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Involvement of multiple distinct *Bordetella* receptor proteins in the utilization of iron liberated from transferrin by host catecholamine stress hormones

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Summary

Bordetella bronchiseptica is a pathogen that can acquire iron using its native alcaligin siderophore system, but can also use the catechol xenosiderophore enterobactin *via* the BfeA outer membrane receptor. Transcription of *bfeA* is positively controlled by a regulator that requires induction by enterobactin. Catecholamine hormones also induce *bfeA* transcription and *B. bronchiseptica* can use the catecholamine norepinephrine for growth on transferrin. In this study, *B. bronchiseptica* was shown to use catecholamines to obtain iron from both transferrin and lactoferrin in the absence of siderophore. In the presence of siderophore, norepinephrine augmented transferrin utilization by *B. bronchiseptica*, as well as siderophore function *in vitro*. Genetic analysis identified BfrA, BfrD and BfrE as TonB dependent outer membrane catecholamine receptors. The BfeA enterobactin receptor was found to not be involved directly in catecholamine utilization; however, the BfrA, BfrD and BfrE catecholamine receptors could serve as receptors for enterobactin and its degradation product 2,3-dihydroxybenzoic acid. Thus, there is a functional link between enterobactin-dependent and catecholamine-dependent transferrin utilization. This investigation characterizes a new *B. bronchiseptica* mechanism for iron uptake from transferrin that uses host stress hormones that not only deliver iron directly to catecholamine receptors, but also potentiate siderophore activity by acting as iron shuttles.

Keywords

Bordetella; iron; catechol; catecholamine; receptor; transferrin

Introduction

The Gram-negative bacterial species *Bordetella bronchiseptica*, *Bordetella pertussis* and *Bordetella parapertussis* are obligate pathogens of mammals (Parkhill *et al.*, 2003; Mattoo and Cherry, 2005). *B. bronchiseptica* typically causes respiratory infections in nonhuman hosts and occasionally in immunocompromised humans, *B. pertussis* is the agent of human whooping cough or pertussis, and *B. parapertussis* causes respiratory disease in humans and sheep. A *B. bronchiseptica*-like strain is thought to be the ancestor of *B. pertussis* and *B. parapertussis*. These bacteria colonize the ciliated cells of the respiratory epithelium where they replicate and produce virulence factors that enable them to persist in the host. Most of the known *Bordetella* virulence factor genes are transcriptionally regulated by a two-

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

Most cells have a requirement for iron and have evolved mechanisms to obtain this element, while at the same time regulating intracellular iron concentrations to avoid iron-catalysed oxidative damage. The mammalian host limits the amount of extracellular iron that may become available to microbial pathogens through homeostatic mechanisms such as production of the iron-binding glycoproteins transferrin (TF) and lactoferrin (LF) (Hentze *et al.*; 2004). For a pathogen, this iron restriction must be overcome for survival and multiplication in the host. Bacterial iron acquisition strategies include the synthesis and utilization of siderophores as well as the production of specific transporters enabling the use of xenosiderophores (Weinberg, 1978; Bullen, 1981; Neilands, 1995; Ratledge and Dover, 2000). In addition, bacteria may have systems allowing the uptake and use of heme iron sources and the direct removal of iron from receptor-bound TF and LF at the bacterial surface (Ratledge and Dover, 2000). Recently, catecholamine neuroendocrine hormones such as epinephrine and norepinephrine (NE) have been reported to remove the iron from TF and LF, and some bacterial species are then able to access this iron (Freestone *et al.*, 2000; 2008; Sandrini *et al.* 2010), providing additional evidence that pathogens may exploit both microbially-produced and host-derived iron sources. To assimilate various iron sources, Gram-negative bacteria use TonB-dependent outer membrane receptors to transfer ferric iron, heme, or ferric chelates across the outer membrane to the periplasm (Postle and Larsen, 2007). Transport from the periplasm to the cytoplasm usually requires a periplasmic binding protein and cognate ATP binding cassette-type transporter.

B. pertussis, *B. parapertussis* and *B. bronchiseptica* produce and utilize the alcaligin siderophore (Moore *et al.*, 1995; Brickman *et al.*, 1996) and *B. pertussis* and *B. bronchiseptica* can also use the xenosiderophores enterobactin (Beall and Sanden, 1995), ferrichrome and desferrioxamine B (Beall and Hoenes, 1997). *B. bronchiseptica* has been reported to use the xenosiderophores ferrichrysin, ferrirubin, aerobactin, protochelin, schizokinen, ferricrocin, vicibactin, and pyoverdin (Pradel and Loch, 2001). These *Bordetella* species have genes encoding many predicted TonB-dependent iron acquisition systems (Table S1); however only three iron source utilization systems have been characterized to date: those for alcaligin, enterobactin and heme. Under iron-replete growth conditions the genes of these three iron systems are repressed by Fur, and under iron starvation conditions each system is positively regulated transcriptionally by a specific regulator with the cognate iron source acting as the inducer (Brickman *et al.*, 2001; Vanderpool and Armstrong, 2003; Anderson and Armstrong, 2004; Brickman and Armstrong, 2009). Mouse respiratory infection studies demonstrated that *B. pertussis* mutants lacking any one of the outer membrane receptors for alcaligin, enterobactin or heme were attenuated for *in vivo* growth (Brickman *et al.*, 2006; 2008; Brickman and Armstrong, 2007). *In vivo* expression studies determined that *B. pertussis* differentially regulates transcription of the alcaligin, enterobactin and heme utilization genes during infection, indicating that these iron sources are present in the host and are used at certain stages of infection (Brickman *et al.*, 2008).

Enterobactin is a siderophore of the catechol structural class (Fig. S1) that is produced primarily by members of the Enterobacteriaceae such as *Escherichia coli*. *Bordetellae* do not produce enterobactin, but can use it to obtain iron *via* the BfeA TonB-dependent outer membrane receptor (Beall and Sanden, 1995). The *bfeA* gene is positively regulated by the AraC-like transcriptional regulator BfeR that requires enterobactin for induction (Anderson and Armstrong, 2004). We determined that other catechol compounds, in addition to enterobactin, were capable of acting as inducers for BfeR. These compounds included the salmochelin and corynebactin siderophores that were less potent inducers than enterobactin,

and the NE, epinephrine and dopamine catecholamines (Fig. S1) that induced high levels of *bfeA* transcription, approximating the induction activity level of enterobactin (Anderson and Armstrong, 2006).

It is not obvious why the respiratory pathogenic *Bordetellae* have evolved the ability to use the enterobactin siderophore produced by gut bacteria, and it is not known whether enterobactin can be found in the respiratory tract. It was hypothesized that perhaps the natural substrate for the BfeA receptor is not enterobactin, but rather, a host-derived molecule such as a catecholamine. NE, when added to iron-depleted cultures containing serum (Anderson and Armstrong, 2006) or iron-loaded TF (Anderson and Armstrong, 2008), stimulated the growth of *B. bronchiseptica*. Although NE induces *bfeA* transcription, efforts to correlate the BfeA receptor with NE utilization or to identify any ferric NE receptor in *B. bronchiseptica* were not successful (Anderson and Armstrong, 2008). In the present study, we report the identification of three previously uncharacterized *B. bronchiseptica* outer membrane ferric catecholamine receptors. Furthermore, we present results indicating that BfeA is not a catecholamine receptor, and that catecholamines can act as iron shuttles, augmenting the activities of siderophores.

