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Transcriptional Analysis of the *Bordetella* Alcaligin Siderophore Biosynthesis Operon

HO YOUNG KANG AND SANDRA K. ARMSTRONG*

Department of Microbiology and Immunology, East Carolina University School of Medicine, Greenville, North Carolina 27858-4354

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The *alc* gene cluster of *Bordetella pertussis* includes three genes, *alcA*, *alcB*, and *alcC*, which are involved in alcaligin siderophore biosynthesis in response to iron starvation. The production of AlcA, AlcB, and AlcC in *Bordetella* cells and the transcriptional organization of *alcA*, *alcB*, and *alcC* were investigated by using a set of three *alc′-′lacZ* gene fusion constructs that were contiguous with the known promoter upstream of *alcA* and extended to fusion junctions within each *alc* cistron. All three *alc′-′lacZ* fusions exhibited iron-repressible reporter gene expression which was abolished by deletion of the 105-bp *alcA* promoter-operator region. In an immunoblot analysis using a monoclonal antibody specific for β-galactosidase, the AlcA-LacZ, AlcB-LacZ, and AlcC-LacZ hybrid proteins were detected in *Bordetella* cells grown under iron-depleted conditions. A *B. pertussis* mutant in which the 105-bp *alcA* promoter-operator region was deleted by allelic exchange was unable to produce detectable levels of siderophore. Hybridization analysis using gene-specific probes showed that *alc*-specific transcript levels in the mutant were negligible compared with those of the wild-type parent. These results confirm that *alcA*, *alcB*, and *alcC* are cotranscribed from an iron-regulated control region immediately upstream of *alcA*. Transcript analysis using hybridization probes representing regions downstream of *alcC* demonstrated that *alc* transcription extends approximately 3.6 kb further downstream from the *alcC* coding region, suggesting the cotranscription of additional, uncharacterized alcaligin system genes.

To establish infection, pathogenic bacteria must successfully compete for a limited iron pool (9, 32). As a defense mechanism to prevent bacterial growth, the mammalian host maintains extremely low levels of free extracellular iron through the action of iron-binding proteins, such as transferrin and lactoferrin (4). One bacterial iron retrieval strategy involves the secretion of high-affinity iron-chelating siderophores (18, 25). Siderophores are produced in response to iron limitation and are capable of removing iron from host sources such as transferrin and lactoferrin (4, 20, 32).

Bordetella pertussis and *Bordetella bronchiseptica* are gram-negative bacterial pathogens that cause respiratory diseases in mammals. The native siderophore of both *B. pertussis* and *B. bronchiseptica* is the macrocyclic dihydroxamate alcaligin (8, 22) which is expressed in low-iron growth conditions and is under the control of the ferric uptake regulator protein, Fur (2, 6). The phenotypes of previously isolated *B. bronchiseptica* siderophore-deficient mutants suggested that multiple genes were involved in alcaligin biosynthesis (1); these mutants were adopted as tools with which to identify the homologous alcaligin biosynthesis genes in *B. pertussis*. Analysis of one class of mutants led to the identification of the *Bordetella odc* gene, which encodes an ornithine decarboxylase catalyzing the conversion of ornithine to putrescine, an essential alcaligin precursor (7). In related studies, a 4.5-kb *Bam*HI-*Sma*I *B. pertussis* genomic DNA fragment which corresponded to the mutated chromosomal regions of three *B. bronchiseptica* siderophore mutants was identified (16). Mutant complementation analysis using subclones of the 4.5-kb region, nucleotide sequence analysis, and protein expression studies suggested the existence of a putative iron-responsive promoter upstream of three alcaligin biosynthesis genes, *alcA*, *alcB*, and *alcC*, which appeared to be organized in an operon (16). The deduced AlcA proteins of *B. pertussis* (16) and *B. bronchiseptica* (15) and the deduced AlcB and AlcC proteins of *B. pertussis* (16) share strong primary amino acid sequence similarities with IucD, IucB, and IucC, respectively, involved in the biosynthesis of the *Escherichia coli* siderophore aerobactin (19, 24). The transcription initiation site of *alcA* was mapped to a position adjacent to a putative Fur repressor binding site (16).

In previous studies, we and others readily visualized the iron-regulated AlcC protein expressed in *B. pertussis* and *B. bronchiseptica*, while expression of AlcA and AlcB was not apparent in either *Bordetella* cells or in *E. coli* by use of a T7 RNA polymerase-promoter protein expression system (16). Although genetic complementation results showing polarity of transposon insertion mutations on downstream *alc* genes and nucleotide sequence data suggested the transcriptional linkage of *alcA*, *alcB*, and *alcC*, conclusive evidence for the cotranscription of these genes was still required, and the 3′ genetic limit of the operon remained unknown. Potentially, the operon may include additional, as-yet-undefined genes downstream of *alcC*. In this study, we examined the *alc* operon, using reporter gene fusion constructs and a *B. pertussis alc* promoter-operator region deletion mutant. We report the expression of AlcA, AlcB, and AlcC hybrid proteins in *Bordetella* cells and establish the existence of an iron-repressible operon transcribed from a promoter upstream of *alcA*.

