

Transcriptional Profiling of the Iron Starvation Response in *Bordetella pertussis* Provides New Insights into Siderophore Utilization and Virulence Gene Expression[∇]§

Timothy J. Brickman,¹ Craig A. Cummings,^{2,4†} Sin-Yee Liew,^{2,4‡}
David A. Relman,^{2,3,4} and Sandra K. Armstrong^{1*}

Department of Microbiology, University of Minnesota Medical School, Minneapolis, Minnesota 55455¹; Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, California 94305²; Department of Medicine, Stanford University School of Medicine, Stanford, California 94305³; and VA Palo Alto Health Care System, Palo Alto, California 94304⁴

Received 22 April 2011/Accepted 30 June 2011

Serological studies of patients with pertussis and the identification of antigenic *Bordetella pertussis* proteins support the hypothesis that *B. pertussis* perceives an iron starvation cue and expresses multiple iron source utilization systems in its natural human host environment. Furthermore, previous studies using a murine respiratory tract infection model showed that several of these *B. pertussis* iron systems are required for colonization and persistence and are differentially expressed over the course of infection. The present study examined genome-wide changes in *B. pertussis* gene transcript abundance in response to iron starvation *in vitro*. In addition to known iron source utilization genes, we identified a previously uncharacterized iron-repressed cytoplasmic membrane transporter system, *fbpABC*, that is required for the utilization of multiple structurally distinct siderophores including alcaligin, enterobactin, ferrichrome, and desferrioxamine B. Expression of type III secretion system genes was also found to be upregulated during iron starvation in both *B. pertussis* strain Tohama I and *Bordetella bronchiseptica* strain RB50. In a survey of type III secretion system protein production by an assortment of *B. pertussis* laboratory-adapted and low-passage clinical isolate strains, iron limitation increased the production and secretion of the type III secretion system-specific translocation apparatus tip protein Bsp22 in all Bvg-proficient strains. These results indicate that iron starvation in the infected host is an important environmental cue influencing not only *Bordetella* iron transport gene expression but also the expression of other important virulence-associated genes.

Iron regulatory mechanisms in the mammalian host serve to limit the amount of free iron available to pathogenic microbes. Host iron homeostasis is controlled by the peptide hormone hepcidin, the production of which is further upregulated during infection and inflammation (38, 41, 62). The action of hepcidin, coupled with the extracellular iron-binding proteins transferrin (Tf) and lactoferrin (Lf), results in an effective innate iron sequestration defense against microbes. Pathogens are able to scavenge iron in the host, and expression studies of a variety of host-pathogen systems have identified iron acquisition genes as a prevalent class of bacterial *in vivo*-expressed genes (51). Bacterial iron acquisition system genes are negatively regulated by Fur, or a functionally similar bacterial repressor, and are derepressed under conditions of iron starvation (11, 40). Microbial iron starvation is a key signal controlling the expression of other known virulence factors

(49, 55). To obtain ferric iron, bacteria may produce siderophores and their cognate transporters or they may produce transporters allowing utilization of xenosiderophores (59). Some organisms have surface receptors allowing direct uptake of heme or the iron from transferrin and lactoferrin (72). In Gram-negative bacteria, TonB-dependent outer membrane receptors are required for transfer of iron chelates and heme to the periplasm, followed by transport to the cytoplasm by ATP-binding cassette (ABC)-type transporters. Inorganic iron in the periplasm is transported to the cytoplasm by membrane transporters such as the Sfu ABC system (6).

Bordetella pertussis, *Bordetella parapertussis*, and *Bordetella bronchiseptica* are highly genetically related respiratory pathogens of mammals (52, 66). *B. pertussis* is a strictly human-adapted species and is the agent of whooping cough or pertussis. *B. parapertussis* causes respiratory infections in humans and sheep, and *B. bronchiseptica* causes respiratory diseases in a range of mammalian hosts, including rare cases in humans. These pathogenic bordetellae attach to the ciliated cells of the host respiratory epithelium by using multiple adhesins and produce a variety of virulence factors including multiple potent toxins, resulting in disease symptoms. Most of the known *Bordetella* virulence factor genes, including the type III secretion system (T3SS) genes, are regulated by a two-component phosphorelay system consisting of the BvgS transmembrane sensor kinase and the BvgA DNA-binding response regulator (8, 86, 91). Expression of the T3SS genes is coregulated by the BtrS

* Corresponding author. Mailing address: University of Minnesota, Department of Microbiology, MMC 196, 420 Delaware St. S.E., Minneapolis, MN 55455-0312. Phone: (612) 625-6947. Fax: (612) 626-0623. E-mail: armst018@umn.edu.

† Present address: Life Technologies, 850 Lincoln Centre Drive, Foster City, CA 94404.

‡ Present address: 39 Deevale Road, Toronto, Ontario M3M 1Z2, Canada.

§ Supplemental material for this article may be found at <http://jbb.asm.org/>.

∇ Published ahead of print on 8 July 2011.

alternative sigma factor, and *btrS* expression is BvgAS dependent (53, 93). T3SS-associated proteins are cytotoxic for a variety of eukaryotic cell types, cause release of hemoglobin from erythrocytes, and have diverse immunomodulatory effects on the host, thus contributing to virulence (1, 34, 46, 54, 92).

In order to acquire iron, *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* produce and utilize the siderophore alcaligin (22, 57, 63). Additionally, *B. pertussis* and *B. bronchiseptica* can use the xenosiderophores enterobactin (14), ferrichrome, and desferrioxamine B (13); *B. bronchiseptica* can also use aerobactin, ferrichrysin, ferricrocin, ferrirubin, protochelin, schizokinene, vicibactin, and pyoverdine (71). The three *Bordetella* species have from 14 to 19 predicted TonB-dependent receptor-mediated transport systems (5, 66), but only the alcaligin, enterobactin, and heme utilization systems have been characterized to date. Expression of each of these systems is repressed by Fur and is also activated under iron starvation conditions by mechanisms involving dedicated positive regulators that require the cognate iron source as the inducer (3, 16, 23, 87, 88).

Several observations indicate that *Bordetella* cells rely on their iron uptake systems for growth *in vivo* and that iron starvation is a key host environmental cue. Iron-starved *B. pertussis* was shown to adhere more effectively to A549 human alveolar epithelial cells compared with iron-replete bacteria (89). A *B. pertussis tonB* mutant was attenuated in a mouse model of infection (70), and a *B. bronchiseptica* alcaligin siderophore biosynthesis mutant showed decreased virulence in neonatal swine (73). Studies of a *B. pertussis* alcaligin receptor mutant showed that alcaligin utilization was critical for bacterial growth in mice throughout infection (20). Similar analyses using a *B. pertussis* heme receptor mutant demonstrated an *in vivo* growth defect in the late stages of infection, suggesting that persistence in the host depends on heme utilization (24). The BfeA enterobactin receptor was required for growth of *B. pertussis* in the early stages of mouse infection (21). Recent studies indicated that *B. pertussis* upregulates expression of the alcaligin, enterobactin, and heme utilization genes *in vivo*, confirming that the mammalian host is an iron-restricted environment and that the cognate iron sources are likely present in the host during infection (21). Expression patterns of these iron systems were correlated with the stage of infection during which the iron systems appear to have their greatest impact on *Bordetella in vivo* fitness. Serum antibodies from pertussis patients recognized numerous *Bordetella* iron-repressible cell envelope proteins including the receptors for alcaligin, enterobactin, and heme. Together, these results suggest that *B. pertussis* is iron starved in its natural human host environment and responds to the availability of diverse iron sources by expressing multiple iron utilization systems.

In the present study, we sought to understand better the responses of *B. pertussis* to the host iron-restricted environment and to identify other *B. pertussis* genes exhibiting increased expression under iron starvation conditions. In addition to identifying the genes responsible for alcaligin, enterobactin, and heme utilization, transcriptome analyses identified a previously uncharacterized cytoplasmic membrane transporter system that we then show is required by *Bordetella* cells for the utilization of multiple siderophores, including alcaligin and enterobactin. These studies also revealed repres-

sion of T3SS genes in *B. pertussis* and *B. bronchiseptica* by iron. Furthermore, we show that production of secreted T3SS proteins, including Bsp22, BteA, BopD, BopB, and BopN, is increased upon iron starvation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *Bordetella* strains and plasmids used in this study are listed in Table 1. *Bordetella* strains were grown on Bordet-Gengou agar plates. *Escherichia coli* strains were grown using Luria-Bertani (LB) broth or LB agar. Stainer-Scholte (SS) medium (76) was used for broth cultures, and all *B. pertussis* SS cultures were supplemented with 0.5% Casamino Acids. Glassware was acid cleaned and rinsed in distilled deionized water prior to use. Iron-replete SS medium contained 36 μ M ferrous sulfate. For iron-depleted SS cultures, SS medium lacking iron supplement was further deferrated using Chelex 100 resin (Bio-Rad Laboratories, Richmond, CA). The chrome azurol S assay (77) was used to monitor alcaligin production as reported previously (9). Final concentrations of antibiotics used for plasmid marker selection were as follows: tetracycline, 15 μ g/ml; ampicillin, 100 μ g/ml; gentamicin, 10 μ g/ml; kanamycin, 50 μ g/ml; and streptomycin, 90 μ g/ml. Growth was monitored spectrophotometrically at a wavelength of 600 nm or by using a Klett colorimeter.

Routine DNA procedures. DNA cloning procedures used standard methods (75). Conjugal transfer of plasmids to *Bordetella* strains, with *E. coli* DH5 α as the plasmid donor strain and DH5 α (pRK2013) as the source of mobilization functions, has been described previously (17). *Bordetella* transconjugants were selected on agar containing the appropriate antibiotics and colicin B (18). PCR was performed using standard procedures and *Bordetella* DNA templates in the presence of 5% dimethyl sulfoxide.

Nucleotide and protein sequence analysis. Annotated *Bordetella* genome sequences (GenBank accession numbers: *B. pertussis* Tohama I, NC_002929; *B. bronchiseptica* RB50, NC_002927) were accessed at the GeneDB website (<http://www.genedb.org/>), developed and maintained by the Sanger Institute's Pathogen Sequencing Unit. Genome sequence features were visualized using the Artemis genome sequence viewer and annotation tool (74) (<http://www.sanger.ac.uk/Software/Artemis/>). Other DNA and protein analyses for sequence data management and PCR primer design relied on the Lasergene software package version 5.53 for Mac OS X (DNASTAR, Inc., Madison, WI).

Growth stimulation assays. *B. bronchiseptica* utilization of iron sources was assessed using an agar bioassay method as described previously (15, 22), with iron-restricted LB agar plates containing the nonutilizable iron chelator EDDA {ethylenediaminedi-[(*o*-hydroxyphenyl)acetic acid]} at a final concentration of 100 μ g/ml. *B. pertussis* LB agar bioassays used a modification of this method described previously (87). Test iron sources included the siderophores alcaligin and enterobactin purified as described previously (3, 22), the siderophores desferrioxamine B mesylate (Desferal) (Ciba Agrochemicals AG, Basel, Switzerland) and ferrichrome (Sigma-Aldrich, St. Louis, MO), ferrous sulfate (Sigma), and bovine hemin chloride (Sigma). Twofold serial dilutions of the iron sources were prepared in water, and five concentrations of each were tested in triplicate: FeSO₄, 2.50, 1.25, 0.63, 0.31, and 0.16 mM; alcaligin, 619, 310, 155, 77, and 39 μ M; enterobactin, 2.50, 1.25, 0.63, 0.31, and 0.16 μ M; and bovine hemin chloride, desferrioxamine B mesylate, and ferrichrome, 100, 50, 25, 13, and 6 μ M. Diameters of bacterial growth zones surrounding the sample wells were measured after 24 h (for *B. bronchiseptica*) or 48 h (for *B. pertussis*) of incubation at 37°C. Linear regression analysis confirmed a strong correlation between the log₁₀ concentration of the iron source and the diameter of the growth zone it produced (correlation coefficients $r \geq 0.99$). The ratio of the iron source concentrations necessary to produce equivalent growth diameters for the wild-type (WT) and mutant strains as determined by linear regression analysis was taken as a measure of their relative iron source utilization ability.

Utilization of lactoferrin (Lf) and transferrin (Tf) was assessed by measuring growth in liquid SS cultures with partially iron-saturated Tf or Lf as the sole iron source. Human Lf and Tf (Sigma) were loaded with ferric iron to 30% saturation as described by Agiata and Dyer (2). The solutions were adjusted to 2 mg of protein per ml in 50 mM Tris (pH 7.5), 150 mM sodium chloride, 20 mM sodium bicarbonate buffer. SS cultures were supplemented with 10 mM sodium bicarbonate and Tf or Lf at a 200- μ g/ml protein concentration, yielding total iron concentrations estimated at 1.5 μ M.

