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Bordetella iron transport and virulence

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Abstract *Bordetella pertussis*, *Bordetella parapertussis*, and *Bordetella bronchiseptica* are pathogens with a complex iron starvation stress response important for adaptation to nutrient limitation and flux in the mammalian host environment. The iron starvation stress response is globally regulated by the Fur repressor using ferrous iron as the co-repressor. Expression of iron transport system genes of *Bordetella* is coordinated by priority regulation mechanisms that involve iron source sensing. Iron source sensing is mediated by distinct transcriptional activators that are responsive to the cognate iron source acting as the inducer.

Keywords *Bordetella* · Iron · Heme · Siderophore

Bordetella species and pathogenesis

Members of the *Bordetella* genus are β-proteobacteria of the family *Alcaligenaceae* and are grouped into eight currently recognized species. The three so-called classical species, *Bordetella pertussis*, *Bordetella parapertussis*, and *Bordetella bronchiseptica*, are highly genetically related respiratory pathogens of mammals (reviewed in Mattoo and Cherry 2005). *B. pertussis* is a strictly human-adapted species and is the etiological agent of whooping cough or pertussis. *B. parapertussis* isolates are divided into two distinct lineages: those that cause a pertussis-like illness in humans and those that infect ovine hosts. *B. bronchiseptica* causes respiratory diseases such as canine kennel cough and swine atrophic rhinitis, but these organisms are also capable of chronic asymptomatic infection of a broad range of mammalian hosts. Based on genomic sequence analysis, *B. bronchiseptica* or a *B. bronchiseptica*-like organism is hypothesized to be the progenitor of *B. pertussis* and *B. parapertussis*. Accordingly, the three classical *Bordetella* species are also referred to as strains of the *B. bronchiseptica* taxonomic cluster. The genome sequences of *B. pertussis* and, to a lesser extent, *B. parapertussis*, are characterized by marked gene loss and rearrangement compared to the genome of *B. bronchiseptica* (Parkhill et al. 2003). This genetic streamlining is likely a consequence of their evolution toward obligate and species-specific pathogens and opposes the strategy of pathogen speciation that results from the acquisition of virulence factors through mobile genetic elements.

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B. pertussis, *B. parapertussis*, and *B. bronchiseptica* colonize the upper respiratory tract of the host, specifically targeting the ciliated cells of the respiratory epithelium. An early event in *Bordetella* colonization is the induction of ciliostasis which, together with epithelial cell damage and mucus accumulation, may prevent efficient clearing of the organisms and may also render the host susceptible to secondary infection (Anderton et al. 2004). Since its isolation in 1906 by Bordet and Gengou (1906), the disease caused by *B. pertussis* has been widely studied. Classical pertussis is a vaccine-preventable disease characterized by coughing fits, which can be followed by sudden involuntary inspiration resulting in an audible whoop. In addition to a prolonged cough, pertussis also results in lymphocytosis (Lagergren 1963) and hypoglycemia (Regan and Tolstoouhov 1936) which are thought to be due to the activity of pertussis toxin released by the bacteria (Munoz et al. 1981; Cherry and Heininger 2004). Immunity to *B. pertussis* appears to be complex, with both branches of adaptive immunity playing a role. Passive immunization studies have demonstrated the protective properties of antibodies in *Bordetella* infection (Granstrom et al. 1991; Bruss and Siber 1999) and experimental infections using athymic mice have demonstrated the importance of cell-mediated immunity (particularly CD4-positive T cells) in combating *B. pertussis* infection (Mills et al. 1993). Since the inception of widespread vaccination during the 1940s using whole-cell preparations, and continuing with more recent immunization using acellular pertussis vaccines, the annual incidence of pertussis in the United States has decreased dramatically (CDC 2002). *B. pertussis* infection is recognized as a cause of persistent cough in adults, who may also serve as an important environmental reservoir for the disease. Waning immunity from childhood vaccination during adolescence and the potential for disease transmission from the adult population has led to recent development and implementation of adult acellular pertussis vaccines (Ward et al. 2005; CDC 2006).

Virulence determinants

Adherence and host colonization by *B. pertussis*, *B. bronchiseptica* and *B. parapertussis* is thought to occur primarily by the actions of cell surface proteins including fimbriae, filamentous hemagglutinin and pertactin. The lipopolysaccharide of these species has typical endotoxic properties and these bacteria also produce several toxins that contribute to their virulence. Tracheal cytotoxin, a disaccharide tetrapeptide that is released during peptidoglycan synthesis, is responsible for much of the respiratory cytopathology found in infected hosts (Flak and Goldman 1999). The toxin causes an increase in host nitric oxide production through activation of the inducible nitric oxide synthase by interleukin-1. Nitric oxide causes intracellular iron depletion and inactivation of iron-containing enzymes important for mitochondrial respiration and DNA synthesis and as a result, epithelial cellular tight junctions are compromised, leading to extrusion of the ciliated cells and denudation of the epithelium. The bifunctional adenylate cyclase is a toxin with both adenylate cyclase and hemolytic activities. Upon internalization by the host cell, the toxin is activated by calmodulin and catalyzes the hyperproduction of cyclic adenosine 3′, 5′-monophosphate, leading to the disruption of cellular regulatory functions. Recent studies indicate that the pore-forming hemolytic activity is also an important element of the cytoxicity of adenylate cyclase (Hewlett et al. 2006). Another toxin produced by *Bordetella* species that causes considerable cytopathology is the dermonecrotic toxin. This toxin enzymatically modifies eukaryotic Rho regulatory GTPases, resulting in their constitutive activation and subsequent alterations in cellular morphology and differentiation. All three classical species of *Bordetella* have the genes encoding pertussis toxin; however only *B. pertussis* produces and secretes the toxin. Pertussis toxin catalyzes the transfer of an ADP ribose group to the alpha subunit of host regulatory GTP-binding proteins, thereby inactivating the subunit and causing dysregulation of host cell function. Pertussis toxin, in toxoided form, is an

essential protective antigen present in all currently used acellular pertussis vaccines.

In *B. pertussis*, *B. bronchiseptica* and *B. parapertussis*, the genes encoding most of the known virulence factors are regulated by the BvgAS two-component phosphorelay system. BvgS is a transmembrane sensor kinase and BvgA is a DNA-binding response regulator (Weiss et al. 1983; Uhl and Miller 1994). Phosphorylated BvgA activates transcription of Bvg^+ phenotypic phase-specific genes such as those encoding adhesins and protein toxins. Some genes are repressed by the BvgR repressor protein, which itself is encoded by a BvgA-activated gene. Although BvgAS mediates the expression of phenotypic phase genes, the natural signals affecting BvgAS activity in the infected host environment are unknown. Transcription of the Bvg-regulated virulence genes has not been reported to be affected by iron starvation.

Bordetella in vivo growth and iron acquisition

After *Bordetella* cells bind to the respiratory epithelium, the ensuing dynamic interaction between host and pathogen is predicted to result in inflammation, activation of immune responses, and host cell injury. In this complex environment, *Bordetella* cells obtain the nutritional iron necessary for growth. It has been demonstrated that the three *Bordetella* species produce the siderophore alcaligin, but they can also utilize xenosiderophores. Therefore, siderophores produced by commensals or temporary colonizers of the nasopharynx may provide additional means of iron retrieval for Bordetellae in the host. As infection progresses, extravasation of immune cells, erythrocytes and serum to the mucosal surface can occur and may be exacerbated by the damaging action of *Bordetella* toxins on epithelial and other host cells. Serum and the molecules released upon host cell lysis may provide iron sources including transferrin and heme compounds such as hemoglobin and haptoglobin, all of which can be used by *Bordetella* species for growth.

The first environment encountered by these bacteria after transmission to a new host is the respiratory mucosa, where lactoferrin acts to bind iron and withhold it from invading microbes. As successful respiratory pathogens, the Bordetellae must be able to exploit lactoferrin as an iron source or defeat its iron-sequestering effect. Redhead and colleagues reported that *B. pertussis* tightly bound lactoferrin, ovotransferrin and transferrin and could use ovotransferrin as a sole iron source (Redhead et al. 1987). Another group found that growth of *B. pertussis* and *B. bronchiseptica* in the presence of human lactoferrin or transferrin did not require bacterial contact with the glycoproteins, suggesting the involvement of a siderophore (most likely alcaligin, the identity of which was unknown at that time) (Menozzi et al. 1991). The authors identified several candidate transferrin- and lactoferrin-binding proteins using affinity chromatography. However, recent analyses of the *B. pertussis, B. parapertussis* and *B. bronchiseptica* genome sequences indicate these organisms lack genes encoding predicted receptors for lactoferrin or transferrin. Therefore, *Bordetella* acquisition of iron from these transferrins likely requires a siderophore—either alcaligin or a xenosiderophore that may be present in host mucus.

Of the known *Bordetella* iron sources, the genetic systems for three have been characterized: the native alcaligin siderophore, the enterobactin xenosiderophore and heme. These organisms can also use other xenosiderophores and have numerous additional genes encoding potential TonB-dependent iron receptors. Thus, *B. pertussis, B. parapertussis* and *B. bronchiseptica* appear to have multiple systems for retrieving iron. It is not known which of the iron systems are operational in the host environment and which iron sources are present in different anatomical regions of the respiratory tract and during different stages of colonization and infection. A key feature of the three known iron retrieval systems is that each is individually up-regulated in the presence of the cognate iron source. This ability to detect and respond to different iron sources implies that these iron sources may be available or used in the host and that the ability to discriminate between iron sources is important to successful *in vivo* growth.

Alcaligin siderophore utilization

Alcaligin is a 20-membered macrocyclic dihydroxamate siderophore [1,8(*S*),11,18(*S*)-tetrahydroxy-1,6,11,16-tetraazacycloeicosane-2,5,12,15-tetrone] (Fig. 1) known to be produced by *B. pertussis* and *B. bronchiseptica* (Moore et al. 1995; Brickman et al. 1996). *B. parapertussis* produces a siderophore (Pradel et al. 1998) likely to be alcaligin, but it has not yet been conclusively identified. Alcaligin was first purified from *Alcaligenes (Achromobacter) denitrificans* subspecies *xylosoxydans* KN3-1 (Nishio et al. 1988; Nishio and Ishida 1990), and the total synthesis of alcaligin has been accomplished by Bergeron and coworkers (Bergeron et al. 1991). Alcaligin is structurally similar to the siderophores bisucaberin, produced by the marine bacterium *Pseudoalteromonas haloplanktis* (Kameyama et al. 1987; Takahashi et al. 1987), and putrebactin produced by *Shewanella putrefaciens* (now classified as species *oneidensis*) (Ledyard and Butler 1997), with the exception that bisucaberin incorporates two residues of *N*-hydroxycadaverine and putrebactin has two residues of *N*-hydroxyputrescine instead of the hydroxyl-substituted *N*-hydroxyputrescine residues of alcaligin (Fig. 1).

