



Published in final edited form as:

*Mol Microbiol.* 2008 October ; 70(1): 3–14. doi:10.1111/j.1365-2958.2008.06333.x.

## Differential expression of *Bordetella pertussis* iron transport system genes during infection

Timothy J. Brickman, Tomoko Hanawa<sup>†</sup>, Mark T. Anderson<sup>‡</sup>, Ryan J. Suhadolc, and Sandra K. Armstrong<sup>\*</sup>

Department of Microbiology, University of Minnesota Medical School, 925 Mayo Memorial Building, 420 Delaware Street, S.E., Minneapolis, MN 55455-0312

### Summary

Temporal expression patterns of the *Bordetella pertussis* alcaligin, enterobactin, and heme iron acquisition systems were examined using *alcaA*-, *bfeA*-, and *bhuR-tnpR* recombinase fusion strains in a mouse respiratory infection model. The iron systems were differentially expressed in vivo, showing early induction of the alcaligin and enterobactin siderophore systems, and delayed induction of the heme system in a manner consistent with predicted changes in host iron source availability during infection. Previous mixed infection competition studies established the importance of alcaligin and heme utilization for *B. pertussis* in vivo growth and survival. In this study, the contribution of the enterobactin system to the fitness of *B. pertussis* was confirmed using wild-type and enterobactin receptor mutant strains in similar competition infection experiments. As a correlate to the in vivo expression studies of *B. pertussis* iron systems in mice, sera from uninfected and *B. pertussis*-infected human donors were screened for antibody reactivity with *Bordetella* iron-repressible cell envelope proteins. Pertussis patient sera recognized multiple iron-repressible proteins including the known outer membrane receptors for alcaligin, enterobactin, and heme, supporting the hypothesis that *B. pertussis* is iron-starved and responds to the presence of diverse iron sources during natural infection.

### Keywords

*Bordetella pertussis*; iron; alcaligin; enterobactin; heme; RIVET

### Introduction

It could be reasoned that the main objective of a pathogen is to survive and multiply. In order to multiply, successful pathogens must be able to scavenge growth-limiting nutrients in the host environment, yet the abundance and source of nutrients may change as an infection progresses, and these changes can decisively affect microbial growth rates and population sizes. One axiom that has emerged from in vivo expression studies of a variety of host-pathogen systems is that nutrient acquisition genes, especially those involved in iron acquisition, represent the prevalent class of in vivo expressed genes (Mahan *et al.*, 2000).

*Bordetella pertussis*, the causative agent of human whooping cough or pertussis, colonizes the ciliated cells of the upper respiratory tract epithelium. The interaction of *B. pertussis*

<sup>\*</sup>E-mail: armst018@umn.edu; Tel. (+1) 612 625 6947; Fax (+1) 612 626 0623.

<sup>†</sup>Present address: Department of Infectious Diseases, Kyorin University School of Medicine, 6-20-2, Shinkawa Mitaka, Tokyo 181-8611, JAPAN

<sup>‡</sup>Present address: Department of Microbiology-Immunology, Feinberg School of Medicine, Northwestern University, 320 East Superior Street, Searle 3-420, Chicago, IL 60611-3010

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 are presumed to alter the array and abundance of iron sources available to *B. pertussis*. The alcaligin siderophore, enterobactin siderophore, and heme utilization systems of *B. pertussis* are genetically characterized and share key regulatory features (Brickman *et al.*, 2001; Vanderpool and Armstrong, 2003; Anderson and Armstrong, 2004) (Table 1). All three systems are repressible by Fur and iron, and maximal expression of each system during iron starvation requires a distinct transcriptional activator that is responsive to the cognate iron source as the inducer. We hypothesize that 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 adaptation to changes in iron source availability during infection. In addition to the outer membrane receptor proteins required for transport of ferric alcaligin (FauA), ferric enterobactin (BfeA) and heme (BhuR), at least nine more genes encoding predicted TonB-dependent receptors of unknown ligand specificity are encoded in the *B. pertussis* genome (Parkill *et al.*, 2003; Anderson and Armstrong, 2008).

Upon initial colonization of the host mucosal surface it is conceivable that *B. pertussis* uses available lactoferrin as an iron source by way of its native siderophore alcaligin. Other siderophores produced by transient microbes or commensals might also deliver iron to *B. pertussis*. As *B. pertussis* multiplies, inflammation and pathologic effects of *Bordetella* toxic factors on the host epithelial barrier could allow iron-loaded cellular and serum components to escape to the mucosal surface (Persson *et al.*, 1991). Ultimately, host cell lysis would release yet additional iron sources such as transferrin and heme proteins that are readily utilized by *B. pertussis*. This postulated series of events spawned the hypothesis that iron utilization systems are differentially expressed in vivo. It was predicted that siderophore-based iron acquisition systems would be essential for *B. pertussis* multiplication and survival during the initial colonization stage for establishment of infection, but that heme utilization would be more important to ecological fitness later in infection once the epithelial barrier is damaged.

Previously, the importance of the alcaligin (Brickman and Armstrong, 2007) and heme (Brickman *et al.*, 2006) systems for multiplication and survival of *B. pertussis* was established using mixed infection competition studies involving co-infection of mice with wild-type and *fauA* or *bhuR* receptor mutant strains. In those studies, the alcaligin system was found to be important for iron acquisition at the initial colonization stage, whereas the heme system was not essential until later in infection, after the peak of bacterial multiplication. Since the two iron systems have different substrates, the distinct phasing of alcaligin and heme system involvement implies that host iron sources changed as the infections progressed. Further investigation was required to determine whether these presumed variations in iron source availability resulted in differential expression of the *B. pertussis* iron systems at the transcriptional level. In this study, temporal expression patterns of key promoters of the *B. pertussis* alcaligin, enterobactin, and heme utilization systems were monitored using recombinase-based in vivo expression technology (RIVET) (Camilli *et al.*, 1994) in mice. The iron systems were differentially expressed during infection in a manner consistent with variations in iron sources, showing early induction of the siderophore-based systems and late induction of the heme system. To augment the transcriptional analysis of the enterobactin utilization system in this study, and complement previous infection studies confirming that the alcaligin and heme iron systems contribute significantly to in vivo fitness of *B. pertussis*, the importance of the enterobactin system for *B. pertussis* growth and survival was established using mixed infection competition experiments in mice. As a correlate to in vivo fitness and expression studies using the mouse

respiratory infection model, sera from normal human donors and pertussis patients were screened for antibody reactivity with *Bordetella* iron-repressible cell envelope proteins. The finding that pertussis patient sera were reactive with numerous iron-repressible cell envelope proteins including iron transport system receptor proteins suggests that *B. pertussis* is iron-starved in its natural human host environment and responds to the presence of diverse iron sources by expression of multiple *B. pertussis* iron utilization systems.

