Temporal signaling and differential expression of *Bordetella* iron transport systems: the role of ferrimones and positive regulators

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Abstract The bacterial respiratory pathogens *Bordetella pertussis* and *Bordetella bronchiseptica* employ multiple alternative iron acquisition pathways to adapt to changes in the mammalian host environment during infection. The alcaligin, enterobactin, and heme utilization pathways are differentially expressed in response to the cognate iron source availability by a mechanism involving substrate-inducible positive regulators. As inducers, the iron sources function as chemical signals termed ferrimones. Ferrimone-sensing allows the pathogen to adapt and exploit early and late events in the infection process.

Keywords *Bordetella* · Iron · Heme · Siderophore · Regulation · Ferrimone

Introduction

Bordetella species

Members of the genus *Bordetella* are Gram-negative β-proteobacteria of the family *Alcaligenaceae*. *Bordetella pertussis*, *Bordetella parapertussis*, and

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Bordetella bronchiseptica, the so-called classical *Bordetella* species*,* are highly genetically related respiratory pathogens of mammals (Mattoo and Cherry 2005; Parkhill et al. 2003). *B. pertussis* is a human-adapted species and is the agent of whooping cough or pertussis. *B. parapertussis* infects humans and other animals including sheep (Mattoo and Cherry 2005) and *B. bronchiseptica* causes respiratory diseases in a variety of nonhuman mammalian hosts. Based on genomic sequence analyses, *B. bronchiseptica* or a *B. bronchiseptica*-like organism is the hypothesized progenitor of *B. pertussis* and *B. parapertussis* (Parkhill et al. 2003).

B. pertussis, *B. parapertussis*, and *B. bronchiseptica* colonize the cilia of the ciliated cells of the host respiratory epithelium where they multiply and elaborate a variety of factors, resulting in disease symptoms. Their virulence factors include adhesins such as filamentous hemagglutinin, a type III secretion system, dermonecrotic toxin and an adenylate cyclase/hemolysin (Confer and Eaton 1982; Fennelly et al. 2008; Hewlett and Wolff 1976; Katada and Ui 1982; Kozak et al. 2007). Tracheal cytotoxin is also released by the bacteria. This toxin is a peptidoglycan-derived disaccharide tetrapeptide that triggers host nitric oxide production that leads to extrusion of the ciliated cells from the epithelium (Flak and Goldman 1999). An additional virulence factor produced only by *B. pertussis* is the ADP-ribosylating pertussis toxin. Production of most of the known *Bordetella* virulence factors is transcriptionally controlled by a

two-component phosphorelay system consisting of the BvgS transmembrane sensor kinase and the BvgA DNA-binding response regulator (Uhl and Miller 1994; Weiss et al. 1983).

Bordetella iron retrieval

During infection, host cells and *Bordetella* cells coordinate their activities in response to one another in a dynamic environment. As both host and bacterial cells are damaged and cellular products are liberated, the nutrients that become available to *Bordetella*, including iron, are likely to fluctuate over the course of the infection. Sensitive iron source-sensing mechanisms allow *Bordetella* to adapt to these fluctuations.

B. pertussis, B. parapertussis and *B. bronchiseptica* have 14–19 genes encoding TonB-dependent outer membrane receptors that are known or predicted to be involved in iron uptake. To obtain iron, these species produce and utilize the siderophore alcaligin (Brickman et al. 1996; Moore et al. 1995). *B. pertussis* and *B. bronchiseptica* are also capable of using the xenosiderophores enterobactin (Beall and Sanden 1995a), ferrichrome and desferrioxamine B (Beall and Hoenes 1997). *B. bronchiseptica* has also been reported to use ferrichrysin, ferrirubin, aerobactin, protochelin, schizokinen, ferricrocin, vicibactin, and pyoverdin (Pradel and Locht 2001). In addition, these *Bordetella* species can also utilize hemin as an iron source (Vanderpool and Armstrong 2001). The *Bordetella* genetic systems for only three known iron sources have been characterized to date: alcaligin, enterobactin and heme. Under

iron-replete growth conditions the genes of these three iron systems are repressed by Fur (Beall and Sanden 1995b; Brickman and Armstrong 1995; Brickman et al. 2004). Importantly, each is also under positive transcriptional regulation that involves induction by the cognate iron source under derepressing conditions.

