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# The Proton Motive Force Drives the Outer Membrane Transport of Cobalamin in *Escherichia coli*

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**Cells of *Escherichia coli* pump cobalamin (vitamin $B_{12}$) across their outer membranes into the periplasmic space, and it was concluded previously that this process is potentiated by the proton motive force of the inner membrane. The novelty of such an energy coupling mechanism and its relevance to other outer membrane transport processes have required confirmation of this conclusion by studies with cells in which cobalamin transport is limited to the outer membrane. Accordingly, I have examined the effects of cyanide and of 2,4-dinitrophenol on cobalamin uptake in *btuC* and *atp* mutants, which lack inner membrane cobalamin transport and the membrane-bound ATP synthase, respectively. Dinitrophenol eliminated cobalamin transport in all strains, but cyanide inhibited this process only in *atp* and *btuC atp* mutant cells, providing conclusive evidence that cobalamin transport across the outer membrane requires specifically the proton motive force of the inner membrane. The coupling of metabolic energy to outer membrane cobalamin transport requires the TonB protein and is stimulated by the ExbB protein. I show here that the *tolQ* gene product can partly replace the function of the ExbB protein. Cells with mutations in both *exbB* and *tolQ* had no measurable cobalamin transport and thus had a phenotype that was essentially the same as TonB$^-$. I conclude that the ExbB protein is a normal component of the energy coupling system for the transport of cobalamin across the outer membrane.**

Cobalamin (Cbl; vitamin $B_{12}$) transport across the outer membrane of the cell envelope of *Escherichia coli* is an active transport process in which the BtuB protein is the Cbl carrier (3, 5, 12). In common with the transport systems for several ferric iron chelates, outer membrane transport of Cbl requires the TonB protein, which indicates that the mechanisms of potentiation of these processes are probably very similar (1, 17). From experiments with *atp* mutants, which lack a functional membrane-bound ATP synthase, it was concluded that the proton motive force generated across the inner membrane was the driving force for the outer membrane Cbl pump (5, 19). However, no experimental evidence was given that proved that the proton motive force was required for Cbl transport in *btuC* mutants, in which outer membrane transport can be studied in isolation because the inner membrane process is inactive. In view of the multiplicity of TonB-dependent systems, it is important that this point be clarified. Accordingly, in this article, I report on the characteristics of Cbl transport in *btuC atp* double mutants. The results support the previous conclusion that the proton motive force is the driving force for Cbl transport across the outer membrane.

In addition to an absolute requirement for the TonB protein, maximal activity of the outer membrane Cbl and ferric siderophore pumps also requires a functional *exbB* gene product (6, 12) and perhaps also the product of the *tolQ* gene. Braun (6) has shown that cells that contain mutations in both *exbB* and *tolQ* are fully resistant to bacteriophages T1 and φ80 and to colicins B, D, and M, yielding a phenotype that is essentially TonB$^-$. I also show here that *exbB tolQ* double mutants have lost the ability to actively transport Cbl.

## MATERIALS AND METHODS

**Radioisotopes.** Cyanocobalamin (CN-Cbl) labeled with $^{57}$Co (CN[$^{57}$Co]Cbl) and $^{35}$S-labeled L-methionine were obtained from the Amersham Corporation, and $^{14}$C-labeled L-proline was obtained from the New England Nuclear Corporation. CN[$^{57}$Co]Cbl was diluted with unlabeled cyanocobalamin to give a 2 μM stock solution that contained 2 μCi of $^{57}$Co per ml. The radioactive amino acids were diluted with the unlabeled compounds to give 200 μM stock solutions containing 10 μCi of radioisotope per ml.

***E. coli* strains.** The *E. coli* strains used are listed in Table 1. Strains CB1101 and CB1111 are *atp* mutants of KBT001 and KBT103, respectively, and were prepared by P1 transduction with DK-8 as the donor strain. A stock of P1*vir* ($3 \times 10^8$ PFU/ml) was obtained from Robert Kadner.

**Growth conditions.** The cells were grown at 37°C on the minimal medium of Davis and Mingioli (8), supplemented with glucose (10 mg/ml) and required amino acids (50 μg/ml). For all experiments, the cells were harvested in mid-log phase, washed once, stored on ice, and used within 3 h.

