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Bordetella pertussis fur Gene Restores Iron Repressibility of Siderophore and Protein Expression to Deregulated *Bordetella bronchiseptica* Mutants

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We report the isolation and preliminary phenotypic characterization of manganese-resistant *Bordetella bronchiseptica* mutants with respect to deregulation of siderophore and iron-regulated protein expression. The *fur* gene of *Bordetella pertussis* was cloned by genetic complementation of this deregulated phenotype and confirmed as *fur* by nucleotide sequence analysis.

In the host, iron-binding proteins withhold iron from disease-causing microorganisms (34), suppressing their proliferation. Hence, the ability of a microorganism to overcome this host defense mechanism can be considered a virulence trait. Furthermore, expression of a number of bacterial products contributing to infectious disease pathology is elevated in response to iron limitation (7, 23, 29).

Mechanisms of iron acquisition by members of the genus *Bordetella* are not well understood. All *Bordetella* species with the exception of *Bordetella avium* have been shown to excrete putative hydroxamate-type siderophores in response to iron starvation (12), and there is evidence in *B. pertussis* and *B. bronchiseptica* of a cell-associated siderophore-independent iron uptake system allowing direct iron removal from the host iron-binding proteins transferrin and lactoferrin (22, 24).

In characterized bacterial systems, iron-regulated gene expression is governed principally by the DNA-binding repressor protein Fur (3). Fur homologs have been reported for at least 13 species of gram-negative bacteria, including *Escherichia coli* (13, 25), *Salmonella typhimurium* (9), *Yersinia pestis* (27, 28), *Vibrio anguillarum* (31), *Vibrio vulnificus* (21), *Vibrio cholerae* (20), *Klebsiella pneumoniae* (33), *Pseudomonas aeruginosa* (23), *Neisseria gonorrhoeae* (5), *Neisseria meningitidis* (17, 30), *Legionella pneumophila* (16), *Campylobacter jejuni* (35), and *B. pertussis* (4; also, this study).

By analogy to other bacterial species, we hypothesized the involvement of a Fur homolog in the regulation of iron acquisition systems in *Bordetella* species. Manganese-resistant mutants of *B. bronchiseptica* B013N (2) were selected on Luria-Bertani agar plates containing 10 mM $MnCl_2$ essentially as described by Hantke (14) for the selection of *fur* mutants of *E. coli* K-12. This procedure yielded three manganese-resistant mutants, designated B013N Mn^r4, B013N Mn^r16, and B013N Mn^r23, that appeared to be completely defective in their ability to repress siderophore expression. Mutants were cultured in parallel under both high-iron and low-iron conditions (2) and tested for constitutive production of siderophore activity by both the chrome azurol S universal siderophore assay (26) and the Csaky assay for hydroxamate class siderophores (8). In addition, iron-regulated protein expression in cell samples taken from the same cultures was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (19), as well as immunoblotting (32) of duplicate gels using murine antiserum raised to the 92-kDa iron-regulated outer membrane protein IR92 (1) of *B. bronchiseptica* B013N. As shown in Fig. 1A, iron-regulated protein expression by the wild-type parent strain B013N was derepressed by iron starvation whereas the mutants expressed iron-regulated proteins constitutively. Deregulated expression of IR92 by the mutants was confirmed by immunoblot analysis as shown in Fig. 1B. Likewise, siderophore production was constitutive (Fig. 1C). This phenotype was therefore consistent with that of *fur* mutants. Preliminary evidence supporting this hypothesis was obtained by functional complementation of the mutant phenotype by the *fur* gene of *E. coli*. Expression of *E. coli fur* (11) in *trans* restored full iron repressibility of siderophore and iron-regulated protein expression to mutant B013N Mn^r4 (data not shown).