Hemolysis assays. Hemolytic activity of *B. bronchiseptica* was measured essentially as described previously (48, 54, 64), using rabbit red blood cells (rRBC, HemoStat Laboratories). Briefly, washed *B. bronchiseptica* RB50 cell suspensions were prepared from 16-h iron-replete and iron-depleted SS cultures, mixed

TABLE 1. *Bordetella* strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Source
<i>B. pertussis</i> strains		
Tohama I	Clinical isolate, ca. 1954	C. Parker
UT25	Clinical isolate, 1975	C. Parker
UT25-90	Degraded, avirulent phase IV derivative of UT25	S. Armstrong
BP6068	Clinical isolate, 1997	J. Miller
165	Office of Biologics, Food and Drug Administration	C. Manclark via C. Parker
114	Office of Biologics, Food and Drug Administration	C. Manclark via C. Parker
3779	Eli Lilly & Co. vaccine strain	C. Parker
10536	Connaught Laboratories vaccine strain	T. Merkel
188	Clinical isolate	T. Merkel
461	Clinical isolate	T. Merkel
<i>B. bronchiseptica</i> strains		
RB50	<i>bvg</i> ⁺ rabbit isolate, wild type	P. Cotter
RB53	<i>bvgS</i> -C3, <i>bvg</i> (Con), <i>Bvg</i> ⁺ phenotypic phase-locked mutant derivative of RB50	P. Cotter
RB54	Δ <i>bvgA5</i> , <i>Bvg</i> ⁻ phenotypic phase-locked mutant derivative of RB50	P. Cotter
Plasmid vectors		
pGEM3Z	2.7-kb cloning vector, Amp ^r	Promega
pRK415	10.5-kb broad-host-range cloning vector, Tet ^r	N. Keen
pRK2013	48-kb helper plasmid for mobilization of non-self-transmissible plasmids, Kan ^r	D. R. Helinski
pEG7	8.0-kb allelic exchange vector derived from pSS1129, Amp ^r Gent ^r	P. Cotter
pSS4245	8.3-kb allelic exchange vector, I-SceI ⁺ , Kan ^r Strep ^r	S. Stibitz
Recombinant plasmids		
pEG7/ <i>fbpA</i> _{Bb}	0.8-kb PCR-generated DNA fragment internal to <i>B. bronchiseptica</i> RB50 <i>fbpA</i> gene cloned into pEG7, BamHI-EcoRI primer/adapters, Amp ^r Gent ^r	This study
pEG7/ <i>fbpA</i> _{Bp}	0.8-kb PCR-generated DNA fragment internal to <i>B. pertussis</i> Tohama I <i>fbpA</i> gene cloned into pEG7, BamHI-EcoRI primer/adapters, Amp ^r Gent ^r	This study
pRK/ <i>fbpA</i> _{Bp}	2.4-kb PCR-generated <i>fbpA</i> ⁺ DNA fragment of <i>B. pertussis</i> Tohama I cloned into pRK415, BamHI-EcoRI primer/adapters, Tet ^r	This study
p3Z/ <i>fbpA</i> _{Bp}	2.4-kb PCR-generated <i>fbpA</i> ⁺ DNA fragment of <i>B. pertussis</i> Tohama I cloned into pGEM3Z, BamHI-EcoRI primer/adapters, Amp ^r	This study
p3Z/ Δ <i>fbpA</i> _{Bp}	p3Z/ <i>fbpA</i> _{Bp} with 0.7-kb in-frame deletion in <i>fbpA</i> generated by whole-plasmid inverse PCR mutagenesis	This study
pSS/ Δ <i>fbpA</i> _{Bp}	1.7-kb BamHI-EcoRI Δ <i>fbpA</i> _{Bp} insert fragment of p3Z/ Δ <i>fbpA</i> _{Bp} subcloned into pSS4245, I-SceI ⁺ , Kan ^r , Strep ^r	This study
pRK/ <i>fbpABC</i> _{Bp}	6.5-kb <i>fpr fbpABC</i> ⁺ PCR-generated DNA fragment of <i>B. pertussis</i> Tohama I cloned into pRK415, HindIII primer/adapters, Tet ^r	This study

with washed rRBC suspensions at various bacterial cell/RBC ratios, and incubated at 37°C for 45 min. Controls included rRBC incubated without bacteria and rRBC lysed with water (100% lysis control).

Insertional mutagenesis of *fbpA* in *B. pertussis* and *B. bronchiseptica*. DNA regions (800 bp) internal to the *fbpA* coding sequences of *B. pertussis* Tohama I (BP1605) and *B. bronchiseptica* RB50 (BB2946) were amplified by PCR using PCR primer/adaptor pair 5'-ggccgatccGTCCGACCAAGCGTTCCTCCCTGAT-3' and 5'-ggccgaattcCGGCGCCGCTGATGTTGACG-3' (underlining indicates restriction sites, and lowercase letters indicate adapter ends). Adapter ends were trimmed using BamHI and EcoRI, and the products were cloned into the mobilizable suicide plasmid pEG7. The resulting pEG7/*fbpA*_{Bp} and pEG7/*fbpA*_{Bb} plasmids were delivered to Tohama I or RB50 recipient strains by conjugation, and *fbpA* insertion mutants were selected on the basis of gentamicin and colicin B resistance.

Cloning the *fbpA* and *fbpABC* genes. Chromosomal DNA regions (2.4 kb) including the 1-kb *fbpA* genes of *B. pertussis* Tohama I and *B. bronchiseptica* RB50 along with approximately 0.7 kb of flanking DNA sequences both upstream and downstream were PCR amplified using PCR primer/adapters 5'-ggccgatccGTCCGCGGTGCCGAACAGATACAGG-3' and 5'-ggccgaattcCGACGCGCCGGCTCCAGTG-3', and the BamHI-EcoRI-digested PCR products were cloned into plasmid vectors pGEM3Z and pRK415 to yield *fbpA*⁺ plasmids p3Z/*fbpA*_{Bp}, p3Z/*fbpA*_{Bb}, pRK/*fbpA*_{Bp}, and pRK/*fbpA*_{Bb}. PCR primer/adapters 5'-ggccaagcttGCGCGCCAAAGAAAACC-3' and 5'-ggccaagcttACGGGGCCGCAATGTCC-3' were used to PCR amplify the 6.5- and 5.5-kb chromosomal DNA regions encompassing the *fbpABC* genes of *B. pertussis*

Tohama I and *B. bronchiseptica* RB50, respectively, and the HindIII-digested products were cloned into plasmid vectors pGEM3Z and pRK415 to yield *fbpABC*⁺ plasmids p3Z/*fbpABC*_{Bp}, p3Z/*fbpABC*_{Bb}, pRK/*fbpABC*_{Bp}, and pRK/*fbpABC*_{Bb}. The *fbpABC* DNA region cloned from Tohama I includes the IS481 insertion sequence located downstream of *fbpC*; this IS element is absent from the 5.5-kb RB50 DNA region.

Construction of a *B. pertussis* Δ *fbp* mutant strain. Plasmid p3Z/*fbpA*_{Bp} was used as the DNA template for *in vitro* deletion mutagenesis by inverse PCR using 5'-phosphorylated DNA oligonucleotide primers 5'-GAAGGCGTCGAGCAGAGG-3' and 5'-TACGCCAGGCCAACTACGA-3'. The reaction products were circularized by ligation using T4 DNA ligase and then digested with DpnI before transformation of *E. coli* to yield plasmids carrying the Δ *fbpA*_{Bp} allele with 729 bp of *fbpA* coding sequences deleted. Putative p3Z/ Δ *fbpA*_{Bp} plasmids were identified by restriction enzyme mapping, and the desired in-frame deletion mutation was confirmed by nucleotide sequencing. The Δ *fbpA*_{Bp} allele was subcloned to allelic exchange plasmid vector pSS4245 (S. Stibitz, unpublished data) to create plasmid pSS/ Δ *fbpA*_{Bp}, and the Δ *fbpA* mutation was transferred to the chromosome of *B. pertussis* strains Tohama I and UT25 by using a method adapted by Stibitz from that of Posfai et al. (69) for use in *B. pertussis*. Briefly, pSS/ Δ *fbpA*_{Bp} was conjugally transferred to *B. pertussis* that had been modulated to *Bvg*⁻ phenotypic phase by growth in the presence of 50 mM MgSO₄. Primary selection for *B. pertussis* pSS/ Δ *fbpA*_{Bp} integrants used kanamycin, streptomycin, and colicin B under modulating (*Bvg*⁻) growth conditions to allow stable maintenance of the integrated plasmid. Integrants were pooled and restreaked on the same selective medium with MgSO₄ and then subcultured to standard BG plates

(Bvg⁺ growth conditions) to allow expression of the plasmid-encoded I-SceI restriction endonuclease, which is under the control of the Bvg-dependent *ptx* promoter. I-SceI production results in cleavage at the pSS4245 plasmid-encoded SceI site to produce a double-stranded break in the chromosome. Repair of this lesion involving recombination between the integrated plasmid and homologous chromosomal sequences results in allelic exchange at high frequency. Colonies were scored for loss of plasmid antibiotic resistance markers, and Δ *fbpA* mutants were confirmed by PCR mapping.

Electrophoretic analysis of *Bordetella* proteins. SDS-PAGE analysis of proteins was performed using standard methods; proteins were visualized by staining with Coomassie blue dye. Extracts enriched in *B. pertussis* periplasmic proteins were produced by the osmotic shock method described by Shouldice and co-workers (79). Cell proteins for immunoblot analysis were prepared from *B. pertussis* strain Tohama I and *B. bronchiseptica* strains RB50 and RB54 cultured in iron-replete and iron-depleted SS media. Cells were suspended at an optical density at 600 nm (OD₆₀₀) of 10 in 1 ml of SDS-PAGE sample buffer containing 2-mercaptoethanol and boiled for 5 min. Samples (40 µg of protein) were separated by SDS-PAGE on 7.5% polyacrylamide gels and transferred to nitrocellulose membranes. Mouse monoclonal antibody 8E7, which is specific for *B. pertussis* Vag8 (10, 35, 36), was used and reactivity detected using goat anti-mouse IgG conjugated to horseradish peroxidase (Cappel) with 4-chloronaphthol as the substrate.

LC-MS/MS analysis of secreted proteins. *Bordetella* cells from SS cultures were pelleted by centrifugation, and the supernatant fluids were clarified by filtration using 0.2-µm pore size, low-protein-binding filters. Proteins were precipitated by addition of 100% trichloroacetic acid (wt/vol) to 15% final concentration, and incubation on ice for 4 h. Protein precipitates were recovered by centrifugation and solubilized by boiling for 5 min in SDS-PAGE sample buffer containing 2-mercaptoethanol. Protein bands were excised from Coomassie blue-stained SDS-PAGE gels and treated with trypsin. Trypsin treatment and reverse-phase liquid chromatography-tandem mass spectrometry (LC-MS/MS) were performed at the University of Minnesota Center for Mass Spectrometry and Proteomics facility. Peptide products were separated on a C₁₈ Nanotrap column (Michrom BioResources, Auburn, CA) and subjected to tandem mass spectrometry using a ThermoFinnigan (ABI, Inc., Foster City, CA) LTQ ion trap mass spectrometer (MS).

Mass spectrometry data were analyzed using Sequest (ThermoFinnigan, version 27, rev. 13) and X! Tandem software (version 2006.04.01.2, The Global Proteome Machine Organization, <http://www.thegpm.org>). X! Tandem and Sequest were set up to search the NCBI *Bordetella* protein sequence database (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=protein>) assuming trypsin cleavage, and with a fragment ion mass tolerance of 1.00 Da and parent ion tolerance of 0.80 Da. The iodoacetamide derivative of cysteine was specified as a fixed modification, and oxidation of methionine as a variable modification. Scaffold (version Scaffold-01_06_19, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 90.0% probability as specified by the Peptide Prophet algorithm (43). Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (60). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

RNA isolation. *B. pertussis* Tohama I or *B. bronchiseptica* RB50 cells were harvested from iron-replete SS cultures at early stationary growth phase, washed extensively with iron-free SS medium, and then subcultured to iron-replete or iron-depleted SS medium at an initial cell density corresponding to an OD₆₀₀ of 0.1. Cultures were grown with rotary shaking at 300 rpm at 37°C. At various times ranging from 2 to 36 h, cultures were sampled and bacterial cells were collected by centrifugation. Supernatant fluids were discarded or collected for recovery of extracellular proteins, and cell pellets were rapidly frozen in liquid nitrogen and stored at -80°C for subsequent RNA isolation. Total RNA was isolated using the RNeasy-4PCR kit (Ambion, Austin, TX) with an additional lysis step using lysozyme (0.4 mg/ml).

