JOURNAL OF BACTERIOLOGY, Jan. 2001, p. 483–489 Vol. 183, No. 2
0021-9193/01/$04.00+0 DOI: 10.1128/JB.183.2.483–489.2001
Copyright © 2001, American Society for Microbiology. All Rights Reserved.

Transcriptional Activation of *Bordetella* Alcaligin Siderophore Genes Requires the AlcR Regulator with Alcaligin as Inducer

TIMOTHY J. BRICKMAN,[1,2] HO YOUNG KANG,[1]† AND SANDRA K. ARMSTRONG[1,2]*

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

Received 24 August 2000/Accepted 27 October 2000

Genetic and biochemical studies have established that Fur and iron mediate repression of *Bordetella* alcaligin siderophore system (*alc*) genes under iron-replete nutritional growth conditions. In this study, transcriptional analyses using *Bordetella* chromosomal *alc-lacZ* operon fusions determined that maximal *alc* gene transcriptional activity under iron starvation stress conditions is dependent on the presence of alcaligin siderophore. Mutational analysis and genetic complementation confirmed that alcaligin-responsive transcriptional activation of *Bordetella* alcaligin system genes is dependent on AlcR, a Fur-regulated AraC-like positive transcriptional regulator encoded within the alcaligin gene cluster. AlcR-mediated transcriptional activation is remarkably sensitive to inducer, occurring at extremely low alcaligin concentrations. This positive autogenous control circuit involving alcaligin siderophore as the inducer for AlcR-mediated transcriptional activation of alcaligin siderophore biosynthesis and transport genes coordinates environmental and intracellular signals for maximal expression of these genes under conditions in which the presence of alcaligin in the environment is perceived.

In the majority of bacterial species characterized to date, the iron starvation stress response is controlled at the transcriptional level by the ferrous iron-activated Fur repressor protein (16, 22). Additional transcriptional regulators have been identified that can act as positive regulators of siderophore biosynthesis and transport gene expression, all of which are Fur controlled and responsive to the presence of the cognate iron chelate (14, 18, 23, 24, 28). The concerted actions of negatively and positively acting regulators in bacterial species capable of utilizing diverse iron sources may ensure that genes encoding specific nutritional iron transport functions are expressed maximally only under appropriate conditions in which the particular iron source is perceived in the environment, thus conserving energy and precursors. This general type of priority regulation is an established function of positive regulators controlling assimilation of available nutrients (35).

Known positive regulators of iron acquisition systems are of three general mechanistic classes: alternative sigma factors, exemplified by the *Escherichia coli* FecI regulator of the ferric citrate utilization system (28), classical two-component sensory transduction systems such as the PfeR-PfeS enterobactin utilization system of *Pseudomonas aeruginosa* (14), and AraC-like transcriptional regulators. AraC-like regulators may be capable of acting positively or negatively depending on the presence or absence of inducers and the position of the regulator binding site on the DNA (20, 35). In *P. aeruginosa*, the AraC-like protein PchR regulates expression of pyochelin siderophore biosynthesis and transport genes; transcriptional activity responds to pyochelin, and induction requires a functional pyochelin receptor (23, 24). Under iron starvation conditions, the *Yersinia pestis* AraC-like regulator YbtA is required for full expression of genes encoding the Psn yersiniabactin siderophore receptor and yersiniabactin siderophore biosynthesis activities (18). The *Bordetella alcR* gene also encodes an AraC-like regulatory protein and is required for maximal expression of alcaligin siderophore biosynthesis (6, 34) and transport activities (6, 10) during iron starvation stress. The mechanism of transcriptional activation by these AraC-like regulators of siderophore genes is thought to involve the cognate siderophore functioning as the inducer.

Bordetella pertussis and *Bordetella bronchiseptica* are gram-negative bacterial pathogens that inhabit the respiratory mucosae of humans and nonhuman mammals. When nutritional iron is limiting in availability, these organisms produce and utilize the macrocyclic dihydroxamate siderophore alcaligin (12, 31). *B. pertussis* and *B. bronchiseptica* are also capable of utilizing iron complexed with the heterologous siderophores enterobactin (4), ferrichrome, and desferrioxamine B (5). Several other iron-regulated genes encoding putative siderophore receptors with undefined specificities have been identified in *B. pertussis* (3) and *B. bronchiseptica* (3, 5), suggesting that the iron-scavenging potential of these organisms may include the ability to utilize additional heterologous siderophores as iron sources. In addition to ferric siderophores, the mammalian host-derived molecules lactoferrin (29, 36), transferrin (29, 36), hemin (1), and hemoglobin (33) have been reported as nutritional iron sources for these bacteria.

The *Bordetella* alcaligin biosynthesis genes *alcABCDE* comprise part of a Fur-regulated operon and encode proteins with amino acid sequence similarities to other known siderophore synthesis enzymes (21, 26, 34). The *alcR* gene encoding the AlcR positive regulator of alcaligin biosynthesis (6, 34) and transport activities is located immediately downstream of *alcABCDE* and is operonic with *alcABCDE* (6, 25). The ma-

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

† Present address: Department of Biology, Washington University, Saint Louis, MO 63130-4899.

