

## *Bordetella* Interspecies Allelic Variation in AlcR Inducer Requirements: Identification of a Critical Determinant of AlcR Inducer Responsiveness and Construction of an *alcR*(Con) Mutant Allele

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Previous studies established the critical roles of AlcR and alcaligin inducer in positive regulation of alcaligin siderophore biosynthesis and transport genes in *Bordetella pertussis* and *Bordetella bronchiseptica*. Transcriptional analyses using plasmid-borne *alcR* genes of *B. pertussis* UT25 and *B. bronchiseptica* B013N to complement the *alcR* defect of *B. bronchiseptica* strain BRM13 ( $\Delta$ *alcR1* *alcA*::mini-Tn5 *lacZ1*) revealed interspecies differences in AlcR inducer requirements for activation of *alcBCDER* operon transcription. Whereas the *B. pertussis* UT25 AlcR protein retained strong inducer dependence when produced from multicopy plasmids, *B. bronchiseptica* B013N *alcR* partially suppressed the alcaligin requirement for transcriptional activation. Functional analysis of AlcR chimeras produced by interspecies domain swapping and interspecies reciprocal site-specific mutagenesis determined that the phenotypic difference in AlcR inducer dependence was due to a single amino acid difference within the proposed inducer-binding and multimerization domain of AlcR. Structural predictions guided the design of a mutant AlcR protein with a single amino acid substitution at this critical position, AlcR(S103T), that was fully constitutive not only when produced from multicopy plasmids but also at a single-copy gene dosage. These results indicate that AlcR residue 103 affects a critical determinant of alcaligin inducer dependence of AlcR-mediated transcriptional activation. The *alcR*(S103T) mutant allele is the first *alcR*(Con) mutant allele identified.

*Bordetella pertussis* and *Bordetella bronchiseptica* are gram-negative mucosal pathogens of humans and nonhuman mammals. In response to iron starvation, these bacteria produce and utilize the macrocyclic dihydroxamate siderophore alcaligin (13, 28). *B. pertussis* and *B. bronchiseptica* can also utilize some nonnative siderophores produced by other bacterial species as nutritional iron sources (3, 4). Apart from these siderophore iron sources, certain host iron sources are also readily utilized by these bacterial pathogens. Vanderpool and Armstrong provided the first genetic evidence of a functional host iron uptake system in *B. pertussis* and *B. bronchiseptica* (38). *B. pertussis* and *B. bronchiseptica* possess a specific transport system for heme iron utilization that is encoded by the *bhu* gene cluster; production of the required heme iron receptor protein BhuR is both iron repressible and heme inducible. Evidence suggesting that *B. pertussis* and *B. bronchiseptica* can utilize the host iron glycoproteins lactoferrin and transferrin (26, 33) has also been reported.

In *B. pertussis* and *B. bronchiseptica*, the iron starvation stress response is regulated transcriptionally by the Fur repressor protein with ferrous iron as the corepressor (7). Fur controls the expression of the *alcBCDER* alcaligin biosynthesis and regulation operon and the *fauA* ferric alcaligin outer membrane receptor gene at three promoter-operator regions within the alcaligin gene cluster (5, 7, 10, 22, 23; T. J. Brickman and

S. K. Armstrong, Abstr. 97th Gen. Meet. Am. Soc. Microbiol., abstr. B-241, p. 70, 1997). AlcR is an AraC family transcriptional regulator (5, 32) that is necessary for maximal expression of alcaligin siderophore biosynthesis and transport genes under iron starvation stress conditions (5, 10, 12). Transcriptional activation by AlcR requires the presence of alcaligin siderophore acting as the inducer (12). Since these *Bordetella* species are capable of utilizing multiple alternative iron sources for growth, priority regulation involving the chelator itself as the inducer ensures that alcaligin biosynthesis and uptake functions are expressed maximally only under conditions in which alcaligin is perceived in the environment (12). We hypothesize that the ability of *Bordetella* spp. to prioritize the expression of their different iron systems may be important for effective adaptation to changes in the host environment during the course of an infection.

AraC family regulators are also known to be involved in the regulation of other bacterial iron transport systems. The AraC-like protein PchR positively regulates expression of the pyochelin siderophore system of *Pseudomonas aeruginosa*; transcriptional activation is responsive to pyochelin, and induction requires a functional ferric pyochelin receptor (18, 19). Similarly, the *Yersinia pestis* AraC family regulator YbtA is required for maximal expression of yersiniabactin siderophore biosynthesis and transport genes (15).

In studies characterizing the role of alcaligin as the inducer of AlcR-mediated transcriptional activation, it was observed that *B. bronchiseptica* AlcR was partially constitutive for activation of a chromosomal *alcA*::mini-Tn5 *lacZ1* fusion gene when AlcR was produced from multicopy plasmids. In contrast, *B. pertussis* AlcR

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TABLE 1. *B. bronchiseptica* strains and *alcR*<sup>+</sup> plasmids used in this study

Strain or plasmid	Relevant genotype, phenotype, or description	Reference or source
BRM13	<i>B. bronchiseptica</i> B013N $\Delta$ <i>alcR1 alcA</i> ::mini-Tn5 <i>lacZ1</i> ; kanamycin <sup>r</sup> AlcR <sup>-</sup> alcaligin deficient	12
BRM13 $\Omega$ ( $\Delta$ <i>alcR1</i> ::pSS/Bp)	pSS/Bp plasmid integrant derivative of BRM13; AlcR <sup>+</sup> kanamycin <sup>r</sup> ampicillin <sup>r</sup> gentamicin <sup>r</sup>	This study
BRM13 $\Omega$ ( $\Delta$ <i>alcR1</i> ::pSS/Bb)	pSS/Bb plasmid integrant derivative of BRM13; AlcR <sup>+</sup> kanamycin <sup>r</sup> ampicillin <sup>r</sup> gentamicin <sup>r</sup>	This study
BRM13 $\Omega$ ( $\Delta$ <i>alcR1</i> ::pSS/Bb-S103T)	pSS/Bp-S103T plasmid integrant derivative of BRM13; AlcR(Con) kanamycin <sup>r</sup> ampicillin <sup>r</sup> gentamicin <sup>r</sup>	This study
pRK415	Mobilizable broad-host-range plasmid cloning vector; tetracycline <sup>r</sup> ; RK2 origin	24
pRK21	pRK415 with 1.6-kb <i>KpnI-PstI B. pertussis</i> UT25 <i>alcR</i> <sup>+</sup> DNA insert fragment; AlcR <sup>+</sup> tetracycline <sup>r</sup> ; formerly designated pP9KP	5, 12
pRK15	pRK415 with 2.3-kb <i>EcoRI-PstI B. bronchiseptica</i> B013N <i>alcR</i> <sup>+</sup> DNA insert fragment; AlcR <sup>+</sup> tetracycline <sup>r</sup>	12
pRK/1.6KP (Bb)	pRK415 with 1.6-kb <i>KpnI-PstI B. bronchiseptica</i> B013N <i>alcR</i> <sup>+</sup> DNA insert fragment; AlcR <sup>+</sup> tetracycline <sup>r</sup>	This study
pRK/2.3EP (Bp)	pRK415 with 2.3-kb <i>EcoRI-PstI B. pertussis</i> UT25 <i>alcR</i> <sup>+</sup> DNA insert fragment; AlcR <sup>+</sup> tetracycline <sup>r</sup>	This study
pBBR1MCS	Mobilizable broad-host-range plasmid cloning vector; chloramphenicol <sup>r</sup>	25
pBB/1.6KP (Bp)	pBBR1MCS with 1.6-kb <i>KpnI-PstI B. pertussis</i> UT25 <i>alcR</i> <sup>+</sup> DNA insert fragment; AlcR <sup>+</sup> chloramphenicol <sup>r</sup>	This study
pBB/1.6KP (Bb)	pBBR1MCS with 1.6-kb <i>KpnI-PstI B. bronchiseptica</i> B013N <i>alcR</i> <sup>+</sup> DNA insert fragment; AlcR <sup>+</sup> chloramphenicol <sup>r</sup>	This study
pBB/2.3EP (Bp)	pBBR1MCS with 2.3-kb <i>EcoRI-PstI B. pertussis</i> UT25 <i>alcR</i> <sup>+</sup> DNA insert fragment; AlcR <sup>+</sup> chloramphenicol <sup>r</sup>	This study
pBB/2.3EP (Bb)	pBBR1MCS with 2.3-kb <i>EcoRI-PstI B. bronchiseptica</i> B013N <i>alcR</i> <sup>+</sup> DNA insert fragment; AlcR <sup>+</sup> chloramphenicol <sup>r</sup>	This study
pRK/N <sub>p</sub> C <sub>b</sub>	pRK415 with 1.2-kb <i>KpnI-PstI</i> DNA insert fragment, N-terminal coding sequences of <i>B. pertussis</i> UT25 <i>alcR</i> spliced with C-terminal coding sequences of <i>B. bronchiseptica</i> B013N <i>alcR</i> by overlap extension PCR; AlcR <sup>+</sup> tetracycline <sup>r</sup>	This study
pRK/N <sub>b</sub> C <sub>p</sub>	pRK415 with 1.2-kb <i>KpnI-PstI</i> DNA insert fragment, N-terminal coding sequences of <i>B. bronchiseptica</i> B013N <i>alcR</i> spliced with C-terminal coding sequences of <i>B. pertussis</i> UT25 <i>lcR</i> by overlap extension PCR; AlcR <sup>+</sup> tetracycline <sup>r</sup>	This study
pRK/N <sub>p</sub> C <sub>p</sub>	pRK415 with 1.2-kb <i>KpnI-PstI</i> DNA insert fragment, N-terminal coding sequences of <i>B. pertussis</i> UT25 <i>alcR</i> spliced with C-terminal coding sequences of <i>B. pertussis</i> UT25 <i>alcR</i> by overlap extension PCR; AlcR <sup>+</sup> tetracycline <sup>r</sup>	This study
pRK/N <sub>b</sub> C <sub>b</sub>	pRK415 with 1.2-kb <i>KpnI-PstI</i> DNA insert fragment, N-terminal coding sequences of <i>B. bronchiseptica</i> B013N <i>alcR</i> spliced with C-terminal coding sequences of <i>B. bronchiseptica</i> B013N <i>alcR</i> by overlap extension PCR; AlcR <sup>+</sup> tetracycline <sup>r</sup>	This study
pRK/Bp-G103S	pRK415 with 1.2-kb <i>KpnI-PstI</i> DNA insert fragment encoding <i>B. pertussis</i> UT25 <i>alcR</i> G103S mutant allele, generated by PCR mutagenesis; AlcR <sup>+</sup> tetracycline <sup>r</sup>	This study
pRK/Bb-S103G	pRK415 with 1.2-kb <i>KpnI-PstI</i> DNA insert fragment encoding <i>B. bronchiseptica</i> B013N <i>alcR</i> S103G mutant allele, generated by PCR mutagenesis; AlcR <sup>+</sup> tetracycline <sup>r</sup>	This study
pRK/Bb-S103T	pRK415 with 1.2-kb <i>KpnI-PstI</i> DNA insert fragment encoding <i>B. bronchiseptica</i> B013N <i>alcR</i> S103T mutant allele, generated by PCR mutagenesis; AlcR(Con) tetracycline <sup>r</sup>	This study
pSS1129	Mobilizable suicide plasmid; ampicillin <sup>r</sup> gentamicin <sup>r</sup> ; ColE1 origin	37
pSS/Bp	pSS1129 with 1.2-kb <i>EcoRI-HindIII</i> DNA insert fragment of pRK/N <sub>p</sub> C <sub>p</sub> ; AlcR <sup>+</sup> ampicillin <sup>r</sup> gentamicin <sup>r</sup>	This study
pSS/Bb	pSS1129 with 1.2-kb <i>EcoRI-HindIII</i> DNA insert fragment of pRK/N <sub>b</sub> C <sub>b</sub> ; AlcR <sup>+</sup> ampicillin <sup>r</sup> gentamicin <sup>r</sup>	This study
pSS/Bb-S103T	pSS1129 with 1.2-kb <i>EcoRI-HindIII</i> DNA insert fragment of pRK/Bb-S103T; AlcR(Con) ampicillin <sup>r</sup> gentamicin <sup>r</sup>	This study