Alcaligin siderophore system

Alcaligin (1,8(S),11,18(S)-tetrahydroxy-1,6,11,16-tetraazacycloeicosane-2,5,12,15-tetrone) is a 20-membered macrocyclic dihydroxamate siderophore (Nishio et al. 1988; Nishio and Ishida 1990). The alcaligin system gene cluster spans a 10.6-kb contiguous region of the *Bordetella* chromosome (Fig. 1), and encodes alcaligin biosynthesis enzymes, the AlcR substrate-inducible transcriptional regulator (Beaumont et al. 1998; Pradel et al. 1998; Brickman et al. 2001), the alcaligin exporter AlcS (Brickman and Armstrong 2005), and the outer membrane FauA receptor protein for transport of ferric alcaligin (Brickman and Armstrong 1999). The organization of the alcaligin system gene cluster is conserved among the classical *Bordetella* species (Parkhill et al. 2003). The genetic system has four transcriptional units: a polycistronic *alcABCDER* transcript, and monocistronic transcripts for *alcR*, *alcS* and *fauA* (Beaumont et al. 1998; Kang and Armstrong 1998; Pradel et al. 1998; Brickman and Armstrong 1999; Brickman and Armstrong 2005).

The *alcABCDER* operon encodes alcaligin biosynthesis and regulatory functions (Armstrong and Clements 1993; Giardina et al. 1995; Kang et al. 1996; Giardina et al. 1997; Kang and Armstrong 1998;

Fig. 1 Genetic organization of the alcaligin, enterobactin, and heme utilization systems in *Bordetella* species. Arrows denote the limits and transcriptional orientations of genes. Locations of Fur-binding sites (rectangles) are shown, and circles represent control regions that are responsive to the cognate positive regulator and iron source inducer
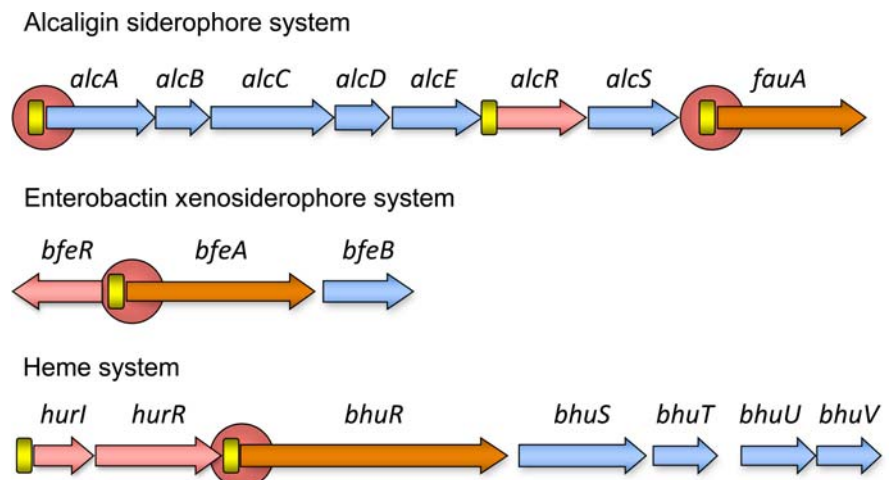
Beaumont et al. 1998; Pradel et al. 1998). The pathway for alcaligin biosynthesis has not been fully elucidated and mechanistic details remain mostly hypothetical (Brickman and Armstrong 1996; Giardina et al. 1995; Kang et al. 1996; Pradel et al. 1998; Challis 2005); however, the initial step in alcaligin biosynthesis in Bordetellae is known to involve decarboxylation of ornithine to produce putrescine, catalyzed by the pyridoxal phosphate-dependent decarboxylase Odc (Brickman and Armstrong 1996). It is believed that putrescine is then converted to N-hydroxyputrescine by AlcA, a putative 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 with succinyl CoA by a process similar to the IucB-dependent acylation of $N6$-hydroxylysine with acetyl CoA in the aerobactin pathway. The product could then undergo C-hydroxylation mediated by AlcE, which has 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, followed by nucleotide triphosphate-dependent macrocyclization to yield alcaligin. The role for AlcD in alcaligin biosynthesis is unclear. Alcaligin binds ferric iron at a 3:2 molar ratio (Fe_2Alc_3) at physiological pH, and the complex has a stability constant estimated at 10^{37} M^{-1} (Nishio et al. 1988).