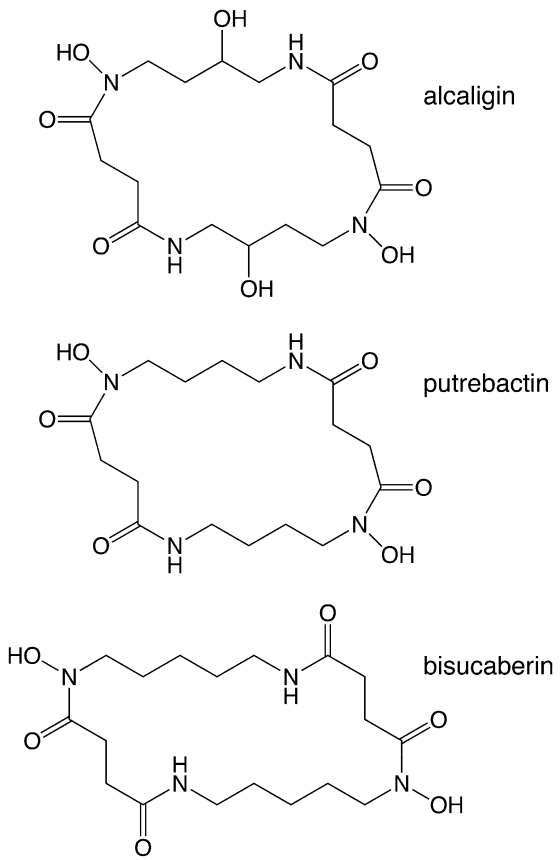

Fig. 1 Molecular structures of alcaligin, putrebactin and bisucaberin

As a tetradentate siderophore, alcaligin binds ferric iron at a 3:2 molar ratio (Fe_2Alc_3) at pH 6.0, whereas at pH 2.0, a less stable monomeric species (FeAlc) is prevalent (Nishio et al. 1988). The Fe_2Alc_3 complex has a stability constant estimated at 10^{37} M^{-1} at pH 6.0 based on EDTA displacement studies (Nishio et al. 1988). Crystallographic analysis of the Fe_2Alc_3 complex (Hou et al. 1996, 1998) elucidated the structural basis for its high stability: the free alcaligin ring exhibits a remarkable degree of structural preorganization for ferric iron binding. The Fe_2Alc_3 complex assumes a monobridged, U-shaped structure comprised of two terminal ligand fragments and one bridging fragment, with each iron atom coordinated in a pseudooctahedral environment involving three hydroxamates. The alcaligin ring of the bridging ligand in the Fe_2Alc_3 complex is significantly twisted from its C_2 molecular symmetry, but the spatial positions of the ring atoms of the two terminal ligands are remarkably similar to those of the free alcaligin ligand. This structural preorganization precludes the need for marked conformational changes to bind iron and provides a 100-fold increase in metal complex stability over ferric complexes formed by the more flexible linear dihydroxamate siderophore rhodotorulic acid.

Studies that address the natural process of iron release from ferric alcaligin complexes have focused on a possible reductive mechanism (Spasojevic et al. 1999), with emphasis on the influence of siderophore denticity and preorganization afforded by cyclization of ferric hydroxamate siderophore complexes on their redox potentials. It was determined that an increase in ligand denticity has a greater effect on redox potential than the closure (cyclization) of the ligand backbone of the same denticity, and that the redox potential of ferric alcaligin might allow reductive iron removal under physiological

conditions. Kinetic analysis of iron release from alcaligin indicated that preorganization of the iron-binding ligands of alcaligin, compared to the lack of structural preorganization of another tetradentate siderophore rhodotorulic acid, also strongly influenced the ligand dissociation paths for their Fe_2L_3 ferric complexes (Boukhalfa et al. 2000; Boukhalfa and Crumbliss 2000).

Alcaligin biosynthesis and export

The contiguous 10.6-kb alcaligin siderophore biosynthesis, regulation and transport gene cluster is depicted in Fig. 2. The same alcaligin gene organization is present in *B. pertussis*, *B. bronchiseptica* and *B. parapertussis* (Parkhill et al. 2003). Alcaligin biosynthesis and regulatory functions are encoded by the *alcABCDER* operon (Table 1) (Armstrong and Clements 1993; Giardina et al. 1995, 1997; Kang et al. 1996; Kang and Armstrong 1998; Beaumont et al. 1998; Pradel et al. 1998). Whereas polypeptide siderophores and most microbial peptide antibiotics are produced by members of the nonribosomal peptide synthetase (NRPS) multienzyme family, certain other siderophores including alcaligin are assembled from alternating dicarboxylic acid and diamine or amino alcohol units that are joined through amide or ester bonds by an NRPS-independent process. The prototypic NRPS-independent pathway yields the siderophore aerobactin, which uses the IucA and IucC synthetases to join the dicarboxylic acid and diamine units containing the functional iron-chelating groups (de Lorenzo et al. 1986; de Lorenzo and Neilands 1986). Several other NRPS-independent siderophore biosynthesis pathways have been identified, all of which involve at least one enzyme with similarity to the aerobactin synthetases. NRPS-independent siderophore biosynthesis pathways and their widespread taxonomic distribution was the subject of a recent review (Challis 2005).

The enzymology of alcaligin biosynthesis has not been fully elucidated and much of the pathway is hypothesized based on similarities of Alc proteins with aerobactin synthetases and other NRPS-independent siderophore biosynthesis enzymes (Brickman and Armstrong 1996; Giardina et al. 1995; Kang et al. 1996; Pradel et al. 1998; Challis 2005). The initial step in alcaligin biosynthesis in Bordetellae is known to involve decarboxylation of ornithine to produce putrescine (**1**) (Fig. 3), catalyzed by the pyridoxal phosphate-dependent decarboxylase encoded by the *odc* gene (Brickman and Armstrong 1996). To date, this is the only alcaligin biosynthesis step that has been confirmed biochemically. It is believed that putrescine is subsequently converted to *N*-hydroxyputrescine (**2**) by AlcA, which likely codes for a FAD-dependent monooxygenase, by a reaction similar to that catalyzed by IucD in aerobactin biosynthesis. AlcB is then thought to mediate the *N*-acylation of the hydroxylamine group in **2** with succinyl CoA by a process similar to the IucB-dependent acylation of *N*6-hydroxylysine with acetyl CoA in the aerobactin pathway. Compound **3** could then undergo C-hydroxylation mediated by AlcE, which shows significant similarity to the ring-hydroxylating dioxygenase family of iron-sulfur cluster proteins. Finally, AlcC, which belongs to a siderophore synthetase family that includes IucC, could catalyze nucleotide triphosphate-dependent dimerization of compound **4**, followed by nucleotide triphosphate-dependent macrocyclization to yield alcaligin (**5**). A role for AlcD in alcaligin biosynthesis is unclear. AlcD shows very limited amino acid sequence similarity with a putative iron reductase of *Vibrio mimicus*, with ArgH, an arginosuccinate lyase of a marine *Synechococcus*

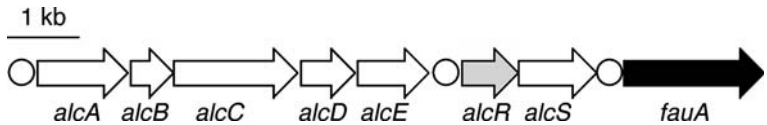

Fig. 2 Genetic organization of the *Bordetella* alcaligin siderophore system. Arrows indicate the transcriptional orientations and spatial limits of the genes, and open circles represent known promoter regions. Known Fur binding sites are located upstream of *alcA*, *alcR* and *fauA*. AlcR-responsive control regions reside upstream of *alcA* and *fauA*

Table 1 *Bordetella* alcaligin system proteins and homologs

Bordetella alcaligin system genes			Homologs			
Protein	Function	Locus Tags	Protein	Function	Genbank Accession Number	Identity
AlcA	Known alcaligin biosynthesis protein	BP2456, BB3893, BPP3443	IucD	Aerobactin biosynthesis protein of *Escherichia coli*, Lysine N6-hydoxylase/L-ornithine N5-oxygenase family	P11295	29% in 428 AA
AlcB	Known alcaligin biosynthesis protein	BP2457, BB3894, BPP3444	IucB	Aerobactin biosynthesis protein of *E. coli*	Q47317	24% in 168 AA
AlcC	Known alcaligin biosynthesis protein	BP2458, BB3895, BPP3445	IucC	Aerobactin biosynthesis protein of *E. coli*	Q47318	29% in 595 AA
AlcD	Hypothetical alcaligin biosynthesis protein	BP2459, BB3896, BPP3446	BAB83801	Hypothetical protein of *Vibrio parahaemolyticus*	AB066099	23% in 223 AA
AlcE	Known alcaligin biosynthesis protein	BP2460, BB3897, BPP3447	RSc2224	Hypothetical protein of *Ralstonia solanacearum*, Dioxygenase (alpha subunit) oxidoreductase family	NP_52035	60% in 370 AA
AlcR	Known AraC/XylS family transcriptional regulator	BP2461, BB3898, BPP3448	PchR	Transcriptional regulator of pyochelin system genes of *Pseudomonas aeruginosa*, AraC/XylS family	P40883	27% in 242 AA
AlcS	Known alcaligin export protein	BP2462, BB3899, BPP3449	Bcr	Sulfonamide and bicyclomycin resistance protein of *E. coli*, Major facilitator family of membrane efflux pumps	P28246	29% in 391 AA
FauA	Known ferric alcaligin receptor protein, TonB-dependent receptor family	BP2463, BB3900, BPP3450	FpvA	Ferric pyoverdine receptor protein of *P. aeruginosa*, TonB-dependent receptor family	A40601	37% in 704 AA

species (Palenik et al. 2003), as well as with a gene encoding a hypothetical iron-sulfur cluster protein that maps adjacent to a *Vibrio parahaemolyticus iutA* ferric aerobactin receptor gene homolog.