A gene library of *B. pertussis* UT25 (6) was conjugally transferred to mutant B013N Mn^r4, and exconjugants were screened for iron-repressible siderophore production. Thirty-six repressed exconjugants identified in this primary screening each carried one of four distinct overlapping *B. pertussis* cosmids, suggesting that an iron-responsive regulatory activity was encoded by the *B. pertussis* DNA shared among these cosmids. Subcloning and complementation analysis localized the putative regulatory activity to an approximately 840-bp *Sal*I DNA fragment of *B. pertussis*. Subclones bearing the 840-bp DNA fragment in the broad-host-range plasmid pRK415 (18) restored wild-type iron repressibility of siderophore and iron-regulated protein expression to B013N Mn^r4 (data not shown). Further, these plasmids were able to functionally complement the *fur* defect of *E. coli* MFR5 (10), a *fur* strain that carries a *lac* transcriptional fusion to the iron-regulated gene *entF*. The putative Fur activity of *B. pertussis* mediated iron-repressible LacZ expression in this heterologous fusion reporter system (data not shown).

Nucleotide sequencing confirmed the identity of the *fur* homolog of *B. pertussis* UT25 (Fig. 2). The *fur* coding region of *B. pertussis* UT25 predicts a 139-amino-acid polypeptide with a molecular mass of 15,717 Da. Most striking is the conservation of a potential metal-binding motif rich in histidines and cysteines (HHXH-X_2-C-X_2-C; amino acid residues 86 to 95; Fig. 2) which has been proposed to provide ligands for iron in the Fur holorepressor complex (15).

The mechanism of manganese selection for the isolation of deregulated iron transport mutants is as yet undefined (14).

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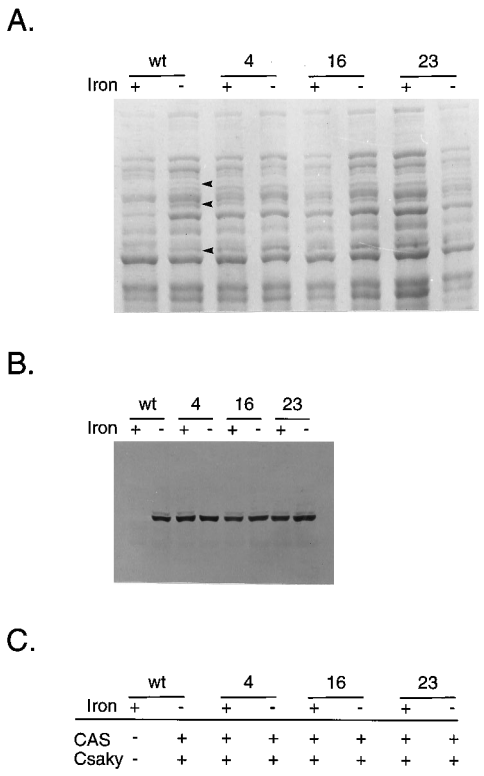

FIG. 1. Deregulated expression of proteins and siderophore activity by manganese-resistant *B. bronchiseptica* mutants. (A) SDS-PAGE analysis of deregulated protein expression by manganese-resistant mutants. Bacterial proteins were resolved on an SDS–7.5% polyacrylamide gel and stained with Coomassie brilliant blue dye. Arrowheads, positions of the 92-, 79-, and 59-kDa apparent molecular mass iron-regulated proteins in the wild-type B013N low-iron sample which are constitutively expressed by manganese-resistant mutants. wt, wild-type B013N; 4, 16, and 23, deregulated manganese-resistant mutants B013N Mn^{r}4, B013N Mn^{r}16, and B013N Mn^{r}23, respectively. + and −, high-iron and low-iron conditions, respectively. (B) Immunoblot analysis of deregulated IR92 expression by manganese-resistant mutants. Cell samples are the same as in panel A, separated on a duplicate polyacrylamide gel, transferred to nitrocellulose, and probed with antisera specific for the iron-regulated outer membrane protein IR92. (C). Deregulated siderophore production by manganese-resistant mutants. Supernatants were taken from the *B. bronchiseptica* cultures in panels A and B and examined for siderophore activity by the chrome azurol S universal siderophore assay and Csaky hydroxamate assay. +, high levels of siderophore activity; −, absence of siderophore activity.

This technique has allowed the isolation of deregulated iron transport mutants of a number of bacterial species, including *E. coli*, *K. pneumoniae*, and *Serratia marcescens* (14), as well as *P. aeruginosa* (23), *V. anguillarum* (31), *Yersinia enterocolitica* (27), and now *B. bronchiseptica* (this study), indicating the likely existence of shared metabolic features influencing iron regulation in microorganisms.