MATERIALS AND METHODS

Bacterial strains and plasmids. *B. bronchiseptica* B013N, a nalidixic acid-resistant derivative of wild-type strain B013 (1), and *B. pertussis* UT25Sm1, a streptomycin-resistant derivative of wild-type *B. pertussis* strain UT25 (12), have been described previously. *E. coli* DH5α [F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*) *U169 endA1 recA1 hsdR17*(r_K^- m_K^+) *deoR thi-1 supE44* λ$^-$ *gyrA96 relA1*] (Gibco BRL, Gaithersburg, Md.) was used as the host for general DNA manipulations. *E. coli* DH5α harboring pRK2013 (13) provided mobilization functions in tripa-

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Biotechnology Building, Room 116, East Carolina University School of Medicine, Greenville, NC 27858-4354. Phone: (919) 816-3125. Fax: (919) 816-3535. E-mail: armstrong@brody.med.ecu.edu.

rental matings. Plasmid vectors pGEM3Z (Promega, Madison, Wis.), pBluescript SK$^+$ (Stratagene, La Jolla, Calif.), and the broad-host-range plasmid vector pBBR1MCS (17) were used for the construction of recombinant plasmids, and suicide plasmid vector pSS1129 (30) was employed for allelic-exchange mutagenesis. β-Galactosidase translational fusions were constructed by using plasmids YIp356, YIp357, and YIp358R (23). Recombinant cosmid pCP1.11 (16) was the source of *B. pertussis* UT25 DNA for alcaligin system gene probes used in hybridization experiments; pBSK+4 contains a 4.5-kb *Bam*HI-*Sma*I *B. pertussis* DNA subfragment of pCP1.11 encompassing *alcA*, *alcB*, and *alcC* (16).

Growth conditions. *E. coli* was grown aerobically on Luria-Bertani (LB) medium (26); *B. bronchiseptica* and *B. pertussis* were cultured on LB agar and Bordet-Gengou agar (5), respectively. Iron-replete or iron-depleted modified Stainer-Scholte (SS) medium (28) was used for liquid culture as described previously (1). Growth was monitored with a Klett-Summerson colorimeter fitted with a no. 54 filter (Klett Manufacturing Co., Long Island City, N.Y.). *Bordetella* cells grown on agar plates were used to inoculate iron-replete SS broth seed cultures. Seed cultures were grown at 37°C with shaking, and the cells were harvested, washed twice with iron-depleted SS broth, and used to inoculate iron-replete or iron-depleted SS media to an initial density of 25 to 30 Klett units. For selection of plasmid-containing strains or selection of mutants constructed by allelic exchange, appropriate antibiotics were added to the culture media at the indicated concentrations (in micrograms per milliliter): ampicillin, 100 for *E. coli* and 50 for *B. pertussis*; chloramphenicol, 30; gentamicin, 10; kanamycin, 50; nalidixic acid, 35; streptomycin, 50; and tetracycline, 15.

General DNA manipulations. Recombinant plasmid isolation, transformation of *E. coli*, restriction endonuclease analysis, and ligation of DNA fragments were performed as described previously (26). Transfer of plasmids from *E. coli* to *Bordetella* cells was carried out by triparental crosses as described by Brickman and Armstrong (7). Nucleotide sequencing using double-stranded plasmid templates was performed by the dideoxy chain termination method (27) as modified by DeShazer and coworkers (11), using [α-^{32}P]dATP (ICN Radiochemicals, Irvine, Calif.) and a Sequenase version 2.0 kit (United States Biochemical Corp., Cleveland, Ohio). Southern and colony DNA hybridizations were performed under high-stringency conditions as described previously (26). Hybridization probes were labelled with [α-^{32}P]dCTP (ICN Radiochemicals) by the random priming method using the Random Primers DNA Labeling System (Gibco BRL). *B. pertussis* chromosomal DNA was isolated by a method described previously (33).

Siderophore detection. The chrome azurol S (CAS) universal siderophore detection assay (29) was performed to monitor siderophore production by *Bordetella* cells grown in iron-replete or iron-depleted SS medium by measuring the decrease in A_{630} of the CAS dye reaction as reported previously (1).

Construction of the *alcA* promoter-operator region deletion plasmid. The 0.7-kb *Bam*HI-*Sph*I DNA fragment was isolated from pBSK+4 and digested with restriction endonucleases *Nde*I and *Ase*I, which generated compatible cohesive ends. The 343-bp *Bam*HI-*Nde*I and 280-bp *Ase*I-*Sph*I fragments were ligated with the vector pGEM3Z digested with *Bam*HI and *Sph*I, resulting in plasmid p3Z17. The resulting *Nde*I-*Ase*I deletion removed the 105-bp *alcA* promoter-operator region containing the putative Fur binding site and transcription start site of *alcA* (16). The correct deletion and ligation were verified by nucleotide sequencing. The original 0.7-kb *Bam*HI-*Sph*I fragment of pBSK+4 was replaced with a 0.6-kb *Bam*HI-*Sph*I deletion derivative fragment isolated from p3Z17, generating plasmid pBSK+5 (see Fig. 1).