TABLE 1. *B. bronchiseptica* strains and plasmids used in this study

Strain or plasmid	Relevant genotype, phenotype, or description	Reference or source
Strains		
B013N	Nalidixic acid-resistant derivative of strain B013, derived from swine isolate B	2
BRM1	B013N *alcA*::mini-Tn*5 lacZ1*; Kan^r; alcaligin siderophore deficient	2
BRM6	B013N *alcC*::mini-Tn*5 lacZ1*; Kan^r; alcaligin siderophore deficient	2
BRM9	B013N *alcA*::mini-Tn*5 lacZ1*, with mini-Tn*5* fusion element in antisense orientation with respect to *alcA* transcription; Kan^r; alcaligin siderophore deficient	2
BRM13	BRM1 Δ*alcR1*	This study
BRM14	BRM6 Δ*alcR1*	This study
BRM15	BRM9 Δ*alcR1*	This study
Plasmids		
pRK415	Mobilizable broad-host-range plasmid cloning vector; Tet^r; RK2 origin	27
pRK21	pRK415 carrying 1.6-kb *Kpn*I-*Pst*I DNA insert fragment of *B. pertussis* UT25; *alcR*$^+$; Tet^r; formerly designated pP9KP	6
pRK15	pRK415 carrying 2.3-kb *Eco*RI-*Pst*I DNA insert fragment of *B. bronchiseptica* B013N; *alcR*$^+$; Tet^r	This study
pRK16	pRK15, except 2.1-kb Δ*alcR1 Eco*RI-*Pst*I DNA insert fragment of *B. bronchiseptica* B013N	This study

jority of *alcR* transcription initiates at the Fur-controlled promoter immediately upstream from *alcA* (T. J. Brickman and S. K. Armstrong, Abstr. 97th Ann. Meet. Am. Soc. Microbiol. 1997, abstr. B-241, p. 70, 1997), but *alcR* is also transcribed from a weaker secondary Fur-regulated promoter located immediately upstream from its own coding region (6, 25).

Although the *Bordetella* alcaligin system genes are repressible by Fur (7; Brickman and Armstrong, Abstr. 97th Gen. Meet. Am. Soc. Microbiol.), as are other microbial siderophore systems (16), AlcR imposes an additional level of control that is required for full expression of the alcaligin siderophore system genes. In the present study, we establish that maximal transcription of the *alc* operon under iron starvation growth conditions is dependent on the AlcR regulator and requires the presence of the cognate siderophore alcaligin acting as the inducer. Furthermore, AlcR-mediated *alc* transcriptional activation is shown to be exquisitely sensitive, responding to extremely low concentrations of inducer.

MATERIALS AND METHODS

Bacterial strains and plasmids. *B. bronchiseptica* strains and *alcR* plasmids used in this study are described in Table 1. The isolation of alcaligin siderophore-deficient mutants BRM1, BRM6, and BRM9, generated by mini-Tn*5 lacZ1* (15) transposon mutagenesis of *B. bronchiseptica* B013N, has been described previously (2). *B. bronchiseptica* Δ*alcR1* mutants BRM13, BRM14, and BRM15, derived from BRM1, BRM6, and BRM9, respectively, were each produced by allelic exchange essentially as described previously for construction of *B. bronchiseptica* B013N Δ*alcR1* mutant BRM11 (6). Presumptive Δ*alcR1* mutants were identified by in situ DNA hybridization analysis using the deleted DNA fragment as the probe; Southern hybridization analysis of genomic DNA samples using appropriate DNA probes confirmed that the wild-type *alcR* allele had been correctly replaced by the Δ*alcR1* mutant allele. *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 strain for routine plasmid construction and propagation, and as the donor strain in conjugal transfer of plasmids to *Bordetella* recipient strains. DH5α (pRK2013) provided plasmid-encoded mobilization functions (19) in triparental matings to transfer plasmid vector pRK415 derivatives to *Bordetella* strains.

Bacterial culture conditions. *B. bronchiseptica* strains were maintained on blood agar plates or standard Luria-Bertani agar plates. Modified Stainer-Scholte (SS) medium (38) was used for broth cultures of *B. bronchiseptica*. Iron-replete and iron-depleted SS culture conditions were achieved by the methods of Armstrong and Clements (2); SS medium was deferrated by batch treatment using Chelex 100 resin (Bio-Rad Laboratories, Richmond, Calif.). Tetracycline was used at 15 μg/ml to select for pRK415 plasmid derivatives, and kanamycin was used at 50 μg/ml for maintenance of pRK2013 and for selection of kanamycin resistance markers of mini-Tn*5 lacZ1* mutants. Ampicillin was used at 100 μg/ml for maintenance of other plasmid cloning intermediates in *E. coli*. In analyses of induction of *alc* gene transcription by alcaligin, SS culture medium was supplemented as required with purified alcaligin siderophore. All glassware was acid cleaned and rinsed repeatedly in distilled deionized water prior to use. 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.).

***Bordetella* alcaligin siderophore purification and detection.** Alcaligin was purified from *B. bronchiseptica* culture supernatants by a modification of the benzyl alcohol-ether extraction method (32) as previously described by Brickman and coworkers (12) and was recrystallized at least eight times from ethanolic solution. The chrome azurol S (CAS) universal siderophore detection assay (39) was used to monitor siderophore production by *B. bronchiseptica* grown in liquid culture as reported previously (2).

Conjugation. Conjugal transfer of pRK415 plasmid derivatives to *Bordetella* strains was accomplished by triparental matings using *E. coli* DH5α as the plasmid donor strain and DH5α(pRK2013) as the source of mobilization functions as described previously (9). Transconjugants were selected on agar plates containing the appropriate selective antibiotics and crude colicin B (8).

Routine DNA procedures. DNA cloning and hybridization analyses were performed using standard methods (37). DNA probes used in nucleic acid hybridizations were radiolabeled by the random-priming method (17) using the Random Primers DNA Labeling System (Gibco BRL) and [α-^{32}P]dCTP (ICN Radiochemicals, Irvine, Calif.). Transformation of *E. coli* was carried out by the $CaCl_2$ method of Cohen and coworkers (13).

