

Isolation and Characterization of *Bordetella bronchiseptica* Mutants Deficient in Siderophore Activity

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Iron acquisition by the gram-negative pathogens *Bordetella bronchiseptica* and *Bordetella pertussis* is thought to occur by hydroxamate siderophore-mediated transport as well as an apparently siderophore-independent process by which host transferrins bind to bacterial surface receptors. We constructed *B. bronchiseptica* mutants deficient in siderophore activity by insertional mutagenesis with miniTn5/*lacZ*1. The mutants could be placed into four distinct complementation groups, as determined from cross-feeding assays which demonstrated restored siderophore synthesis. Mutants deficient in siderophore activity were BRM1, BRM6, and BRM9, exhibiting approximately 36 to 41% of wild-type siderophore levels, and BRM3 and BRM8, which appeared to produce very little or no detectable siderophore. Mutant BRM4 was found to be a leucine auxotroph, while mutants BRM2 and BRM7 could synthesize siderophore only in low-iron medium which was supplemented with various amino acids. Evaluation of all transcriptional fusions revealed an apparent lack of iron-regulated *lacZ* expression. Genomic regions flanking the transposable element in the siderophore mutants were homologous with *B. pertussis* chromosomal DNA, while bioassays suggested siderophore cross-feeding between *B. pertussis* and *B. bronchiseptica*. These results indicate probable similarity between the siderophore biosynthetic and transport systems of the two species.

Complex high-affinity iron transport systems are expressed by microorganisms in response to suboptimal availability of this nutrient. The prototypic transport system involves excretion of low-molecular-weight siderophores for mobilization of iron to the intracellular compartment (30). Recent studies have described a direct contact uptake mechanism in species of *Neisseria* (24, 38, 43) and *Haemophilus* (28, 37) which appears to rely solely on the physical interaction of bacterial surface receptors and host iron-binding proteins such as transferrin or lactoferrin.

Little is known about iron acquisition in members of the genus *Bordetella*, which consists of gram-negative respiratory pathogens of a variety of animal species (6, 18, 31). *Bordetella pertussis*, the etiologic agent of human whooping cough, and *Bordetella bronchiseptica*, which primarily infects nonhuman mammals, exhibit a strong affinity for several transferrins (25, 33, 34). The assimilation of iron from cell-bound transferrin by *B. pertussis* suggested the existence of a direct-contact iron-sequestering system similar to that used by the *Neisseria* and *Haemophilus* species (33). Demonstration that *B. pertussis* grew well when separated from iron-loaded transferrin by a dialysis membrane (19) suggested siderophore excretion. The assay of Schwyn and Neilands (39) was used to detect siderophore activity in iron-restricted *B. pertussis* culture supernatants which also yielded positive results with the Csaky assay for hydroxamic acids (11), suggesting that the chelator may be of the hydroxamate siderophore class (19). Iron-stressed *B. bronchiseptica* and *Bordetella parapertussis* also produce siderophore activity with a putative hydroxamate structure (19).

Bordetella species are usually considered obligate pathogens restricted to host respiratory epithelia, yet a report

describing the ability of *B. bronchiseptica* to grow in lake water (32) suggests more diverse ecological niches for this organism. In addition, *B. bronchiseptica* has been reported to cause bacteremia in immunocompromised humans (5, 22). Such environmental versatility would require adaptation to a considerably wide variety of physical and nutritional conditions. The source of iron, existing as insoluble oxyhydroxides in external environments or coordinated by animal host proteins, may differentially influence the expression of siderophore genes and those required for the putative direct-contact iron retrieval mechanism.

As an initial step in our study of iron transport in *Bordetella* species, we used a transposon reporter element to generate transcriptional fusions in *B. bronchiseptica*. In this report, we describe mutants defective in siderophore production on low-iron medium and demonstrate potential relatedness of the siderophore systems of *B. bronchiseptica* and *B. pertussis* by genetic homologies and bioassays.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. bronchiseptica* B013, derived from swine isolate strain B (15), was from Robert Goodnow through Charlotte Parker and was the source of the nalidixic acid-resistant strain B013N. Virulent-phase *B. pertussis* UT25 has been described elsewhere (16). *Escherichia coli* S17-1(λ pir) (*thi thr leu tonA lacY supE recA::RP4-Tc::Mu-Km::Tn7* λ pirR6K) (27, 40) was obtained from Kenneth Timmis via the laboratory of Mark McIntosh and was used as the donor in mating experiments. *E. coli* DH5 α [F⁻ ϕ 80d*lacZ* Δ M15 Δ (*lacZYA-argF*)U169 *endA1 recA1 hsdR17*(r_K⁻ m_K⁺) *deoR thi-1 supE44* λ ⁻ *gyrA96 relA1*] (Bethesda Research Laboratories, Gaithersburg, Md.) was used as a host for routine plasmid construction. *E. coli* LG1315 (*ara entA lac mtl proC rpsL supE thi tonA trpE xyl* [pColV-K30]) (45) and LG1522 (*ara fepA lac leu mtl proC rpsL supE thi tonA trpE xyl* [pColV-K30 *iuc*])

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(10) were used for siderophore bioassays. *B. bronchiseptica* and *B. pertussis* were cultured on blood agar or Bordet-Gengou plates (6, 16) unless otherwise stated; *E. coli* was grown on Luria-Bertani (LB) or M9 agar (26). High-iron culture conditions for *B. bronchiseptica* were achieved by use of LB broth or modified Stainer-Scholte (SS) defined minimal medium containing 36 μ M iron (36, 41). Transconjugants were cultured in the presence of antibiotics at the following concentrations: nalidixic acid, 35 μ g/ml; and kanamycin, 30 μ g/ml. Propagation of *E. coli* strains containing plasmids was in the presence of appropriate antibiotics: ampicillin at 100 μ g/ml and kanamycin at 70 μ g/ml.

