Mutual Inhibition of Cobalamin and Siderophore Uptake Systems Suggests Their Competition for TonB Function

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Vitamin B12 (CN-Cbl) and iron-siderophore complexes are transported into Escherichia coli occurs in two energy-dependent steps. The first step is mediated by substrate-specific outer membrane transport proteins and the energy-coupling TonB protein complex, and the second step uses separate periplasmic permeases for transport across the cytoplasmic membrane. Genetic and biochemical evidence suggests that the TonB-dependent outer membrane transporters contact TonB directly, and thus they might compete for limiting amounts of functional TonB. The transport of iron-siderophore complexes, such as ferrichrome, causes a partial decrease in the rate of CN-Cbl transport. Although CN-Cbl uptake does not inhibit ferrichrome uptake in wild-type cells, in which the amount of the outer membrane ferrichrome transporter FhuA far exceeds that of the cobalamin transporter BtuB, CN-Cbl does inhibit ferrichrome uptake when BtuB is overexpressed from a multicopy plasmid. This inhibition by CN-Cbl is increased when the expression of FhuA and TonB is repressed by growth with excess iron and is eliminated when BtuB synthesis is repressed by CN-Cbl. The mutual inhibition of CN-Cbl and ferrichrome uptake is overcome by increased expression of TonB. Additional evidence for interaction of the Cbl and iron transport systems is provided by the strong stimulation of the BtuB- and TonB-dependent transport of CN-Cbl into a nonexchangable, presumably cytoplasmic pool by precinubcation of cells with the iron(II) chelator 2,2'-dipyridyl. Other metal ion chelators inhibited CN-Cbl uptake across the outer membrane. Although the effects of chelators are multiple and complex, they indicate competition or interaction among TonB-dependent transport systems.

Uptake of vitamin B12 (cyanocobalamin, [CN-Cbl]) and iron-siderophore complexes in Escherichia coli occurs by sequential energy-dependent transport across both cell membranes. The unusual operation of specific transport systems across the outer membrane is necessary for acquisition of these valuable nutrients, whose passage through the porin channels is restricted by their size and low concentrations in natural environments (reviewed in references 6, 18, 27, and 28). Outer membrane proteins BtuB and FhuA mediate high-affinity binding and transport of cobalamin and ferrichrome (Fc), respectively. The TonB-ExbB-ExbD protein complex couples the action of these transporters to the proton motive force across the cytoplasmic membrane (3, 11). The TonB protein is anchored in the cytoplasmic membrane through a nonpolar segment near its N-terminal end (12, 16, 32) and extends across the periplasmic space to interact directly with the outer membrane transporters (2, 33, 35).

The btuCD and fluCDB loci encode the ATP-driven periplasmic permeases for unidirectional transport of CN-Cbl and Fc, respectively, across the cytoplasmic membrane (7, 8, 10, 34). In a btuC mutant, CN-Cbl is accumulated in the periplasmic space by BtuB and TonB action and is released in unaltered form during a chase with an unlabeled substrate (31). Cobalamin and siderophore uptake is inhibited both by agents that dissipate the proton motive force and by those that decrease the ATP pool, consistent with the operation of serial transport systems with different modes of energy coupling (3, 5, 30, 31).

Since TonB appears to be present in lower amounts than the transporters it activates (29), the TonB-dependent cobalamin and siderophore uptake systems might compete for the limiting amounts of functional TonB protein and be affected by the level and stability of TonB. Inhibition of general protein synthesis or of TonB expression results in a decline of all TonB-dependent activities with a half-time of 15 to 20 min that is accelerated by the presence of TonB-dependent transport substrates (20). Conversely, overexpression of TonB from multicopy plasmids also results in reduced TonB function (23). Overexpressed TonB protein in excess of the level of ExbB is metabolically labile, but TonB produced in normal amounts is stable with a half-life of >60 min (9, 36). Competition among TonB-dependent transport systems may explain the reduction in the rate of CN-Cbl uptake caused by the concomitant uptake of Fc and other siderophores (20). However, the converse inhibition of siderophore uptake by covalamins was not seen. We show here that cobalamin transport inhibits ferrichrome uptake when the level of BtuB is amplified.

