Alcaligin Siderophore Production by *Bordetella bronchiseptica* Strain RB50 Is Not Repressed by the BvgAS Virulence Control System

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A previous study found that alcaligin siderophore production by *Bordetella bronchiseptica* strain RB50 is Bvg repressed. In contrast, we report that alcaligin production by RB50 does not require Bvg phenotypic phase modulation and that isogenic Bvg(Con) and Bvg[−] phase-locked mutants both produce alcaligin in response to iron starvation.

Bordetella species are gram-negative respiratory pathogens of humans and other animals (26) that can undergo virulence-associated phenotypic alterations in response to environmental signals. This phenotypic modulation is mediated by the BvgAS sensory transduction system (1, 33, 34). Most virulence-associated traits are expressed in the Bvg⁺ phenotypic phase, and in laboratory cultures the switch from the Bvg⁺ to the Bvg[−] phase can be induced by supplementation of culture medium with nicotinic acid (NA) (28, 29) or MgSO₄ (20) or by culturing at a low temperature (20).

Siderophores are low-molecular-mass, high-affinity iron chelators of microbial origin that are produced coordinately with their cognate receptors and transporters in response to iron starvation (21). *Bordetella pertussis* and *Bordetella bronchiseptica* produce the potent siderophore alcaligin (10, 24). Transcription of alcaligin biosynthesis and transport genes is negatively regulated by Fur and iron (3, 8, 9, 18, 19) and positively regulated by AlcR (4, 27) by using the alcaligin siderophore as the inducer (11).

A previous study reported that the *B. bronchiseptica* rabbit isolate strain RB50 produced alcaligin siderophore only in the Bvg[−] phenotypic phase (17). The additional observations that the isogenic Bvg[−] phase-locked mutant RB54 (13) did not require phenotypic modulation for alcaligin production and that the Bvg(Con) phase-locked mutant RB53 (13) did not produce detectable alcaligin under any growth condition provided compelling evidence for Bvg repression of alcaligin production in *B. bronchiseptica* RB50. A subsequent phenotypic survey in that study, involving 114 other *B. bronchiseptica* strains, led to the conclusion that Bvg repression of alcaligin production was significantly associated with phylogenetic lineage and mammalian host source. Although this is the only published report of Bvg repression of alcaligin production in *B. bronchiseptica*, it has frequently been cited in scientific publications, including several review articles (5, 7, 12, 16, 23, 25,

31), as providing an important example of a Bvg-repressed trait, with implications for pathogenicity and host specificity.

In contrast with key findings of that previous study, we report that (i) siderophore production by wild-type *B. bronchiseptica* strain RB50 does not require modulation to the Bvg[−] phase and is independent of Bvg phenotypic phase, and (ii) isogenic Bvg(Con) and Bvg[−] phase-locked mutant derivatives of RB50 both produce siderophores at wild-type levels in response to iron starvation. Furthermore, biological and biochemical analysis confirmed that RB50 (Bvg⁺ phase), RB53, and RB54 all produce siderophores with properties identical to those of alcaligin.

Siderophore production by the wild-type *B. bronchiseptica* strain RB50 does not require phenotypic modulation to Bvg[−] phase. *B. bronchiseptica* strains (Table 1) were cultured at 37°C on Bordet-Gengou (6) agar plates containing 20% defibrinated sheep blood. After 24 h of growth on Bordet-Gengou agar plates, bacteria were transferred to iron-replete Stainer-Scholte broth (SS) (29, 32) and cultured at 37°C with shaking for 24 h. These iron-replete seed cultures were harvested by centrifugation, washed with iron-depleted SS, and seeded to iron-replete and iron-depleted SS cultures as described previously (2), at an initial cell density corresponding to an optical density at 600 nm of 0.02. After 24 h, cleared culture supernatants were quantitatively assayed for alcaligin by the chrome azurol S (CAS) method (30), using highly purified alcaligin (10) as the standard. The wild-type strain RB50 was cultured for siderophore production assays under nonmodulating (Bvg⁺) conditions. RB50 produced very high levels (circa 150 μM, or about 60 μg/ml) of alcaligin in the Bvg⁺ phase in response to iron starvation (Table 2).