Construction of protein fusions. For the construction of *alcA′-′lacZ*, *alcAB′-′lacZ*, and *alcABC′-′lacZ* translational fusion plasmids, the 1.5-kb *Bam*HI-*Pst*I, 2.3-kb *Bam*HI-*Sph*I, and 3.6-kb *Bam*HI-*Eco*RI *alc* DNA fragments isolated from pBSK+4 were ligated upstream of the promoterless *′lacZ* genes of the vectors YIp357, YIp356, and YIp358R, respectively. Similarly, to construct the corresponding *alcA* promoter-operator region deletion derivatives, the 1.4-kb *Bam*HI-*Pst*I, 2.2-kb *Bam*HI-*Sph*I, and 3.5-kb *Bam*HI-*Eco*RI DNA fragments isolated from deletion plasmid pBSK+5 were ligated in frame with the *′lacZ* gene of the vectors YIp357, YIp356, and YIp358R, respectively. Because these vectors harbor the ColE1 origin of replication for maintenance in *E. coli*, the *alc′-′lacZ* fusions were subcloned as *Bam*HI-*Apa*I fragments into the broad-host-range vector pBBR1MCS (17) for use in *Bordetella* species. The resultant plasmids were named pBB8, pBB15, pBB9, pBB11, pBB16, and pBB12 (see Fig. 2). In-frame fusion of each *alc′-′lacZ* construct was verified by nucleotide sequencing.

β-Galactosidase assays. β-Galactosidase assays were performed by the method of Miller (21). *B. bronchiseptica* cells grown in iron-replete or iron-depleted SS medium were permeabilized with chloroform-sodium dodecyl sulfate (SDS). The enzyme activities were measured by cleavage of the chromogenic substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG) and expressed in Miller units.

Immunoblot analysis. Cultures grown under iron-replete and iron-depleted conditions were concentrated by centrifugation and each adjusted to an optical density at 600 nm equivalent to 5.0. A 50-μl volume of cell suspension was treated by being boiled for 5 min in digestion buffer consisting of 0.65% SDS, 6.26% glycerol, 6.25% 2-mercaptoethanol, 0.0025% bromophenol blue, 3% urea, and 0.125 M Tris (pH 6.8). Proteins were separated by SDS-polyacrylamide gel electrophoresis using 7.5% polyacrylamide gels containing 3% urea (28), transferred electrophoretically to nitrocellulose membranes as described by Towbin et al. (31), and processed as described previously (14). The membranes were blocked with 3% bovine serum albumin in 10 mM Tris–0.9% NaCl (pH 7.4) and incubated with a 1:5,000 dilution of mouse monoclonal antibody specific for β-galactosidase (Promega) and then a 1:2,000 dilution of peroxidase-conjugated goat anti-mouse immunoglobulin G (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.). The positive control was 1 μl of high-molecular-mass protein standards product (Bio-Rad Laboratories, Hercules, Calif.) containing β-galactosidase.

RNA preparation and analysis. Total RNA of *B. pertussis* UT25Sm1 and mutant PM-4 was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method (10) from cells grown under iron-replete or iron-depleted conditions as previously described (16).

For Northern hybridization, 50-μg samples of RNA were subjected to electrophoresis on 1.2% agarose-formaldehyde gels and transferred to nitrocellulose membranes as described elsewhere (26). For dot blot hybridizations, twofold dilutions of RNA samples (20 to 0.63 μg) were applied to a nitrocellulose membrane by using a 96-well vacuum manifold apparatus, and each well was rinsed twice with 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The membranes were baked at 80°C for 90 min, prehybridized, and incubated at 42°C with radiolabelled gene- or region-specific DNA probes in a solution containing 5× Denhardt's solution, 50% formamide, 6× SSPE (1× SSPE is 0.15 M NaCl, 0.01 M NaH_2PO_4, and 1.25 mM EDTA), 0.5% SDS, and 100 μg of denatured sheared salmon sperm DNA per ml. Transcript levels were quantitated with a PhosphorImager (model 425E; Molecular Dynamics, Sunnyvale, Calif.). Alternatively, membranes were subjected to autoradiography, and quantitation of signal intensities was performed on a Macintosh PowerPC computer using the public domain NIH Image version 1.61 software package (developed at the National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Construction of the *B. pertussis alcA* promoter-operator deletion mutant. The 3.5-kb *Bam*HI-*Eco*RI DNA fragment isolated from deletion plasmid pBSK+5 was subcloned to the suicide vector pSS1129 (30) and conjugally transferred to *B. pertussis* UT25Sm1 to transfer the mutation to the chromosome by homologous recombination. Presumptive mutants lacking the 105-bp *alcA* promoter-operator region were identified by colony hybridization using the 105-bp *Nde*I-*Ase*I DNA fragment isolated from pBSK+4 as a probe. Correct allelic exchange in mutant PM-4 was verified by Southern hybridization analysis of chromosomal DNA using probes spanning the deletion junction.

RESULTS

Iron-regulated expression of *alcA*, *alcB*, and *alcC*. Since expression of the *alcA* and *alcB* gene products was not detected in an earlier study (16), a set of β-galactosidase translational fusions was constructed. Each fusion carried DNA sequences contiguous with the known promoter upstream of *alcA* and extending to fusion junctions within each *alc* cistron. To investigate the potential cotranscription of *alcA*, *alcB*, and *alcC* directed by the promoter-operator located upstream of *alcA*, a 105-bp deletion (positions −100 to +5 relative to the *alcA* transcription start site) (Fig. 1) was introduced into each *alc′-′lacZ* fusion construct to produce the corresponding deletion set of fusions (Fig. 2). The 105-bp deletion encompasses the putative Fur binding and transcriptional start sites, yet does not impinge on the *alcA* coding region.