exhibited normal alcaligin inducer dependence even when over-produced. Elucidation of the molecular basis of this difference in multicopy suppression phenotype, a single amino acid substitution in the predicted inducer-binding and multimerization domain of AlcR, led to the identification of a key determinant of AlcR function. Structural predictions directed the construction of a mutant AlcR protein that was fully constitutive for activation of transcription even at a single-copy gene dosage.

#### MATERIALS AND METHODS

**Bacterial strains and plasmids.** *B. bronchiseptica* strains, plasmid cloning vectors, and *alcR*<sup>+</sup> plasmids used in this study are listed in Table 1. The construction

and phenotypic characterization of the *B. bronchiseptica*  $\Delta$ *alcR1 alcA*::mini-Tn5 *lacZ1* mutant strain BRM13 have been described previously (12). *Escherichia coli* DH5 $\alpha$  [F<sup>-</sup>  $\phi$ 80d *lacZ* $\Delta$ M15  $\Delta$ (*lacZYA-argF*)U169 *endA1 recA1 hsdR17*( $\tau_K^-$  m $\kappa^+$ ) *deoR thi-1 supE44*  $\lambda^-$  *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* recipients. The broad-host-range plasmid cloning vectors pRK415 (24) and pBBR1MCS (25) were used in the construction of *alcR*<sup>+</sup> multicopy plasmids used in complementation analyses; the suicide plasmid cloning vector pSS1129 (37) was used to deliver cloned *alcR* alleles to the *B. bronchiseptica* chromosome by homologous recombination. DH5 $\alpha$ (pRK2013) provided plasmid-encoded mobilization functions (16) in triparental matings to transfer pRK415 and pSS1129 plasmid derivatives to *B. bronchiseptica*. Plasmids pRK21 and pRK15 have been described previously (5, 12). Plasmids pRK/1.6KP (Bb) and pRK/2.3EP (Bp) were con-

TABLE 2. Oligonucleotide primers used in *alcR* PCR splicing and mutagenesis

Primer	Nucleotide sequence (5' to 3') <sup>a</sup>	Nucleotide positions <sup>b</sup>
N1 ( <i>KpnI</i> )	ggccGGTACCTGAGCAACATGGGAGAGCAG	1–26
C2( <i>PstI</i> )	ggccctgcagGAATGGGATGGTCAATGCCGCAATG	1189–1162
N2	CAGTCGCGCCAGGTCATCCAGAGTATGG	909–882
C1	CCATACTCTGGATGACCTGGCGCGACTG	882–909
N2P(G103)	CCGGATGAACGCCCGTCATGGCGGAC	538–514
C1P(G103)	GTCGCCATGACGGGCGTTCATCCGG	514–538
N2B(S103)	CCGGATGAACGCTCGTCATGGCGGAC	538–514
C1B(S103)	GTCGCCATGACGAGCGTTCATCCGG	514–538
N2B(T103)	CCGGATGAACGGTCTCGTCATGGCGGAC	538–514
C1B(T103)	GTCGCCATGACGACCGTTCATCCGG	514–538

<sup>a</sup> Uppercase letters represent *alcR* sequences; lowercase letters represent synthetic primer GC clamp and restriction site adapter sequences.

<sup>b</sup> Nucleotide positions correspond to positions in the reported *alcR* region nucleotide sequence (GenBank accession number AF018255) of *B. pertussis* strain UT25 (5).

structed by subcloning the 1.6-kb *KpnI-PstI alcR*<sup>+</sup> DNA region of *B. bronchiseptica* B013N from plasmid pRK15 (12) and the 2.3-kb *EcoRI-PstI alcR*<sup>+</sup> DNA region of *B. pertussis* UT25 from cosmid pCP1.11 (23), respectively, to plasmid vector pRK415. The same *alcR*<sup>+</sup> 1.6-kb *KpnI-PstI* and 2.3-kb *EcoRI-PstI* DNA fragments of *B. bronchiseptica* B013N and *B. pertussis* UT25 were also subcloned to plasmid vector pBBR1MCS to produce plasmids pBB/1.6KP (Bb), pBB/1.6KP (Bp), pBB/2.3EP (Bb), and pBB/2.3EP (Bp).

