JOURNAL OF BACTERIOLOGY, May 1977, p. 750-758
Vol. 130, No. 2

Printed in U.S.A.

Functional Stability of the *bfe* and *tonB* Gene Products in *Escherichia coli*

PHILIP J. BASSFORD, JR.,[1] CARL A. SCHNAITMAN, AND ROBERT J. KADNER*

Department of Microbiology, University of Virginia School of Medicine, Charlottesville, Virginia 22901

Received for publication 23 December 1976

The expression of several functional properties of the products of the *bfe* and *tonB* genes in *Escherichia coli* was measured after the specific termination of the synthesis of the products of these genes. This was accomplished by the use of a temperature-sensitive amber suppressor mutation, which allowed control, by manipulation of the growth temperature, of the level of product formed from suppressible mutant alleles of the *bfe* or *tonB* gene. The *bfe* product is an outer membrane receptor protein for vitamin B_{12}, the E-colicins, and bacteriophage BF23. The identity of the *tonB* product is unknown, but it is necessary for a subsequent step of uptake of vitamin B_{12}, iron chelates, all of the group B colicins, and bacteriophages T1 and ϕ80. Results from a different experimental system had shown that the termination of expression of the *bfe* locus was rapidly followed by loss of sensitivity to colicins E2 and E3 and, subsequently, to bacteriophage BF23. This was confirmed with this experimental system. Receptors that were no longer functional for colicin or phage uptake remained fully effective for B_{12} uptake, showing that receptors are stable on the cell surface. This supports previous contentions for the presence of different functional states for colicin receptors. The functional properties of the *tonB* product, measured by B_{12} uptake or sensitivity to the group B colicin D, were unstable, declining extensively after cessation of its synthesis.

The uptake of vitamin B_{12} by *Escherichia coli* is a biphasic process. The first phase consists of the rapid energy-independent binding of B_{12} to a receptor located in the outer membrane (9, 10, 25). The receptor is a protein of 60,000 daltons that is encoded by the *bfe* locus and was initially characterized as being the cell surface receptor for the three E-colicins and bacteriophage BF23 (5, 6, 11, 15, 17, 21). The secondary phase of B_{12} uptake responsible for transporting B_{12} from the cell surface to the cytoplasm is an energy-dependent process driven by the protonmotive force (4, 9).

The activity of the secondary phase is dependent on an intact *tonB* gene product, although B_{12} binding to strains harboring *tonB* mutations is not affected (2). The various siderophore-mediated iron transport systems, which also involve outer membrane components, are also dependent on an intact *tonB* gene product (12, 14, 20, 23). The role of *tonB* in these transport systems is unkown, but transport systems not involving outer membrane components are normal in *tonB* mutants (2, 12), as is the uptake of maltose (2), which also is associated with an outer membrane protein. In addition to manifesting a defect in B_{12} and iron transport, *tonB* mutants are resistant to phages T1 and ϕ80 and to all of the group B colicins, all of which absorb to receptors located in the outer membrane (7, 13).

tonB mutants are sensitive to the E-colicins and phage BF23 (7). In addition, strains tolerant to the E-colicins as a result of mutations at any of a number of genetic loci are still phage BF23 sensitive and can still transport B_{12} normally (16). Evidence such as this suggests that the uptake mechanisms for B_{12}, the E-colicins, and phage BF23 deoxyribonucleic acid must differ, despite their utilization of a common receptor on the cell surface. This was further illustrated in a recent communication (3), in which a comparison was made of the kinetics of expression of colicin E3 and phage BF23 sensitivity or resistance by the recipient cell after the conjugal entry of either the wild-type or mutant *bfe* allele. The results suggested that newly synthesized receptors were fully capable of lethally adsorbing either colicin E3 or phage BF23, but these receptors subsequently lost, first, the capability to lethally adsorb the colicin and, later, the capability to lethally adsorb the phage, although receptor activity was pre-

[1] Present address: Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115.

sumably still present. Thus, it appeared that the *bfe* gene product can exist in different functional states in the outer membrane. This present study was initiated to confirm the previous findings and to demonstrate the continued presence of receptor on the cell surface after specific termination of receptor synthesis.

Oeschger and Woods (19) recently described the isolation of a temperature-sensitive amber suppressor mutant that permits the control of the level of synthesis of specific proteins simply by manipulation of the growth temperature. In this study, strains carrying both this suppressor and amber mutations in either the *bfe* or *tonB* gene were constructed. After the synthesis of the *bfe* and *tonB* gene products was specifically terminated by increasing the growth temperature of these strains, the activity of products synthesized prior to the temperature shift was followed. The results show that the *bfe*-coded receptor protein remains functional for B_{12} transport for a considerable period after its synthesis. This transport activity is dependent on the continued synthesis of the *tonB* gene product. This study also shows that only newly synthesized *bfe*-coded receptor is functional for the productive adsorption of the E-colicins and phage BF23.

MATERIALS AND METHODS

Bacterial strains and media. The strains of *E. coli* K-12 employed in this study are listed in Table 1. Except where specified, the minimal growth medium used throughout was medium A of Davis and Mingioli (8), supplemented with glucose (0.5%), required amino acids (100 μg/ml), and thiamine (1 μg/ml). Medium in plates was solidified with 2% agar; that in overlays contained 0.7% agar. Most chemicals were obtained from Sigma Chemical Co.