Wild-type *B. bronchiseptica* B013N harboring the fusion plasmid constructs was cultured in iron-replete or iron-depleted growth conditions to detect the expressed Alc-LacZ hybrid proteins by measurement of β-galactosidase fusion protein activity (Fig. 2). The iron starvation status of the cultures was monitored by measurement of siderophore activity in supernatants (data not shown). *Bordetella* cells containing pBB8, pBB15, and pBB9 expressed approximately 34-, 17-, and 20-fold increases in levels of β-galactosidase activity, respectively, under iron starvation growth conditions compared with iron-replete conditions. This iron-regulated expression of the Alc-LacZ hybrid proteins in *Bordetella* cells confirms the in vivo expression of the proteins encoded by *alcA*, *alcB*, and *alcC*. Moreover, deletion of the 105-bp *alcA* promoter-operator region in the *alc* fusion constructs (derivatives pBB11, pBB16, and pBB12) abolished the expression of β-galactosidase activities in *B. bronchiseptica* cells grown under iron-depleted conditions, indicating that the 105-bp region is required for iron-

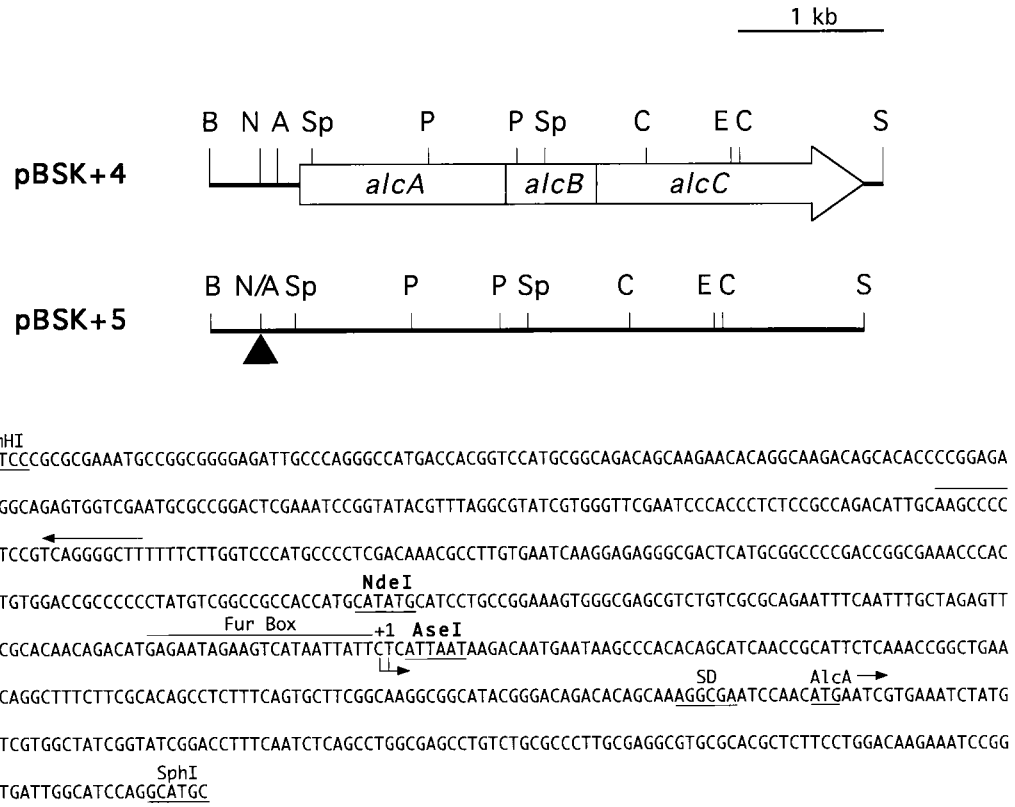

FIG. 1. Schematic overview of the genetic organization of *B. pertussis alcABC* genes and the direction of transcription. The nucleotide sequence of the 723-bp *Bam*HI-*Sph*I DNA region used in the construction of the *Nde*I-*Ase*I deletion of the *alcA* promoter-operator is shown below the diagram. The putative Fur repressor binding site (Fur Box), the *alcA* transcription initiation site determined previously (16) (+1), the position of a Shine-Dalgarno-like sequence (SD) upstream from the *alcA* open reading frame, and the upstream position of a putative transcription terminator (converging arrows) are indicated. Plasmid pBSK+5 carries the same insert DNA fragment as pBSK+4 but has the 105-bp *Nde*I-*Ase*I region upstream of *alcA* deleted (triangle). Abbreviations for restriction endonuclease sites: A, *Ase*I; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; N, *Nde*I; P, *Pvu*II; S, *Sma*I; Sp, *Sph*I; N/A, *Nde*I-*Ase*I deletion junction.

responsive transcription of not only *alcA*, but of *alcB* and *alcC* as well. This mutation also resulted in increased LacZ expression in cells carrying fusions pBB11 (encoding AlcA-LacZ) and pBB12 (AlcC-LacZ) under iron-replete, versus iron-depleted, growth conditions. However, cells carrying pBB16 (AlcB-LacZ), in which the same 105-bp DNA region was deleted, expressed negligible β-galactosidase activity, regardless of the iron status of the growth medium.