The effect of metal ion chelators on TonB-dependent transport was investigated to test whether deprivation of iron or other ions affects cobalamin uptake. The effect of chelators on outer membrane transport processes must be interpreted with caution since metal ions such as Ca2+ and Mg2+ are required for integrity of the outer membrane and its barrier properties (25). In addition, Ca2+ is necessary for binding of CN-Cbl to BtuB (4). Thirdly, chelators can decrease the level of available iron and result in derepression of the iron-repressible transport systems and a reduction in the amounts of their substrates. This competition between added chelators and siderophores for iron could also affect cobalamin transport activity by de-
increasing the competition by the siderophore transporters for TonB.

MATERIALS AND METHODS

Bacterial strains and plasmids. Competition between Fc uptake and cobalamin uptake was measured in Escherichia coli AB2847 (aroB malT txy thr). The plasmids used in this study included vector plasmid pBR322; pkH3-8, which is pBR322 with a 2.2-kb insert carrying 

\[
\text{btuB}
\]

and pkHIM2, which is pASYC177 with a 1.7-kb insert carrying 

\[
\text{btuC}
\]

and pkHIM2 were grown in iron-limiting conditions, and uptake of be the only form of Cbl present in a BtuC

as200moleculesperhaploidcell,whichisroughlythenumberofBtuBmole-

The BtuB-bound Cbl in a cell is assumed to equal the sum of three pools: the Cbl bound to BtuB plus exchangeable Cbl plus nonexchangeable Cbl. The BtuB-bound Cbl pool is immediately released upon a chase with unlabeled CN-Cbl and is defined to BtuB plus exchangeable Cbl plus nonexchangeable Cbl. The BtuB-bound Cbl pool is released from the cell and retained radioactivity was measured in a liquid scintillation counter. Uptake was calculated by correction for binding of substrate to filters in the absence of cells.

The kinetics of formation of the exchangeable and nonexchangeable pools of transported cobalamin (Cbl) was calculated as follows. The total amount of labeled Cbl in a cell is assumed to equal the sum of three pools: the Cbl bound to BtuB plus exchangeable Cbl plus nonexchangeable Cbl. The BtuB-bound Cbl pool is immediately released upon a chase with unlabeled CN-Cbl and is defined to be 200 molecules per haploid cell, which is roughly the number of BtuB molecules. The exchangeable Cbl pool is assumed to represent periplasmic Cbl and to be the only form of Cbl present in a BtuC

strain. The size of this pool is determined from a plot of the rate of exchange of label from untreated BtuC

cells versus the total amount of label present at the start of the chase period. The nonexchangeable Cbl pool is calculated by subtraction of the BtuB-bound Cbl and exchangeable Cbl pools from the total cell-associated label. This calculated value of exchangeable Cbl was similar to the amount of Cbl label remaining in the cells after a 12-min chase.

RESULTS

Inhibition of Fc uptake by Cbl. In previous work, we showed that transport of the TonB-dependent substrate Fe or enterobactin inhibited Cbl uptake (20). To determine whether the lack of inhibition of Fc uptake by CN-Cbl was the result of the much lower content of BtuB and correspondingly lower transport activity for Cbl relative to that of Fc, transport was measured in strains in which BtuB was overexpressed from a multicopy 

\[
\text{btuB}
\]

plasmid. Cells of strain AB2847 carrying various plasmids were grown in iron-limiting conditions, and uptake of \(^{55}\text{Fe}\)Fc was assayed in the absence or presence of 10 \(\mu\)M unlabeled CN-Cbl. In cells carrying vector plasmid pBR322, Fc uptake was not affected by the presence of CN-Cbl as expected (20). In the strain carrying \(\text{btuB}^*\) plasmid pKH3-8, Fc uptake occurred at a rate similar to that in the control strain but was markedly inhibited by the presence of CN-Cbl (Fig. 1B). Half-maximal inhibition of Fc uptake occurred at about 10 nM CN-Cbl (data not shown).

