Norepinephrine Mediates Acquisition of Transferrin-Iron in *Bordetella bronchiseptica*†

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Previous research demonstrated that the sympathoadrenal catecholamine norepinephrine could promote the growth of *Bordetella bronchiseptica* in iron-restricted medium containing serum. In this study, norepinephrine was demonstrated to stimulate growth of this organism in the presence of partially iron-saturated transferrin but not lactoferrin. Although norepinephrine is known to induce transcription of the *Bordetella bfeA* enterobactin catechol xenosiderophore receptor gene, neither a *bfeA* mutant nor a bfr regulator mutant was defective in growth responsiveness to norepinephrine. However, growth of a *tonB* mutant strain was not enhanced by norepinephrine, indicating that the response to this catecholamine was the result of high-affinity outer membrane transport. The *B. bronchiseptica* genome encodes a total of 19 known and predicted iron transport receptor genes, none of which, when mutated individually, were found to confer a defect in norepinephrine-mediated growth stimulation in the presence of transferrin. Labeling experiments demonstrated a TonB-dependent increase in cell-associated iron levels when bacteria grown in the presence of 55Fe-transferrin were exposed to norepinephrine. In addition, TonB was required for maximum levels of cell-associated norepinephrine. Together, these results demonstrate that norepinephrine facilitates *B. bronchiseptica* iron acquisition from the iron carrier protein transferrin and this process may represent a mechanism by which some bacterial pathogens obtain this essential nutrient in the host environment.

The acquisition of essential nutrients, such as iron, in the host environment is key to successful infection by bacterial pathogens. However, much of the extracellular iron in the host is bound by high-affinity iron-chelating glycoproteins belonging to the transferrin family. Transferrin and lactoferrin are two members of this family that are predominantly found in serum and mucosal secretions, respectively (26, 34, 47). These proteins play an important role in iron homeostasis and sequestration, but several bacterial species are able to exploit them as sources of nutritional iron. Bacterial utilization of these host iron-binding proteins can occur via dedicated surface receptors, as exemplified by the TbpAB and LbpAB transporter complexes of *Neisseria* species (15). Siderophores produced and excreted by many microbes can also contribute to iron acquisition by stripping the iron from transferrins and delivering it to microbial recipients through ferric siderophore transport machinery (29).

The respiratory pathogens *Bordetella bronchiseptica* and *Bordetella pertussis* have multiple characterized mechanisms of iron acquisition, including transport of heme-iron (55), biosynthesis and utilization of the alcaligin siderophore (10, 27), and uptake of catechol xenosiderophores, such as enterobactin (4, 8). In addition, there are other putative outer membrane iron transporters encoded in the *Bordetella* genome for which the ligand is unknown (41). *Bordetella* species can obtain the iron from transferrin (transferrin-iron) and lactoferrin (45); however, neither the *B. bronchiseptica* nor *B. pertussis* genome appears to code for TbpAB or LbpAB receptor homologs. Two low-molecular-mass *Bordetella* proteins that bound transferrin and lactoferrin were previously isolated, but the identity and function of these proteins remain unknown (35). Even though both transferrin and lactoferrin associate tightly with the *B. pertussis* cell surface (45), direct contact is reportedly not essential for internalization of the iron (25, 35). It is therefore likely that *Bordetella* iron acquisition from transferrin and lactoferrin is mediated by siderophores or other iron-chelating molecules.

The catecholamines epinephrine, norepinephrine, and dopamine are widely distributed throughout plant and animal species. In humans, epinephrine is synthesized in the adrenal medulla and released into the bloodstream as a result of impulses from the central nervous system. Norepinephrine is synthesized and stored primarily in peripheral sympathetic nerve endings. Dopamine is a neurotransmitter of the central nervous system but is also present in sympathetic nerves and the adrenal medulla (58). All three molecules are effectors of the mammalian sympathetic nervous system, and there is increasing evidence that these catecholamines are also perceived by bacterial pathogens (48, 52). One bacterial response that has been attributed to catecholamines is the ability to stimulate growth in the presence of serum, transferrin, or lactoferrin (13, 21, 31). Free-stone et al. originally identified the growth-promoting serum component as transferrin and although the mechanism remains unclear, it was found that norepinephrine liberates iron from transferrin and lactoferrin, making it available for growth of *Escherichia coli* (23). Complexes of norepinephrine with ferric transferrin or ferric lactoferrin were demonstrated, but a
stable ferric norepinephrine complex was not observed. However, it is known that catecholamines are capable of binding iron, and norepinephrine-iron complexes have been analyzed in other neurochemical studies (40, 51).