Isolation of amber mutations in *bfe* and *tonB*. Strain PB501 (*metE supD43*) is a B_{12}-methionine auxotroph that carries the temperature-sensitive amber suppressor mutation described by Oeschger and Woods (19). After its mutagenesis with 2-aminopurine (as described by Miller [18]), strain PB501 was cultured overnight in L-broth (18) at 42°C (17). Phage BF23-resistant isolates were selected at 42°C and then screened for the ability to use 5 nM B_{12} as a methionine source at 30°C but not at 42°C. Such mutants were cloned several times and tested for sensitivity to colicin E3 and phage BF23 at 30 and 42°C. Finally, these putative *bfe* amber mutations were moved into strain MX364 ($SupD^-$) by cotransduction with either *arg* or *rpoB*, and the transductants were tested for resistance to colicin E3 and phage BF23 after growth at 30°C. Amber mutations in *tonB* were isolated and characterized in an identical manner, except that the initial selection was made for resistance at 42°C to phage *φ80vir* and colicin D as previously described (2), and that the potential *tonB* amber mutations were moved into strain MX364 by cotransduction with *trp*. By this procedure, 30 amber mutations in *bfe* and 6 amber mutations in *tonB* were obtained. That these mutations were in the *bfe* or *tonB* locus was shown by their characteristic cotransduction frequencies with *argH* or *trp*, respectively. Their suppressible nature was verified by the appropriate responses to B_{12} and the phages or colicins of strains carrying these mutations and either no suppressor (*supD*), a temperature-independent suppressor ($SupD^+$ = *supD32*), or a temperature-sensitive suppressor (SupD(Ts) = *supD43*). The amber mutations eventually used in this study were those that, when moved into a strain carrying the temperature-sensitive suppressor *supD43*, allowed the highest rate of B_{12} uptake at the permissive temperature of 21°C. Temperature-sensitive suppression was also tested in these strains by suppression of amber mutations in *trp* and *lacZ*.

Transport assays. For the measurement of B_{12} uptake, portions of cells were removed directly from the culture flask and mixed with [^{57}Co]cyanocobalamin (specific activity, 20.24 Ci/mmol; Amersham/Searle Co.) at a final concentration of 5 nM. At the indicated times, a 0.20-ml portion of the uptake mixture was transferred to the center of a membrane filter (0.45-μm pore size, Millipore Corp.), filtered, washed with 5 ml of medium A, immediately removed from the filtration apparatus, and air-dried. The radioactivity retained on the filter was measured in a scintillation counter with toluene-Omnifluor (New England Nuclear Corp.). All values were corrected for the binding or trapping

TABLE 1. *Bacterial strains*

Strain	Genotype[a]	Source
MX364	F^- *arg his trp*(Am) *lacZ*(Am)	M. Oeschger
MX383	F196 *his*$^+$ *supD32*/*his arg trp*(Am) *lacZ*(Am) *recA nalA*	B. Bachmann
MX419	Hfr KL96 *lacZ*(Am) *nalA rpsL supD43,74*(Ts)	B. Bachmann
PB501	As MX419 but *metE*	This study
PB521	As MX364 but *rpoB bfe-451*(Am)	This study
PB527	As PB521 but *his*$^+$ *supD43,74*(Ts)	This study
PB583	As PB521 but *his*$^+$ *supD32*	This study
PB564	As MX364 but *trp*$^+$ *tonB456*(Am)	This study
PB568	As PB564 but *his*$^+$ *supD43,74*(Ts)	This study
PB572	As PB564 but *his*$^+$ *supD32*	This study

[a] Gene symbols are those proposed by Bachmann et al. (1), with allele isolation numbers assigned by B. Bachmann, Coli Genetic Stock Center.

of B_{12} to the filter in the absence of cells. In experiments in which B_{12} uptake was expressed in terms of picomoles per microliter of cell water, 1 μl was assumed to be equal to 250 μg of protein. All transport assays were performed at room temperature (21°C) so that all uptake activities would be comparable. The loss of suppression by thermal inactivation in the SupD(Ts) strains is not reversible over the assay period.

Preparation and standardization of colicins and bacteriophage. Colicin E3 and phage BF23 were prepared, diluted, and standardized with respect to one another as previously described (3). The colicin E3 stock preparation contained 10^6 killing units as defined by Sabet and Schnaitman (21). Phage BF23 was employed at a concentration of 2×10^{11} plaque-forming units per ml.

Colicin D was also prepared as previously described (2). The colicin D stock preparation contained 10^4 killing units and was diluted with L-broth.

RESULTS

B_{12} uptake in *bfe* and *tonB* amber mutants. Strains carrying amber mutations in *bfe* and *tonB* were isolated by selection for resistance at 42°C to the appropriate phage and colicin followed by screening for the ability to utilize B_{12} at the permissive temperature. The uptake of B_{12} by SupD$^-$ strains harboring an amber mutation in either *bfe* or *tonB* was measured (Fig. 1). There was no detectable binding or transport of B_{12} by strain PB521 [Bfe(Am) SupD$^-$]. Strain PB564 (TonB(Am) SupD$^-$) totally lacked the secondary phase of B_{12} uptake while retaining parental levels of the initial phase. The parental strain MX364 possessed both phases characteristic of B_{12} uptake by wild-type *E. coli* K-12 (9).

