

## Transport of Vitamin B<sub>12</sub> in *tonB* Mutants of *Escherichia coli*

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It is known that the *tonB* mutation in *Escherichia coli* is responsible for a defect in the transport of iron chelates. These are transported by systems that involve outer membrane components. We found that *tonB* mutants were also deficient in the secondary, energy-dependent phase of vitamin B<sub>12</sub> transport, although the mutants have normal levels of B<sub>12</sub> receptors on their cell surface. In addition, *tonB* mutants derived from vitamin B<sub>12</sub> auxotrophs required elevated levels of B<sub>12</sub> for normal growth. Maltose uptake, mediated by another transport system involving an outer membrane component, was unaffected by the *tonB* mutation.

Receptor proteins in the outer membrane of cells of *Escherichia coli* and *Salmonella typhimurium* are intimately involved in the uptake of vitamin B<sub>12</sub> and iron chelates. The *bfe* gene product of *E. coli* is an outer membrane protein of 60,000 molecular weight which is obligatory for the cellular adsorption of phage BF23, the three E colicins, and B<sub>12</sub> (2, 6, 14, 15). The energy-independent binding of B<sub>12</sub> to the *bfe* product has been shown to be the first step in the transport of this vitamin by *E. coli*. Numerous mutations that affect the uptake of iron chelates are known. According to the available evidence, the *tonA* product is an 85,000-molecular-weight protein that binds phages T1, T5, and  $\phi$ 80, colicin M, and iron-ferrichrome (1, 11, 12, 23). A separate protein, which may be the product of the *feu* locus, appears to function as the initial receptor for colicins B and D and for the ferric-enterochelin complex (13, 24). Finally, the uptake of maltose and maltodextrins is facilitated by the function of the product of the *lamB* gene, the phage lambda receptor (18).

Mutations in the *tonB* locus of *E. coli* confer resistance to bacteriophages T1 and  $\phi$ 80 and all of the group B colicins (3). In addition, *tonB* mutants are chromium sensitive (22), resistant to the siderophore antibiotic albomycin (12), hyperexcrete enterochelin (9), and are totally deficient in the uptake of all iron chelates. However, these mutants are still capable of reversibly binding phages T1 and  $\phi$ 80, and they retain receptor activity for the colicins and iron chelates (3, 11, 16). These observations, together with the finding that no outer membrane proteins appear to be missing in *tonB* mutants (3), suggest that the *tonB* product is involved in some step of colicin or ferric-entero-

chelin uptake subsequent to binding to the receptor.

The activities of the uptake systems for a number of amino acids and inorganic phosphate were normal in *tonB* mutants (8). However, none of these transport systems appears to employ components in the outer membrane. Hence, the effect of alterations in *tonB* on the function of other transport systems utilizing outer membrane components was investigated. This paper will demonstrate that *tonB* mutants are totally deficient in the energy-dependent uptake phase of vitamin B<sub>12</sub> transport but are unaffected in maltose transport, even at low external concentrations of the substrate.

### MATERIALS AND METHODS

**Bacterial strains and media.** The strains of *E. coli* K-12 employed in this study are listed in Table 1. Selection for phage- or colicin-resistant mutants was as described (15). Mutants in *tonB* were selected for simultaneous resistance to phage  $\phi$ 80*vir* and colicin D. A small percentage of these (less than 5%) simultaneously acquired a tryptophan auxotrophy; these are designated as  $\Delta$ *tonB-trp*. All presumptive *tonB* mutants were tested for their sensitivity to phages T5 and BF23. Mutants in *tonA* were characterized as those resistant to phages T5 and  $\phi$ 80*vir* but sensitive to phage BF23 and colicin D. A number of colicin D-insensitive mutants that were sensitive to phages T5,  $\phi$ 80*vir*, and BF23 were isolated and tested.

Mutants in *lamB* were obtained as those resistant to phage  $\lambda$ cI which were still Mal<sup>+</sup> on MacConkey maltose plates (19). Cloned isolates were tested for their resistance to phage  $\lambda$ cI and their sensitivity to phages  $\lambda$ h80 (to eliminate possible  $\lambda$  lysogens) and BF23.