All glassware used for low-iron cultures was treated with 1 M sulfuric acid to decrease residual iron contamination. SS basal medium was deferrated by passage through a column packed with Chelex100 (Bio-Rad, Richmond, Calif.). The SS vitamin supplement lacked iron and was also deferrated by using Chelex100. $MgCl_2$ and $CaCl_2$ in distilled H_2O were added to the medium (final concentrations of 0.5 and 0.2 mM, respectively) to replace the magnesium and calcium ions removed during deferration. Amino acid supplements were prepared in distilled water, deferrated by using Chelex100, and filter sterilized. Liquid medium was inoculated with cells grown previously in high-iron broth and washed three times in deferrated SS basal medium. Growth was at 37°C on an orbital shaker and was measured as optical density (OD) with a spectrophotometer or a Klett-Summerson colorimeter equipped with a no. 54 filter (Klett Mfg. Co., Long Island City, N.Y.).

High-iron cyclodextrin solid medium (CSM) was prepared as described previously (21), using Molecusol MB cyclodextrin from Pharmatec Inc. (Alachua, Fla.). Low-iron CSM was produced by treatment of molten CSM medium with Chelex100 prior to autoclaving and was supplemented with a mixture of 20 deferrated L-amino acids.

Chrome azurol S (CAS) agar for the evaluation of *B. bronchiseptica* siderophore production was a modification of the formulation of Schwyn and Neilands (39). Briefly, a 150-ml volume of sterile CAS-iron dye reagent was added to 850 ml of autoclaved SS (pH 7.0) containing 1.5% agar and the usual SS vitamin supplement to produce the blue medium. *B. bronchiseptica* siderophore excretion was evident on CAS agar after incubation for 48 h at 37°C.

Bioassays. For siderophore bioassays, the *B. pertussis* indicator strain was applied as a lawn to the surface of low-iron CSM. Supernatants from test strains of *B. bronchiseptica* grown in low-iron SS without antibiotics and containing deferrated L-amino acids were filter sterilized, and 200- μ l volumes were placed in wells cut into the agar. To correct for differences in the final ODs of the cultures, the supernatants were diluted accordingly with deferrated SS. Other bioassays on low-iron M9 agar (supplemented with 20 deferrated L-amino acids) involved patching test organisms onto the *E. coli* LG1522 indicator lawn prior to incubation.

Bioassays in low-iron liquid medium were performed by adding 3-ml volumes of filter-sterilized culture supernatants (derived from late-logarithmic-stage bacteria cultured in low-iron SS) to 7 ml of low-iron SS. *B. bronchiseptica* strains were inoculated at low density (20 Klett units) and monitored for growth. Controls included no additions to 10 ml of low-iron SS and addition of $FeCl_3$ (to 36 μ M) to 10 ml of low-iron SS.

Cross-feeding assays on CAS agar were performed by cross-streaking *B. bronchiseptica* strains on the agar or by supplying (on filter disks) each streaked strain with low-iron culture supernatants concentrated 10-fold by lyophilization.

Mutagenesis. Transcriptional fusions were constructed by using plasmid pUT containing miniTn5/*lacZ1* (gift of Kenneth Timmis) (12, 20). *B. bronchiseptica* B013N was grown overnight on LB agar containing 35 μ g of nalidixic acid per ml, while the donor strain, *E. coli* S17-1(λ pir) carrying pUT::miniTn5/*lacZ1*, was grown overnight on LB agar containing kanamycin. Bacteria were harvested from the plates and washed in 10 mM NaCl. The cell density was adjusted to an OD at 600 nm (OD_{600}) of 1.0 in LB broth, and the cells were mixed in a ratio of 1:1. After 0.5 ml of the cell mixture was spread onto LB agar containing 20 mM $MgSO_4$ (44), the cells were dried onto the surface of the medium and incubated for 6 h at 37°C. The cells were harvested and plated onto LB agar containing kanamycin and nalidixic acid and also spread with 30 to 50 μ l of a colicin B-enriched bacterial lysate (approximately 5 mg of total protein per ml) to additionally discourage growth of *E. coli* donors (9). Transconjugants were patched onto CAS agar for assessment of siderophore activity.

Determination of siderophore in culture supernatants. Mutants lacking siderophore haloes on CAS agar were grown in high-iron and low-iron SS broth, with amino acid supplements if appropriate. Supernatants were harvested at intervals and tested for siderophore activity by the CAS assay (39) in which the reactions were allowed to proceed for 4 h prior to measurement of OD_{630} . The assay of Arnow (3) was used to detect phenolate-based siderophore compounds, while the determination of hydroxamates was accomplished by the Csaky protocol, using hydroxylamine hydrochloride as a standard (11). Reported values for all siderophore detection assays were calculated with the measurements from 4 to 14 separate experiments.

Spectral analysis of culture supernatants of iron-starved cells was performed by using a Beckman DU-65 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) at wavelengths ranging continuously from 200 to 700 nm. Filter-sterilized supernatants were analyzed both without and with $FeCl_3$ added to a final concentration of 74 μ M.

DNA methods. Plasmid DNA was isolated by routine procedures (35), while genomic DNA was recovered from *B. bronchiseptica* and *B. pertussis* by the method described by Brown and Parker (8). Restriction endonuclease digestions and ligations were performed with products from Promega (Madison, Wis.) or United States Biochemical (Cleveland, Ohio) in accordance with the manufacturers' instructions.