**Cbl uptake.** The harvested cells were washed once with and resuspended in 100 mM potassium phosphate (pH 6.6). The methods for measuring Cbl uptake were essentially those described previously (14) and consisted of incubating *E. coli* cells with CN[$^{57}$Co]Cbl and separating the cells from the reaction mixtures by filtration through Millipore filters. The precise conditions are given in the figure legends. Samples (1 ml) were removed at timed intervals and filtered through Millipore filters (type HAWP; 0.45-μm pore size, 25-mm diameter). The filters were washed twice with 10 ml of 100 mM lithium chloride, dried in an oven at about 90°C, and placed in 3-ml Filmware tubes (Sybron/Nalge Corp.) together with 100 μl of toluene containing 0.4% 2,5-diphenyloxazole and 0.01% 1,4-*bis*[2-(4-methyl-5-phenyloxazolyl)]benzene. Radioactivity was counted in a Beckman LS-100C liquid scintillation counter. The results are expressed as picomoles of CN-Cbl taken up by 1 ml of cells with an optical density at 660 nm ($OD_{660}$) of 0.6, which is ca. $10^9$ cells.

TABLE 1. *E. coli* strains used in this study

| Strain | Relevant genotype | Source |
|---|---|---|
| KBT001 | F⁻ *leu pro lysA trp purE metE str lac fhuA* | This laboratory |
| KBT103 | KBT001 *btuC* | This laboratory |
| CB1101 | KBT001 *atp ilv*::Tn*10* | This study |
| CB1111 | KBT103 *atp ilv*::Tn*10* | This study |
| DK8 | Δ*atpB-C ilv*::Tn*10 bglR rel-1* Hfr Pol | Robert D. Simoni |
| GM1 | *ara* Δ(*pro-lac*) *thi* F *pro-lac* | Kathleen Postle |
| KP1037 | GM1 *exbB*::Tn*10* | Kathleen Postle |
| TPS13 | GM1 *tolQ* | Kathleen Postle |
| KP1038 | TPS13 *exbB*::Tn*10* | Kathleen Postle |
| KP1082 | GM1 Δ(*trp-tonB*) | Kathleen Postle |

## RESULTS

**Generation of *atp* mutants.** Mutants lacking the membrane-bound ATP synthase were prepared by generalized transduction with bacteriophage P1*vir*. The donor strain was *E. coli* DK-8, which has a large deletion in the *atp* operon and a transposon Tn*10* inserted in the nearby *ilv* locus. The recipient strains were *E. coli* KBT001 and KBT103, which possess wild-type and mutant alleles, respectively, of *btuC*. The *atp* mutant derivatives of these strains were isolated by resistance to tetracycline and by growth that was slower with succinate than with glucose as the energy source. The auxotrophic requirements of these isolates were checked, and like the parent recipient strains, they required Ade, Leu, Lys, Met, Pro, and Trp. The methionine requirement could be satisfied by CN-Cbl, indicating retention of the lesion in *metE*. In addition, a requirement for isoleucine but not for valine was found, presumably as the result of the Tn*10* insertion in *ilv*. These properties further indicate that the Tn*10* insertion must be in *ilvA*, inactivating the threonine dehydratase.

The presumptive *atp* mutant status of the isolates was further investigated by measuring the effects of cyanide and dinitrophenol on the rates of transport of L-[U-$^{14}$C]proline and L-[$^{35}$S]methionine. Although *E. coli* strains have more than one transport system for each of these amino acids (15, 22), the concentrations in the reaction mixtures were only 10 μM so that only the high-affinity systems would show appreciable activity. As has been shown by others, high-affinity proline transport is potentiated by the proton motive force, whereas that of methionine is specifically ATP dependent (2, 16). In the absence of a functional membrane-bound ATP synthase, proton motive force-dependent transport should become more sensitive to inhibition by cyanide, whereas ATP-dependent transport should not. The results (not shown) confirmed that strains CB1101 and CB1111 are *atp* mutants. Thus, proline transport was inhibited strongly by both 1 mM KCN and 2 mM dinitrophenol in all strains, but the inhibition was much more complete in the presumptive *atp* mutants. In contrast, methionine transport was much less sensitive to these inhibitors in the *atp* mutant strains, with dinitrophenol even stimulating uptake by about 20%.