The *alcS* gene (previously, *orfX* or *bcr*) (Beaumont et al. 1998; Pradel et al. 1998) encodes a permease of the major facilitator superfamily class of proton motive force-dependent membrane efflux pumps (Brickman and Armstrong 2005). Expression of *alcS* is independent of the *alcABCDER* control region and is not repressible by iron (Kang and Armstrong 1998). AlcS exports monomeric alcaligin from the *Bordetella* cell and is important for the excretion of newly synthesized alcaligin (Brickman and Armstrong 2005). In liquid culture, *alcS* null mutants produce less extracellular alcaligin than wild-type bacteria, but exhibit significantly elevated levels of cell-associated alcaligin. *alcS* mutants also demonstrate a growth defect under iron starvation conditions. Normal growth of *alcS* mutants is restored by mutation of the *alcA* biosynthesis gene and the poor growth phenotype is subsequently recapitulated by genetic complementation of the *alcA* mutation to restore alcaligin production. Thus,

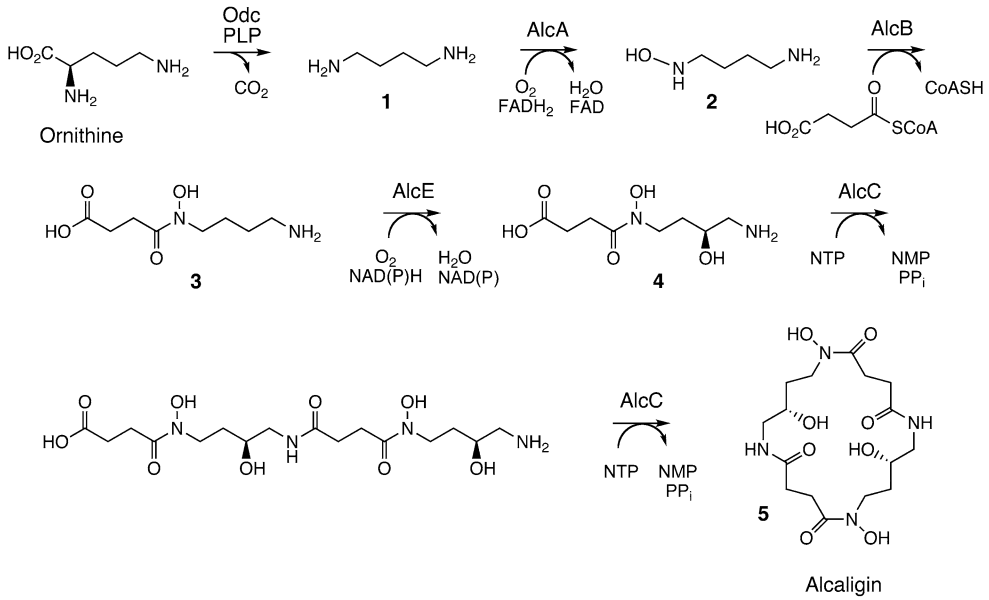

Fig. 3 Alcaligin biosynthesis. A hypothetical pathway for the biosynthesis of alcaligin in *Bordetella* species is depicted, based on similarities between alcaligin biosynthesis proteins and enzymes of other nonribosomal peptide synthase-independent siderophore biosynthesis pathways, particularly the aerobactin system of *E. coli*

intracellular accumulation of alcaligin, due to *alcS* mutation, is deleterious for growth. Transcription studies established that the exporter activity of AlcS is required to maintain optimal intracellular alcaligin levels for activation of the AlcR positive transcriptional regulator of alcaligin system genes. Hence, AlcS is central to the complex regulatory process that controls alcaligin gene expression.

Ferric alcaligin transport

Ferric alcaligin uptake in the classical *Bordetella* species requires the TonB system (Nicholson and Beall 1999; Pradel et al. 2000) and only one *tonB* gene has been identified in each of these species (Parkhill et al. 2003). FauA is the 79-kDal TonB-dependent outer membrane receptor protein required for ferric alcaligin uptake (Brickman and Armstrong 1999). *fauA* is transcribed from its own Fur-regulated promoter and is dependent on the AlcR regulator and alcaligin inducer for maximal expression. Since the molar ratio of iron to alcaligin in the ferric alcaligin complex is 2:3 under the conditions in which these *Bordetella* species are thought to grow in the host, the FauA receptor is predicted to recognize the Fe_2Alc_3 complex. The fate of ferric alcaligin after it transits the outer membrane is unknown. No other ferric alcaligin-specific transporters or permeases have been identified in *Bordetella* species, but candidate cytoplasmic membrane transporters have been predicted from the *Bordetella* nucleotide sequence databases at The Wellcome Trust Sanger Institute.

Regulation of alcaligin system genes

Genetic and biochemical studies established that Fur and iron mediate repression of *Bordetella* alcaligin siderophore system genes. *B. bronchiseptica fur* mutants were originally identified by screening for constitutive alcaligin production after selection for manganese resistance (Brickman and Armstrong 1995). The *B. pertussis fur* gene was cloned by complementation of the *fur* mutant phenotype restoring iron-repressible alcaligin production, and its identity was confirmed by nucleotide sequencing. At the same time, another group also identified and characterized the *B. pertussis fur* gene (Beall and Sanden 1995a). The alcaligin genetic system is comprised of four distinct transcriptional units: *alcABCDER*, *alcR*, *alcS* and *fauA* (Beaumont et al. 1998; Kang and Armstrong 1998; Pradel

et al. 1998; Brickman and Armstrong 1999, 2005). The *alcS* alcaligin exporter gene appears to be constitutively expressed (Kang and Armstrong 1998; Brickman and Armstrong 2005). The *alcABCDER* genes are cotranscribed from a Fur and iron-repressible promoter upstream of *alcA* (Brickman and Armstrong 1995; Kang et al. 1996; Kang and Armstrong 1998). In gel mobility shift experiments using purified *B. pertussis* Fur protein, the *alcA* upstream DNA region was found to have multiple high-affinity Fur-binding sites that display a significant degree of positive cooperativity. The *alcR* gene is also expressed from a monocistronic transcript originating at a weak promoter immediately upstream of the *alcR* gene. Metallated Fur protein specifically binds the *alcR*-upstream DNA region; this secondary *alcR* promoter is strongly repressed by Fur and iron, but is independent of AlcR activation (Beaumont et al. 1998). A third Fur-binding region resides upstream of the *fauA* receptor gene (Brickman and Armstrong 1999). The *fauA* transcript is monocistronic and as with *alcABCDER* regulation, *fauA* transcripts originate from a Fur and iron-repressible promoter that is activated by AlcR in response to alcaligin induction. Thus, Fur and iron-mediated negative control of transcription is exerted at three known promoter–operator regions within the alcaligin system gene cluster-upstream of the *alcABCDER* operon, upstream of *alcR* and upstream of *fauA*.

When Fur repression is relieved by iron starvation, expression of *fauA* and the *alcABCDER* operon is activated by the AlcR regulator by a mechanism requiring induction by alcaligin (Brickman et al. 2001). AlcR (Beaumont et al. 1998; Pradel et al. 1998) is a member of the AraC/XylS family of transcriptional regulators (Gallegos et al. 1997), and is most similar to the *Bordetella* BfeR regulator of enterobactin utilization genes (Anderson and Armstrong 2004), and to the known AraC-like regulators PchR of *Pseudomonas aeruginosa* and YbtA of *Yersinia pestis*, which are involved in regulation of the pyochelin and yersiniabactin iron transport systems, respectively. AlcR is a 36-kDal protein that requires activation by alcaligin (Brickman et al. 2001; Brickman and Armstrong 2002), and has the *N*-terminal inducer-binding and multimerization domain and C-terminal DNA-binding domain structure typical of most AraC/XylS family regulators. Alcaligin does not induce transcription under iron-replete conditions, indicating that Fur derepression is a prerequisite for induction and activation of transcription (Brickman et al. 2001).

Alcaligin-sensing

AlcR-mediated transcriptional activation is exquisitely sensitive, allowing the bacterium to perceive and respond to extremely low concentrations of inducer. AlcR has a nanomolar threshold concentration for activation of transcription in iron-starved *Bordetella* cells (Brickman et al. 2001). This alcaligin concentration is more than 1000-fold less than the concentration required for measurable growth stimulation in feeding assays (Brickman et al. 1996; Brickman and Armstrong 1999). *Bordetella fauA* and *tonB* null mutants that cannot transport ferric alcaligin are equally responsive to induction as wild type strains (Brickman and Armstrong, unpublished observations), indicating that high-affinity receptor-mediated uptake of ferric alcaligin *via* FauA or another TonB-dependent outer membrane receptor is not required for induction. Since AlcR is an AraC-type regulator and likely requires direct interaction with the inducer, the siderophore must gain access to the cell cytoplasm for interaction with AlcR. It is possible that deferri-alcaligin could cross the outer membrane by passive diffusion through porins; however the mechanism of inducer uptake across the cytoplasmic membrane remains to be determined. All evidence obtained to date supports the hypothesis that it is the iron-free form of alcaligin that has inducer activity.

Alcaligin-sensing varies among *Bordetella* species. It was found that overproduction of *B. bronchiseptica* AlcR partially suppressed its inducer requirement, whereas *B. pertussis* AlcR retained strict alcaligin inducer dependence when overproduced (Brickman and Armstrong 2002). This suppression phenotype was due to a single amino acid difference in the *N*-terminal inducer-binding and multimerization domain of the AlcR proteins of the two species. Based on that information, a mutant AlcR protein was

constructed that was fully constitutive (i.e., inducer-independent) for activation of transcription at single-copy gene dosage, thus defining a critical AlcR determinant of inducer responsiveness. Since overproduction of *B. bronchiseptica* AlcR suppresses its inducer requirement but overproduction of the *B. pertussis* protein does not, it is thought that the inactive conformation of the *B. bronchiseptica* AlcR protein adopts the active conformation more readily than *B. pertussis* AlcR. The interspecies difference in AlcR signal requirements for activation may have biological relevance for signal perception. The fact that modest AlcR overproduction results in inducer independent AlcR function in *B. bronchiseptica* suggests that the alcaligin inducer may normally be required only for initial activation of transcription of the alcaligin system genes. Once activated, elevated levels of AlcR resulting from increased expression of the *alcABCDER* operon would suppress any additional need for inducer. In contrast, *B. pertussis* AlcR may require the persistence of signal from the inducer to maintain AlcR in an active conformation. Therefore, if inducer were no longer perceived by *B. pertussis*, activation of alcaligin system gene transcription would cease and the bacterium's energy and precursors could be appropriately channeled to the utilization of alternative sources of iron. The more stringent genetic regulation of the alcaligin siderophore system by the AlcR protein of *B. pertussis* may contribute to the success of *B. pertussis* as an obligate pathogen.