In iron-starved Bordetella cells, enterobactin induces transcription of its cognate receptor gene bfaA in a process that is dependent on the BfeR AraC-type positive regulator (3). Analysis of other catechol compounds demonstrated that dopamine, norepinephrine, and epinephrine also induced transcription of bfaA, and norepinephrine was additionally capable of stimulating Bordetella growth in iron-limiting medium containing serum (4). The localization of norepinephrine in peripheral tissues suggests that bacterial inhabitants of the respiratory tract, such as Bordetella species, are more likely to encounter this catecholamine during infection than dopamine or epinephrine. Based on our experimental observations and evidence from the literature that transferrin and lactoferrin potentiate norepinephrine-mediated growth stimulation of bacteria (23), we sought to further characterize the Bordetella growth response to norepinephrine.

MATERIALS AND METHODS

Bacterial strains and plasmids. B. bronchiseptica strains B013N (5) and RB50 (17) were used as wild-type strains for this study. The BRM18 AfaA (10), BRM24 ΔbfeA (3), BRM26 ΔFadA (9), and BRM31 tonA:BpsSt2 (4) mutants derived from B. bronchiseptica strain B013N have been previously described. E. coli DH5α (Invitrogen, Carlsbad, CA) was used as the host strain for routine cloning purposes and was the plasmid donor in tripartite matings. DNA mobilization functions for conjugations using the DH5α donor strain were provided by plasmid pRK2013 (20) or were chromosomally encoded by strain S17-1 (50). Plasmid pGEMZS (Promega, Madison, WI) was used as a general cloning vector in E. coli. The tonB and exbB plasmid pBPh4 (3) was constructed by subcloning a DNA fragment containing the TonB system genes of B. bronchiseptica from previously described plasmid pRK11 (39) to broad-host-range plasmid pBBR1MCS-1 (30). Suicide plasmids pSS1129 (54) and its derivative pEG18 (18) were used in the construction of B. bronchiseptica mutants by allelic exchange or plasmid integration.

Culture conditions. Luria-Bertani broth and agar (46) were used for routine cultivation of E. coli, and B. bronchiseptica strains were grown on Luria-Bertani agar or blood agar (Becton, Dickinson and Co., Franklin Lakes, NJ). Stainer-Scholte (SS) medium (53), modified as described previously (49), was used as a chemically defined medium for the cultivation of B. bronchiseptica strains in growth stimulation assays. SS basal medium was rendered iron depleted by treatment with Chelex 100 (Bio-Rad, Richmond, CA) and iron replete by the addition of 36 M FeSO4. Each culture additionally contained either 50 M FeSO4 and 50 M norepinephrine and 36 M FeSO4. Triplicate aliquots of a 0.9% NaCl solution containing either 50 M FeSO4 or FeSO4 and 50 M norepinephrine were used for Fe and [3H]norepinephrine binding assays. Preparation of radiolabeled transferrin was based on a previously published method (23). Apotransferrin (Sigma-Aldrich) solutions were prepared in 0.1 M sodium citrate and 0.1 M sodium bicarbonate buffer. A mixture of FeCl3 and FeCl2 (PerkinElmer, Boston, MA) was added to the transferrin solution to achieve an iron saturation level of 50% and an activity of 5 µCi/µg protein. Ferric transferrin complexes were allowed to form at 37°C for 5 h followed by separation of unbound iron using Sephadex G25 gel filtration. B. bronchiseptica strains were grown in iron-replete SS medium for 24 h followed by growth for 16 h in iron-depleted SS medium. Bacteria were harvested from frozen cultures and resuspended in 0.85% NaCl solution.