**Cbl transport.** The results shown in Fig. 1 indicate that the *atp* mutant strains CB1101 and CB1111 have retained the Cbl transport phenotypes of their parents, strains KBT001 and KBT103, respectively. Cells of each strain were incubated with limited amounts of CN[$^{57}$Co]Cbl, so that ca. 80 to 90% would be taken up by the cells within the first few minutes. The incubations were continued for 50 min, after which a

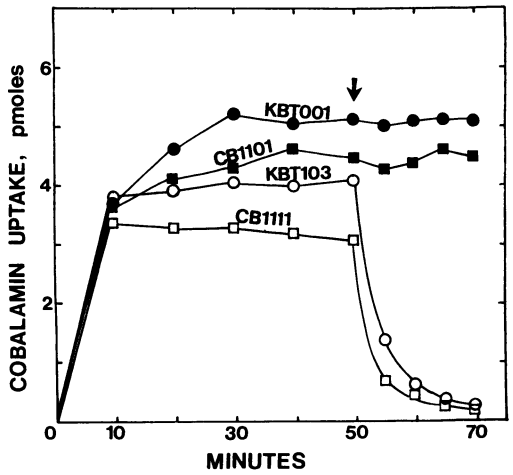


FIG. 1. Exchangeability of the Cbl taken up by various *E. coli* strains. Each 10-ml reaction mixture contained ca. 5 × $10^8$ *E. coli* cells, 25 mM glucose, 3 nM CN[$^{57}$Co]Cbl, and 100 mM potassium phosphate (pH 6.6) and was incubated in a water bath shaker at 37°C and 300 rpm. There was a 10-min preincubation before addition of the CN[$^{57}$Co]Cbl. Samples (1 ml) were removed at timed intervals and filtered through Millipore filters; the filters were washed and dried, and radioactivity was counted in a liquid scintillation counter. At 50 min (shown by the arrow), unlabeled CN-Cbl, to give a final concentration of 360 nM, was added to each reaction mixture, and sampling was continued. The results are expressed as picomoles of CN[$^{57}$Co]Cbl associated with 1 ml of cells at an $OD_{660}$ of 0.6. The *E. coli* strains used were KBT001 (●), KBT103 (*btuC*) (○), CB1101 (*atp*) (■), and CB1111 (*atp btuC*) (□).

100-fold excess of unlabeled CN-Cbl was added to each reaction mixture. This is a diagnostic test for distinguishing *btuC* mutants (19). Cbl transport across the inner membrane is defective in *btuC* mutant strains, which permits measurements of outer membrane Cbl transport in isolation. The Cbl transported across the outer membrane accumulates in the periplasmic space and is exchangeable with Cbl in the medium. As shown (Fig. 1), essentially all of the labeled CN-Cbl was released from strains KBT103 and CB1111, confirming that CB1111 has retained the *btuC* lesion of its KBT103 parent. Virtually none of the CN[$^{57}$Co]Cbl was released from the cells of strains KBT001 and CB1101, indicating that both of these strains possess the wild-type phenotype for Cbl transport. The results therefore confirm the following expected genotypes for these *E. coli* strains: KBT001, *atp*$^+$ *btuC*$^+$; KBT103, *atp*$^+$ *btuC*; CB1101, *atp btuC*$^+$; and CB1111, *atp btuC*. It should also be noted that under the given experimental conditions and in the absence of metabolic inhibitors, the *atp* mutation has no significant effect on Cbl transport.

**Inhibition of Cbl transport.** Figure 2 shows the effects of 1 mM potassium cyanide and of 2 mM 2,4-dinitrophenol on the uptake of CN[$^{57}$Co]Cbl by wild-type cells and by *atp* and *btuC* mutant cells of *E. coli*. In each case, dinitrophenol eliminated Cbl transport and limited uptake to the level of Cbl binding to the BtuB protein in the outer membrane. This inhibition in the *btuC* mutant strains is in itself strong evidence that the proton gradient is required for Cbl transport across the outer membrane. More conclusive are the results with cyanide, which inhibited Cbl uptake only in the *atp* mutant strains.