Enterobactin xenosiderophore utilization

Bordetella cells do not produce enterobactin but they use the enterobactin excreted by other bacterial species in a type of siderophore piracy. Beall and Sanden identified the 2.6-kb *B. pertussis* *bfeA* gene that encodes the outer membrane enterobactin receptor (Beall and Sanden 1995b) (Fig. 4). BfeA exhibits strong amino acid sequence similarity with other enterobactin receptors including *Pseudomonas aeruginosa* PfeA, *Salmonella enterica* IroN, and *Escherichia coli* FepA. BfeA orthologs are encoded in the genomes of *B. pertussis*, *B. bronchiseptica*, and *B. parapertussis* (Table 2) (Beall and Sanden 1995b; Parkhill et al. 2003).

Enterobactin is a catechol siderophore consisting of a cyclic trimer of 2,3-dihydroxybenzoylserine. Iron is coordinated in a hexadentate interaction with enterobactin and the complex possesses an extremely high stability constant of 10^{49} (Loomis and Raymond 1991). Several members of the *Enterobacteriaceae*, including *E. coli* (O'Brien and Gibson 1970) and other species such as certain *Streptomyces* isolates (Fiedler et al. 2001), have the capacity to synthesize enterobactin. In addition to the *Bordetella* species, *Neisseria gonorrhoeae* (Carson et al. 1999), *Neisseria meningitidis* (Rutz et al. 1991), *Haemophilus parainfluenzae*, and *Haemophilus paraphrophilus* (Williams et al. 1990) are other extraintestinal mucosal colonizers that can utilize enterobactin as a xenosiderophore. The acquisition of a limiting nutrient without the energetic cost of siderophore biosynthesis and secretion is predicted to be a beneficial aspect of enterobactin xenosiderophore utilization by these bacterial species. The ability to utilize enterobactin may be additionally beneficial given its superior iron-chelating capability, which could potentially render weaker siderophores ineffective if competing for the same iron pool.

Downstream of the *bfeA* gene is the 0.9-kb *bfeB* open reading frame. The *bfeB* gene is predicted to be cotranscribed and translationally coupled to the *bfeA* transcript since only two nucleotides separate the genes. BfeB is a member

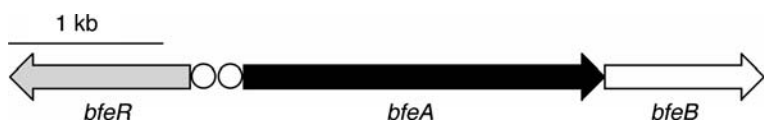

Fig. 4 Genetic organization of the *Bordetella* enterobactin utilization system. Arrows indicate the spatial limits and transcriptional orientations of the genes. Circles represent the positions of predicted Fur-regulated promoters upstream of *bfeR* and *bfeA*

Table 2 *Bordetella* enterobactin xenosiderophore utilization system proteins and homologs

Bordetella enterobactin system genes			Homologs			
Protein	Function	Locus tags	Protein	Function	Genbank Accession Number	Identity
BfeR	Known regulator of enterobactin transport and utilization, AraC/XylS family protein	BP2900, BB1943, BPP2496	AlcR	Regulator of alcaligin siderophore system genes of *Bordetella pertussis*, *Bordetella bronchiseptica*, and *Bordetella parapertussis*, AraC/XylS family protein	AAC38169	34% in 243 AA
BfeA	Known ferric enterobactin receptor protein, TonB-dependent receptor family	BP2901, BB1942, BPP2495	PfeA	Ferric enterobactin receptor protein of *Pseudomonas aeruginosa*, TonB-dependent receptor family	Q05098	52% in 740 AA
BfeB	Hypothetical ferric enterobactin esterase/hydrolase	BP2902, BB1941, BPP2494	IroE	Predicted esterase/hydrolase of *Salmonella enterica* subsp. *enterica*, Esterase/hydrolase of $\alpha\beta$ superfamily	AAC46182	30% in 241 AA

of the α/β superfamily of hydrolases (COG2891) and contains the GXSXGG serine active site esterase family signature (Interpro: IPR000801). Proteins within this family that exhibit amino acid sequence similarity to BfeB include *P. aeruginosa* PfeE, *S. enterica* IroE, and the *E. coli* Fes protein. The genes encoding all of these proteins are adjacent to known enterobactin or catechol siderophore transport genes, suggesting a common function in catechol siderophore utilization. IroE and PfeE have been demonstrated to hydrolyze ferric salmochelin and enterobactin *in vitro* and *iroE* mutants exhibit a growth defect when salmochelin is provided as the sole iron source (Zhu et al. 2005). The *E. coli* Fes protein cleaves the ester bonds of enterobactin in a process that is required for release of the bound iron (Langman et al. 1972; Brickman and McIntosh 1992). BfeB and IroE are predicted periplasmic proteins based on the presence of a secretion signal. A periplasmic localization would distinguish these enzymes from the well-characterized cytoplasmic esterase Fes and may indicate that these hydrolases have additional roles in enterobactin transport across the cytoplasmic membrane.

Regulation of enterobactin utilization

Divergently transcribed from *bfeA* is the 1.0-kb *bfeR* gene. *bfeR* encodes a regulatory protein belonging to the iron subfamily of AraC/XylS-type transcriptional regulators, which includes *Bordetella* AlcR (Beaumont et al. 1998; Pradel et al. 1998; Brickman et al. 2001), *P. aeruginosa* PchR (Heinrichs and Poole 1993; Heinrichs and Poole 1996), *Y. pestis* YbtA (Fetherston et al. 1996), and *Sinorhizobium meliloti* RhrA (Lynch et al. 2001). Enterobactin-inducible transcription of *bfeA-lacZ* was observed in iron-starved *B. pertussis* and *B. bronchiseptica*, consistent with Fur-mediated repression exerted at three putative Fur-binding sites in the *bfeR* and *bfeA* intergenic region. The BfeR regulator was required for this enterobactin-responsive transcription (Anderson and Armstrong 2004). Since the BfeR protein is a transcriptional regulator and lacks a secretion signal, enterobactin inducer interaction is hypothesized to occur in the bacterial cytoplasm. However, similar to AlcR regulation of alcaligin

system genes, high-affinity siderophore transport does not appear to be required for uptake of the inducer. Both BfeA and TonB are dispensable for transcriptional activation of *bfeA*, despite the strict requirement for both proteins in ferric enterobactin utilization (Anderson and Armstrong 2004, 2006).

Bfe recognition of multiple catechols

Structurally similar siderophores have the potential to be recognized and transported by a single iron acquisition system, thus foregoing the need for independent uptake mechanisms. This is true in *S. enterica* where the IroN and FepA receptors are both capable of transporting multiple catechol siderophores (Rabsch et al. 1999). Similarly, salmochelin (Bister et al. 2004) and corynebactin (Budzikiewicz et al. 1997), two catechol siderophores with structural similarity to enterobactin, were found to stimulate growth of iron-restricted *B. bronchiseptica* cells. Two synthetic enterobactin analogs, TRENCAM (Rodgers et al. 1987) and MECAM (Venuti et al. 1979; Weitl and Raymond 1979), also stimulated *Bordetella* growth. Growth stimulation by these compounds was *bfeA*-dependent, indicating that BfeA facilitates utilization of multiple catechol siderophores (Anderson and Armstrong 2006). Salmochelin and MECAM were additionally able to induce transcription of *bfeA* although not as well as enterobactin (Table 3).

Several host compounds have structural similarity to enterobactin, including the neuroendocrine catecholamines epinephrine, norepinephrine, and dopamine. Epinephrine and norepinephrine stimulate expression of certain *E. coli* locus of enterocyte effacement and flagellar genes (Sperandio et al. 2003) and norepinephrine was reported to induce *E. coli* FepA enterobactin receptor production (Burton et al. 2002). Analysis of *Bordetella bfeA* transcription demonstrated that epinephrine, norepinephrine, and dopamine induced *bfeA* expression levels by more than 28-fold (Table 3). Induction by norepinephrine was found to be iron-regulated and dependent on the BfeR regulator, similar to induction by enterobactin. Norepinephrine also stimulated growth of *B. bronchiseptica* cells in iron-restricted and serum-supplemented medium (Anderson and Armstrong 2006). This growth stimulation is siderophore-independent and could be the result of norepinephrine-mediated release of nutritional iron from the transferrin complexes in serum, as reported by Freestone and colleagues (Freestone et al. 2000). In addition to potentially serving as an *in vivo* iron acquisition mechanism, the ability of *Bordetella* cells to sense and respond to host catecholamines indicates that these molecules may be perceived in the context of an infection during which catecholamine recognition may serve as a host environmental cue.

Table 3 Induction of *bfeA* transcription by catechol compounds

Compound	*bfeA-lacZ* transcriptional activity[a]		Fold induction[b]
	– Inducer	+ Inducer	
Enterobactin	43 ± 5	1532 ± 124	36.0
Salmochelin	57 ± 5	183 ± 12	3.2
Corynebactin	55 ± 8	91 ± 19	1.7
MECAM	27 ± 3	460 ± 25	16.8
TRENCAM	59 ± 8	89 ± 8	1.5
Epinephrine	24 ± 20	866 ± 37	36.7
Norepinephrine	32 ± 28	905 ± 61	28.2
Dopamine	17 ± 15	834 ± 39	48.5

[a] Miller Units ± 1 standard deviation, determined by β-galactosidase assay of *B. bronchiseptica* carrying a *bfeA-lacZ* fusion plasmid and grown in iron-depleted medium in the absence (–) or presence (+) of the indicated inducer at 50 μM concentration.

[b] $LacZ_{+Inducer}/LacZ_{-Inducer}$

Heme utilization

B. pertussis was originally taxonomically included in the genus *Haemophilus* because of its cellular and colonial morphological similarities with those of other *Haemophilus* species and its apparent growth requirement for blood. Subsequent studies determined that blood prevented the growth inhibition of *B. pertussis* on agar medium (by binding inhibitors such as peroxides and fatty acids) and these organisms, as well as *B. parapertussis* and *B. bronchiseptica*, were not auxotrophic for heme. However, optimal growth of virulent-phase *Bordetella* strains on solid medium is still

best accomplished using blood-containing media such as the agar of Bordet and Gengou (Bordet and Gengou 1906). More recent work demonstrated that *B. pertussis* could use hemin (Agiato and Dyer 1992; Nicholson and Beall 1999) and hemoglobin (Nicholson and Beall 1999) as sole sources of iron, and utilization of these compounds by *B. pertussis* and *B. bronchiseptica* was demonstrated to be dependent on TonB (Nicholson and Beall 1999; Pradel et al. 2000). Research from our laboratory identified the *B. pertussis* and *B. bronchiseptica* gene clusters associated with heme utilization (Vanderpool and Armstrong 2001).

