Direct Measurements of the Outer Membrane Stage of Ferric Enterobactin Transport

POSTUPTAKE BINDING

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For binding and transport experiments, we subcultured strains at 1% from stationary phase LB cultures into iron-free MOPS minimal medium (30) with vigorous aeration at 37 °C for 5.5 h to an approximate A600 nm of 0.8–0.9, which derepressed the Fur-regulated ferric siderophore transport systems. The experiments compared BN1071 (F−, entA, pro, trp, B1) (31), which contains a wild-type FeEnt uptake system, with its site-directed deletion derivatives OKN1 (ΔtonB) (32), OKN3 (ΔfepA) (32), OKN13 (ΔtonB, ΔfepA) (32), OKN4 (ΔfepB), OKN6 (ΔfepC), OKN11 (ΔfepD), OKN12 (ΔfepG), OKN34 (ΔfepA, ΔfepB), and OKN422 (ΔfepB, ΔtolC). We constructed the latter six strains by allelic replacement, creating precise, complete, in-frame deletions by transformation with linear PCR-generated DNA fragments (33), and verified the mutations by DNA sequence analysis of chromosomal PCR products. For some experiments, we introduced pFepAG54C or pFepAS271C (32) in OKN34 by electroporation.

· We purified enterobactin (28) and apofer- richrome (27) from cultures of E. coli and Ustilago sphaerogena, respectively, and formed their iron complexes with 56Fe or 59Fe. We purified FeEnt and Fc by chromatography over Sephadex LH20 (Amersham Biosciences) (34) or DE52 (Whatman) (35), respectively. Both 56Fe- and 59Fe-labeled enterobactin were either prepared fresh or repurified for each experiment.

Postuptake Binding (PUB) Measurements of OM Transport—The passage of a single molecule of FeEnt through FepA occurs in 10–15 s (23, 27, 28). If bacterial cells are saturated with 56FeEnt at 0 °C (which allows its binding to FepA but not transport) and rapidly shifted to 37 °C for 1 min (which allows transport), then the FepA proteins that internalize the metal complex will become vacant and available for binding of 59FeEnt. Because each FepA protein adsorbs a single molecule of FeEnt (8), the extent of subsequent 59FeEnt binding reveals the number of receptors that were vacated because they successfully transported 56FeEnt. Hence, PUB assays directly determine the quantity and fraction of active FepA proteins during the incubation at 37 °C, by afterward measuring their binding of 59FeEnt (supplemental Fig. S1).

We performed this technique, which exclusively monitors OM transport, as follows. (i) We deposited 1 ml of freshly grown, late exponential phase MOPS cultures (10^9 cells) into microcentrifuge tubes, chilled them on ice for 20 min, and added 56FeEnt to 100 nm. After pelleting the bacteria by centrifugation at 4 °C for 1 min, we removed excess siderophore by carefully aspirating the supernatant and resuspended the cells in 100 μl of ice-cold MOPS medium. (ii) We allowed uptake of the bound 56FeEnt by adding 900 μl of MOPS medium at 42 °C (instantaneously warming the cells to 37 °C) and incubating them 1 min in a 37 °C water bath. (iii) We chilled the cells on ice, pelleted and resuspended them in 1 ml of MOPS medium at 0 °C, diluted 100-μl aliquots into 10 ml of ice-cold MOPS medium containing varying concentrations of 59FeEnt, collected the bacteria on nitrocellulose filters, and counted the filters to determine the extent of 59FeEnt binding (10). This approach required two control experiments: (i) normal binding assays to measure 59FeEnt adsorption at 0 °C to cells that were not previously exposed to 56FeEnt (which measured the total FeEnt binding capacity); (ii) blocked binding assays that measured 59FeEnt adsorption at 0 °C to cells previously satu-
**PUB Determinations of OM Transport**

FepAS271C-FM and its ultimate reversion by FeEnt transport (36).