Microarray hybridization and analysis. *Bordetella* RNA was reverse transcribed into Cy5-labeled cDNA and then cohybridized along with a Cy3-labeled mixture of *B. pertussis*, *B. bronchiseptica*, and *B. parapertussis* genomic DNA to a *Bordetella* microarray representing 97.4% of the *B. pertussis* Tohama I open reading frames (ORFs), 97.9% of the *B. parapertussis* strain 12822 ORFs, and 98.5% of the *B. bronchiseptica* RB50 ORFs, as previously described (25). The use of a common reference across different cDNA microarray experiments improves the reproducibility of hybridization signals and allows comparisons of gene expression levels determined in separate experiments (80). Arrays were scanned

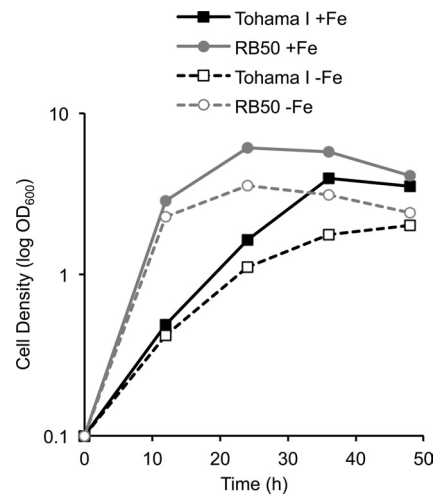


FIG. 1. Growth of *B. pertussis* Tohama I and *B. bronchiseptica* RB50 in iron-replete (+Fe) and iron-depleted (-Fe) SS media. Typical growth curves (as monitored spectrophotometrically at 600 nm wavelength over a 48-h culture period) are shown.

using a GenePix 4000B scanner and the GenePix Pro 5.0 software (Axon Instruments, Union City, CA). By using the GenePix Pro 6.0 software (Axon Instruments), spots were screened visually to exclude the low-quality spots, and background-subtracted Cy5/Cy3 intensity ratios were calculated. Data were filtered to include only spots containing more than 30 pixels and having a mean background-subtracted Cy3 signal greater than 150 U. Cy5/Cy3 ratios were averaged for replicate spots, log transformed, and normalized by calculating the median log ratio and subtracting the value obtained from each data point.

For the analysis of the iron starvation response in Tohama I and RB50, significance analysis of microarrays (SAM; version 1.0) (85) was performed for the subset of array elements whose data passed filtering criteria for at least 50% of the arrays in the experiment. For this analysis, data were assigned to two groups, iron replete and iron depleted, in order to identify transcripts with significant differences in abundance between the two nutritional growth conditions. Only time points subsequent to a detectable iron depletion response (16 h and later for RB50, 24 h and later for Tohama I) were used in the SAM analysis.

Hierarchical clustering of data was performed with Cluster (version 2.11.0.1) (32), using only those array elements whose data passed filtering criteria for at least 80% of the arrays. Transcript abundance was considered to vary significantly according to iron availability if there was a difference of at least 2 between the maximum and minimum log values across the data set (4-fold variation). Results were displayed using Treeview (version 1.60). Functional classifications of genes whose transcript abundances varied significantly in response to iron availability were as assigned by Parkhill and coworkers (66), and by the descriptive multifunctional classification of Serres and Riley (78).

Microarray data accession numbers. Microarray data have been deposited in ArrayExpress under accession numbers E-MEXP-3263 (*B. pertussis* Tohama I) and E-MEXP-3076 (*B. bronchiseptica* RB50).

RESULTS

***B. pertussis* transcriptional response to iron restriction.** To analyze the *B. pertussis* transcriptional response to iron limitation, cDNA microarray expression analysis using *Bordetella* genomic DNA as the common reference was employed. Strain Tohama I was cultured in parallel in iron-replete and iron-depleted SS media and RNA was isolated at time points from 2 h to 36 h, during the different stages of growth (Fig. 1 and 2; also see Tables S1 and S2 in the supplemental material). This sampling scheme allowed growth phase-related changes in gene expression to be distinguished from those related to the iron starvation response. *B. pertussis* genes showing increased transcript abundance under iron-replete growth conditions

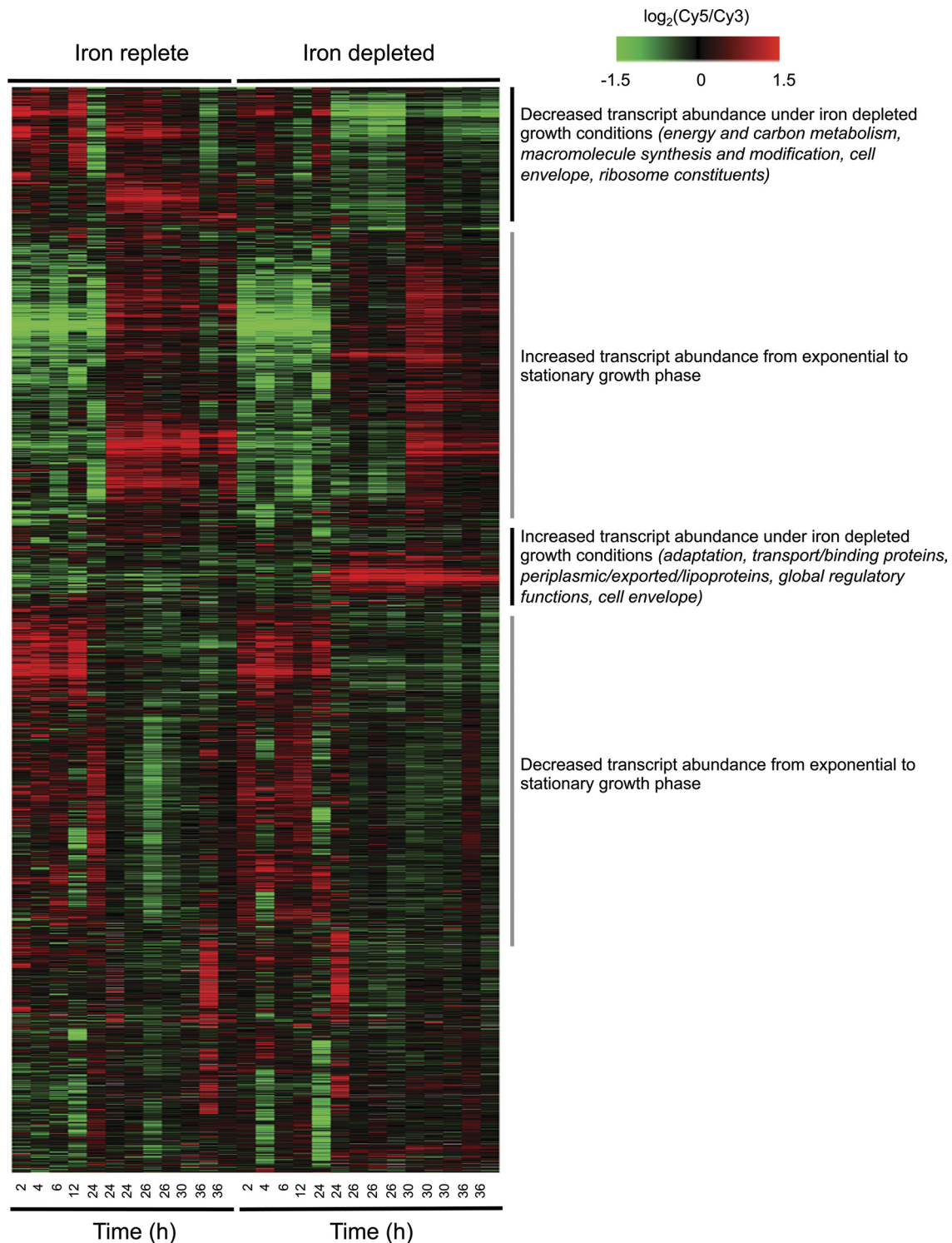


FIG. 2. Gene transcript abundance patterns in *B. pertussis* Tohama I. Gene transcript abundance patterns in *B. pertussis* Tohama I are shown for cultures grown under iron-replete and iron-depleted conditions. Rows correspond to array elements, and major functional categories for groups of genes and their predicted products are indicated on the right. Some genes are represented by multiple array elements. Gray indicates missing data. The elapsed time since the culture was started is indicated at the bottom.

(downregulated by iron limitation) included genes encoding 30S and 50S ribosomal proteins, ATP synthase subunits, and iron-containing proteins including Nuo subunits of the NADH-ubiquinone oxidoreductase, cytochromes, and cata-

lase. Transcripts for capsular biosynthesis genes and two bacterioferritin genes, the products of which are predicted to play an important role in the storage of iron, were also increased in abundance in the presence of iron.

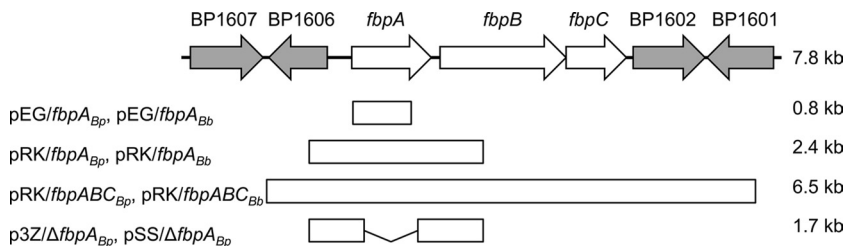


FIG. 3. Genetic organization of the *B. pertussis* *fbpA* chromosomal region. The arrows in the uppermost diagram represent genetic limits and orientations of genes within the *B. pertussis* Tohama I DNA region that includes the *fbpABC* genes (white arrows): *fbpA* (BP1605), ferric iron-binding protein (periplasmic component of an ABC-type Fe³⁺ transport system); *fbpB* (BP1604), putative cytoplasmic membrane permease; *fbpC* (BP1603), probable ATP-binding component of ABC transporter. Flanking genes were as follows: BP1606, putative ferredoxin-NADP⁺ reductase; BP1607, probable LysR-family transcriptional regulator; BP1602, transposase; BP1601, conserved hypothetical protein. Cloned DNA fragments used for insertional mutagenesis (0.8 kb), complementation (2.4 kb and 6.5 kb), and construction of in-frame *fbpA* deletion mutations are shown. In *B. bronchiseptica*, the fragment analogous to the 6.5-kb region is only 5.5 kb in size because this region lacks the *IS481* element.

As anticipated, iron-repressed genes (upregulated by iron limitation) included the alcaligin, enterobactin, and heme iron acquisition system genes, *tonB* and *exbBD*, and genes encoding predicted TonB-dependent receptors. The alcaligin, enterobactin, and heme utilization system genes are known to be iron repressible by Fur, in addition to being positively regulated (3, 16, 23, 87, 88). Of the 12 genes encoding uncharacterized TonB-dependent receptors, *bfrB* (BP2016) and *bfrC* (BP3663) were significantly upregulated upon iron starvation *in vitro*. Other genes predicted to be involved in iron transport, BP1136, BP1139, and BP1140, were also upregulated when iron was restricted. BP1136 encodes an extracytoplasmic function sigma factor; this gene is an ortholog of the *B. bronchiseptica* *ecfI* gene adjacent to and required for expression of *bfrH* (BB3658/BP1138), encoding a TonB-dependent receptor of unknown ligand specificity (27). Other upregulated genes adjacent to predicted TonB-dependent iron source receptor genes included BP1961 near *bfrI* (BP1962) and BP0454 near *hemC* (BP0456). BP1605, a gene encoding a predicted periplasmic ferric iron-binding component of an ABC superfamily transporter system, also showed increased expression during iron starvation. In this report, a detailed functional analysis of this gene is presented.

Some of the *B. pertussis* genes that were increased in expression under conditions of iron limitation were not anticipated to be regulated in such a fashion. For example, *fhaB* (BP1879), *ptlC* (BP3790), and *ptlI* (BP3792) were classified as iron repressed. In *B. pertussis*, *fhaB* is monocistronic (29, 50) and the pertussis toxin structural (*ptx*) and secretion (*ptl*) genes are cotranscribed from a promoter upstream of *ptxA* (12, 45). Analysis of the *B. pertussis* Tohama I derivative BP953 (81), carrying chromosomal *fhaB-lacZ* and *ptx-phoA* transcriptional fusions, revealed only modest elevation of *fhaB* and *ptxA* expression under iron-depleted growth conditions as compared with iron-replete conditions (46% and 14% increases in reporter activity after 48 h of culturing, respectively) (data not shown). Also among the *B. pertussis* genes showing elevated transcript abundance under iron starvation conditions were Bvg-dependent genes of the T3SS, which have been extensively analyzed in *B. bronchiseptica* (26, 37, 47, 48, 53, 54, 64, 65, 92, 93). We examined the influence of iron starvation on *Bordetella* T3SS gene expression and protein production in greater detail (see below).