The effect of Fc and CN-Cbl on the uptake of the other substrates was measured in cells grown in the presence of citrate, ferric citrate, or CN-Cbl conditions which affect the level of the transport components (Table 1). In the haploid strain with plasmid pBR322, \(^{55}\text{Fe}\)Fc uptake was not affected by unlabeled CN-Cbl whether the cells were grown under iron-limiting (no supplement or with citrate) or iron-replete (with ferric citrate) conditions, even though the rate of Fc uptake was repressed about sixfold in the iron-replete cells. Uptake of \(^{3}H\)CN-Cbl in this strain had the low rate typical of haploid cells and was reduced by about 50% by 10 \(\mu\)M Fc in cells grown under iron-limiting or iron-replete conditions.

In cells carrying multicopy \(\text{btuB}\) grown under iron-limiting conditions (no supplement or with citrate), \(^{55}\text{Fe}\)Fc uptake activity was slightly reduced relative to that of the vector-bearing strain and was inhibited by unlabeled CN-Cbl to a modest degree (24 to 38%). When this strain was grown under iron-replete conditions (with ferric citrate), Fc uptake activity was repressed about threefold and the presence of CN-Cbl was substantially more inhibitory (72% inhibition) than in iron-limited cells. This increased inhibition of Fc uptake by CN-Cbl in iron-repressed cells could result from the reduced ratio of FhuA relative to BtuB and from the increased competition for the iron-repressed levels of TonB protein. Expression of TonB is repressed two- to threefold by iron under aerobic conditions (26, 37). When these cells were grown in the presence of both ferric citrate and CN-Cbl, CN-Cbl transport was repressed to about the level in a haploid cell, and CN-Cbl no longer inhibited Fc uptake.

The rate of \(^{3}H\)CN-Cbl uptake was increased five- to eightfold by amplification of the BtuB content in the presence of pKH3-8 (Table 1). In iron-limited cells, Fe still inhibited CN-Cbl uptake, but to a lower degree than in cells with a single copy of \(\text{btuB}\) (20 to 36% inhibition versus 43 to 53% inhibition in haploid cells). In iron-repressed cells of AB2847(pKH3-8), CN-Cbl uptake was reduced three- to fourfold and Fe caused little if any inhibition of CN-Cbl uptake. Since iron supplementation had no effect on CN-Cbl uptake activity in haploid cells, its effect in BtuB-overexpressing cells may reflect the repression of TonB levels. Cells grown in the presence of repressing levels of both iron and CN-Cbl exhibited repressed rates of transport of both substrates and a low degree of cross-inhibition. These patterns of transport activities are consistent with the competition of the TonB-dependent transport systems for limiting amounts of functional TonB protein.

Effect of \(\text{tonB}\) amplification. To test whether genetic amplification of TonB could reduce the competition between TonB-dependent transport systems, the transport activities of AB2847 cells carrying \(\text{tonB}\) alone or both \(\text{btuB}\) and \(\text{tonB}\) on compatible plasmids were determined. Fc transport was reduced two- to fourfold by the presence of amplified levels of TonB (Fig. 1C and Table 1), as expected (23). CN-Cbl had only
as described in Fig. 1. Micromanipulations were compared. Uptake in btuC-dependent CN-Cbl transport across the cytoplasmic membrane was inhibited by EDTA or EGTA (ethylene glycol-bis-[β-aminoethyl ether] N,N,N',N'-tetraacetic acid) at 5 mM in some experiments and 0.5 to 0.7 mM EDTA. Some metal ion chelators stimulate CN-Cbl transport. Other metal ion chelators did not inhibit CN-Cbl transport. Precipitation of cells with nitrilotriacetic acid and EDDP (ethylendiamine-N,N'-diacetic acid-N,N'-di-β-propiolic acid) at 5 mM resulted in moderate stimulation in some experiments and a slight effect on Fc transport in cells with amplified tonB but haploid btuB. Notably, in AB2847(pKH3-8/pBJM2) with multicopy amplification of BtuB and TonB, CN-Cbl significantly stimulated Fc uptake, in contrast to the inhibition seen in cells with haploid tonB.