Results

NE-mediated acquisition of iron from TF and LF

Our previous studies demonstrated that the alcaligin deficient *B. bronchiseptica* Δ *alcA* strain BRM26 grew poorly in our iron-depleted defined SS medium culture system; addition of partially iron saturated TF or LF to the medium exacerbated this growth defect, presumably by chelation of residual iron in the system (Anderson and Armstrong, 2008). In that study, we found that *B. bronchiseptica* could utilize NE to obtain growth-promoting iron from TF by a mechanism that did not require the alcaligin siderophore, but NE-mediated iron acquisition from LF could not be demonstrated. Here, the ability of the *B. bronchiseptica* Δ *alcA* strain BRM26 to use NE to retrieve LF iron was reexamined, using apo-LF that we iron-saturated to 70%. In contrast with our 2008 results that used commercially available partially iron-saturated LF, new studies indicate that *B. bronchiseptica* can use NE to obtain the iron from LF (Fig. 1A). *B. pertussis* and *B. parapertussis* however demonstrated little, if any, NE-dependent growth stimulation with TF as the iron source (Fig. 1B, Fig. 1C), and no apparent growth promotion by LF (data not shown). Testing of other strains of these two *Bordetella* species yielded similar results (data not shown). These results indicate that NE-mediated acquisition of both TF- and LF-bound iron in *B. bronchiseptica* can be siderophore-independent, and that *B. pertussis* and *B. parapertussis* are comparatively deficient in NE utilization.

NE is a soluble iron chelator

Catecholamines and catechol monomers are known to chelate iron (Green *et al.*, 1956; Jewett *et al.*, 1997), although complex formation is not as effective as that of siderophores such as enterobactin (Harris *et al.*, 1979; Gerard *et al.*, 1999). In our iron depleted culture system, NE is not able to scavenge iron sufficient for *Bordetella* growth promotion (Anderson and Armstrong, 2008). When growth yields of alcaligin-deficient mutant BRM26 were determined using defined SS medium supplemented with various concentrations of NE and having 30% iron-saturated TF as the sole iron source (Fig. 2), a NE dose-dependent growth response was observed. At 24 h, BRM26 exhibited the best growth in the presence of 100 μ M NE, whereas growth at concentrations ranging from 0 – 12.5 μ M was minimal. By 48 h, good growth yields were obtained using 50 μ M and 100 μ M NE, compared to that in iron replete (36 μ M Fe) medium, and the lowest NE concentration at which statistically significant growth stimulation could be detected was 6.25 μ M. In these studies, growth

stimulation was NE-dependent, since the *Bordetella* strain lacked the ability to produce the alcaligin siderophore and cultures without NE supplementation failed to grow. Iron replete control cultures supplemented with TF had high growth yields comparable to those of iron replete cultures lacking TF (data not shown).

It was predicted that NE can function as a soluble chelator that removes iron from TF and subsequently diffuses to *Bordetella* cells for iron uptake and utilization. Early reports noted that TF and LF are able to bind to the *Bordetella* cell surface but no TF- or LF-specific receptor proteins have been identified (Redhead *et al.*, 1987; Menozzi *et al.*, 1991). To determine whether the bacteria require direct contact with TF for efficient NE-mediated iron uptake, *B. bronchiseptica* Δ alcA strain BRM26 was cultured in iron-depleted SS medium with TF that was either free in the medium or partitioned within a dialysis bag (8,000 dal molecular mass cutoff) immersed in the culture medium (Fig. 3). Without siderophore or NE supplementation of the culture medium, the bacteria showed no substantial growth regardless of whether the TF was free or sequestered in the dialysis bag (Fig. 3, -Fe samples). As predicted, addition of the alcaligin siderophore to the medium stimulated growth in both the free-TF and sequestered-TF cultures. Importantly, addition of NE alone to both free- and sequestered-TF cultures also stimulated growth, although NE promoted higher levels of growth in the free-TF than the sequestered-TF cultures. When both alcaligin and NE were supplied to TF-sequestered cultures, remarkably high levels of growth stimulation were observed, suggestive of a synergistic effect. Overall, these results showed that even in the absence of a siderophore, NE-mediated TF utilization did not require direct cell contact with TF; thus, NE is capable of functioning as a diffusible iron chelator for *Bordetella* cells. Although contact of the bacteria with TF was not necessary for NE-stimulated growth, direct contact resulted in higher growth yields. Regardless of whether TF binding to *Bordetella* cells is specific, interaction of TF with the bacterial surface may facilitate more efficient iron retrieval *via* siderophores or NE, perhaps simply by virtue of favorable diffusion kinetics associated with TF proximity to the cell.

The putative synergistic enhancement of *B. bronchiseptica* TF utilization by siderophore and NE together suggested that NE may augment siderophore function, or *vice versa*. A similar amplified *B. bronchiseptica* growth response was noted in our previous study using TF-containing cultures supplied with NE plus the enterobactin siderophore (Anderson and Armstrong, 2008). The chrome azurol S (CAS) universal siderophore assay was used to determine whether NE had iron-chelating activity similar to that of a siderophore and to examine whether NE or alcaligin had any functional influence on one another (Fig. 4). In the CAS assay (Schwyn and Neilands, 1987), siderophores can remove iron from the blue-colored CAS dye complex, resulting in a blue-to-orange color change that is measured as a decrease in absorbance at 630 nm wavelength. In this functional siderophore assay, the weak chelator, 5-sulfosalicylic acid (SSA), is a shuttle routinely used to speed the iron exchange to equilibrium. Purified alcaligin exhibited characteristic iron-binding activity in this assay and addition of the SSA shuttle increased the initial rate of alcaligin-mediated iron removal, consistent with previous results (Brickman *et al.*, 1996). NE alone was not able to remove the iron from the CAS dye complex; however, when added along with the siderophore, it acted as a shuttle for alcaligin that was much more effective than SSA. These results suggest that although NE may not function as a *bona fide* siderophore in this assay system, it can augment the iron-binding activity of the alcaligin siderophore. Direct NE-mediated iron removal from TF is likely to be mechanistically different than iron removal from the CAS dye complex. In other experiments, *Bordetella* *alcA* mutant cultures supplied with NE did not produce a CAS-active compound, indicating that NE is not being used by *Bordetella* cells as a precursor for synthesis of another siderophore (data not shown). Taken together, the results suggest that NE can function as a pseudosiderophore to remove iron from TF (and likely LF) and deliver it to *B. bronchiseptica* directly or it can function as a shuttle to

facilitate iron transfer to alcaligin or another utilizable siderophore, whereupon it is taken up by the cognate siderophore transport apparatus.