**Electrophoresis and Western Immunoblots**—For SDS-PAGE (37), samples were prepared in sample buffer with 3% β-mercaptoethanol, boiled for 5 min, and electrophoresed at 30 mA. For Western immunoblots, the proteins were transferred to nitrocellulose paper, which was blocked for 10 min with TBS (50 mM Tris chloride, pH 7.5, 0.9% NaCl) plus 1% gelatin, and incubated with appropriate mouse or rabbit primary antisera in the same buffer. For visualization of FepA, we used mouse monoclonal antibody (mAb) 45 (38); for FepB we used mouse mAbs 2, 4, 23, and 28 (19); for TonB, we used rabbit polyclonal antibodies (23). After incubation and washing 5 times with tap water, the filter was incubated with alkaline phosphatase-conjugated goat anti-mouse or goat anti-rabbit IgG or 125I-labeled protein A. The paper was washed five times with tap water, and the immunoblot was developed by the addition of nitro blue tetrazolium and bromochloroindoyl phosphate or visualized on a StormScanner (Amersham Biosciences), respectively.

**FepA-FepB Co-immunoprecipitation**—To test the possibility that FepB interacts with FepA at the periplasmic interface of the OM, we immunoprecipitated FepA with an IgG2b monoclonal antibody in the presence of FepB and FeEnt and analyzed the precipitate by SDS-PAGE. Although the binding of mAb 45, which recognizes an epitope in FepA L4, near residue 329 (39) inhibits FeEnt adsorption and uptake (38), flow cytometric analyses showed that the binding of FeEnt to FepA does not block the adsorption of mAb 45 (data not shown). Before the addition to the reaction mixture, the component solutions of FepA, mAb 45, and FepB were centrifuged at 18,000 × g for 5 min to remove precipitates. After preliminary experiments to determine the optimum order and concentrations of reagents for formation of an immune complex, we incubated FepA (4.5 μg) with or without FeEnt (10 nM) for a few s before the addition of FepB (15.4 μg) and anti-FepA mAb 45 (30 μg), in a final volume of 0.5 ml of TBS. We allowed the suspension to sit overnight 4 °C, and in the morning we added 50 μl of protein A-agarose (Pierce immobilized protein A plus) contains protein A at 3 mg/ml resin and allowed the mixture to incubate for an additional 2 h at room temperature. Immune complexes were pelleted by centrifugation at 5000 × g for 5 min, solubilized in sample buffer, and analyzed by SDS-PAGE.

**Determination of the Activation Energy of FeEnt Transport through FepA**—FeEnt binding to FepAS271C-FM quenches its fluorescence emissions (40), but as live bacteria deplete the ferric siderophore from solution by transport, the fluorescence rebounds. From such spectroscopic measurements (36) we determined the rate constants for the FeEnt OM transport reaction in two ways. In the first case (time to depletion threshold method), we initially saturated the cells with 10 nM FeEnt and measured the elapsed time for them to deplete it from solution.

\[
\begin{align*}
V & = \frac{1}{2} k_2 [\text{FepA-FeEnt}] \\
\text{REACTION 1}
\end{align*}
\]

FeEnt was in excess during this period, so \( V = {V_{\text{max}}} \) and \([\text{FepA-FeEnt}] = [\text{FepA}] \). Therefore, the following was true.

\[
V_{\text{max}} = k_2 [\text{FepA}] \\
\text{(Eq. 2)}
\]

To determine \( V_{\text{max}} \) by this method we measured the time (s) from the point of the FeEnt addition until \( F/F_o \) inflected upward (to an arbitrary value of 0.4), at which point \( V \approx 20 \) pmol of FeEnt were transported by the cells. From \( V_{\text{max}} \) at each temperature and the concentration of FeEnt (5 nM), we found \( k_2 \).

In the second case (depletion rate method), after the bacteria reached the depletion threshold, as transport continued and [FeEnt] further decreased, FepAS217C-FM underwent a linear unquenching that reverted fluorescence to its original level. At the midpoint of the unquenching curve, FepA was half-saturated with FeEnt, so the following was true.

\[
V = \frac{V_{\text{max}}}{2} = \frac{k_2 [\text{FepA}]}{2} \\
\text{(Eq. 3)}
\]

In this case, the slope of the reversion curve was proportional to \( v \), and from the concentration of FepA (5 nM), we found \( k_2 \). We plotted \( \log (k_2) \) against \( 1/T \) and obtained the activation energy \( (E_a) \) from the Arrhenius equation, \( k = A e^{-E_a/RT} \) (41). These calculations were approximations, but whether \( k_2 \) derived from the elapsed time to the depletion threshold or from the depletion rates at half-saturation, it proportionately reflected the temperature dependence of FeEnt transport.