The heme utilization genetic system

The *B. pertussis* and *B. bronchiseptica* genetic system encoding heme utilization and regulatory functions is comprised of the *hurIR bhuRSTUV* genes (Fig. 5, Table 4) (Vanderpool and Armstrong 2001). The more distantly related avian pathogen *Bordetella avium* has an orthologous gene cluster, *rhuIR bhuRSTUV*, that is required for heme utilization (Murphy et al. 2002). The *Bordetella bhu* genes encode heme transport and utilization proteins that are similar to those of heme uptake systems of other gram-negative pathogens including *P. aeruginosa*, *Y. pestis, Yersinia enterocolitica, Shigella dysenteriae and Vibrio cholerae.*

BhuR is the outer membrane receptor for hemin, hemoglobin-haptoglobin, heme-bovine serum albumin and human hemoglobin, as well as hemoglobins from other animal sources, including turkey, rabbit, cow and pig (Vanderpool and Armstrong 2001). It has a TonB box C motif and the FRAP/NPNL amino acid sequence motif that is conserved among members of the bacterial heme receptor family. BhuR also contains an *N*-terminal extension that is absent in the other bacterial heme receptors but is found in receptors whose expression is regulated by ECF σ factors, such as the *E. coli* ferric citrate receptor FecA and the *P. putida* WCS358 ferric pseudobactin receptor PupB. This *N*-terminal extension is required for the signaling process that leads to transcription initiation by the ECF σ factor (Kim et al. 1997; Braun and Mahren 2005).

The BhuT, BhuU and BhuV proteins are the predicted periplasmic heme-binding protein, cytoplasmic membrane permease and ATP binding proteins, respectively, required for the uptake of heme. Homologs of the BhuS protein are found in other bacterial heme systems and although little is known of the function of these proteins, the ShuS protein of *S. dysenteriae* is the best characterized example. ShuS binds heme and has been hypothesized to sequester internalized heme, thus preventing toxicity associated with its cytoplasmic accumulation (Wyckoff et al. 2005). A *shuS* mutant was deficient in heme utilization, and overexpression of *shuS* protected both the mutant and parental strains from heme toxicity.

The *Bordetella* HurI protein is an ECF σ factor that is highly similar to FecI of the *E. coli* ferric citrate uptake system (Enz et al. 2000). HurR has a predicted membrane-spanning region and contains the three conserved tryptophan residues shared by *E. coli* FecR and its homologs. In the ferric citrate system, the FecI σ factor is controlled by direct interaction with the cytoplasmic membrane FecR anti-σ factor. FecI initiates transcription of genes encoding the ferric citrate transport machinery only under conditions of iron-limitation when ferric citrate binds the FecA outer membrane receptor. The *Bordetella bhu* genes are positively regulated by HurIR, BhuR and heme in a mechanistically similar manner.

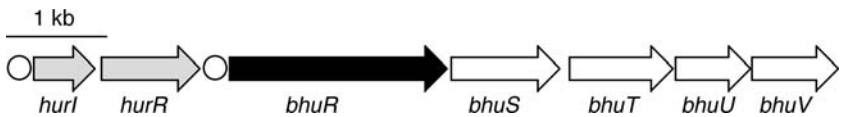

Fig. 5 Genetic organization of the *Bordetella* heme utilization system. Arrows indicate the transcriptional orientations and spatial limits of the genes, and open circles designate the positions of predicted promoter regions. A Fur-binding site resides upstream of *hurI*, and a predicted ECF σ factor-dependent promoter is upstream of *bhuR*

Table 4 *Bordetella* heme iron utilization system proteins and homologs

Bordetella heme iron system genes			Homologs			
Protein	Function	Locus tags	Protein	Function	Genbank Accession Number	Identity
HurI	Known ECF σ factor	BP0349, BB4653, BPP4183	FecI	Known ECF σ factor of *Escherichia coli*	JV0111	46% in 153 AA
HurR	Known sensor/regulator	BP0348, BB4654, BPP4184	FecR	Known sensor/regulator of *Escherichia coli*	B37804	29% in 332 AA
BhuR	Known heme receptor protein, TonB-dependent receptor family	BP0347, BB4655, BPP4185	PhuR	Predicted TonB-dependent heme receptor of *Pseudomonas aeruginosa*	AAC13289	28% in 787 AA
BhuS	Predicted heme utilization protein	BP0346, BB4656, BPP4186	ShuS	Known heme chaperone protein of *Shigella dysenteriae*	YP405020	37% in 338 AA
BhuT	Predicted periplasmic heme binding protein	BP0345, BB4657, BPP4187	HemT	Predicted periplasmic heme binding protein of *Yersinia enterocolitica*	S54437	37% in 248 AA
BhuU	Predicted heme permease	BP0344, BB4658, BPP4188	HemU	Predicted heme permease of *Yersinia enterocolitica*	CAA54863	49% in 254 AA
BhuV	Predicted ATPase	BP0343, BB4659, BPP4189	HemV	Predicted ATPase of *Yersinia enterocolitica*	CAA54864	35% in 250 AA

Transcriptional regulation of the *bhu* genes

Early observations indicated that production of the BhuR heme receptor in iron-starved *Bordetella* cells was increased significantly in response to hemin (Vanderpool and Armstrong 2001) and this response was abrogated by mutation of *hurI* (Vanderpool and Armstrong 2003). Concordantly, *hurI* mutants are deficient in heme utilization; the mutants can be complemented by *hurI* in multicopy, which also results in heme-inducible overproduction of BhuR. Analyses of *bhuR-lacZ* transcriptional fusions in *B. bronchiseptica* demonstrated that heme-responsive *bhuR* transcription requires *hurI*. Expression of *hurI* at high copy number suppressed the heme inducer requirement, resulting in heme-independent *bhuR-lacZ* expression. If HurR acts as an anti-σ factor, *hurI* overexpression may cause in an imbalance in HurI-HurR stoichiometry and loss of the negative influence of HurR.

Upstream of *hurI* lies a DNA region containing a predicted σ^{70}-like promoter and a Fur-binding site that has strong Fur-binding activity (Vanderpool and Armstrong 2001) in an *in vivo* Fur titration assay (Stojiljkovic et al. 1994). In contrast, the *Bordetella bhuR* promoter region has little or no functional Fur binding activity and lacks predicted Fur-binding sequences. Accordingly, transcription from the *hurI* promoter was found to be iron-regulated and heme-independent, while expression from the *bhuR* promoter is HurI-dependent, requiring both iron-starvation and the heme inducer (Vanderpool and Armstrong 2004). Primer extension and mutagenesis studies indicated that iron-regulated transcription from *hurI* through the *hurR-bhuR* intergenic region contributes to *bhuR* expression in the absence of heme. Thus, under iron starvation conditions, polycistronic *hurIRbhuRSTUV* transcription occurs from the *hurI* promoter. Subsequent exposure of the iron-starved bacteria to heme results in HurI-mediated *bhuRSTUV* transcription and increased production of the Bhu heme utilization machinery. Similar to results with the FecA ferric citrate receptor (Harle et al. 1995) and consistent with the proposed role for BhuR in signal transduction, mutation of *bhuR* results not only in loss of heme uptake but also in

loss of heme-responsive *bhuR* transcription (measured as *bhuR-lacZ* transcription) (Vanderpool and Armstrong 2003). Similarly, in the *B. avium* heme system the ECF σ factor RhuI activates transcription of *bhuR* in response to hemin and this activation requires the BhuR heme receptor (Kirby et al. 2001).

The *Bordetella* heme utilization system is a hybrid system that combines the functionality of the heme uptake apparatus found in gram-negative pathogens with regulators of the iron starvation subfamily of ECF regulators. Based on information from the *E. coli* Fec system and results from *Bordetella* experiments, a model for *bhu* gene regulation was developed (Fig. 6). When *Bordetella* strains are grown under iron-limiting conditions, Fur derepression at the *hurI* promoter leads to transcription of *hurIR* and read-through transcription of *bhuRSTUV* to allow basal levels of production of the heme transport machinery, including the BhuR receptor needed for heme sensing as well as uptake. Under these conditions, the HurR cytoplasmic membrane anti-σ factor is predicted to bind HurI, maintaining it in an inactive state. When the BhuR outer membrane receptor binds heme, this receptor occupancy signal is transmitted across the periplasm via physical contact of its specialized *N*-terminal extension with HurR. A productive BhuR-HurR interaction allows the release of HurI, enabling it to associate with RNA polymerase to promote transcription initiation at the *bhuR* promoter. In this manner, the *bhuRSTUV* genes are up-regulated only when the cognate heme iron sources are present in the *Bordetella* environment.

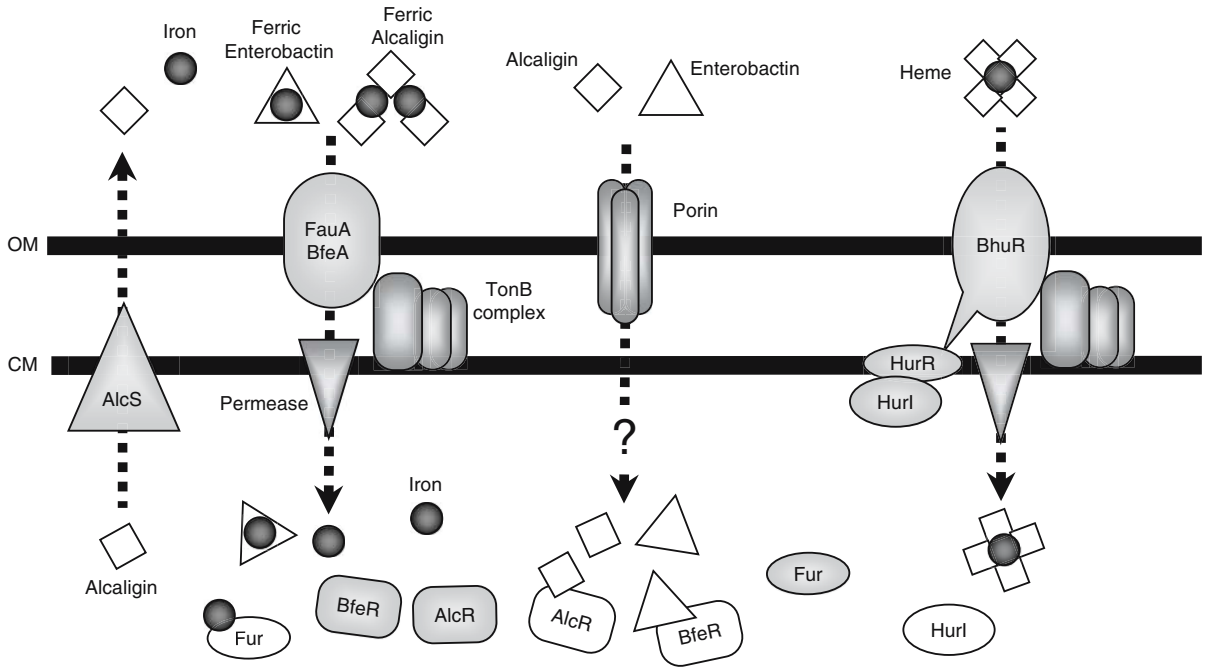

Fig. 6 Model for ferrimone sensing. The adaptive response to fluctuations in iron availability is controlled by a hierarchical regulatory scheme involving the Fur repressor and the positive transcriptional regulators AlcR, BfeR and HurIR. The production of the positive regulators is repressible by Fur, subject to the availability of iron corepressor, and the activity of the positive regulators is modulated by the presence of their specific ferrimone inducers. In the case of the AraC family regulators AlcR and BfeR, the inducing deferrisiderophore signals are perceived intracellularly, whereas the ECF σ factor HurI is activated by transduction of the extracellular BhuR heme receptor occupancy signal. BfeR and AlcR respond to inducers that are internalized by an unknown mechanism independent of their respective TonB-dependent outer membrane receptors, BfeA and FauA. The alcaligin export activity of AlcS is central to the alcaligin sensing process since it influences the intracellular concentration of the inducer