Preliminary phenotypic characterization of manganese-resistant *B. bronchiseptica* mutants in this study confirmed that they were fully deregulated with respect to both siderophore production and normal iron-repressible protein expression, in agreement with mutations in *fur*. Support for *fur* involvement was obtained by complementation of the deregulated phenotype of *B. bronchiseptica* mutants by the *E. coli fur* gene. Conversely, we demonstrated that the *fur* gene of *B. pertussis* was functional in *E. coli*. Functional complementation of *E. coli fur* mutants has been used to identify *fur* homologs of such diverse species as *N. gonorrhoeae* (5), *Y. pestis* (28), *L. pneumophila* (16), *V. cholerae* (20), and *V. anguillarum* (31). In light of taxonomic divisions and differences in nucleotide composition between these bacterial species and *E. coli*, given the fact that Fur repression at iron-regulated promoters depends upon specific Fur-DNA interactions, it seems that Fur-binding DNA sequences, as well as Fur protein structural determinants critical to DNA recognition and binding, are functionally conserved to a considerable degree. One might predict the conservation of essential features of such a fundamental biological activity as the regulation of the response to environmental fluctuations of the crucial biometal iron.

Nucleotide sequence accession number. The nucleotide sequence in Fig. 2 has been assigned GenBank accession number U11699.

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                    SalI                                                M   S   D   Q
   1                GTCGACCATCGACCCCCCATGCCACCGCGGATGGAGAGCGATTACACC ATG AGC GAC CAA

       S   E   L   K   N   M   G   L   K   A   T   F   P   R   L   K   I   L   D   I
  61   AGC GAA CTG AAA AAC ATG GGC CTG AAG GCG ACG TTT CCC CGC CTG AAG ATC CTT GAC ATT

       F   R   K   S   D   L   R   H   L   S   A   E   D   V   Y   R   A   L   I   A
 121   TTT CGT AAA TCC GAC CTC CGC CAC CTC AGC GCG GAA GAT GTC TAT CGC GCG CTG ATC GCC

       E   N   V   E   I   G   L   A   T   V   Y   R   V   L   T   Q   F   E   Q   A
 181   GAG AAT GTG GAA ATC GGG CTG GCG ACG GTA TAT CGC GTG CTG ACG CAG TTC GAG CAG GCG

       G   I   L   T   R   S   Q   F   D   T   G   K   A   V   F   E   L   N   D   G
 241   GGC ATC CTC ACG CGT AGC CAG TTC GAT ACC GGC AAG GCG GTG TTC GAG CTG AAT GAT GGC

       D   H   H   D   H   L   I   C   T   N   C   G   T   V   F   E   F   S   D   P
 301   GAT CAC CAC GAT CAC CTC ATC TGC ACC AAT TGC GGT ACG GTA TTC GAG TTT TCG GAT CCC

       D   I   E   K   R   Q   Y   K   V   A   K   D   N   G   F   V   L   E   S   H
 361   GAT ATT GAG AAG CGT CAA TAC AAG GTG GCG AAG GAC AAC GGC TTT GTG CTC GAA AGC CAT

       A   M   V   L   Y   G   I   C   G   N   C   Q   K   G   R   *
 421   GCC ATG GTG TTG TAC GGC ATT TGC GGA AAC TGT CAG AAG GGC CGC TGA TCCTCCGGCTCTACC

 484   AACCCTGCCTCCAGGCGCAAGCCTGGAGGTTTTTTTTTGCGGTTTGGTAGCAGG
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FIG. 2. Nucleotide and deduced primary amino acid sequences of the *B. pertussis* UT25 *fur* gene. The sense strand of the *B. pertussis fur* region is shown. The deduced primary amino acid sequence in the one-letter code is given above the respective codons. The upstream *Sal*I recognition site used in subcloning the *fur* region is indicated, and a unique *Bam*HI site internal to the *fur* coding region is underlined. Arrows, downstream inverted repeat sequences. Nucleotide positions of the 547-bp region sequenced in this study are indicated on the left.

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