A more detailed examination of the effects of cyanide is shown in Fig. 3, in which the relative rates of Cbl transport

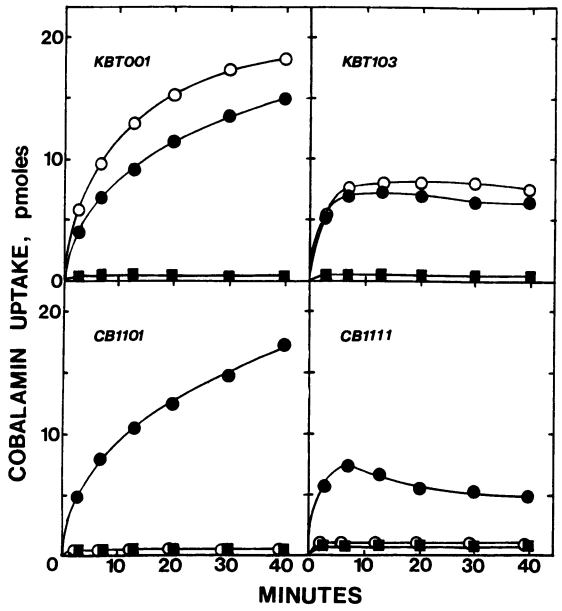


FIG. 2. Effects of cyanide and dinitrophenol on CN[$^{57}$Co]Cbl uptake by *E. coli* cells. Each 10-ml reaction mixture contained ca. 3 × $10^8$ cells, 25 mM glucose, 10 nM CN[$^{57}$Co]Cbl, other additions, and 100 mM potassium phosphate (pH 6.6). The incubation conditions were 37°C and 300 rpm, with a 10-min preincubation before addition of the CN[$^{57}$Co]Cbl. Samples (1 ml) were removed at timed intervals and treated as described in the legend to Fig. 1. The *E. coli* strains used are indicated in each panel. Other additions to the reaction mixtures: none (●); 1 mM potassium cyanide (○); and 2 mM 2,4-dinitrophenol (■). The results are expressed as picomoles of CN[$^{57}$Co]Cbl taken up by 1 ml of cells at on $OD_{660}$ of 0.6.

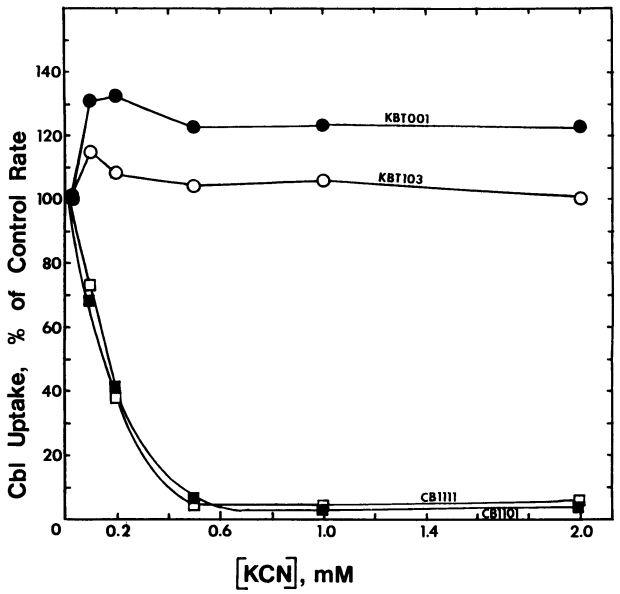


FIG. 3. Effects of various cyanide concentrations on CN[$^{57}$Co] Cbl uptake by *E. coli* cells. The reaction and incubation conditions were the same as those described for Fig. 2 except that the concentrations of potassium cyanide were varied. Samples (1 ml) were removed at timed intervals, and the rates of CN[$^{57}$Co]Cbl transport during the first 3 min were determined after subtraction of the amount of CN[$^{57}$Co]Cbl bound to the cell surface. These rates are expressed as percentages of the control rate, obtained in the absence of added cyanide, and are plotted against the cyanide concentration in the reaction mixtures. The *E. coli* strains used were KBT001 (●), KBT103 (○), CB1101 (■), and CB1111 (□).

are plotted against the concentrations of cyanide in the reaction mixtures. Each rate is the amount of Cbl taken up in 3 min less the amount of Cbl bound to the BtuB protein, expressed as a percentage of the control value measured in the absence of cyanide. The amount of Cbl bound to the BtuB protein was taken as the amount of CN[$^{57}$Co]Cbl associated with the cells in the presence of 2 mM 2,4-dinitrophenol. Again, inhibition by cyanide was seen only in the *atp* mutants, with 50% inhibition given by ca. 0.1 mM KCN. Thus, when respiratory electron transport is inhibited by cyanide, the potentiation of Cbl transport requires a functional membrane-bound ATP synthase in both *btuC*$^+$ and *btuC* strains. The inevitable conclusion is that the proton motive force of the inner membrane is the driving force for active Cbl transport across the outer membrane.