BP1605 encodes a ferric iron binding protein. Compared with expression levels during iron-replete growth conditions, the BP1605 gene of Tohama I showed a 4.8-fold increase in transcript abundance under conditions of iron limitation. BP1605 is predicted to encode a 37.4-kDa protein, referred to here as FbpA, that is similar to ferric iron-binding proteins including those involved in utilization of Lf- and Tf-bound iron by *Neisseria gonorrhoeae* and *Neisseria meningitidis* (FbpA, 28% identity) (30) and *Haemophilus influenzae* (HitA, 30% identity) (44), as well as the *Serratia marcescens* Sfu protein (28% identity) (6) involved in the uptake of inorganic ferric iron and FbpA of *Campylobacter jejuni* (35% identity) (see Fig. S1 in the supplemental material). The *B. pertussis* FbpA is a predicted periplasmic solute-binding component of an ABC superfamily transporter system. The crystal structures of the apo- and holo-forms of *B. pertussis* FbpA were determined previously by Tom-Yew and coworkers (83), and used to aid the refinement of the structure of the ferric iron-binding protein of *C. jejuni* (83), but no functional studies of *B. pertussis* FbpA have been reported. Adjacent to *fbpA* (Fig. 3), the downstream *fbpB* (BP1604) and *fbpC* (BP1603) genes encode putative inner membrane permease and ATPase components of the ABC transporter system, respectively. The *fbpABC* genes are predicted to be cotranscribed from a putative Fur-repressible promoter located upstream of *fbpA*. BP1603 is annotated as a pseudogene that is interrupted by an *IS481* element in *B. pertussis* Tohama I; however, alignment of the deduced BP1603 product with the protein sequence of the *B. bronchiseptica* RB50 FbpC ortholog (locus tag BB2948) indicates that the IS element does not impinge on the coding sequence and the 265-amino-acid BP1603 product is intact but has 6 amino acids (FbpC_{Bp} amino acid residues 119 to 124, inclusive) that are absent in FbpC_{Bb}. BP1604 was absent from the array data set, so its expression patterns are unknown; however, BP1603 also showed an increase in transcript abundance during iron limitation, albeit less than 2-fold. Since *fbpABC* were iron repressed and encoded a predicted ABC-type transporter system, we hypothesized that these genes could potentially be involved in alcaligin or enterobactin utilization. No cytoplasmic membrane transporter functions are encoded within the alcaligin and enterobactin system gene clusters, and none had been identified until the present study.

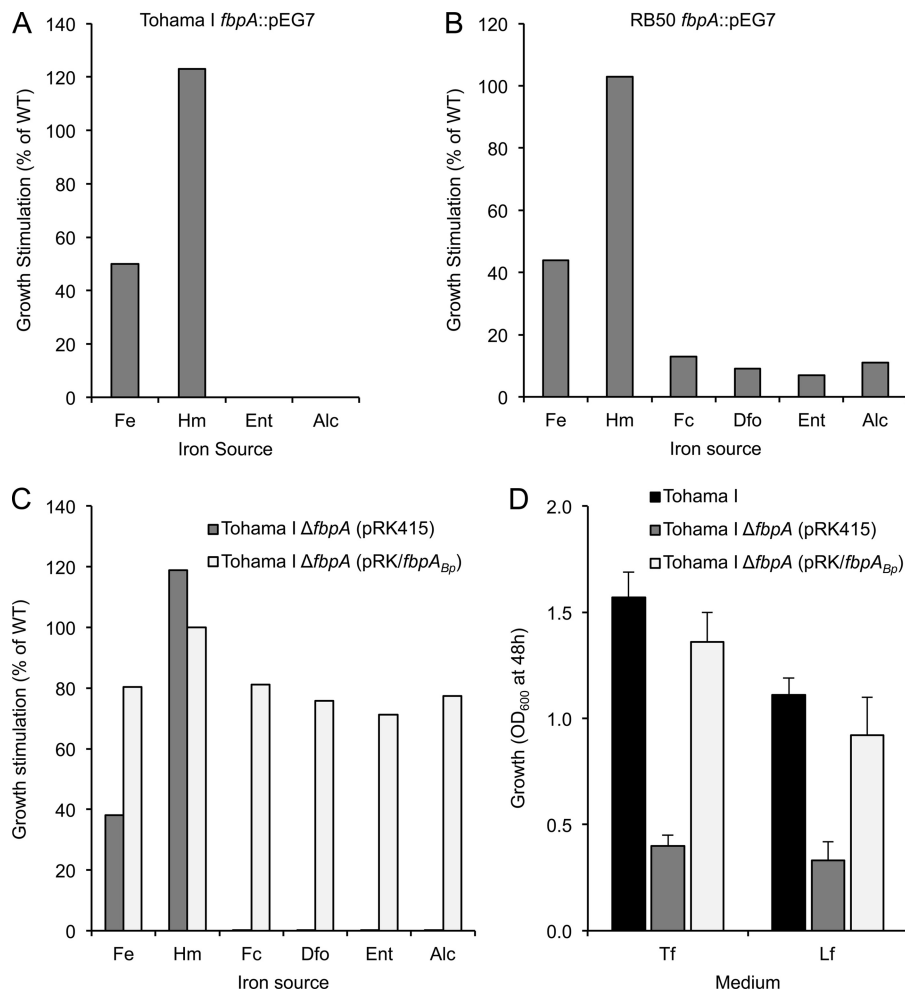


FIG. 4. Iron source utilization by WT and *fbpA* mutant strains of *B. pertussis* and *B. bronchiseptica*. (A) Growth stimulation of *B. pertussis* Tohama I *fbpA::pEG7* mutant is shown relative to that of the WT parental strain. The mutant showed no measurable growth stimulation by enterobactin or alcaligin. (B) Growth stimulation of *B. bronchiseptica* RB50 *fbpA::pEG7* mutant is shown relative to that of the WT parental strain. (C) Growth stimulation of *B. pertussis* Tohama I $\Delta fbpA$ (pRK415) ($\Delta fbpA$) and the complemented strain Tohama I $\Delta fbpA$ (pRK/*fbpA*_{Bp}) ($\Delta fbpA$ /*fbpA*⁺) compared to that of the WT parental strain. The $\Delta fbpA$ mutant showed no measurable growth stimulation by any siderophore tested. (D) Growth of *B. pertussis* in SS medium with Tf or Lf as the sole source of iron. Growth yield was measured as the optical density at 600 nm (mean \pm SD, $n = 3$) after 48 h of culture. Iron sources: Fe, ferrous sulfate; Hm, heme chloride; Fc, ferrichrome; Dfo, desferrioxamine B; Ent, enterobactin; Alc, alcaligin; Tf, transferrin; Lf, lactoferrin.

***fbpA* mutants are defective in the utilization of multiple siderophores.** The *fbpA* gene of *B. pertussis* Tohama I was insertionally inactivated and the resulting mutant was tested for the ability to utilize a variety of known iron sources. Relative to the wild-type parent strain, the *B. pertussis* *fbpA::pEG7* insertion mutant had severe defects in utilization of alcaligin and enterobactin and also showed reduced growth stimulation by iron (Fig. 4A). The mutant retained wild-type heme utilization levels, indicating that FbpA is not required for heme uptake; presumably that function is provided by the BhuT periplasmic heme binding protein encoded in the heme system gene cluster (87). *fbpA::pEG7* insertion mutations were also constructed in *B. pertussis* strain UT25 and in *B. bronchiseptica* RB50. The *B. pertussis* UT25 (data not shown) and *B. bronchiseptica* RB50 (Fig. 4B) mutants had similar defective iron utilization phenotypes as did Tohama I *fbpA::pEG7*, indicating that these *fbpABC*-related functions are conserved in at least

two *B. pertussis* strains and in these two *Bordetella* species. Other growth assays showed that, in addition to alcaligin and enterobactin, RB50 *fbpA::pEG7* was also defective in the utilization of the siderophores ferrichrome and desferrioxamine B (Fig. 4B). Although *fbpA* disruption nullified growth stimulation by siderophores in *B. pertussis*, the *B. bronchiseptica* RB50 *fbpA::pEG7* mutant retained limited siderophore utilization. The *fbpA* insertion mutants were complemented using plasmids carrying wild-type *fbpABC* genes but not by plasmids bearing *fbpA* alone (data not shown), suggesting that the *fbpA::pEG7* insertion mutations exert polar effects on the downstream *fbpB* and *fbpC* genes.

The requirement for FbpA in the TonB-dependent utilization of siderophores was not anticipated since no other bacterial ferric iron-binding protein with this particular function has been described previously. To further establish the role of FbpA in siderophore utilization by *B. pertussis*, nonpolar in-

frame *fbpA* deletion mutations were constructed in strains Tohama I and UT25. Both *B. pertussis* Δ *fbpA* mutants had the same defective phenotype as did the *B. pertussis* *fbpA*::pEG7 insertion mutants; however, unlike the insertion mutants, the Δ *fbpA* mutants could be complemented in *trans* by the *fbpA* gene alone to greater than 70% of wild-type ability (Fig. 4C). These studies confirmed that *fbpA* is important for assimilation of iron delivered by multiple catecholate- and hydroxamate-type siderophores, and complementation results are consistent with predictions that *fbpB* and *fbpC* are operonic with *fbpA*.

FbpA is required for utilization of Tf and Lf as iron sources.

To retrieve the iron from Tf and Lf, *Bordetella* cells must use a siderophore such as alcaligin. Since *fbpA* is essential for the utilization of alcaligin by *B. pertussis*, it was hypothesized that *fbpA* mutants would display a growth defect in cultures having Lf or Tf as the sole iron source. Wild-type Tohama I was cultured in parallel with the Δ *fbpA* mutant, Tohama I Δ *fbpA* (pRK415 vector), and the complemented mutant, Tohama I Δ *fbpA*(pRK/*fbpA*_{Bp}), in iron-depleted SS medium supplemented with partially iron-saturated human Tf or Lf. Under these iron-limited conditions, growth yields for the Δ *fbpA* mutant with Tf or Lf were significantly reduced compared with those of the wild-type parent (approximately 25% and 30% of wild-type growth levels, respectively) (Fig. 4D), despite production of alcaligin at normal levels by both wild-type and Δ *fbpA* strains; alcaligin concentrations consistently ranged from 11 to 16 μ M in supernatant fluids of both wild-type and Δ *fbpA* strains after 48 h growth under iron-limited conditions. Growth with Tf or Lf was restored to the mutant to greater than 80% of the wild-type level by *trans*-complementation using *fbpA*. Since the ability to use a siderophore such as alcaligin to acquire iron from Lf and Tf is predicted to be important for host colonization, *fbpABC* may play a key role in this process.

FbpA is localized to the periplasm and its production is iron repressible. Osmotic shock fluids enriched in periplasmic components were prepared from *B. pertussis* cells and analyzed by SDS-PAGE. In support of the gene transcript abundance data, an iron-regulated periplasmic protein of ca. 37-kDa molecular mass (FbpA) was produced by *B. pertussis* Tohama I (Fig. 5). FbpA was not produced by Tohama I Δ *fbpA*(pRK415), and FbpA production was restored to the mutant by plasmid pRK/*fbpA*_{Bp}. Expression of *fbpA* from multicopy plasmid pRK/*fbpA*_{Bp} resulted in modest overproduction of FbpA by iron-starved *B. pertussis* and in detectable production of FbpA by iron-replete cells. FbpA was the most abundant periplasmic protein in iron-starved *B. pertussis* cells, accounting for approximately 50% to 70% of the total protein released by osmotic shock.

Effects of iron limitation on type III secretion system gene expression. Gene expression patterns from microarray studies revealed that certain *B. pertussis* Tohama I T3SS *bsc* cluster genes, including *bsp22*, *bopN*, *bopB*, and *bcrH2*, as well as the T3SS effector gene *bteA* (also known as *bopC*), were significantly upregulated during iron limitation (1.8- to 2.7-fold increase in gene transcript abundance) (Fig. 2; also see Table S1 in the supplemental material). Since Tohama I had been reported to be deficient in T3SS protein production (34, 53), the potential iron regulation of T3SS gene expression and protein production was examined in *B. bronchiseptica* RB50, the strain in which the Bvg-dependent

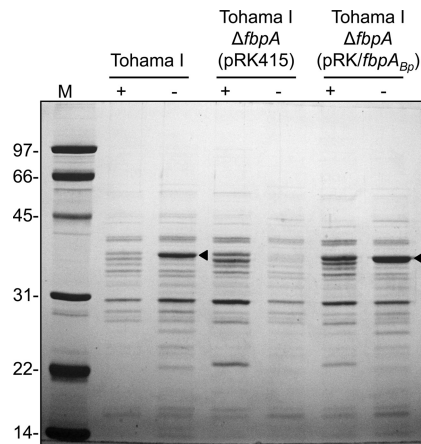


FIG. 5. Proteins released from *B. pertussis* cells by osmotic shock treatment. Shock fluids enriched in periplasmic proteins were prepared from cells harvested from iron-replete (+) and iron-depleted (-) SS cultures. A Coomassie blue-stained 12% polyacrylamide gel is shown. Sample loads were normalized to cell numbers based on optical density. Sizes of molecular mass (M) markers are shown in kDa. The arrowheads indicate the position of FbpA.