**AlcR domain swapping by overlap extension PCR.** The *B. pertussis* UT25 and *B. bronchiseptica* B013N *alcR* N-terminal (amino acids 1 to 223) and C-terminal (amino acids 224 to 303) domain coding sequences (5) were exchanged by overlap extension PCR essentially as described by Horton et al. (21). The coding sequences for the AlcR N-terminal and C-terminal domains of *B. pertussis* UT25 and *B. bronchiseptica* B013N were each PCR amplified using primer pairs N1(*KpnI*) and N2, and C1 and C2(*PstI*) (Table 2), yielding domain-specific products with overlapping termini spanning the N- and C-terminal coding sequence splicing junction. PCR primers N1(*KpnI*) and C2(*PstI*) were designed to include GC clamps and restriction enzyme site adapters (Table 2) to facilitate directional cloning of the fusion products as *KpnI-PstI* DNA fragments to the plasmid cloning vector pRK415. PCR primers N2 and C1, which were internal to the *alcR* coding sequence, were complementary and spanned the *alcR* domain splicing junction. PCR mixtures contained 100 ng of *B. pertussis* UT25 or *B. bronchiseptica* B013N genomic DNA, 10 mM KCl, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 20 mM Tris-HCl (pH 8.75), 2 mM MgSO<sub>4</sub>, 0.1% Triton X-100, 100 μg of bovine serum albumin/ml, 200 μM each deoxynucleoside triphosphate, primer pairs at 0.5 μM each, 5% dimethyl sulfoxide, and 2.5 U of *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, Calif.) in a 100-μl reaction volume. Reactions were performed by using a thermal cycler programmed for 30 cycles of denaturation at 96°C for 1 min, primer annealing at a temperature 5°C below the primer melting temperature (*T<sub>m</sub>*) for 1 min, and primer elongation at 72°C for 1 min.

To splice the *alcR* domain coding sequences by overlap extension PCR, the N- and C-terminal domain-specific PCR products were combined in equimolar proportions, denatured, and reannealed to allow strand reassociation via base pairing within the 28-nucleotide (nt) overlap region to form partial duplex molecules. The recessed termini of the partial duplex molecules were extended with *Pfu* Turbo DNA polymerase and deoxynucleoside triphosphates to yield full-length 1.2-kb *alcR* DNA duplexes that were PCR amplified by using primers N1(*KpnI*) and C2(*PstI*). PCR conditions were essentially the same as those for production of the individual *alcR* domain coding sequences. Combining *B. pertussis* UT25 and *B. bronchiseptica* B013N N- and C-terminal domain coding sequences yielded interspecies hybrid *alcR* genes with an in-frame fusion at the splicing junction. As controls for the splicing procedure, the wild-type *alcR* genes of both species were reconstituted by joining N-terminal and C-terminal domain coding sequences derived from the same-species *alcR* gene.

*alcR* DNA fragments produced by the splicing reactions were purified, digested with *KpnI* and *PstI* to produce cohesive termini at the synthetic adapter sequences, and ligated with *KpnI-PstI*-digested pRK415 plasmid vector DNA. The cloning procedures resulted in two interspecies hybrid *alcR*<sup>+</sup> plasmids, pRK/N<sub>b</sub>C<sub>b</sub> and pRK/N<sub>p</sub>C<sub>p</sub>, and two reconstituted wild-type *alcR*<sup>+</sup> plasmids, pRK/N<sub>p</sub>C<sub>p</sub> and pRK/N<sub>b</sub>C<sub>b</sub> (Table 1).

**Site-specific PCR mutagenesis.** The *alcR* genes of *B. pertussis* UT25 and *B. bronchiseptica* B013N were reciprocally altered to the nucleotide sequence of the other species' gene by an overlap extension PCR mutagenesis procedure (20). Site-specific PCR mutagenesis was performed essentially as described for AlcR

domain swapping by overlap extension PCR using *B. pertussis* UT25 and *B. bronchiseptica* B013N genomic DNA templates, but with complementary pairs of mutagenic primers [N2P(G103) and C1P(G103) or N2B(S103) and C1B(S103) (Table 2)] which overlapped the *alcR* DNA region spanning the codon specifying amino acid position 103 in the *B. pertussis* UT25 or *B. bronchiseptica* B013N *alcR* open reading frame. The spliced products were cloned to plasmid vector pRK415 using the N1(*KpnI*) and C2(*PstI*) primer-encoded *KpnI* and *PstI* adapter sites to produce *alcR* mutant plasmids pRK/Bp-G103S and pRK/Bb-S103G (Table 1) for phenotypic analysis in BRM13. Similarly, PCR mutagenesis using the mutagenic primers N2B(T103) and C1B(T103) (Table 2) was performed to mutate the *B. bronchiseptica alcR* gene to produce the *alcR*(S103T) mutant allele, which was cloned to produce plasmid pRK/Bb-S103T. Predictions of the functional consequences of amino acid substitutions at AlcR position 103, leading to the choice of a threonine substitution, were made by using Dayhoff's mutation odds matrix (14), the amino acid substitution matrices of Bordo and Argos (6) for the identification of structurally tolerated substitutions, and the SIFT (30) sequence homology-based protein engineering tool (Fred Hutchinson Cancer Research Center [http://blocks.fhcrc.org/~pauline/SIFT\_seq\_submit.html]) for the prediction of tolerated and deleterious amino acid substitutions. All mutations were confirmed by nucleotide sequencing.

**Bacterial culture conditions.** *E. coli* and *B. bronchiseptica* strains were cultured on Luria-Bertani agar plates supplemented as required with antibiotics. Modified Stainer-Scholte (SS) medium (36) 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). 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 the kanamycin resistance marker of mini-Tn5 *lacZ1* mutant strain BRM13. Ampicillin was used at 100 μg/ml, and gentamicin was used at 10 μg/ml, for maintenance of plasmid vector pSS1129 derivatives in *E. coli* and for selection for insertions of pSS1129-borne *alcR* alleles at the chromosomal *ΔalcR1* locus of *B. bronchiseptica* strain BRM13. In analyses of induction of *alcABCDER* operon transcription by alcaligin, SS culture medium was supplemented with purified alcaligin siderophore at a 20-μg/ml final concentration, which approximates the inducer requirement for maximal activation of transcription of the *alcABCDER* operon by AlcR under iron-depleted SS culture conditions (12). All glassware was acid cleaned and rinsed repeatedly in distilled deionized water prior to use.

**Bordetella alcaligin siderophore purification.** Alcaligin was purified from *B. bronchiseptica* culture supernatants by a modification of the benzyl alcohol/ether extraction method of Neilands (29) as previously described by Brickman and coworkers (13) and was recrystallized from ethanolic solution.

**Routine DNA procedures.** General genetic techniques were performed essentially as described previously (34). *E. coli* was transformed by electroporation using standard methods (Bio-Rad, Hercules, Calif.). Conjugal transfer of pRK415 and pSS1129 plasmid derivatives to *B. bronchiseptica* involved triparental matings using *E. coli* DH5α as the plasmid donor host strain and DH5α(pRK2013) as the source of mobilization functions as described previously (9). Transconjugants or plasmid integrants were selected on agar plates containing the appropriate selective antibiotics and crude colicin B (8).

**β-Galactosidase assays.** *B. bronchiseptica alcA::mini-Tn5 lacZ1* fusion strains were assayed for β-galactosidase activity by the method of Miller (27) as modified by Brickman and coworkers (11) after culture in iron-replete or iron-depleted SS medium, or in iron-depleted SS medium supplemented with purified

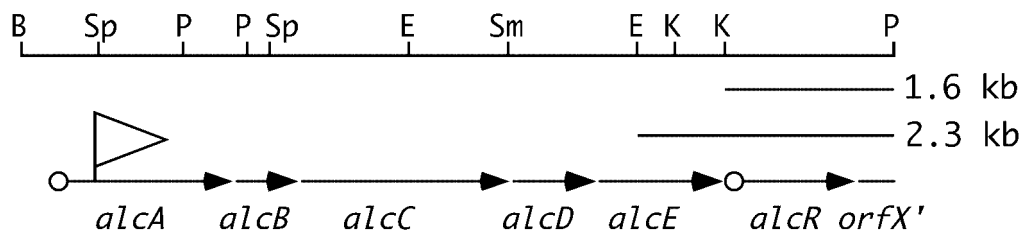


FIG. 1. Genetic organization of the *Bordetella alcABCDE* alcaligin siderophore operon. The diagram represents an approximately 8-kb *Bam*HI-*Pst*I chromosomal DNA subregion of the alcaligin gene cluster of *B. bronchiseptica* B013N. Filled arrows indicate the genetic limits and transcriptional orientations of cistrons comprising the *alcABCDE* operon, and open circles represent the locations of known Fur-regulated promoter-operator regions upstream of *alcA* and *alcR*. The large open arrowhead indicates the position and transcriptional orientation of the promoterless *lacZ* reporter gene associated with the *alcA*::mini-Tn5 *lacZ*I transposon insertion of alcaligin-deficient *B. bronchiseptica* mutant strain BRM1 (2) and its  $\Delta$ *alcR*I derivative strain BRM13 (12). The spatial limits of the 1.6-kb *Kpn*I-*Pst*I and 2.3-kb *Eco*RI-*Pst*I subregions used to construct *alcR*<sup>+</sup> multicopy plasmids are indicated. Abbreviations: B, *Bam*HI; Sp, *Sph*I; P, *Pst*I; E, *Eco*RI; Sm, *Sma*I; K, *Kpn*I.

alcaligin at a 20- $\mu$ g/ml final concentration.  $\beta$ -Galactosidase activities represent means from triplicate assays ( $n = 3$ )  $\pm$  1 standard deviation.

