expression experiments consistently identified IR59 as the protein encoded by *alcC*, although the predicted polypeptide has a size of nearly 70 kDa. A labelled protein of 51 kDa observed in the T7 protein expression

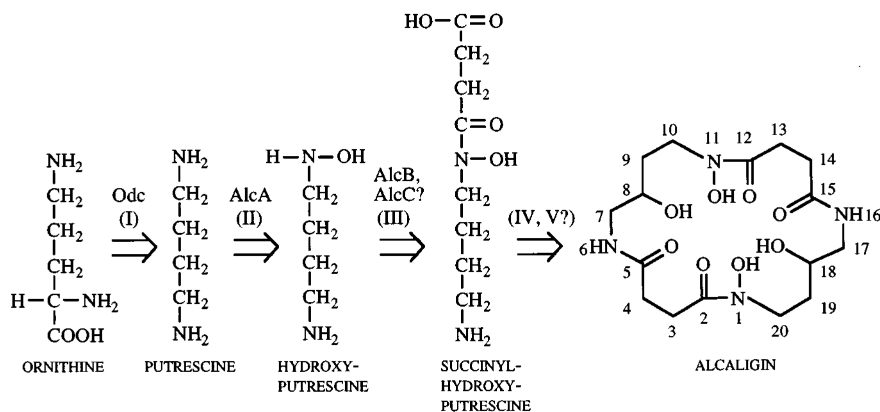

FIG. 7. Hypothetical pathway for alcaligin biosynthesis in *Bordetella* spp.

experiments has a size similar to that of the deduced AlcA protein, but a polypeptide corresponding to the predicted 23-kDa AlcB size was not detected in those studies.

Information based on our experimental data and comparison with other siderophore systems allows the prediction of a hypothetical alcaligin biosynthetic pathway (Fig. 7). The initial reaction (I) yields putrescine from ornithine by the activity of Odc ornithine decarboxylase, characterized previously (10). The *odc* gene does not map near *alcABC*, and its protein activity is not iron repressible; hence, it is not likely to be a dedicated step in alcaligin biosynthesis although it supplies an essential precursor. We propose subsequent hydroxylation of putrescine (II) by the AlcA oxygenase and an acylation step (III) involving succinate, perhaps catalyzed by a homolog of the aerobactin system enzyme IucB (potentially encoded by *alcB*). Given the structure of alcaligin, we hypothesize the involvement of an activity catalyzing the condensation of the ring (IV) and yet another activity (V) resulting in hydroxyl additions at positions 8 and 18 in the ring. The order of these postulated steps is unknown at this time; alternative biosynthetic schemes could be envisioned but are likely to require similar numbers of enzymatic activities. Since microbial siderophore biosynthesis genes are usually clustered on the chromosome, the coding regions for the uncharacterized alcaligin biosynthesis activities may be located near *alcABC.*

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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. **Alexeyev, M. F.** 1995. Three kanamycin resistance gene cassettes with different polylinkers. BioTechniques **18:**52–56.
3. **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.
4. **Arico, B., J. F. Miller, C. Roy, S. Stibitz, D. Monack, S. Falkow, R. Gross, and R. Rappuoli.** 1989. Sequences required for expression of *Bordetella pertussis* virulence factors share homology with prokaryotic signal transduction proteins. Proc. Natl. Acad. Sci. USA **86:**6671–6675.
5. **Armstrong, S. K.** Unpublished data.
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. **Blanton, K. J., G. D. Biswas, J. Tsai, J. Adams, D. W. Dyer, S. M. Davis, G. G. Koch, P. K. Sen, and P. F. Sparling.** 1990. Genetic evidence that *Neisseria gonorrhoeae* produces specific receptors for transferrin and lactoferrin. J. Bacteriol. **172:**5225–5235.
8. **Bordet, J., and O. Gengou.** 1906. Le microbe de la coqueluche. Ann. Inst. Pasteur (Paris) **20:**731–741.
9. **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.
10. **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.
11. **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.
12. **Brown, D. R., and C. D. Parker.** 1987. Cloning of the filamentous hemagglutinin of *Bordetella pertussis* and its expression in *Escherichia coli*. Infect. Immun. **55:**154–161.
13. **Bullen, J. J.** 1981. The significance of iron in infection. Rev. Infect. Dis. **3:**1127–1138.
14. **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.
15. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
16. **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.
17. **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.
18. **DeShazer, D., G. E. Wood, and R. L. Friedman.** 1994. Boiling eliminates artifact banding when sequencing double-stranded templates. BioTechniques **17:**288–290.
19. **Field, L. H., and C. D. Parker.** 1979. Differences observed between fresh isolates of *Bordetella pertussis* and their laboratory-passaged derivatives, p. 124–132. *In* C. R. Manclark and J. C. Hill (ed.), International Symposium on Pertussis. U.S. Department of Health, Education, and Welfare, Washington, D.C.
20. **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.
21. **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.
22. **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.
23. **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.
24. **Grant, C. C. R., and M. L. Vasil.** 1986. Analysis of transcription of the exotoxin A gene of *Pseudomonas aeruginosa*. J. Bacteriol. **168:**1112–1119.
25. **Hawley, D. K., and W. R. McClure.** 1983. Compilation and analysis of *Escherichia coli* promoter DNA sequences. Nucleic Acids Res. **11:**2237–2255.