Cyanide gave some consistent stimulation of Cbl transport in the *atp*$^+$ strains, with the effect being more pronounced at KCN concentrations below 0.5 mM. In an earlier study with KBT001 cells (5), no significant stimulation by low levels of cyanide was observed. A difference in the two sets of experiments was that the cells in the earlier work had been starved by incubation with dinitrophenol for several hours, to deplete endogenous sources of energy. In the present study, the cells were not starved because I wanted to test Cbl transport in cells that contained more-normal levels of components that would be found in growing cells.

**Cbl transport in *exbB*, *tolQ*, and *tonB* mutants.** As has been known for some time, the TonB protein is required for penetration through the outer membrane of a variety of nutrients and infectious agents, including Cbl, several ferric siderophores, some colicins, and bacteriophages. Evidence has since been obtained that retention of full activity by the TonB protein requires functional ExbB and ExbD proteins, which can be replaced to some extent by the TolQ and TolR proteins, respectively (6, 9, 13, 17, 21, 24). Hantke and Zimmermann (13) showed that Cbl transport was impaired in *exbB* mutants, but its possible dependence on the *tol* genes has not been studied. Because of Braun's observations (6) that *exbB tolQ* double mutants had a TonB$^-$-like phenotype with respect to resistance to bacteriophages T1 and φ80 and to colicins B, D, and M, I tested the involvement of the *exbB* and *tolQ* gene products in Cbl transport. The results are shown in Fig. 4.

Cbl transport in cells containing the single mutations was lower than that in wild-type cells but was much more severely impaired in the *exbB* mutant. Even in this strain, the rate of uptake was much more than adequate to keep up with the Cbl requirements of *metE* mutant cells grown with Cbl but no methionine in the medium. However, the double *exbB tolQ* mutant showed no significant active transport of Cbl and had the same Cbl transport phenotype as TonB$^-$ cells and of wild-type cells incubated in the presence of 2 mM 2,4-dinitrophenol.

## DISCUSSION

It was shown some time ago with *atp* mutant cells that the proton motive force is absolutely required for measurable Cbl transport in *E. coli* (5). However, since these experiments were done with cells that were wild type with respect to Cbl transport, they did not permit any conclusion about

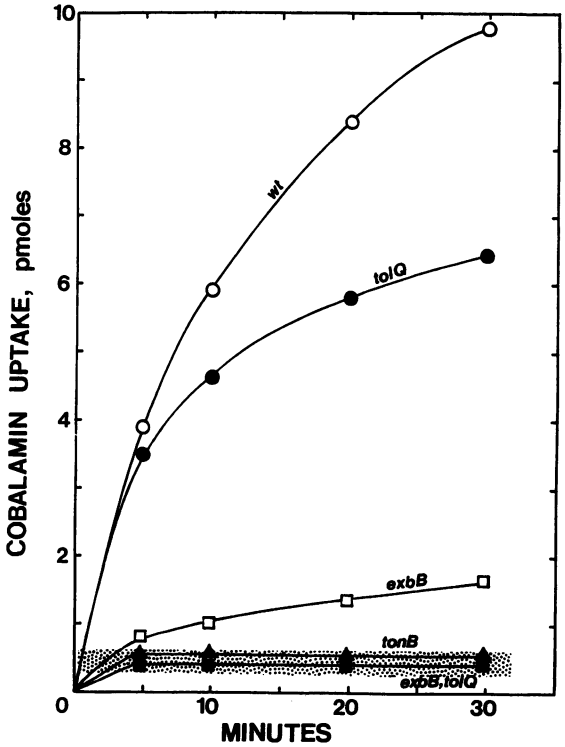


FIG. 4. Uptake of CN[$^{57}$Co]Cbl by *exbB*, *tolQ*, and *tonB* mutants of *E. coli*. Each 10-ml reaction mixture contained ca. $5 \times 10^8$ cells per ml, 25 mM glucose, and 10 nM CN[$^{57}$Co]Cbl in 100 mM potassium phosphate (pH 6.6) and was incubated at 37°C. Samples (1 ml) were removed at timed intervals and treated as described in the legend to Fig. 1. The results are expressed as picomoles of CN[$^{57}$Co]Cbl per milliliter of cells at an $OD_{660}$ of 0.6. The strains used were GM1 (○), TPS13 (*tolQ*) (●), KP1037 (*exbB*) (□), KP1083 (*tonB*) (▲), and KP1038 (*exbB tolQ*) (■). A second series of reaction mixtures differed from those above only in the inclusion of 2 mM 2,4-dinitrophenol. All of the uptake values for all of the strains in that series fell within the shaded area.