B_{12} uptake in SupD$^+$ strains. The temperature-sensitive amber suppressor mutation *supD43* was introduced into these mutants by transduction. The transport of B_{12} was measured in the resulting SupD(Ts) strains after their growth at the permissive temperature (21°C) and at the nonpermissive temperature (42°C). To insure that the suppressed gene product was not, itself, temperature sensitive, B_{12} transport was also measured in strains with the same amber mutation but carrying the temperature-independent suppressor mutation *supD32*.

When strain PB527 [Bfe(Am) Sup(Ts)] was grown at 42°C, there was no detectable accumulation of B_{12} over a 30-min period (Fig. 2A). However, when this strain was grown at 21°C, B_{12} uptake was observed, corresponding to about 3% of that in the *bfe*$^+$ parental strain. This low level of B_{12} uptake was inhibited by the presence of the energy poison dinitrophenol (data not shown). Since transport was not observed after growth of strain PB527 at 42°C or in strain PB521 grown at any temperature, it was concluded that such accumulation repre-

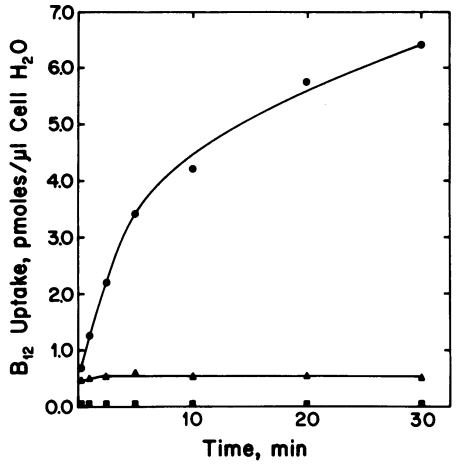

FIG. 1. *B_{12} uptake in bfe and tonB amber mutants. The uptake of B_{12} was assayed with exponentially growing cells of strains MX364 (ton$^+$ bfe$^+$, ●), PB521 (bfe-451, ■), and PB564 (tonB456, ▲).*

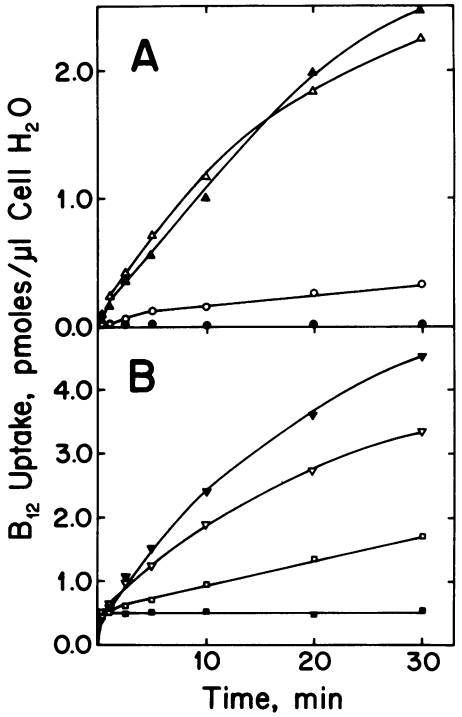

FIG. 2. *Suppression by SupD of bfe and tonB amber mutations. (A) Vitamin B_{12} uptake in the bfe-451 strains PB527 [SupD(Ts)] grown at 21°C (○) or 42°C (●) and PB533 (SupD$^+$) grown at 21°C (△) or 42°C (▲). TonB456 strains PB568 [SupD(Ts)] grown at 21°C (□) or 42°C (■) and PB572 (SupD$^+$) grown at 21°C (▽) or 42°C (▼).*

sented active transport responsive to the level of the suppressed *bfe*(Am) gene product. B_{12} uptake in strain PB533 (Bfe(Am) SupD$^+$) was similar whether growth was at either 21 or 42°C (Fig. 2A). Thus, the suppressed *bfe*(Am) gene product, once synthesized, was not inactivated at 42°C. It should be noted here that the efficiency of suppression by the temperature-sensitive suppressor *supD43* is only a fraction of that observed with the temperature-independent suppressor *supD32*, even at a growth temperature of 21°C. This inefficiency had also been found for the suppression of amber mutations by this temperature-sensitive suppressor allele (19).

When strain PB568 [TonB(Am) SupD(Ts)] was grown at 42°C, its B_{12} binding was normal, but there was no detectable B_{12} transport activity (Fig. 2B). When this strain was grown at 21°C, B_{12} transport activity was observed, corresponding to about 20% of that of the *ton*$^+$ parental strain. Transport activity for strain PB572 [TonB(Am) SupD$^+$] grown at 21 or 42°C was similar, indicating that the suppressed *tonB*(Am) gene product was not temperature labile at 42°C (Fig. 2B).

Turnover of the B_{12} receptor protein. From the above-mentioned results, it was concluded that the synthesis of either the *bfe*(Am) or *tonB*(Am) gene products could be specifically terminated by shifting a culture of the respective strains from 21 to 42°C, without affecting the activity of these gene products, which were synthesized prior to the temperature shift. Hence, the effect of a temperature shift on the B_{12} uptake activity of a culture of PB527 grown at 21°C was investigated. As a control, a parallel culture of PB533 was treated in an identical manner.