Southern hybridization analysis was performed as described previously (34), using Biotrace RP nylon membranes (Gelman Sciences, Ann Arbor, Mich.). Immobilized genomic DNA was examined for the presence of transposon sequences by using a ^{32}P -labeled probe obtained from pUT::miniTn5/*lacZ1* as a ca. 5.0-kb *Bam*HI-*Eco*RI fragment which contains virtually the entire transposable element. Probes were labeled by random priming with the Promega Prime-a-Gene product. For detection of delivery plasmid-specific sequences, a ca. 2.0-kb *Bam*HI fragment from pUT::miniTn5/*lacZ1* was used as the source of probe DNA. Other probes were isolated as described in the text. Hybridization was performed at 68°C in 6 \times SSPE (0.9 M NaCl, 0.06 M $NaH_2PO_4 \cdot H_2O$, 6 mM EDTA). Stringent conditions were maintained by washing the blots at 68°C in 0.1 \times SSC-0.1% sodium dodecyl sulfate (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

Transposon markers were cloned from *B. bronchiseptica* mutants by using conventional methods (35). Genomic DNA was digested with *Eco*RI or *Sal*I, and the fragments were size selected (on the basis of Southern hybridization data) and ligated into the appropriately restricted vector pGEM3Z

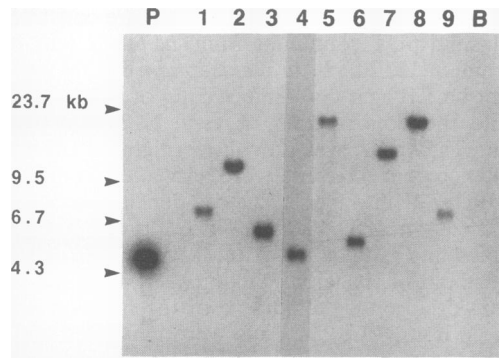


FIG. 1. Southern hybridization analysis of *B. bronchiseptica* mutants. Chromosomal DNA samples from mutants and the parental strain were digested with *EcoRI*, electrophoresed on a 0.5% agarose gel, and transferred to a nylon membrane prior to probing with a ca. 5-kb ^{32}P -labeled fragment specific for miniTn5/*lacZ1*. Lanes: P, ca. 5-kb fragment from miniTn5/*lacZ1* from which the probe was derived; 1 to 9, chromosomal DNA from mutants BRM1 to BRM9, respectively; B, genomic DNA from parental strain B013N. Size markers are indicated at the left.

(Promega). *E. coli* DH5 α transformants were selected on ampicillin- and kanamycin-containing LB agar.

β -Galactosidase measurements. β -Galactosidase activity of the mutants was determined by the method of Miller (26). The assay was performed several times (each in triplicate) on cells grown in high-iron and low-iron SS media. Qualitative evaluation of β -galactosidase activity was accomplished by growth on high- and low-iron CSM agar containing 0.04 mg of 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal) per ml.

RESULTS

Isolation of *B. bronchiseptica* insertion mutants. Approximately 9,000 nalidixic acid- and kanamycin-resistant *B. bronchiseptica* transconjugants were patched onto CAS agar to detect organisms deficient in siderophore excretion. Randomly chosen transconjugants as well as the apparent siderophore mutants carried no pUT::miniTn5/*lacZ1* plasmid DNA. Southern hybridization experiments confirmed that the element alone and not the entire plasmid had integrated into the chromosomes of putative siderophore mutants (data not shown).

Chromosomal DNA isolated from siderophore-deficient mutants (designated BRM1 to BRM9) was digested with *EcoRI* (which does not cut within the miniTn5/*lacZ1* element) and analyzed by Southern hybridization using a transposon-specific probe (Fig. 1). Each of the nine mutants demonstrated a single hybridizing DNA fragment. Digestion of genomic DNA with both *EcoRI* and *Bam*HI followed by hybridization with the same probe distinguished BRM1 from BRM9, whereas analysis with these and other restriction enzymes indicated that BRM5 and BRM8 were siblings (data not shown). Therefore, eight independent insertion events defined the collection of mutants. Cryptic plasmid DNA isolated from the mutants failed to hybridize with the miniTn5/*lacZ1*-specific probe (data not shown).

Analysis of siderophore deficiency. The mutants were tested several additional times on the blue CAS agar with consistent results (Fig. 2). The parent strain B013N exhibited a distinct wide yellow halo indicative of siderophore

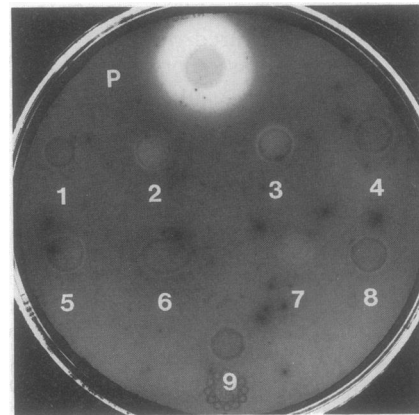


FIG. 2. Growth and siderophore production on CAS agar. Suspensions of washed bacteria were spotted onto CAS agar and incubated for 48 h at 37°C. The letter P is located near the growth and siderophore halo of parental strain B013N; the numbers 1 to 9 identify mutants BRM1 to BRM9, respectively.

excretion, while the mutants grown on the same agar demonstrated no apparent siderophore activity.