**RESULTS**

**Proportion of FepA Proteins That Transport FeEnt**—The discrepant cell envelope concentrations of FepA and TonB indicate that at any given time only a fraction of FepA proteins actively transport FeEnt, which potentially explains the receptor’s low turnover number (about 3–5 min⁻¹) (10, 23, 28). We employed PUB experiments to clarify this point. When the population of FepA proteins is saturated with 56FeEnt, if only a fraction of the transporters participates in its uptake, then only that fraction will become vacant and capable of binding 59FeEnt. If, on the other hand, all of the FepA proteins with bound 56FeEnt transport it, then they will all be free to bind 59FeEnt. PUB experiments measure the number of FepA proteins that transport FeEnt through the OM, without dependence on subsequent uptake of the ligand through the IM into the cytoplasm.

For these studies, it was necessary to measure the dissociation of bound FeEnt from FepA in live bacteria. Transfer of cells that are saturated with FeEnt into fresh medium results in re-equilibration of the receptor-ligand interaction, as dictated by their concentrations and the affinity of the association. In practice, the cells release some of the bound ligand, and we measured this quantity for strain BN1071, which expresses wild type FeEnt transport components from single copy chromosomal genes (20, 31). Cells of BN1071 were chilled on ice and incubated with 100 nM 56FeEnt at 0 °C, which saturated FepA proteins (\( K_D = 0.2 \) nM) (10, 39). The iced cells were incapable of actively transporting the ligand through the OM, and when cells with bound 56FeEnt at 0 °C were collected by centrifugation and resuspended in ice-cold buffer, approximately one-third of their FepA proteins became free to adsorb 59FeEnt; i.e.
$^{56}$FeEnt dissociated from a third of the FepA proteins during the manipulations, and two-thirds of the receptor proteins retained the bound ligand and therefore did not adsorb $^{59}$FeEnt (blocked binding; Fig. 1). We observed little variation in the $^{56}$FeEnt dissociation-retention level for any individual strain. Against this background, we performed the same procedures, except that after saturation with $^{56}$FeEnt at 0 °C, centrifugation, and resuspension in cold buffer, we jumped the temperature to 37 °C for 1 min to allow transport of bound ligand before recooling to 0 °C and assay with $^{59}$FeEnt. In BN1071, all FepA proteins with bound $^{56}$FeEnt were functional during the incubation at 37 °C, in that they internalized it and then adsorbed $^{59}$FeEnt to full capacity (Fig. 1). The difference between the blocked binding and the PUB values revealed the extent of FeEnt OM transport. Non-functional FepA proteins (in the TonB-deficient strain OKN1 (H9004/tonB) or in CCCP-treated BN1071) did not transport $^{56}$FeEnt after the temperature jump and hence did not bind additional $^{59}$FeEnt.

Initial Rate of FeEnt Uptake by FepA—Standard radioisotopic iron uptake assays quantify the accumulation of the metal across the IM into the cytoplasm, but because the OM stage is rate-limiting in the overall process, such determinations reflect OM transport rates. We sought an independent measurement of the low FepA and FhuA turnover numbers and employed PUB assays to directly monitor the OM transport reaction. Uptake began immediately and continued until all FepA proteins were vacated (about 90 s; Fig. 2). The transport reaction was a first order process with $k_1$ = 1.2 min$^{-1}$, which was consistent with, although slightly slower (3-fold) than, the previously measured rate. However, whereas previous determinations evaluated bacteria during exponential growth at 37 °C, in PUB assays, the cells were initially chilled on ice, exposed to FeEnt, and then rewarmed to physiological temperature. We suspected that the 3-fold slower rate derived from this difference in protocols and performed conventional $^{56}$FeEnt uptake assays with cells chilled on ice prior to assay at 37 °C to test this supposition; $V_{max}$ was 83 pmol/10$^9$ cells/min for the first 20 s (compared with 208 pmol/10$^9$ cells/min for exponentially growing cells at 37 °C (23)), and the rate approximately doubled to 172 pmol/10$^9$ cells/min for the succeeding 40 s (data not shown). These data translated into turnover numbers of 1.2/ min for the first 20 s, 2.6/min for the next 40 s, and a mean value.
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![Figure 3. Temporal dependence of FeEnt uptake. A, conventional assays of 59FeEnt accumulation. BN1071 was grown in MOPS medium to midlog, excess 59FeEnt (10 μM) was added, and aliquots were removed and counted over a 90-min period. The accumulation time course was triphasic (fitting curves: ---, ---, ---). B, PUB determinations of FeEnt transport rates. Bacteria were grown as in A, and 59FeEnt was added to 10 μM at t = 0. Before its addition and at 5 and 20 min afterward, aliquots of cells were collected by centrifugation, resuspended in fresh MOPS medium for 1 min at 37 °C, and assayed for PUB of 59FeEnt over a range of concentrations. We measured normal binding (●), blocked binding (▲), and PUB at t = 0 (○), 5 (●), and 25 min (▲). The experiment found three different rates of FeEnt uptake at t = 0 (--), 5 (- - - -), and 20 min (– – – –). Error bars, S.E.](https://example.com/figure3)