To visualize the AlcA-LacZ, AlcB-LacZ, and AlcC-LacZ hybrid proteins expressed in *B. bronchiseptica*, proteins from solubilized cells from the same cultures used for the β-galactosidase assays were separated by SDS-polyacrylamide gel electrophoresis and subjected to immunoblot analysis using a monoclonal antibody specific for β-galactosidase. The immunoreactive AlcA-LacZ, AlcB-LacZ, and AlcC-LacZ hybrid proteins were detected in cells grown under iron-depleted con-

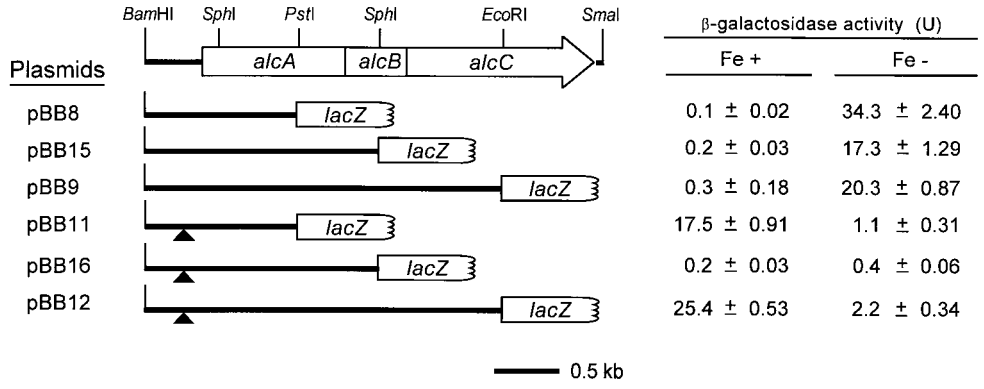

FIG. 2. Iron-regulated expression and cotranscription of *alcA*, *alcB*, and *alcC*. Genetic maps of *alcA'-'lacZ*, *alcAB'-'lacZ*, and *alcABC'-'lacZ* translational fusions carried on the designated plasmids are shown. Deletions of the 105-bp *Nde*I-*Ase*I fragment containing the *alcA* promoter-operator region shown in Fig. 1 are indicated (triangles). Levels of β-galactosidase expressed in wild-type *B. bronchiseptica* B013N containing *alc'-'lacZ* fusion plasmids in response to iron-replete (Fe+) and iron-depleted (Fe−) growth conditions are reported in Miller units (21) and are expressed as means of triplicate measurements ($n = 3$) ± standard deviations.

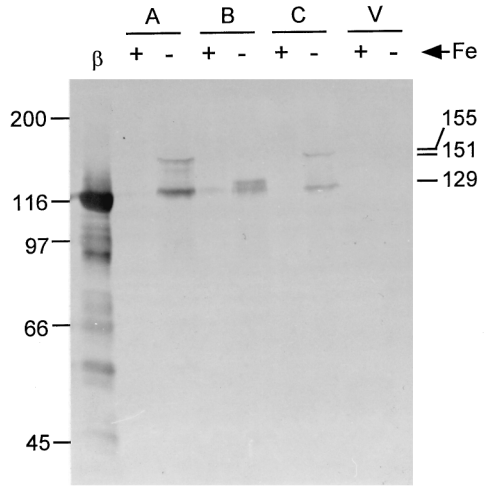

FIG. 3. Immunoblot analysis of *B. bronchiseptica* B013N containing fusion plasmids. Cells grown in iron (Fe)-replete (+) and iron-depleted (−) conditions were subjected to immunoblot analysis as described in Materials and Methods. The Alc-LacZ hybrid proteins were visualized by reactivity with anti-β-galactosidase monoclonal antibody. Lanes: β, β-galactosidase positive control; A, cells carrying *alcA'-'lacZ* plasmid pBB8; B, *alcAB'-'lacZ* (pBB15); C, *alcABC'-'lacZ* (pBB9); V, vector plasmid pBBR1MCS. The relative migration positions of protein standards (left) and estimated molecular masses of Alc-LacZ hybrid proteins (right) are indicated in kilodaltons.

ditions, and their apparent molecular masses were approximately 151, 129, and 155 kDa, respectively (Fig. 3). Little or no antibody reactivity was detected in cells grown in high-iron medium or in plasmid vector control samples. Densitometric analysis of the immunoblots showed that levels of fusion protein expression were proportional to the β-galactosidase enzyme activities measured in the cells (data not shown).

On the basis of the nucleotide sequences of the *alc'-'lacZ* fusions, the calculated molecular masses of AlcA-LacZ, AlcB-LacZ, and AlcC-LacZ are 152.1 kDa (1,332 amino acid residues), 128.8 kDa (1,133 amino acid residues), and 155.2 kDa (1,366 amino acid residues), respectively. The observed and calculated molecular masses determined from these studies are therefore consistent with the predicted translation start codons for the *alcA*, *alcB*, and *alcC* open reading frames identified in our previous studies (16). The immunoreactive species of approximately 120 kDa detected in cells containing the *alc'-'lacZ* constructs is hypothesized to be a degradation product and may correspond to the LacZ portion of the fusion proteins.

Construction and transcriptional analysis of a *B. pertussis alcA* promoter-operator region deletion mutant. To further establish the role of the *alcA* promoter in directing cotranscription of *alcABC* and to evaluate transcription of the entire operon, the 105-bp *alcA* promoter-operator region deletion was introduced into the chromosome of *B. pertussis* by allelic exchange. The mutant, PM-4, was unable to produce detectable levels of alcaligin siderophore (data not shown), indicating that the chromosomal deletion of 105 bp upstream of *alcA* abrogated the expression of alcaligin biosynthesis genes, consistent with the results observed in the *alc'-'lacZ* reporter gene plasmid experiments. Supplying *alcABC* in *trans* as the 4.5-kb *Bam*HI-*Sma*I fragment restored siderophore activity to PM-4 (data not shown).