## Results and Discussion

### Construction of *B. pertussis* iron transport system promoter-resolvase fusion strains

Three different *B. pertussis* iron transport system promoter-*tnpR* fusion strains were constructed for monitoring expression of the alcaligin, enterobactin, and heme utilization systems by placing *tnpR* expression under the control of the substrate-inducible *alcA* (Kang and Armstrong, 1998; Brickman *et al.*, 2001), *bfeA* (Anderson and Armstrong, 2004) and *bhuR* (Vanderpool and Armstrong, 2004) promoters (Fig. 1). The TnpR recombinase can then catalyze the excision of the *resI* site-flanked kanamycin resistance marker on the integrated fusion plasmid, resulting in a stable kanamycin-sensitive phenotype that can be scored in *B. pertussis* populations recovered from infected mice as an indicator of prior expression of a particular iron system. The *B. pertussis* RIVET system (Veal-Carr and Stibitz, 2005) can employ various combinations of the *tnpR* resolvase and *res* site variant alleles devised previously to make the reporter system amenable to use with promoters having low-to-moderate basal expression levels (Lee *et al.*, 1999). In the current study, preliminary in vitro experiments using the attenuated *tnpR135* and *tnpR168* alleles to construct *B. pertussis* RIVET strains found that the reduced TnpR production levels from these mutant alleles were insufficient to catalyze resolution of a *resI* site-flanked marker when expressed in *B. pertussis* from the iron system promoters (data not presented), whereas production of TnpR using the wild-type allele was associated with normally regulated expression and high resolution frequencies under culture conditions known to activate the promoters. Accordingly, all gene fusions in this study used the wild-type *tnpR* allele in conjunction with the *resI* resolvase substrate. Resolvase substrates were stably maintained during in vitro cloning and strain construction procedures by supplementing culture media with additional iron over the standard formulations. In addition, since the *bhuR* heme receptor gene promoter is induced by heme under iron-limiting growth conditions (Vanderpool and Armstrong, 2001), heme-free Stainer-Scholte charcoal agar medium (SCMB) was used instead of Bordet-Gengou agar (BG) (Bordet and Gengou, 1906) in *bhuR-tnpR B. pertussis* strain construction procedures.

### Analysis of *B. pertussis* iron system promoter activities in vitro

The alcaligin, enterobactin, and heme utilization systems of *B. pertussis* have been studied extensively, and their genetic and transcriptional organizations, substrate and inducer specificities, and regulatory features are well characterized (Brickman *et al.*, 2004; Brickman *et al.*, 2007). To confirm that the iron promoter-*tnpR* fusion genes were transcriptionally responsive to iron nutritional status and substrate induction, resolution frequencies were determined under controlled growth conditions in vitro. It was anticipated that the resolution frequencies would be influenced primarily by the iron co-repressor concentration and its effect on the intensity of the iron starvation signal, positive regulator gene expression levels and effective inducer concentrations, and intrinsic iron system promoter strength.

After growth to post-exponential phase (48 h) under iron-replete (*i.e.*, repressing) growth conditions, mean resolution frequencies were 7% or less for all three fusion strains. Under iron starvation growth conditions in the absence of inducer supplements, the *bfeA-tnpR* and

*bhuR-tnpR* fusion strains showed moderate elevation of mean resolution frequencies to 26% and 36%, respectively, consistent with relief of Fur repression. In contrast, the *alcA-tnpR* strain had a mean resolution frequency of 96%, indicative of strong *alcA* promoter activation known to occur during iron starvation in the presence of the autogenously produced alcaligin siderophore inducer (Brickman *et al.*, 2001). Likewise, when supplied with enterobactin or heme inducers under iron starvation conditions, promoter activation in the *bfeA-tnpR* and *bhuR-tnpR* fusion strains resulted in mean resolution frequencies of 91% and 92%, respectively, consistent with their known inducer requirements for maximal promoter activation (Vanderpool and Armstrong, 2003; Anderson and Armstrong, 2004). These *in vitro* RIVET assays established that resolution frequencies for all fusion strains correlated well with previously characterized transcriptional activities associated with iron-repressed, derepressed, and induced expression levels of the *alcA*, *bfeA*, and *bhuR* iron system promoters (Brickman *et al.*, 2001; Vanderpool and Armstrong, 2003; Anderson and Armstrong, 2004).

### Differential expression of iron transport system promoters *in vivo*

The three *B. pertussis* RIVET strains were used individually in mouse respiratory infection experiments for temporal analysis of alcaligin, enterobactin, and heme system gene expression. Since the use of heme-free medium was necessary to prevent induction of the heme-inducible *bhuR-tnpR* fusion in strain TohI-*res-R-bhuR*, the same heme-free SCMB medium was used for the other two fusion strains as well. Plating efficiencies of *B. pertussis* fusion strains on SCMB were found to be similar to those on BG, with mean CFU values differing by no more than 6% when determined in parallel (data not presented).

The total CFU and basal resolution frequencies for the mouse input inocula were determined to be  $2.06 \times 10^6$  CFU (3.9% resolution frequency) for TohI-*res-R-alcA*,  $2.15 \times 10^6$  CFU (1.9% resolution frequency) for TohI-*res-R-bfeA*, and  $1.02 \times 10^6$  CFU (2.9% resolution frequency) for strain TohI-*res-R-bhuR*. Over the 7d course of the infection study, all three strains showed total CFU levels that are typical of *B. pertussis* Tohama I mouse respiratory infections (Fig. 2A, C, E). All fusion strains showed a significant increase in resolution by 1d post-infection compared with the base-line resolution frequencies of the input population; however, the temporal changes in resolution frequencies varied considerably among strains through day 7 (Fig. 2B, D, F). The *alcA-tnpR* alcaligin system promoter fusion strain resolved to 21% by day 1, further increased to 55% mean resolution by day 3, then displayed a progressive increase in resolution through day 5 to reach 91% by day 7. The changes in mean resolution frequencies were statistically significant for all consecutive sampling time intervals for the *alcA-tnpR* fusion strain (*P* values ranged from 0.001 to 0.047). The *bhuR-tnpR* heme system fusion strain resolved to 18% by day 1, displayed a limited increase to 24% and 28% resolution by days 3 and 5, but experienced a significant increase in resolution between days 5 and 7 (*P* = 0.022), achieving 44% resolution on day 7. The *bfeA-tnpR* enterobactin system fusion strain resolved to 16% by day 1, increased significantly again to 23% by day 3 (*P* = 0.013), held at 23% and 22% resolution at days 3 and 5, and reached only 25% by 7 days post-infection. The fold-change in resolution relative to the basal resolution frequency of the input population provides a numerical value we have termed the resolution index (Fig. 3). The resolution index is a useful measure for comparing temporal changes in iron system gene expression, normalized for differences in basal transcriptional activity and intrinsic promoter strength. The resolution index increased steadily for the *alcA-tnpR* strain over the entire 7d experiment, whereas the resolution index values for the *bfeA-tnpR* strain increased during the first day, and between days 1 and 3, but were maximal at day 3. The *bhuR-tnpR* strain showed a significant increase in resolution frequency during the first day, then did not increase significantly until between days 5 and 7.

Most notably, the timing of alcaligin and heme iron system induction by RIVET analysis approximated the phasing of changes in competitive indices (CIs) that were observed in previous competition infection studies in mice (Brickman *et al.*, 2006; Brickman and Armstrong, 2007). Comparison of the relative fitness of *B. pertussis* wild-type and *fauA* alcaligin receptor mutant strains revealed the attenuating effect of the mutation early in infection. Concordantly, in the current RIVET analysis *alcA* transcription was significantly elevated over repressed expression levels by 1d post-infection, and was heightened to an induced level by as early as 3 days post-infection. In contrast, previous infection studies found that a *bhuR* heme receptor mutant showed a competitive defect only later in infection. The delay in CI reduction until after the peak of infection indicated that heme utilization contributes little to initial survival and multiplication of *B. pertussis*. This would imply that the heme resource or host condition responsible for the competitive exclusion of the *bhuR* mutant was unavailable or inactive in the host niche at the initiation of infection, but was made accessible during more advanced stages of infection, presumably by the action of *Bordetella* toxic factors on the host. The delayed induction of *bhuR* transcription seen in RIVET analyses is consistent with the hypothesis that heme is not prevalent in the host niche initially, but becomes available to *B. pertussis* later in infection. Early expression of the *bfeA-tnpR* fusion in RIVET analysis indicates that enterobactin or another catechol inducer (Anderson and Armstrong, 2006; Anderson and Armstrong, 2008) is present in the host niche early in infection.