**Nucleotide sequencing.** Nucleotide sequencing of *B. bronchiseptica alcR* used the dideoxy chain termination method (35) and double-stranded plasmid DNA templates, with nucleotide sequencing services provided by the University of Minnesota Microchemical Facility and the University of Minnesota Advanced Genetic Analysis Center. DNA oligonucleotide primers were purchased from Invitrogen Life Technologies (Grand Island, N.Y.). Management and analysis of nucleotide sequence data used the Lasergene sequence analysis software system for the Macintosh PowerPC computer (DNASTAR, Inc., Madison, Wis.).

**Phenotypic characterization of AlcR expressed from wild-type and mutant *alcR* alleles in single copy.** *alcR*<sup>+</sup> integrant strains were constructed by integration of pSS1129 suicide plasmid-borne *alcR* alleles at the  $\Delta$ *alcR*I locus of BRM13 by homologous recombination. The wild-type *alcR* genes of *B. pertussis* UT25 and *B. bronchiseptica* B013N, as well as the *B. bronchiseptica alcR*(S103T) mutant allele, were subcloned from their respective pRK415 derivatives as 1.2-kb *Eco*RI-*Hind*III fragments to suicide plasmid pSS1129 to produce plasmids pSS/Bp, pSS/Bb, and pSS/Bb-S103T (Table 1). Each pSS1129 suicide plasmid derivative was conjugally transferred to *B. bronchiseptica* BRM13, and plasmid integrants were selected and maintained on the basis of ampicillin and gentamicin resistance associated with the plasmid vector. Transcriptional activation by AlcR was monitored as  $\beta$ -galactosidase activity produced from the BRM13 *alcA*::mini-Tn5 *lacZ*I fusion gene in response to iron starvation and the presence of alcaligin inducer.

**Nucleotide sequence accession number.** The GenBank accession number assigned to the *B. bronchiseptica* B013N *alcR* gene is AF426103.

## RESULTS

**Interspecies variation in the alcaligin inducer requirement of AlcR for activation of *alcABCDE* operon transcription.** AlcR is a Fur-regulated AraC-like transcriptional regulator encoded within the alcaligin gene cluster (Fig. 1) of *B. pertussis* and *B. bronchiseptica* (5, 32). The *alcR* gene of *B. pertussis* was first identified by phenotypic complementation of the alcaligin utilization and production defects of a *B. bronchiseptica* mutant selected on the basis of streptonigrin resistance (5). Genetic and biochemical studies established the requirement of AlcR for maximal expression of the *alcABCDE* operon (5, 12, 22) and the *fauA* gene encoding the outer membrane receptor for ferric alcaligin under iron starvation stress conditions (10). Transcriptional activation by AlcR was shown to be dependent on the presence of the cognate siderophore alcaligin acting as the inducer (12). Subsequent studies aimed at further analysis of the inducer requirement of AlcR involved construction and comparative phenotypic characterization of AlcR produced from *B. pertussis* and *B. bronchiseptica alcR*<sup>+</sup> plasmids. Gene fusion analyses using *alcR*<sup>+</sup> plasmids to activate *alcABCDE*

operon transcription in the AlcR<sup>-</sup> and alcaligin-deficient *B. bronchiseptica* strain BRM13 revealed that the AlcR proteins of *B. pertussis* UT25 and *B. bronchiseptica* B013N exhibited different apparent requirements for inducer when produced from multicopy plasmids (Fig. 2). *B. bronchiseptica* AlcR displayed a partial constitutive [AlcR(Con)] phenotype; expression of *B. bronchiseptica* B013N *alcR* from the pRK415-based multicopy plasmid pRK15 partially suppressed the alcaligin inducer requirement of AlcR normally needed for maximal *alcABCDE* operon transcription under iron starvation stress conditions. In iron-depleted cultures, comparison of *alcABCDE* operon transcriptional activities of uninduced cultures with those of cultures supplemented with alcaligin provides a useful measure of the degree of inducer dependence of transcriptional activation by AlcR (Fig. 2B). In iron-depleted cultures of BRM13(pRK15) without alcaligin inducer supplementation, *alcA*::mini-Tn5 *lacZ*I transcriptional activity was 76% of the maximal transcriptional activity that was associated with alcaligin-induced cultures of the same strain. In contrast, *B. pertussis* AlcR produced from plasmid pRK21 exhibited strong dependence on alcaligin inducer when produced in BRM13; transcription of the *alcA*::mini-Tn5 *lacZ*I fusion gene in uninduced BRM13(pRK21) cultures was only 6% of the transcriptional activity of alcaligin-induced cultures.

The *B. bronchiseptica alcR*<sup>+</sup> plasmid pRK15, with a 2.3-kb *Eco*RI-*Pst*I DNA insert fragment (Fig. 1), includes an additional 0.7 kb of *alcR* upstream DNA sequence compared with the cloned 1.6-kb *Kpn*I-*Pst*I *B. pertussis alcR*<sup>+</sup> DNA region of pRK21. When multicopy *alcR*<sup>+</sup> plasmids pRK/2.3EP (Bp) and pRK/1.6KP (Bb), which carry the corresponding 2.3-kb *Eco*RI-*Pst*I and 1.6-kb *Kpn*I-*Pst*I genetic regions of these species as plasmids pRK15 and pRK21, were constructed and analyzed in BRM13, the same interspecies difference in the AlcR inducer requirement was observed (Fig. 2). Similarly, the partial constitutivity of AlcR produced from plasmid-borne copies of *B. bronchiseptica* B013N *alcR* was associated with the same 1.6-kb *Kpn*I-*Pst*I and 2.3-kb *Eco*RI-*Pst*I cloned *alcR*<sup>+</sup> genetic regions carried in a different plasmid vector, pBBR1MCS, that are present in plasmids pBB/1.6KP (Bb) and pBB/2.3EP (Bb) (data not shown). The corresponding pBBR1MCS-derived *B. pertussis* UT25 plasmids, pBB/1.6KP (Bp) and pBB/2.3EP (Bp), did not suppress the alcaligin inducer requirement of AlcR. Thus, the interspecies difference in AlcR function was