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Cell-associated norepinephrine levels were determined using bacterial cultures prepared and inoculated as described above for the iron transport assay. Experimental cultures consisted of iron-depleted SS medium containing 200 µg/ml partially iron-saturated transferrin supplemented with either 50 M norepinephrine or 50 M FeSO4. Each culture additionally contained 1.67 µCi/ml of [3H]norepinephrine (GE Healthcare, Buckinghamshire, England). Cell-associated radioactivity was determined as described above for triplicate wells as previously described (23).
TABLE 1. *B. bronchiseptica* TonB-dependent receptor genes

| Locus tag | Gene name and/or reference | Primer sequence (5′→3′) |
|-----------|---------------------------|-------------------------|
| BB0078    | *bfrC* (6)                | GGCCGAATTCGGGGTGCAGAAG  |
| BB0078    |                          | CACATGAC                 |
| BB0078    |                          | GGCCAGTTGGGGCCGCGCAGAG  |
| BB0078    |                          | AACTG                   |
| BB0189    | This study                | GGCCGAATTCGGGGGCGCTCAG  |
| BB0189    |                          | GGCCGATG                 |
| BB0832    | 41                        | GGCCGAATTCGGGGGCGCTCAG  |
| BB0832    |                          | GGCGAGATG                |
| BB0832    |                          | GGGCGATCCTGGCGGCGAAGT   |
| BB0832    |                          | CCCAGATG                |
| BB1294    | *bfrG* (41)              | GGCCGAATTCGGGGGCGCTCAG  |
| BB1294    |                          | GGCCGAGT                 |
| BB1294    |                          | GGGCGATCCTGGCGGCGAAGT   |
| BB1294    |                          | CCCAGATG                |
| BB1785    | *bfrI* (41)              | GGCCGAATTCGGGGGCGCTCAG  |
| BB1785    |                          | GGCGGATG                 |
| BB1785    |                          | GGGCGATCCTGGCGGCGAAGT   |
| BB1785    |                          | CCCAGATG                |
| BB1846    | *bfrB* (6)               | GGCCGAATTCGGGGGCGCTCAG  |
| BB1846    |                          | GGCGGATG                 |
| BB1846    |                          | GGGCGATCCTGGCGGCGAAGT   |
| BB1846    |                          | CCCAGATG                |
| BB1905    | 41                        | GGCCGAATTCGGGGGCGCTCAG  |
| BB1905    |                          | GGCGGATG                 |
| BB1905    |                          | GGGCGATCCTGGCGGCGAAGT   |
| BB1905    |                          | CCCAGATG                |
| BB1942    | *bfrA* (8)               | GGCCGAATTCGGGGGCGCTCAG  |
| BB1942    |                          | GGCGGATG                 |
| BB1942    |                          | GGGCGATCCTGGCGGCGAAGT   |
| BB1942    |                          | CCCAGATG                |
| BB2179    | This study                | GGCCGAATTCGGGGGCGCTCAG  |
| BB2179    |                          | GGCGGATG                 |
| BB2179    |                          | GGGCGATCCTGGCGGCGAAGT   |
| BB2179    |                          | CCCAGATG                |
| BB3658    | *bfrH* (41)              | GGCCGAATTCGGGGGCGCTCAG  |
| BB3658    |                          | GGCGGATG                 |
| BB3658    |                          | GGGCGATCCTGGCGGCGAAGT   |
| BB3658    |                          | CCCAGATG                |
| BB3825    | *bfrE* (41)              | GGCCGAATTCGGGGGCGCTCAG  |
| BB3825    |                          | GGCGGATG                 |
| BB3825    |                          | GGGCGATCCTGGCGGCGAAGT   |
| BB3825    |                          | CCCAGATG                |
| BB3826    | *bfrD* (42)              | GGCCGAATTCGGGGGCGCTCAG  |
| BB3826    |                          | GGCGGATG                 |
| BB3826    |                          | GGGCGATCCTGGCGGCGAAGT   |
| BB3826    |                          | CCCAGATG                |
| BB3900    | *faeA* (10)              | NA                      |
| BB3900    |                          | GGCGGATTCGGGGGCGCTCAG   |
| BB3900    |                          | GGCGGATCCTCGGGCGCGAGCT  |
| BB3900    |                          | GCCACAC                 |
| BB4122    | *bfrF* (41)              | NA                      |
| BB4122    |                          | GGCGGATTCGGGGGCGCTCAG   |
| BB4122    |                          | GGCGGATCCTCGGGCGCGAGCT  |
| BB4122    |                          | GCCACAC                 |
| BB4457    | This study                | NA                      |
| BB4457    |                          | GGCGGATTCGGGGGCGCTCAG   |
| BB4457    |                          | GGCGGATCCTCGGGCGCGAGCT  |
| BB4457    |                          | GCCACAC                 |
| BB4655    | *bhuR* (55)              | NA                      |
| BB4655    |                          | GGCGGATTCGGGGGCGCTCAG   |
| BB4655    |                          | GGCGGATCCTCGGGCGCGAGCT  |
| BB4655    |                          | GCCACAC                 |
| BB4744    | *bfrZ* (44)              | NA                      |
| BB4744    |                          | GGCGGATTCGGGGGCGCTCAG   |
| BB4744    |                          | GGCGGATCCTCGGGCGCGAGCT  |
| BB4744    |                          | GCCACAC                 |
| BB4761    | *bfeA* (7)               | NA                      |
| BB4761    |                          | GGCGGATTCGGGGGCGCTCAG   |
| BB4761    |                          | GGCGGATCCTCGGGCGCGAGCT  |
| BB4761    |                          | GCCACAC                 |
| BB4956    | *hemC* (41)              | NA                      |
| BB4956    |                          | GGCGGATTCGGGGGCGCTCAG   |
| BB4956    |                          | GGCGGATCCTCGGGCGCGAGCT  |
| BB4956    |                          | GCCACAC                 |