Amplification of the tonB gene dosage also resulted in reduction of [3H]CN-Cbl uptake, but the reduction in cells with amplified BtuB was much less severe than was the reduction in Fc uptake in the same cells or in CN-Cbl uptake in cells with normal levels of BtuB. This result is consistent with the hypothesis that the reduced function and stability of overexpressed TonB are partially prevented by an increase in the number of TonB-dependent receptors (6). Whereas Fc inhibited CN-Cbl uptake in cells with normal levels of TonB, Fc had little effect on CN-Cbl uptake in cells with overexpressed TonB, suggesting that the mutual inhibition of Fc and CN-Cbl uptake is overcome by increased production of TonB protein.

Some metal ion chelators inhibit CN-Cbl transport. Isogenic E. coli strains that were proficient or deficient in btuC-dependent CN-Cbl transport were compared. Uptake in btuC mutant RK4385 reflects transport across the outer membrane into the periplasmic space, whereas uptake in btuC" strain RK4386 includes, in addition, unidirectional uptake of CN-Cbl into the cytoplasm. Several different responses were seen. Preincubation for 10 min with EDTA or EGTA [ethylene glycol-bis-[β-aminoethyl ether] N,N,N',N'-tetraacetic acid] at 5 mM resulted in almost complete inhibition of CN-Cbl uptake in the BtuC" strain (Fig. 2A). Uptake in the BtuC" strain was inhibited to a lesser degree (Fig. 2B), suggesting that these chelators acted primarily or exclusively to inhibit CN-Cbl transport across the outer membrane but not across the cytoplasmic membrane. The inhibition of transport by EDTA was reversed by addition of 10 mM MgCl₂ or CaCl₂ or by suspension of the EDTA-treated cells in media lacking the chelator (data not shown). Half-maximal inhibition of transport in a BtuC" or BtuC+ strain occurred with 0.5 to 0.7 mM EDTA.

**TABLE 1. Effects of growth conditions and plasmid content on competition between Fc and CN-Cbl uptake**

<table>
<thead>
<tr>
<th>Plasmid(s)</th>
<th>Addition to growth medium</th>
<th>Rate of uptake of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>[55Fe]Fc (kcpm/5 min)</td>
</tr>
<tr>
<td></td>
<td>No addition</td>
<td>With 1 μM CN-Cbl</td>
</tr>
<tr>
<td>pBR322</td>
<td>None</td>
<td>10.6</td>
</tr>
<tr>
<td></td>
<td>Citrate</td>
<td>14.8</td>
</tr>
<tr>
<td></td>
<td>Fe citrate</td>
<td>2.2</td>
</tr>
<tr>
<td>pKH3-8</td>
<td>None</td>
<td>9.8</td>
</tr>
<tr>
<td></td>
<td>Citrate</td>
<td>8.7</td>
</tr>
<tr>
<td></td>
<td>Fe citrate</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>Fe citrate + B₁₂</td>
<td>3.2</td>
</tr>
<tr>
<td>pKH3-8 + pBJM2</td>
<td>None</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>Citrate</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>Fe citrate</td>
<td>0.3</td>
</tr>
<tr>
<td>pBJM2</td>
<td>None</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>Citrate</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>Fe citrate</td>
<td>0.3</td>
</tr>
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</table>

* Cells of strain AB2847 carrying the plasmid(s) indicated were grown in M63 salts with glucose, Casamino Acids, aromatic supplements, and the addition(s) indicated. Cells were harvested in the mid-log phase, washed, and assayed for uptake of [55Fe]Fc or [3H]CN-Cbl in the absence or presence of the inhibitor indicated, as described in Fig. 1.

* The rate of uptake was calculated from the increase in radioactivity from 5 to 10 min for Fc uptake and from 5 to 15 min for CN-Cbl uptake. Activity is expressed as 10⁻³ cpm/10⁹ cells. Percent activity is the relative rate of uptake in the presence or absence of the inhibitor.