All RIVET strains resolved to a significant degree over the input population, confirming that all three iron system promoters were active *in vivo*. Furthermore, the strains resolved to different levels and with different kinetics, indicating that the iron system promoters were differentially expressed *in vivo*. Temporal changes in iron system gene expression by RIVET could be correlated with the infection stage during which the iron systems appear to have their greatest impact on *in vivo* fitness. The moderate but significant increase in resolution frequencies for all fusion strains by 1d post-infection likely reflects the initial adaptation of *B. pertussis* to iron starvation in the host environment, causing relief of Fur repression with concomitant increases in iron system promoter activities. Since *in vitro* studies have established that maximal transcription from the *alcA*, *bfeA*, and *bhuR* promoters requires induction and activation subsequent to derepression, it is likely that the increased resolution frequencies observed after day 1 reflect the actions of the system-specific positive regulators AlcR, BfeR, and Hurl in response to cognate iron source availability.

### **BfeA system function contributes to *in vivo* fitness of *B. pertussis* in mice**

The resolution frequencies for the enterobactin system RIVET strain rose significantly during the first 3 days of infection but did not increase thereafter. The 25% resolution frequency at day 7 was relatively low compared with that of the *alcA-tnpR* and *bhuR-tnpR* strains. This lower level of resolution in the *bfeA-tnpR* strain may reflect differences in induction, or differences in intrinsic promoter strength under conditions that prevail in the infected mouse respiratory tract. Nonetheless, under conditions known to maximize transcriptional activity of *bfeA*, the same *bfeA-tnpR* fusion strain resolved to 91% *in vitro*. Since *bfeA-tnpR* expression was relatively low *in vivo*, mixed infection competition experiments were performed to evaluate the contribution of the enterobactin utilization system to multiplication and survival in the mouse respiratory infection model. *In vivo* fitness of *B. pertussis* *bfeA* enterobactin receptor mutant strain PM19 was determined relative to the wild-type parent strain UT25Sm1. The UT25Sm1 genetic background was used instead of the Tohama I background used in RIVET experiments to allow direct comparison with previously published mixed infection competition studies of *fauA* (Brickman and Armstrong, 2007) and *bhuR* (Brickman *et al.*, 2006) iron receptor mutants.



In growth competition studies in vitro, enterobactin receptor mutant PM19 and UT25Sm1 showed no significant difference in relative fitness when co-cultured for 48 h in iron-replete SS. In contrast, the wild-type strain had a distinct competitive advantage over the *bfeA* mutant when co-cultured in iron-depleted SS supplemented with ferric enterobactin as the sole source of iron; the mean CI value was  $0.17 \pm 0.06$  (mean  $\pm$  SD,  $n = 3$ ). For in vivo competition studies, mice were infected intranasally with a 1:1 mixed strain suspension of UT25Sm1 and PM19, and total CFU (Fig. 4A) and competitive index values (CI) (Fig. 4B) were determined for the *B. pertussis* populations recovered in respiratory tract tissues at 3d, 7d, 14d, and 21d post-infection. At 3 days post-infection, the mean CI value had decreased significantly from the reference value of 1.00 to 0.77 ( $P < 0.05$ ) indicating an early fitness cost associated with inactivation of the enterobactin utilization system. The mean CI decreased further to 0.50 by day 7, which constitutes a significant reduction from the mean CI at day 3 ( $P < 0.05$ ). At subsequent time points, no additional significant change in mean CI was observed, but the mean CI remained lower than the reference value of 1.00 (mean CI = 0.44 and 0.69 at days 14 and 21, respectively;  $P < 0.05$ ).

As with the alcaligin and heme utilization systems of *B. pertussis*, there is general agreement between the stage of infection at which the enterobactin system appears to be most important for in vivo fitness by the mixed infection competition approach, and the stage associated with a significant increase in enterobactin system transcriptional activity by RIVET. Further, the competition infection results confirm that although *bfeA* transcriptional activity was insufficient to drive TnpR production to levels necessary for high-frequency resolution in the RIVET strain, BfeA function significantly favors the in vivo growth and survival of *B. pertussis* during primary infection of the mouse respiratory tract, and is most important under conditions that exist in the host environment at the early stage of infection.

The mean CI values for the enterobactin system study (Fig. 4) and from similar, previously published infection studies of the alcaligin and heme systems were used to estimate the relative importance of the three iron systems for in vivo fitness of *B. pertussis* in the mouse respiratory infection model (Fig. 5). It is reasonable to hypothesize that the host niche conditions that influenced competition of the *B. pertussis* strains during these co-infection studies were related to limiting iron resources since co-infecting strains differed only in their production of iron receptors. Given that proposition, a value we have termed the fitness index (FI, calculated as  $1 - \text{mean CI}$ ) provides a measure of the relative in vivo importance or fitness contribution of a particular iron system for multiplication and survival of *B. pertussis* under the existing host niche conditions. These data support the hypothesis that the alcaligin system has a crucial role in iron assimilation both in initial colonization and during the entire infection process. The enterobactin system also contributes significantly to *B. pertussis* growth at the initial stage of infection but to a lesser extent than the alcaligin system, and its role is diminished after the peak of bacterial multiplication. Conversely, the heme system appears to be dispensable for growth until after *B. pertussis* has reached its maximum growth level, at which time it is essential for iron acquisition.

### **Pertussis patient sera recognize *B. pertussis* iron transport system receptor proteins**

The RIVET experiments have determined that the *B. pertussis* alcaligin, enterobactin, and heme utilization systems are expressed in vivo, and competition studies established the importance of these iron systems individually for *B. pertussis* growth and survival during primary infection in mice. Since the mouse is not a natural host for *B. pertussis* and thus, has its limitations as an infection model, we investigated the expression of *B. pertussis* iron systems in the natural human host by screening human sera for antibody reactivity with the three iron source receptors. Representative immunoblot results are shown in Figure 6A and C. Cell envelope preparations from iron replete, iron-starved and iron source-induced *Bordetella* cells were probed blindly using a panel of human sera obtained from normal

adult donors and from *B. pertussis* culture-positive donors (Mobberley-Schuman *et al.*, 2003). Use of *B. bronchiseptica* mutant strains lacking *fauA*, *bfeA*, or *bhuR* (data not presented) and strains over-expressing those genes (Fig. 6C) aided identification of the three receptors in immunoblot analyses. Although this limited survey found no consistent pattern of serum reactivity with *Bordetella* envelope proteins, most sera from *B. pertussis* culture-positive donors (8 out of 11) reacted with multiple iron-repressible proteins from both *Bordetella* species, including FauA, BfeA, and BhuR (Fig. 6C), whereas 3 out of 10 serum samples from uninfected donors reacted strongly with these receptor proteins. It is possible that this serum reactivity could be due to antibodies against other TonB-dependent receptors that happen to crossreact with FauA, BfeA, and BhuR; however, this is not likely since it is the surface-exposed, substrate-specific receptor domains that are the most antigenic regions of these outer membrane receptors. In addition to the three known receptors, other iron-repressible proteins of unknown identity also reacted strongly with *B. pertussis* culture-positive donor sera (Fig. 6B).

The presence of iron receptor-specific antibodies in pertussis patient sera is presumptive evidence that the proteins are produced by *B. pertussis* in response to the cognate iron source inducer during natural infection. It is unknown whether antibodies specific for known outer membrane iron receptors or other iron-repressible envelope proteins have any functional importance in protective immunity against *B. pertussis* infection. Since the alcaligin and enterobactin siderophore-dependent iron systems are important in the early stages of infection, and *B. pertussis*-infected humans appear to respond immunologically to the FauA and BfeA siderophore receptors, immunity to those receptors might inhibit colonization. Furthermore, BhuR-specific immunity could interfere with heme utilization and hasten clearance of *B. pertussis* from the host.