26. **Hein, J. J.** 1990. Unified approach to alignment and phylogenies. Methods Enzymol. **183:**626–645.
27. **Herrero, M., V. de Lorenzo, and J. B. Neilands.** 1988. Nucleotide sequence of the *iucD* gene of the pColV-K30 aerobactin synthesis operon and topology of its product studied with *phoA* and *lacZ* gene fusions. J. Bacteriol. **170:** 56–64.
28. **Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger.** 1988. Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. Gene **70:**191–197.
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. **Lankford, C. E.** 1973. Bacterial assimilation of iron. Crit. Rev. Microbiol. **2:**273–331.
31. **Litwin, C. M., and S. B. Calderwood.** 1993. Role of iron in regulation of virulence genes. Clin. Microbiol. Rev. **6:**137–149.
32. **Lory, S.** 1986. Effect of iron on accumulation of exotoxin A-specific mRNA in *Pseudomonas aeruginosa*. J. Bacteriol. **168:**1451–1456.
33. **Martinez, J. L.** 1994. Unpublished data.
34. **Mei, B., A. D. Budde, and S. A. Leong.** 1993. *sid1*, a gene initiating siderophore biosynthesis in *Ustilago maydis*: molecular characterization, regulation by iron, and role in phytopathogenicity. Proc. Natl. Acad. Sci. USA **90:**903–907.
35. **Mietzner, T. A., and S. A. Morse.** 1994. The role of iron-binding proteins in the survival of pathogenic bacteria. Annu. Rev. Nutr. **14:**471–493.
36. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
37. **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.
38. **Morton, D. J., and P. Williams.** 1990. Siderophore-independent acquisition of transferrin-bound iron by *Haemophilus influenzae* type b. J. Gen. Microbiol. **136:**927–933.
39. **Musser, J. M., E. L. Hewlett, M. S. Peppler, and R. K. Selander.** 1986. Genetic diversity and relationships in populations of *Bordetella* spp. J. Bacteriol. **166:**230–237.
40. **Neilands, J. B.** 1995. Siderophores: structure and function of microbial iron transport compounds. J. Biol. Chem. **270:**26723–26726.
41. **Nishio, T., and Y. Ishida.** 1990. Production of dihydroxamate siderophore alcaligin by *Alcaligenes denitrificans* subsp. *xylosoxydans*. Agric. Biol. Chem. **54:**1837–1839.
42. **Nishio, T., N. Tanaka, J. Hiratake, Y. Katsube, Y. Ishida, and J. Oda.** 1988. Isolation and structure of the novel dihydroxamate siderophore alcaligin. J. Am. Chem. Soc. **110:**8733–8734.
43. **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.
44. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
45. **Scarlato, V., A. Prugnola, B. Arico, and R. Rappuoli.** 1991. The *bvg*-dependent promoters show similar behaviour in different *Bordetella* species and share sequence homologies. Mol. Microbiol. **5:**2493–2498.
46. **Schmitt, M. P., and R. K. Holmes.** 1991. Iron-dependent regulation of diphtheria toxin and siderophore expression by the cloned *Corynebacterium diphtheriae* repressor gene *dtxR* in *C. diphtheriae* C7 strains. Infect. Immun. **59:**1899–1904.
47. **Schneider, D. R., and C. D. Parker.** 1982. Effect of pyridines on phenotypic properties of *Bordetella pertussis*. Infect. Immun. **38:**548–553.
48. **Schwyn, B., and J. B. Neilands.** 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. **160:**47–56.
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. **Stibitz, S.** 1994. Use of conditionally counterselectable suicide vectors for allelic exchange. Methods Enzymol. **235:**458–465.
51. **Stibitz, S., and J. F. Miller.** 1994. Coordinate regulation of virulence in *Bordetella pertussis* mediated by the *vir* (*bvg*) locus, p. 407–422. *In* V. L. Miller, J. B. Kaper, D. A. Portnoy, and R. R. Isberg (ed.), Molecular genetics of bacterial pathogenesis. American Society for Microbiology, Washington, D.C.
52. **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.
53. **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.
54. **Tsai, J., D. W. Dyer, and P. F. Sparling.** 1988. Loss of transferrin receptor activity in *Neisseria meningitidis* correlates with inability to use transferrin as an iron source. Infect. Immun. **56:**3132–3138.
55. **Visca, P., A. Ciervo, and N. Orsi.** 1994. Cloning and nucleotide sequence of the *pvdA* gene encoding the pyoverdin biosynthetic enzyme L-ornithine N^5-oxygenase in *Pseudomonas aeruginosa*. J. Bacteriol. **176:**1128–1140.
56. **Weinberg, E. D.** 1995. Acquisition of iron and other nutrients in vivo, p. 79–93. *In* J. A. Roth, C. A. Bolin, K. A. Brogden, F. C. Minion, and M. J. Wannemuehler (eds.), Virulence mechanisms of bacterial pathogens, 2nd ed. American Society for Microbiology, Washington, D.C.