Further assessment of the mutants confirmed the extent of their siderophore deficits (Table 1). After growth of parental strain B013N and the mutants in low-iron SS, cell-free culture supernatants were analyzed by a quantitative CAS siderophore assay. Supernatant from B013N demonstrated a large reduction in OD₆₃₀ as the result of strong iron-chelating activity. B013N grown in high-iron SS did not produce CAS-reactive material. The mutants showed only a small

TABLE 1. Production of siderophore activity by *B. bronchiseptica* mutants grown in deferrated minimal SS medium

Strain	CAS activity ^a (OD ₆₃₀ ± SD)	Csaky assay ^b (mM standard)	Absorption spectra ^c
B013N	0.14 ± 0.04 (100)	0.32 ± 0.04	+
BRM1	0.43 ± 0.03 (14)	0	-
BRM2	0.46 ± 0.03 (3)	0	-
BRM3	0.48 ± 0.02 (0)	0	-
BRM4 ^d	0.47 ± 0.05 (1)	0	-
BRM6	0.44 ± 0.02 (10)	0.05 ± 0.02	-
BRM7	0.42 ± 0.06 (17)	0	-
BRM8	0.46 ± 0.02 (4)	0	-
BRM9	0.42 ± 0.03 (17)	0	-
None (uninoculated medium)	0.47 ± 0.01 (0)	0	-

^a Cell-free culture supernatants of bacteria grown in low-iron medium to late logarithmic stage were tested by using the CAS assay as detailed in Materials and Methods. A low OD₆₃₀ relative to that of uninoculated medium indicates siderophore activity; values in parentheses represent the percent of the parental level of activity as calculated from the mean. Strain B013N cultured under iron-replete conditions yielded an OD₆₃₀ of 0.63.

^b Cell-free supernatants of iron-deficient bacteria were subjected to the Csaky assay as described in Materials and Methods. An increase in OD₅₀₀ relative to uninoculated medium and as compared with a standard indicates the presence of hydroxamic acids; the value given is the concentration of hydroxylamine hydrochloride equivalent and is the average of at least three independent experiments. Strain B013N cultured under iron-replete conditions routinely gave a value of 0.

^c Cell-free supernatants of bacteria were analyzed by spectroscopy as described in Materials and Methods. +, spectra characteristic of siderophores (for *B. bronchiseptica* B013N, a λ_{max} shift from 305 to 425 nm upon ferration); -, lack of absorbance at wavelengths in the range of 290 to 700 nm.

^d Displayed poor growth in SS.

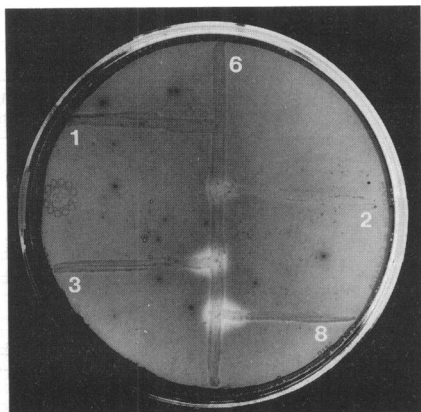


FIG. 3. Phenotypic cross-complementation on CAS agar. *B. bronchiseptica* mutants were cross-streaked on CAS agar and incubated for 48 h at 37°C. Results of the analysis of BRM6 (vertical primary streak) with BRM1, BRM2, BRM3, and BRM8 are shown.

reduction in OD₆₃₀, signifying a lack of siderophore activity in the culture supernatants. These mutants were analyzed throughout the entire growth phase with similar results.

Culture supernatant from iron-starved B013N grown to late exponential phase and tested for the presence of hydroxamic acids yielded the equivalent of 0.32 mM hydroxylamine hydrochloride standard. Hydroxamic acids were absent in the supernatants of all similarly cultured mutants with the exception of BRM6, which produced trace levels of hydroxylamine hydrochloride equivalent (0.05 mM). B013N cultured in high-iron SS did not produce detectable hydroxamates.

As siderophores and their ferric chelates display characteristic absorption spectra (30), supernatants of iron-stressed cells were analyzed spectrophotometrically. Supernatant from B013N or wild-type B013 demonstrated maximal absorbance at 305 nm which shifted to 425 nm when iron was added. Similar tests performed on iron-free or ferrated supernatants from the iron-starved mutants showed no measurable absorption in the range of 290 to 700 nm.

CAS cross-feeding analysis. Cross-feeding assays were performed to determine whether any mutant produced and excreted an intermediate of siderophore biosynthesis which

could be used by another mutant to alleviate its presumed block in the biosynthetic pathway. In four independent experiments, distinct zones of siderophore production were observed at the junctions where certain mutants were cross-streaked onto CAS agar (Fig. 3). Strong siderophore activity was produced at the junctions where BRM3 and BRM8 were cross-streaked with BRM6. Slight activity was observed at the area where BRM2 and BRM6 intersected, while the combination of BRM1 and BRM6 demonstrated no siderophore halo. Compilation of the data revealed that the eight mutants could be divided into four phenotypic complementation groups (Table 2). To determine which mutants supplied the siderophore-stimulating compounds and which mutants were fed by such compounds, concentrated cell-free supernatants were used to test each mutant. Group I mutants were fed by no others but supplied stimulating factors to members of groups II and III. Group II mutants were fed by group I but were unable to supply any other mutant. Group III mutants were fed by group I organisms and stimulated the group IV mutant, BRM4. Supernatant cross-feeding experiments between groups II and III produced variable results which could not be unequivocally determined. BRM4 was fed weakly by group III organisms and appeared incapable of feeding any other mutant.

Effect of amino acids on growth and siderophore production. The mutants displayed variable rates of growth in low-iron SS liquid medium (Table 3). Experiments consistently showed the mean generation times of BRM1, BRM6, and BRM9 to be similar to that of the parent strain. BRM3 and BRM8 displayed somewhat diminished growth capacity which was greater than that of BRM2 and BRM7. Both mutant and wild-type cells demonstrated increased growth in high-iron SS, but the rates of BRM3, BRM8, BRM2, and BRM7 were still below that of the wild-type (data not shown). BRM4 failed to grow in high-iron or low-iron SS unless a large inoculum was used, in which case the growth was still rather poor (data not shown). Inclusion of 20 deferrated L-amino acids in the medium (Table 3) dramatically enhanced the growth of BRM4, suggesting amino acid auxotrophy; CAS and Csaky (not shown) assays detected parental levels of siderophore activity and hydroxamate, respectively, in the BRM4 supernatant. Amino acid supplementation slightly improved the growth of BRM2 and BRM7 and also restored siderophore production. Similar amino acid additions, although not enhancing the growth of BRM1,