## Experimental procedures

### Bacterial strains and in vitro growth conditions

*Bordetella pertussis* strains Tohama I (Kasuga *et al.*, 1954), UT25 (Field and Parker, 1979), UT25Sm1 (Brickman and Armstrong, 1996), and PM19 were grown on BG. *Bordetella bronchiseptica* B013N (Armstrong and Clements, 1993) and its derivatives were cultured on LB agar (Sambrook *et al.*, 1989) or blood agar plates with selective antibiotics. SCMB was used as solid agar growth medium in *B. pertussis* RIVET experiments. To prepare SCMB, SS was supplemented with 66  $\mu$ M iron, 0.5% powdered activated charcoal, 0.1% methylated  $\beta$ -cyclodextrin, 1% casamino acids, 0.15% bovine serum albumin, and 1.5% agar. Modified Stainer-Scholte medium (SS) (Schneider and Parker, 1982; Stainer and Scholte, 1970) was used as chemically defined broth culture medium for *Bordetella* cultures.

*B. pertussis* and *B. bronchiseptica* growth in SS was monitored as optical density using a spectrophotometer or a Klett-Summerson colorimeter equipped with a #54 filter (Klett Mfg. Co.). *Escherichia coli* DH5 $\alpha$  (Bethesda Research Laboratories) was used as the host for routine plasmid propagation and DNA cloning procedures, and as the donor strain in tri-parental matings to transfer plasmids to *Bordetella* strains. *E. coli* strains were grown in Luria-Bertani (LB) broth or on LB agar plates. Antibiotics were used at the indicated final concentrations: ampicillin, 100  $\mu$ g/ml; gentamicin, 5  $\mu$ g/ml; kanamycin, 10  $\mu$ g/ml; chloramphenicol, 30  $\mu$ g/ml; and tetracycline, 15  $\mu$ g/ml.

### General genetic methods

Conjugal transfer of plasmids to *Bordetella* strains was performed using previously described methods (Brickman and Armstrong, 1996), and transformation of *E. coli* strains by electroporation used standard methods. General genetic techniques were performed

essentially as described previously (Sambrook *et al.*, 1989). DNA and protein sequence analysis used the Lasergene sequence analysis software system for the Macintosh PowerPC computer (DNASTAR, Inc.). Synthetic DNA oligonucleotides were purchased from Integrated DNA Technologies, and nucleotide sequencing was performed by the University of Minnesota BioMedical Genomics Center.

### Recombinase fusion plasmid construction

TnpR fusion plasmids were constructed as described previously using plasmid vector pSS3110-TnpR (Veal-Carr and Stibitz, 2005). The *alcA*, *bfeA* and *bhuR* promoter regions were PCR-amplified from Tohama I genomic DNA templates using primer-adapters (Table 2) to add *EcoRI* and *Sall* restriction sites to the products' termini. The *EcoRI*- and *Sall*-digested PCR products were ligated with *EcoRI-Sall*-digested pSS3110-TnpR plasmid DNA, and then the 3.2-kb *NotI-PspOM1 res1-neo-res1* cassette of pRES1 was inserted at the unique *NotI* site of the resulting plasmids to produce the pSS3110-TnpR-*res-alcA*, -*bfeA*, and -*bhuR* promoter fusion plasmids. Cloned *Bordetella* promoter DNA fragments were confirmed by nucleotide sequencing. Nucleotide coordinates of Tohama I (The Wellcome Trust Sanger Institute, <ftp://ftp.sanger.ac.uk/pub/pathogens/bp/>) iron transport system promoter fragments cloned into pSS3110-TnpR for RIVET were *alcA*: 2597374 -> 2597813 (440 bp), *bfeA*: 3076704 -> 3077139 (436 bp), and *bhuR*: 351402 <- 350898 (505 bp).

### RIVET strain construction

The pSS3110-TnpR-*res*-promoter plasmids were delivered to *B. pertussis* Tohama I by triparental mating using *E. coli* DH5 $\alpha$  as the donor, with helper functions supplied by pRK2013. Exconjugants were selected on BG supplemented with selective antibiotics, 30  $\mu$ M FeSO<sub>4</sub>, and crude colicin B, except for the *bhuR-tnpR* fusion strain, which was selected on SCMB instead of BG. Integration of recombinase fusion plasmids at the appropriate *B. pertussis* Tohama I chromosomal site was confirmed by PCR analysis of genomic DNA using primers BP0771 and 3110junc (Table 2), yielding the *B. pertussis* Tohama I derivative strains TohI-*res-R-alcA*, TohI-*res-R-bfeA*, and TohI-*res-R-bhuR*.

### In vitro resolution assays

*B. pertussis* Tohama I derivative strains TohI-*res-R-alcA* and TohI-*res-R-bfeA*, carrying the integrated *alcA-tnpR* and *bfeA-tnpR* promoter fusion plasmids with their resolvase substrates, were cultured for 2 days on BG supplemented with selective antibiotics and 30  $\mu$ M FeSO<sub>4</sub>. The *bhuR-tnpR* strain TohI-*res-R-bhuR* was cultured similarly, except using SCMB instead of BG to prevent induction of *bhuR* transcription by the heme present in BG, which contains defibrinated sheep blood. Plate growth was used to inoculate 10-ml SS cultures supplemented with selective antibiotics and 66  $\mu$ M FeSO<sub>4</sub> at an initial optical density of approximately 0.1 OD<sub>600</sub>. SS cultures of the *bhuR-tnpR* strain from SCMB growth were also supplemented with 1% casamino acids. After 18 to 24 h growth at 37°C, bacteria were washed using iron-free SS, and subcultured to 10-ml volumes of iron-replete and iron-depleted SS. In resolution assays with TohI-*res-R-bfeA*, 6- $\mu$ M apo-enterobactin inducer was added to the iron-depleted culture at 15 h, and for TohI-*res-R-bhuR*, 1.25- $\mu$ M bovine hemin chloride inducer was added to the iron-depleted culture at 8 h. The *alcA* promoter of the TohI-*res-R-alcA* strain responds to the autogenously produced inducer alcaligin, thus requires no inducer supplement. At various times, cultures were sampled for differential CFU determinations in triplicate on BG with kanamycin and gentamicin versus BG with gentamicin to determine resolution frequencies, except that TohI-*res-R-bhuR* CFU counts used heme-free SCMB instead of BG. Resolution frequencies (% Kan<sup>S</sup> CFU) were calculated as  $[(\text{Gent}^{\text{R}} \text{ CFU} - \text{Gent}^{\text{R}} \text{ Kan}^{\text{R}} \text{ CFU}) / \text{Gent}^{\text{R}} \text{ CFU}] \times 100$ .



## RIVET analysis in mice

All research involving experimental animals was performed in accordance with federal guidelines and institutional policies. *B. pertussis* RIVET strains were cultured for 48 h at 37°C on SCMB with selective antibiotics. Bacterial growth was washed using SS supplemented with 1% casamino acids and 66 µM iron, and used to inoculate SS supplemented with 66 µM iron, 1% casamino acids, and gentamicin (5 µg/ml) plus kanamycin (10 µg/ml) at an initial cell density corresponding to an OD<sub>600</sub> of 0.1. Cultures were grown at 37°C for 18 h, washed using cold phosphate-buffered saline (PBS), and resuspended in cold PBS to an OD<sub>600</sub> of 0.1, which corresponds to approximately  $2 \times 10^8$  CFU/ml. The total CFU in the inocula were enumerated by standard plate counting on SCMB agar (gentamicin, 5 µg/ml), and in parallel on BG agar to determine the relative plating efficiencies of *B. pertussis* on SCMB versus BG. Resolution frequencies in the inoculum suspensions were calculated as described for the in vitro resolution assays after differential CFU counting on SCMB.