In cultures of strains PB527 and PB533 growing at 21°C, B_{12} uptake activity increased concomitantly with the increase in optical density (Fig. 3). When strain PB533 (SupD$^+$) was shifted to 42°C, uptake activity continued to increase (Fig. 3A). In contrast, when strain PB527 [SupD(Ts)] was shifted to 42°C, the total B_{12} uptake activity in the culture remained at a constant level after the shift, even though the cells increased in number at an increased rate in accordance with the elevated growth temperature (Fig. 3B). This B_{12} uptake activity remained quite constant over several generations, with the typical decline in activity as the culture entered the stationary phase.

Effect of amino acid starvation on the activity of the B_{12} receptor protein. It was shown previously that the inhibition of general protein synthesis was followed by a fairly rapid and specific depression in the secondary phase

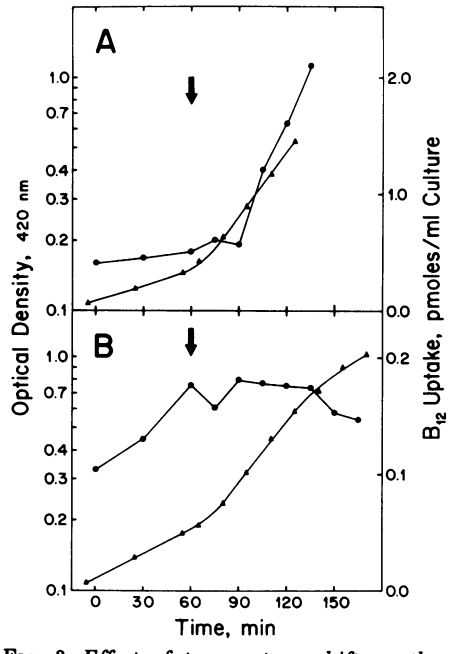

FIG. 3. *Effect of temperature shift on the suppressed B_{12} uptake activity in Bfe(Am) strains. (A) Strain PB533 [SupD$^+$ Bfe(Am)]; (B) strain PB527 [SupD(Ts) Bfe(Am)]. Cells growing in minimal medium at 21°C were assayed for optical density (▲) and B_{12} uptake activity (●). B_{12} uptake represents the amount of substrate accumulated after 10 min less that accumulated after 1 min and is expressed in terms of picomoles of B_{12} accumulated per milliliter of culture. The culture was shifted to 42°C at the time indicated by the arrow (60 min).*

of B_{12} transport, without loss of surface B_{12} binding activity (16). It was of interest to determine whether this depression in transport activity was manifested at the level of the outer membrane receptor protein, or whether the requirement for continued protein synthesis occurred at some subsequent step in the B_{12} uptake process.

The removal of arginine from a culture of PB527 growing at 21°C resulted in a gradual decline in B_{12} uptake activity as the cells became starved for this required amino acid (Fig. 4). The depression in uptake activity was not as rapid as previously observed (16), perhaps due to the fact that the cells were growing much more slowly here as compared with the earlier experiments in which cells were grown at 37°C. After a period of arginine starvation, during which B_{12} uptake activity decreased to about 30% of the prestarvation level, protein synthesis was restored by the addition of arginine to the culture, which was simultaneously shifted to 42°C so that no new B_{12} receptor proteins could be synthesized. B_{12} uptake activity

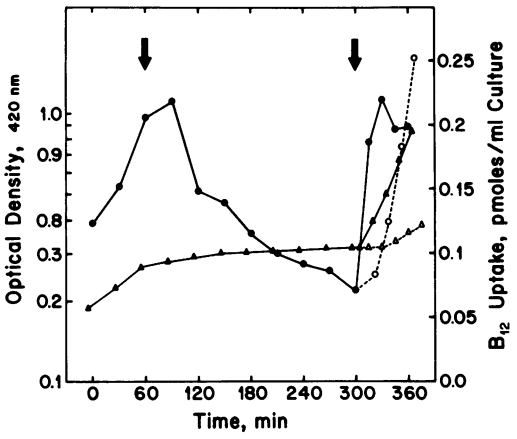

FIG. 4. *Effect of arginine deprivation and temperature shift on B_{12} uptake activity. Cells of strain PB527 [SupD(Ts) Bfe(Am)] were grown at 21°C. At the time indicated by the first arrow, the cells were washed once and suspended in the original volume of minimal growth medium lacking arginine at 21°C. At the time indicated by the second arrow, arginine (100 μg/ml) was restored to the culture. The culture was simultaneously shifted to 42°C (●, ▲); a duplicate culture was maintained at 21°C (○, △). As described in the legend of Fig. 3, the culture was assayed at the indicated times for optical density (▲, △) and B_{12} uptake (●, ○).*

quickly returned to its prestarvation level and remained there as the cells resumed logarithmic growth at 42°C. When arginine was added to a parallel culture that remained at 21°C, the prestarvation level of B_{12} uptake was soon exceeded as the culture resumed logarithmic growth (Fig. 4). Thus, the inhibition of protein synthesis did not permanently uncouple preformed B_{12} receptor protein from participating in the B_{12} uptake process.