the specific part of the process that required the proton motive force. Subsequently, when *btuC* mutant cells were used to show that Cbl was actively transported across the outer membrane in the absence of measurable inner membrane Cbl transport, it was concluded that the proton motive force must be required for outer membrane Cbl transport, but no conclusive data were given (19). The results reported here with cells carrying mutations in both *atp* and *btuC* show unambiguously that Cbl transport across the outer membrane of *E. coli* has an absolute requirement for the proton motive force of the inner membrane.

The demonstration that the *tonB* gene product was required for Cbl transport indicated a fundamental similarity between this process and that of the transport of several ferric iron siderophores (1, 10–12, 23, 25). The available evidence indicates that Cbl is actively transported across the outer membrane and that the TonB protein functions in energy transduction, coupling outer membrane transport to the consumption of the respiratory proton gradient across the inner membrane. It is reasonable to conclude that the TonB-dependent transport systems for the ferric siderophores are also outer membrane pumps, a conclusion that is supported by the demonstration by Wooldridge et al. (26) that ferric aerobactin is actively transported across the outer membrane of *E. coli*. On the other hand, a recent report by Rutz et al. (20) has suggested that the TonB-dependent transport systems are gated channels, which implies facilitated diffusion, but it should be noted that their results with FepA do not, in fact, rule out active transport as the mechanism of outer membrane transport of ferric enterobactin. The TonB protein is tethered to the inner membrane and also interacts directly with the carrier proteins of the TonB-dependent transport processes (7, 14, 18). It has been proposed previously that the interaction of the TonB protein with the BtuB protein modulates the affinity of the BtuB protein for Cbl via a conformational change in a calcium-binding site (4). Maximal activity of TonB-dependent systems evidently also requires two other inner membrane proteins, the products of the *exbB* and *exbD* genes, which appear to have, at a minimum, a role in stabilizing the TonB protein (9, 13, 21). There is also evidence that the functions of these two proteins can be replaced to some extent by the *tolQ* and *tolR* gene products (6). Thus, while *exbB* mutants showed reduced transport of ferric ferrichrome and Cbl, the double *exbB tolQ* mutant had a phenotype that was essentially the same as TonB$^-$ with respect to resistance to bacteriophages T1 and φ80.

I have examined this situation with regard to Cbl transport. As shown previously by Hantke and Zimmermann (13), Cbl transport is much reduced in *exbB* mutant cells, but appreciable levels of Cbl are still taken up. Cbl transport was only slightly impaired in *tolQ exbB*$^+$ cells, although the rate of uptake slowed more quickly than in wild-type cells. Skare and Postle reported previously (21) that mutations in *tolQ* affect neither the chemical nor the functional stability of the TonB protein. In a single experiment (results not shown), I saw no significant difference in the rates of decay of Cbl transport after chloramphenicol was added to *tolQ* mutant and wild-type cells, which agrees with Skare and Postle's conclusion that a lack of TolQ does not alter the functional stability of the TonB protein. The somewhat lower Cbl transport found in *tolQ exbB*$^+$ cells (Fig. 4) could be explained if there is normally not quite enough ExbB protein to interact with all of the TonB present and the TolQ protein associates with the excess TonB. This would result in no difference in the relative rates of decay of TonB in the two strains, but the *tolQ* mutant cells would start at a somewhat lower level of activity. In cells containing mutations in both *exbB* and *tolQ*, I found no measurable Cbl transport, and uptake was limited to that bound to the BtuB protein, yielding the same phenotype as TonB$^-$. This clearly agrees with the observations of Braun (6) on the resistance of *tolQ exbB* mutant strains to bacteriophages φ80 and T1.

The evidence presently available indicates that, for optimal coupling of the proton motive force to the outer membrane transport processes, the TonB, ExbB, and ExbD proteins are required. While the requirement for TonB is absolute, the functions of the ExbB and ExbD proteins can be replaced to a limited extent by the TolQ and TolR proteins.

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