Female BALB/cAnNHsd mice (10–20 g) (Harlan Sprague Dawley, Inc.) were mildly sedated by isoflurane inhalation and infected intranasally with  $\sim 2 \times 10^6$  CFU of a single strain in a 10-µl volume. At 1d, 3d, 5d, and 7d post-infection, 4 mice from each strain infection group were euthanized, respiratory tissues (lungs and trachea) were aseptically collected, and homogenates were immediately prepared in ice-cold SS liquid medium supplemented with 66 µM iron and 1% casamino acids. Homogenates were diluted in the same medium, and 100-µl volumes of undiluted homogenates and  $10^{-1}$  to  $10^{-4}$  10-fold serial dilutions were plated in parallel on SCMB medium with gentamicin versus SCMB medium with gentamicin plus kanamycin for total CFU, differential CFU counting, and determination of resolution frequencies as described for the in vitro resolution assays. For each strain infection group, Student's *t*-test was used to determine whether any changes in mean resolution frequencies between consecutive time points were significant; probabilities (*P*) of  $\leq 0.05$  were considered significant.

## Mixed infection competition experiments

To construct the *B. pertussis* enterobactin receptor mutant strain PM19, two non-contiguous segments of the *B. pertussis* UT25 *bfeA* region were PCR-amplified and joined by cloning into the allelic exchange vector pSS1129, generating a *bfeA* allele with a 920-nt deletion that was replaced by a *Hind*III restriction site. A kanamycin resistance cassette from plasmid pBSL86 (Alexeyev, 1995) was inserted at the *Hind*III site, and the  $\Delta bfeA::kan$  allele was crossed to the chromosome of *B. pertussis* strain UT25Sm1 by allelic exchange.

Inocula for mixed infection competition experiments in mice were prepared as described previously (Brickman *et al.*, 2006; Brickman and Armstrong, 2007). Wild-type *B. pertussis* strain UT25Sm1 and the isogenic  $\Delta bfeA::kan$  enterobactin siderophore receptor mutant strain PM19 were subcultured from BG to separate SS cultures. After 36 h, bacteria were subcultured to SS medium containing 18 µM FeSO<sub>4</sub> and grown for an additional 32 hours. Bacteria were washed, resuspended in PBS, and the two strain suspensions were combined at a 1:1 strain ratio to prepare a mixed strain suspension determined by CFU counting to have  $7 \times 10^7$  CFU/ml.

Female BALB/cAnNHsd mice (10–20 g) (Harlan Sprague Dawley, Inc.) were mildly sedated by isoflurane inhalation, and infected with  $\sim 7 \times 10^5$  total CFU of a 1:1 mixture of UT25Sm1 and PM19 (10-µl volume instilled intranasally). At 3d, 7d, 14d, and 21d post-infection, 5 mice were euthanized, and respiratory tissue (lungs and trachea) homogenates were plated in the presence of appropriate antibiotics for differential enumeration of bacterial strains. The CI was calculated as the mutant/wild-type CFU ratio in the output

recovered at each time point divided by the mutant/wild-type CFU ratio in the input inoculum. Each mean CI value is the mean of 4 to 5 independent mouse infections. Student's *t*-test was used to determine whether the mean CI at each time point differed significantly from the hypothesized mean value of 1.00 (the predicted mean CI if there was no difference in fitness between the two strains), and from the mean CI at the preceding time point. Probabilities (*P*) of  $\leq 0.05$  were considered significant.

### **Immunoreactivity of pertussis patient sera with iron-regulated envelope proteins of *B. pertussis***

All research involving human subjects was performed in accordance with federal guidelines and institutional policies. Sera from *B. pertussis* culture-positive donors (aged 6 to 17 years old, previously vaccinated) and a control group of normal adult donors not known to have been infected with *B. pertussis* were analyzed by immunoblotting (Towbin *et al.*, 1979) for antibody reactivity with *Bordetella* cell envelope proteins. Serum samples were provided by Dr. Alison Weiss, University of Cincinnati (Mobberley-Schuman *et al.*, 2003), or were from our laboratory collection.

Total cell envelope proteins for immunoblot analysis were prepared from *B. pertussis* strain UT25 (Field and Parker, 1979) and *B. bronchiseptica* strain B013N (Armstrong and Clements, 1993) bacterial cells cultured in iron-replete and iron-depleted SS. Total cell envelope fractions were also prepared from *B. bronchiseptica* strains BRM18(pBB24) (Brickman and Armstrong, 1999), B013N(pBB32) (S. K. Armstrong, unpublished results), and BRM23(pRK37) (Vanderpool and Armstrong, 2003), which overproduce the iron receptor proteins FauA, BfeA, and BhuR, respectively. For BhuR and BfeA overproduction by *B. bronchiseptica* B013N(pBB32) and BRM23(pRK37), iron-depleted SS cultures were supplemented with the inducers, 1.25  $\mu$ M hemin chloride or 3.25  $\mu$ M enterobactin, respectively. FauA overproduction by BRM18(pBB24) was responsive to the autogenously produced inducer, alcaligin; thus, it needed no inducer supplement.

*Bordetella* cell fractionation for isolation of total cell envelopes used a modification of the Schnaitman method (Schnaitman, 1971). *Bordetella* cells were suspended in 50 mM *N*-2-hydroxyethylpiperazine-*N*9-2-ethanesulfonic acid (HEPES; pH 7.4), frozen and thawed, then disrupted using a French pressure cell (American Instrument Company). The pressates were centrifuged at 5000  $\times g$  at 4°C for 5 min to sediment unbroken cells, and the resulting supernatant fluids were centrifuged at 100,000  $\times g$  at 4°C for 1 h to obtain the insoluble cell fractions. The insoluble residue was washed twice using 10 mM Tris (pH 8)/1 mM EDTA buffer, then suspended in 50 mM HEPES buffer (pH 7.4) to yield the total cell envelope fraction.

For immunoblots, protein samples (adjusted to approximately 40  $\mu$ g/lane) were separated by 0.1% sodium dodecyl sulfate-7.5% polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Human sera were used at 1:400 dilution, and detection used goat anti-human IgGAM F(ab')<sub>2</sub> conjugated to horseradish peroxidase (1:2000) (Cappel) with 4-chloronaphthol as the substrate.

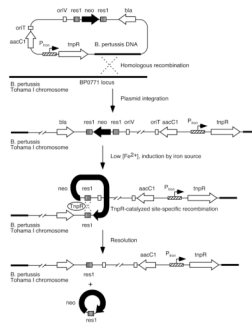
### **Acknowledgments**

We are grateful to Scott Stibitz and Wendy Veal-Carr for sharing RIVET plasmids pSS3110-TnpR, pSS3110-TnpR135, and pSS3110-TnpR168, for pRES1 originally obtained from Andrew Camilli, and for helpful discussions about RIVET studies in *B. pertussis*. We are thankful to Alison Weiss for providing samples of normal human sera and pertussis patient sera, and to Carin Vanderpool for help with interpretation of immunoblot results. This work was supported by Public Health Service grant AI-31088 from the National Institute of Allergy and Infectious Diseases.