AlcR is a 36-kDal protein of the AraC/XylS family of transcriptional regulators (Gallegos et al. 1997) that has high similarity to the Bordetella BfeR regulator that activates enterobactin utilization genes in response to enterobactin and certain other catechol compounds (Anderson and Armstrong 2004). During iron starvation, transcription of fauA and alcABCDER is activated by AlcR by a mechanism requiring induction by alcaligin (Brickman et al. 2001; Brickman and Armstrong 2002). Thus, AlcR functions as a substrate-inducible regulator of alcaligin biosynthesis and transport genes. Expression of alcR itself is subject to positive autoregulation. AlcR-mediated transcriptional activation is ultrasensitive, allowing the bacterium to sense and respond to extremely low concentrations of inducer in its environment (Brickman et al. 2001).

Export of monomeric alcaligin from the bacterial cell requires AlcS, a permease of the major facilitator superfamily class of proton motive force-dependent membrane efflux pumps (Brickman and Armstrong 2005). The exporter activity of AlcS is also critical for maintenance of appropriate intracellular alcaligin substrate levels for normal AlcR-dependent induction of alcaligin system gene expression. The alcS gene is expressed constitutively from a monocistronic transcript that is produced independent of cellular iron status (Kang and Armstrong 1998; Brickman and Armstrong 2005).

Bordetella alcaligin system genes are repressible by Fur and iron (Beall and Sanden 1995b; Brickman and Armstrong 1995) (Fig. 1) acting at three control regions. The alcABCDER operon is transcribed from a Fur and iron-repressible promoter upstream of alcA (Brickman and Armstrong 1995; Kang et al. 1996; Kang and Armstrong 1998). The alcR gene is also expressed as a monocistronic transcript originating at another Fur-regulated promoter immediately upstream of the alcR gene; the activity of this secondary alcR promoter is independent of the autoregulatory influence of AlcR acting at the alcABCDER control region (Beaumont et al. 1998). A third Fur-controlled promoter-operator region resides upstream of the fauA outer membrane receptor gene (Brickman and Armstrong 1999).

Enterobactin xenosiderophore system

Enterobactin has an extremely high ferric ion stability constant of 10^{49} M^{-1} (Loomis and Raymond 1991) and is commonly produced by members of the Enterobacteriaceae. As obligate mucosal pathogens, B. pertussis and B. bronchiseptica have no known external environmental reservoirs; however, they can use enterobactin to obtain iron and their expression of enterobactin transport systems implies that they naturally encounter enterobactin. The ability to use this high-affinity siderophore may confer to them a competitive advantage in the host.

The Bordetella BfeA outer membrane receptor is required for transport and utilization of ferric enterobactin (Beall and Sanden 1995a). Downstream of bfeA is the predicted cotranscribed and translationally coupled bfeB gene that encodes a member of the α/β superfamily of hydrolases (Fig. 1). BfeB exhibits significant amino acid sequence similarity to the periplasmic Salmonella enterica IroE protein involved in hydrolytic cleavage of salmochelin, the glucosylated form of enterobactin (Zhu et al. 2005). Divergently transcribed from bfeA is the bfeR gene

that encodes an AraC/XylS-like regulator that is highly similar to *Bordetella* AlcR involved in alcaligin gene regulation, with the greatest similarity residing in the putative C-terminal DNA binding domain. Three Fur repressor DNA-binding sites had been predicted upstream of *bfeA* in *B. pertussis* (Beall and Sanden 1995a) and functional binding of Fur to the 0.4 kb *bfeA-bfeR* intergenic region in *E. coli* was demonstrated using the Fur titration assay (Anderson and Armstrong 2004).