a Primer sequences for PCR amplification of internal gene fragments used in the construction of insertion mutations.

b NA, not applicable.

RESULTS

Iron acquisition using siderophores and norepinephrine.

Norepinephrine was previously shown to stimulate growth of *B. bronchiseptica* in iron-depleted SS medium containing serum (4). Since it was reasoned that transferrin was the likely serum iron source responsible for this growth enhancement, the ability of *B. bronchiseptica* to obtain iron from transferrin via norepinephrine was examined. *B. bronchiseptica* aca1 mutant strain BRM26 is unable to synthesize alcaligin and therefore allowed for the analysis of norepinephrine- and transferrin-mediated growth stimulation without the influence of the native siderophore on iron acquisition. In iron-replete medium at a low inoculum of ~100 CFU/ml, strain BRM26 exhibited an extended lag phase but was able to achieve significantly elevated growth levels over the course of the experiment (Fig. 1A). In the absence of any inorganic iron source or transferrin, strain BRM26 showed a similarly long lag phase but then relatively poor growth, regardless of the presence of norepinephrine or enterobactin. These results indicate that norepinephrine itself is not a growth-stimulating nutrient and that an iron source must be available in the culture system.

Addition of norepinephrine to BRM26 cultures in iron-depleted medium containing partially iron-saturated transferrin resulted in a reduced lag phase and an increase in growth yield compared to parallel cultures that contained transferrin alone (Fig. 1B). These results are similar to those from our previous *B. bronchiseptica* growth studies using serum (4) and demonstrate that transferrin can substitute for serum in norepinephrine-mediated growth stimulation assays. Remarkably, norepinephrine alone was capable of stimulating the growth of *B. bronchiseptica* in the presence of transferrin; no siderophore was required to achieve this effect. This norepinephrine feeding effect was also observed using the RB50 strain of *B. bronchiseptica* (data not shown). Norepinephrine-mediated growth of *E. coli* has been reported to involve the native enterobactin siderophore (13, 21), and since Bonetella species use enterobactin as a xenosiderophore, its influence on growth stimulation by norepinephrine was investigated. In the presence of transferrin, enterobactin facilitated robust growth compared to growth of unsupplemented cultures, and only a modest increase in growth yield was observed when cultures were additionally supplemented with norepinephrine (Fig. 1B).

When lactoferrin was used as the iron source, growth of *B. bronchiseptica* BRM26 was inhibited and addition of norepinephrine failed to stimulate growth (Fig. 1C). The siderophores enterobactin (Fig. 1C) and alcaligin (data not shown) relieved the bacteriostatic effects of lactoferrin. As observed with transferrin, norepinephrine only moderately enhanced the growth of lactoferrin-containing BRM26 cultures supplemented with enterobactin or alcaligin. The bacteriostatic effect of lactoferrin on *B. bronchiseptica* resulted from iron sequestration, since addition of excess FeSO4 relieved the growth inhibition. Although the iron-binding affinity of lactoferrin is higher than that of transferrin (1), these results indicate that both glycoproteins can be utilized as iron sources by *B. bronchiseptica*. However, only transferrin was
effectively used as a substrate for norepinephrine-mediated growth stimulation.