β-Galactosidase assays. *B. bronchiseptica alc*::mini-Tn*5 lacZ1* fusion strains were assayed for β-galactosidase activity by the method of Miller (30) as modified by Brickman and coworkers (11) after culture in iron-replete or iron-depleted SS medium. In experiments examining the responsiveness of *alc* gene transcription to alcaligin siderophore, SS cultures were supplemented with purified alcaligin at the specified final concentrations, ranging from 0 to 50 μg/ml. β-Galactosidase activities presented are means from triplicate assays.

RESULTS

***alc* operon transcriptional activity is not increased by iron starvation in alcaligin siderophore-deficient mutants.** *B. bronchiseptica* mini-Tn*5 lacZ1* insertion mutants BRM1, BRM6, and BRM9 have been identified previously on the basis of alcaligin siderophore production defects resulting from *alc* biosynthesis gene disruption (2). BRM1 carries a chromosomal mini-Tn*5 lacZ1*-encoded *lacZ* transcriptional fusion to the *alcA* cistron of the *alc* operon, and BRM6 carries a similar fusion to the downstream *alcC* cistron (Fig. 1 and Table 1). The positions and orientations of the fusion elements in both of these mutants place the promoterless *lacZ* reporter genes under the control of the *alc* operon control region located immediately upstream of *alcA*. The mini-Tn*5 lacZ1* fusion

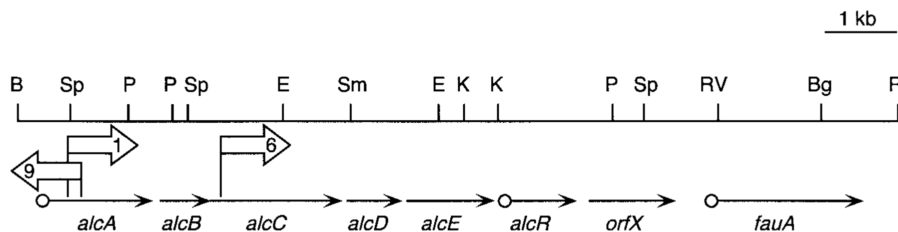

FIG. 1. Spatial organization of the *Bordetella alcABCDER* alcaligin siderophore operon. The linear genetic map depicts an approximately 8-kb *Bam*HI-*Pst*I chromosomal DNA region of *B. bronchiseptica* B013N. Arrows indicate the transcriptional orientations and genetic limits of genes of the *alcABCDER* operon, and open circles represent the locations of known Fur-regulated promoter-operator regions upstream from *alcA, alcR,* and *fauA*. Numbered open arrows indicate the positions and transcriptional orientations of the promoterless *lacZ* reporter genes associated with the mini-Tn*5 lacZ1* transposon insertions of alcaligin-deficient mutants BRM1, BRM6, and BRM9. Abbreviations: B, *Bam*HI; Sp, *Sph*I; P, *Pst*I; E, *Eco*RI; Sm, *Sma*I; K, *Kpn*I; RV, *Eco*RV; Bg, *Bgl*II.

element of BRM9 is inserted into *alcA* at a position approximately 200 bp downstream from the insertion site in BRM1, but the element is oriented antisense to *alc* operon transcription (Fig. 1 and Table 1). Although the BRM1, BRM6, and BRM9 mini-Tn*5 lacZ1* insertions served to define genes required for alcaligin siderophore production (2) and led to the discovery of the alcaligin siderophore gene clusters of *B. bronchiseptica* and *B. pertussis* (26), iron-regulated *lacZ* reporter gene expression associated with the mini-Tn*5 lacZ1* operon fusion elements was not observed. These data were inconsistent with findings that *alc* transcription monitored by RNA hybridization methods (25, 26) and alcaligin siderophore production (2) were strongly iron repressible in the alcaligin-producing parent strain B013N. Subsequent genetic and biochemical studies determined that the AlcR regulator protein was required for full expression of alcaligin biosynthesis and transport activities (6) and that the *alc* genes were cotranscribed from a Fur- and iron-regulated promoter-operator region located immediately upstream from *alcA* (25, 26; Brickman and Armstrong, Abstr. 97th Gen. Meet. Am. Soc. Microbiol.). Although the *alcR* regulatory gene is transcribed at a low level from another Fur-controlled promoter immediately upstream from the *alcR* coding sequences, the majority of *alcR* expression results from transcription emanating from the *alc* operon promoter (6, 25; Brickman and Armstrong, Abstr. 97th Gen. Meet. Am. Soc. Microbiol.). Therefore, we hypothesized that the failure to observe elevated *alc* operon transcriptional activity under iron starvation conditions using the chromosomal *alc*::mini-Tn*5 lacZ1* transcriptional fusions as reporters was likely due to polar effects of the transposon insertions in *alcA* or *alcC* on expression of the downstream *alcR* regulatory gene.

The *alcR*$^+$ plasmid pRK21 complements the alcaligin biosynthesis and transport defects of *B. bronchiseptica* and *B. pertussis alcR* mutants (6). We introduced *alcR* as plasmid pRK21 to *B. bronchiseptica* fusion strains BRM1, BRM6, and BRM9 to ascertain whether *alcR* provided in *trans*, thus circumventing the hypothesized polar influence of the mini-Tn*5 lacZ1* insertions on the chromosomal copy of *alcR*, could result in elevated expression of the *alc-lacZ* operon fusions under iron-depleted growth conditions. Transcriptional activity of the *alc* operon in the BRM1 and BRM6 mini-Tn*5 lacZ1* fusion strains was not significantly altered by the presence of plasmid pRK21 compared with that in fusion strains bearing the control plasmid vector pRK415 (Fig. 2). Elevated β-galactosidase reporter gene activities were not observed when bacteria were cultured under iron-depleted conditions compared with iron-replete conditions. Thus, relief of polar effects of insertions on *alcR* by the *alcR*$^+$ plasmid pRK21 was insufficient to effect iron-regulated *alc* transcription in fusion strains BRM1 and BRM6 carrying mini-Tn*5 lacZ1* fusion elements in the *alc* sense orientation.