TABLE 2. Phenotypic cross-complementation of *B. bronchiseptica* siderophore mutants

Complementation group	Mutant	Siderophore produced when streaked against BRM mutants ^a	Produces siderophore when fed by BRM mutants ^b	Supplies siderophore stimulus to BRM mutant(s) ^b
I	BRM1	2, 3, 7, 8	None	2, 3, 7, 8
	BRM6	2, 3, 7, 8	None	2, 3, 7, 8
	BRM9	2, 3, 7, 8	None	2, 3, 7, 8
II	BRM2	1, 3, 6, 8, 9	1, 6, 9 ^c	None
	BRM7	1, 3, 6, 8, 9	1, 6, 9 ^c	None
III	BRM3	1, 2, 4, 6, 7, 9	1, 6, 9 ^d	4
	BRM8	1, 2, 4, 6, 7, 9	1, 6, 9 ^d	4
IV	BRM4	3, 8 ^e	3, 8 ^e	None

^a Mutants were cross-streaked on CAS agar and evaluated for siderophore synthesis on the basis of results such as those depicted in Fig. 3; results were consistent in four independent experiments.

^b Mutants were streaked onto CAS agar and supplied with concentrated cell-free supernatants derived from BRM mutants cultured in low-iron SS minimal medium. Siderophore production was scored by the presence or absence of characteristic yellow haloes surrounding filter disks containing the supernatants. Results are from three experiments, each performed in duplicate.

^c Results with BRM3 and BRM8 supernatants were too variable to be conclusive.

^d Results with BRM2 and BRM7 supernatants were too variable to be conclusive.

^e Weak siderophore activities were observed with both BRM3 and BRM8 and their supernatants.

TABLE 3. Mutant growth and effect of medium composition on siderophore activity

Complementation group	Strain (generation time [h]) ^a	CAS activity ^b (OD ₆₃₀ ± SD) in deferrated minimal SS plus:			
		No additions ^c	All amino acids ^d	Leucine	All amino acids except leucine
Wild type	B013N (3.8)	0.14 ± 0.04 (100)	0.14 ± 0.03 (100)	0.15 ± 0.04 (100)	0.11 ± 0.04 (100)
I	BRM1 (4.0)	0.43 ± 0.03 (14)	0.33 ± 0.05 (41)	— ^e	—
	BRM6 (4.2)	0.44 ± 0.02 (10)	0.35 ± 0.06 (36)	—	—
	BRM9 (4.0)	0.42 ± 0.03 (17)	0.34 ± 0.05 (39)	—	—
II	BRM2 (9.5)	0.46 ± 0.03 (3)	0.17 ± 0.03 (91)	0.15 ± 0.06 (100)	0.14 ± 0.06 (91)
	BRM7 (11.3)	0.42 ± 0.06 (17)	0.16 ± 0.02 (92)	0.18 ± 0.02 (90)	0.15 ± 0.05 (88)
III	BRM3 (5.5)	0.48 ± 0.02 (0)	0.46 ± 0.02 (3)	—	—
	BRM8 (6.4)	0.46 ± 0.02 (4)	0.45 ± 0.03 (5)	—	—
IV	BRM4 (no growth)	0.47 ± 0.05 ^f (1)	0.18 ± 0.02 (87)	0.18 ± 0.04 (90)	0.52 ± 0.05 ^f (0)

^a Mean generation time during growth in the usual low-iron minimal SS with no amino acid supplements.

^b Culture supernatants of bacteria grown in low-iron medium were tested by using the CAS assay as detailed in Materials and Methods. A low OD₆₃₀ relative to that of uninoculated medium indicates siderophore activity; values in parentheses represent the percent of the parental level of activity as calculated from the mean.

^c Values taken from Table 1 for comparison.

^d Each of 20 deferrated L-amino acids was added to a final concentration of 50 µg/ml. For other experiments, individual amino acids were used at the same concentration.

^e —, not determined.

^f Little or no growth from small inocula was observed.

BRM6, and BRM9, did result in increased siderophore production to approximately 36 to 41% of the wild-type level. BRM3 and BRM8 were unaffected by addition of amino acids to the medium; their growth remained unchanged, and they still failed to produce significant siderophore activity. Neither the mutants nor the wild-type strain, regardless of low-iron medium composition, demonstrated phenolate types of siderophore compounds as determined by the assay of Arnow (3) (data not shown).

Testing of specific amino acids revealed that BRM4 growth and subsequent siderophore production were dependent upon an exogenous supply of leucine only (Table 3). Although BRM2 and BRM7 could grow in the absence of leucine, this amino acid enhanced their growth and elicited wild-type levels of siderophore activity. A mixture of amino acids lacking leucine also had the capacity to stimulate siderophore production in these mutants. In other experiments (data not shown), a number of amino acids, alone or in combination, potentiated siderophore production in BRM2 and BRM7. These included tryptophan, tyrosine, serine, asparagine, aspartic acid, glutamine, methionine, phenylalanine, proline, and the combinations isoleucine-valine and glycine-histidine-lysine.

β-Galactosidase expression. Qualitative examination of all BRM mutants on X-Gal-containing low- and high-iron CSM agar failed to reveal any iron-regulated reporter gene activity, as they appeared to process similar amounts of chromogenic substrate. Quantitative evaluation of the siderophore mutants in groups I and III demonstrated generally equivalent or lower β-galactosidase levels upon culture in low-iron conditions compared with those obtained after growth in high-iron medium. For example, mutants BRM3 and BRM8 demonstrated the following β-galactosidase levels (mean values, in Miller units) in high- and low-iron media, respectively: BRM3, 239 and 137; and BRM8, 1,106 and 857. Analysis of group II and group IV mutants also revealed a lack of iron-regulated *lacZ* expression (data not shown).