Minimal salts medium A (4) was supplemented with glucose at 0.5%, required amino acids (100  $\mu$ g/

TABLE 1. List of bacterial strains and phage stocks employed

Strain	Genotype	Source
RK4126	<i>proC lysA metE argH strA</i>	RK4101 (15)
1485F <sup>-</sup>	Wild-type K-12	B. Bachman, and cured of F with acridine orange.
RK4129	as RK4126, but <i>tonB15</i>	
RK4128	as RK4126, but <i>tonB2</i>	
RK4127	as RK4126, but <i>bfe</i>	
RK4130	as RK4127, but <i>tonB2</i>	
CA23	Colicinogenic for colicin D	T. Pugsley
RK1044	<i>entA ilv nalA</i>	AN248 from G. Cox
Phage stocks		
BF23		R. Benzinger
φ80vir		J. R. Johnson
λcI (W30)		M. Gottesman
λh80 (W248)		M. Gottesman
T5		Laboratory stock

ml), and thiamine (1 μg/ml). Strains were induced for maltose transport by growth with 0.4% maltose as carbon source.

**Transport assays.** The uptake of cyanocobalamin ( $B_{12}$ ) was assayed by the procedure described by DiGirolamo and Bradbeer (5). Cells grown at 37°C were harvested in the exponential phase, washed with medium A or 0.1 M potassium phosphate, pH 6.6. Washed cells at ca.  $5 \times 10^8$ /ml were incubated in the presence of glucose with [ $^3$ H]cyanocobalamin. At intervals, portions were withdrawn, passed through membrane filters (Millipore Corp., 0.45-μm pore size) and washed with 5 ml of buffer. Correction was made for the binding or trapping of substrate by the filter in the absence of cells. Measurement of  $B_{12}$  binding to whole cells employed cells incubated for 10 min with dinitrophenol (5 mM) (5).

The assay for maltose transport was similar to the fast assay described by Szmelcman and Hofnung (18), except that the buffer for washing and suspension of the cells was medium A. Induced cells were washed once in medium A and suspended in medium A containing chloramphenicol (40 μg/ml) for 15 min before assay. After addition of [ $^{14}$ C]maltose (7.9 Ci/mol) to final concentrations of 3.5 or 35 μM, portions were filtered after 30, 60, 90, and 120 s, as described above. Filters were washed with 5 ml of medium A, air-dried, and counted in a scintillation counter with toluene-based fluor.

**Chemicals.** Colicin D was prepared following growth of the colicinogenic strain (CA 23) in L broth to a density of  $4 \times 10^9$ /ml. The culture was induced by exposure to 0.2 μg of mitomycin C per ml and incubated at 37°C for 90 min, after which chloroform was added. The culture supernatant solution was clarified by centrifugation and stored at 4°C, at which temperature it was stable for several weeks.

Radioactively labeled amino acids were obtained from New England Nuclear Corp. Labeled maltose and cyanocobalamin were from Amersham/Searle Co. Other chemicals were from Sigma Chemical Co.

## RESULTS

**$B_{12}$  uptake in *tonB* mutants.** Mutants altered at *tonB* are defective in the uptake of ferric chelates (8, 21, 22). For the sake of comparison, the uptake of  $B_{12}$  was measured in several independent *tonB* mutants derived from each of three parental strains. This collection of mutants included those that had acquired a tryptophan auxotrophy, associated with a deletion from *tonB* at least through to the nearby *trp* locus. All of the *tonB* mutants tested were totally deficient in the secondary, concentration phase of  $B_{12}$  uptake (Fig. 1). The data for only two unrelated *tonB* mutants and their parental strains are shown, but identical results were obtained for 18 additional mutants, including some point mutants, as detailed below. The initial phase of uptake, corresponding to binding to the outer membrane receptor, was measured in cells treated with the energy poison, dinitrophenol. The level of  $B_{12}$  binding by whole cells was essentially unaltered in all of the *tonB* mutants relative to that in the parental strains (Table 2). These results are consistent with the full sensitivity of *tonB* mutants to phage BF23 and the E colicins which share the  $B_{12}$  receptor.

Frost and Rosenberg (8) showed that *tonB* mutants are not altered in other nutrient transport systems not employing outer membrane constituents, such as arginine, serine, proline,