Expression of resistance to colicin E3 and phage BF23. In parallel with its B_{12} uptake activity, strain PB527 is sensitive to colicin E3 and phage BF23 when grown at the permissive temperature and is resistant to them when grown at the restrictive temperature. Accordingly, the kinetics of appearance of resistance to colicin E3 and phage BF23 after cessation of receptor synthesis was determined.

When strain PB527 [Bfe(Am) SupD(Ts)] was grown at 21°C, nearly 40% of the cell population was colicin E3 resistant under these experimental conditions, and nearly 25% of the cell population was phage BF23 resistant (Fig. 5). This does not indicate that a large percentage of the cells in the culture were incapable of synthesizing the receptor (e.g., Sup^-). When a drop of an identical concentration of colicin or phage used in this experiment was spotted onto a lawn of strain PB527 grown at the permissive temperature, a completely clear zone of inhibition resulted. Longer incubation times of colicin or phage with cells were not required, since a very high multiplicity of colicin and phage particles per cell (greater than 100,000:1) was employed, and longer adsorption periods did not significantly alter the results (see Discussion).

When the culture of strain PB527 was shifted to 42°C, the proportion of cells resistant to colicin and phage rapidly increased (Fig. 5). For example, during the 20-min period immediately after the shift, the percentage of colicin E3-resistant cells in the culture increased from 38 to 61%. In terms of actual numbers, there was a much greater increase in colicin-resistant cells than in total cells over the same 20-min period. Within one generation time after the shift, essentially 100% of the cell population had developed resistance to colicin E3. Simi-

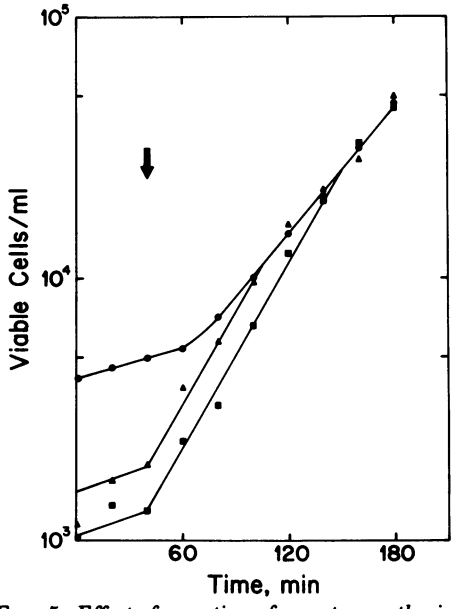

FIG. 5. *Effect of cessation of receptor synthesis on insensitivity to colicin E3 and phage BF23. A culture of strain PB527 [Sup(Ts) Bfe(Am)] was grown at 21°C to a density of approximately 4 × 10^8 cells per ml and then diluted by a factor of 10^5 in the same medium. At the indicated intervals, 0.1 ml of culture was mixed with 0.1 ml of medium (●), or with 0.1 ml of either a 1:125 dilution of colicin E3 (▲) or 0.1 ml of phage BF23 (2 × 10^{11} plaque-forming units per ml (■), and incubated for 5 min at 42°C. These samples were then mixed with 3 ml of medium A soft agar and poured onto minimal agar plates, which were immediately incubated at 42°C. Colony counts were determined 48 h later. The arrow indicates the time at which the growth temperature of the culture was shifted from 21 to 42°C.*

larly, within two generation times after the shift, greater than 95% of the population was resistant to phage BF23.

Turnover of the *tonB* gene product. When strains PB568 [TonB(Am) SupD(Ts)] and PB572 (TonB(Am) SupD$^-$) were grown at 21°C, both B_{12} transport and binding activity continued to increase in proportion to the optical density of the culture (Fig. 6). When strain PB572 was shifted to 42°C, B_{12} transport and binding continued to increase (Fig. 6A). When strain PB568 [SupD(Ts)] was shifted to 42°C, binding activity increased normally (Fig. 6B). Immediately after the temperature shift, however, there occurred a fairly rapid decline in B_{12} transport activity. Within one generation time after the shift, transport activity had declined to less than 25% of that at the time of shift. The declince continued, albeit more slowly, until no transport activity was discernible by the time the culture began to enter stationary phase, 2.5 generation times after the shift.

Effect of amino acid starvation on the activity of the *tonB* gene product. These and previous experiments suggested that the specific depression in the secondary phase of B_{12} uptake after the inhibition of protein synthesis was not the result of the inhibition of synthesis of the *bfe* product. The possibility that this depression of B_{12} uptake resulted from the specific inhibition of synthesis of the *tonB* product was investigated.

When a culture of PB568 [TonB(Am) SupD(Ts)] growing at 21°C was starved for arginine for several hours, B_{12} transport activity declined to 40% of that present prior to the onset of starvation (Fig. 7). The culture was then shifted to 42°C, and protein synthesis was restored by the addition of arginine. In contrast to the earlier results obtained with strain PB527 [Bfe(Am) SupD(Ts)], the inhibition of B_{12} transport activity was not relieved. Rather, the decline in B_{12} transport activity continued as the culture resumed logarithmic growth at 42°C. In a parallel culture that remained at 21°C after arginine addition, there was a slow recovery of B_{12} transport activity upon the resumption of growth (Fig. 7). This suggested that such recovery was dependent on the synthesis of a new *tonB* gene product.