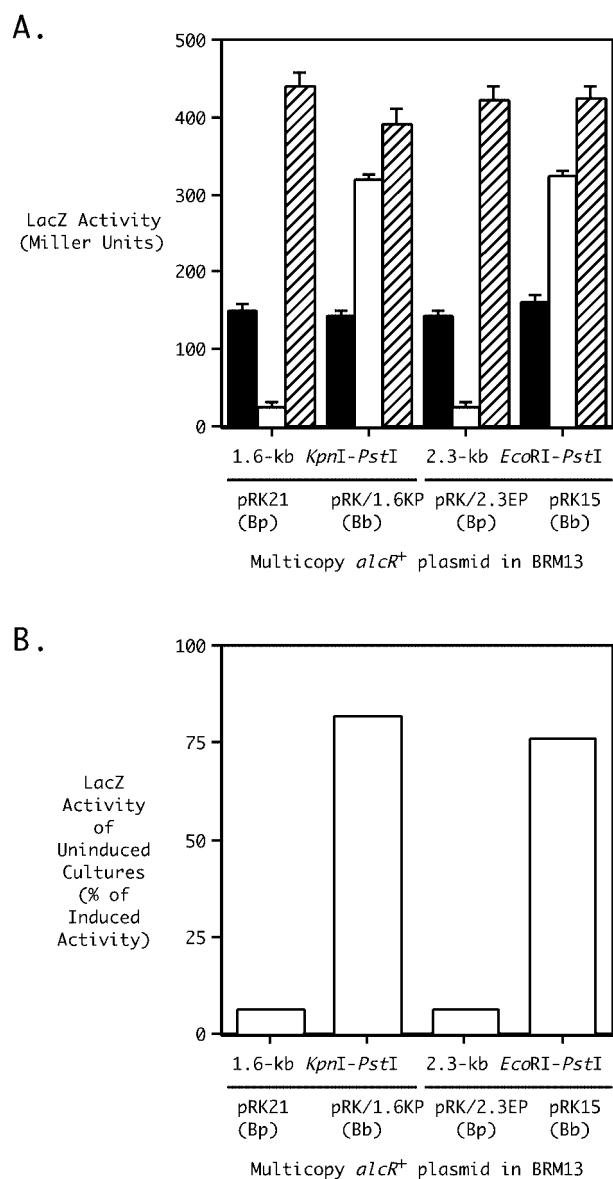


FIG. 2. Interspecies allelic variation in AlcR requirements for alcaligin inducer: partial constitutivity of *B. bronchiseptica* AlcR associated with production from multicopy plasmids. (A)  $\beta$ -Galactosidase reporter activities associated with expression of the *alcA*::mini-Tn5 *lacZ1* fusion element of *B. bronchiseptica*  $\Delta$ *alcR1* mutant strain BRM13 complemented either with the multicopy *B. bronchiseptica* *alcR*<sup>+</sup> plasmids pRK/1.6KP(Bb) and pRK15 or with the analogous *B. pertussis* *alcR*<sup>+</sup> plasmids pRK21 and pRK/2.3EP(Bp). The genetic limits of the cloned DNAs (1.6-kb *KpnI*-*PstI* and 2.3-kb *EcoRI*-*PstI*) carried by *B. pertussis* and *B. bronchiseptica* *alcR*<sup>+</sup> plasmids are indicated.  $\beta$ -Galactosidase activities were measured for cells cultured in iron-replete SS medium (solid bars), iron-depleted SS medium (open bars), or iron-depleted SS medium supplemented with 20  $\mu$ g of purified alcaligin/ml (hatched bars).  $\beta$ -Galactosidase activities are expressed in Miller units (mean  $\pm$  1 standard deviation;  $n = 3$ ). (B) The relative degree of constitutivity of AlcR produced from multicopy *B. bronchiseptica* or *B. pertussis* *alcR*<sup>+</sup> plasmids in BRM13 under iron-depleted culture conditions was quantified by expressing the  $\beta$ -galactosidase activities produced without alcaligin inducer as percentages of the induced activities.

apparently not related to differences in the spatial limits of the cloned *alcR* sequences, nor was it specific to expression of *alcR* from pRK415-based plasmids.

Despite this consistent interspecies variation in AlcR inducer dependence associated with production of AlcR from multicopy plasmids, AlcR of *B. pertussis* UT25 and AlcR of *B. bronchiseptica* B013N were found to activate *alcA*::mini-Tn5 *lacZ1* transcription in BRM13 to approximately the same levels in alcaligin-induced cultures (Fig. 2A). Thus, although the two species' AlcR proteins exhibit different degrees of inducer dependence when produced in this genetic context, both proteins are equally capable of activating *alcABCDER* operon transcription to the maximal levels normally associated with growth of *Bordetella* under iron-depleted conditions in the presence of inducer.

**AlcR domain swapping: role of the AlcR N-terminal domain in interspecies variation in the alcaligin inducer requirement associated with *alcR* expression from multicopy plasmids.** Given the strong conservation of structural features among members of the AraC family of transcriptional regulators (17), AlcR determinants responsible for the observed interspecies variation in the inducer requirement were hypothesized to be associated with the N-terminal domain of the activator protein. This hypothesis was tested by examining the inducer requirements of interspecies AlcR chimeras for activation of *alcABCDER* operon transcription in strain BRM13. Chimeras were generated from the *alcR* genes of *B. pertussis* UT25 and *B. bronchiseptica* B013N by reciprocal exchange of the N- and C-terminal coding sequences by overlap extension PCR. Four spliced *alcR* products were generated and cloned to the multicopy plasmid vector pRK415 to yield the chimeric *alcR*<sup>+</sup> plasmids pRK/N<sub>p</sub>C<sub>b</sub> and pRK/N<sub>b</sub>C<sub>p</sub>, as well as wild-type *alcR*<sup>+</sup> plasmids of both species, pRK/N<sub>p</sub>C<sub>p</sub> and pRK/N<sub>b</sub>C<sub>b</sub>, as splicing reaction controls (Table 1).

In BRM13, the chimeric plasmid pRK/N<sub>b</sub>C<sub>p</sub> encoding AlcR with the *B. bronchiseptica* N-terminal domain conferred the partial constitutive regulatory phenotype associated with production of the wild-type *B. bronchiseptica* AlcR protein from multicopy plasmids (Fig. 3). The transcriptional activity of the *alcA*::mini-Tn5 *lacZ1* fusion gene in uninduced iron-depleted cultures of BRM13(pRK/N<sub>b</sub>C<sub>p</sub>) was 83% of the alcaligin-induced transcriptional activity. Concordantly, control plasmid pRK/N<sub>b</sub>C<sub>b</sub>, bearing a spliced version of the wild-type *B. bronchiseptica* *alcR* gene, also conferred the partial AlcR(Con) phenotype in BRM13. In contrast, AlcR proteins with the *B. pertussis* N-terminal domain, produced from the chimeric plasmid pRK/N<sub>p</sub>C<sub>b</sub> or the wild-type *B. pertussis* plasmid pRK/N<sub>p</sub>C<sub>p</sub>, exhibited the normal inducer dependence observed previously for the wild-type *B. pertussis* AlcR protein, with uninduced iron-depleted cultures displaying only 5 and 6% of the alcaligin-induced transcriptional activities, respectively. This phenotypic characterization of AlcR interspecies chimeras strongly suggested that the interspecies variation in the AlcR inducer requirement for transcriptional activation was attributable to undefined determinants associated with the N-terminal portions of the AlcR proteins.

**The *alcR* coding sequences of *B. pertussis* UT25 and *B. bronchiseptica* B013N differ by a single nucleotide.** The nucleotide sequence of the *alcR* gene of *B. pertussis* strain UT25 (GenBank accession number AF018255) was reported previously by

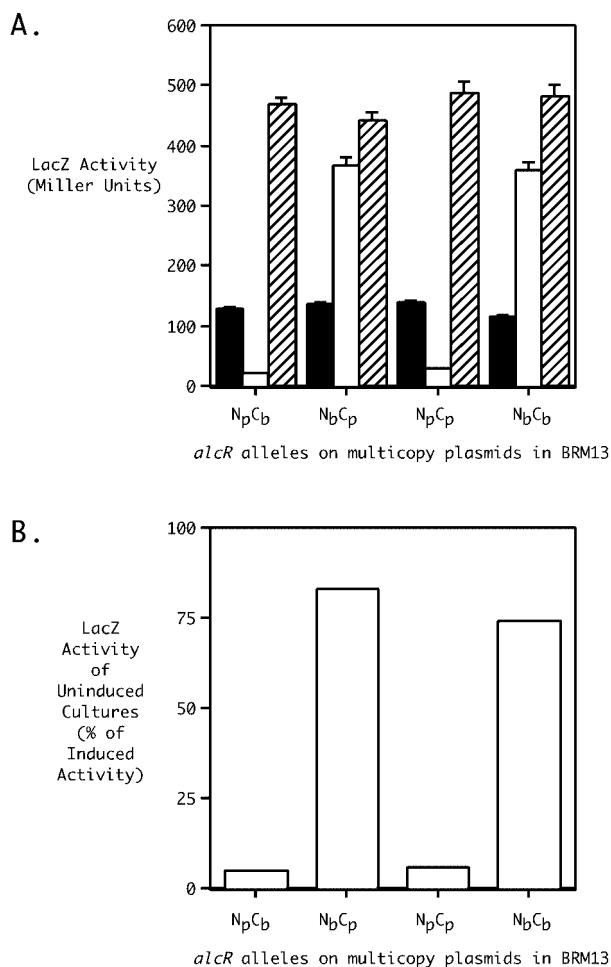


FIG. 3. Alcaligin inducer requirements of wild-type and interspecies chimeric AlcR proteins produced from *alcR* alleles borne on multicopy plasmids. (A) *alca::mini-Tn5 lacZ1* transcriptional activities were monitored in the *alcR* mutant strain BRM13 complemented with wild-type and interspecies chimeric *alcR*<sup>+</sup> plasmids constructed by overlap extension PCR.  $\beta$ -Galactosidase activities were measured for cells cultured in iron-replete SS medium (solid bars), iron-depleted SS medium (open bars), or iron-depleted SS medium supplemented with 20  $\mu$ g of purified alcaligin/ml (hatched bars).  $\beta$ -Galactosidase activities are expressed in Miller units (mean  $\pm$  1 standard deviation;  $n = 3$ ). (B) The relative degree of constitutivity of AlcR is depicted as in Fig. 2B. Multicopy *alcR*<sup>+</sup> plasmids: N<sub>p</sub>C<sub>b</sub>, pRK/N<sub>p</sub>C<sub>b</sub>; N<sub>b</sub>C<sub>p</sub>, pRK/N<sub>b</sub>C<sub>p</sub>; N<sub>p</sub>C<sub>p</sub>, pRK/N<sub>p</sub>C<sub>p</sub>; N<sub>b</sub>C<sub>b</sub>, pRK/N<sub>b</sub>C<sub>b</sub>.