## References

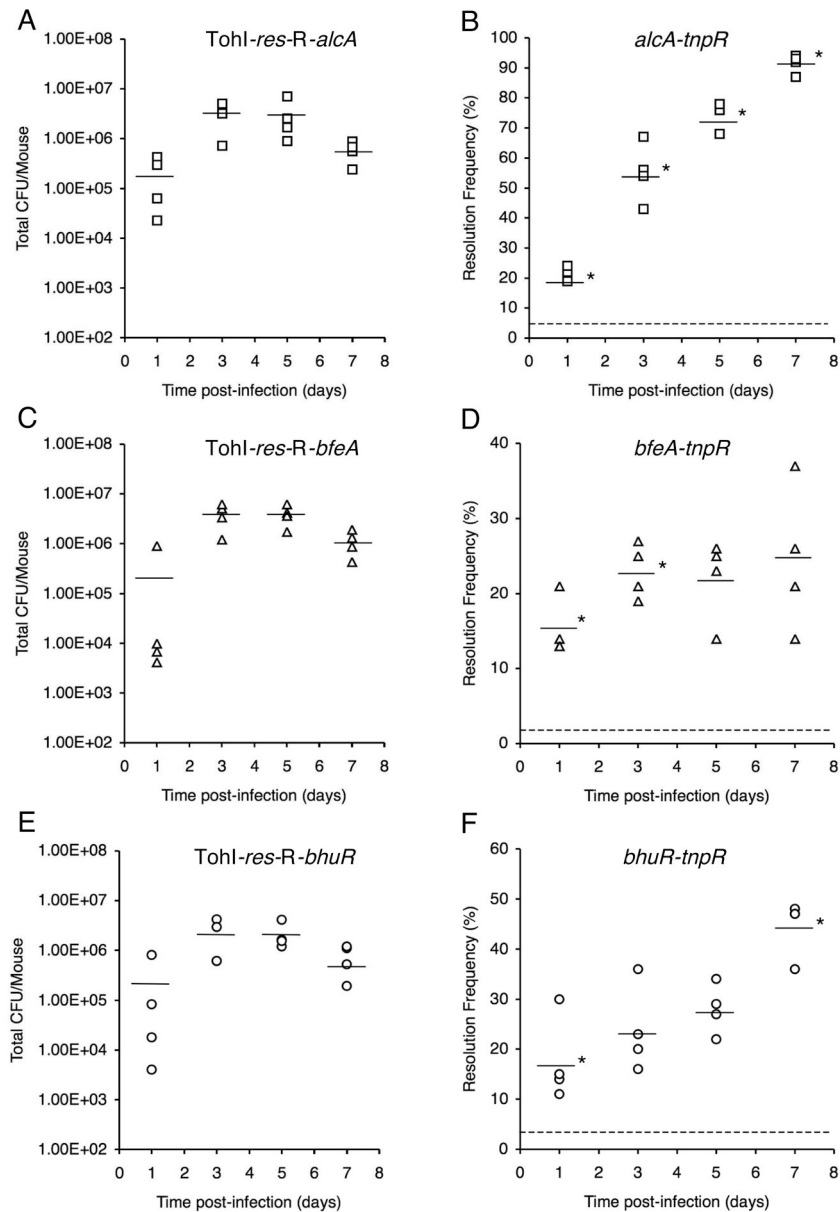
- Anderson MT, Armstrong SK. The BfeR regulator mediates enterobactin-inducible expression of *Bordetella* enterobactin utilization genes. *J Bacteriol.* 2004; 186:7302–7311. [PubMed: 15489442]
- Anderson MT, Armstrong SK. The *Bordetella bfe* system: growth and transcriptional response to siderophores, catechols, and neuroendocrine catecholamines. *J Bacteriol.* 2006; 188:5731–5740. [PubMed: 16885441]
- Anderson MT, Armstrong SK. Norepinephrine mediates acquisition of transferrin-iron in *Bordetella bronchiseptica*. *J Bacteriol.* 2008; 190:3940–3947. [PubMed: 18390651]
- Armstrong SK, Clements MO. Isolation and characterization of *Bordetella bronchiseptica* mutants deficient in siderophore activity. *J Bacteriol.* 1993; 175:1144–1152. [PubMed: 8381782]
- Beaumont FC, Kang HY, Brickman TJ, Armstrong SK. 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.* 1998; 180:862–870. [PubMed: 9473040]
- Bordet J, Gengou O. Le microbe de la coqueluche. *Ann Inst Pasteur (Paris).* 1906; 20:731–741.
- Brickman TJ, Armstrong SK. The ornithine decarboxylase gene *odc* is required for alcaligin siderophore biosynthesis in *Bordetella* spp.: putrescine is a precursor of alcaligin. *J Bacteriol.* 1996; 178:54–60. [PubMed: 8550442]
- Brickman TJ, Kang HY, Armstrong SK. Transcriptional activation of *Bordetella* alcaligin siderophore genes requires the AlcR regulator with alcaligin as inducer. *J Bacteriol.* 2001; 183:483–489. [PubMed: 11133941]
- Brickman TJ, Vanderpool CK, Armstrong SK. *Bordetella*. Iron Transport in Bacteria. 2004:311–328.
- Brickman TJ, Vanderpool CK, Armstrong SK. Heme transport contributes to in vivo fitness of *Bordetella pertussis* during primary infection in mice. *Infect Immun.* 2006; 74:1741–1744. [PubMed: 16495546]
- Brickman TJ, Armstrong SK. Impact of alcaligin siderophore utilization on in vivo growth of *Bordetella pertussis*. *Infect Immun.* 2007; 75:5305–5312. [PubMed: 17724074]
- Brickman TJ, Anderson MT, Armstrong SK. *Bordetella* iron transport and virulence. *Biometals.* 2007; 20:303–322. [PubMed: 17295050]
- Camilli A, Beattie DT, Mekalanos JJ. Use of genetic recombination as a reporter of gene expression. *Proc Natl Acad Sci U S A.* 1994; 91:2634–2638. [PubMed: 8146167]
- Field, LH.; Parker, CD. Differences observed between fresh isolates of *Bordetella pertussis* and their laboratory-passaged derivatives. In: Manclark, CR.; Hill, JC., editors. International Symposium on Pertussis. Washington, D.C.: U.S. Dept. of Health, Education and Welfare, Public Health Service; 1979. p. 124-132.
- Kang HY, Armstrong SK. Transcriptional analysis of the *Bordetella* alcaligin siderophore biosynthesis operon. *J Bacteriol.* 1998; 180:855–861. [PubMed: 9473039]
- Kasuga T, Nakase Y, Ukishima K, Takatsu K. Studies on *Haemophilus pertussis*. V. Relation between the phase of bacilli and the progress of the whooping-cough. *Kitasato Arch Exp Med.* 1954; 27:57–62. [PubMed: 13296323]
- Lee SH, Hava DL, Waldor MK, Camilli A. Regulation and temporal expression patterns of *Vibrio cholerae* virulence genes during infection. *Cell.* 1999; 99:625–634. [PubMed: 10612398]
- Mahan MJ, Heithoff DM, Sinsheimer RL, Low DA. Assessment of bacterial pathogenesis by analysis of gene expression in the host. *Annu Rev Genet.* 2000; 34:139–164. [PubMed: 11092824]
- Mobberley-Schuman PS, Connelly B, Weiss AA. Phagocytosis of *Bordetella pertussis* incubated with convalescent serum. *J Infect Dis.* 2003; 187:1646–1653. [PubMed: 12721945]
- Parkhill J, Sebahia M, Preston A, Murphy LD, Thomson N, Harris DE, et al. Comparative analysis of the genome sequences of *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica*. *Nat Genet.* 2003; 35:32–40. [PubMed: 12910271]
- Persson CG, Erjefalt I, Alkner U, Baumgarten C, Greiff L, Gustafsson B, Luts A, Pipkorn U, Sundler F, Svensson C, et al. Plasma exudation as a first line respiratory mucosal defence. *Clin Exp Allergy.* 1991; 21:17–24. [PubMed: 2021873]