Expression of colicin D resistance. *tonB* mutants are resistant to all group B colicins,

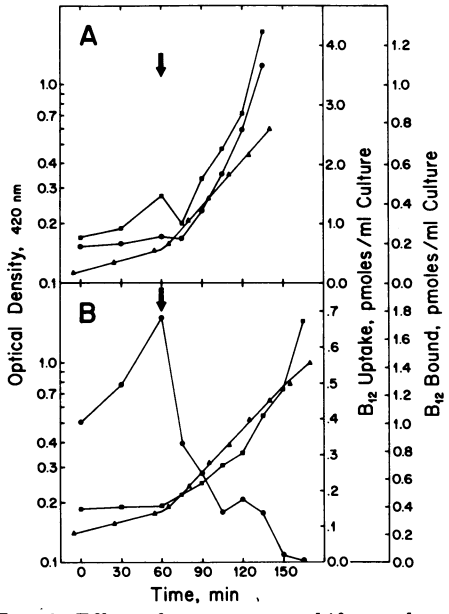

FIG. 6. *Effect of temperature shift on the suppressed B_{12} uptake activity in TonB(Am) strains. Cells of strains (A) PB572 [Sup$^+$ TonB(Am)] and (B) PB568 [Sup(Ts) TonB(Am)] were grown in minimal medium at 21°C and shifted to 42°C at the time indicated by the arrow. At the indicated intervals, the culture was assayed for optical density (▲), picomoles of B_{12} bound and transported after 1 min per milliliter of culture (■), and the amount of B_{12} accumulated after 10 min less that at 1 min, again expressed as picomoles per milliliter of culture (●).*

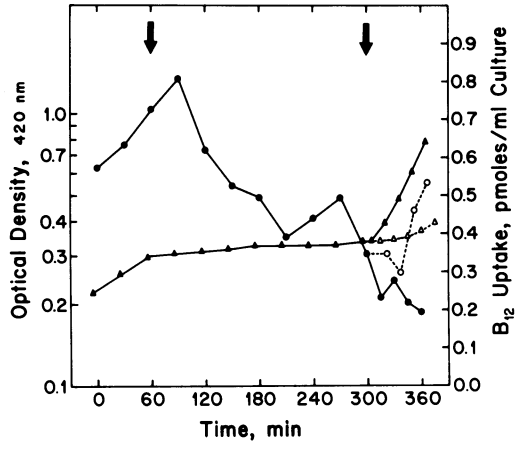

FIG. 7. *Effect of arginine deprivation and temperature shift on B_{12} uptake activity. Cells of strain PB568 [Sup(Ts) TonB(Am)] were grown at 21°C. At the time indicated by the first arrow, the cells were washed once and suspended in the original volume of minimal growth medium lacking arginine at 21°C. At the time indicated by the second arrow, arginine (100 μg/ml) was restored to the culture. The culture was simultaneously shifted to 42°C (●, ▲); a duplicate culture was maintained at 21°C (○, △). As described in the legend of Fig. 3, the culture was assayed at the indicated times for optical density (▲, △) and B_{12} uptake (●, ○).*

including colicin D. When a culture of strain PB568 was grown at 21°C, greater than 98% of the cell population was sensitive to colicin D under the experimental conditions employed (Fig. 8). When a culture was shifted to 42°C, the proportion of colicin D-resistant cells increased markedly over a relatively short period of time. For example, nearly 25% of the cell population expressed resistance to colicin D only 20 min after the shift. Within 60 min of the temperature shift, during which the total cell population had increased approximately 60%, almost 100% of the cell population was resistant to killing by colicin D.

DISCUSSION

The results presented here confirm our earlier finding that the *bfe* receptor is functional for the effective adsorption of colicin E3 or phage BF23 for only a short period after its synthesis. This result had been based on the rapid acquisition of resistance by cells after the introduction by conjugation of a mutant *bfe* allele. In this paper, the specific termination of expression of the *bfe* locus was accomplished by the use of a temperature-sensitive suppression system. Shortly after the temperature shift, there was the typical rapid loss of insensitivity to colicin E3 and phage BF23. Postulation of an asymmetrical distribution of receptor molecules to daughter cells after cell division could not account for the observed rapidity of loss. As seen before (3), the appearance of colicin E3 insensitivity preceded that of phage BF23 insensitivity. Over the same time span after cessation of receptor synthesis, there was no decline in receptor-dependent B_{12} uptake. The difficulty with this experimental system was that the level of suppression by the temperature-sensitive suppressor allele was quite low. Hence, direct measurement of the receptor level by a B_{12}-binding assay was not possible. Nonetheless, the uptake activity was reflective of the receptor level, as shown by the consistency of these results with those obtained by other techniques.