T3SS has been most extensively characterized. The effect of iron limitation on the protein secretion profiles of wild-type strain RB50 and its isogenic Bvg phenotypic phase-locked derivatives RB53 (Bvg^c) and RB54 (Bvg⁻) (28) were analyzed by SDS-PAGE. Notably, iron limitation significantly altered the secretion profiles of RB50 (Fig. 6A) and RB53 (data not shown), resulting in the elevated production of prominent proteins of approximately 22-kDa and 39-kDa apparent molecular masses. In contrast, the secretion profiles of Bvg⁻ strain RB54 were much less complex than those of Bvg⁺ strain RB50 and showed an overall reduction in total secreted protein levels under both iron-replete and iron-depleted conditions. However, a unique protein of an approximately 44-kDa apparent molecular mass appeared to be produced primarily by RB54 under iron-replete conditions.

Based on published secretion profiles of strain RB50 (92, 93), it was hypothesized that the major ca. 22-kDa protein that was produced under both iron-replete and iron-depleted growth conditions was the T3SS tip complex structural protein, Bsp22. To confirm the identities of selected proteins secreted by *B. bronchiseptica*, protein bands were excised from one-dimensional SDS-PAGE gels (Fig. 6A) and analyzed (Fig. 6C). Reverse-phase liquid chromatography-tandem mass spectrometry (LC-MS/MS) confirmed the identity of the 22-kDa major extracellular protein as Bsp22 (22.1 kDa; BB1617) (bands 1 and 2). The second most abundant protein in iron-depleted culture supernatant fluids (band 3) was BopN (38.9 kDa; BB1616), the product of the *bopN* gene that is divergently transcribed from *bsp22* in the T3SS *bsc* gene cluster. Bsp22 and BopN are typically the most abundant T3SS proteins secreted by *B. bronchiseptica* (48, 93). Analysis of density profile plots of stained SDS-PAGE gels determined that iron limitation resulted in an approximately 10-fold increase in Bsp22 protein levels in RB50 culture supernatant fluids when normalized to cell density (data not shown). Iron-regulated protein band 4 was identified as

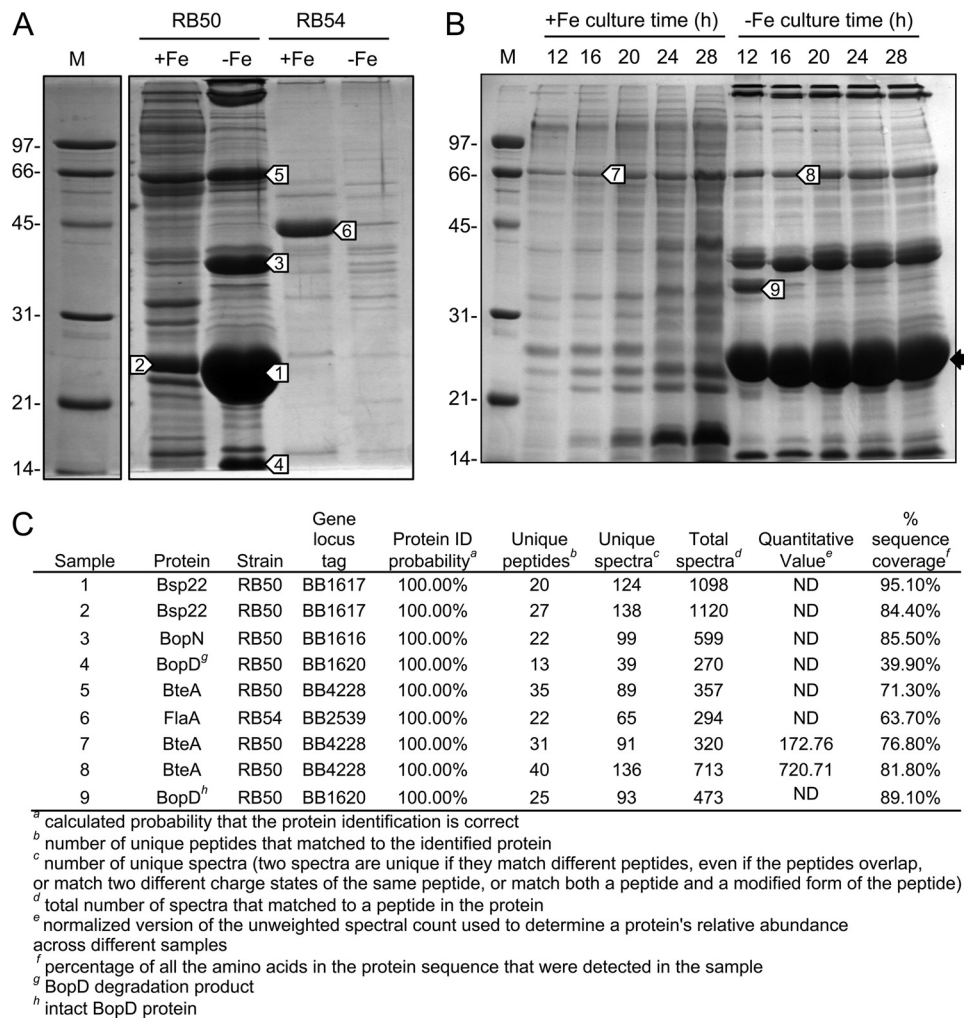


FIG. 6. Secretion profiles of *B. bronchiseptica* strain RB50 and isogenic Bvg^- phenotypic phase-locked derivative RB54. Proteins were recovered from supernatant fluids of *B. bronchiseptica* strains cultured for 16 h in iron-replete (+Fe) or iron-depleted (-Fe) SS medium and separated by SDS-PAGE. Sizes of molecular mass markers (M) are given in kDa. (A) Secreted protein profiles of RB50 and RB54. Normalized sample loads represent proteins recovered from 1.2 ml of supernatant fluid (cell densities $[\text{OD}_{600}]$ of cultures: RB50 +Fe, 5.52; RB50 -Fe, 2.80; RB54 +Fe, 8.89; RB54 -Fe, 2.18). Bands (white arrows numbered 1 to 6) were excised from replicate gels for identification by LC-MS/MS. (B) Secreted proteins from RB50 cultures used as RNA sources in microarray analysis. The black arrow indicates the Bsp22 protein band in -Fe samples. Normalized sample loads represent proteins recovered from 1 ml of supernatant fluid. Bands (white arrows numbered 7 to 9) were excised from replicate gels for identification by LC-MS/MS. (C) Protein identities as determined by LC-MS/MS. Relative abundance of BteA (also known as BopC) in 16-h +Fe and -Fe sample bands was estimated by spectral counting (quantitative value). ND, not determined.

a degradation product of BopD (31.8 kDa intact; BB1620), as previously reported by Kuwae and coworkers (47). Band 5 was found to contain the T3SS effector protein BteA (BB4228) along with pertactin (both ca. 69 kDa in monomeric form). BteA is also thought to form large homomultimeric protein complexes or aggregates of greater than 200-kDa molecular mass (37, 47, 65). The extracellular protein (band 6) produced by the Δbvg mutant strain RB54 under iron-replete growth conditions was identified as flagellin (FlaA, 40.4 kDa; BB2539). FlaA levels in RB54 culture supernatant fluids were dramatically reduced under iron starvation growth conditions.

***B. bronchiseptica* shows elevated hemolytic activity during iron starvation.** *B. bronchiseptica* Bsp22 is required for T3SS cytotoxicity and hemolytic activity that was previously shown by mutational analysis to be independent from that of adenylate

cyclase (54). Medhekar and coworkers (54) showed that deletion of the *cyxA* adenylate cyclase toxin gene had no effect on hemolytic activity of *B. bronchiseptica* RB50 in this assay, whereas a *cyxA bcsN* double mutant, also lacking the T3SS ATPase activity, showed no hemolytic activity. Consistent with the elevated production of T3SS proteins during iron starvation, iron-starved RB50 cultures showed increased hemolytic activity against rabbit RBCs *in vitro* by the assay method of Medhekar and coworkers (54), compared with bacteria from iron-replete cultures (Fig. 7). The maximal hemolytic activity of iron-starved RB50 bacteria was 81% at a bacterial cell:RBC ratio of approximately 14:1, relative to that of water-lysed RBCs (100%, positive control). In contrast, the hemolytic activity of *B. bronchiseptica* cultured under iron-replete conditions was 26% at the same bacterial cell:RBC ratio.

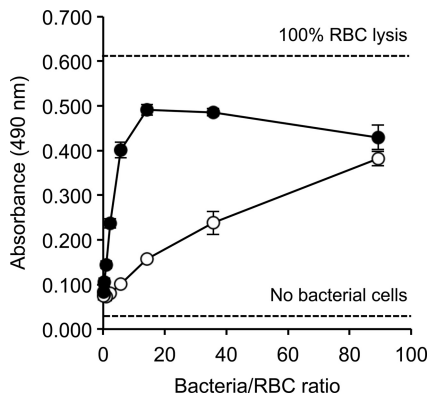


FIG. 7. Effect of iron starvation on T3SS-dependent hemolytic activity of *B. bronchiseptica* RB50. Dilutions of normalized bacterial suspensions prepared from iron-replete (open circles) and iron-depleted (filled circles) SS cultures were incubated with rabbit RBC, and liberated hemoglobin was measured by absorbance at a wavelength of 490 nm. Mean absorbance values ($n = 3$, ± 1 SD) are shown. Dashed lines show control absorbance values corresponding to hemoglobin released in the absence of bacteria (no bacterial cells) and by hypotonic lysis of rabbit RBC using water (100% RBC lysis).

***B. bronchiseptica* iron limitation is associated with increased T3SS gene expression.** A limited analysis of iron-regulated T3SS gene expression patterns in *B. bronchiseptica* RB50 was performed using microarrays. Multiple RNA sample sets were generated from RB50 cultured in parallel in iron-replete and iron-depleted SS media at various times of the growth cycle (Fig. 1). As cells were collected for RNA isolation, secreted proteins were recovered from supernatant fluids and analyzed by SDS-PAGE (Fig. 6B). High-level production of Bsp22 by iron-starved RB50 was clearly evident by 8 h to 12 h of culturing (during early to mid-exponential growth phase) and thereafter up to 28 h (stationary growth phase), whereas Bsp22 production by iron-replete bacteria was comparatively low. In addition, a protein band (Fig. 6B, band 9) that had not been observed previously in this study was evident only in the 12-h sample (mid-exponential growth phase) from the iron-depleted culture. LC-MS/MS identified that protein as the intact BopD protein (31.8 kDa; BB1620) (Fig. 6C). The BopD protein complexes with BopB to form the pore in the host plasma membrane for translocation of T3SS effectors. Protein bands predicted to contain BteA were excised from the 16 h iron-replete and iron-depleted sample lanes (Fig. 6B, bands 7 and 8) and analyzed by LC-MS/MS. Estimates of BteA production levels based on spectral counting indicated that the specific level of BteA was more than 4-fold greater in the iron-depleted culture sample than in the iron-replete culture sample (Fig. 6C). Since protein gel loads had been normalized to culture volume and not culture cell density (OD_{600} at 16 h: iron replete, 3.94; iron depleted, 2.67), BteA production by iron-starved *B. bronchiseptica* on a per-cell basis is estimated to be more than 6-fold higher at 16 h than that by iron-replete cells.

B. bronchiseptica RB50 microarray expression analysis showed increased transcript abundance of T3SS genes similar to results obtained for *B. pertussis* (see Fig. S2 in the supplemental material). Expression of T3SS genes was strongly up-regulated during iron starvation, with almost every gene in the T3SS cluster showing a large increase in transcript abundance

over iron-replete levels. These findings are consistent with the proteomics-based evidence for elevated production of T3SS proteins by *B. bronchiseptica* under iron starvation conditions, and confirm that iron regulation of *B. bronchiseptica* T3SS gene expression occurs at the transcriptional level. Furthermore, the T3SS effector protein gene *bteA* (47, 65) and the *vag8* gene BB1864, which encodes a T3SS accessory factor (53), were found to be coordinately up-regulated with the RB50 T3SS locus. To corroborate the *vag8* transcription results, Vag8 protein levels in both *B. bronchiseptica* and *B. pertussis* were examined by immunoblot analysis using Vag8-specific mouse monoclonal antibody 8E7 (see Fig. S3 in the supplemental material). Although Vag8 was detectable in the *B. pertussis* Tohama I samples, there was no significant effect of iron starvation on Vag8 production; similar results were obtained using *B. pertussis* strain UT25 (data not shown). In contrast, Vag8 production by *B. bronchiseptica* RB50 was estimated to be at least 3-fold higher in iron-starved cells than in cells from iron-replete cultures. As predicted, Vag8 protein production by the Bvg^- strain RB54 was not detected in cells regardless of iron status.