NE utilization requires TonB

Since the experimental results indicated that NE can act as a pseudosiderophore, it was postulated that a *Bordetella* TonB-dependent cell surface receptor may be involved in the uptake of the iron itself or of the ferric NE complex. Our previous study examined the ability of a *B. bronchiseptica* *tonB* mutant to grow in the presence of NE with TF as the sole iron source (Anderson and Armstrong, 2008). Although a growth defect was observed, it was thought that since the *Bordetella* strain was capable of alcaligin production, and that alcaligin would be predicted to remove any iron from NE, then the observed defect could be attributed to abrogation of ferric alcaligin uptake which depends on the TonB-dependent receptor protein FauA, rather than an effect on uptake via a putative NE transporter. In the present study, the *B. bronchiseptica* *alcA* mutant BRM26 was made *tonB*. The resulting double mutant, BRM46, was defective in alcaligin production and the ability to use hemin, alcaligin and enterobactin, consistent with a *tonB* defect (data not shown). BRM46 was tested for the ability to use NE for growth with TF as the iron source (Fig. 5). Whereas BRM26 showed typical NE-mediated growth in the absence of alcaligin production, NE-supplemented BRM46 cultures exhibited poor growth yields similar to those of TF-containing cultures that lacked NE. Genetic complementation of BRM46 with a wild-type copy of *tonB* restored high-level growth on TF *via* NE. These results implicated a TonB-dependent outer membrane receptor in NE-mediated TF utilization in *Bordetella*.

Identification of TonB-dependent outer membrane receptors

Annotation by the Sanger Centre, as well as our own bioinformatic analysis, predicted that *B. bronchiseptica* has 19 TonB-dependent receptor genes, *B. pertussis* has 15 and *B. parapertussis* has 14 (Table S1) (Parkhill *et al.*, 2003; Anderson and Armstrong, 2008). In our previous study, each of the predicted *B. bronchiseptica* TonB-dependent receptor genes was insertionally inactivated and the resulting mutants tested for their ability to utilize NE to obtain TF-iron (Anderson and Armstrong, 2008). No single mutant was found to be defective. In the present study, given the possibility that the functional alcaligin siderophore system of the mutant strains might mask any NE utilization defects, a new bank of 19 insertion mutants in the BRM26 *alcA* background was constructed and tested for the ability to use NE to grow with TF as the sole iron source. It was predicted that the *bfeA* enterobactin receptor mutant would be defective for growth since NE and enterobactin share similar iron-binding catechol moieties and NE induces *bfeA* transcription. However, all 19 of the receptor mutants were able to grow in the NE-supplemented TF culture system (data not shown), indicating that there were multiple TonB dependent receptors for ferric NE.

Constructing strains with all possible combinations of multiple receptor gene mutations was not a realistic approach to identify the NE receptors. It was reasoned that since *B. bronchiseptica* had an exalted ability to use NE compared with *B. pertussis* and *B. parapertussis*, this difference could be due to receptors encoded by *B. bronchiseptica*-specific genes that are absent in the other two species. There are four predicted TonB-dependent receptor genes that are apparently unique to *B. bronchiseptica*: *bfrA* (BB4761), *bfrZ* (BB4744), BB1905, and BB0832. Plasmid-borne copies of these *B. bronchiseptica* genes were transferred by conjugation to *B. pertussis* strain UT25Sm1. The *B. pertussis* strain carrying the plasmid vector control demonstrated the typical modest growth yields in SS medium supplemented with TF and NE (Fig. 6). Of the four tested genes, only *bfrA* conferred the ability to promote NE utilization when carried by *B. pertussis* (Fig. 6 and data not shown); however, NE-dependent growth yields for the *BfrA_{Bb}⁺* *B. pertussis* strain by 48 h were not as high as those typically observed for *B. bronchiseptica*.

With the identification of BfrA as a presumptive NE receptor, and the likelihood that the BfeA enterobactin receptor was also a NE receptor candidate, a *B. bronchiseptica* *bfeA bfrA* mutant was constructed in the BRM26 *alcA* background. Strains BRM65 (*alcA bfeA*) and BRM66 (*alcA bfeA bfrA*) were examined for the ability to grow using NE and TF (Fig. 7). As predicted, iron-replete cultures of both strains grew well over the 44 h culture period and cultures lacking NE grew poorly. Compared with the BfrA⁺ strain BRM65, the *bfrA* mutant strain BRM66 exhibited a diminished growth rate and yield in SS medium containing both NE and TF. Interestingly, despite its decreased growth, the BRM66 *alcA bfeA bfrA* mutant did not demonstrate a null NE-dependent growth phenotype. This result indicated not only that BfrA was not the sole NE receptor, but that even if BfeA were also involved, there were still additional NE receptors.

Of the four *B. bronchiseptica*-specific, putative NE receptor genes tested- *bfrA* (BB4761), *bfrZ* (BB4744), BB1905, and BB0832- only the *bfrA* gene conferred NE utilization upon *B. pertussis*. This result suggested that *B. pertussis* has catecholamine receptors in common with *B. bronchiseptica* but they are insufficiently expressed in the *in vitro* culture system used. Expressing the cloned *B. bronchiseptica* *bfeRAB* enterobactin transport gene cluster in *B. pertussis* failed to impart enhanced NE utilization (data not shown), suggesting that *B. bronchiseptica* BfeA is likely not an NE receptor. In depth bioinformatic analyses were repeated on all of the *B. bronchiseptica* TonB-dependent receptor genes. As noted by Beall and Hoenes (1997), BfrA was found to be most similar to the IrgA and Cir receptors for enterobactin and its catechol derivatives including the biosynthetic intermediate 2,3-dihydroxybenzoic acid (DHBA) and the enterobactin degradation product 2,3-dihydroxybenzoylserine (DHBS) (Table S2) (Hantke, 1990; Mey *et al.*, 2002). The IrgA and Cir receptors are distinct from the well-characterized FepA-type enterobactin receptors (Bäumler *et al.*, 1998; Rabsch *et al.*, 1999). The *Bordetella* BfrD and BfrE putative receptors showed remarkable similarity to the *E. coli* Fiu receptor for monomeric catechols such as DHBS (Hantke, 1990). Based on amino acid sequence similarities of predicted *Bordetella* receptors with known catechol and enterobactin receptors, after BfrA, the top NE receptor candidates were identified as BfrD, BfrE and BfeA.

Combinations of single, double, triple and quadruple mutations were constructed in the BRM26 Δ *alcA* background (Table S3). The *bfrD* and *bfrE* genes are adjacent to one another on the chromosome, allowing both genes to be easily deleted simultaneously. The mutants were tested in NE growth stimulation assays with TF as the iron source (Fig. 8). Similar to the BRM66 *alcA bfeA bfrA* strain results (Fig. 7), an *alcA* mutant lacking BfeA, BfrD and BfrE (BRM68) was still able to utilize NE. Subsequent inactivation of *bfrA* in BRM68, yielding quadruple receptor gene mutant strain BRM69 (*alcA bfeA bfrA bfrDE*), finally resulted in the elimination of the NE-dependent TF utilization phenotype. To confirm that BfeA, BfrA, BfrD and BfrE were involved in NE utilization, BRM69 was genetically complemented with the individual *B. bronchiseptica* genes carried on plasmids (data not shown). Individually, the *bfrA*, *bfrD*, and *bfrE* genes were able to restore NE utilization to mutant BRM69. However, the *bfeA* enterobactin receptor gene did not complement the mutant. Based on this result, a strain that was *alcA bfrA bfrDE* (BRM71) was constructed and shown to be fully defective in the use of NE (Fig. 9). Since it was possible that BfeA could be a receptor for ferric catecholamines other than NE, BfeA⁺ (BRM71, *alcA bfrA bfrDE*) and BfeA⁻ (BRM69, *alcA bfeA bfrA bfrDE*) strains were compared in growth studies that used TF as the iron source (data not shown). The alcaligin-deficient parent strain BRM26 was able to use epinephrine, dopamine and L-3,4-dihydroxyphenylalanine (L-DOPA) for growth, but the two mutant strains BRM71 and BRM69 could not use these catecholamines regardless of the presence or absence of the BfeA receptor. These experiments identified BfrA, BfrD and BfrE as receptors functioning in catecholamine

utilization in *B. bronchiseptica* and established that the BfeA enterobactin receptor is not involved.