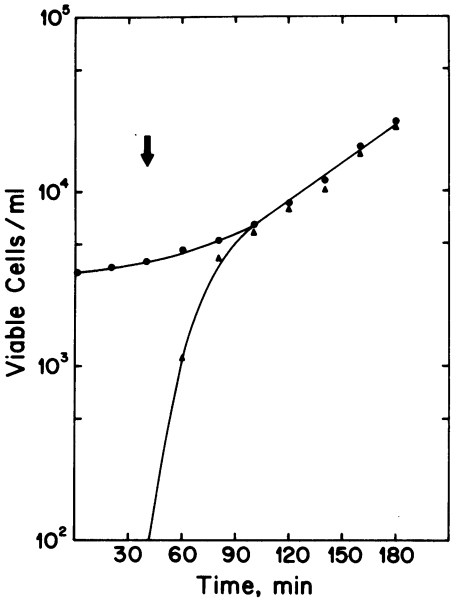

FIG. 8. *Acquisition of insensitivity to colicin D after cessation of synthesis of the tonB gene product. A culture of strain PB568 [Sup(Ts) TonB(Am)] was grown at 21°C in M63 medium (16) supplemented with glucose (0.5%), arginine (100 μg/ml), and thiamine (1 μg/ml) to a density of ca. 4 × 10⁸ cells per ml and diluted by a factor of 10⁵ in the same medium. (M63 was employed here because the citrate in medium A interferes with the lethal adsorption of colicin D [22].) At the indicated intervals, 0.1 ml of culture was mixed with either 0.1 ml of medium (●) or with 0.1 ml of a 1:25 dilution of colicin D (▲), incubated for 5 min at 42°C, and then treated as described in the legend of Fig. 5.*

These results showed that, in growing cells, *bfe* receptor molecules are able to interact with the system(s) responsible for subsequent steps of uptake of colicin E3 or phage BF23 deoxyribonucleic acid for only a short period after their appearance on the cell surface. However, receptors appear to be capable of continuously interacting with the system (*tonB?*) responsible for B_{12} uptake. In other words, the *bfe* product is functionally stable, but its interaction with the subsequent components of the colicin uptake system is not. It does appear that colicins E2 and E3 must enter the cytoplasm for their lethal action. However, the mechanism of colicin uptake and even the nature of the components of this process remain unknown.

The existence of two functional classes of receptor can explain the observation that a large proportion of the culture of strain PB527 [Bfe(Am) SupD(Ts)] grown at the permissive temperature were insensitive to colicin E3 and phage BF23. A wild-type cell possesses approximately 200 B_{12} receptors (21, 25). Since strain PB527 exhibits about 3% of the wild-type rate of B_{12} uptake, there should be roughly six receptors per cell. Hence, some of these receptors must be nonfunctional. The observation that 40% of the cells in such a culture are insensitive to colicin E3 can be simply explained by the assumption that only 10% of the receptors are "newly synthesized", i.e., effective for colicin uptake. These results also suggest that sensitivity to phage BF23 is not solely dependent on the presence of receptor. Uptake of phage deoxyribonucleic acid also appears to call for

newly synthesized receptor, but this uptake system is not the same as that for the colicins, as shown by the phage BF23 sensitivity of mutants tolerant to the E-colicins (16).

That the *tonB* product is functionally unstable is indicated by the rapid loss of B_{12} uptake capacity upon cessation of *tonB* expression. This was confirmed by the simultaneous loss of sensitivity to colicin D, whose action is *tonB* dependent (7). This instability of the *tonB* product could well account for the decrease in B_{12} uptake after inhibition of general protein synthesis (16). In support of this, it has been found that cells whose protein synthesis has been inhibited are markedly less sensitive to the *tonB*-dependent colicins D (22), Ia, and B (R. J. Kadner, manuscript in preparation). Growth inhibition has no effect on sensitivity to the *tonB*-independent colicin E1 (16). It would be of interest to determine whether siderophore-mediated iron transport is likewise depressed by growth inhibition.

Experiments now in progress are attempting to define the role of the *tonB* gene product. It has been suggested that the *tonB* product might be involved in maintaining the proper orientation of certain outer membrane receptors with sites, presumably in the cytoplasmic membrane, for the subsequent uptake of their respective substrates (2). Since all of these uptake processes are energy dependent, it could also be that the *tonB* product functions to couple energy to the outer membrane or to specific outer membrane components (13). The observation that the *tonB* product must be continually synthesized to function in B_{12} transport suggests that it does not interact specifically with each outer membrane receptor protein but, rather, functions in some nonspecific manner. This need for continued synthesis is also compatible with its consumption during energy-dependent transport processes.

The orientation of the outer membrane with the inner membrane may also provide the physiological basis underlying the demonstration that the B_{12} receptor can apparently exist in different states. It could be that only receptors newly inserted into the outer membrane possess the proper orientation for the lethal adsorption of the E-colicins and phage BF23. As the outer membrane continues to grow, this orientation is lost, first with respect to the colicins and then with respect to the phage. However, the *bfe* product can continue to function in the B_{12} uptake process as long as the *tonB* product is continually synthesized, although the *tonB* product plays no role in the lethal adsorption of the colicins and phage that utilize the same receptor.

Attempts in this laboratory to identify the *tonB* product by polyacrylamide gel electrophoresis of various fractions of the *E. coli* envelope have thus far been unsucessful. This may be due, in part, to rapid turnover of the *tonB* product. However, it would appear that an understanding of the nature, function, and location of the *tonB* product is absolutely essential to an understanding of a number of processes involving the outer membrane of *E. coli* K-12.

ACKNOWLEDGMENTS

R.J.K. wishes to dedicate this paper to his former advisor, Joseph F. Nyc, on the occasion of his retirement from the University of California School of Medicine, Los Angeles.

This work was supported by Public Health Service grant GM19078 from the National Institute of General Medical Sciences (to R.J.K.) and by grant BMS-04973 from the National Science Foundation (to C.A.S.). R.J.K. is the recipient of Public Health Service Research Career Development Award GM00019 from the National Institute of General Medical Sciences.