During iron starvation, expression of *bfeA,* and likely *bfeB,* is stimulated in the presence of the transport system substrate, enterobactin, by a BfeR-dependent mechanism (Anderson and Armstrong 2004). Since BfeR is a predicted cytoplasmic AraC family regulator, its activation likely requires direct contact with enterobactin. Interestingly, although growth stimulation by ferric enterobactin requires the BfeA receptor, transcriptional activation of the *bfeA* gene by BfeR and enterobactin requires neither BfeA (Anderson and Armstrong 2004) nor TonB (Anderson and Armstrong 2006). These observations indicate that, as with the FauA- and TonB-independent induction of the alcaligin system by alcaligin, the enterobactin inducer also enters the cell by a route that does not rely on the ferric siderophore's outer membrane receptor or any other TonB dependent receptor.

Since the upper respiratory tract is the natural colonization site for *B. pertussis* and *B. bronchiseptica*, yet enterobactin is produced primarily by inhabitants of the intestinal tract, the utilization of enterobactin by *Bordetella* is obscure. It is possible that certain respiratory tract commensals produce enterobactin or a structurally similar siderophore, or that members of the *Enterobacteriaceae* can transiently colonize the host upper respiratory tract and release enterobactin. Alternatively, the authentic ligand for BfeA may be a host molecule that bears structural similarity to enterobactin.

The ability of other catechol siderophores to be transported into *Bordetella* cells *via* BfeA was examined. The *B. bronchiseptica* BfeA receptor protein could transport the ferric catechol siderophores salmochelin and corynebactin to promote bacterial growth in iron-restricted medium. When these siderophores were tested for the ability to activate BfeR-mediated transcription of *bfeA*, salmochelin was found to have inducing activity but there

was little, if any, induction by corynebactin. In comparison, enterobactin was the most potent inducer and stimulator of *Bordetella* growth (Anderson and Armstrong 2006).

Host neuroendocrine catecholamines such as epinephrine, norepinephrine, and dopamine bear structural similarity to the iron-binding ligands of enterobactin and also have the ability to bind iron (Jewett et al. 1997; Raymond et al. 1976). Norepinephrine was reported to induce *E. coli* FepA enterobactin receptor production (Burton et al. 2002). Previous studies have examined the role of norepinephrine in removing the iron from transferrin and lactoferrin, making it available for growth of *E. coli* (Burton et al. 2002; Freestone et al. 2003) and *Salmonella enteriditis* (Methner et al. 2008). Epinephrine, norepinephrine, and dopamine were found to induce *bfeA* expression levels in *Bordetella* by more than 28-fold (Anderson and Armstrong 2006). Induction by norepinephrine is iron-regulated and dependent on the BfeR regulator, similar to induction by enterobactin. Since catecholamine-induced *bfeA* transcription may be solely due to structural similarity to enterobactin and not necessarily a biologically relevant observation, the ability of norepinephrine to provide iron for *Bordetella* growth was examined.

Growth of *B. bronchiseptica* in iron-depleted and serum-supplemented medium was enhanced by addition of norepinephrine (Anderson and Armstrong 2006). This growth promotion was siderophore-independent. When iron-depleted culture medium was supplemented with partially iron-saturated transferrin instead of serum, growth of *B. bronchiseptica* was dramatically enhanced by norepinephrine (Anderson and Armstrong 2008). Most importantly, *B. bronchiseptica alcA* alcaligin biosynthesis mutants that do not produce siderophore can obtain transferrin-bound iron via norepinephrine. These observations contrast with those showing that norepinephrine-mediated growth of *E. coli* or *S. enterica* requires either enterobactin or breakdown products of enterobactin or salmochelin (Burton et al. 2002; Methner et al. 2008; Freestone et al. 2003). In *Bordetella*, norepinephrine-mediated transferrin-iron uptake requires neither the BfeA enterobactin receptor nor the alcaligin siderophore or its FauA receptor (Anderson and Armstrong 2008). Preliminary evidence indicates involvement of the TonB system in norepinephrine-mediated iron acquisition (Anderson and Armstrong 2008). Since *Bordetella* cells