of 2 for the 1-min assay period. Thus, chilling the cells retarded the uptake of FeEnt during the first min of rewarming, explaining the lower rate found by the PUB tests.

**Kinetics of 59FeEnt Accumulation**—We exposed BN1071 to 59FeEnt at 10 μM, a sufficiently high concentration to avoid depletion during transport at V_max for ~2 h, and measured the time course of FeEnt accumulation by conventional radiotopic measurements over a 90-min period. These data (Fig. 3) showed three uptake stages by the chromosomally encoded FeEnt transport system: an initial phase at maximum rate during the first 30 s (V_max = 150 pmol/min/10^9 cells), which was consistent with previous measurements (10, 28); a secondary phase in the ensuing 10 min with an intermediate rate (V_max = 78 pmol/min/10^9 cells); and a final, apparently steady phase of lowest rate (V_max = 37 pmol/min/10^9 cells) that persisted to the end of the 90-min period. To further understand the triphasic time course, we made PUB assays of FepA activity during the three stages, at 15 s and at 5 and 25 min. The results confirmed the existence of three different uptake rates at the three time points (Fig. 3). Alterations in the amount of FepA in the OM did not explain the stepwise 2-fold and 5-fold decrease in rate that occurred by t = 20 min, because quantitative immunoblot determinations of FepA concentration (10) showed no significant variation at the three sequential time points (data not shown). These results indicated that a decrease in the transport rate through FepA caused the drop in overall FeEnt accumulation into the cytoplasm (19), suggesting that other cell envelope or intracellular processes regulate the OM transport activity of FepA. The FeEnt uptake rate was inversely proportional to the amount accumulated by the cells and reached a steady state within about 10 min.

**FepA-mediated FeEnt Uptake in Bacteria Devoid of FepB, FepD, or FepG**—We genetically engineered (33) in-frame deletions of the fepB, fepD, and fepG loci in BN1071 and verified the expected structures of the deletions by DNA sequence analysis. The mutant strains were inactive in siderophore nutrition tests with FeEnt and unable to accumulate any 59FeEnt in conventional uptake assays (data not shown). We also employed PUB assays to measure FepA transport, and none of the four strains measurably internalized bound FeEnt through the OM (Fig. 4). Although we anticipated their inability to accumulate FeEnt into the cytoplasm, we did not expect their complete lack of FepA-mediated OM transport.

The defects in FeEnt uptake as a result of ΔfepB raised the possibility of an interaction between the binding protein and FepA during the OM transport reaction. To test this idea, we immunoprecipitated FepA with an IgG2b antibody that recognizes an epitope in surface loop 4 (anti-FepA mAb 45) (38, 39) in the presence or absence of FepB and FeEnt (10 nM). The immunochemical reaction precipitated FepA from solution, but FepB did not co-precipitate, whether or not FeEnt was present. These data (supplemental Fig. S2) argued against the notion that FepB actively mediates the passage of FeEnt into the periplasm by a direct interaction with FepA. We repeated the experiment but in this case precipitated FepA in OM fragments (42) by ultracentrifugation, in the presence of FepB and FeEnt, with the same results (supplemental Fig. S3); FepB did not detectably bind to FepA under any conditions.