**FIG. 2. Effect of metal ion chelators on uptake of CN-Cbl.** Cells of strain RK4385 (btuC−) and RK4386 (btuC+) were grown in medium A with required amino acids, glucose, and Casamino Acids. Cells were harvested in the mid-log phase, washed twice, and suspended in N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-glucose medium. Portions of cells were incubated with chelators for 10 min at 37°C, after which [57Co]CN-Cbl (0.25 μCi/55 nM) was added. Samples were withdrawn at the times indicated, filtered through Millipore membranes, and washed with 5 ml of medium A. The chelators added at 5 mM were EDTA (●), EGTA (▲), nitrilotriacetic acid (●), and DP (△). No chelator added. Similar results were obtained when uptake was assayed in medium A.
no effect in others. The iron(II) chelator DP (2,2'-dipyridyl) strongly and reproducibly stimulated CN-Cbl transport in both BtuC<sup>−</sup> and BtuC<sup>+</sup> strains to comparable levels (Fig. 2). The effect of DP on transport activity was studied further.

The stimulatory effect of DP became apparent after about 5 min, when uptake in the BtuC<sup>−</sup> strain reached a steady-state level (Fig. 2). To test whether the action of DP is induced only after a preincubation period, 5 mM DP was added to BtuC<sup>−</sup> cells simultaneously with or 10 min before addition of labeled CN-Cbl (Fig. 3). Preincubation with DP resulted in stimulation of uptake after the control culture had reached a steady state. Simultaneous addition of DP resulted in initial inhibition of CN-Cbl uptake, followed by stimulation to a rate and final level similar to those in cells preincubated with DP. Thus, DP appears to have multiple effects. Maximal stimulation of uptake was obtained at about 5 mM DP, and higher concentrations were inhibitory (data not shown). Growth of cells was blocked by concentrations of DP above 0.6 mM and was restored by iron supplementation.

The stimulatory effect of DP on CN-Cbl uptake was prevented by exposure to EDTA (Fig. 4A and B) or sodium citrate (data not shown). Cells treated with EDTA plus DP had lower rates of CN-Cbl uptake activity than did cells treated with EDTA alone. These results are consistent with the proposal that DP has an initial inhibitory effect on CN-Cbl transport across the outer membrane which is followed by inducible stimulation.

Strains lacking the <i>btuB</i> or <i>tonB</i> function are defective in CN-Cbl transport across the outer membrane (1). Uptake in <i>btuB</i> or <i>tonB</i> mutant strains was not stimulated by preincubation with either EDTA or DP (data not shown), showing that DP-stimulated uptake still requires the BtuB-TonB transport system but not BtuC-dependent transport across the cytoplasmic membrane.

**Effect of DP on CN-Cbl pools.** To identify the step that was affected by DP, we compared its effect on the rates of CN-Cbl uptake into the periplasm and the cytoplasm by examining the release of labeled CN-Cbl following a chase with an unlabeled substrate (Fig. 4C and D). In BtuC<sup>−</sup> strain RK4386, addition of unlabeled CN-Cbl after 20 min of uptake of <sup>57</sup>CoCN-Cbl resulted in release only of an amount of label corresponding to that bound to BtuB, with retention of the remaining intracellular label (Fig. 4C). This result was expected, since most of the transported CN-Cbl enters the cytoplasm in a unidirectional process. Treatment with DP had no apparent effect on the rate or extent of release of label. Untreated BtuC<sup>−</sup> cells showed the expected rapid chase of accumulated CN-Cbl from the periplasm, but DP-treated BtuC<sup>−</sup> cells showed the slow release and extensive retention of label seen in BtuC<sup>−</sup> cells (Fig. 4D). These results suggested that DP stimulates transport by accelerating transport of CN-Cbl into the cytoplasm or another site, where it was not available for exchange, by inhibiting the rate of exchange across the outer membrane, or both.