Beaumont and coworkers (5). The UT25 *alcR* coding sequence spans residues 220 to 1131, numbered as in the GenBank database record, and specifies a 304-amino-acid protein with a molecular mass of approximately 34 kDa. In an effort to define the molecular basis of the interspecies variation in AlcR function, the nucleotide sequence of the *alcR* gene of *B. bronchiseptica* B013N was determined and compared with the known *alcR* sequence of *B. pertussis* strain UT25. The *alcR* coding sequence of *B. bronchiseptica* B013N was found to differ from *alcR* of *B. pertussis* UT25 by a single nucleotide, corresponding to position 526 in the *B. pertussis* UT25 *alcR* GenBank record. As a result, *B. bronchiseptica* B013N AlcR carries a serine

residue (encoded by an AGC triplet) at amino acid position 103, whereas a glycine residue (encoded by a GGC triplet) is found at that position in the N-terminal domain of the UT25 protein, as deduced from the nucleotide sequence. The *alcR* upstream sequences of *B. bronchiseptica* B013N, including the Fur- and iron-regulated promoter-operator control region defined in previous studies (5; T. J. Brickman and S. K. Armstrong, Abstr. 97th Gen. Meet. Am. Soc. Microbiol.) as well as putative AlcR translation initiation control sequences, were found to be identical to the known *B. pertussis* UT25 *alcR* region sequence extending to the upstream *KpnI* recognition site (Fig. 1). By analogy with other members of the AraC family of transcriptional regulators, this amino acid sequence difference at position 103 resides in the structural domain putatively involved in inducer-binding and multimerization and thus might be predicted to influence these functions of the AlcR protein. Since no other nucleotide sequence differences were identified, these data suggested that the identity of amino acid residue 103 had a profound influence on AlcR function when the activator was produced in this genetic context.

**Phenotypic characterization of the inducer requirements of mutant AlcR proteins produced from reciprocal interspecies *alcR* mutant alleles borne on multicopy plasmids.** To confirm the hypothesized role of amino acid 103 in the interspecies variation in AlcR inducer dependence, site-directed mutagenesis was used to generate mutant *B. bronchiseptica* B013N and *B. pertussis* UT25 *alcR* alleles carrying single reciprocal nucleotide changes associated with the wild-type genes of the alternate species. That is, the mutated *alcR* genes were designed to produce a mutant *B. bronchiseptica* AlcR protein with a serine-to-glycine change at position 103, Bb-S103G, and a mutant *B. pertussis* AlcR protein with a glycine-to-serine change at position 103, Bp-G103S; thus, each mutant corresponded to the opposing species' AlcR protein sequence. The *alcR* mutant alleles generated by site-directed mutagenesis were cloned to the pRK415 multicopy plasmid for phenotypic characterization of the mutant AlcR proteins in strain BRM13. Phenotypic analysis supported the predicted role of amino acid residue 103 in interspecies differences in alcaligin inducer requirements for AlcR-mediated transcriptional activation (Fig. 4); the mutant AlcR proteins each exhibited the degree of constitutivity that was normally associated with the alternate species' wild-type AlcR protein. In BRM13, the *B. pertussis* AlcR(G103S) mutant protein produced from plasmid pRK/Bp-G103S activated *alca::mini-Tn5 lacZ1* transcription under uninduced iron-depleted conditions to a level equivalent to 71% of the induced level of transcription, similar to the partial AlcR(Con) phenotype associated with overproduction of the *B. bronchiseptica* wild-type AlcR protein. Conversely, *alcaBCDER* operon transcriptional activities in uninduced cultures of BRM13(pRK/Bb-S103G) producing the *B. bronchiseptica* AlcR(S103G) mutant protein averaged only 6% of induced levels, similar to the phenotype associated with the wild-type *B. pertussis* AlcR regulator. Since each of the mutated *alcR* alleles differed from the corresponding wild-type *alcR* sequence by only a single nucleotide in the codon for amino acid 103, the interspecies phenotypic variation in AlcR inducer requirements was attributed to the amino acid difference at position 103 in the N-terminal protein domain.

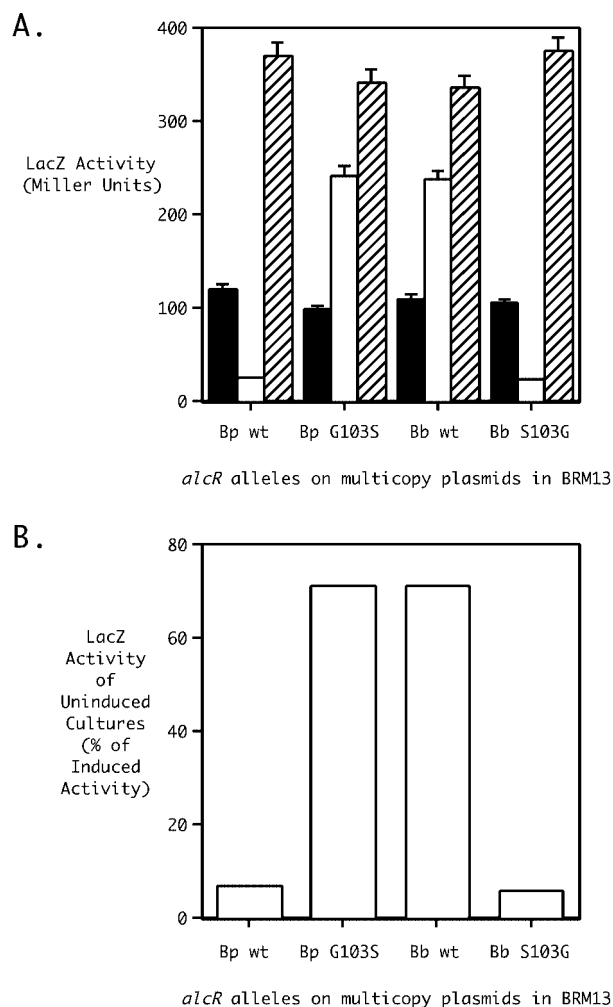
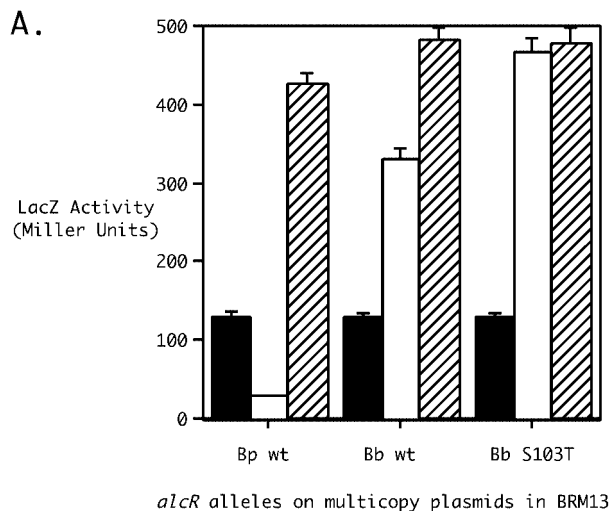


FIG. 4. Alcaligin inducer requirements of *B. pertussis* UT25 and *B. bronchiseptica* B013N mutant AlcR proteins produced from reciprocally mutated *alcR* alleles borne on multicopy plasmids. (A) *alcA::mini-Tn5 lacZ1* transcriptional activities were monitored in strain BRM13 complemented with wild-type and mutated *alcR*<sup>+</sup> plasmids constructed by overlap extension PCR. Each species' *alcR* genes were altered by a single nucleotide to the nucleotide sequence of the wild-type *alcR* gene of the other species.  $\beta$ -Galactosidase activities were measured for cells cultured in iron-replete SS medium (solid bars), iron-depleted SS medium (open bars), or iron-depleted SS medium supplemented with 20  $\mu$ g of purified alcaligin/ml (hatched bars).  $\beta$ -Galactosidase activities are expressed in Miller units (mean  $\pm$  1 standard deviation;  $n = 3$ ). (B) The relative degree of constitutivity of AlcR is depicted as in Fig. 2B. Multicopy *alcR*<sup>+</sup> plasmids: Bp wt, pRK/N<sub>p</sub>C<sub>p</sub>; Bb wt, pRK/N<sub>b</sub>C<sub>b</sub>; Bp G103S, pRK/Bp-G103S; Bb S103G, pRK/Bb-S103G.