Modulation to the Bvg[−] phenotypic phase does not result in elevated siderophore production by wild-type strain RB50 compared with Bvg⁺-phase organisms. In phenotypic modulation experiments, RB50 was cultured for siderophore production (i) in the presence of NA at concentrations ranging from 0.1 to 16.0 mM, (ii) in the presence of 50 mM MgSO₄, or (iii) at 20°C, in parallel with nonmodulated cultures. All modulating (Bvg[−]) culture conditions (except for iron-depleted cultures with ≥8.0 mM NA, which did not grow) resulted in the

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TABLE 1. *B. bronchiseptica* strains and bacteriophage

Strain or phage	Relevant genotype and phenotype	Reference or source
RB50	<i>bvg</i> ⁺ rabbit isolate, wild type ^a	13
RB53	<i>bvgS</i> -C3, <i>bvg</i> (Con), <i>Bvg</i> ⁺ phenotypic phase-locked mutant derivative of RB50 ^a	13
RB54	Δ <i>bvgAS</i> , <i>Bvg</i> ⁺ phenotypic phase-locked mutant derivative of RB50 ^a	13
B013N	Nalidixic acid-resistant derivative of wild-type swine isolate strain B (15), proficient in alcaligin production and utilization, source of purified alcaligin	Robert Goodnow via Charlotte Parker
BRM1	<i>alcA</i> ::mini-Tn5 <i>lacZ</i> I derivative of B013N, defective in alcaligin production	2
BRM17	Δ <i>fauA</i> :: <i>kan</i> derivative of B013N, defective in alcaligin transport and utilization	9
BP3c	<i>Bvg</i> ⁺ phase-specific bacteriophage ^b	22; Liu et al. ^c

^a *Bvg* phase-related phenotypes were confirmed on the basis of colony morphology, hemolytic activity on BG, and sensitivity to the *Bvg*⁺-phase-specific bacteriophage BP3c.

^b *Bvg* phase specificity was confirmed by determining titers of bacteriophage lysates on *Bvg*⁺- versus *Bvg*⁺-phase *B. bronchiseptica*.

^c M. Liu, J. Parkhill, S. Baker, P. Davis, M. Simmonds, A. Preston, D. J. Maskell, P. A. Cotter, and J. F. Miller, Abstr. 100th Gen. Meet. Am. Soc. Microbiol., abstr. B-185, p. 83, 2000.

production of high levels of siderophore under iron starvation conditions and showed no significant increase over levels produced by nonmodulated, *Bvg*⁺-phase RB50 organisms (Table 2). The *Bvg* phenotypic phase of modulated and nonmodulated alcaligin-producing bacteria recovered from iron-depleted cultures was confirmed by a bacteriophage adsorption assay as described previously (22) using the *Bvg*⁺-phase-specific bacteriophage BP3c (data not shown).

***Bvg*(Con) and *Bvg*⁺ phase-locked mutants both produce siderophores.** Isogenic *Bvg* phase-locked mutant strains RB53 and RB54 both produced alcaligin at levels comparable to the parental wild-type strain RB50 (Table 2) in response to iron starvation, providing genetic evidence for *Bvg*-independent alcaligin production in this strain lineage. These data are consistent with our RB50 phenotypic modulation experiments, which showed equivalent alcaligin production by RB50 in both *Bvg*⁺ and *Bvg*⁺ phenotypic phases.

RB50, RB53, and RB54 culture supernatants stimulate growth of alcaligin indicator strains. In iron-restricted growth stimulation bioassays (10), supernatants from iron-depleted cultures (nonmodulating SS) of RB50, RB53, and RB54 stimulated the growth of alcaligin-deficient mutant BRM1 to equivalent levels (Table 3), but no supernatants stimulated the growth of the ferric alcaligin receptor (*fauA*) mutant BRM17, indicating that the growth-stimulating substance present in the supernatants was alcaligin. Control iron sources were human hemoglobin (Sigma) and purified alcaligin (10).