The effect of DP on the sizes of the exchangeable and non-exchangeable pools was investigated from the rates of exchange of labeled CN-Cbl from BtuC<sup>−</sup> and BtuC<sup>+</sup> cells after various periods of uptake (Fig. 5A and B). Uptake was measured over a longer period of time than in previous assays to accentuate the effect of DP stimulation. The rate of exchange was calculated from the amount of label lost 6 min after the addition of unlabeled CN-Cbl. The rate of exchange from BtuC<sup>−</sup> strain RK4385 in the absence of DP was a linear function of the total amount of label in the cell after subtraction of the amount bound to the BtuB protein. The linear relationship between the rate of exchange and the size of the exchangeable pool in the BtuC<sup>−</sup> strain should allow calculation of the size of the exchangeable pools under other conditions from the rate of exchange, if all of the label in these cells is exchangeable (31) and if the exchange rate is a linear function of the amount of CN-Cbl in the exchangeable pool over the entire concentration range achieved. By using this correlation, the size of the exchangeable pool under the other three conditions was calcu-
FIG. 5. Effect of DP on the rate of exchange of CN-Cbl. Cells of strains RK4386 (A) and RK4385 (B) were assayed for uptake of [57Co]CN-Cbl in replicate tubes either without (circles) or with (squares) 5 mM DP added at −10 min. Unlabeled CN-Cbl (10 μM) was added at the times indicated, and release of radioactivity was determined (open symbols). Rate of efflux was calculated from the amount of radioactivity lost 6 min after the addition of unlabeled CN-Cbl. The rate of efflux for mutant strain RK4385 in the absence of DP was a linear function of the amount of radioactivity in the cells at the onset of the chase period. This linear relationship was used to calculate the concentration of exchangeable CN-Cbl for the other three conditions, as shown in panel C. Symbols: ○, RK4386 without DP; ●, RK4386 plus DP; △, RK4385 without DP; ▲, RK4385 with DP. The difference between the total cellular radioactivity and the exchangeable pool was calculated to be the nonexchangeable pool of CN-Cbl and is shown in panel D with the same symbols as in panel C.

luted (Fig. 5C), showing that the size of this pool remained fairly constant, regardless of the buTC genotype. DP had a modest inhibitory effect on the initial stages of transport, i.e., binding to BtuB or formation of the exchangeable pool, but had no effect on the size of this pool after 30 min. It also prevented a decrease in the size of this pool during the second hour of transport.

The size of the nonexchangeable pool was calculated by subtracting the amount of label in the nonexchangeable pool and the amount associated with BtuB from the total radioactivity in the cell at each time point (Fig. 5D). The rate of formation of this pool increased progressively during the period of uptake in the BtuC+ strain and was increased about threefold by the presence of DP in both BtuC+ and BtuC− strains. Thus, DP appears to stimulate Cbl uptake by increasing the rate of formation of the nonexchangeable pool rather than by stimulating transport across the outer membrane.

**DISCUSSION**

The TonB protein spans the periplasmic space to contact specific proteins in the cytoplasmic and outer membranes. Missense changes or substitutions in the N-terminal transmembrane segment of TonB eliminate transport function, suggesting that this region is not just a membrane anchor but is involved in interaction with cytoplasmic membrane proteins, including the ExbBD complex (21, 35). Evidence for interaction of TonB with outer membrane transport proteins has been indicated by the allele-specific suppression by TonB variants of energy-uncoupled mutants with amino acid substitutions in a region conserved among TonB-dependent transporters (2, 15, 33). Biochemical evidence for this interaction includes the stabilization of overexpressed TonB protein by overexpressed FhuA (6) and the in vivo chemical cross-linking of TonB and the FepA transporter protein with formaldehyde (35, 36). Although TonB levels have not been quantified, they are likely to be much less than the numbers of transport proteins (26, 29).

Competition among TonB-dependent transporters is suggested by our findings that uptake of iron siderophores inhibits CN-Cbl uptake (20) and that CN-Cbl inhibits Fe uptake when the level of the BtuB protein is amplified. Fe uptake was only slightly affected by overexpression of BtuB when CN-Cbl was not present, which is consistent with the hypothesis that TonB interacts with the outer membrane transporters only when they contain a bound substrate. The inhibitory effect of CN-Cbl on Fe uptake was further increased when cells were grown with iron supplementation, which partially represses (three- to five-fold) both FhuA and TonB levels. The decreased level of both proteins should exacerbate the competition of FhuA with BtuB for TonB.

CN-Cbl transport activity is normally not affected by iron supplementation (Table 1). However, when BtuB was overexpressed, growth with excess iron resulted in a fourfold reduction in CN-Cbl uptake with no significant change in CN-Cbl binding. This repression is likely to reflect the Fur-mediated repression of TonB levels, which has been demonstrated with the use of transcriptional fusions and immunquantitation of the TonB protein (26, 37). It was not possible previously to demonstrate iron repression of TonB function because of the concomitant Fur-mediated repression of the iron transporters. Apparently, the repressed level of TonB allows full CN-Cbl uptake activity when BtuB is at its normal low levels but is limiting when BtuB is overexpressed.