alc **operon transcription in siderophore-deficient mutants responds to alcaligin as an inducer under iron starvation conditions.** At the time that *alcR* was identified as a key regulator of alcaligin siderophore biosynthesis and transport activities, nucleotide sequencing revealed it to be a member of the AraC family of transcriptional regulators (6). With the observation that relief of the polarity of *alc*::mini-Tn*5 lacZ1* insertions by the *alcR* plasmid pRK21 was insufficient to result

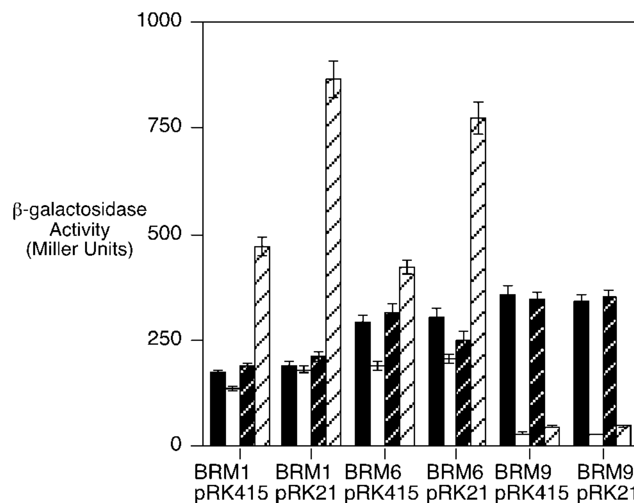

FIG. 2. Transcriptional activity of the *alc* operon promoter. Alcaligin siderophore-deficient *alc*::mini-Tn*5 lacZ1* mutants BRM1, BRM6, and BRM9, each carrying the *alcR*$^+$ plasmid pRK21 or the control plasmid vector pRK415, were cultured in iron-replete or iron-depleted SS medium with or without supplementation of the SS medium with 20 μg of purified alcaligin/ml. Transcriptional activities of the fusion genes were monitored using β-galactosidase assays, with results expressed in Miller units (means ± 1 standard deviation, $n = 3$). Solid bars, iron-replete cultures; hatched solid bars, iron-replete cultures supplemented with alcaligin; open bars, iron-depleted cultures; hatched open bars, iron-depleted cultures supplemented with alcaligin.

in elevated *alc-lacZ* fusion gene activity in mutants BRM1 and BRM6 under iron starvation growth conditions, it was hypothesized that as an AraC-like regulator, AlcR might require a small-molecule inducer in order to function as a transcriptional activator of *alc* gene expression. Because other known positive regulators of microbial iron acquisition systems appear to respond to their cognate chelator as the inducer, it was further hypothesized that AlcR might respond to alcaligin siderophore. Since the *alc*::mini-Tn*5 lacZ1* fusion reporter strains are alcaligin siderophore deficient, replicate sets of iron-replete and iron-depleted β-galactosidase assay cultures were supplemented with purified alcaligin at a final concentration of 20 μg/ml to assess the responsiveness of *alc* transcription to alcaligin. Supplementation of the culture medium with alcaligin resulted in robust elevation of *alc*::mini-Tn*5 lacZ1* transcription in BRM1 and BRM6 under iron-depleted growth conditions, but, as predicted, β-galactosidase activity in BRM9, which carries the *lacZ* reporter gene fusion in the *alc* antisense orientation (Fig. 2), was not increased but instead was significantly decreased, likely due to antisense transcription of *lacZ*. Moreover, supplying *alcR* in *trans* as plasmid pRK21 augmented alcaligin-responsive *alc*::mini-Tn*5 lacZ1* transcriptional activity in BRM1 and BRM6, possibly by relief of the polarity of *alc*::mini-Tn*5 lacZ1* insertions on *alcR* as well as by increased *alcR* expression resulting from multicopy gene dosage (Fig. 2). Although the $alcR^+$ plasmid pRK21 enhanced *alc* operon transcription in BRM1 and BRM6 in response to alcaligin, pRK21 did not relieve the absolute requirement for alcaligin as an inducer of *alc* operon transcription, even though overexpression of some AraC-like regulators due to multicopy gene dosage may suppress the regulator's inducer requirements for activation (35). CAS siderophore detection assays of supernatants from cultures used for β-galactosidase assays confirmed that pRK21 did not complement the alcaligin siderophore biosynthesis defects of *alc*::mini-Tn*5 lacZ1* mutants BRM1, BRM6, and BRM9; thus, induction of *alc* transcription in β-galactosidase assays was dependent on the exogenously supplied alcaligin. In control CAS siderophore detection assays, *alcR* overexpression due to multicopy gene dosage did not result in deregulated alcaligin production in the wild-type alcaligin-producing parent strain B013N; B013N(pRK21) produced alcaligin at normal levels and only under iron-depleted culture conditions. These results indicate that *alc* operon transcription under iron-depleted growth conditions is dependent on the presence of alcaligin and that *alc* operon transcriptional activity is AlcR responsive. Alcaligin supplementation did not increase *alc* transcriptional activity under iron-replete culture conditions (Fig. 2), indicating that derepression of Fur- and iron-repressible *alc* transcription in response to an iron starvation signal is a prerequisite for alcaligin inducer responsiveness and AlcR-mediated activation of *alc* transcription.