The mechanistic basis of the association between iron starvation stress and upregulation of T3SS gene expression in *B. pertussis* and *B. bronchiseptica* is unknown. A bioinformatics-based approach was used to identify potential Fur repressor binding sites within the T3SS *bsc* and *btr* gene clusters that might account for the apparent iron regulation of T3SS genes, but no predicted strong Fur binding sites could be identified in the *bsp22-bopN* intergenic region or upstream of the *bcr* and *btr* loci. Four potential weak overlapping Fur binding sites (57.9 to 62.5% similarity with the *E. coli* consensus Fur binding site, 5'-GATAATGATAATCATTATC-3') were identified in the DNA region located 20 to 61 nucleotides (nt) upstream of *btrS* (BP2234/BB1638), which encodes an extracytoplasmic function sigma factor required for transcription of the *bsc* T3SS genes (5'-AAACCGGCAGTCAGTATCTTGGTGACGTGAATTATCACC-3'). This *btrS* upstream DNA region (identical in *B. pertussis* and *B. bronchiseptica*) was tested for Fur binding activity using a functional Fur repressor titration assay (82), but it had no significant Fur binding activity in this assay (data not shown).

***B. pertussis* clinical and laboratory-adapted strains show increased production of T3SS proteins during iron limitation.** It was reported that *B. pertussis* strain Tohama I does not produce detectable Bsp22 or other T3SS proteins (33, 53). In a later study, Bsp22 was identified in low-passage clinical *B. pertussis* isolates but not the laboratory-adapted Tohama I strain (34). Importantly, none of the studies to date has examined the production of *B. pertussis* T3SS proteins during iron starvation. Since the current microarray study found that T3SS gene transcription was up-regulated during iron starvation in both *B. pertussis* Tohama I and *B. bronchiseptica* RB50, it was hypothesized that secreted T3SS proteins might be detected in iron-starved Tohama I cultures. In initial studies, neither *B. pertussis* Tohama I nor the low-passage strain UT25 produced T3SS proteins by 16 to 18 h of growth in iron-replete or iron-depleted SS medium (data not shown). However, subsequent cultures showed that by 24 h *B. pertussis* UT25 produced multiple iron-regulated proteins with electrophoretic mobilities similar to those of the RB50 T3SS proteins. Tohama I also

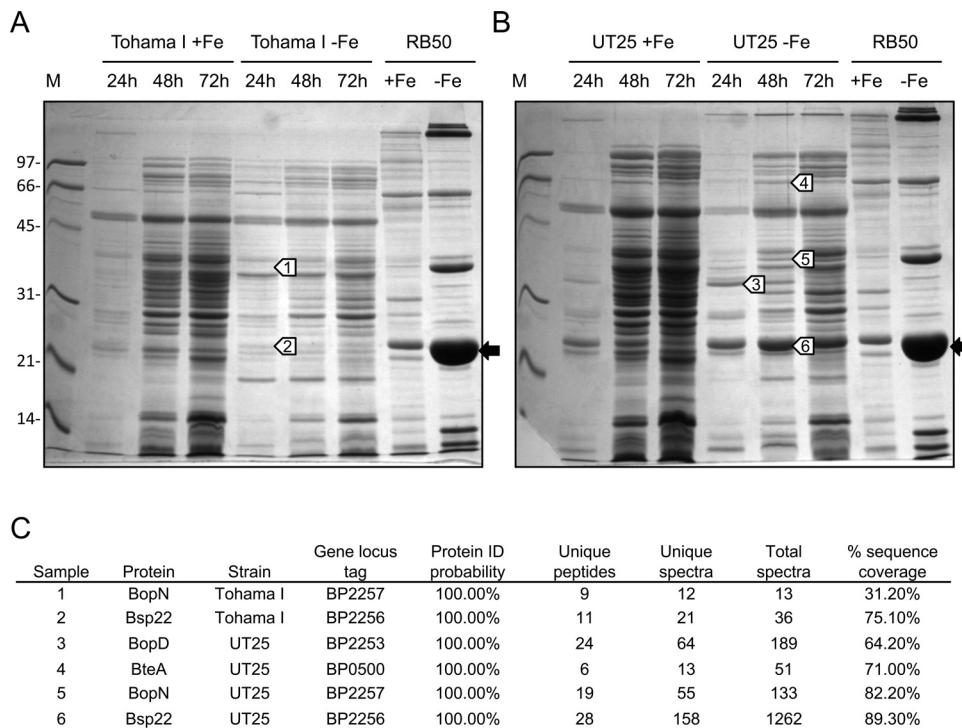


FIG. 8. Secretion profiles of *B. pertussis* strains Tohama I (A) and UT25 (B) cultured in iron-replete (+Fe) or iron-depleted (-Fe) SS medium. Supernatant fluids were collected at various culture times and the secreted proteins were recovered as described in Materials and Methods. Coomassie blue-stained 12% polyacrylamide gels are shown, with sizes of molecular mass (M) markers given in kDa. Sample loads were normalized to culture cell density. *B. bronchiseptica* RB50 secreted proteins are shown for comparison. The prominent bands (black arrows) in the RB50 -Fe lanes are Bsp22. Bands (white arrows numbered 1 to 6) were excised from replicate gels and subjected to LC-MS/MS analysis. (C) Protein identities as determined by LC-MS/MS.

appeared to produce at least two putative T3SS proteins during iron starvation, including the major protein Bsp22, albeit at levels much lower than those of UT25 or RB50 (Fig. 8A and B). Putative *B. pertussis* T3SS proteins (Fig. 8A and B, bands 1 to 6) were excised from gels and analyzed by LC-MS/MS. UT25 protein bands were confirmed to be Bsp22, BopN, BopD, and BteA (Fig. 8C). Tohama I protein bands were identified as the major T3SS proteins Bsp22 and BopN. Despite low-level production of Bsp22 and BopN by Tohama I, protein identities could be assigned with the highest degree of statistical confidence (Fig. 8C). For all *B. pertussis* proteins that were identified by LC-MS/MS, spectra included ion fragments unique to *B. pertussis* T3SS proteins compared with their *B. bronchiseptica* orthologs (data not shown).

Since the low-passage isolate UT25 produced T3SS proteins at higher levels than the relatively high-passage Tohama I strain, a limited survey of various *B. pertussis* strains (Table 1) was performed to assess whether high-level T3SS protein production was a trait common only to low-passage isolates and whether T3SS protein production was increased by iron starvation in other *B. pertussis* strains. Strains were cultured in iron-replete and iron-depleted SS media for 46 h; all strains grew to similar levels and produced high levels of alcaligin in iron-depleted SS medium (data not shown). Using Bsp22 as a hallmark of T3SS production, all *B. pertussis* strains examined except for the avirulent, laboratory-adapted derivative UT25-90 produced T3SS proteins, albeit at an apparent level

lower than that of *B. bronchiseptica* RB50 (Fig. 9). There was no strict correlation between T3SS protein production level and the extent of laboratory passage. Of the strains examined, strains 3779 (high passage) and UT25 (low passage) appeared to produce the highest levels of Bsp22, whereas Tohama I produced the lowest level. All *B. pertussis* strains that produced Bsp22 appeared to produce elevated levels of it under iron-limited growth conditions.

DISCUSSION

The natural host environment of *B. pertussis* is an exquisitely iron-depleted one in which homeostatic mechanisms severely limit the amount of extracellular iron available to microbes (38, 41, 62). Expression of the *Bordetella* alcaligin, enterobactin, and heme receptor genes in infected mice and production of those receptors in human hosts indicate a natural state of iron starvation stress *in vivo* (21). In the current study, *B. pertussis* genes encoding the anticipated alcaligin biosynthesis and heme uptake functions, the BfeR regulator of the *bfeA* enterobactin receptor gene, TonB system genes, and genes encoding TonB-dependent receptors of unknown specificity showed increased transcript abundance under iron starvation conditions.

In previous *Bordetella* studies, increased transcript abundance for *alcABC* and *hurI* was associated with glutamate starvation (58). Nakamura and colleagues (58) also detected

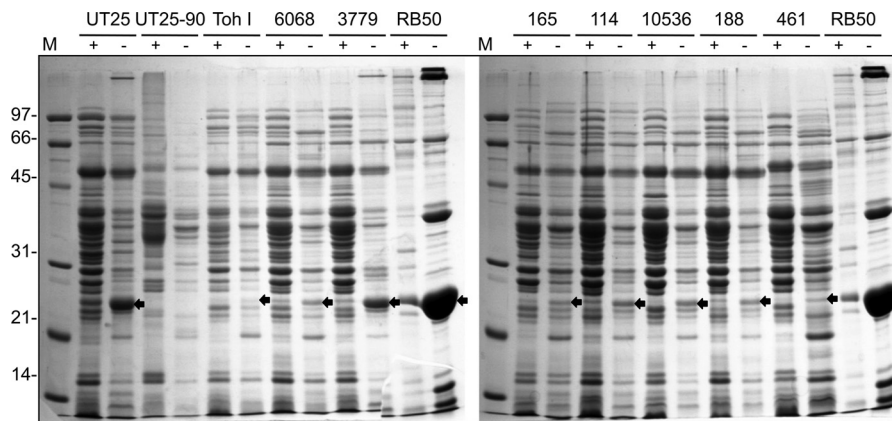


FIG. 9. Secretion profiles of various *B. pertussis* strains. Low-passage and high-passage strains of *B. pertussis* (Table 1) were cultured for 46 h in iron-replete (+) or iron-depleted (–) SS medium. Secreted proteins were recovered as described in Materials and Methods and analyzed using SDS-PAGE. Molecular mass (M) markers are shown in kDa. The black arrows indicate the positions of Bsp22. *B. bronchiseptica* RB50 secreted proteins are shown for comparison. Sample loads were normalized to culture cell density and represent proteins recovered from a volume of supernatant fluid originating from 1.0 OD₆₀₀ of bacterial cells.

increased transcript abundance for other iron-related genes (including *bfrB*, *bfrC*, *bfrH*, *bhuR*, *hurIR*, and *alcABDE*) upon transition from logarithmic to stationary growth phase, when the bacteria begin to respond to nutrient scarcity. Results from the current study using specific iron-depleted growth conditions indicate that *B. pertussis* responds to iron starvation stress in a manner distinct from that of cells transitioning to stationary growth. Production of the *Bordetella* TonB-dependent receptor BfrD is both Bvg activated and iron repressed (7, 67). Others have reported genes encoding several TonB-dependent receptors of unknown ligand specificity as variably Bvg activated or Bvg repressed (29, 61). The significance of the differential expression of those genes in Bvg-related array studies is uncertain, since the cultures were not specifically starved for iron to allow Fur derepression. Absolute transcript abundance would likely be minimal under standard, iron-replete growth conditions regardless of Bvg phenotypic phase, and only relative expression levels were determined in those studies.

Genes with elevated transcript abundance during iron-replete, versus iron-restricted, growth included those involved in energy production, ribosome structure, iron storage, and oxidative stress. Iron response array studies using bacteria such as *Campylobacter jejuni* (42), *Burkholderia* (84) and *Neisseria* (31, 39) spp., *Helicobacter pylori* (56), and *Pasteurella multocida* (68) have shown similar expression patterns, with genes such as those encoding ferritin, NADH dehydrogenase, cytochromes *c*, ribosomal proteins, and oxidative stress functions identified as having increased expression under iron-replete, versus depleted, growth conditions. Typically, many of these genes encode proteins with iron or heme cofactors; thus, their reduced expression during iron starvation may in part represent an iron conservation strategy.

The present study is, to our knowledge, the first global analysis of *Bordetella* gene transcription in response to iron starvation. A 2007 analysis of *B. pertussis* proteins produced during iron-replete versus iron-depleted conditions (90) identified some of the same iron-repressed proteins specified by genes

found in our analysis: the BhuS heme utilization protein, the BfrB TonB-dependent receptor protein, AlcC alcaligin biosynthesis enzyme, FumC fumarate hydratase, and the BP1605 product that we describe herein as FbpA. Those investigators noted similarity of the BP1605 protein to FbpA family members and of the predicted products of BP1604 and BP1603 to the permease and ATPase components of an ABC transporter, respectively.