Specific sites within FepA are chemically modifiable during binding and transport of FeEnt (32, 43), and chemical modification of the substitution G54C occurs during active FeEnt uptake. Located in the N-domain of FepA, G54C is susceptible to fluoresceination during FeEnt transport (32) but not labeled in OKN4, FepA internalized FeEnt, but without FepB in the periplasm, we did not expect their complete lack of FepA-mediated OM transport.

Because the PUB assays and spectroscopic experiments gave different results, we performed additional studies on the retention of 59FeEnt by wild-type (BN1071), ΔtonB (OK1N), and ΔfepB (OKN4) bacteria, comparable with those conducted by Bradbeer (44) with BtuB. BN1071 rapidly acquired 59FeEnt from solution (Fig. 5B) and retained it even if subsequently exposed to excess 56FeEnt. On the other hand, OKN4 absorbed little 59FeEnt, and excess 56FeEnt released most of the radiolabeled siderophore from the strain (Fig. 5, B and C). These data indicated that without periplasmic binding, FeEnt exchanged across the OM, as postulated for vitamin B_12 (44). Thus, in PUB assays of OKN4, FepA internalized FeEnt, but without FepB in the periplasm to bind it, the iron complex leaked out and...
rebound to FepA, creating a futile cycle that prevented subsequent adsorption of $^{59}$FeEnt.

Besides its involvement in antibiotic export, TolC was implicated in the export of newly synthesized enterobactin from *E. coli* (45). We generated a ΔfepB ΔtolC derivative (OKN422) and subjected it to the same experiment. Unlike OKN4, OKN422 acquired nearly as much $^{59}$FeEnt as cells with an intact FeEnt uptake system, and it retained it when exposed to excess $^{56}$FeEnt (Fig. 5B). Thus, the ΔtolC mutation prevented exchange of FeEnt across the OM. Again unlike OKN4, PUB assays of OKN422 revealed FepA-mediated uptake of FeEnt into the periplasm (Fig. 5D). These data confirmed that in the absence of FepB, FepA transported FeEnt across the OM, but without the binding protein to adsorb it, the metal complex escaped from the cells. The results also identified the protein responsible for FeEnt release through the OM: TolC.

**Simultaneous TonB-dependent Uptake of Two Ferric Siderophores**—TonB functions in the uptake of all ferric siderophores through the OM. The fact that it physically contacts the OM receptor proteins implies that simultaneous transport of different ferric siderophores will competitively inhibit their individual rates of ligand internalization. Using both conventional radioisotopic assays and PUB determinations, we studied the effect of concomitant ferrichrome uptake at $V_{\text{max}}$ on the kinetics of FeEnt transport, and vice-versa. Saturating concentrations of Fc had little discernible effect on the uptake of $^{59}$FeEnt in standard uptake assays (Fig. 6); $K_m$ and $V_{\text{max}}$ of the FeEnt acquisition reaction were unchanged relative to the same parameters in the absence of Fc. PUB measurements, on the other hand, showed a 20% decrease in the $V_{\text{max}}$ of FeEnt uptake when Fc was present. When the situation was reversed, saturating FeEnt markedly reduced $V_{\text{max}}$ of $^{59}$Fc transport (by about 50%), which was apparent even in conventional radioisotopic uptake assays (Fig. 6).

**Activation Energy of FeEnt Transport through the OM**—From fluorescence spectroscopic observations of FeEnt uptake at different temperatures (36), we used the Arrhenius equation to calculate the activation energy of the OM stage of FeEnt transport. When bacteria expressing FepAS271C-FM were exposed to 10 nM FeEnt, binding of the ferric siderophore quenched fluorescence emissions, but as the cells transported the ligand, they depleted it from solution, ultimately reversing the quenching effect. We determined the temperature dependence of the uptake rate in two ways: from the time required to deplete FeEnt (10 nM) from solution (elapsed time to the depletion threshold) and from the steady-state uptake rate at half-saturation. In the former case, we monitored the time from the point of FeEnt addition (maximum quenching) until $F/F_o$ inflected upward and reached a value of 0.4 (Fig. 7). In the latter case, we measured the rate at which fluorescence rebounded when transport depleted the externally supplied FeEnt; as the concentration of ferric siderophore decreased, FepAS271C-FM underwent a linear unquenching that reverted fluorescence to its original level (Fig. 7). Calculations by both methods resulted in a linear dependence of log $k_2$ versus $1/T$, and the two approaches corroborated each other; the depletion threshold analysis gave a slope of $-17,997$ K, and a calculated activation energy of 35.8 kcal/mol; the depletion rate analysis resulted in a slope of $-16,670$ K and a calculated activation energy of 32.7 kcal/mol.