Induction of *alc* operon transcription by alcaligin is AlcR dependent. To extend the observations that *alc* transcriptional activity was alcaligin dependent and responsive to AlcR, it was necessary to establish whether induction of transcription by alcaligin was absolutely dependent on AlcR function. The *B. bronchiseptica* Δ*alcR1* mutant allele was crossed into *alc*::mini-Tn*5 lacZ1* mutants BRM1, BRM6, and BRM9 to create isogenic Δ*alcR1* mutant derivatives BRM13, BRM14, and BRM15, respectively, for transcriptional analysis (Table 1).

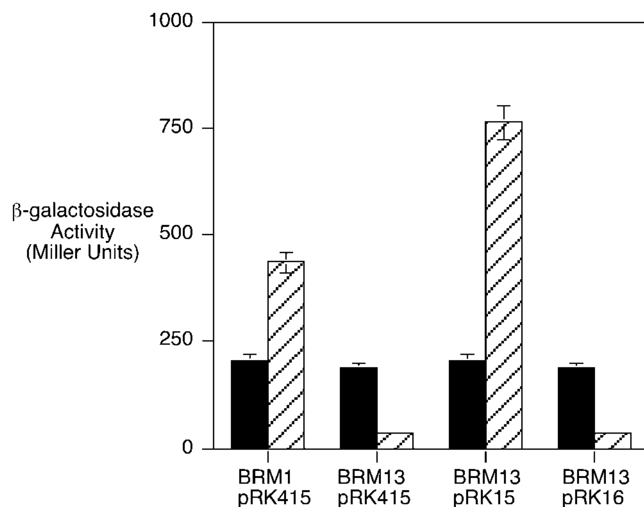

FIG. 3. *alcR*-dependent induction of *alc* operon transcription. β-Galactosidase reporter activities associated with expression of the *alcA*::mini-Tn*5 lacZ1* fusion element of BRM1(pRK415) were compared with those of the isogenic Δ*alcR1* derivative BRM13 carrying the control plasmid vector pRK415, the $alcR^+$ plasmid pRK15, or the Δ*alcR1* mutated plasmid pRK16. β-Galactosidase activities were measured for cells cultured in iron-replete SS medium or in iron-depleted SS medium supplemented with 20 μg of purified alcaligin/ml. β-Galactosidase activities are expressed in Miller units (means ± 1 standard deviation; $n = 3$). Solid bars, iron-replete cultures; hatched bars, iron-depleted cultures supplemented with alcaligin.

The Δ*alcR1* mutation is a nonpolar in-frame deletion mutation created by deletion of a 264-bp *Ngo*AIV fragment internal to the *B. bronchiseptica alcR* gene (6). The same mutation was previously introduced into *B. bronchiseptica* strain B013N and *B. pertussis* UT25 to construct Δ*alcR1* mutant strains BRM11 and PM10, respectively (6). The regulatory defect of Δ*alcR1* mutant strains can be complemented using plasmid pRK15, which carries a 2.3-kb *Eco*RI-*Pst*I *B. bronchiseptica* $alcR^+$ insert DNA fragment, but not by the related plasmid derivative pRK16, which carries the corresponding 2.1-kb *Eco*RI-*Pst*I Δ*alcR1* mutated insert DNA fragment (Table 1). In β-galactosidase assays monitoring *alc* transcription in response to iron starvation, the presence of the Δ*alcR1* mutation in the *alcA*::mini-Tn*5 lacZ1* strain BRM13 abrogated the transcriptional responsiveness of the fusion gene to alcaligin inducer that was observed with the parental $alcR^+$ strain BRM1 (Fig. 3). Furthermore, responsiveness to alcaligin inducer was restored to BRM13 by the $alcR^+$ plasmid pRK15, but not by the Δ*alcR1* plasmid derivative pRK16 encoding a defective AlcR regulator. Alcaligin-responsive expression of the *alcC*::mini Tn*5 lacZ1* fusion of BRM14 was likewise found to be *alcR* dependent, and expression of the antisense-oriented reporter gene of *alcA*::mini Tn*5 lacZ1* control strain BRM15 was negligible, as predicted (data not shown). These results indicate that iron-regulated *alc* transcription is alcaligin and AlcR dependent; thus, alcaligin participates in a positive autogenous control circuit regulating its own production and utilization through the action of the AlcR regulator.

Relationship between alcaligin inducer concentration and *alc* operon transcriptional activity. Alcaligin siderophore concentrations measured in iron-depleted SS culture supernatants

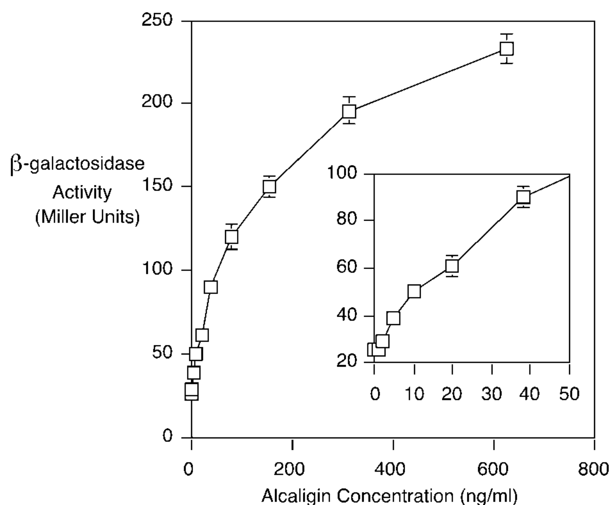

FIG. 4. Transcriptional activity of the *alc* operon varies with alcaligin inducer concentration. BRM13(pRK21) cells were cultured in iron-depleted SS medium supplemented with different concentrations (0, 1, 2, 5, 10, 20, 39, 79, 156, 313, and 625 ng/ml) of purified alcaligin, and transcriptional activity of the *alcA*::mini-Tn*5 lacZ1* fusion gene was monitored by β-galactosidase assay. (Inset) Enlarged scale in the concentration range from 0 to 50 ng of alcaligin/ml. β-Galactosidase activities, expressed in Miller units, are shown as means ± 1 standard deviation ($n = 3$).