The *Bordetella* outer membrane alcaligin and enterobactin receptor genes are located within their cognate system gene clusters (3, 4, 14, 19). However, there are no genes in either cluster that are predicted to encode a cytoplasmic membrane transport apparatus, and none could be reliably predicted from the genome sequences. The present study identified *fbpA*, encoding a member of the family of periplasmic ferric iron-binding proteins. *Bordetella* FbpA is required for optimal utilization of inorganic iron. In addition, *B. pertussis* and *B. bronchiseptica* *fbpA* mutants were unable to utilize not only the alcaligin siderophore but also the xenosiderophores enterobactin, ferrichrome, and desferrioxamine B. Therefore, FbpA, and likely FbpB and FbpC, appear to constitute a binding protein-dependent ABC transporter for the uptake of iron. Since the use of these structurally diverse siderophores requires the Fbp system, it is likely that the iron is removed from the siderophores after transit to the periplasm. The *Bordetella* Fbp system may represent a critical nodal point in iron assimilation in these pathogens, providing an activity essential for iron delivery via siderophores including alcaligin and enterobactin, which are known to be important for multiplication *in vivo* (20, 21).

The *B. pertussis* T3SS genes specifying BcrH2, BopB, Bsp22, and BopN were also upregulated upon iron starvation. Comparative analyses showed that *B. bronchiseptica* T3SS proteins were produced and secreted at increased levels under iron starvation growth conditions; Bsp22 and BopN in particular were secreted in remarkable abundance. *B. bronchiseptica* gene expression profiling also indicated increased transcription of a number of T3SS-related genes. Included in this group were *vag8*, a Bvg-activated gene encoding an outer membrane pro-

tein implicated in secretion of type III substrates, and the T3SS effector gene *bteA*. Examination of secreted proteins from iron-replete versus iron-starved *B. pertussis* Tohama I confirmed the microarray results, revealing increased production of Bsp22 and BopN. In *B. pertussis*, *vag8* was not iron regulated, nor were *Vag8* production levels influenced by the iron status; however, in *B. bronchiseptica*, *Vag8* production was enhanced during growth under iron-limited conditions.

A survey of *B. pertussis* strains revealed that they all produced and secreted the T3SS protein Bsp22 and perhaps others, and this production was enhanced by iron limitation. Some strains, including Tohama I, secreted modest levels of T3SS proteins and others secreted significantly higher levels; none of the *B. pertussis* strains produced the high Bsp22 levels observed in *B. bronchiseptica* RB50. It is possible that T3SS expression in *B. pertussis* is a strain- or lineage-variable trait, as has been proposed for *B. bronchiseptica* (26). No obvious correlation was observed between highly passaged laboratory-adapted *B. pertussis* isolates versus those that have been minimally passaged.

Another unusual observation was that significant levels of the FlaA flagellin subunit were found in the spent culture supernatant of the *B. bronchiseptica* RB54 Δ *bvgS* mutant grown under iron-replete, but not iron-depleted, conditions. Neither the *flaA* gene of *B. pertussis* nor that of *B. bronchiseptica* RB50 was transcriptionally upregulated specifically under iron-replete growth conditions. The RB54 FlaA result may represent an artifact of growth under high-iron conditions that are not encountered *in vivo* or be related to the *Bvg*⁻ status of the mutant that leads to constitutive production of flagellin, the excess of which is found in the culture supernatant. The potential contribution of post-transcriptional regulation has not been examined.

Iron repression of the *Bordetella* T3SS has not been described previously, and the biological significance and mechanisms of iron regulation of the system are unknown; however, iron starvation does not appear to be associated with increased expression of all *Bvg*-dependent virulence genes. *Bordetella* type III proteins are required for a hemolytic activity that is distinct from that caused by the adenylate cyclase toxin (48, 54, 64), and the present study showed that hemolytic activity was increased in iron-starved *Bordetella* cells. It is possible that the T3SS plays a role in iron acquisition by making intracellular heme or iron available, necessitating iron-mediated control of the system. Alternatively, iron starvation may be an important host environmental cue affecting expression of the *Bordetella* T3SS. These observations illuminate a potentially important intersection of the *Bvg* virulence regulon and the iron starvation response. This response allows *Bordetella* cells to perceive a primary nutritional stress signal that not only triggers mechanisms to obtain essential iron but also informs the organism that it is in a host, whereupon appropriate bacterial virulence factors may be deployed.

ACKNOWLEDGMENTS

Support for this study was provided by Public Health Service grants AI057188 and AI54970 (to D.A.R.) and AI31088 (to S.K.A.) from the National Institute of Allergy and Infectious Diseases.

We thank Scott Stibitz and Noel Keen for plasmids and Peggy Cotter, Tod Merkel, Jeff F. Miller, and Charlotte Parker for *Bordetella*

strains. We also thank Bruce Witthuhn for help with analysis of mass spectrometry data.

REFERENCES

1. Abe, A., K. Nagamatsu, and M. Watanabe. 2008. The *Bordetella* type III secretion system: its application to vaccine development. *Microbiol. Immunol.* **52**:128–133.
2. Agiato, L. A., and D. W. Dyer. 1992. Siderophore production and membrane alterations by *Bordetella pertussis* in response to iron starvation. *Infect. Immun.* **60**:117–123.
3. Anderson, M. T., and S. K. Armstrong. 2004. The BfeR regulator mediates enterobactin-inducible expression of *Bordetella* enterobactin utilization genes. *J. Bacteriol.* **186**:7302–7311.
4. Anderson, M. T., and S. K. Armstrong. 2006. The *Bordetella bfe* system: growth and transcriptional response to siderophores, catechols, and neuroendocrine catecholamines. *J. Bacteriol.* **188**:5731–5740.
5. Anderson, M. T., and S. K. Armstrong. 2008. Norepinephrine mediates acquisition of transferrin-iron in *Bordetella bronchiseptica*. *J. Bacteriol.* **190**:3940–3947.
6. Angerer, A., G. Gaisser, and V. Braun. 1990. Nucleotide sequences of the *sfuA*, *sfuB*, and *sfuC* genes of *Serratia marcescens* suggest a periplasmic-binding-protein-dependent iron transport mechanism. *J. Bacteriol.* **172**:572–578.
7. Antoine, R., et al. 2000. New virulence-activated and virulence-repressed genes identified by systematic gene inactivation and generation of transcriptional fusions in *Bordetella pertussis*. *J. Bacteriol.* **182**:5902–5905.
8. Arico, B., et al. 1989. Sequences required for expression of *Bordetella pertussis* virulence factors share homology with prokaryotic signal transduction proteins. *Proc. Natl. Acad. Sci. U. S. A.* **86**:6671–6675.
9. Armstrong, S. K., and M. O. Clements. 1993. Isolation and characterization of *Bordetella bronchiseptica* mutants deficient in siderophore activity. *J. Bacteriol.* **175**:1144–1152.
10. Armstrong, S. K., and C. D. Parker. 1986. Heat-modifiable envelope proteins of *Bordetella pertussis*. *Infect. Immun.* **54**:109–117.
11. Bagg, A., and J. B. Neilands. 1987. Ferric uptake regulation protein acts as a repressor, employing iron (II) as a cofactor to bind the operator of an iron transport operon in *Escherichia coli*. *Biochemistry* **26**:5471–5477.
12. Baker, S. M., A. Masi, D.-F. Liu, B. K. Novitsky, and R. A. Deich. 1995. Pertussis toxin export genes are regulated by the *ptx* promoter and may be required for efficient translation of *ptx* mRNA in *Bordetella pertussis*. *Infect. Immun.* **63**:3920–3926.
13. Beall, B., and T. Hoenes. 1997. An iron-regulated outer-membrane protein specific to *Bordetella bronchiseptica* and homologous to ferric siderophore receptors. *Microbiology* **143**:135–145.
14. Beall, B., and G. N. Sanden. 1995. A *Bordetella pertussis* *fepA* homologue required for utilization of exogenous ferric enterobactin. *Microbiology* **141**:3193–3205.
15. Beaumont, F. C., H. Y. Kang, T. J. Brickman, and S. K. Armstrong. 1998. Identification and characterization of *alcR*, a gene encoding an AraC-like regulator of alcaligin siderophore biosynthesis and transport in *Bordetella pertussis* and *Bordetella bronchiseptica*. *J. Bacteriol.* **180**:862–870.
16. Brickman, T. J., and S. K. Armstrong. 1995. *Bordetella pertussis* *fur* gene restores iron repressibility of siderophore and protein expression to deregulated *Bordetella bronchiseptica* mutants. *J. Bacteriol.* **177**:268–270.
17. Brickman, T. J., and S. K. Armstrong. 1996. The ornithine decarboxylase gene *odc* is required for alcaligin siderophore biosynthesis in *Bordetella* spp.: putrescine is a precursor of alcaligin. *J. Bacteriol.* **178**:54–60.
18. Brickman, T. J., and S. K. Armstrong. 1996. Colicins B and Ia as novel counterselective agents in interspecies conjugal DNA transfers from colicin-sensitive *Escherichia coli* donors to other gram-negative recipient species. *Gene* **178**:39–42.
19. Brickman, T. J., and S. K. Armstrong. 1999. Essential role of the iron-regulated outer membrane receptor FauA in alcaligin siderophore-mediated iron uptake in *Bordetella* species. *J. Bacteriol.* **181**:5958–5966.
20. Brickman, T. J., and S. K. Armstrong. 2007. Impact of alcaligin siderophore utilization on *in vivo* growth of *Bordetella pertussis*. *Infect. Immun.* **75**:5305–5312.
21. Brickman, T. J., T. Hanawa, M. T. Anderson, R. J. Suhadolc, and S. K. Armstrong. 2008. Differential expression of *Bordetella pertussis* iron transport system genes during infection. *Mol. Microbiol.* **70**:3–14.
22. Brickman, T. J., J. G. Hansel, M. J. Miller, and S. K. Armstrong. 1996. Purification, spectroscopic analysis and biological activity of the macrocyclic dihydroxamate siderophore alcaligin produced by *Bordetella pertussis* and *Bordetella bronchiseptica*. *Biometals* **9**:191–203.
23. Brickman, T. J., H. Y. Kang, and S. K. Armstrong. 2001. Transcriptional activation of *Bordetella* alcaligin siderophore genes requires the AlcR regulator with alcaligin as inducer. *J. Bacteriol.* **183**:483–489.
24. Brickman, T. J., C. K. Vanderpool, and S. K. Armstrong. 2006. Heme transport contributes to *in vivo* fitness of *Bordetella pertussis* during primary infection in mice. *Infect. Immun.* **74**:1741–1744.
25. Brinig, M. M., et al. 2006. Significant gene order and expression differences