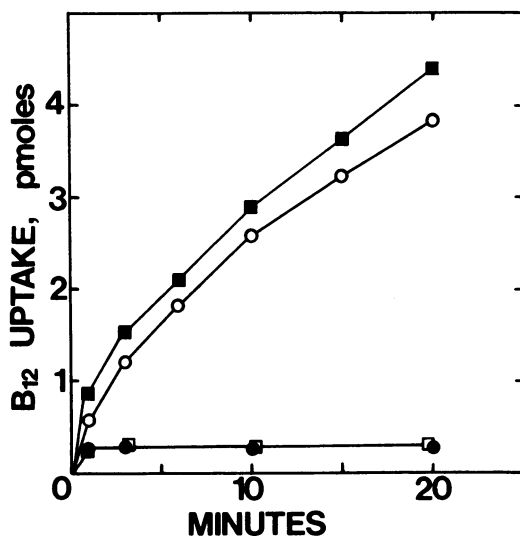


FIG. 1. Uptake of  $B_{12}$  in *tonB* mutants. The strains used were RK4126 (○), RK4128 (●), 1485F<sup>-</sup> (■), and 1485F<sup>-</sup> Δ*tonB*-*trp* (□). Cells were grown in minimal growth medium to mid-logarithmic phase and assayed as described in Materials and Methods. Samples are expressed as pmol of  $B_{12}$  taken up per  $10^9$  cells. Cells were counted with a Petroff-Hauser slide.

TABLE 2.  $B_{12}$  binding by dinitrophenol-treated *tonB* mutants

Strain	Relevant genotype	$B_{12}$ bound <sup>a</sup> (pmol/10 <sup>9</sup> cells)	Receptors/ cell <sup>b</sup>
RK4126	<i>ton</i> <sup>+</sup>	0.26	158
RK4128	<i>tonB2</i>	0.22	134
RK4129	<i>tonB15</i>	0.19	115
1485F <sup>-</sup>	<i>ton</i> <sup>+</sup>	0.35	211
1485F <sup>-</sup>	$\Delta$ <i>tonB-trp</i>	0.21	127

<sup>a</sup> External  $B_{12}$  concentration was 8.6 nM.

<sup>b</sup> As determined from  $B_{12}$  binding.

and inorganic phosphate. The uptake of glutamine, histidine, and proline was normal in the four *tonB* mutants examined by us. Leucine uptake was also normal except for that in a strain carrying a  $\Delta$ *tonB-trp* deletion. This strain had only 20% of the wild-type level of leucine transport. Thorne and Corwin (20) had shown that analogous deletions in *S. typhimurium* were defective in leucine uptake.

The possibility existed that the defect observed in vitamin  $B_{12}$  uptake in *tonB* mutants did not result from a defective *tonB* gene product, but rather resulted from the high levels of enterochelin known to be excreted into the culture medium by *tonB* mutants (9). It seemed unlikely that enterochelin was competing with  $B_{12}$  for a component of the specific  $B_{12}$  transport system, since both these substrates utilize different receptors on the cell surface. Two experiments were done which eliminated this possibility. First, it was shown that vitamin  $B_{12}$  transport was not restored in *tonB* mutants by growth in the presence of 100  $\mu$ M  $FeSO_4$ , which is more than sufficient to repress enterochelin biosynthesis, even in a *tonB* mutant (21). Second, *tonB* mutants were isolated in a strain (RK1044) that carried an *entA* mutation. Young et al. (25) had shown that *entA* mutants were specifically blocked in the pathway leading to the biosynthesis of enterochelin. When  $B_{12}$  transport was examined in *entA tonB* double mutants, the secondary phase of  $B_{12}$  transport was still not detectable.

Other mutations affecting iron uptake did not affect  $B_{12}$  uptake. Mutations in *tonA*, which abolish cellular receptor activity for iron-ferrichrome (23), had no effect on the uptake or binding of vitamin  $B_{12}$  in nine independent mutants tested. Similarly, four colicin D-insensitive mutants, which were still sensitive to all of the phages tested, had normal  $B_{12}$  transport. Thus, the elimination of the secondary phase of  $B_{12}$  uptake appears to be a result of the *tonB* mutation.

Growth on  $B_{12}$  by *tonB* mutants. Since

*tonB* mutants are completely devoid of  $B_{12}$  uptake by the usual assay, it seemed likely that they would be unable to utilize  $B_{12}$  as a growth-limiting nutrient. Hence, *tonB* mutants were selected in *metE* strains, which require either methionine or  $B_{12}$  for growth (4). The *tonB*<sup>+</sup> *metE* parental strain (RK4126) responds well on plates to  $B_{12}$  concentrations as low as  $5 \times 10^{-10}$  M, with partial growth at  $5 \times 10^{-11}$  M. A *bfe* derivative, RK4127, grows well only at  $5 \times 10^{-6}$  M  $B_{12}$  and poorly at  $5 \times 10^{-7}$  M (Table 3).