Receptor substrate preference

To determine if there was selectivity for different catecholamines among the BfrA, BfrD and BfrE receptors, the BRM69 quadruple receptor gene mutant strain carrying individual plasmid-borne receptor genes was examined for its ability to utilize NE, epinephrine, dopamine and L-DOPA (Fig. 10). As predicted, the BRM26 parent strain grew in the presence of TF and all of the aforementioned catecholamines, and the BRM69 null mutant carrying the plasmid vector control did not grow. Each of the *bfrA*, *bfrD*, and *bfrE* genes was able to complement BRM69, promoting growth in all of the catecholamine cultures and indicating that each of the three receptors can utilize all four of the catecholamines tested. Epinephrine was significantly less effective ($P \leq 0.0129$) than either NE, dopamine, or L-DOPA in mediating TF utilization; NE, dopamine, and L-DOPA yielded comparable growth with TF.

Since the newly identified receptors share significant similarity with *E. coli* receptors for enterobactin and other catechols, the growth of *Bordetella* mutants differentially expressing the receptor genes was examined in catechol-containing cultures. In SS medium containing TF, enterobactin and NE, the BRM26 parent strain grew well, as did a *bfeA* mutant derivative (BRM65) and a *bfrA bfrDE* mutant (BRM71) (Fig. 11A). BRM26 growth yield in the presence of both siderophore and NE was enhanced compared with the growth observed in cultures containing only NE (at 36 h, a 32% increase) (Fig. 11B) or enterobactin (at 36 h, a 26% increase) (Fig. 11C), consistent with previous results (Anderson and Armstrong, 2008) and the proposed NE-siderophore synergism. As predicted, in cultures containing TF and NE, only strains producing catecholamine receptors were able to grow (Fig. 11B). In medium containing TF and only enterobactin (*i.e.*, lacking NE), the receptor-proficient BRM26 grew, as did the strains producing any of the known enterobactin or catecholamine receptors, including the *bfeA* enterobactin receptor mutant BRM65 (Fig. 11C). The BRM69 mutant lacking all known enterobactin and catecholamine receptors showed poor growth in all of the media. All four strains grew poorly in TF-containing medium that lacked NE or enterobactin supplementation (Fig. 11D). Together, these experiments demonstrated that there is reciprocity between enterobactin-dependent and catecholamine-dependent TF utilization: NE augments enterobactin- and BfeA receptor-dependent TF utilization and conversely, enterobactin enhances delivery of TF iron *via* the catecholamine-dependent uptake system. Furthermore, that a *bfeA* enterobactin receptor mutant could still grow using enterobactin, but the quadruple receptor mutant could not, suggested a role for BfrA, BfrD and/or BfrE in utilization of enterobactin or its breakdown products.

In growth yield experiments using cultures supplemented with TF and enterobactin, the BRM65 *bfeA* enterobactin receptor mutant grew as in the previously described experiments, as did strains lacking 1) *bfeA* and *bfrA* (BRM66) and 2) *bfeA* and *bfrDE* (BRM68) (Fig. 12A). Similar patterns of growth for these strains were noted when the cultures contained DHBS rather than enterobactin. These results indicate that BfrA, BfrD and/or BfrE can serve as enterobactin and DHBS receptors in *B. bronchiseptica*. The receptor-proficient BRM26 strain was incapable of utilizing the enterobactin biosynthetic precursor DHBA to obtain iron from TF (data not shown), indicating that either DHBA is not recognized by any of the four catechol receptors or that DHBA is unable to remove the iron from TF in this experimental system.

To determine whether enterobactin and DHBS utilization in the BRM66 *alcA bfeA bfrA* mutant was due to BfrD, BfrE or both receptors, the quadruple receptor gene mutant BRM69 carrying *bfeA*⁺, *bfrA*⁺, *bfrD*⁺ or *bfrE*⁺ plasmids was assessed for TF-dependent

growth mediated by enterobactin or DHBS (Fig. 12B). BRM69 expressing plasmid-borne *bfeA*, *bfrA*, *bfrD* or *bfrE* genes was able to use enterobactin to obtain growth-promoting iron from TF, compared with BRM69 carrying the plasmid vector control. In the DHBS-containing cultures, all strains grew except BRM69 (vector control) and BRM69 carrying the *bfeA*⁺ plasmid, indicating that the BfeA enterobactin receptor is incapable of DHBS transport in this culture system and is specific for the tris-catecholate enterobactin. In sum, *B. bronchiseptica* cells producing BfeA, BfrA, BfrD or BfrE can use enterobactin for growth on ferric TF as the sole iron source. BfrA, BfrD and BfrE, the newly discovered catecholamine receptors, are also able to recognise the DHBS catechol.

Discussion

Lyte made the early observations of the growth-enhancing effects of catecholamines on bacteria (Lyte and Ernst, 1992) and coined the term “microbial endocrinology” to describe the intersection of the fields of microbiology and neurobiology (Lyte, 2004). The association of sepsis and increased susceptibility to infection with catecholamine treatment has been observed clinically in human patients as well as in animal models of infection (Lyte, 2004; Freestone *et al.*, 2008). Catecholamines are produced in the adrenal medulla and concentrations in the pM to nM range can be found in plasma, with higher concentrations at neuronal synapses and in highly innervated tissues such as the gut. To our knowledge, the levels of catecholamines on the respiratory mucosal surface are not precisely known, although it is known that serum exudes from the circulatory system onto the respiratory epithelial surface in both healthy and diseased states (Persson *et al.*, 1991). NE production, excretion and responsiveness have been reported in a variety of immune cell types, and stimulation of neutrophils and alveolar macrophages by lipopolysaccharide induces transcription of catecholamine synthesis genes and the release of NE and epinephrine (Flierl *et al.*, 2008). Our NE titration experiments showed *B. bronchiseptica* *in vitro* growth promotion at concentrations as low as 6.25 μ M. It is possible that on the respiratory epithelium, *Bordetella* cells may be exposed to nutritionally relevant catecholamine concentrations in the local microenvironment. Therefore, respiratory pathogens such as *B. bronchiseptica* may be exposed to catecholamines during infection, either on the mucosal surface *via* serum exudation or through interaction with immune cells.