inhabit the respiratory mucosa, one may reason that lactoferrin would be the host glycoprotein most commonly encountered. During infection, host catecholamines as well as transferrin from the bloodstream may access epithelial surfaces damaged by *Bordetella* toxins; thus it is conceivable that the *Bordetella* catecholamine iron retrieval mechanism may be biologically relevant to growth in the host.

Heme system

B. pertussis and *B. bronchiseptica* can use hemin and hemoglobin as sole sources of iron, and this utilization requires the TonB system (Nicholson and Beall 1999; Pradel et al. 2000). The *B. pertussis* and *B. bronchiseptica hurIR bhuRSTUV* gene cluster (Fig. 1) associated with heme utilization and regulatory functions has been identified and characterized (Vanderpool and Armstrong 2001). The bird pathogen *Bordetella avium* is a more genetically distant species and has an orthologous heme utilization gene cluster, *rhuIR bhuRSTUV* (Murphy et al. 2002). BhuR is the outer membrane receptor for hemin; it has a predicted TonB box C motif and the amino acid sequence motif (FRAP/NPNL) that is conserved among members of the bacterial heme receptor family (Vanderpool and Armstrong 2001). For the transport of heme, BhuT, BhuU and BhuV are the predicted periplasmic heme-binding protein, cytoplasmic membrane permease and ATP binding proteins, respectively. The possible function of BhuS is not clear. The homologous ShuS protein of *Shigella dysenteriae* can bind heme and is thought to function as a chaperone to sequester internalized heme, thus preventing heme toxicity (Wyckoff et al. 2005).

The BhuR outer membrane heme receptor has an N-terminal extension of 70 amino acids that is similar to the specialized N-terminal regions of members of the *E. coli* ferric citrate receptor FecA superfamily. These proteins are involved in iron source-inducible extracytoplasmic function (ECF) sigma factor-dependent regulation of iron genes. The receptor N-terminal extension is essential for the signaling cascade that leads to transcription initiation via a cognate ECF σ factor (Kim et al. 1997; Braun and Mahren 2005). In *Bordetella* cells, the other participants in the signaling cascade are the homologs of *E. coli* FecI and FecR proteins, HurI (ECF sigma factor) and the HurR anti-sigma membrane sensor (Vanderpool and Armstrong 2001). Initial studies demonstrated that in iron-starved *Bordetella* cells, production of the BhuR heme receptor was significantly elevated in response to addition of hemin to the culture (Vanderpool and Armstrong 2001). Increased BhuR production was transcriptionally controlled and required a functional *hurI* gene (Vanderpool and Armstrong 2003). As predicted, *hurI* mutants are unable to use heme effectively but can be complemented by *hurI*. Expression of *hurI* from a high copy number plasmid suppressed the heme inducer requirement, resulting in heme-independent *bhuR* expression.

Transcriptional activation of the *bhu* genes in response to heme is dose-dependent and specific for heme; other heme structural analogs such as chlorophyll *a*, protoporphyrin IX and zinc-protoporphyrin IX did not activate *bhu* gene expression (Vanderpool and Armstrong 2004). The promoters controlling expression of the *hurIR bhuRSTUV* genes were mapped (Vanderpool and Armstrong 2004). The *hurI* promoter has similarity to σ^{70}-like promoters and its transcription is Fur- and iron-repressible and heme independent. Results from those studies indicate that transcription originating at the *hurI* promoter reads through the *hurR–bhuR* region, contributing to *bhuR* expression under iron-starvation conditions in the absence of heme. Heme-responsive transcription originating at *bhuR* is HurI-dependent. A recent report by the Connell group showed that HurP, a predicted RseP protease homolog encoded outside of the *hur–bhu* gene cluster, is required for heme utilization and heme-inducible *bhuR* expression and is proposed to function by modifying the HurR protein (King-Lyons et al. 2007).