- in *Bordetella pertussis* despite limited gene content variation. *J. Bacteriol.* **188**:2375–2382.
26. **Buboltz, A. M., T. L. Nicholson, L. S. Weyrich, and E. T. Harvill.** 2009. Role of the type III secretion system in a hypervirulent lineage of *Bordetella bronchiseptica*. *Infect. Immun.* **77**:3969–3977.
 27. **Burgos, J. M., N. D. King-Lyons, and T. D. Connell.** 2010. Expression of BfrH, a putative siderophore receptor of *Bordetella bronchiseptica*, is regulated by iron, FurI, and the extracellular function sigma factor Ecfl. *Infect. Immun.* **78**:1147–1162.
 28. **Cotter, P. A., and J. F. Miller.** 1994. BvgAS-mediated signal transduction: analysis of phase-locked regulatory mutants of *Bordetella bronchiseptica* in a rabbit model. *Infect. Immun.* **62**:3381–3390.
 29. **Cummings, C. A., H. J. Bootsma, D. A. Relman, and J. F. Miller.** 2006. Species- and strain-specific control of a complex, flexible regulon by *Bordetella* BvgAS. *J. Bacteriol.* **188**:1775–1785.
 30. **Dhungana, S., D. S. Anderson, T. A. Mietzner, and A. L. Crumbliss.** 2005. Kinetics of iron release from ferric binding protein (FbpA): mechanistic implications in bacterial periplasm-to-cytosol Fe³⁺ transport. *Biochemistry* **44**:9606–9618.
 31. **Ducey, T. F., M. B. Carson, J. Orvis, A. P. Stintzi, and D. W. Dyer.** 2005. Identification of the iron-responsive genes of *Neisseria gonorrhoeae* by microarray analysis in defined medium. *J. Bacteriol.* **187**:4865–4874.
 32. **Eisen, M. B., P. T. Spellman, P. O. Brown, and D. Botstein.** 1998. Cluster analysis and display of genome-wide expression patterns. *Proc. Natl. Acad. Sci. U. S. A.* **95**:14863–14868.
 33. **Fauconnier, A., et al.** 2001. Characterization of the type III secretion locus of *Bordetella pertussis*. *Int. J. Med. Microbiol.* **290**:693–705.
 34. **Fennelly, N. K., et al.** 2008. *Bordetella pertussis* expresses a functional type III secretion system that subverts protective innate and adaptive immune responses. *Infect. Immun.* **76**:1257–1266.
 35. **Finn, T. M., and D. F. Amsbaugh.** 1998. Vag8, a *Bordetella pertussis* bvg-regulated protein. *Infect. Immun.* **66**:3985–3989.
 36. **Frank, D. W., and C. D. Parker.** 1984. Isolation and characterization of monoclonal antibodies to *Bordetella pertussis*. *J. Biol. Stand.* **12**:353–365.
 37. **French, C. T., et al.** 2009. The *Bordetella* type III secretion system effector BteA contains a conserved N-terminal motif that guides bacterial virulence factors to lipid rafts. *Cell. Microbiol.* **11**:1735–1749.
 38. **Ganz, T.** 2009. Iron in innate immunity: starve the invaders. *Curr. Opin. Immunol.* **21**:63–67.
 39. **Grifantini, R., et al.** 2003. Identification of iron-activated and -repressed Fur-dependent genes by transcriptome analysis of *Neisseria meningitidis* group B. *Proc. Natl. Acad. Sci. U. S. A.* **100**:9542–9547.
 40. **Hantke, K.** 1981. Regulation of ferric iron transport in *Escherichia coli* K12: isolation of a constitutive mutant. *Mol. Gen. Genet.* **182**:288–292.
 41. **Hentze, M. W., M. U. Muckenthaler, and N. C. Andrews.** 2004. Balancing acts: molecular control of mammalian iron metabolism. *Cell* **117**:285–297.
 42. **Holmes, K., et al.** 2005. *Campylobacter jejuni* gene expression in response to iron limitation and the role of Fur. *Microbiology* **151**:243–257.
 43. **Keller, A., A. I. Nesvizhskii, E. Kolker, and R. Aebersold.** 2002. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal. Chem.* **74**:5383–5392.
 44. **Khan, A. G., et al.** 2007. High-affinity binding by the periplasmic iron-binding protein from *Haemophilus influenzae* is required for acquiring iron from transferrin. *Biochem. J.* **404**:217–225.
 45. **Kotob, S. I., S. Z. Hausman, and D. L. Burns.** 1995. Localization of the promoter for the *pil* genes of *Bordetella pertussis*, which encode proteins essential for secretion of pertussis toxin. *Infect. Immun.* **63**:3227–3230.
 46. **Kozak, N. A., E. M. Panina, and J. F. Miller.** 2007. Type III secretion in *Bordetella* subspecies, p. 119–139. In C. Locht (ed.), *Bordetella* molecular microbiology. Horizon Bioscience, Norfolk, United Kingdom.
 47. **Kuwaie, A., et al.** 2006. BopC is a novel type III effector secreted by *Bordetella bronchiseptica* and has a critical role in type III-dependent necrotic cell death. *J. Biol. Chem.* **281**:6589–6600.
 48. **Kuwaie, A., M. Ohishi, M. Watanabe, M. Nagai, and A. Abe.** 2003. BopB is a type III secreted protein in *Bordetella bronchiseptica* and is required for cytotoxicity against cultured mammalian cells. *Cell. Microbiol.* **5**:973–983.
 49. **Litvin, C. M., and S. B. Calderwood.** 1993. Role of iron in regulation of virulence genes. *Clin. Microbiol. Rev.* **6**:137–149.
 50. **Locht, C., M. C. Geoffroy, and G. Renaud.** 1992. Common accessory genes for the *Bordetella pertussis* filamentous hemagglutinin and fimbriae share sequence similarities with the *papC* and *papD* gene families. *EMBO J.* **11**:3175–3183.
 51. **Mahan, M. J., D. M. Heithoff, R. L. Sinsheimer, and D. A. Low.** 2000. Assessment of bacterial pathogenesis by analysis of gene expression in the host. *Annu. Rev. Genet.* **34**:139–164.
 52. **Mattoo, S., and J. D. Cherry.** 2005. Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to *Bordetella pertussis* and other *Bordetella* subspecies. *Clin. Microbiol. Rev.* **18**:326–382.
 53. **Mattoo, S., M. H. Yuk, L. L. Huang, and J. F. Miller.** 2004. Regulation of type III secretion in *Bordetella*. *Mol. Microbiol.* **52**:1201–1214.
 54. **Medhekar, B., R. Shrivastava, S. Mattoo, M. Gingery, and J. F. Miller.** 2009. *Bordetella* Bsp22 forms a filamentous type III secretion system tip complex and is immunoprotective *in vitro* and *in vivo*. *Mol. Microbiol.* **71**:492–504.
 55. **Mekalanos, J. J.** 1992. Environmental signals controlling expression of virulence determinants in bacteria. *J. Bacteriol.* **174**:1–7.
 56. **Merrell, D. S., et al.** 2003. Growth phase-dependent response of *Helicobacter pylori* to iron starvation. *Infect. Immun.* **71**:6510–6525.
 57. **Moore, C. H., L. A. Foster, D. G. Gerbig, Jr., D. W. Dyer, and B. W. Gibson.** 1995. Identification of alcaligin as the siderophore produced by *Bordetella pertussis* and *B. bronchiseptica*. *J. Bacteriol.* **177**:1116–1118.
 58. **Nakamura, M. M., et al.** 2006. Growth phase- and nutrient limitation-associated transcript abundance regulation in *Bordetella pertussis*. *Infect. Immun.* **74**:5537–5548.
 59. **Neilands, J. B.** 1995. Siderophores: structure and function of microbial iron transport compounds. *J. Biol. Chem.* **270**:26723–26726.
 60. **Nesvizhskii, A. I., A. Keller, E. Kolker, and R. Aebersold.** 2003. A statistical model for identifying proteins by tandem mass spectrometry. *Anal. Chem.* **75**:4646–4658.
 61. **Nicholson, T. L.** 2007. Construction and validation of a first-generation *Bordetella bronchiseptica* long-oligonucleotide microarray by transcriptional profiling the Bvg regulon. *BMC Genomics* **8**:220.
 62. **Nicolas, G., et al.** 2001. Lack of hepcidin gene expression and severe tissue iron overload in upstream stimulatory factor 2 (USF2) knockout mice. *Proc. Natl. Acad. Sci. U. S. A.* **98**:8780–8785.
 63. **Nishio, T., et al.** 1988. Isolation and structure of the novel dihydroxamate siderophore alcaligin. *J. Am. Chem. Soc.* **110**:8733–8734.
 64. **Nogawa, H., A. Kuwaie, T. Matsuzawa, and A. Abe.** 2004. The type III secreted protein BopD in *Bordetella bronchiseptica* is complexed with BopB for pore formation on the host plasma membrane. *J. Bacteriol.* **186**:3806–3813.
 65. **Panina, E., et al.** 2005. A genome-wide screen identifies a *Bordetella* type III secretion effector and candidate effectors in other species. *Mol. Microbiol.* **58**:267–279.
 66. **Parkhill, J., et al.** 2003. Comparative analysis of the genome sequences of *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica*. *Nat. Genet.* **35**:32–40.
 67. **Passerini de Rossi, B. N., et al.** 2003. Vir90, a virulence-activated gene coding for a *Bordetella pertussis* iron-regulated outer membrane protein. *Res. Microbiol.* **154**:443–450.
 68. **Paustian, M. L., B. J. May, and V. Kapur.** 2001. *Pasteurella multocida* gene expression in response to iron limitation. *Infect. Immun.* **69**:4109–4115.
 69. **Posfai, G., V. Kolisnychenko, Z. Bereckzki, and F. R. Blattner.** 1999. Markerless gene replacement in *Escherichia coli* stimulated by a double-strand break in the chromosome. *Nucleic Acids Res.* **27**:4409–4415.
 70. **Pradel, E., N. Guiso, F. D. Menozzi, and C. Locht.** 2000. *Bordetella pertussis* TonB, a Bvg-independent virulence determinant. *Infect. Immun.* **68**:1919–1927.
 71. **Pradel, E., and C. Locht.** 2001. Expression of the putative siderophore receptor gene *bfrZ* is controlled by the extracytoplasmic-function sigma factor BupI in *Bordetella bronchiseptica*. *J. Bacteriol.* **183**:2910–2917.
 72. **Ratledge, C., and L. G. Dover.** 2000. Iron metabolism in pathogenic bacteria. *Annu. Rev. Microbiol.* **54**:881–941.
 73. **Register, K. B., T. F. Ducey, S. L. Brockmeier, and D. W. Dyer.** 2001. Reduced virulence of a *Bordetella bronchiseptica* siderophore mutant in neonatal swine. *Infect. Immun.* **69**:2137–2143.
 74. **Rutherford, K., et al.** 2000. Artemis: sequence visualization and annotation. *Bioinformatics* **16**:944–945.
 75. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
 76. **Schneider, D. R., and C. D. Parker.** 1982. Effect of pyridines on phenotypic properties of *Bordetella pertussis*. *Infect. Immun.* **38**:548–553.
 77. **Schwyn, B., and J. B. Neilands.** 1987. Universal chemical-assay for the detection and determination of siderophores. *Anal. Biochem.* **160**:47–56.
 78. **Serres, M. H., and M. Riley.** 2000. MultiFun, a multifunctional classification scheme for *Escherichia coli* K-12 gene products. *Microb. Comp. Genom.* **5**:205–222.
 79. **Shouldice, S. R., et al.** 2003. High resolution structure of an alternate form of the ferric ion binding protein from *Haemophilus influenzae*. *J. Biol. Chem.* **278**:11513–11519.
 80. **Sterrenburg, E., R. Turk, J. M. Boer, G. B. van Ommen, and J. T. den Dunnen.** 2002. A common reference for cDNA microarray hybridizations. *Nucleic Acids Res.* **30**:e116.
 81. **Stibitz, S.** 1994. Mutations in the *bvgA* gene of *Bordetella pertussis* that differentially affect regulation of virulence determinants. *J. Bacteriol.* **176**:5615–5621.
 82. **Stojilkovic, I., A. J. Baumler, and K. Hantke.** 1994. Fur regulon in gram-negative bacteria. Identification and characterization of new iron-regulated *Escherichia coli* genes by a Fur titration assay. *J. Mol. Biol.* **236**:531–545.
 83. **Tom-Yew, S. A., D. T. Cui, E. G. Bekker, and M. E. Murphy.** 2005. Anion-independent iron coordination by the *Campylobacter jejuni* ferric binding protein. *J. Biol. Chem.* **280**:9283–9290.
 84. **Tuanyok, A., et al.** 2005. Genome-wide expression analysis of iron regulation

- in *Burkholderia pseudomallei* and *Burkholderia mallei* using DNA microarrays. FEMS Microbiol. Lett. **252**:327–335.
85. **Tusher, V. G., R. Tibshirani, and G. Chu.** 2001. Significance analysis of microarrays applied to the ionizing radiation response. Proc. Natl. Acad. Sci. U. S. A. **98**:5116–5121.
86. **Uhl, M. A., and J. F. Miller.** 1994. Autophosphorylation and phosphotransfer in the *Bordetella pertussis* BvgAS signal transduction cascade. Proc. Natl. Acad. Sci. U. S. A. **91**:1163–1167.
87. **Vanderpool, C. K., and S. K. Armstrong.** 2001. The *Bordetella bhv* locus is required for heme iron utilization. J. Bacteriol. **183**:4278–4287.
88. **Vanderpool, C. K., and S. K. Armstrong.** 2003. Heme-responsive transcriptional activation of *Bordetella bhv* genes. J. Bacteriol. **185**:909–917.
89. **Vidakovics, M. L., et al.** 2007. Iron stress increases *Bordetella pertussis* mucin-binding capacity and attachment to respiratory epithelial cells. FEMS Immunol. Med. Microbiol. **51**:414–421.
90. **Vidakovics, M. L., et al.** 2007. Profiling the *Bordetella pertussis* proteome during iron starvation. J. Proteome Res. **6**:2518–2528.
91. **Weiss, A. A., E. L. Hewlett, G. A. Myers, and S. Falkow.** 1983. Tn5-induced mutations affecting virulence factors of *Bordetella pertussis*. Infect. Immun. **42**:33–41.
92. **Yuk, M. H., E. T. Harvill, P. A. Cotter, and J. F. Miller.** 2000. Modulation of host immune responses, induction of apoptosis and inhibition of NF-kappaB activation by the *Bordetella* type III secretion system. Mol. Microbiol. **35**:991–1004.
93. **Yuk, M. H., E. T. Harvill, and J. F. Miller.** 1998. The BvgAS virulence control system regulates type III secretion in *Bordetella bronchiseptica*. Mol. Microbiol. **28**:945–959.