Xenosiderophores and TonB-dependent receptors

Analysis of the genome sequences of *B. pertussis, B. bronchiseptica* and *B. parapertussis* has revealed multiple genes encoding potential iron acquisition systems. In addition to the genes encoding the FauA, BfeA and BhuR receptors for ferric alcaligin, ferric enterobactin and heme, respectively, there are 13 other *Bordetella* genes potentially encoding TonB-dependent receptors (Table 5). All 13 genes are present in *B. bronchiseptica*, whereas some of the genes are absent or exist as nonfunctional pseudogenes in *B. pertussis* and *B. parapertussis*. It is possible that some of the putative TonB-dependent receptors are involved in the transport of compounds other than iron sources. It is also conceivable that some of the receptors, though once used by an ancestor that lived in the external environment, are produced but no longer serve a function for the species that has evolved in a host environment. However, the genes encoding such receptors would be predicted to acquire mutations over time, eventually giving rise to pseudogenes. An alternative view holds that since the *Bordetella* receptor genes still appear to be intact, their expression must impart some benefit or be important for the ability of the organisms to grow in the host or survive transmission.

B. pertussis and *B. bronchiseptica* are capable of TonB-dependent utilization of the xenosiderophores ferrichrome and desferroxamine B (Beall and Hoenes 1997). In addition, *B. bronchiseptica* has been reported to use coprogen, schizokinen, ferricrocin, vicibactin, ferrichrysin, ferrirubin, protochelin, aerobactin and several pyoverdins (Pradel and Locht 2001). However, the only genetic system with a known xenosiderophore specificity is the *bfe* system for the utilization of enterobactin (Beall and Sanden 1995b) and related catechols (Anderson and Armstrong 2006). The *bfrA* (Beall and Hoenes 1997), *bfrB* and *bfrC* (Beall 1998) genes were identified using a *B. bronchiseptica* Tn*phoA* insertion mutant library in a screen for iron-repressible *phoA* fusion expression. Using feeding assays, the iron source substrates for the putative receptors encoded by these genes could not be identified: mutants retained the ability to use heme, enterobactin, desferrioxamine B and ferrichrome.

Table 5 *Bordetella* TonB-dependent outer membrane receptors

Receptor	Locus Tag [a]	Iron Source, Homolog, Reference[b]
Characterized receptors of known specificity		
FauA	BP2463, BPP3450, BB3900	ferric alcaligin (Brickman and Armstrong 1999)
BfeA	BP2901, BPP2495, BB1942	ferric enterobactin (Beall and Sanden 1995b)
BhuR	BP0347, BPP4185, BB4655	heme (Vanderpool and Armstrong 2001)
Putative receptors of unknown specificity		
BfrA	BB4761	*E. coli* Iha catechol receptor ABB17254 (Beall and Hoenes 1997)
BfrB	BP2016, BPP2396, BB1846	*Y. enterocolitica* ferrichrome receptor FcuA, Q05202 (Beall 1998)
BfrC	BP3663, BPP0079, BB0078	*Vibrio anguillarum* FatA ferric anguibactin receptor P11461 (Beall 1998)
BfrD	BP0856, BPP3376, BB3826	*P. aeruginosa* Fiu Pa4514 (Passerini de Rossi et al. 2003)
BfrE	BP0857, BPP3375*[c], BB3825	*P. aeruginosa* Fiu Pa4514
BfrF	BP0736, BPP3688, BB4122	*E. coli* FhuA NP_414692
BfrG	BP2922, BPP1078, BB1294	*E. coli* FhuA NP_414692
BfrH	BP1138, BPP3206, BB3658	*E. coli* FhuA; *Burkholderia cepacia* OrbA, AF013993
BfrI	BP1962, BPP2334, BB1785	*P. aeruginosa* FiuA AF051691; *Salmonella typhimurium* FhuA Y14025
BfrZ	BB4744	COG1629[d] (Pradel and Locht 2001)
HemC	BP0456, BPP4370*, BB4956	*Haemophilus ducreyi* TdhA heme receptor AAC35765
–	BPP2457, BB1905	COG1629[d]; *Serratia marcescens* HasR hemophore receptor CAE46936
–	BP3101*, BPP0746*, BB0832	COG1629[d]

[a] genome sequence database, Wellcome Trust Sanger Institute (http://www.sanger.ac.uk); prefix designations: BP, *B. pertussis*; BPP, *B. parapertussis*; BB, *B. bronchiseptica*

[b] in parentheses, reference describing the *Bordetella* receptor

[c] * denotes a pseudogene

[d] COG1629: Outer membrane receptor proteins, mostly Fe transport

Passerini de Rossi and colleagues identified a 90-kDal outer membrane protein in wild-type *B. pertussis* that was absent in an isogenic *bvgS* mutant (Passerini de Rossi et al. 1999). Interestingly, this protein was later identified as the TonB-dependent receptor BfrD and expression of *bfrD* was determined to be activated by the BvgAS signal transduction system involved in virulence gene regulation (Antoine et al. 2000). No substrate specificity could be ascribed to BfrD (Passerini de Rossi et al. 2003) or another recently described putative *B. bronchiseptica* receptor, BfrZ (Pradel and Locht 2001).

Virulence and iron acquisition

B. pertussis is an obligate human pathogen, however the mouse has served as the animal model of infection for many years. For *B. bronchiseptica*, a variety of mammals including mice, rats, rabbits, dogs and swine are natural hosts and can serve as experimental animal models. Several published studies employing animal infections using defined iron system mutants have established the importance of iron acquisition in *Bordetella* pathogenesis. One technical aspect that may influence the outcome of these types of virulence studies is the method of preparation of the infecting inoculum. In some studies, the bacteria are cultivated on blood-containing Bordet-Gengou agar and used directly as inocula. Therefore the bacteria are pre-loaded with both heme and iron at the outset of infection and may need to deplete their internal stores of these compounds before initiating their iron transport functions. In other experiments, *Bordetella* cells are first grown on Bordet-Gengou agar, then passaged in defined liquid medium prior to use as inocula. These bacteria may also be iron-loaded if the commonly-used Stainer Scholte liquid medium (containing 36 μM iron) is used for their cultivation (Stainer and Scholte 1970). The ideal situation for experimental infections would be to simulate the iron-metabolic state of the bacteria as they are expelled from the host and transmitted to a new host. Unfortunately, the natural metabolic iron status of *Bordetella* cells during transmission remains unknown.

In vitro, *B. bronchiseptica* and *B. pertussis tonB* mutants are defective in the uptake of multiple iron sources (Nicholson and Beall 1999; Pradel et al. 2000). In mouse infections, wild-type *B. pertussis* exhibited good growth in the lung in the first week of infection, followed by gradual clearing of the organism over the course of 20 days. In contrast, an isogenic *B. pertussis tonB exbB* mutant was unable to efficiently colonize and grow in the lungs of mice, although it was gradually cleared by the host at rate similar to that of the parental strain (Pradel et al. 2000).

Two studies have examined the *Bordetella* alcaligin system *in vivo*. In one mouse study, a *B. pertussis alcR* mutant lacking the ability to upregulate alcaligin system genes demonstrated no defect in the ability to colonize the lung (Pradel et al. 1998). However, it is known that *alcR* strains retain some ability to produce and utilize alcaligin (Beaumont et al. 1998). In a different study, neonatal swine infected with a *B. bronchiseptica alcA* siderophore biosynthesis mutant demonstrated significantly reduced colonization of the nasal turbinates, trachea and lung, compared with the wild-type parent strain (Register et al. 2001). Furthermore, animals infected with this *alcA* mutant exhibited either mild or absent nasal pathology compared with the pathology usually associated with swine atrophic rhinitis. These results indicate that the *Bordetella* alcaligin siderophore is required for maximal virulence in this animal model.

The Bhu heme utilization system was demonstrated to be important for *B. pertussis* pathogenesis in mixed infection competition experiments using a mouse model (Brickman et al. 2006). A *bhuR* mutant unable to use heme was co-inoculated intranasally into mice with the wild-type parent strain. Enumeration of the bacterial populations isolated from the infected lungs and trachea during the course of infection allowed calculation of competitive indices as measures of *in vivo* fitness of the heme uptake mutant compared with the wild-type parent. Within the first week of infection, the mutant was recovered from respiratory tissues in numbers similar to those of the parental strain. However, after one week, the *bhuR* mutant exhibited a marked impairment in colonization ability, suggesting that later in

infection heme utilization is critical for growth in the host. In the later stages of infection, there would be increased damage to host epithelial cells due to the action of *Bordetella* virulence factors and released heme may be readily available. The *bhuR* mutant is able to produce and use its alcaligin siderophore. Since siderophore production does not appear to benefit the *bhuR* mutant late in infection, alcaligin may not be stable or serve as an effective scavenger of iron in the host environment where extensive tissue damage has occurred. The more distantly-related species *Bordetella avium* does not produce a siderophore, but possesses a heme uptake system homologous to that of the three classical *Bordetella* species. In competition infection experiments using co-inoculated *B. avium* wild-type and *bhuR* mutant strains, the BhuR heme receptor was demonstrated to be important in tracheal colonization of turkey poults (Murphy et al. 2002).