Roles of the enterobactin and alcaligin receptors in the norepinephrine response. Since enterobactin and norepinephrine can induce transcription of the bfeA ferric enterobactin receptor gene by a BfeR-dependent mechanism (3, 4), it was hypothesized that norepinephrine-mediated acquisition of transferrin-iron would also require the BfeA receptor. When iron-depleted and transferrin-supplemented cultures of \( \textit{B. bronchiseptica} \) \( \textbf{bfeA} \) mutant strain BRM33 were provided with norepinephrine, an approximately twofold increase in growth yield was observed over that of cultures lacking norepinephrine (Fig. 2A). Since a similar norepinephrine-mediated increase in growth was observed for the wild-type strain B013N, this suggested that if BfeA were a ferric norepinephrine receptor, it is not the sole receptor. The levels of norepinephrine-stimulated growth for both strains were nearly equivalent to the growth observed in medium containing 36\( \mu \)M iron. The \( \textbf{bfeR} \) mutant strain BRM24 also exhibited wild-type levels of norepinephrine-stimulated growth (Fig. 2B), indicating that BfeR-dependent induction of \( \textbf{bfeA} \) transcription by norepinephrine is not required for this growth response.

The presence of the alcaligin siderophore did not influence the ability of norepinephrine to promote the growth of \( \textit{B. bronchiseptica} \), since an alcaligin mutant (Fig. 1) as well as alcaligin proficient strains (Fig. 2) responded similarly to the catecholamine. Furthermore, a \( \textbf{fauA} \) alcaligin receptor mutant strain also exhibited a similar growth response to norepinephrine compared to the wild-type, \( \textbf{bfeA} \), and \( \textbf{bfeR} \) mutant strains (data not shown). Together, these results do not suggest a role for either alcaligin or the alcaligin receptor in transferrin-iron acquisition via norepinephrine.

TonB-dependent norepinephrine utilization. To determine whether \( \textit{B. bronchiseptica} \) iron acquisition by norepinephrine required a TonB-dependent transport system, the growth responsiveness of \( \textbf{tonB} \) mutant strain BRM31 was tested. The growth levels of strain BRM31 in iron-depleted SS medium containing transferrin were similarly low both in the presence and absence of norepinephrine (Fig. 3). The requirement for TonB was confirmed by genetic complementation in \( \textit{trans} \) using \( \textbf{tonB}^+ \textbf{exbBD}^+ \) plasmid pBB41, restoring the ability of norepinephrine to stimulate BRM31 growth to near wild-type
levels. The tonB mutation had no effect on growth of *B. bronchiseptica* iron-replete control cultures. The growth yield of the tonB strain was decreased in iron-restricted medium lacking norepinephrine compared to those of the wild-type and complemented mutant strains. This result likely reflects the contribution of alcaligin-mediated acquisition of transferrin-iron to the growth of *B. bronchiseptica*, since ferric alcaligin transport is TonB dependent (39).

**Mutational analysis of putative TonB-dependent receptor genes.** The fact that norepinephrine utilization was TonB dependent suggested the requirement for a TonB-dependent outer membrane receptor. There are 16 annotated TonB-dependent outer membrane receptor genes in the published genome sequence of *B. bronchiseptica* strain RB50 (Table 1) (41). Three of these receptors, BfeA (enterobactin) (8), BhuR (heme) (55), and FauA (alcaligin) (10) are well characterized, while the ligands for the remaining 13 receptor proteins have not been identified. In the present study, three additional putative TonB-dependent receptors (BB0189, BB2179, and BB4457) were identified in the *B. bronchiseptica* genome sequence, based on the presence of a TonB-dependent receptor motif (Pfam 00593) and the receptor plug domain (Pfam 07715). Each of the 17 receptor genes not yet analyzed in this study was disrupted, in either the B013N or RB50 parental strain background, and the resulting mutants were tested for enhanced growth in response to norepinephrine in the ferric transferrin utilization assay. No single TonB-dependent receptor mutant was found to be defective in norepinephrine-mediated growth stimulation compared with its parental control strain, nor was there an observable growth stimulation difference between the two parent strains (data not shown). The growth phenotype of each mutant in the iron acquisition assay was similar to that of the ΔfeA and ΔfaA mutants. These data suggest that more than one TonB-dependent receptor is capable of facilitating transport of iron from transferrin via norepinephrine.