Catecholamines have been reported not only to possess *in vitro* growth-promoting activity for bacteria, but also to influence bacterial virulence. For example, pretreatment of chicks with NE renders them more susceptible to colonization by *Salmonella enterica* (Methner *et al.*, 2008) and NE was found to increase adherence of *E. coli* 0157:H7 to bovine intestinal mucosa (Vlissidou *et al.*, 2004) and to colonic mucosae of pigs (Green *et al.*, 2004). NE and epinephrine also activate the quorum sensing autoinducer-3 QseBC system of enterohemorrhagic *E. coli* that regulates the expression of certain virulence genes (Clarke *et al.* 2006). Both growth and expression of type III secretion system-1 genes in *Vibrio parahaemolyticus* are enhanced by NE (Nakano *et al.*, 2007) and in *Borrelia burgdorferi*, expression of the membrane protein OspA, important for tick colonization, is increased in the presence of NE or epinephrine (Scheckelhoff *et al.* 2007).

Catecholamines bind iron *in vitro* (Green *et al.*, 1956; Jewett *et al.*, 1997; Gerard *et al.*, 1999) and they participate in feedback inhibition of their own biosynthesis by binding a ferric iron cofactor of tyrosine hydroxylase (Andersson *et al.*, 1988). Bidentate catechols similar to NE can remove iron from TF and transfer it to the siderophore desferrioxamine (Devanur *et al.*, 2008), and NE was demonstrated to remove the iron from TF and LF (Freestone *et al.*, 2000). Previous studies demonstrated a role for catecholamines such as NE for growth of *E. coli* (Burton *et al.*, 2002; Freestone *et al.*, 2003) and *Salmonella enteritidis* (Methner *et al.*, 2008) in serum-containing medium. However, use of NE by these organisms

required enterobactin, DHBA or DHBS. It is possible that in these studies, NE acted as a shuttle to augment siderophore function, consistent with our results and a hypothesis proffered by Freestone and coworkers (Freestone *et al.*, 2003). Recently, NE was demonstrated to form a complex with TF or LF and remove the iron by a reductive mechanism (Sandrini *et al.*, 2010). The study also reported that *E. coli* mutants defective in either TonB or enterobactin biosynthesis showed decreased NE-mediated iron uptake compared with the wild-type strain. In contrast, the present investigation demonstrated that a siderophore is not required by *Bordetella* cells to utilize NE to obtain iron from TF. Thus, for *B. bronchiseptica*, NE acts as a soluble iron chelator, in a manner that is functionally similar to that of a siderophore. *Listeria monocytogenes*, which produces no known siderophores, was reported to utilize catecholamines to obtain iron from a weak chemical chelator, tropolone; the mechanism appeared to involve iron reduction at the cell surface (Coulanges *et al.*, 1997). *Campylobacter jejuni* also does not produce siderophores, but exhibits CfrA enterobactin receptor-dependent growth using NE on agar containing desferrioxamine as an iron chelator (Zeng *et al.*, 2009). A recent study described *Staphylococcus aureus* siderophore mutants that could use catecholamines to obtain growth-promoting iron from TF, and showed that the Sst cytoplasmic membrane transporter for catechol siderophores was involved in this process (Beasley *et al.*, 2011). Our report describes *Bordetella* outer membrane receptors for catecholamines; however the presumed required cytoplasmic membrane transport proteins remain unknown and no predicted candidates are encoded near the receptor genes *bfrA*, *bfrD* or *bfrE*.

Under iron starvation growth conditions, *Bordetella bfeA* transcription and production of the BfeA enterobactin receptor is increased in the presence of enterobactin (Anderson and Armstrong, 2004; 2006). This induction response, requiring the transcriptional regulator BfeR, can also be stimulated by catecholamines. Based on these induction studies and the structural similarity between catecholamines and enterobactin, it was hypothesized that BfeA may also function in iron uptake mediated by NE and other catecholamines. Surprisingly, the present analysis demonstrated that the BfeA receptor is not involved in ferric catecholamine utilization, although catecholamines clearly augment enterobactin-mediated TF utilization (Anderson & Armstrong, 2008; this study). That catecholamines induce transcription of the *Bordetella* enterobactin receptor gene may simply reflect the structural similarity of these catechol compounds to the enterobactin inducer, acting via the positive regulator BfeR, or may indicate that catecholamines serve as host cues to induce *bfeA* transcription. Since the *B. pertussis* BfeA receptor was found to be important for growth in a mouse model of respiratory infection (Brickman *et al.*, 2008), *in vivo* induction of *bfeA* transcription by stress hormones may be advantageous to the pathogen.

The present study identified BfrA, BfrD and BfrE as catecholamine receptors. *bfrA* was first reported as a *B. bronchiseptica* iron-repressed gene encoding a putative TonB-dependent outer membrane receptor that is absent in *B. pertussis* and *B. parapertussis* (Beall and Hoenes, 1997). That study noted that a *bfrA* mutant was still able to use alcaligin, enterobactin or DHBS as iron sources. BfrA is most similar to outer membrane catechol receptors of the IrgA group and to the *E. coli* Cir receptor for monomeric catechols such as DHBS and DHBA (Hantke, 1990). In *Vibrio cholerae*, IrgA is a receptor for enterobactin and possibly its intermediates or breakdown products (Mey *et al.*, 2002). The *C. jejuni* CfrA enterobactin receptor is another member of the IrgA catechol receptor group (Zeng *et al.*, 2009), as is the Iha receptor, found in strains of uropathogenic *E. coli* (Leveille *et al.*, 2006). The *Bordetella* BfrD and BfrE catecholamine receptors are most similar to the *E. coli* Fiu receptor for DHBS and DHBA (Hantke, 1990). BfrD is an outer membrane protein produced by virulent Bvg⁺ phenotypic phase, but not Bvg⁻ phase, *B. pertussis* strains (Passerini de Rossi *et al.*, 1999; Antoine *et al.*, 2000). The adjacent *bfrE* gene was identified as transcriptionally Bvg independent, and encoding a predicted receptor protein with 56.7%

identity to BfrD (Antoine *et al.*, 2000). A subsequent study verified that BfrD production was Bvg activated and modestly iron repressed, although it was noted that *bfrD* does not have a well-conserved predicted Fur repressor binding site (Passerini de Rossi *et al.*, 2003). Consistent with these observations, subsequent *B. pertussis* and *B. bronchiseptica* microarray analyses did not identify *bfrD* or *bfrE* as iron-repressed genes (Brickman *et al.*, 2011) but confirmed that *bfrD* is a member of the BvgAS regulon (Cummings *et al.*, 2006). Similarity searches of sequences from *Achromobacter xylosoxidans* A8 and *Achromobacter piechaudii*, which are taxonomically related to *Bordetella* species, revealed each *Achromobacter* species to have a single gene (AXYL01473; ZP_06686349, respectively) encoding a predicted receptor with highest similarity to BfrE (80% identity), rather than BfrD (57% identity). *Bordetella petrii* also has a single gene (Bpet1692) that encodes a protein 80% identical to BfrE and 56% identical to BfrD. No *Bordetella avium* receptor gene was identified that specified a protein with high-scoring similarity to either BfrD or BfrE. Based on the presence of a single *bfrE* - like gene in *Achromobacter* species and in *B. petrii*, it is likely that the *bfrE* gene was shared among these species as well as the progenitor of the mammalian-pathogenic *Bordetella* species, whereupon the gene was duplicated in the pathogen ancestor and evolved to become the Bvg-regulated *bfrD* gene. Since both *B. pertussis* and *B. paraptussis* have an intact *bfrD* gene and *B. pertussis* has *bfrE* (*B. paraptussis* *bfrE* is an annotated pseudogene), it is unknown why these species utilize catecholamines relatively poorly in comparison to *B. bronchiseptica*. It is possible that they lack the requisite cytoplasmic membrane transport machinery to internalize ferric catecholamines. It is also conceivable that the *bfrD* genes of *B. pertussis* and *B. paraptussis*, as well as *B. pertussis* *bfrE*, are not sufficiently expressed so as to confer effective catecholamine utilization, at least in the experimental system used in this study.