Results from this study and previous work (16) for the alcaligin gene cluster were consistent with a polycistronic transcriptional organization for *alcA*, *alcB*, and *alcC*. To provide biochemical evidence for the proposed operonic structure of the *alcABC* region, transcript analysis was performed using total RNA isolated from both wild-type *B. pertussis* and *B. pertussis alc* promoter-operator mutant PM-4 grown in high- and low-iron medium.

(i) Northern blot analysis. RNA samples from wild-type cells and PM-4 were subjected to Northern hybridization analysis using the following DNA probes (Fig. 4A) specific for each *alc* gene: *alcA*, 0.5-kb *Sph*I-*Pst*I fragment; *alcB*, 0.2-kb *Pst*I-*Sph*I fragment; and *alcC*, 0.6-kb internal *Cla*I fragment. While strong hybridization signals were detected for the RNA samples isolated from wild-type cells grown in low-iron conditions, no signal was observed for RNA samples from wild-type cells grown in high-iron conditions or from the promoter deletion mutant grown in either low- or high-iron conditions (data not shown). However, the sizes of the RNA transcripts from iron-starved wild-type cells could not be determined with confidence because of apparent rapid turnover of *alc* mRNA. Therefore, RNA dot hybridization using gene- and region-specific probes was employed as an alternative approach to quantitate *alc*-specific messages.

(ii) Cotranscription of *alcA*, *alcB*, and *alcC* from the *alcA* promoter. RNA samples isolated from both wild-type cells and the promoter deletion mutant PM-4 grown in low- and high-iron media were hybridized with the DNA probes specific for *alcA*, *alcB*, and *alcC*. In the RNA samples from wild-type cells grown in low-iron conditions, *alcA*, *alcB*, and *alcC* transcripts were detected, whereas few or no *alc*-specific transcripts were detected in RNA preparations from these cells grown in high-iron conditions. RNA samples isolated from mutant PM-4 cultured under either low- or high-iron conditions hybridized weakly, if at all, with the *alcA*, *alcB*, and *alcC* probes (Fig. 4A). Densitometric analysis of the autoradiograms showed at least a 3- to 10-fold increase in the levels of *alcA*, *alcB*, and *alcC* transcripts from iron-starved wild-type cells versus those grown in high-iron conditions, confirming the iron-regulated transcription of *alcA*, *alcB*, and *alcC* noted in our previous studies (16). Further, these results establish that *alcA*, *alcB*, and *alcC* are cotranscribed from an iron-regulated promoter-operator region upstream of *alcA*. Deletion of this promoter region abrogates transcription of these three *alc* genes, which comprise all or part of the known alcaligin biosynthesis operon.

(iii) Determination of the 3′ genetic limit of the *alcABC*-containing operon. To determine the 3′ limit of the *alcABC*-containing operon, RNA dot hybridization was performed using the RNA from wild-type *B. pertussis* and mutant PM-4 with DNA probes representing genetic regions downstream of *alcC*. Probes derived from a 0.7-kb *Sac*I-*Eco*RI DNA region (0.6 kb downstream from *alcC*) and a 0.4-kb *Eco*RI-*Sac*I DNA region (1.3 kb downstream of *alcC*) hybridized with the RNA samples in an iron-repressible pattern essentially the same as that observed in the dot blots using the *alcA*, *alcB*, and *alcC* probes. Iron-regulated transcripts were detected in wild-type cells but were negligible in *B. pertussis* mutant PM-4 RNA samples (Fig. 4B, probes D and E). Densitometric analysis of the autoradiograms also revealed at least a three- to fivefold increase in levels of *alc* region transcripts from wild-type cells grown in low-iron over high-iron conditions, similar to the patterns observed for *alcA*, *alcB*, and *alcC* transcripts.

We have obtained the nucleotide sequence of a 1.6-kb *Kpn*I-*Pst*I fragment located 2 kb downstream of *alcC* and identified a gene, *alcR*, which is involved in the regulation of alcaligin siderophore system genes (3). A DNA probe derived from a 0.5-kb *Sma*I-*Pvu*II fragment internal to *alcR* hybridized strongly with RNA from wild-type cells grown in low-iron conditions compared with the results for transcripts from iron-starved mutant PM-4 (Fig. 4B, probe F). Quantitative analysis of mul-