**Association of norepinephrine and iron with Bordetella cells.** If norepinephrine mediates transport of transferrin-iron for *B. bronchiseptica* cells, then cellular localization of iron should increase in a norepinephrine-dependent manner. Apotransferrin was loaded with a mixture of FeCl₃ and ⁵⁵FeCl₃, and used as the bacterial iron source in binding experiments. Wild-type *B. bronchiseptica* provided with norepinephrine exhibited a significantly higher level of cell-associated ⁵⁵Fe label than cultures that lacked the catecholamine, suggesting that norepinephrine-mediated growth stimulation is the result of iron transport (Fig. 4A). The nearly twofold increase in cell association of ⁵⁵Fe decreased dramatically when ³Hnorepinephrine was supplemented with either 200 µg/ml transferrin plus 50 µM norepinephrine (shaded bars), or 200 µg/ml ⁵⁵Fe-transferrin plus 50 µM norepinephrine plus 36 µM FeSO₄ (open bars). (B) *B. bronchiseptica* strains B013N (solid bars), BRM31(pBBR1MCS-1) (shaded bars), and BRM31(pBB41) (open bars) were used. Iron-depleted basal medium containing ³Hnorepinephrine was supplemented with either 200 µg/ml transferrin plus 50 µM norepinephrine (FeTf + NE) or 200 µM transferrin plus 50 µg/ml norepinephrine plus 36 µM FeSO₄ (FeTf + NE + FeSO₄). Cell-associated levels of ⁵⁵Fe or ³Hnorepinephrine were determined by scintillation counting and normalized based on the optical density at 600 nm (OD₆₀₀) after 24 h of incubation. Values represent means ± 1 standard deviation (error bars) from triplicate determinations.
iron in a TonB-dependent manner, we examined whether norepinephrine itself became cell associated during the iron acquisition process. In iron-depleted conditions in the presence of transferrin, the amount of [3H]norepinephrine bound to the tonB mutant cells was significantly lower than that associated with the wild-type strain or the genetically complemented tonB mutant strain (Fig. 4B). However, in the presence of excess iron, cell-associated [3H]norepinephrine levels were similar among all three strains, indicating that the TonB system is not important for the association of norepinephrine with iron-replete bacteria. The reason underlying the increased binding of [3H]norepinephrine to the tonB mutant under iron-replete versus iron-depleted conditions is presently unknown. In sum, these binding studies indicate that both norepinephrine and the iron from transferrin localize to B. bronchiseptica cells in a tonB-dependent manner that is consistent with a receptor-driven transport process.

**DISCUSSION**

Examples of bacterial responses to sympathoadrenal catecholamines, such as norepinephrine, have been reported in the scientific literature. Epinephrine and norepinephrine induce production of the tick colonization factor OspA from the Lyme disease pathogen *Borrelia burgdorferi* (48). Both compounds also activate, via the QseC histidine kinase, the autoinducer-3 quorum-sensing system of enterohemorrhagic *E. coli*, which is responsible for the controlled expression of several virulence genes (14). Other bacterial responses to catecholamines are seemingly related to iron transport, such as the induced production of the *E. coli* enterobactin receptor protein FepA by norepinephrine (13) and induced transcription of the *B. bronchiseptica* bfeA enterobactin receptor gene by epinephrine, norepinephrine, and dopamine (4). The growth of several gram-negative and gram-positive bacterial species in iron-restrictive culture conditions is stimulated by catecholamines (19, 22, 28, 31, 37). With this report, we have established that *B. bronchiseptica* utilizes norepinephrine as an iron source in the presence of transferrin and that utilization of norepinephrine appears to require high-affinity TonB-dependent transport.