of wild-type *B. bronchiseptica* normally range from approximately 25 to 50 μg/ml (12). The relationship between alcaligin inducer concentration and *alc* operon transcriptional activity was examined using BRM13(pRK21), by monitoring *alcA*::mini-Tn*5 lacZ1* fusion gene expression under iron-depleted conditions in the presence of various concentrations of alcaligin ranging from 0 to 50 μg/ml. Transcriptional activity of the *alcA-lacZ* fusion of BRM13(pRK21) increased as a function of alcaligin inducer concentration, approaching a maximum reporter gene activity at approximately 20 μg of alcaligin/ml (data not shown). Induction of *alc* operon transcription by much lower alcaligin concentrations, ranging between 0 and 625 ng/ml, is shown in Fig. 4. A 10-ng/ml threshold concentration of alcaligin inducer nearly doubled the transcriptional activity of the *alcA-lacZ* fusion gene compared with the basal level of expression under iron-depleted conditions without alcaligin supplement. These findings reveal that *alc* transcriptional responsiveness to alcaligin inducer is extremely sensitive and that measurable induction of *alc* transcription occurs at an alcaligin concentration that approximates the minimal concentration required for detectable transport of ferric alcaligin in quantitative [^{55}Fe]ferric alcaligin uptake assays (12). Remarkably, the threshold concentration of alcaligin for induction of *alc* operon transcription is more than 3 orders of magnitude lower than the concentration of alcaligin required to effect measurable growth stimulation in siderophore bioassays (10, 12).

DISCUSSION

The experimental results obtained in this study establish the roles of the regulatory protein AlcR and alcaligin siderophore in the control of alcaligin siderophore system gene expression in *Bordetella* species. Strong iron-regulated expression of *alc* transcriptional fusions was achieved by circumventing the polar effects of mini-Tn*5 lacZ1* insertions on the *alcR* gene and by exogenously supplying the alcaligin siderophore inducer that was lacking in the *B. bronchiseptica alc*::mini-Tn*5 lacZ1* fusion strains. AlcR is an AraC-like transcriptional regulator that is necessary for maximal expression of alcaligin siderophore biosynthesis and transport activities under iron starvation stress conditions, and AlcR function requires the presence of alcaligin siderophore acting as the inducer.

The precise mechanism for siderophore induction of transcription involving any of the known iron-related AraC-like regulators YbtA, PchR, and AlcR remains unknown at this time. Since AlcR is an AraC-like protein with a predicted DNA-binding helix-turn-helix structural motif (6), it is presumed that its function involves a sequence-specific DNA-binding activity. It is further hypothesized that the role of alcaligin as an inducer is to modify AlcR activity or DNA-binding site selection to effect transcriptional activation of alcaligin system genes under the appropriate conditions, although it is formally unknown whether alcaligin functions as a coactivator by direct interactions with AlcR. Examination of the nucleotide sequence near the *alc* operon promoter region revealed the presence of two copies of an 11-nucleotide direct repeat sequence, 5′-TTCTTCGCACA-3′, occupying nucleotide positions −41 to −31 and +71 to +81 with respect to the *alc* operon major transcription initiation site (Fig. 5). The position of the upstream copy of the repeat with respect to the *alc* operon promoter is consistent with a potential role (20) in AlcR binding and transcriptional activation of the *alc* operon; the downstream copy is centered in the *alcA* initial transcribed region, separated from the upstream copy by 10 integral B-DNA turns. Although it is unknown whether either of these sequences represents actual AlcR-binding sites, the phasing of these two sequences on the DNA helix could potentially allow an interaction between proteins bound at both of these DNA positions. Alternatively, the upstream copy of the putative AlcR-binding site may be directly involved in *alc* transcriptional activation, and the downstream copy could function as an enhancer-like sequence serving to recruit AlcR to the vicinity of the *alc* operon promoter. At this time, no evidence exists for AlcR binding to these sequences, and no other candidate AlcR-binding sequences have been identified by nucleotide sequence analysis or mutation. Experimental evidence suggests that expression of the AraC-like regulators YbtA and PchR is negatively autoregulated (18, 24). Examination of potential AlcR autoregulation using an *alcR*::mini-Tn*5 lacZ1* fusion plasmid that placed *lacZ* reporter gene expression under the control of the secondary promoter-operator region immediately upstream of *alcR* (Fig. 1) revealed that *alcR-lacZ* fusion gene expression was not significantly altered in a *B. bronchiseptica* Δ*alcR1* mutant host strain compared with that in an *alcR*$^+$ strain, regardless of nutritional iron status or the presence of alcaligin inducer in the culture (data not shown). Lack of evidence for AlcR autoregulation acting at the *alcR* upstream promoter is consistent with the absence of putative AlcR-binding sequences or other identifiable nucleotide sequence similarities shared by the *alcA* and *alcR* upstream regions, with the exception of the Fur-binding sites (Brickman and Armstrong, Abstr. 97th Gen. Meet. Am. Soc. Microbiol.).