Ferrimone-inducible iron acquisition

When nutritional iron is limiting, *Bordetella* cells can produce alcaligin along with its requisite transport and utilization functions, and can also produce proteins required for the utilization of host heme-iron compounds and certain xenosiderophores, including the potent and ubiquitous siderophore enterobactin. Each of these distinct iron-scavenging systems is controlled by a different Fur- and iron-repressible transcriptional activator that can sense and respond to the presence of the cognate iron source, ultimately resulting in elevated expression of the genes involved in its assimilation. In their role as inducers, alcaligin, heme and enterobactin function as signaling molecules termed ferrimones. Ferrimone sensing is used to integrate environmental and intracellular signals to prioritize expression of the iron transport systems (Fig. 6). It is postulated that the ability of *Bordetella* species to selectively activate the expression of the different iron systems contributes to their capacity to effectively adapt and multiply in the dynamic host environment during the course of infection.

The full iron-scavenging potential of *Bordetella* species is undetermined. In addition to the three known outer membrane receptor proteins required for utilization of ferric alcaligin (FauA), ferric enterobactin (BfeA) and heme-iron (BhuR), at least nine more genes encoding TonB-dependent receptor homologs of unknown specificity can be identified in the genomes of the classical *Bordetella* species. Further research is needed to characterize the key elements of ferrimone-inducible regulation of *Bordetella* iron transport systems and to assess their contributions to pathogenesis in the mammalian host.

References

Agiato LA, Dyer DW (1992) Siderophore production and membrane alterations by *Bordetella pertussis* in response to iron starvation. Infect Immun 60:117–123

Anderson MT, Armstrong SK (2004) The BfeR regulator mediates enterobactin-inducible expression of *Bordetella* enterobactin utilization genes. J Bacteriol 186:7302–7311

Anderson MT, Armstrong SK (2006) The *Bordetella* Bfe system: growth and transcriptional response to siderophores, catechols, and neuroendocrine catecholamines. J Bacteriol 188:5731–5740

Anderton TL, Maskell DJ, Preston A (2004) Ciliostasis is a key early event during colonization of canine tracheal tissue by *Bordetella bronchiseptica*. Microbiology 150:2843–2855

Antoine R, Alonso S, Raze D 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

Armstrong SK, Clements MO (1993) Isolation and characterization of *Bordetella bronchiseptica* mutants deficient in siderophore activity. J Bacteriol 175:1144–1152

Beall B (1998) Two iron-regulated putative ferric siderophore receptor genes in *Bordetella bronchiseptica* and *Bordetella pertussis*. Res Microbiol 149:189–201

Beall B, Hoenes T (1997) An iron-regulated outer-membrane protein specific to *Bordetella bronchiseptica* and homologous to ferric siderophore receptors. Microbiology 143(Pt 1):135–145

Beall B, Sanden GN (1995a) A *Bordetella pertussis fepA* homologue required for utilization of exogenous ferric enterobactin. Microbiology 141:3193–3205

Beall BW, Sanden GN (1995b) Cloning and initial characterization of the *Bordetella pertussis fur* gene. Curr Microbiol 30:223–226

Beaumont FC, Kang HY, Brickman TJ, Armstrong SK (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

Bergeron RJ, McManis JS, Perumal PT, Algee SE (1991). The total synthesis of alcaligin. J Org Chem 56:5560–5563

Bister B, Bischoff D, Nicholson GJ et al (2004) The structure of salmochelins: C-glucosylated enterobactins of *Salmonella enterica*. Biometals 17:471–481

Bordet J, Gengou O (1906) Le microbe de la coqueluche. Ann Inst Pasteur (Paris) 20:731–741

Boukhalfa H, Brickman TJ, Armstrong SK, Crumbliss AL (2000) Kinetics and mechanism of iron(III) dissociation from the dihydroxamate siderophores alcaligin and rhodotorulic acid. Inorg Chem 39:5591–5602

Boukhalfa H, Crumbliss AL (2000) Multiple-path dissociation mechanism for mono- and dinuclear tris(hydroxamato)iron(III) complexes with dihydroxamic acid ligands in aqueous solution. Inorg Chem 39:4318–4331

Braun V, Mahren S (2005). Transmembrane transcriptional control (surface signalling) of the *Escherichia coli* Fec type. FEMS Microbiol Rev 29:673–684

Brickman TJ, Armstrong SK (1995) *Bordetella pertussis fur* gene restores iron repressibility of siderophore and protein expression to deregulated *Bordetella bronchiseptica* mutants. J Bacteriol 177:268–270

Brickman TJ, Armstrong SK (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

Brickman TJ, Armstrong SK (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

Brickman TJ, Armstrong SK (2002). *Bordetella* interspecies allelic variation in AlcR inducer requirements: identification of a critical determinant of AlcR inducer responsiveness and construction of an *alcR*(-Con) mutant allele. J Bacteriol 184:1530–1539

Brickman TJ, Armstrong SK (2005). *Bordetella* AlcS transporter functions in alcaligin siderophore export and is central to inducer sensing in positive regulation of alcaligin system gene expression. J Bacteriol 187:3650–3661

Brickman TJ, Hansel JG, Miller MJ, Armstrong SK (1996) Purification, spectroscopic analysis and biological activity of the macrocyclic dihydroxamate siderophore alcaligin produced by *Bordetella pertussis* and *Bordetella bronchiseptica*. Biometals 9:191–203

Brickman TJ, Kang HY, Armstrong SK (2001) Transcriptional activation of *Bordetella* alcaligin siderophore genes requires the AlcR regulator with alcaligin as inducer. J Bacteriol 183:483–489

Brickman TJ, McIntosh MA (1992) Overexpression and purification of ferric enterobactin esterase from *Escherichia coli*. Demonstration of enzymatic hydrolysis of enterobactin and its iron complex. J Biol Chem 267:12350–12355

Brickman TJ, Vanderpool CK, Armstrong SK (2006) Heme transport contributes to in vivo fitness of *Bordetella pertussis* during primary infection in mice. Infect Immun 74:1741–1744

Bruss JB, Siber GR (1999) Protective effects of pertussis immunoglobulin (P-IGIV) in the aerosol challenge model. Clin Diagn Lab Immunol 6:464–470

Budzikiewicz H, Bössenkamp A, Taraz K, Pandey A, Meyer JM (1997) Corynebactin, a cyclic catecholate siderophore from *Corynebacterium glutamicum* ATCC 14067 (*Brevibacterium* sp. DSM 20411). Z Naturforsch 52:551–554

Burton CL, Chhabra SR, Swift S et al (2002). The growth response of *Escherichia coli* to neurotransmitters and related catecholamine drugs requires a functional enterobactin biosynthesis and uptake system. Infect Immun 70:5913–5923

Carson SDB, Klebba PE, Newton SM, Sparling PF (1999) Ferric enterobactin binding and utilization by *Neisseria gonorrhoeae*. J Bacteriol 181:2895–2901

Centers, for, Disease, Control, and, Prevention (2002) Pertussis – United States, 1997–2000. MMWR Morb Mortal Wkly Rep 51:73–76

Centers, for, Disease, Control, and, Prevention (2006) Preventing tetanus, diphtheria, and pertussis among adolescents: use of tetanus toxoid, reduced diphtheria toxoid and acellular pertussis vaccines: recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Morb Mortal Wkly Rep 55:1–46

Challis GL (2005) A widely distributed bacterial pathway for siderophore biosynthesis independent of nonribosomal peptide synthetases. Chembiochem: Eur J Chem Biol 6:601–611

Cherry JD, Heininger U (2004) Pertussis and other *Bordetella* infections. In: Feigin RD, Cherry JD, Demmler GJ, Kaplan S (eds) Textbook of pediatric infectious diseases, 5th edn. The W. B. Saunders Co, Philadelphia, pp 1588–1608

de Lorenzo V, Bindereif A, Paw BH, Neilands JB (1986) Aerobactin biosynthesis and transport genes of plasmid ColV-K30 in *Escherichia coli* K-12. J Bacteriol 165:570–578

de Lorenzo V, Neilands JB (1986) Characterization of *iucA* and *iucC* genes of the aerobactin system of plasmid ColV-K30 in *Escherichia coli*. J Bacteriol 167:350–355

Enz S, Mahren S, Stroeher UH, Braun V (2000) Surface signaling in ferric citrate transport gene induction: interaction of the FecA, FecR, and FecI regulatory proteins. J Bacteriol 182:637–646

Fetherston JD, Bearden SW, Perry RD (1996) YbtA, an AraC-type regulator of the *Yersinia pestis* pesticin/yersiniabactin receptor. Mol Microbiol 22:315–325

Fiedler HP, Krastel P, Muller J, Gebhardt K, Zeeck A (2001) Enterobactin: the characteristic catecholate siderophore of *Enterobacteriaceae* is produced by *Streptomyces* species. FEMS Microbiol Lett 196:147–151

Flak TA, Goldman WE (1999) Signalling and cellular specificity of airway nitric oxide production in pertussis. Cell Microbiol 1:51–60

Freestone PP, Lyte M, Neal CP, Maggs AF, Haigh RD, Williams PH (2000) The mammalian neuroendocrine hormone norepinephrine supplies iron for bacterial