An examination of the ability of over 300 *tonB* mutants to grow on  $5 \times 10^{-9}$  M  $B_{12}$  revealed the existence of two types of responses. The major class, comprising about 75% of the mutants and including all of the  $\Delta$ *ton-trp* deletion strains, gave no growth on this level of  $B_{12}$ . The minority class was capable of nearly normal growth under these conditions. Members of the majority class, characterized by strain RK4128, could grow well on plates containing  $5 \times 10^{-7}$  M  $B_{12}$ . The introduction of a *bfe* mutation to this strain, yielding strain RK4130, further reduced the response to  $B_{12}$  to the level seen in the *ton*<sup>+</sup> *bfe* strain RK4127. Members of the minority class, such as strain RK4129, were only partially impaired in their response to  $B_{12}$ .

Despite these differences in growth response, there was no detectable difference in  $B_{12}$  uptake properties of the strains tested in the usual transport assay. There was no secondary-phase activity detected in either class of mutant. As before, the extent of  $B_{12}$  binding in energy-poisoned cells was normal in all strains tested. This result is in keeping with other observations that the ability of cells to utilize  $B_{12}$  for growth is a more sensitive assay of its uptake than is the usual transport assay with isotopically labeled substrate (15). However, even by this more sensitive criterion, the *tonB* product is clearly implicated in the uptake of  $B_{12}$ .

$B_{12}$ -utilizing revertants from *tonB* mutants. Further information on the relationship of *tonB* to  $B_{12}$  uptake was obtained from the isolation of  $B_{12}$ -utilizing revertants of many of the *tonB* strains. Revertants from the major class of *tonB* mutants, which were unable to respond to  $5 \times 10^{-9}$  M  $B_{12}$ , were selected on  $5 \times 10^{-10}$  M  $B_{12}$ . Two classes of revertants could be discerned. One type was generated by all the *tonB* mutants tested, including *tonB-trp* deletions, and were recovered at high frequencies ranging from  $10^{-4}$  to  $10^{-5}$  per cell plated. These form small, mucoid colonies on  $5 \times 10^{-10}$  M  $B_{12}$  and are still resistant to colicin D and phage  $\phi 80vir$ . In addition, they are quite unstable, such that growth of cloned isolates on L plates or minimal medium with methionine instead of

TABLE 3. Growth of *tonB* mutants with  $B_{12}$  as methionine source

Strain	Relevant genotype	Methio- nine (100 $\mu$ g/ml)	Response on minimal agar plates with <sup>a</sup>					
			$B_{12}$ (M)					
			$5 \times 10^{-6}$	$5 \times 10^{-7}$	$5 \times 10^{-8}$	$5 \times 10^{-9}$	$5 \times 10^{-10}$	$5 \times 10^{-11}$
RK4126	<i>metE</i>	3+	3+	3+	3+	3+	3+	+
RK4127	<i>metE bfe</i>	3+	3+	+				
RK4128	<i>metE tonB2<sup>b</sup></i>	3+	3+	3+	+			
RK4129	<i>metE tonB15<sup>c</sup></i>	3+	3+	3+	3+	2+	+	
RK4130	<i>metE tonB2 bfe</i>	3+	3+	+				

<sup>a</sup> Determined by relative colony size compared with strain RK4126 on methionine after 48 h of growth on minimal growth medium with the indicated supplements.

<sup>b</sup> This strain is a representative of the major class of *tonB* mutant; it appears to be a point mutation, but deletion strains behave identically.

<sup>c</sup> This is a representative of the minor class of *tonB* mutants.

$B_{12}$  results in a rapid loss of the ability to utilize  $B_{12}$ . These strains are similar to a class of revertants of *bfe* strains that will be discussed in a future communication. In essence, these result from a secondary lesion producing an alteration in the outer membrane increasing its permeability for  $B_{12}$ , thereby bypassing the usual receptor-mediated route of entry.

A second class of revertants was derived from only a few *tonB* mutants. This class formed large colonies on  $5 \times 10^{-10}$  M  $B_{12}$ ; members of this group were sensitive to colicin D and phage  $\phi 80vir$  and exhibited normal, wild-type levels of  $B_{12}$  binding and uptake. These are presumably revertants to  $TonB^+$ . These are not generated by any of the  $\Delta tonB-trp$  strains or by a number of  $Trp^+$  strains, which may have been generated by short deletions. Presumably, point mutants in *tonB* are able to give rise to this class of true revertants. This confirms that it is the loss of the *tonB* product alone that is responsible for the defect in  $B_{12}$  uptake, rather than the loss by deletion of some adjacent locus.