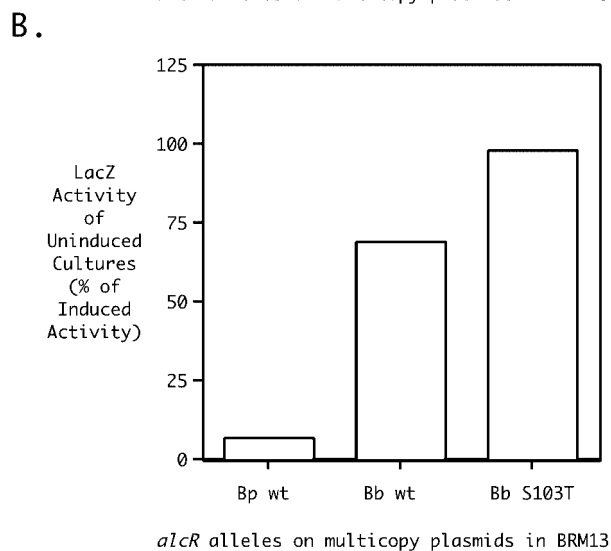
**A threonine substitution at amino acid position 103 in the *B. bronchiseptica* AlcR protein renders it fully constitutive for transcriptional activation.** The striking interspecies difference in the AlcR inducer requirement suggested that amino acid position 103 was associated with an important functional determinant of inducer dependence. The functional importance of AlcR amino acid residue 103 was further probed by site-directed mutagenesis. Prediction of the functional consequences of amino acid substitutions at AlcR position 103 using Dayhoff's mutation odds matrix (14) suggested that a threo-

nine substitution for either species' naturally occurring serine or glycine would be well tolerated structurally, since these three amino acids have been observed to share similar properties in proteins. The amino acid substitution matrices of Bordo and Argos (6) were applied for the prediction of amino acid substitutions that would be least likely to disrupt local protein structure or protein folding, so as to allow informative probing of the functional significance of the substituted residue. These matrices predicted that threonine could be substituted for serine at *B. bronchiseptica* AlcR amino acid position 103 with 95% confidence that local protein structure would not be radically perturbed, yet the substitution might influence specific aspects of protein function potentially related to inducer requirements for transcriptional activation. A relatively new tool for protein engineering and functional analysis, SIFT (30), predicts structurally tolerated amino acid substitutions in proteins based on sequence homologies and also predicts whether a particular amino acid substitution will be likely to specifically affect protein function. SIFT analysis of AlcR predicted that a serine-to-threonine change at position 103 would be well tolerated and not deleterious to protein function yet was likely to alter the phenotype associated with the protein. Based on the general concurrence of the three prediction tools and the partial AlcR(Con) phenotype associated with the wild-type *B. bronchiseptica* AlcR protein carrying a serine residue at position 103, it was hypothesized that a physicochemically similar threonine substitution at position 103 would alter *B. bronchiseptica* AlcR inducer dependence. PCR-based site-specific mutagenesis was performed using mutagenic primers N2B(T103) and C1B(T103) with *B. bronchiseptica* genomic DNA as the template to generate the *alcR*(S103T) mutant allele, which was cloned to plasmid vector pRK415 to produce plasmid pRK/Bb-S103T. Phenotypic analysis of the AlcR(S103T) mutant protein produced from pRK/Bb-S103T in BRM13 confirmed that the threonine substitution, as predicted, had a significant effect on the inducer requirement of the protein for transcriptional activation (Fig. 5). The AlcR(S103T) mutant protein exhibited a greater degree of constitutivity than the partially constitutive (69%) wild-type *B. bronchiseptica* AlcR protein assayed in parallel (Fig. 5B); the threonine substitution rendered the mutant AlcR activator fully constitutive (98%). Notably, the AlcR(S103T) mutant protein activated *alcABCDER* operon transcription to approximately the same maximal level as the wild-type activators of both species (Fig. 5A); thus, the phenotypic effect of the mutation appeared to be confined to the inducer dependence function of the protein.

Since the *alcR*(S103T) mutation had a strong influence on AlcR inducer requirements when produced from multicopy plasmids, it was predicted that the *alcR*(S103T) mutant allele might confer an altered inducer dependence phenotype even at a single-copy gene dosage. The *B. pertussis* UT25 and *B. bronchiseptica* B013N wild-type *alcR* genes and the *alcR*(S103T) mutant allele were delivered to the chromosome of BRM13 by homologous recombination as described in Materials and Methods and were assayed for inducer dependence. All integrated *alcR* alleles were capable of activating *alcA::mini-Tn5 lacZ1* transcription to similar maximal levels under inducing conditions (Fig. 6). Analysis of the AlcR inducer requirements for transcriptional activation confirmed



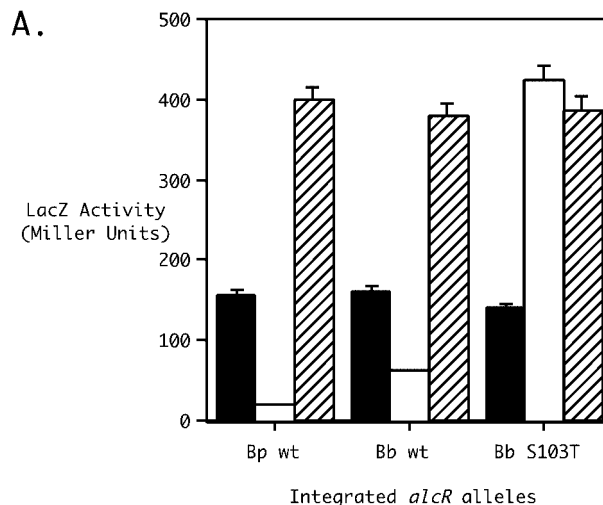
*alcR* alleles on multicopy plasmids in BRM13



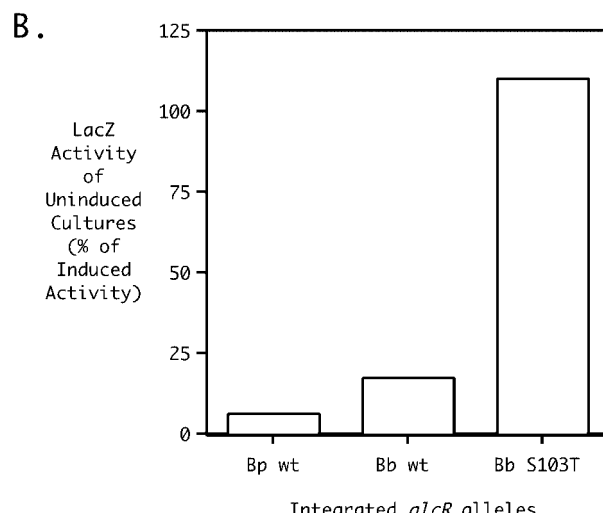
*alcR* alleles on multicopy plasmids in BRM13

FIG. 5. Alcaligin inducer requirement of the AlcR(S103T) mutant protein produced from the *alcR*(S103T) mutant allele borne on a multicopy plasmid. (A) *alcA*::mini-Tn5 *lacZ*I transcriptional activities were monitored in strain BRM13 complemented with wild-type and mutated *alcR*<sup>+</sup> plasmids constructed by overlap extension PCR. β-Galactosidase activities were measured for cells cultured in iron-replete SS medium (solid bars), iron-depleted SS medium (open bars), or iron-depleted SS medium supplemented with 20 µg of purified alcaligin/ml (hatched bars). β-Galactosidase activities are expressed in Miller units (mean ± 1 standard deviation; n = 3). (B) The relative degree of constitutivity of AlcR is depicted as in Fig. 2B. Multicopy *alcR*<sup>+</sup> plasmids: Bp wt, pRK/N<sub>p</sub>C<sub>p</sub>; Bb wt, pRK/N<sub>b</sub>C<sub>b</sub>; Bb S103T, pRK/Bb-S103T.

that the wild-type AlcR proteins of both species displayed strong inducer dependence for transcriptional activation when expressed from single-copy genes, in contrast to the pronounced interspecies variation in AlcR function associated with production from multicopy plasmids. When produced from integrated *alcR*<sup>+</sup> alleles in strain BRM13 during iron starvation in the absence of inducer, *B. pertussis* UT25 AlcR activated *alcA*::mini-Tn5 *lacZ*I transcription to a level that averaged only 6% of induced transcriptional activity, and *B. bronchiseptica* B013N AlcR activated transcription to a level