The mechanism of iron acquisition via norepinephrine remains unknown. However, the catechol structure of norepinephrine suggests that it may be able to form a bidentate interaction with ferric iron. *Borrelia* cells can utilize norepinephrine for iron retrieval from transferrin in the absence of a siderophore, suggesting that a TonB-dependent receptor recognizes and transports ferric norepinephrine. Early work demonstrated that transferrin bound tightly to *Borrelia* cell surfaces to the degree that it copurified with outer membrane fractions (45). Therefore, it is unlikely that norepinephrine-mediated iron chelation from transferrin would need to occur at a distance. Rather, hypothesized ternary interactions between transferrin, iron, and norepinephrine (23) in close association with the bacterial surface may be sufficient for disruption of ferric transferrin complexes and bacterial assimilation of the liberated iron. In contrast, norepinephrine-mediated growth stimulation of *E. coli* apparently requires enterobactin (13, 21), and for *Salmonella enterica*, it requires either the enterobactin precursor 2,3-dihydroxybenzoic acid or the enterobactin or salmochelin monomeric breakdown products 2,3-dihydroxybenzoylserine and SX, respectively (36). The ability to use norepinephrine for iron retrieval has important implications in pathogenesis, especially since pretreatment with norepinephrine significantly enhanced the colonization and dissemination of *S. enterica* in infected mice (36, 55).

*Bordetella* iron sources, such as enterobactin (3), alcaligin (12), and heme-iron (55, 56) induce transcription of their cognate transport genes. Since norepinephrine is a transcriptional inducer of the bfeA receptor gene required for utilization of enterobactin and other catechol siderophores (3), we hypothesized that BfeA also facilitates iron transport via norepinephrine. However, no apparent defect in iron acquisition by norepinephrine was detected in two different bfeA mutant strains (Fig. 2A and data not shown). The transcriptional response to norepinephrine may be due simply to its structural similarity to enterobactin, a hypothesis that is supported by the finding that the presence of a catechol group is a key structural element of inducer recognition by BfeR (4). The requirement for TonB in norepinephrine-mediated growth strongly suggests the existence of at least one outer membrane receptor for ferric norepinephrine. Of the 16 annotated TonB-dependent receptors in the published *B. bronchiseptica* genome (41) and the three predicted TonB-dependent receptors identified in this work, no single receptor mutant exhibited a norepinephrine utilization defect. We hypothesize that *Bordetella* norepinephrine utilization likely involves multiple TonB-dependent transporters, one of which could still be BfeA. A recent study of *E. coli* reported that each of the three known catechol siderophore receptor genes (*fepA, irn*, and *cir*) was involved in growth stimulation by norepinephrine (57).

*B. bronchiseptica* inhabits the upper respiratory mucosa of the host, and it is well-known that the lactoferrin in that environment sequesters extracellular iron from invading microbial pathogens (33, 34). We have demonstrated that both alcaligin and enterobactin were able to stimulate *Bordetella* growth in iron-depleted medium containing lactoferrin. In the absence of siderophores, lactoferrin was bacteriostatic, emphasizing the importance of siderophore-mediated iron acquisition to *Bordetella* cells colonizing that anatomical site. Although norepinephrine did not stimulate *B. bronchiseptica* growth in the presence of lactoferrin, others have shown that both transferrin and lactoferrin can serve as substrates for *E. coli* iron acquisition via norepinephrine (23).

Although transferrin is primarily located in serum (1) and *B. bronchiseptica* is generally considered a noninvasive mucosal pathogen (24), *Bordetella* cells can acquire transferrin-bound iron by way of siderophores or norepinephrine. Another pathogen that inhabits a mucosal surface, *Neisseria gonorrhoeae*, was unable to initiate urethritis in human volunteers without a functional transferrin-iron uptake system, suggesting that sufficient transferrin is naturally present to support infection (16). Plasma exudation through intact respiratory epithelium occurs in response to the presence of various stimuli, including microbial pathogens (43), and may result in the presence of transferrin on respiratory surfaces. Therefore, *Bordetella* species may encounter transferrin during the course of infection. Norepinephrine-mediated transport of transferrin-associated iron may represent an important mechanism by
which bacteria are able to obtain an essential nutrient in the host environment.

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