**DISCUSSION**

The PUB experiments directly measured the transport activity of FepA, allowing us to study some unresolved aspects of a TonB-dependent uptake system. The energy dependence of
OM transport was one such consideration. Natural environmental amounts of ferric siderophores do not exceed micromolar levels, but typical cytoplasmic iron concentrations are in the millimolar range (46–48). Hence, the energy dependence of iron uptake by *E. coli* B/r (49) was not surprising because it was originally presumed relevant to the IM transport stage. Without explicit demonstrations, nevertheless, subsequent results suggested that OM metal transport also required energy. The facts that T1 and φ80 needed energy for irreversible adsorption to FhuA (TonA) (50) and that vitamin B₁₂ uptake across the OM was inhibited by energy poisons (44, 51, 52), inferred that TonB-dependent transporters catalyzed active OM transport. However, general porins (OmpF, OmpC, PhoE, etc.) in the OM create >10⁵ water-filled, 10-Å diameter channels per cell (53), eliminating the possibility of a trans-OM ion gradient as a driving force for metal uptake. This constraint led to the idea that TonB itself transduces energy to OM proteins, by membrane fusion (54) or intra-cell envelope protein-protein interactions (13, 14, 55–57) or by rotational motion (22, 23, 58–60). With some exceptions (32, 36), prior descriptions of ferric siderophore acquisition observed OM transport as the rate-limiting step in overall uptake through the cell envelope (10, 28, 51–53, 61–63), whose attributes were revealed by analysis of cytoplasmic iron accumulation. PUB determinations, on the other hand, observed internalization of the metal complex by FepA, ultimately emphatically demonstrating that both ΔtonB and proton motive force depletion prevent OM transport of FeEnt.

The amount of energy needed for the proton motive force- and TonB-dependent OM stage of iron uptake was unknown. Regarding FepA-FeEnt transport, Arrhenius calculations converged on an activation energy of 33–36 kcal/mol. It is a high value for a biochemical reaction, which translates into a *Q*₁₀ value of 6–7 (64). Reactions with *Q*₁₀ > 2 usually involve significant conformational changes, and the higher value for a TonB-dependent transporter presumptively reflects the need to rearrange its N-domain, or to expel it from the transmembrane channel, during ligand internalization. The noted bioenergetic quantity equates to hydrolysis of about 4 ATPs per molecule of FeEnt transported through FepA. Estimates of the amount of iron associ-
C-terminus (24) probably initiates the OM transport process. Therefore, the relative abundances of OM transporters and TonB may influence metal uptake rates as a result of competition among ligand-bound receptors for the limited number of TonB proteins. Decreased OM uptake rates during simultaneous transport of FeEnt and Fc support this idea. The 50% inhibition of FhuA-mediated Fc transport by concomitant activity of FepA was consistent with the much higher abundance of the ferric catecholate transporter in the OM. \(^{59}\)FeEnt capacity and quantitative immunoblots estimate the maximum concentration of chromosomally derived FepA as 35,000/cell in bacteria with derepressed iron acquisition systems (23). Measurements of TonB concentration show a maximum of 1000 copies/cell (26), indicating a 35-fold difference in the amounts of FepA and TonB. These calculations imply that at any instant, only about 3% of the OM-resident FepA may associate with TonB, which raises the question of what fraction of the FepA population actually transports FeEnt? If only this small percentage of total FepA proteins are active, then its \(V_{\text{max}}\) from traditional uptake assays (5–6/min) (23) underestimates an individual protein’s transport rate as much as 30-fold. PUB experiments showed that when bacteria were shifted to 37 °C, the population of FeEnt-saturated FepA molecules exponentially decayed at a rate of 1.2/min and was depleted within 80 s. Hence, all FepA proteins with bound ligand transported it, implying that TonB identified and functionally interacted with all of them during that time. These data eliminate the possibility that only a fraction of the receptor population is functionally active. Therefore, within 80 s, each TonB protein located and facilitated transport of ~30 FepA proteins. It is conceivable that this identification/facilitation activity by TonB is the rate-limiting step of metal transport, potentially explaining the low overall turnover numbers of ferric siderophore transporters.