- Pradel E, Guiso N, Loch C. Identification of AlcR, an AraC-type regulator of alcaligin siderophore synthesis in *Bordetella bronchiseptica* and *Bordetella pertussis*. *J Bacteriol.* 1998; 180:871–880. [PubMed: 9473041]
- Sambrook, J.; Fritsch, EF.; Maniatis, T. *Molecular cloning : a laboratory manual.* Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory; 1989.
- Schnaitman CA. Solubilization of the cytoplasmic membrane of *Escherichia coli* by Triton X-100. *J Bacteriol.* 1971; 108:545–552. [PubMed: 4941569]
- Schneider DR, Parker CD. Effect of pyridines on phenotypic properties of *Bordetella pertussis*. *Infect Immun.* 1982; 38:548–553. [PubMed: 6292105]
- Stainer DW, Scholte MJ. A simple chemically defined medium for the production of phase I *Bordetella pertussis*. *J Gen Microbiol.* 1970; 63:211–220. [PubMed: 4324651]
- Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A.* 1979; 76:4350–4354. [PubMed: 388439]
- Vanderpool CK, Armstrong SK. The *Bordetella bhv* locus is required for heme iron utilization. *J Bacteriol.* 2001; 183:4278–4287. [PubMed: 11418569]
- Vanderpool CK, Armstrong SK. Heme-responsive transcriptional activation of *Bordetella bhv* genes. *J Bacteriol.* 2003; 185:909–917. [PubMed: 12533466]
- Vanderpool CK, Armstrong SK. Integration of environmental signals controls expression of *Bordetella* heme utilization genes. *J Bacteriol.* 2004; 186:938–948. [PubMed: 14761988]
- Veal-Carr WL, Stibitz S. Demonstration of differential virulence gene promoter activation in vivo in *Bordetella pertussis* using RIVET. *Mol Microbiol.* 2005; 55:788–798. [PubMed: 15661004]

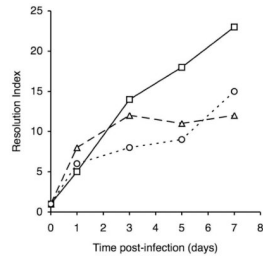


**Fig. 1.** Schematic representation of RIVET analysis of *B. pertussis* iron transport system genes. Transcriptional activity of *alca* (alcaligin), *bfeA* (enterobactin) and *bhuR* (heme) iron system promoters (designated  $P_{Iron}$ ) in vivo drives production of the site-specific resolvase TnpR, which catalyzes excision of the linked kanamycin resistance marker (*neo*) by recombination between the *resI* sites. Loss of kanamycin resistance serves as a stable heritable marker of prior gene expression.

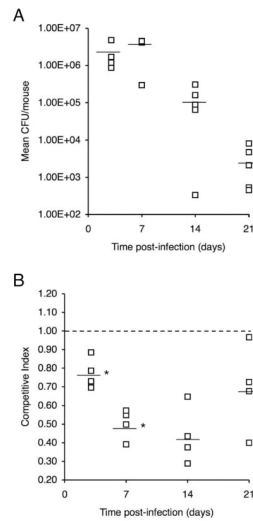




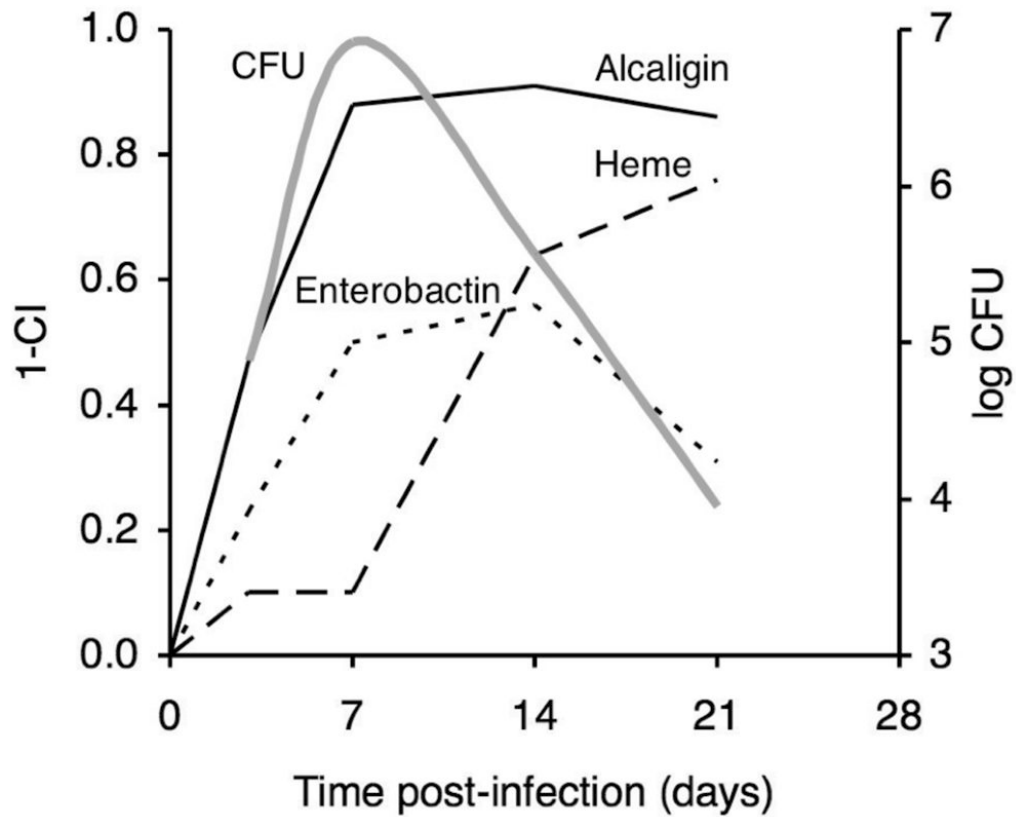
**Fig. 2.** Total CFU (A, C, E) and TnpR-mediated resolution frequencies (B, D, F) at various times post-infection for RIVET strains carrying the indicated fusion genes. Bars represent the mean total CFU/mouse or mean resolution frequencies ( $n = 4$  mice per time point), and each symbol represents the total CFU or the resolution frequency for the *B. pertussis* population recovered from an individual mouse. An asterisk indicates that the mean resolution frequency differed significantly from the mean at the preceding time point. The dashed lines in panels B, D, and F correspond to the base-line resolution frequencies of the inocula.



**Fig. 3.** Resolution index values for RIVET strains at various times post-infection. Values represent the fold-change in mean resolution frequency compared with the base-line resolution frequency determined for the input inocula. Strains: TohI-res-R-*alcA* (squares), TohI-res-R-*bfeA* (triangles), and TohI-res-R-*bhuR* (circles).