Members of the Enterobacteriaceae have multiple TonB-dependent receptors for the uptake of ferric catechols; these include FepA (enterobactin, myxochelin C, DHBS), IroN (salmochelin, enterobactin, bacillibactin, DHBS), Iha (enterobactin, DHBS), and the Cir and Fiu receptors that transport ferric catechols such as DHBS. For example, a single *Salmonella* strain can produce several of these receptors (Rabsch *et al.*, 1999), suggesting that a diverse catechol receptor repertoire may be needed for successful growth in a host. Many bacterial species that do not synthesize enterobactin have evolved receptors to use it as well as its degradation products (Thulasiraman *et al.*, 1998). *Bordetella* cells use enterobactin via BfeA, which exhibits highest similarity to FepA and our present analysis identified three receptors for ferric catecholamines. Thus the presence of four catechol receptors in the obligate pathogen *B. bronchiseptica* suggests that catechols, either derived from the resident flora or provided by the host, are present in the respiratory tract. Furthermore, the co-regulation of *bfrD* with other Bvg-controlled virulence genes suggests that this receptor is important *in vivo*.

LF is the principal iron-binding glycoprotein on host mucosal surfaces, and TF is also present on the respiratory tract mucosae of humans (Stites *et al.*, 1995). *Bordetella* toxins, such as the tracheal cytotoxin, are known to kill host cells and damage the respiratory epithelium (Flak and Goldman, 1999). It is reasonable to hypothesize that during infection, *Bordetella* cells have access to heme, as well as LF and TF, catecholamine hormones, and any xenosiderophores produced by microbial flora. Our studies revealed that NE has strong iron shuttling activity and can potentiate the activity of siderophores in the presence of ferric TF. In the host, *Bordetella* cells may initially use existing xenosiderophores to obtain iron from LF and TF until they can synthesize effective concentrations of alcaligin. In this environment, host catecholamines may function as shuttles that enhance ferric iron uptake from any utilizable siderophores that are available. In the absence of siderophores, catecholamines would be predicted to remove the iron from TF and LF and provide it directly to *B. bronchiseptica* via any or all of the newly identified receptors.

Experimental Procedures

Bacterial strains and culture conditions

Bordetella strains used in this study are listed in Table S3. *E. coli* DH5 α (Invitrogen, Carlsbad, CA) was used in routine cloning and in conjugations. Luria Bertani broth and agar (Sambrook *et al.*, 1989) were used for the cultivation of *E. coli*. *Bordetella* strains were grown on Bordet Gengou agar (Bordet and Gengou, 1906) containing 20% (*B. pertussis*, *B. parapertussis*) or 7.5% (*B. bronchiseptica*) sheep blood, or Luria Bertani agar (*B. bronchiseptica*). Modified Stainer-Scholte (SS) medium (Schneider and Parker, 1982; Stainer and Scholte, 1970) was used as a chemically defined medium for the cultivation of *Bordetella* strains. SS basal medium was rendered iron-depleted by treatment with Chelex 100 (BioRad, Richmond, CA) and iron-replete by supplementation with 36 μ M FeSO $_4$ (Armstrong and Clements, 1993). The siderophores alcaligin (Brickman *et al.*, 1996) and enterobactin (Anderson and Armstrong, 2004) were purified as described previously. Monomeric 2,3-dihydroxybenzoylserine (DHBS) was from EMC Microcollections (Tuebingen, Germany) and 2,3-dihydroxybenzoic acid (DHBA) was obtained from Sigma-Aldrich (St. Louis, MO). Stock solutions of norepinephrine bitartrate salt, epinephrine, dopamine and L-3,4-dihydroxyphenylalanine (L-DOPA) (Sigma-Aldrich) were made fresh and added to SS cultures at the specified concentrations. The purified apo- forms of human TF and LF were purchased from Sigma-Aldrich and loaded with ferric iron as described (Agiato and Dyer, 1992). *Bordetella* growth in SS medium was monitored by optical density using a spectrophotometer (at 600 nm wavelength) or a Klett-Summerson colorimeter. Media were supplemented with the following concentrations of antibiotics as appropriate: ampicillin, 100 μ g/ml; chloramphenicol, 30 μ g/ml; gentamicin, 10 μ g/ml; kanamycin, 50 μ g/ml; nalidixic acid, 35 μ g/ml; tetracycline, 15 μ g/ml.

Siderophore assay

Siderophore activity was determined using the chrome azurol S (CAS) assay (Schwyn and Neilands, 1987). Reaction mixtures contained various combinations of 60 μ M alcaligin, 500 μ M 5-sulfosalicylic acid (SSA) shuttle and 500 μ M norepinephrine with the CAS dye reagent. The absorbance of the reaction mixtures at 630 nm wavelength was monitored over time. Results typical of two independent experiments are shown.

Growth stimulation assays

Catecholamine-mediated growth stimulation by TF (30% iron saturated) or LF (70% iron saturated) was measured as growth in liquid SS cultures. Where noted, cultures contained alcaligin (123.7 μ M), enterobactin (5 μ M), DHBS (50 μ M) or DHBA (50 μ M). *B. bronchiseptica* strains were grown on agar for 24 h, then subcultured into iron-replete SS medium. After growth of this seed culture for 18 hours at 35°C in a shaking incubator (250 rpm), the cells were harvested and washed with SS basal medium lacking iron. The cells were then subcultured to an initial optical density (600nm) of 0.01 to iron-replete medium or iron-depleted medium containing 10 mM sodium bicarbonate and 200 μ g/ml TF or 200 μ g/ml LF (approximately 2.5 μ M TF or LF), with or without 50 μ M catecholamine (NE, epinephrine, dopamine or L-DOPA). For Fig. 3 experiments, the 30% iron saturated TF (1 ml of a 3 mg/ml solution) was placed within a sterile dialysis bag (8,000 molecular mass cutoff) that was then submerged in 15 ml of the iron-depleted SS medium. For *B. pertussis* strain UT25Sm1 and *B. parapertussis* strain ONT1, seed cultures were initiated using inocula from agar plates, and grown in iron replete SS for 24 hours, shaking, at 35°C. The cells were harvested, washed, and subcultured to an initial O.D.₆₀₀ of 0.02 to iron-replete or iron-depleted medium containing TF or LF, with or without catecholamine, as described above for *B. bronchiseptica*. The optical densities of the *Bordetella* cultures were measured 24 and 48 hours post inoculation. The growth yield results reported are the means of

triplicate cultures and each experiment was performed at least twice independently. Statistical analysis of growth yield data used Student's *t*-test (paired, two-tailed, hypothesized difference = 0), with StatView version 4.51 software (Abacus Concepts, Inc.). The dialysis membrane and growth curve experiments are representative of at least two experimental trials.