LITERATURE CITED

1. **Bachmann, B. J., K. B. Low, and A. L. Taylor.** 1976. Recalibrated linkage map of *Escherichia coli* K-12. Bacteriol. Rev. 40:116–167.
2. **Bassford, P. J., Jr., C. Bradbeer, R. J. Kadner, and C. A. Schnaitman.** 1976. Transport of vitamin B_{12} in *tonB* mutants of *Escherichia coli*. J. Bacteriol. 128:242–247.
3. **Bassford, P. J., Jr., R. J. Kadner, and C. A. Schnaitman.** 1977. Biosynthesis of the outer membrane receptor for vitamin B_{12}, E colicins, and bacteriophage BF23 by *Escherichia coli:* kinetics of phenotypic expression after the introduction of *bfe*$^+$ and *bfe* alleles. J. Bacteriol. 129:265–275.
4. **Bradbeer, C., and M. L. Woodrow.** 1976. Transport of vitamin B_{12} in *Escherichia coli:* energy dependence. J. Bacteriol. 128:99–104.
5. **Bradbeer, C., M. J. Woodrow, and L. I. Khalifah.** 1976. Transport of vitamin B_{12} in *Escherichia coli*: common receptor system for vitamin B_{12} and bacteriophage BF23 on the outer membrane of the cell envelope. J. Bacteriol. 125:1035–1039.
6. **Buxton, R. S.** 1971. Genetic analysis of *Escherichia coli* K-12 mutants resistant to bacteriophage BF23 and the E-group colicins. Mol. Gen. Genet. 113:154–156.
7. **Davies, J. K., and P. Reeves.** 1975. Genetics of resistance to colicins in *Escherichia coli* K-12: cross-resistance among colicins of group B. J. Bacteriol. 123:96–101.
8. **Davis, B. D., and E. S. Mingioli.** 1950. Mutants of *Escherichia coli* requiring methionine or vitamin B_{12}. J. Bacteriol. 60:17–28.
9. **DiGirolamo, P. M., and C. Bradbeer.** 1971. Transport of vitamin B_{12} in *Escherichia coli*. J. Bacteriol. 106:745–750.
10. **DiGirolamo, P. M., R. J. Kadner, and C. Bradbeer.** 1971. Isolation of vitamin B_{12} transport mutants of *Escherichia coli*. J. Bacteriol. 106:751–757.
11. **DiMasi, D. R., J. C. White, C. A. Schnaitman, and C. Bradbeer.** 1973. Transport of vitamin B_{12} in *Escherichia coli:* common receptor sites for vitamin B_{12} and the E colicins on the outer membrane of the cell envelope. J. Bacteriol. 115:506–513.
12. **Frost, G. E., and H. Rosenberg.** 1975. Relationship between the *tonB* locus and iron transport in *Esche-*

richia coli. J. Bacteriol. 124:704–712.

13. **Hancock, R. E. W., and V. Braun.** 1976. Nature of the energy requirement for the irreversible adsorption of bacteriophages T1 and ϕ80 to *Escherichia coli*. J. Bacteriol. 125:409–415.
14. **Hantke, K., and V. Braun.** 1975. Membrane receptor dependent iron transport in *Escherichia coli*. FEBS Lett. 49:301–305.
15. **Jasper, P. E., E. Whitney, and S. Silver.** 1972. Genetic locus determining resistance to phage BF23 and colicins E_1, E_2 and E_3 in *Escherichia coli*. Genet. Res. 19:305–312.
16. **Kadner, R. J., and P. J. Bassford, Jr.** 1977. Relation of cell growth and colicin tolerance to vitamin B_{12} uptake in *Escherichia coli*. J. Bacteriol. 129:254–264.
17. **Kadner, R. J., and G. L. Liggins.** 1973. Transport of vitamin B_{12} in *Escherichia coli:* genetic studies. J. Bacteriol. 115:514–521.
18. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Oeschger, M. P., and S. L. Woods.** 1976. A temperature-sensitive suppressor enabling the manipulation of the level of individual proteins in intact cells. Cell 7:205–212.
20. **Pugsley, A. P., and P. Reeves.** 1976. Iron uptake in colicin B-resistant mutants of *Escherichia coli* K-12. J. Bacteriol. 126:1052–1062.
21. **Sabet, S. F., and C. A. Schnaitman.** 1973. Purification and properties of the colicin E3 receptor of *Escherichia coli*. J. Biol. Chem. 248:1797–1806.
22. **Timmis, K., and A. J. Hedges.** 1972. The killing of sensitive cells by colicin D. Biochim. Biophys. Acta 262:200–207.
23. **Wang, C. C., and A. Newton.** 1971. An additional step in the transport of iron defined by the *tonB* locus of *Escherichia coli*. J. Biol. Chem. 246:2147–2151.
24. **Wayne, R., K. Frick, and J. B. Neilands.** 1976. Siderophore protection against colicins M, B, V, and Ia in *Escherichia coli*. J. Bacteriol. 126:7–12.
25. **White, J. C., P. M. DiGirolamo, M. L. Fu, Y. A. Preston, and C. Bradbeer.** 1973. Transport of vitamin B_{12} in *Escherichia coli:* location and properties of the initial binding site. J. Biol. Chem. 248:3978–3986.