Revertants of the minor class of *tonB* mutants that were able to grow on moderately low levels of  $B_{12}$  were difficult to obtain and characterize. These mutants presumably represent missense mutations resulting in the presence of an altered but still partially functional *tonB* gene product. In spot tests, these mutants are still slightly sensitive to phage  $\phi 80vir$ , although not to colicin D. All of these results support the conclusion that the *tonB* product must be functional to allow the uptake of  $B_{12}$  or ferric chelates.

**Maltose transport in *tonB* mutants.** Szmelcman and Hofnung (18) have shown that the product of the *lamB* gene, the phage  $\lambda$  receptor, participates in the uptake of maltose at low concentrations. The basic piece of evidence for this, which we have confirmed, was that some *lamB* mutants exhibited greatly de-

creased rates of uptake of maltose at low external concentrations ( $3.5 \mu M$ ) relative to the parental strain. Uptake in the mutant at higher external concentrations approached that of the parent strain. Missense mutants in *lamB* were less severely affected in maltose uptake than were nonsense or deletion mutants. The uptake of  $3.5 \mu M$  maltose was measured over a 2-min time period in *tonB* and *tonB* derivatives of *lamB* and *lamB* strains. There was no significant effect of the state of the *ton* locus on maltose uptake at either  $3.5$  or  $35 \mu M$  maltose. Several of the *tonB* mutants studied here carried a  $\Delta tonB-trp$  deletion, and all were totally deficient in vitamin  $B_{12}$  uptake. Thus, the *tonB* product, although required for uptake of  $B_{12}$  or ferric chelates, is apparently not involved in maltose uptake, even under conditions where that uptake is dependent on an outer membrane component.

## DISCUSSION

Several studies have implicated the *tonB* product as being a common component of the various iron chelate transport systems (8, 21, 22), based primarily on the loss of all measured iron chelate transport activities. The *tonB* product would appear not to be the initial receptor for the iron chelates for several reasons. First, distinct mutants have been found which appear to be defective in individual iron chelate uptake systems, such as that for ferric-ferrichrome (12, 23) and for ferric-enterochelin (13). In fact, the *tonA* product has been strongly implicated as being the cell surface receptor for ferric-ferrichrome. Secondly, mutants in *tonB* retain receptor activity for phages T1, T5, and  $\phi 80$  (11), all the group B colicins, and for ferric-enterochelin (3, 16).

However, from the results presented in this paper, it is clear that the *tonB* product is also required for the uptake of vitamin  $B_{12}$ , which



has never before been shown to be related to any of the iron uptake systems. Along this line, neither phage BF23 nor the three E colicins that share the B<sub>12</sub> receptor are affected by the *tonB* mutation.

Explanations for these observations may well be expressed in terms of the architecture of the outer membrane and, possibly, in specialized regions of communication of the outer membrane receptors with the putative transport systems in the cytoplasmic membrane. Results of Haller and colleagues (10) and Rosenbusch (17) are suggestive that outer membrane proteins are arranged in complex asymmetric arrays. It is not inconsistent with the available evidence that the *tonB* product could be involved in maintenance of the proper orientation of receptors in the outer membrane with their sites for subsequent uptake. It would not be unexpected that transport systems totally dependent on the function of an outer membrane receptor might have very strict requirements for the proper orientation of its components between the two membranes.

The failure of the *tonB* mutation to adversely affect maltose uptake is, in fact, consistent with this hypothesis. On the basis of the current evidence, the maltose uptake system appears to be quite distinct from that for B<sub>12</sub> and the iron chelates. Unlike the case for vitamin B<sub>12</sub> or the iron chelates, there is no detectable binding of maltose or any higher maltodextrin to the phage λ receptor, either in vivo or in vitro (18a). Furthermore, maltose uptake is only dependent on the function of the *lamB* product at very low external concentrations (18). Even under these conditions, the uptake of maltose is absolutely dependent on its binding to the maltose-binding protein located in the periplasm. Thus, no specific orientation of the *lamB* product in the outer membrane with any component in the cytoplasmic membrane need be invoked, since the substrate must appear in the periplasm before uptake. Although the genetics of B<sub>12</sub> or iron uptake are not as advanced as that for the maltose pathway, there is so far no evidence in the former two systems for the obligate involvement of periplasmic proteins.

Experiments are now in progress to attempt to identify the nature and function of the *tonB* product, information on which has not yet been obtained.

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