Based on results from our studies (Vanderpool and Armstrong 2001, 2003, 2004), those from Connell and colleagues (King et al. 2005; King-Lyons et al. 2007; Kirby et al. 2001; Murphy et al. 2002), and knowledge from the *E. coli* Fec system (Harle et al. 1995; Ochs et al. 1995, 1996), a model of *Bordetella bhu* gene regulation has emerged. Upon iron-depletion, Fur derepression at the *hurI* promoter leads to *hurIR* transcription and read-through transcription of *bhuRSTUV*, resulting in basal production levels of the heme transport proteins. Under these conditions, the HurR sensor is predicted to bind HurI. When BhuR binds heme, this signal is transmitted across the

periplasm via contact of its specialized N-terminus with HurR. BhuR–HurR interaction allows release and/or activation of HurI, enabling it to associate with RNA polymerase to promote transcription at the *bhuR* promoter. HurP may serve to proteolytically alter HurR to modulate the signaling cascade. This *Bordetella* mechanism of cell surface signaling by heme ensures that *bhuRSTUV* are up-regulated only when the cognate heme source is present.

Temporal signaling and the infection process

On the upper respiratory tract epithelium, the interaction of *B. pertussis* with its human host characteristically results in inflammation, activation of immune responses, and injury to host tissues. Over the course of infection, changes in host niche conditions alter the array and abundance of iron sources available to the organism. The involvement of the substrate-inducible positive regulators AlcR (Beaumont et al. 1998; Pradel et al. 1998), BfeR (Anderson and Armstrong 2004), and HurI (Vanderpool and Armstrong 2001) ensures that the iron systems can be differentially expressed, allowing for effective adaption to changes in iron source availability during infection.

The importance of the alcaligin (Brickman and Armstrong 2007), heme (Brickman et al. 2006) and enterobactin (Brickman et al. 2008) systems for in vivo growth of *B. pertussis* was established in mixed infection competition experiments using a wild-type strain together with an isogenic *fauA*, *bhuR* or *bfeA* receptor mutant in a mouse respiratory infection model system. Alcaligin, enterobactin and heme receptor mutants were each attenuated for virulence, indicating that each iron utilization system makes a distinct and essential contribution to in vivo multiplication and survival of *B. pertussis*, albeit at different stages of infection. The alcaligin system had a crucial role in initial colonization and over the entire course of infection. The enterobactin system contributed significantly to initial growth but to a lesser extent than the alcaligin system, and its role diminished after peak bacterial multiplication. Interestingly, the heme system was not needed for growth until after the peak of *B. pertussis* growth, at which time it was essential. Notably, the phasing of alcaligin, enterobactin and heme system involvement implies that host iron sources indeed change as infection progresses and

that *B. pertussis* can adapt to those changes by deploying different iron acquisition systems.

Temporal expression patterns of the alcaligin, enterobactin and heme systems were examined using recombinase-based in vivo expression technology (RIVET) in a mouse respiratory infection model (Brickman et al. 2008). The three systems were found to be differentially expressed in vivo, showing early induction of the alcaligin and enterobactin siderophore systems and delayed induction of the heme system. These results correlated with those from our competition infection experiments indicating the importance of siderophores early in infection and the heme system at later stages of infection. These RIVET transcription studies confirmed that *B. pertussis* expresses these three iron acquisition systems in a host and the relevant iron sources are present, while the competition infection experiments demonstrated that *B. pertussis* requires these iron acquisition systems in vivo. Overall, the results render a pattern of *Bordetella* iron acquisition in the host that is characterized by early reliance on siderophores and subsequent dependence on heme.