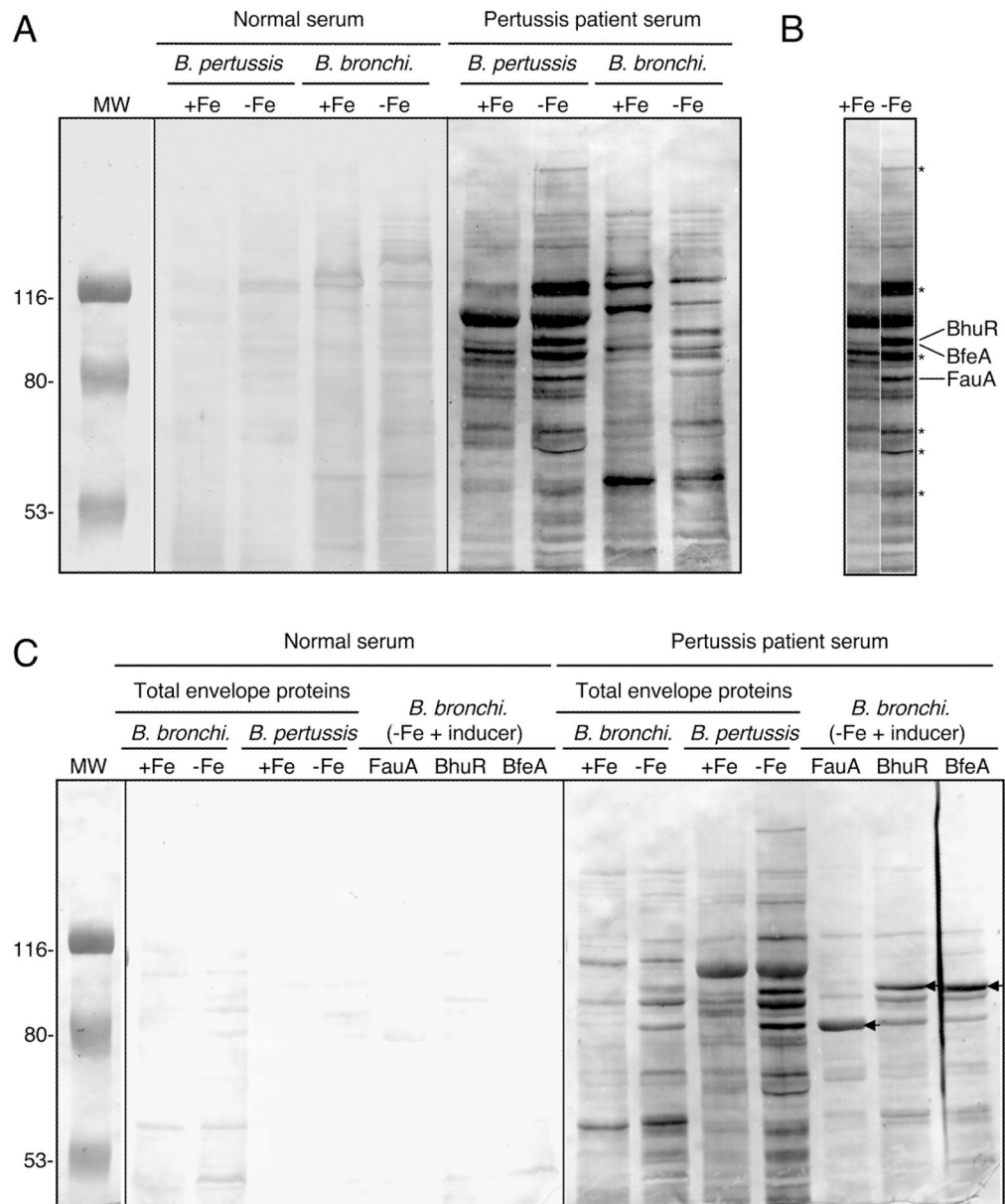


**Fig. 4.** Relative in vivo fitness of a *B. pertussis* enterobactin receptor mutant during primary infection in mice. Mice were infected intranasally with a 1:1 mixture of wild-type strain UT25Sm1 and enterobactin receptor mutant strain PM19. (A) Total CFU over the course of the infection experiment. (B) Competitive index values were calculated as the mutant/wild-type CFU ratio in the output recovered at each time point divided by the mutant/wild-type CFU ratio in the input inoculum. CI values are shown relative to the reference CI of 1.00 (dashed line). Bars represent mean values, and each symbol represents the total CFU or CI value determined for an individual mouse. An asterisk indicates that the mean CI differed significantly from the mean at the preceding time point.



**Fig. 5.**

Relative importance of iron systems for in vivo fitness of *B. pertussis* at various phases of infection. Mean competitive index values from mixed infection competition experiments involving co-infection of mice with wild-type and alcaligin (Brickman and Armstrong, 2007), enterobactin (see Fig. 4), or heme (Brickman *et al.*, 2006) receptor mutant strains were used to calculate a fitness index value (FI, calculated as  $1 - \text{mean CI}$ ), at various times post-infection. The FI provides a measure of the relative in vivo importance or fitness contribution an iron system for multiplication and survival of *B. pertussis* under the existing host niche conditions. Solid line, alcaligin system; dotted line, enterobactin system; dashed line, heme system. A plot (grey line) showing CFU levels that are typical of *B. pertussis* mouse respiratory infections is overlaid for reference.



**Fig. 6.** Immunoblot reactivity of human sera with *Bordetella* iron-repressible envelope proteins. (A) Total envelope proteins prepared from *B. pertussis* UT25 and *B. bronchiseptica* B013N cultured under iron-replete (+Fe) and iron-depleted (-Fe) conditions were probed in parallel using sera from an uninfected human donor (normal serum) and a *B. pertussis* culture-positive human donor (pertussis patient serum). (B) Juxtaposition of *B. pertussis* sample lanes from panel A. Known positions of BhuR, BfeA and FauA are indicated, and asterisks designate positions of select iron-repressible envelope proteins. (C) The same antigens analyzed in panel A, and total envelope proteins from FauA, BhuR or BfeA receptor-overproducing *B. bronchiseptica* (*B. bronchi.*) strains were probed using serum from different donors than those in panel A. Positions of FauA, BhuR and BfeA are indicated by the arrows.



**Table 1**Relevant features of *B. pertussis* iron systems analyzed using RIVET

<b>Iron system</b>	<b>Promoter<sup>a</sup></b>	<b>Activator</b>	<b>Inducer</b>
Alcaligin siderophore biosynthesis, transport and Utilization	<i>alcA</i>	AlcR (AraC/XylS family)	Alcaligin
Enterobactin siderophore transport and utilization	<i>bfeA</i>	BfeR (AraC/XylS family)	enterobactin, other Catechols
Heme transport and utilization	<i>bhuR</i>	HurI (Extracytoplasmic function sigma factor)	heme compounds

<sup>a</sup> inducible promoter driving *mpR* expression in a RIVET strain

**Table 2**

Oligonucleotide primers used for PCR cloning of *B. pertussis* promoter regions and for genomic mapping of integrated *tnpR* fusion plasmids

Name	Nucleotide sequence	Base positions <sup>a</sup>
<i>alcA-EcoRI</i>	GGCCgaattcAGACATTGCAAGCCCCTGAT <sup>b</sup>	2597374..2597393
<i>alcA-SalI</i>	GGCCgtcgacGTCCGATACCGATAGCCACGAA	2597813..2597792
<i>bfeA-EcoRI</i>	GGCCgaattcGCGCAGGCCGGGCTTGAGTTC	3076704..3076724
<i>bfeA-SalI</i>	GGCCgtcgacGGGGGTGGACATGCGGCTTCT	3077139..3077119
<i>bhuR-EcoRI</i>	GGCCgaattcCGCCGACAACCGCACCCACTC	351402..351382
<i>bhuR-SalI</i>	GGCCgtcgacGCCCTGATCGCAAGCGTAAACCAT	350898..350921
BP0771 <sup>c</sup>	CCGCCGCGCCGTAGTTCACCAC	792956..792978
3110junc	CGCGGACCACAGCCCCATCACCAG	NA <sup>d</sup>

<sup>a</sup>Nucleotide coordinates of the *B. pertussis* Tohama I genome sequence (The Wellcome Trust Sanger Institute, <ftp://ftp.sanger.ac.uk/pub/pathogens/bp/>).

<sup>b</sup>Nucleotides in lower case represent restriction enzyme recognition site adapters incorporated for cloning to pSS3110-TnpR. Underlined nucleotides are complementary to *B. pertussis* chromosomal DNA sequences.

<sup>c</sup>complementary to the *B. pertussis* chromosomal site of pSS3110-TnpR integration

<sup>d</sup>Not applicable, primer is complementary to the pSS3110-TnpR plasmid vector