Cloning of genomic DNA containing miniTn5/*lacZ* insertions. The chromosomal regions containing miniTn5/*lacZ* insertions of the siderophore-deficient mutants were cloned for use as probes and to allow comparison of the flanking DNA regions of members in each phenotypic complementa-

tion group (Fig. 4). Restriction endonuclease maps, along with results of Southern hybridizations using probes generated from the clones (data not shown), revealed the insertions of group I mutants to be on the same ca. 15-kb *SalI* chromosomal fragment. Analysis of a *SalI* fragment isolated from group III mutants BRM3 and BRM5 (identical to BRM8) showed that the insertions were 200 to 300 bp from one another.

Comparative analyses with *B. pertussis*. Southern hybrid-

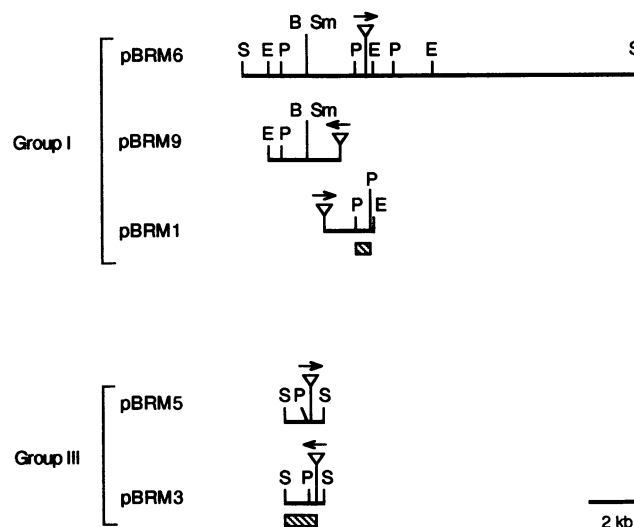


FIG. 4. Partial restriction maps of cloned genomic regions from siderophore-deficient mutants. The name of each plasmid which contains the insert identifies the mutant from which the cloned DNA originated. Triangles indicate the positions of miniTn5/*lacZ*; arrows denote direction of transcription of the *trp'*-*lacZ* reporter gene. Maps were constructed on the basis of restriction enzyme digestions. Group-specific maps were aligned by using information derived from restriction enzyme maps and Southern hybridizations. Striped boxes show fragments used as probes for the Southern hybridizations shown in Fig. 5. Abbreviations: B, *Bam*HI; E, *Eco*RI; P, *Pst*I; S, *Sal*I; Sm, *Sma*I.

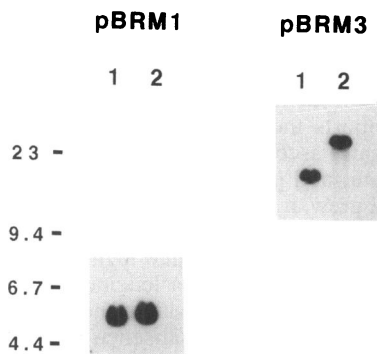


FIG. 5. Southern hybridization analysis of chromosomal DNA from *B. bronchiseptica* B013 (lanes 1) and *B. pertussis* UT25 (lanes 2). The DNA was digested with *Eco*RI and processed as described for Fig. 1, using probes derived from pBRM1 and pBRM3, as indicated. Size markers (in kilobases) are indicated at the left.

izations at high stringency and using probes (shown in Fig. 4) from genomic regions flanking the insertions in mutants BRM1 and BRM3 showed cross-hybridization with *B. pertussis* UT25 chromosomal DNA (Fig. 5). Similarly sized 5.2- to 5.3-kb *Eco*RI fragments from *B. bronchiseptica* B013 and *B. pertussis* UT25 hybridized with the BRM1 probe. The BRM3 probe, a ca. 1.0-kb fragment derived from pBRM3, hybridized to a ca. 17-kb *Eco*RI fragment from *B. bronchiseptica* B013 and a fragment greater than 23 kb from *B. pertussis* UT25. The analogous 1.0-kb probe from pBRM5 showed the same hybridization patterns with B013 and UT25 chromosomal DNA (data not shown).

If the siderophore systems of *B. bronchiseptica* and *B. pertussis* were identical or very similar, it might be expected that their siderophores or excreted siderophore intermediates would cross-feed each other in low-iron environments. Supernatants of B013N and BRM mutants (grown in low-iron SS with amino acid supplements to circumvent the nutritional deficits of group II and group IV mutants) were tested for the ability to relieve the initial iron stress of *B. pertussis* UT25 in bioassays on low-iron CSM agar. Supernatants from siderophore-producing *B. bronchiseptica* strains were able to maximally enhance iron-restricted growth of *B. pertussis* UT25 (Table 4). Group III mutant supernatants were less capable of doing so, displaying weak or no haloes of UT25 growth stimulation. There was no halo formation around wells containing uninoculated medium. The equivalent experiments on high-iron CSM produced an even lawn of UT25 growth on the entire agar surface, with no haloes surrounding the supernatant wells (not shown). Experiments using *B. bronchiseptica* strains patched onto the *B. pertussis* lawn yielded similar results: as the siderophore-producing strains grew, they excreted compounds which enhanced the growth of *B. pertussis*; there was no halo formation around *B. pertussis* UT25 itself spotted onto the agar.