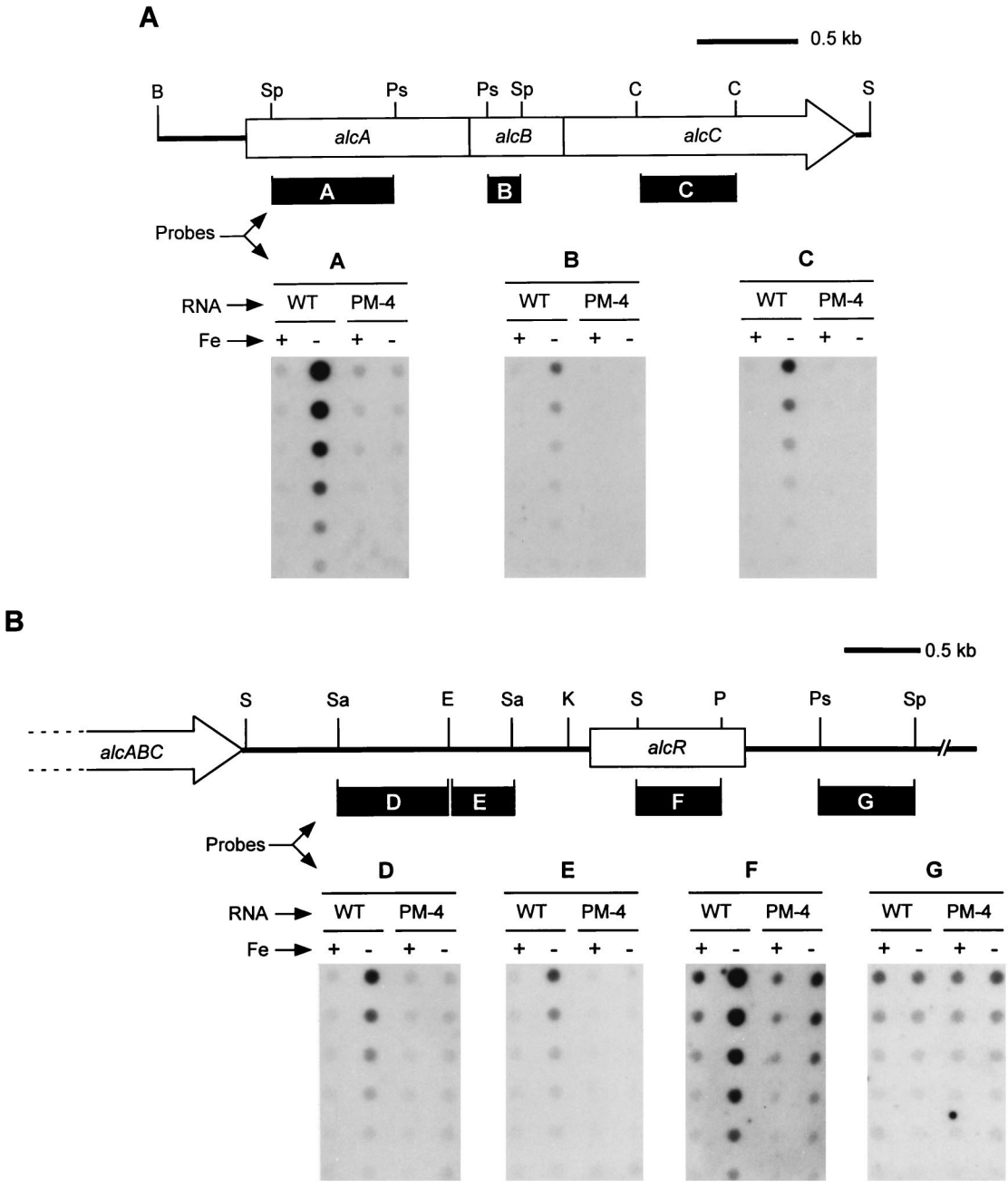

FIG. 4. RNA dot hybridization showing transcriptional linkage of *alcA*, *alcB*, and *alcC* and the 3′ limit of the *alc* operon. RNA was isolated from wild-type *B. pertussis* UT25Sm1 (WT) and its isogenic *alcA* promoter deletion mutant (PM-4) cultured in parallel under high- and low-iron conditions (Fe+ or −, respectively). Twofold serial dilutions of denatured RNA samples (from 20 to 0.63 μg) were applied to the nitrocellulose membranes. (A) DNA fragments used as probes and derived from each *alc* gene are indicated (solid bars): A, 770-bp *Sph*I-*Pst*I fragment; B, 220-bp *Pst*I-*Sph*I fragment; and C, 620-bp *Cla*I fragment. (B) Physical map of the DNA region downstream of *alcABC*, including the *alcR* gene. DNA fragments representing subregions downstream from *alcC* which were used as probes are indicated (solid bars): D, 700-bp *Sac*I-*Eco*RI fragment; E, 400-bp *Eco*RI-*Sac*I fragment; F, 530-bp *Sma*I-*Pvu*II fragment; G, 600-bp *Pst*I-*Sph*I fragment. Abbreviations: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; K, *Kpn*I; P, *Pvu*II; Ps, *Pst*I; S, *Sma*I; Sa, *Sac*I; Sp, *Sph*I.

tiple hybridization experiments (including the data set shown in Fig. 4B) consistently showed that the levels of transcripts detected in iron-starved wild-type cells were elevated approximately fourfold over transcripts detected in iron-starved mutant PM-4 (data not shown), indicating that transcription of *alcR* is also under control of the *alcA* promoter. Interestingly, although the level of *alcR* transcripts detected in mutant PM-4 was reduced due to deletion of the *alcA* promoter-operator region, significant residual iron-regulated *alcR* transcription was consistently observed. Approximately twofold-higher levels of *alcR* transcripts were observed in low-iron conditions than in high-iron conditions in the absence of a functional *alcA* promoter. These observations strongly suggest that *alcR* transcription is directed from the *alcA* promoter as well as from an iron-regulated secondary promoter unaffected by the deletion mutation in PM-4.