Integrated *alcR* alleles



Integrated *alcR* alleles

FIG. 6. Alcaligin inducer requirement of the AlcR(S103T) mutant protein produced from the *alcR*(S103T) mutant allele at a single-copy gene dosage. (A) *alcR*<sup>+</sup> integrant strains were constructed by integration of pSS1129 suicide plasmid-borne *alcR* alleles at the  $\Delta$ *alcR*I locus of BRM13 by homologous recombination, and *alcA*::mini-Tn5 *lacZ*I transcriptional activities were monitored by β-galactosidase assays of cells cultured in iron-replete SS medium (solid bars), iron-depleted SS medium (open bars), or iron-depleted SS medium supplemented with 20 µg of purified alcaligin/ml (hatched bars). β-Galactosidase activities are expressed in Miller units (mean ± 1 standard deviation; n = 3). (B) The relative degree of constitutivity of AlcR is depicted as in Fig. 2B. Plasmid integrant strains: Bp wt, BRM13Ω( $\Delta$ *alcR*I::pSS/Bp); Bb wt, BRM13Ω( $\Delta$ *alcR*I::pSS/Bb); Bb S103T, BRM13Ω( $\Delta$ *alcR*I::pSS/Bb-S103T).

that averaged only 17% of induced levels. Remarkably, the AlcR(S103T) mutant protein was fully constitutive at a single-copy gene dosage, with uninduced transcriptional activity averaging 110% of induced transcriptional activity. Thus, the *alcR*(S103T) mutant allele is a true *alcR*(Con) allele that encodes a mutant AlcR(Con) protein capable of activating *alcABCDER* operon transcription in an inducer-independent manner at a single-copy gene dosage.



## DISCUSSION

Expression of alcaligin siderophore biosynthesis and transport genes of *B. pertussis* and *B. bronchiseptica* is subject to both negative and positive transcriptional control. Transcription of the *alcABCDER* operon and the ferric alcaligin outer membrane receptor gene *fauA* is Fur and iron repressible (7, 10, 22, 23; Brickman and Armstrong, Abstr. 97th Gen. Meet. Am. Soc. Microbiol.) but is activated by the alcaligin-inducible AraC-like regulator AlcR under iron starvation stress conditions (5, 12). In studies detailed in this report, gene fusion analyses revealed that the AlcR regulator proteins of *B. pertussis* and *B. bronchiseptica*, when produced from multicopy plasmids, exhibited different apparent requirements for the inducer to activate transcription of the *alcABCDER* operon. Further examination of the basis of this phenomenon led to the identification of a key protein determinant of AlcR inducer responsiveness. This discovery guided the design and construction of a mutated gene encoding a fully constitutive AlcR mutant protein.

When produced from either pRK415- or pBBR1MCS-based multicopy plasmids, *B. bronchiseptica* AlcR activity was found to be partially constitutive, whereas *B. pertussis* AlcR retained its normal alcaligin inducer dependence. Phenotypic characterization of interspecies AlcR chimeras generated by domain swapping confirmed the predicted involvement of the AlcR N-terminal domain in the interspecies variation in inducer dependence. Nucleotide sequencing of the *B. bronchiseptica* B013N *alcR* gene revealed that it differed from the known *B. pertussis* UT25 *alcR* nucleotide sequence by a single nucleotide, resulting in a substitution at amino acid position 103 in the N-terminal domain. Site-directed mutagenesis was used to generate mutant *B. bronchiseptica* and *B. pertussis* *alcR* genes carrying single reciprocal nucleotide changes associated with the wild-type genes of the other species. Phenotypic analysis of the mutant AlcR proteins confirmed the hypothesized role of AlcR amino acid residue 103 in the interspecies difference in alcaligin inducer requirements for activation.

In this study, the *alcR* coding sequence of *B. bronchiseptica* strain B013N was found to be identical to the reported *alcR* coding sequence of *B. bronchiseptica* strain BB1015 (32). In the published report of the BB1015 *alcR* nucleotide sequence (GenBank accession number AJ000061), the authors noted the same single-nucleotide sequence difference between the *alcR* coding sequences of *B. bronchiseptica* BB1015 and *B. pertussis* strain BPSM, and they deduced the serine-to-glycine substitution in the *B. pertussis* BPSM protein sequence. The conservative nature of this serine-glycine amino acid substitution led to the prediction that the AlcR proteins would have the same function (32). Based on the findings obtained in the present study, we propose that there are significant phenotypic effects of the serine-glycine substitutions in the *B. pertussis* UT25 and *B. bronchiseptica* B013N AlcR proteins that influence their inducer requirements.

The functional importance of AlcR amino acid residue 103 was further analyzed by site-directed mutagenesis. The mutagenesis strategy was assisted primarily by the SIFT protein engineering and functional analysis tool of Ng and Henikoff (30) for prediction of structurally tolerated amino acid substitutions likely to have phenotypic effects on protein function.

SIFT analysis predicted that a serine-to-threonine substitution at *B. bronchiseptica* AlcR position 103 would be well tolerated structurally yet likely to alter the phenotype associated with the protein; thus, it was hypothesized that the protein in which threonine was substituted would display altered inducer dependence for activation of transcription. In phenotypic analyses, the AlcR(S103T) mutant protein was found to be fully constitutive for transcriptional activation not only at a multicopy but also at a single-copy gene dosage in BRM13 integrant strains. In addition, differences in the inducer dependence of the wild-type *B. pertussis* and *B. bronchiseptica* AlcR proteins were measurable even when the proteins were produced from single-copy genes in plasmid integrant strains.

The difference in inducer dependence between *B. bronchiseptica* B013N and *B. pertussis* UT25 AlcR regulators produced from multicopy plasmids is remarkable given their single amino acid difference. Both species' AlcR proteins require alcaligin for activation of transcription when produced from chromosomal genes. The partial constitutivity of AlcR results when the *B. bronchiseptica* *alcR* copy number is elevated to an estimated 5 to 8 copies/cell (31) on pRK415-based plasmids. However, despite the striking suppression of the inducer requirement associated with this relatively modest increase in the *B. bronchiseptica* *alcR* gene dosage, strict inducer dependence is retained with *B. pertussis* *alcR*, even at approximately 30 to 40 copies/cell (1) on the pBBR1MCS-based plasmids. These results suggest that natural levels of AlcR production are critical for the controlled induction of AlcR-mediated transcriptional activation by the siderophore. In an exhaustive review of the AraC/XylS family of transcriptional regulators and their mechanisms of action, Gallegos et al. (17) proposed an explanation for suppression of the regulators' inducer requirements, based largely on detailed analyses of the *Pseudomonas putida* XylS regulator. Consistent with the proposed mechanisms of regulation by AraC type proteins and the role of the inducer in activation (17), a tentative hypothesis explaining the suppression of the inducer requirement associated with *B. bronchiseptica* AlcR overproduction can be proffered. We propose that an inactive AlcR protein conformation exists in equilibrium with an active AlcR conformation that is proficient for transcriptional activation. The postulated role of alcaligin inducer is to shift that equilibrium toward the active AlcR conformation by binding to the inactive regulator protein. Overproduction of the regulator protein would also be predicted to increase the concentration of the active conformer, thus suppressing the requirement for the inducer.

Since overproduction of *B. bronchiseptica* AlcR suppresses its inducer requirement but overproduction of the *B. pertussis* protein does not, it is also reasonable to hypothesize that the *B. bronchiseptica* AlcR protein shifts from the inactive conformation to the active conformation more readily than *B. pertussis* AlcR. This might explain the observed difference in the AlcR inducer requirement between these species at a single-copy gene dosage with normal levels of AlcR production. These hypothetical differences in AlcR structure result from substitutions at amino acid position 103. In the absence of crystallographic data, it is not known whether the inducer actually contacts AlcR amino acid position 103 in the active regulator.

We speculate that the interspecies difference in AlcR signal requirements for activation may have biological relevance for

signal perception and transduction in priority regulation of the alcaligin siderophore system in the two related species. The fact that a modest increase in copy number results in inducer-independent AlcR function in *B. bronchiseptica* suggests that alcaligin inducer may normally be required only to “prime the pump” for initial activation of transcription of the alcaligin system genes in *B. bronchiseptica*. Once activated, elevated levels of AlcR production resulting from increased expression of the *alABCDEF* operon may suppress any additional need for inducer. In contrast, *B. pertussis* AlcR may require the persistence of the signal from the inducer to maintain AlcR in a conformation that is proficient to activate transcription. Thus, if the inducer is no longer perceived by *B. pertussis*, activation of alcaligin system gene transcription ceases, and the bacterium’s energy and precursors can be appropriately channeled to the utilization of alternative sources of nutritional iron. It is an intriguing possibility that the stringent genetic regulation of the alcaligin siderophore system by the AlcR protein of *B. pertussis* has relevance for the success of *B. pertussis* as an obligate pathogen. Ongoing genetic and biochemical analyses are aimed at further elucidation of structure-function relationships of AlcR.

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