To evaluate the reciprocal cross-feeding, B013N was tested for the ability to use *B. pertussis* UT25 CAS-positive low-iron supernatant to relieve iron stress. Because the siderophore-proficient *B. bronchiseptica* B013N proved difficult to test on low-iron CSM (it always grew as a lawn, with no haloes even around an iron source), experiments were performed by using low-iron liquid cultures supplemented with filter-sterilized spent culture supernatants containing putative siderophore material. In liquid medium, cross-

TABLE 4. Stimulation of *B. pertussis* growth on low-iron agar by *B. bronchiseptica* culture supernatants^a

Complementation group	<i>B. bronchiseptica</i> supernatant	<i>B. pertussis</i> growth stimulation level
Wild type	B013N	+++
I	BRM1	++
	BRM6	++
	BRM9	++
	BRM2	++
II	BRM7	++
	BRM3	-/+
III	BRM8	-/+
	BRM4	++

^a *B. pertussis* UT25 was swabbed onto low-iron CSM agar, and *B. bronchiseptica* filter-sterilized culture supernatants (from late-logarithmic-stage bacteria cultured in low-iron SS with amino acid supplements) were added to wells in the agar. Haloes of *B. pertussis* UT25 growth were measured around the wells after incubation for 72 h. The following halo measurements represent mean diameters and exclude the 8 mm contributed by the sample well: +++, a dense halo with a diameter range of 17 to 22 mm; ++, a moderately dense halo with a diameter in the range of 9 to 16 mm; -/+, no stimulation ranging to a faintly visible halo of 1 to 5 mm. Reported results are from three to four separate measurements. Uninoculated medium did not stimulate *B. pertussis* growth.

feeding would be manifested as enhanced initial growth due to inhibition of the normally observed low-iron growth lag. Such experiments consistently showed that *B. bronchiseptica* B013N, inoculated at low density into low-iron SS, displayed stimulated growth upon addition of CAS-positive supernatants from *B. pertussis* UT25 and B013N itself (Fig. 6). Organisms in unsupplemented medium demonstrated the usual extended lag phase during culture, while those in high-iron SS grew to the greatest final optical density.

B. bronchiseptica was analyzed to determine whether it could cross-feed *E. coli*. Bioassays on low-iron M9 minimal agar showed that neither *B. bronchiseptica* B013N nor its CAS-positive supernatants could relieve the iron stress of the indicator *E. coli* strain LG1522 (an aerobactin biosynthetic mutant still able to transport aerobactin). In contrast, the aerobactin-producing *E. coli* strain LG1315 stimulated a large growth halo of LG1522 on the same medium (data not shown).

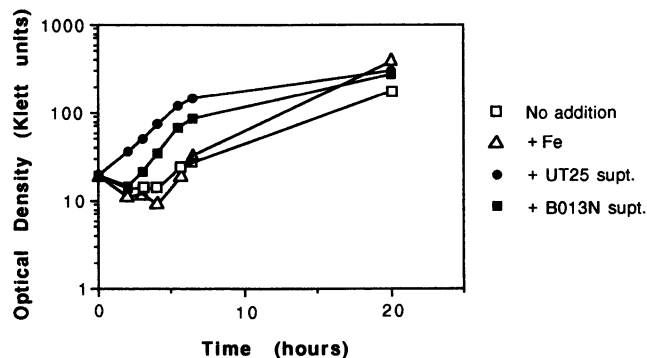


FIG. 6. Growth of *B. bronchiseptica* B013N in low-iron SS. Strain B013N was inoculated at 20 Klett units into low-iron SS containing no additions, 36 μ M iron, or low-iron CAS-positive culture supernatants (supt.) from *B. pertussis* UT25 or *B. bronchiseptica* B013N.

DISCUSSION

We selected the element miniTn5/*lacZ*1 for construction of transcriptional fusions in *B. bronchiseptica* to facilitate the study of iron-regulated siderophore gene expression, which in *E. coli* is primarily mediated by the Fur repressor protein and is maximal during iron stress and repressed under iron-replete conditions (4, 7, 14). In contrast, our siderophore mutants displayed somewhat similar levels of β -galactosidase regardless of iron concentration of the culture medium. *E. coli* strains carrying plasmids pBRM3 and pBRM5 (Fig. 4) also failed to demonstrate iron-regulated *lacZ* expression (data not shown). One interpretation of these results is that while siderophore production is iron regulated, the gene which is mutated is not. Alternatively, the iron-responsive element or regulatory signal may be inactivated by the mutation. As the orientation and position of the insertion with respect to the transcriptional unit are unknown, sequencing of the flanking DNA regions as well as transcript analysis may provide insight into the lack of expected regulation.

The mutants isolated in this study were initially identified by the absence of a siderophore halo after growth on SS-based minimal agar incorporating the iron-binding dye CAS (39). Supernatants of these bacteria cultured in deferrated minimal SS also did not react significantly with the CAS dye. As the color change of the dye corresponds with loss of the iron, siderophore iron-chelating activity rather than the siderophore chemical structure itself is detected. Other criteria such as absorption spectrum analysis and the Csaky assay confirmed the paucity of hydroxamate compounds in the mutants.

Mutants capable of feeding one another siderophore biosynthetic precursors to alleviate pathway defects have been described for the enterobactin (17, 46) and aerobactin (13) siderophore systems. CAS agar cross-feeding assays performed in this study clearly showed that siderophore was produced only when certain strains comprising distinct complementation groups were streaked in contact with one another. Throughout this study, members of each group were found to share siderophore production and growth characteristics, and the mutations of members of each of groups I and III were found in close proximity on discrete genomic fragments. To distinguish the factor-producing from the factor-using strains on CAS agar, filter-sterilized concentrated supernatants from iron-starved mutants were used. That group I siderophore-deficient mutants supplied factors to groups II and III but could not be fed by any others suggests that these mutants are blocked in a later step in the siderophore biosynthetic pathway. In light of the amino acid supplementation experiments, it is likely that for mutants BRM2, BRM4, and BRM7, the siderophore-stimulating factors that they required were amino acids or their biosynthetic intermediates. It is unknown why BRM4 was not cross-fed by group I mutants. Cross-feeding between groups II and III was difficult to determine, as the results varied considerably. These organisms may be blocked at early steps in siderophore synthesis, as they appear incapable of feeding the group I siderophore mutants. It is possible that groups II and III need to cross-feed each other simultaneously. Alternatively, the stimulating factor may have a short half-life and would need to be continually produced by growing organisms for the cross-feeding effect. Because group II and group IV mutants did not grow optimally in the minimal medium, their supernatants may have lacked suffi-

cient concentrations of the putative siderophore intermediates for adequate feeding of the other mutants.