If the TonB-dependent transporters compete for limiting TonB protein, amplification of TonB levels should reduce or eliminate this competition. Although amplification of TonB results in a substantial decrease in all TonB-dependent activities (23), the competition between Fe- and CN-Cbl uptake systems was eliminated. The presence of one transport substrate usually increased the rate of uptake of the other substrate. These results are consistent with our original proposal (20, 23) that overexpressed TonB is not only unstable but actually inhibitory. The inhibitory species could be proteolytic fragments of TonB formed by the turnover of the excess TonB that is not stabilized by interaction with ExbB or could be the TonB that has not been activated by the rate-limiting energization process. The presence of additional TonB-dependent transport substrates reduced this competition, either by accelerating the rate of turnover of excess TonB protein or its fragments or by providing additional sites for binding of the inhibitory TonB species. We predict from the lack of effect of excess unligated transporters that TonB binds preferentially to the outer membrane transporters complexed with their substrates. TonB can be chemically cross-linked to FepA but not to other TonB-dependent transporters (35). It is possible that TonB has the unusual ability to recognize unligated FepA or that the cells produce enterobactin or a precursor that binds to FepA to allow its interaction with TonB. Although amplification of buTC results in an increased amount of BtuB protein and CN-Cbl binding, the overall transport rate of CN-Cbl is not increased proportionately. The amount of TonB in plasmid-bearing cells is not strongly increased owing to its increased liability. The amount of BtuB-TonB complexes has not been determined, and they are not detectable in normal cells (35).
The effects of metal ion chelators on TonB-dependent CN-Cbl transport indicated a mutual interaction between CN-Cbl uptake and siderophore uptake. The comparison of BuTC\(^{2+}\) and BuTC\(^{3+}\) strains differentiates chelator effects on transport across the cytoplasmic membrane or intracellular metabolism. EDTA and the calcium chelator EGTA strongly reduced CN-Cbl uptake across the outer membrane in the BuTC\(^{3+}\) strain but apparently not transport across the cytoplasmic membrane. This inhibition results, in part, from disruption of the outer membrane structure, since removal of calcium and magnesium ions results in loss of a fraction of the cellular lipopolysaccharide and substantial changes in outer membrane barrier function (22). In addition, calcium ions increase the affinity of BtuB for CN-Cbl (4).

CN-Cbl uptake was consistently stimulated by DP, an iron-binding compound with preference for Fe(II). DP causes an initial inhibition of uptake, followed after 5 to 10 min by a strong stimulation. Stimulation by DP required the function of BtuB and TonB but was independent of BtuC and was abrogated by simultaneous exposure to EDTA or other chelators. These results indicated that DP acts at a step after CN-Cbl binds to BtuB and is transported across the outer membrane. It cannot be determined whether protein synthesis is required for induction of this stimulation, because protein synthesis inhibitors reduce TonB function (19).

Measurement of the effect of DP treatment on the rate of exchange of labeled CN-Cbl indicated that DP did not affect the kinetics of the exchangeable CN-Cbl pool but stimulated the formation of the nonexchangeable pool. This result suggests that DP treatment stimulates entry of CN-Cbl into the cytoplasm even in BuTC\(^{3+}\) strains which lack the cytoplasmic membrane Cbl transport system, assuming that the nonexchangeable Cbl is in the cytoplasm. The possibility that DP treatment reduces the exchange activity of BtuB is unlikely because the rates of exchange were similar in DP-treated and control cells. Another possible explanation is that the rate of Cbl exchange is not a direct indicator of the size of the periplasmic Cbl pool because the rate of exchange was saturated at the DP-stimulated periplasmic content. Nonetheless, these results indicate that iron depletion mediated by DP results in increased CN-Cbl transport activity. It remains to be seen whether this potential effect on iron levels affects CN-Cbl transport through changes in the level or activity of TonB or at a subsequent transport or metabolic step.

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