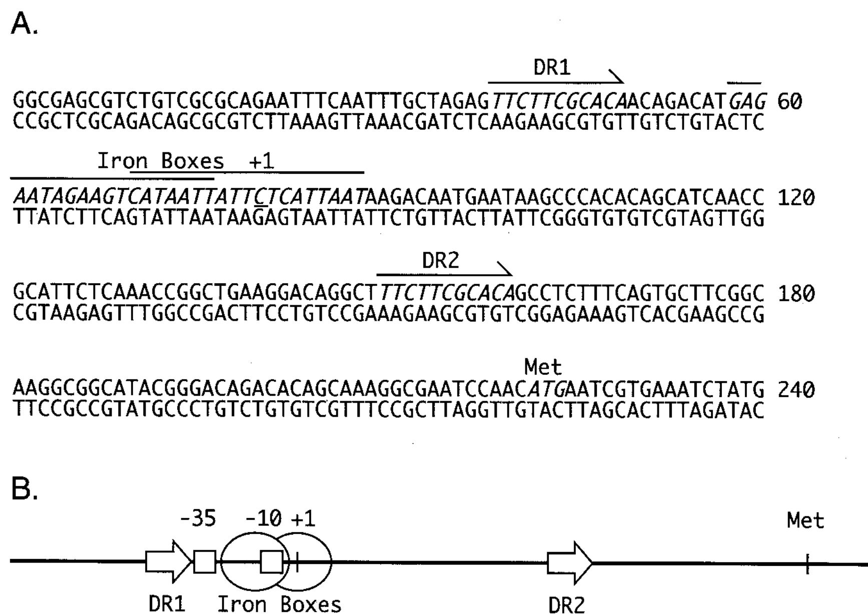

FIG. 5. The *alc* operon control region of *B. bronchiseptica* B013N. (A) Nucleotide sequence of a 240-bp DNA segment representing nucleotide positions −79 to +161 with respect to the *alc* operon major transcription initiation site (underlined C residue designated +1) (26). Key nucleotide sequence features are italicized. Overlapping nucleotide sequences with similarity to Fur-binding sites (Iron Boxes) are overlined. Two copies of an 11-nucleotide direct repeat sequence (5′-TTCTTCGCACA-3′), designated DR1 and DR2, occupy nucleotide positions −41 to −31 and +71 to +81. The predicted translation initiation methionine (Met) codon for *alcA* is indicated. (B) Simplified schematic representation of the 240-bp region shown in panel A, showing the spatial relationships of known and predicted regulatory elements. Direct repeat sequences DR1 and DR2 (arrows) and Fur-binding sites (Iron Boxes, designated by ovals) are shown in relation to the major *alc* operon −35 and −10 promoter determinants (squares) and the transcription initiation site (+1). The relative position of the proposed *alcA* translation initiation codon (Met) is also indicated.

Thus, although *alcR* expression is negatively regulated by Fur acting at the *alcABCDER* operon control region as well as at the secondary promoter-operator in the *alcR* upstream region, no evidence for negative autoregulation of *alcR* was observed in this study. AlcR positively regulates its own expression by activating transcription of the *alcABCDER* operon. For the YbtA-regulated *psn* promoter of *Y. pestis*, two 18-bp inverted repeat sequences located 48 and 68 bp upstream from the transcription initiation site have been implicated as YbtA-binding sequences (18) by mutational analysis of a *psn-lacZ* fusion construct. In the *psn* system, it appears that the promoter-proximal copy of the repeat that overlaps the −35 promoter region alone is sufficient for significant YbtA-mediated activation of transcription but that both repeats are required for maximal expression of *psn*. Other potential YbtA-binding sequences were identified upstream of *irp2*, a gene involved in yersiniabactin biosynthesis, as well as in the *ybtA* initial transcribed region. Positional effects of the two putative YbtA-binding sites in the *ybtA* initial transcribed region are thought to be responsible for the observed negative autoregulation of *ybtA*. Experiments in progress are aimed at examination of the putative DNA-binding activity of AlcR, and the influence of alcaligin on AlcR DNA binding and transcriptional activation.

It was established in this study that activation of *alc* operon transcription by AlcR can occur at extremely low concentrations of alcaligin inducer. This suggests that *Bordetella* species, and perhaps other bacterial species having similar regulatory mechanisms controlling iron uptake systems, have evolved a remarkable capacity to sense and respond to the presence of siderophores in their environment. Since a siderophore produced by one bacterium might be sensed by another bacterium of the same or different species expressing the cognate positive regulator, siderophores as small diffusible molecules can mediate a type of intercellular and interspecies communication. Transcriptional activation of the chelate-specific iron transport system in the sensing cell occurs in response to the perceived presence of the siderophore in a manner analogous to the responsiveness of transcriptional regulators involved in perception and response to classical intercellular signaling molecules.

Transcription of the *alc* operon has previously been shown to be Fur and iron repressible, (7, 25, 26; Brickman and Armstrong, Abstr. 97th Gen. Meet. Am. Soc. Microbiol.) and is now known to be alcaligin and AlcR dependent. Thus alcaligin, as the end product of the siderophore biosynthesis pathway, is a key participant along with AlcR in a positive autogenous control circuit regulating its own production and transport. Since AlcR production is Fur controlled, this positive regulatory mechanism can be viewed simply as subroutine of the global Fur- and iron-regulated negative-control circuit in which the essential nutrient iron, as corepressor with Fur, participates directly in the genetic control of its own assimilation. A major role of positive control of transcription initiation is to establish priorities between pathways that serve the same final purpose (35). Priority regulation of iron acquisition system gene expression could be particularly important when bacteria that are capable of utilizing a variety of potential iron sources are confronted with a mixture of those iron sources,

some of which may be more effectively utilized than others in that particular microenvironment. The predominant role of chelate-specific positive regulators may be to allow bacteria to sample their environment, perceive which iron source is available, and selectively activate expression of genes involved in assimilation of the effective iron source. Such regulatory mechanisms may be common to many bacterial species capable of utilizing multiple alternative sources of nutritional iron.