The *B. bronchiseptica* mutants displayed various rates of growth in low-iron SS. This Tris-buffered medium, which routinely supports the growth of *Bordetella* spp., contains salts, phosphate, ascorbate, nicotinamide, glutathione, cystine, glutamate, and proline (36, 41). Members of group II and group III grew at an intermediate rate in deferrated SS, while the only group IV member, BRM4, failed to grow from small inocula in both low-iron and high-iron SS. Our experiments suggest that BRM4 is a leucine auxotroph, and its inability to produce detectable siderophore may be related to extremely poor growth in the absence of leucine. Another consideration is that the siderophore molecular structure itself may be derived from leucine. It was noted that BRM2 and BRM7, while not obvious auxotrophs, produced siderophore upon the addition of various amino acids which are synthesized by different biochemical pathways. The mutations of these group II members may affect some central or regulatory aspect of amino acid metabolism to which biosynthesis of the siderophore is secondary. Under low-iron conditions in minimal SS, the siderophore would not be produced in these mutants, as other biosynthetic needs must be met first. This would explain the inability of group II supernatants to feed group III mutants on CAS agar.

Group I mutants exhibited reduced siderophore output, as under the best conditions, the maximal level of activity was only 36 to 41% of that of the parent strain B013N. The leaky phenotype may be due to defective siderophore excretion or to polar effects of the insertion on a siderophore biosynthetic gene or one involved in the positive regulation of biosynthetic genes. The ability of group I mutants to grow as well as wild-type B013N under low-iron conditions may be due to a postulated naturally low requirement for iron (19) coupled with the observed low levels of siderophore production. The pronounced siderophore deficiency of group III mutants BRM3 and BRM8 was unaffected by the amino acid composition of the growth medium, suggesting insertional inactivation of a genetic locus directly required for siderophore biosynthesis or one involved in positive regulation of synthesis. As the transposon elements in these mutants were chromosomally located, it may be presumed that, unlike ColV plasmid-encoded aerobactin genes (13), the genes encoding *B. bronchiseptica* siderophore function probably do not reside on the cryptic plasmid found in strain B013.

Gorringe et al. first identified in culture supernatants of iron-starved *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* the CAS and Csaky activities characteristic of hydroxamate siderophores (19). A subsequent study confirmed the presence of siderophore activity in *B. pertussis* supernatants and characterized it by spectral analysis (1). The reported absorbance maximum of ferrated *B. pertussis* supernatant at 450 nm differed from our 425-nm value for similarly tested *B. bronchiseptica*. It is unknown whether the *B. pertussis* supernatant material, termed bordetellin by Agiato and Dyer (1), is identical to that which we have described for *B. bronchiseptica* B013. Purification and chemical analysis of the compounds from each organism will clarify their molecular structures. The *B. bronchiseptica* siderophore probably does not resemble the hydroxamate siderophore aerobactin, as CAS-positive supernatants were unable to cross-feed an aerobactin-utilizing strain of *E. coli*. Our finding that *B. pertussis* and *B. bronchiseptica* are cross-fed by each other in low-iron cultures suggests that each produces a siderophore which can also be used by the other species. The occasionally observed faint level of *B.*

pertussis low-iron growth enhancement by group III siderophore mutants may be due to their excretion of siderophore biosynthetic intermediates or other stimulatory metabolites or of compounds which may condition the medium by inactivating inhibitors in the agar to which *B. pertussis* is sensitive (21). The observation that *B. pertussis* DNA is homologous with the DNA regions flanking the insertions in BRM3 and BRM8 further strengthens the concept of the same or a similar siderophore system in both species. One line of evidence which does not appear to support the idea of an identical siderophore produced by both species involves iron-regulated outer membrane proteins which might function in the transport of the ferrisiderophore complex. Menozzi et al. reported iron-repressed *B. pertussis* outer membrane proteins of 70 and 27 kDa, yet the analogous proteins in *B. bronchiseptica* were 79.5, 73.5, 32, and 30 kDa (25). Agiato and Dyer found *B. pertussis* iron-repressed outer membrane proteins with molecular sizes of 93, 77, and 63 kDa (1). Considering the high degree of genetic relatedness between *B. pertussis* and *B. bronchiseptica* (23, 29), if these organisms synthesized identical siderophores, one might expect more similarly sized putative transport proteins.

In *Bordetella* spp., the *vir* genetic locus (*bvgAS*) coordinates the regulation of virulence-associated genes by a proposed two-component type of mechanism (2, 42). *Bordetella* iron acquisition genes do not appear to be regulated by *bvgAS*, as Gorringer et al. found that avirulent-phase *B. pertussis* produced siderophore activity equivalent to that of virulent strains (19). Redhead et al. found avirulent *B. pertussis* capable of binding transferrins as well as virulent-phase bacteria (34), while Menozzi et al. showed that iron-repressed outer membrane protein production in *B. pertussis* and *B. bronchiseptica* was unaffected by nicotinic acid-induced phenotypic modulation (25).

B. pertussis and *B. bronchiseptica* are thought to utilize not only a siderophore-mediated iron uptake system but also a putatively siderophore-independent mechanism involving the direct binding of transferrin and lactoferrin. The *B. bronchiseptica* siderophore mutants obtained in this study provide an opportunity to ascertain the contribution of each system to iron acquisition under different in vitro and in vivo conditions and to determine whether the direct-contact mechanism is truly independent of siderophore function.

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