Pertussis patients mount an antibody response to *Bordetella* iron receptors. A collection of human sera obtained from normal uninfected adults and from *B. pertussis* culture-positive patients was screened for antibody reactivity with iron-regulated *Bordetella* membrane proteins (Brickman et al. 2008). Eight of ten sera from infected donors reacted with multiple iron-repressible proteins, including the receptors for alcaligin (FauA), enterobactin (BfeA) and heme (BhuR), whereas 3 of 10 samples from uninfected donors reacted strongly with these receptor proteins. This survey supplied evidence that *B. pertussis* produces these receptors in the human host during natural infection, confirms that *B. pertussis* experiences iron starvation in vivo, and implies that the cognate iron source inducers are present in the human host environment.

Iron source 'ferrimones' integrate environmental and intracellular signals to prioritize expression of the iron transport systems

Intercellular chemical signaling enables a population of bacteria to regulate gene expression collectively in response to environmental signals produced by the same or different species, and in response to host
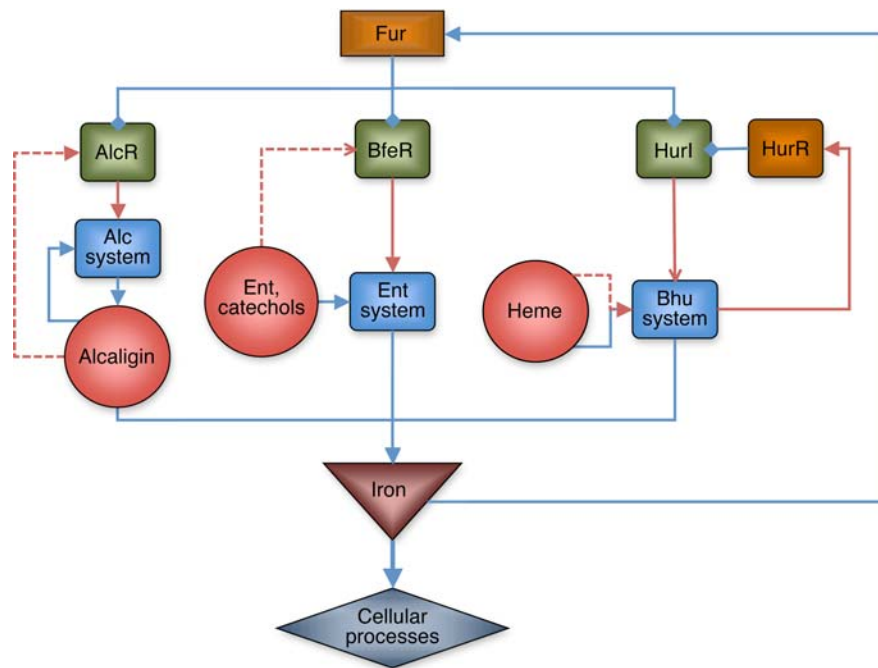
Fig. 2 Regulatory circuits controlling expression of the alcaligin, enterobactin, and heme utilization systems in *Bordetella* species. Fur and iron control the expression of three parallel regulatory circuits that respond to the cognate iron source via distinct substrate-inducible positive regulators (AlcR, BfeR, and HurI). The circuits are activated independently, and are not subject to interference or signal antagonism. All three circuits converge to supply the requisite nutrient iron. When the bacterium's iron demands are satisfied, iron activates the DNA-binding activity of the Fur repressor and iron transport system expression is globally repressed

signals and environmental cues. *Bordetella* species possess multiple ultrasensitive iron source-sensing circuits organized in parallel (Fig. 2). In their role as inducers, the alcaligin, heme and enterobactin iron sources function as signaling molecules we have termed ferrimones that activate the cognate iron transport system through the action of the specific substrate-inducible positive regulators, AlcR, BfeR and HurIR. Ferrimone-sensing underlies the bacterium's ability to discriminate and integrate multiple iron source availability cues to coordinate expression of the iron transport systems. As the iron acquisition systems are activated independently, they are not subject to interference or signal antagonism, and all circuits converge to supply the requisite nutrient iron. When the bacterium's iron demands are satisfied, iron activates the DNA-binding activity of the Fur repressor and iron transport system expression is globally repressed. Ferrimone-sensing thus acts to support, promote, and exploit early and late events in the infection process to assure growth and survival of the pathogen.

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