General genetic methods

Standard methods were used for the construction of recombinant plasmids (Sambrook *et al.*, 1989). Plasmid DNA was transferred to *E. coli* strains by electroporation and to *Bordetella* strains by conjugation using *E. coli* DH5 α as the donor strain, with mobilization functions provided by plasmid pRK2013 (Figurski and Helinski, 1979). Broad host range plasmid pRK415 (Keen *et al.*, 1988) was the cloning vector used in *Bordetella* strains. Plasmids pGEM3Z, pGEM7Z (Promega, Madison, WI) and pBluescript (Stratagene/Agilent Technologies, Santa Clara, CA) were used as general cloning vectors in *E. coli*. Construction of *B. bronchiseptica* mutants by allelic exchange employed suicide plasmids pEG7 (Cotter and Miller, 1997) or pRE112 (Edwards *et al.*, 1998), and construction of *B. bronchiseptica* mutants by plasmid integration used pEG7 or pSS1129 (Stibitz, 1994). *Bordetella* transconjugants were selected on agar containing the appropriate antibiotics and colicin B as described previously (Brickman and Armstrong, 1996a). Oligonucleotide primers (Table S4) were purchased from Integrated DNA Technologies (Coralville, IA). For expression of *B. bronchiseptica* genes in *B. pertussis*, the sequences of the *B. bronchiseptica* *bfeRAB* genes and those encoding the predicted TonB-dependent receptors BfrA, BfrZ and BB0832 and BB1905 were amplified from strain RB50 by polymerase chain reaction (PCR), subcloned to pRK415 and the resulting plasmids conjugated to *B. pertussis* strain UT25Sm1. *B. bronchiseptica* genes *tonB*, *bfrA* and *bfrE* were amplified by PCR, cloned to pRK415, and used in mutant complementation studies. *bfrD* was expressed from the 238 bp *bfrE* promoter region (primers pbfrE1 and pbfrE2) cloned upstream of the coding region. The *Bordetella* genome sequences (Genbank accession numbers: *B. pertussis* Tohama I, NC_002929; *B. parapertussis* NC_002928; *B. bronchiseptica* RB50, NC_002927) were accessed at the GeneDB website (<http://www.genedb.org/>), developed and maintained by the Sanger Institute's Pathogen Sequencing Unit. BLASTP analyses were conducted using the server at the National Center for Biotechnology Information (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). DNA and protein analyses and PCR primer design used the Lasergene software package version 5.53 (DNASTAR, Inc., Madison, WI).

Mutant construction

Insertion mutants—*B. bronchiseptica* mutants defective in each known or predicted TonB-dependent receptor gene were constructed using plasmids described previously (Brickman and Armstrong, 1999; Anderson and Armstrong, 2008). Suicide plasmids were introduced to the *alcA* mutant strains BRM1 (Armstrong and Clements, 1993) or BRM26 by conjugation, and integrants were selected based on gentamicin resistance. Integration sites were confirmed by PCR analysis.

***tonB* mutant**—An in-frame 627-bp *tonB* deletion was made by PCR amplification of a region comprising 1022-bp upstream of *tonB* (BB1980) with 89-bp of the 5' end of the gene from RB50 chromosomal DNA and cloning this fragment into pGEM3Z as an SstI-BamHI fragment, yielding plasmid p3Z164. A PCR product comprising of 1095-bp downstream of *tonB* with 91-bp at the 3' end of the gene was cloned into p3Z164 as a BamHI-XbaI fragment to make plasmid p3Z165. The resulting Δ *tonB* allele was subcloned to pRE112 as an SstI-XbaI fragment. The plasmid was delivered to *B. bronchiseptica* *alcA* mutant BRM26 and following allelic exchange, mutant BRM46 was isolated and confirmed to have the *tonB* mutation by PCR.

bfeA mutant—Construction of the allelic exchange plasmid carrying *bfeA* with a 582-bp in-frame deletion mutation has been described (Anderson and Armstrong, 2008). The plasmid was transferred to *B. bronchiseptica* BRM26 and allelic exchange mutant BRM65 was isolated and confirmed to have the *bfeA* mutation by PCR.

bfrA mutant—A region containing *bfrA* along with flanking sequence on either side was PCR amplified and cloned into pBluescript II KS+, yielding plasmid pKS+/bfrA1#4. An in-frame 810-bp *bfrA* deletion was made by NcoI restriction digestion and religation of plasmid pKS+/bfrA1#4. The resulting $\Delta bfrA$ allele was subcloned to pEG7 as a BamHI-HindIII fragment along with the *sacBR* cassette as a BamHI fragment. The plasmid was delivered to *B. bronchiseptica* strains and following allelic exchange, mutants were isolated and their *bfrA* mutations confirmed by PCR.

bfrDE mutant—The *bfrD* and *bfrE* genes are adjacent on the chromosome and a single plasmid construct was designed to mutate both genes simultaneously. A region consisting of the first 76 bp of the *bfrD* gene and extending 1681 bp upstream was PCR amplified and cloned into pGEM7Z as an XbaI-HindIII fragment, resulting in plasmid p7Z5. A PCR product comprising 1160-bp downstream of *bfrE* and 34-bp of the 3' end of the gene was cloned into p7Z5 as a HindIII-SstI fragment, yielding p7Z6. The resulting $\Delta bfrDE$ fragment possessed a deletion mutation spanning both genes, removing 4393 bp from the coding sequences. The $\Delta bfrDE$ fragment was subcloned to pBluescript II KS+ as an SstI-PstI fragment then to the allelic exchange vector pEG7 as an EcoRI fragment along with the *sacBR* cassette from pEG18.3 as a BamHI fragment. The plasmid was conjugated to *B. bronchiseptica* strains and putative mutants were isolated and their *bfrDE* mutations confirmed by PCR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. NE mediated utilization of LF or TF iron

A. Siderophore-deficient $\Delta alcA$ *B. bronchiseptica* strain BRM26 was grown in iron replete Stainer Scholte (SS) defined medium (+Fe) or in iron-depleted SS medium with human LF (200 $\mu\text{g/ml}$) (-Fe +LF), or iron-depleted SS medium additionally supplemented with 50 μM NE (-Fe +LF +NE). Growth yields are reported as the means $\pm$ standard deviation from triplicate cultures measured (by optical density at 600 nm) after 48 h of incubation. NE supplementation of LF cultures results in a significant increase in mean growth yield over LF alone ($P = 0.0098$).

B. Growth yields of *B. pertussis* strain UT25Sm1. UT25Sm1 was cultured in iron replete SS medium (+Fe) or in iron-depleted SS medium with human TF (200 $\mu\text{g/ml}$) (-Fe +TF) or with additional 50 μM NE supplementation (-Fe +TF +NE). Mean growth yields after 48 h are shown. Error bars indicate one standard deviation for triplicate cultures. Statistical significance : ns, not significant.

C. Growth yields of *B. paraptussis* strain ONT-1. ONT-1 was cultured as for *B. pertussis* in (B). Mean growth yields after 48 h are shown. Error bars indicate one standard deviation for triplicate cultures. Statistical significance : *, significant, $P = 0.0368$.

Fig. 2.