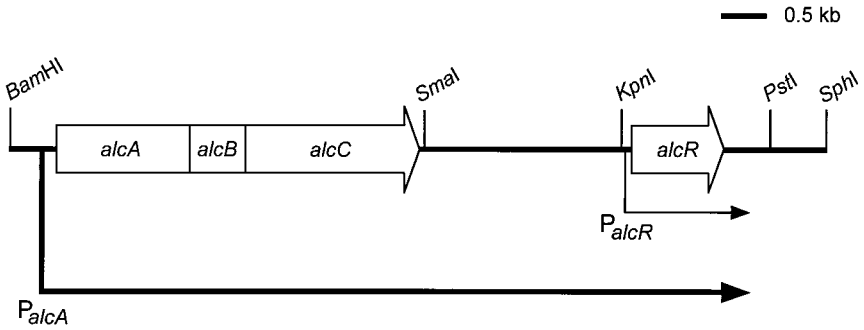

FIG. 5. Transcriptional organization of the alcaligin biosynthesis operon. Transcription from the *alcA* promoter (P_{alcA}) extends 3.6 kb downstream from *alcC*. *alcR* is the last gene contained in the *alc* operon and is also transcribed from its own promoter (P_{alcR}) (3).

Downstream of *alcR*, a 0.6-kb *Pst*I-*Sph*I DNA fragment probe hybridized to all RNA samples isolated from both wild-type *B. pertussis* and mutant PM-4, regardless of iron status (Fig. 4B, probe G). This result indicates that *alcR* is most likely the last gene transcribed from the *alcA* promoter and is monocistronic with respect to the putative secondary promoter (Fig. 5).

DISCUSSION

Previous studies indicated that the three *Bordetella* alcaligin biosynthesis genes, *alcA*, *alcB*, and *alcC*, were carried on a 4.5-kb *B. pertussis Bam*HI-*Sma*I DNA fragment and were likely organized as a polycistronic transcriptional unit (16). Because we were able to visualize only the AlcC protein in *Bordetella* cell preparations, in this study we constructed Alc-LacZ protein fusions to confirm the expression of the three Alc proteins. *B. bronchiseptica* cells harboring *alc'-'lacZ* translational fusions expressed iron-regulated hybrid proteins which were detected by β-galactosidase activity assays and immunoblot analysis using anti-β-galactosidase monoclonal antibody. Although these *alc* genes are cotranscribed, different levels of β-galactosidase activity and Alc-LacZ fusion proteins were observed. This result is likely due to differences in translation initiation efficiencies of each *alc* cistron or differential stabilities or enzymatic activities of the Alc-LacZ hybrid proteins. The observed molecular masses of the fusion proteins corresponded to the predicted masses of the native AlcA, AlcB, and AlcC proteins based on nucleotide sequence predictions (16).

The results of the present study unambiguously confirmed the transcriptional linkage of *alcA*, *alcB*, and *alcC* and localized the 3′ genetic limit of the *alc* operon. Deletion of the 105-bp DNA region encompassing the *alcA* promoter-operator abolished *alcA'-'lacZ*, *alcAB'-'lacZ*, and *alcABC'-'lacZ* reporter gene expression under iron-depleted growth conditions. However, this deletion had variable effects on Alc-LacZ hybrid protein activities under high-iron conditions. Deletion of the *alcA* promoter-operator region did not result in the apparent formation of a functional promoter from newly juxtaposed sequences at the deletion junction, and there are no apparent promoters located upstream. The variable β-galactosidase fusion expression levels observed under high-iron conditions may reflect differential stabilities of the three Alc-LacZ transcripts or hybrid proteins in the *Bordetella* host background. In the direct analysis of RNA transcripts, negligible levels of *alcA*, *alcB*, and *alcC* transcripts were observed in RNA isolated from the *alcA* promoter deletion mutant PM-4 grown in low-iron medium. Together, the results confirm that the three alcaligin biosynthesis genes, *alcA*, *alcB*, and *alcC*, are cotranscribed from the iron-regulated *alcA* promoter-operator region.

RNA analyses suggested that the regulatory gene *alcR*, located 2.1 kb downstream of *alcC*, is included in the *alc* operon, because deletion of the *alcA* promoter region resulted in significantly lower abundance of *alcR* transcripts. A 0.6-kb *Pst*I-*Sph*I DNA probe immediately downstream of *alcR* hybridized uniformly to all RNA samples isolated from both the wild type and PM-4, regardless of iron status, indicating that *alcR* is likely to represent the 3′-terminal gene of the *alc* operon. The fact that iron-regulated transcription of *alcR* was decreased in PM-4 but not abrogated (as was observed with the upstream *alcABC* genes) suggested that it has its own secondary promoter. Indeed, primer extension analysis of *alcR* revealed iron-regulated transcription from an initiation site immediately upstream of this gene and adjacent to potential Fur binding sequences (3). The 2.1-kb region between *alcC* and *alcR* has not yet been fully characterized. On the basis of the hypothetical alcaligin biosynthesis pathway, at least one other enzyme activity is predicted to be required for the complete synthesis of alcaligin (16). Procaryotic genes encoding activities which function in related cellular processes are most often organized in polycistronic operons where transcription is most efficiently regulated from a single control region. Therefore, it is likely that these predicted enzyme activities are encoded in the region downstream of *alcC*, making this iron-responsive operon dedicated to alcaligin biosynthesis and regulatory functions.

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ADDENDUM IN PROOF

After submission of this paper, a study reporting the transcriptional linkage of the *B. bronchiseptica alcABC* genes was published (P. C. Giardina, L.-A. Foster, S. I. Toth, B. A. Roe, and D. W. Dyer, Gene **194:**19–24, 1997). Pradel and coworkers have also identified the *Bordetella alcR* gene and determined the nucleotide sequence of the *alcC-alcR* intergenic region (E. Pradel, N. Guiso, and C. Locht, J. Bacteriol. **180:**871–880, 1998).

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