growth in the presence of transferrin or lactoferrin. J Bacteriol 182:6091–6098
Gallegos MT, Schleif R, Bairoch A, Hofmann K, Ramos JL (1997) Arac/XylS family of transcriptional regulators. Microbiol Mol Biol Rev 61:393–410
Giardina PC, Foster L-A, Toth SI, Roe BA, Dyer DW (1995) Identification of *alcA*, a *Bordetella bronchiseptica* gene necessary for alcaligin production. Gene 167:133–136
Giardina PC, Foster LA, Toth SI, Roe BA, Dyer DW (1997) Analysis of the *alcABC* operon encoding alcaligin biosynthesis enzymes in *Bordetella bronchiseptica*. Gene 194:19–24
Granstrom M, Olinder-Nielsen AM, Holmblad P, Mark A, Hanngren K (1991) Specific immunoglobulin for treatment of whooping cough. Lancet 338:1230–1233
Harle C, Kim I, Angerer A, Braun V (1995) Signal transfer through three compartments: transcription initiation of the *Escherichia coli* ferric citrate transport system from the cell surface. EMBO J 14:1430–1438
Heinrichs DE, Poole K (1993) Cloning and sequence analysis of a gene (*pchR*) encoding an AraC family activator of pyochelin and ferripyochelin receptor synthesis in *Pseudomonas aeruginosa*. J Bacteriol 175:5882–5889
Heinrichs DE, Poole K (1996) PchR, a regulator of ferripyochelin receptor gene (*fptA*) expression in *Pseudomonas aeruginosa*, functions both as an activator and as a repressor. J Bacteriol 178:2586–2592
Hewlett EL, Donato GM, Gray MC (2006) Macrophage cytotoxicity produced by adenylate cyclase toxin from *Bordetella pertussis*: more than just making cyclic AMP! Mol Microbiol 59:447–459
Hou Z, Raymond KN, O'Sullivan B, Esker TW, Nishio T (1998) A preorganized siderophore: thermodynamic and structural characterization of alcaligin and bisucaberin, microbial macrocyclic dihydroxamate chelating agents (1). Inorg Chem 37:6630–6637
Hou Z, Sunderland CJ, Nishio T, Raymond KN (1996) Preorganization of ferric alcaligin, Fe_2L_3. the first structure of a ferric dihydroxamate siderophore. J Amer Chem Soc 118:5148–5149
Kameyama T, Takahashi A, Kurasawa S et al (1987) Bisucaberin, a new siderophore, sensitizing tumor cells to macrophage-mediated cytolysis. I. Taxonomy of the producing organism, isolation and biological properties. J Antibiot 40:1664–1670
Kang HY, Armstrong SK (1998) Transcriptional analysis of the *Bordetella* alcaligin siderophore biosynthesis operon. J Bacteriol 180:855–861
Kang HY, Brickman TJ, Beaumont FC, Armstrong SK (1996) Identification and characterization of iron-regulated *Bordetella pertussis* alcaligin siderophore biosynthesis genes. J Bacteriol 178:4877–4884
Kim I, Stiefel A, Plantor S, Angerer A, Braun V (1997) Transcription induction of the ferric citrate transport genes via the N-terminus of the FecA outer membrane protein, the Ton system and the electrochemical potential of the cytoplasmic membrane. Mol Microbiol 23:333–344
Kirby AE, Metzger DJ, Murphy ER, Connell TD (2001) Heme utilization in *Bordetella avium* is regulated by RhuI, a heme-responsive extracytoplasmic function sigma factor. Infect Immun 69:6951–6961
Lagergren J (1963) The white blood cell count and the erythrocyte sedimentation rate in pertussis. Acta Paediatr 52:405–409
Langman L, Young IG, Frost GE, Rosenberg H, Gibson F (1972). Enterochelin system of iron transport in *Escherichia coli*: mutations affecting ferric-enterochelin esterase. J Bacteriol 112:1142–1149
Ledyard KM, Butler A (1997). Structure of putrebactin, a new dihydroxamate siderophore produced by *Shewanella putrefaciens*. J Biol Inorg Chem 2:93–97
Loomis LD, Raymond KN (1991) Solution equilibria of enterobactin and metal-enterobactin complexes. Inorg Chem 30:906–911
Lynch D, O'Brien J, Welch T et al (2001) Genetic organization of the region encoding regulation, biosynthesis, and transport of rhizobactin 1021, a siderophore produced by *Sinorhizobium meliloti*. J Bacteriol 183:2576–2585
Mattoo S, Cherry JD (2005) Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to *Bordetella pertussis* and other *Bordetella* subspecies. Clin Microbiol Rev 18:326–382
Menozzi FD, Gantiez C, Locht C (1991) Identification and purification of transferrin- and lactoferrin-binding proteins of *Bordetella pertussis* and *Bordetella bronchiseptica*. Infect Immun 59:3982–3988
Mills KH, Barnard A, Watkins J, Redhead K (1993) Cell-mediated immunity to *Bordetella pertussis*: role of Th1 cells in bacterial clearance in a murine respiratory infection model. Infect Immun 61:399–410
Moore CH, Foster LA, Gerbig DG Jr., Dyer DW, Gibson BW (1995) Identification of alcaligin as the siderophore produced by *Bordetella pertussis* and *B. bronchiseptica*. J Bacteriol 177:1116–1118
Munoz JJ, Arai H, Bergman RK, Sadowski PL (1981) Biological activities of crystalline pertussigen from *Bordetella pertussis*. Infect Immun 33:820–826
Murphy ER, Sacco RE, Dickenson A et al (2002) BhuR, a virulence-associated outer membrane protein of *Bordetella avium*, is required for the acquisition of iron from heme and hemoproteins. Infect Immun 70:5390–5403
Nicholson ML, Beall B (1999) Disruption of tonB in *Bordetella bronchiseptica* and *Bordetella pertussis* prevents utilization of ferric siderophores, hemin and hemoglobin as iron sources. Microbiology (Reading, United Kingdom) 145:2453–2461
Nishio T, Ishida Y (1990) Production of dihydroxamate siderophore alcaligin by *Alcaligenes xylosoxidans* subsp. *xylosoxidans*. Agr Biol Chem 54:1837–1839
Nishio T, Tanaka N, Hiratake J, Katsube Y, Ishida Y, Oda J (1988) Isolation and structure of the novel dihydroxamate siderophore alcaligin. J Amer Chem Soc 110:8733–8734
O'Brien IG, Gibson F (1970) The structure of enterochelin and related 2,3-dihydroxy-N-benzoylserine conjugates

from *Escherichia coli*. Biochim Biophys Acta 215:393–402

Palenik B, Brahamsha B, Larimer FW et al (2003) The genome of a motile marine *Synechococcus*. Nature 424:1037–1042

Parkhill J, Sebaihia M, Preston A et al (2003) Comparative analysis of the genome sequences of *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica*. Nat Genet 35:32–40

Passerini de Rossi BN, Friedman LE, Belzoni CB et al (2003) Vir90, a virulence-activated gene coding for a *Bordetella pertussis* iron-regulated outer membrane protein. Res Microbiol 154:443–450

Passerini de Rossi BN, Friedman LE, Gonzalez Flecha FL, Castello PR, Franco MA, Rossi JP (1999) Identification of *Bordetella pertussis* virulence-associated outer membrane proteins. FEMS Microbiol Lett 172:9–13

Pradel E, Guiso N, Locht C (1998) Identification of AlcR, an AraC-type regulator of alcaligin siderophore synthesis in *Bordetella bronchiseptica* and *Bordetella pertussis*. J Bacteriol 180:871–880

Pradel E, Guiso N, Menozzi FD, Locht C (2000) *Bordetella pertussis* TonB, a Bvg-independent virulence determinant. Infect Immun 68:1919–1927

Pradel E, Locht C (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

Rabsch W, Voigt W, Reissbrodt R, Tsolis RM, Baumler AJ (1999) *Salmonella typhimurium* IroN and FepA proteins mediate uptake of enterobactin but differ in their specificity for other siderophores. J Bacteriol 181:3610–3612

Redhead K, Hill T, Chart H (1987) Interaction of lactoferrin and transferrins with the outer membrane of *Bordetella pertussis*. J Gen Microbiol 133:891–898

Regan JC, Tolstoouhov A (1936) Relations of acid base equilibrium to the pathogenesis and treatment of whooping cough. N Y State J Med 36:1075–1087

Register KB, Ducey TF, Brockmeier SL, Dyer DW (2001) Reduced virulence of a *Bordetella bronchiseptica* siderophore mutant in neonatal swine. Infect Immun 69:2137–2143

Rodgers SJ, Lee C, Ng CY, Raymond KN (1987) Ferric ion sequestering agents. 15. Synthesis, solution chemistry, and electrochemistry of a new cationic analogue of enterobactin. Inorg Chem 26:1622–1625

Rutz JM, Abdullah T, Singh SP, Kalve VI, Klebba PE (1991) Evolution of the ferric enterobactin receptor in gram-negative bacteria. J Bacteriol 173:5964–5974

Spasojevic I, Armstrong SK, Brickman TJ, Crumbliss AL (1999) Electrochemical behavior of the Fe(III) complexes of the cyclic hydroxamate siderophores alcaligin and desferrioxamine E. Inorg Chem 38:449–454

Sperandio V, Torres AG, Jarvis B, Nataro JP, Kaper JB (2003) Bacteria-host communication: the language of hormones. Proc Natl Acad Sci USA 100:8951–8956

Stainer DW, Scholte MJ (1970) A simple chemically defined medium for the production of phase I *Bordetella pertussis*. J Gen Microbiol 63:211–220

Stojiljkovic I, Baumler AJ, Hantke K (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

Takahashi A, Nakamura H, Kameyama T et al (1987) Bisucaberin, a new siderophore, sensitizing tumor cells to macrophage-mediated cytolysis. II. Physicochemical properties and structure determination. J Antibiot 40:1671–1676

Uhl MA, Miller JF (1994). Autophosphorylation and phosphotransfer in the *Bordetella pertussis* BvgAS signal transduction cascade. Proc Natl Acad Sci USA 91:1163–1167

Vanderpool CK, Armstrong SK (2001) The *Bordetella bhu* locus is required for heme iron utilization. J Bacteriol 183:4278–4287

Vanderpool CK, Armstrong SK (2003) Heme-responsive transcriptional activation of *Bordetella bhu* genes. J Bacteriol 185:909–917

Vanderpool CK, Armstrong SK (2004) Integration of environmental signals controls expression of *Bordetella* heme utilization genes. J Bacteriol 186:938–948

Venuti MC, Rastetter WH, Neilands JB (1979) 1,3,5-Tris(N, N′, N″-2,3-dihydroxybenzoyl)aminomethylbenzene, a synthetic iron chelator related to enterobactin. J Med Chem 22:123–124

Ward JI, Cherry JD, Chang SJ et al (2005) Efficacy of an acellular pertussis vaccine among adolescents and adults. N Engl J Med 353:1555–1563

Weiss AA, Hewlett EL, Myers GA, Falkow S (1983) Tn*5*-induced mutations affecting virulence factors of *Bordetella pertussis*. Infect Immun 42:33–41

Weitl FL, Raymond KN (1979) Ferric ion sequestering agents. 1. Hexadentate O-bonding N,N′,N″-tris(2,3-dihydroxybenzoyl) derivatives of 1,5,9-triazacyclotridecane and 1,3,5-triaminomethylbenzene. J Amer Chem Soc 101:2728–2731

Williams P, Morton DJ, Towner KJ, Stevenson P, Griffiths E (1990) Utilization of enterobactin and other exogenous iron sources by *Haemophilus influenzae*, *H. parainfluenzae* and *H. paraphrophilus*. J Gen Microbiol 136:2343–2350

Wyckoff EE, Lopreato GF, Tipton KA, Payne SM (2005) *Shigella dysenteriae* ShuS promotes utilization of heme as an iron source and protects against heme toxicity. J Bacteriol 187:5658–5664

Zhu M, Valdebenito M, Winkelmann G, Hantke K (2005) Functions of the siderophore esterases IroD and IroE in iron-salmochelin utilization. Microbiology 151:2363–2372