NE dose-responsive growth yields of $\Delta alcA$ *B. bronchiseptica* strain BRM26. Cells were grown in iron-depleted SS medium with TF (200 $\mu\text{g}/\text{ml}$) and graded concentrations of NE (-Fe +TF +NE). Iron replete control cultures were grown in SS medium with 36 μM iron (+Fe). Growth yields were measured at 24h and 48h (means $\pm$ standard deviation from triplicate cultures). At 48 h, the mean growth yields of all NE-containing cultures, except the 3.13 μM NE culture ($P = 0.6784$), were significantly greater than the cultures with no added NE ($P \leq 0.0069$).

Fig. 3.

NE-mediated TF utilization does not require cell contact with TF. *B. bronchiseptica* $\Delta alcA$ strain BRM26 was cultured in iron depleted SS medium (-Fe) with 123.7 μM alcaligin (-Fe +Alc), or 50 μM NE (-Fe +NE), or both (-Fe +NE +Alc), with TF either free in the medium (200 $\mu\text{g/ml}$) or contained within a dialysis bag (same total amount of TF as when free in the medium). Growth yields were measured after 48h. Iron replete control cultures were grown in SS medium containing TF (200 $\mu\text{g/ml}$) and 36 μM iron (+Fe).

Fig. 4.

NE shuttles iron to alcaligin. The rate of iron removal from a chrome azurol S (CAS) dye-iron complex was monitored *in vitro* as a decrease in absorbance at 630 nm wavelength. 5-sulfosalicylic acid (SSA) is used in the standard CAS siderophore assay as a shuttle to increase the rate of iron exchange from the dye reagent to the siderophore. The CAS dye-iron complex reactions contained: no additions (Blank), 60 μ M alcaligin (+Alc), 500 μ M 5-sulfosalicylic acid shuttle (+SSA), 500 μ M NE (+NE), 60 μ M alcaligin and 500 μ M 5-sulfosalicylic acid (+Alc +SSA), 60 μ M alcaligin and 500 μ M NE (+Alc +NE).

Fig. 5.

TonB is required for NE-mediated TF utilization. *B. bronchiseptica* strains BRM26 (*alcA*), BRM46 (*alcA tonB*) carrying the pRK415 plasmid vector and BRM46 (*alcA tonB*) carrying the *tonB⁺* plasmid pRKton, were cultured in iron-depleted SS medium with TF (200 μ g/ml), either with 50 μ M NE (-Fe +TF +NE) or without NE (-Fe +TF) supplement. Iron replete control cultures contained 36 μ M iron (+Fe). Mean growth yields were determined after 48h. Error bars indicate one standard deviation for triplicate cultures. Statistical significance: ns, not significant; ***, $P \leq 0.0006$.

Fig. 6. The *B. bronchiseptica* *bfrA* gene promotes NE-mediated TF utilization in *B. pertussis*. *B. pertussis* strain UT25Sm1 carrying the pRK415 plasmid vector or the plasmid-borne *bfrA* gene (pRK/bfrA) was grown in iron-depleted SS medium with TF (200 μ g/ml) (-Fe +TF), or medium additionally supplemented with 50 μ M NE (-Fe +TF +NE). Iron replete control cultures contained 36 μ M iron and grew to an OD₆₀₀ of approximately 4.0 (data not shown). Mean growth yields were determined after 48h. Error bars indicate one standard deviation for triplicate cultures. Statistical significance: ***, $P = 0.0005$.

Fig. 7.

A. B. bronchiseptica mutant lacking BfrA exhibits reduced growth in the presence of TF and NE. Strains BRM65 (*alcA bfeA*) and BRM66 (*alcA bfeA bfrA*) were cultured in iron-depleted SS medium with TF (200 μ g/ml), either with (-Fe + TF+ NE) or without (-Fe +TF) 50 μ M NE supplementation. Iron replete control cultures contained 36 μ M iron (+Fe). Growth was measured by optical density over a 44-h time period.

Fig. 8. Identification of a *B. bronchiseptica* receptor mutant unable to utilize NE. Strains BRM68 (*alcA bfeA bfrDE*) and BRM69 (*alcA bfeA bfrA bfrDE*) were cultured in iron replete SS medium (+Fe) and in iron-depleted SS medium with TF (200 µg/ml) (-Fe +TF), or additionally supplemented with 50 µM NE (-Fe +TF +NE). Growth yields were measured after 48h (mean +/- standard deviation from triplicate cultures).

Fig. 9.

A *B. bronchiseptica* mutant lacking BfrA, BfrD and BfrE is unable to utilize NE: the BfeA enterobactin receptor is not involved. Parent strain BRM26 and receptor mutant derivatives BRM65, BRM67, BRM70, and BRM71 were cultured in iron replete SS medium (+Fe) and in iron-depleted SS medium with TF (200 μ g/ml) (-Fe +TF), or with both TF and 50 μ M NE (-Fe +TF +NE). Genotypes are designated for each strain. Growth yields were measured after 48h (mean $\pm$ standard deviation from triplicate cultures). Statistical significance: *P* values for all strains' mean growth yields (-Fe + TF + NE), ranged from 0.0001 to 0.0007.

Fig. 10.

The BfrA, BfrD and BfrE receptors are each capable of utilizing NE, epinephrine, dopamine and L-DOPA. Parent strain BRM26 and the null receptor mutant BRM69 (*alcA bfeA bfrA bfrDE*) carrying plasmids bearing *bfrA*, *bfrE* or *bfrD* were cultured in iron replete SS medium (+Fe) and in iron-depleted SS medium with TF (200 µg/ml) (-Fe +TF), or with both TF and 50 µM NE (-Fe +TF +NE), TF and 50 µM epinephrine (-Fe +TF +Epi), TF and 50 µM dopamine (-Fe +TF +Dop), and TF and 50 µM L-DOPA (-Fe +TF +L- DOPA). Growth yields were measured after 48h (means +/- standard deviation from triplicate cultures). Epinephrine was significantly less effective than either NE, dopamine, or L-DOPA in mediating TF utilization ($P \leq 0.0129$)

Fig. 11. Enterobactin-dependent and catecholamine-dependent TF utilization. Parent strain BRM26 and mutant derivatives BRM65, BRM71 and BRM69 were cultured in iron-depleted SS medium with TF (200 $\mu\text{g}/\text{ml}$) as the sole iron source. Those cultures were further supplemented with both 5 μM enterobactin and 50 μM NE (A), NE alone (B), enterobactin alone (C), or were not supplemented (D). Growth was measured by optical density over a 36-h time period.

Fig. 12.

The BfrA, BfrD and BfrE catecholamine receptors function in both enterobactin and DHBS utilization of TF iron. Parent strain BRM26 and mutant derivatives BRM65, BRM66, BRM68 and BRM69 were grown in the SS test media (A). BRM26 and the mutant BRM69 (*alcA bfeA bfrA bfrDE*) carrying plasmids bearing *bfeA*, *bfrA*, *bfrE* or *bfrD* were cultured in SS media (B). Culture media: iron replete SS medium (+Fe) and in iron-depleted SS medium with TF (200 µg/ml) (-Fe +TF), or with both TF and 50 µM DHBS (-Fe +TF +DHBS), TF and 5 µM enterobactin (-Fe +TF +Ent). Mean growth yields after 48 h are shown. Error bars indicate one standard deviation for triplicate cultures.