The protein framework underlying Gram-negative bacterial metal acquisition encompasses several layers of complexity in the trilaminar cell envelope: an OM receptor protein, the IM/OM-spanning TonB-ExbB-ExbD complex that may energize the OM stage, a periplasmic binding protein, an IM ABC transporter, and in some cases an N-terminal extension of the OM transporter that is tied to transcriptional regulation (65, 66). The PUB approach resolved the FeEnt OM transport process into three temporal phases: a rapid initial phase that persisted for about 30 s, a secondary stage that occurred from 0.5 to 10 min, and an ultimate, steady-state rate that continued indefinitely, from 10 to 90 min. These previously unobserved phases may reflect mechanistic connections to TonB-ExbBD, FepB, and FepCDG-Fes. The rate measurements begin with TonB presumably unassociated with (ligand-free) FepA or other metal transporters and neither FepB nor FepCDG-Fes occupied by FeEnt. In the second stage, at least TonB and FepB become saturated by their binding partners, and the binding protein transfers the ferric siderophore to the IM permease complex. In the final stage, all transport system proteins are saturated by FeEnt or FeEnt-bound proteins. The limited amounts of TonB (10, 26), FepB (19), and FepCDG-Fes in the cell envelope, relative to that of FepA, suggests that at steady state, a functional interaction among these proteins may

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**FIGURE 6. Concomitant transport of FeEnt and Fc.** BN1071 was grown in MOPS medium. A, conventional measurements of \(^{59}\)FeEnt uptake in the presence of Fc. The bacteria were assayed for \(^{59}\)FeEnt uptake over a range of concentrations in the absence (○) and presence (●) of 100 \(\mu\)M Fc, which had no apparent effect by this protocol. B, PUB measurements of FeEnt uptake. The bacteria were tested for \(^{59}\)FeEnt binding in normal (○) or blocked (●) conditions or in PUB assays in the presence of 100 \(\mu\)M Fc. C, conventional measurements of \(^{59}\)Fc uptake in the presence of FeEnt. Cells were tested for \(^{59}\)Fc uptake over a range of concentrations in the absence (○) and presence (●) of 100 \(\mu\)M FeEnt, which decreased \(V_{\text{max}}\) of the hydroxamate siderophore ~50%. Error bars, S.E.
become rate-limiting, but existing data do not yet identify this subreaction.

The absence of FeEnt uptake through FepA in strains lacking FepB, -D, or -G was unexpected and without obvious explanation, intimating that FepA was non-functional without a periplasmic binding protein or an active IM permease complex. Bacteria lacking these components were superficially equivalent to ΔtonB mutants: no stimulation by FeEnt in siderophore nutrition tests, no 59FeEnt acquisition in standard uptake assays, and no measurable OM transport. Bradbeer (44) found that after [57Co]vitamin B12 enters the OM through BtuB, without IM transport the periplasmic pool exchanges with vitamin B12 outside the cell. We also saw release of adsorbed 59FeEnt from both ΔfepB and ΔtonB bacteria. However, exposure of the ΔfepB strain to FeEnt at 37 °C made FepA residue G54C accessible (32) to fluoresceination, whereas in ΔtonB bacteria, which bind but do not transport, the same residue was inaccessible to chemical modification. Even without FepB, therefore, FepA had activity associated with FeEnt transport, whereas without TonB, it was mechanistically inactive. PUB experiments were decisive to the realization that indeed FepA internalized FeEnt in the ΔfepB strain, but in the absence of a binding protein in the periplasm, the ligand escaped through TolC and rebound to FepA. This futile transport/leakage cycle underscored the indispensability of periplasmic binding proteins to metal transport. All TonB-dependent OM uptake systems require periplasmic binding proteins (67), so this conclusion about FeEnt acquisition probably generalizes to other ferric siderophore transporters as well. Finally, the discharge of FeEnt through the TolC channel demonstrates the influence of this pathway on the concentrations of even non-antibiotic, nutritite solutes in the periplasm.

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