ACKNOWLEDGMENT

This work was supported by Public Health Service grant AI-31088 from the National Institute of Allergy and Infectious Diseases.

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. **Armstrong, S. K., and M. O. Clements.** 1993. Isolation and characterization of *Bordetella bronchiseptica* mutants deficient in siderophore activity. J. Bacteriol. **175:**1144–1152.
3. **Beall, B.** 1998. Two iron-regulated putative ferric siderophore receptor genes in *Bordetella bronchiseptica* and *Bordetella pertussis*. Res. Microbiol. **149:**189–201.
4. **Beall, B., and G. N. Sanden.** 1995. A *Bordetella pertussis fepA* homologue required for utilization of exogenous ferric enterobactin. Microbiology **141:** 3193–3205.
5. **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.
6. **Beaumont, F. C., H. Y. Kang, T. J. Brickman, and S. K. Armstrong.** 1998. Identification and characterization of *alcR*, a gene encoding an AraC-like regulator of alcaligin siderophore biosynthesis and transport genes in *Bordetella pertussis* and *Bordetella bronchiseptica.* J. Bacteriol. **180:**862–870.
7. **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.
8. **Brickman, T. J., and S. K. Armstrong.** 1996. Colicins B and Ia as novel counterselective agents in interspecies conjugal DNA transfers from colicin-sensitive *Escherichia coli* donors to other gram-negative recipient species. Gene **178:**39–42.
9. **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.
10. **Brickman, T. J., and S. K. Armstrong.** 1999. Essential role of the iron-regulated outer membrane receptor FauA in alcaligin siderophore-mediated iron uptake in *Bordetella* species. J. Bacteriol. **181:**5958–5966.
11. **Brickman, T. J., B. A. Ozenberger, and M. A. McIntosh.** 1990. Regulation of divergent transcription from the iron-responsive *fepB-entC* promoter-operator regions in *Escherichia coli*. J. Mol. Biol. **212:**669–682.
12. **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.
13. **Cohen, S. N., A. C. Y. Chang, and L. Hou.** 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* R-factor DNA. Proc. Natl. Acad. Sci. USA **69:**2110–2114.
14. **Dean, C. R., and K. Poole.** 1993. Expression of the ferric enterobactin receptor PfeA of *Pseudomonas aeruginosa*: involvement of a two-component regulatory system. Mol. Microbiol. **8:**1095–1103.
15. **de Lorenzo, V., M. Herrero, U. Jakubzik, and K. N. Timmis.** 1990. Mini-Tn*5* transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J. Bacteriol. **172:**6568–6572.
16. **Escolar, L., J. Perez-Martin, and V. de Lorenzo.** 1999. Opening the iron box: transcriptional metalloregulation by the Fur protein. J. Bacteriol. **181:**6223–6229.
17. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
18. **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.
19. **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.
20. **Gallegos, M.-T., C. Michan, and J. L. Ramos.** 1993. The XylS/AraC family of regulators. Nucleic Acids Res. **4:**807–810.
21. **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.
22. **Hantke, K.** 1981. Regulation of ferric iron transport in *Escherichia coli* K-12: isolation of a constitutive mutant. Mol. Gen. Genet. **182:**288–292.
23. **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.
24. **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.
25. **Kang, H. Y., and S. K. Armstrong.** 1998. Transcriptional analysis of the *Bordetella* alcaligin siderophore biosynthesis operon. J. Bacteriol. **180:**855–861.
26. **Kang, H. Y., F. C. Beaumont, T. J. Brickman, and S. K. Armstrong.** 1996. Identification and characterization of iron-regulated *Bordetella pertussis* alcaligin siderophore biosynthesis genes. J. Bacteriol. **178:**4877–4883.
27. **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.
28. **Kim, I., A. Stiefel, S. Plantör, A. Angerer, and V. Braun.** 1997. Transcription induction of the ferric citrate transport genes via the N-terminus of the FecA outer membrane protein, the Ton system and the electrochemical potential of the cytoplasmic membrane. Mol. Microbiol. **23:**333–344.
29. **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.
30. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
31. **Moore, C. H., L. A. Foster, J. 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.
32. **Neilands, J. B.** 1952. A crystalline organo-iron pigment from a rust fungus (*Ustilago sphaerogena*). J. Am. Chem. Soc. **74:**4846–4847.
33. **Nicholson, M. L., and B. Beall.** 1999. Disruption of *tonB* in *Bordetella bronchiseptica* and *Bordetella pertussis* prevents utilization of ferric siderophores, haemin and haemoglobin as iron sources. Microbiology **145:**2453–2461.
34. **Pradel, E., N. Guiso, and C. Locht.** 1998. Identification of AlcR, an AraC-type regulator of alcaligin siderophore synthesis in *Bordetella bronchiseptica* and *Bordetella pertussis*. J. Bacteriol. **180:**871–880.
35. **Raibaud, O., and M. Schwartz.** 1984. Positive control of transcription initiation in bacteria. Annu. Rev. Genet. **18:**173–206.
36. **Redhead, K., T. Hill, and H. Chart.** 1987. Interaction of lactoferrin and transferrins with the outer membrane of *Bordetella pertussis*. J. Gen. Microbiol. **133:**891–898.
37. **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.
38. **Schneider, D. R., and C. D. Parker.** 1982. Effect of pyridines on phenotypic properties of *Bordetella pertussis*. Infect. Immun. **38:**548–553.
39. **Schwyn, B., and J. B. Neilands.** 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. **160:**47–56.