TABLE 2. Alcaligin production by *B. bronchiseptica* strains cultured in iron-depleted SS under various conditions of phenotypic modulation

Strain	Alcaligin concn (μ M) ^a			
	No modulation	MgSO ₄ ^b	NA ^c	20°C
RB50 (<i>bvg</i> ⁺)	152 $\pm$ 2	149 $\pm$ 1	154 $\pm$ 4	107 $\pm$ 0 ^d
RB53 [<i>bvgS</i> -C3, <i>bvg</i> (Con)]	153 $\pm$ 4	150 $\pm$ 0	ND	ND
RB54 (Δ <i>bvgAS</i>)	151 $\pm$ 3	149 $\pm$ 0	ND	ND

^a Values are means $\pm$ standard deviations obtained after 24 h of culture based on the standard curve determined for purified alcaligin in the CAS assay ($r = 0.996$). Alcaligin was not produced at measurable levels in any iron-replete cultures grown in parallel with iron-depleted cultures. ND, not determined.

^b Cultures were supplemented with 50 mM MgSO₄.

^c Cultures were supplemented with 4 mM NA (similar alcaligin levels were measured in cultures supplemented with 0.1 to 4.0 mM NA [157 $\pm$ 8 μ M, $n = 15$]).

^d Poor growth at 24 h.

Siderophores of RB50, RB53, and RB54 are purified by the alcaligin extraction method, are ferric iron reactive, and comigrate with purified alcaligin in thin-layer chromatography.

Siderophores were extracted from 3-ml volumes of supernatants from iron-depleted cultures (nonmodulating SS) of RB50, RB53, and RB54 by using a scaled-down benzyl alcohol-ether extraction procedure (10). Purified siderophores were analyzed by silica gel thin-layer chromatography against a purified alcaligin standard as described previously (10) by using an *n*-butanol-acetic acid-H₂O (4:1:5, upper phase) solvent system (Fig. 1). Red ferric iron-reactive species were visualized by spraying the plates with 1% FeCl₃ in 0.1 mM HCl. All three strains produced a single ferric iron-reactive species that comigrated with authentic alcaligin.

Summary. Conflicting results regarding a relationship between *Bvg* phenotypic phase and alcaligin production in *B. bronchiseptica* strain RB50 were obtained in this study compared with the previous report of Giardina et al. (17), using the same RB50-related bacterial strains and culture conditions. Although the previous study used the Csaky method (14) in primary screening for hydroxamate siderophore production, strains that were Csaky negative for alcaligin production were further tested with the CAS siderophore assay, so the discrepancy between reports cannot be simply explained by differences in assay methods. Most importantly, we find that (i) RB50 produces high levels of alcaligin under nonmodulating (*Bvg*⁺ phase) conditions, and (ii) the *Bvg*(Con) phase-locked mutant RB53 also produces high levels of alcaligin. These are

TABLE 3. Growth stimulation of iron source indicator strains

Culture supernatant source (genotype) or iron source (concn)	Growth zone diam (mm) of indicator strain (phenotype) ^a	
	BRM1 (alcaligin ⁺)	BRM17 (alcaligin transport ⁺)
RB50 (<i>bvg</i> ⁺)	17	NS
RB53 [<i>bvgS</i> -C3, <i>bvg</i> (Con)]	17	NS
RB54 (Δ <i>bvgAS</i>)	17	NS
Alcaligin (125 μ g/ml)	19	NS
Human hemoglobin (4 μ M)	18	18

^a Diameter of growth zone surrounding a 6-mm well in an indicator strain-seeded Luria-Bertani agar plate containing the iron chelator ethylenediaminedi-[(*o*-hydroxyphenyl)acetic acid] (EDDA) at 100 μ g/ml. NS, no growth stimulation of indicator strain.

FIG. 1. Analytical silica gel thin-layer chromatography of siderophores purified from iron-depleted SS culture supernatants of RB50 (*Bvg*⁺ phase), RB53, and RB54. Dots at the lower edge of the chromatogram indicate the origin for each sample. The arrowhead indicates the position of a red spot ($R_f = 0.53$) corresponding to ferric alcaligin complexes (10). Alcaligin control sample, 5 μ g.

positive results that argue strongly that alcaligin production is not *Bvg* repressed in these strains.

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