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Cloning and Expression of the Gene for the Vitamin B_{12} Receptor Protein in the Outer Membrane of *Escherichia coli*

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The transport of cyanocobalamin (vitamin B_{12}) in cells of *Escherichia coli* is dependent on a receptor protein (BtuB protein) located in the outer membrane. A 9.1-kilobase pair *Bam*HI fragment carrying the *btuB* gene was cloned from a specialized transducing phage into multicopy plasmids. Insertions of transposon Tn*1000* which prevented production of the receptor localized *btuB* to a 2-kilobase pair region. Further subcloning allowed isolation of this region as a 2.3-kilobase pair *Sau*3A fragment. The BtuB$^+$ plasmids were shown by maxicell analysis to encode a polypeptide with a molecular weight of 66,000 in the outer membrane. This polypeptide was missing in cells with Tn*1000* insertions in *btuB* and was reduced in amount upon growth of plasmid-bearing cells in repressing concentrations of vitamin B_{12}. Several Tn*1000* insertions outside the 5′ end of the coding region exhibited reduced production of receptor. A deletion at the 3′ end of *btuB* resulted in formation of an altered receptor. Amplified production of this polypeptide was associated with increased levels of binding of the receptor's ligands (vitamin B_{12} and phage BF23), increased rates of vitamin B_{12} uptake, and altered susceptibility to the group E colicins. Deficiency in various major outer membrane proteins did not affect production of the *btuB* product, and the amplified levels of this protein partially reversed the tolerance to E colicins seen in these mutants.

Vitamin B_{12} (cyanocobalamin) and ferri-siderophore complexes are taken up by *Escherichia coli* cells by means of a group of unusual transport systems which are dependent on specific outer membrane receptor proteins and on the TonB and ExbB functions (2, 24, 26). The vitamin B_{12} receptor is coded by the *btuB* gene, located at 89.6 min on the *E. coli* genetic map (1, 6, 11, 16). This protein is also employed as the receptor for phage BF23, the E colicins, and, in conjunction with OmpF porin, colicin A (6, 7). The lethal action of these agents and their binding to cells is competitively blocked by vitamin B_{12}, suggesting the existence of a common binding site.

The *btuB* product has been identified as an outer membrane protein with a molecular weight of approximately 60,000 which is missing from *btuB* mutants (14, 27) and present in reduced amounts after growth in the presence of vitamin B_{12} (15). The protein has been purified by several techniques. Purification is complicated by the existence of complexes between the receptor and porin proteins (14). The low level of production of the receptor (200 to 300 molecules per cell) poses another problem for biochemical investigations (11).

To initiate studies into the topology of this protein, its function in the transport process, and its possible interaction with the *tonB* product, the *btuB* gene was cloned and its expression was determined. The genetic selection for acquisition of plasmids carrying *btuB*$^+$ made use of the fact that *metE* mutants require either methionine or vitamin B_{12}, owing to the absence of the vitamin B_{12}-independent homocysteine methyltransferase (10). Whereas *metE btuB*$^+$ strains grow well at vitamin B_{12} concentrations as low as 10^{-11} M, strains deficient in *btuB* or *tonB* or both respond only at vitamin B_{12} concentrations greater than 10^{-6} M (3). The *btuB* region was cloned from the specialized transducing phage, λ d*argECBH13*, which has been isolated by Mazaitis et al. (21) and carries the chromosomal genes *ppc*, *argECBH*, and *btuB*. The approximate location of *btuB* on the bacterial insert in this phage has been determined by heteroduplex mapping (21). A partial restriction map of the insert has been obtained previously (23). In this report is described the cloning of the *btuB* gene from λ d*argECBH13* into plasmid vectors and the expression of the cloned gene. The accompanying paper (13) presents the nucleotide sequence of the *btuB* region.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used were derived from *E. coli* K-12 and are described in Table 1. Plasmids are shown in Fig. 1 and were derived from pBR322 and pACYC184. The bacterial strain carrying λy199 and λ d*arg*13 (21) was kindly provided by W. K. Maas. All other strains were from the stock collection at the University of Virginia Medical School.

Minimal growth medium was medium A, as described by Davis and Mingioli (10), supplemented with thiamine (1 μg/ml), glucose (0.5%), required amino acids (100 μg/ml), and vitamin B_{12} at indicated concentrations. For cloning studies, selection was for growth on minimal medium with 5 nM vitamin B_{12} in place of methionine. Rich medium was L broth. All plasmid-bearing strains were grown on medium containing the appropriate antibiotic at 25 μg/ml.

Transformations were carried out as described by Dagert and Ehrlich (8). Transformants were selected on L agar plates with the appropriate antibiotic and then replica-plated onto minimal medium with 5 nM vitamin B_{12}. The BtuB$^+$ phenotype is defined by sensitivity to BF23 and the ability of

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TABLE 1. *E. coli* K-12 strains used

Strain	Properties
MC4100	F⁻ Δ(*argF-lac*)*U169 araD139 relA1 rpsL150 flb-5301 deoC1 tonA21 thi*
CS1129	*ompR151 lac recA*/F′ ts114 *lac*⁺ *zzf*::Tn*10*
RK5173	MC4100 *ton*⁺ *gyrA219 non metE70*
RK4793	RK5173 Δ*btuB*
RK4783	RK5173 Δ*ompC zeh*::Tn*10*
RK4784	RK4793 Δ*ompC zeh*::Tn*10*
RK4785	RK5173 *ompF*::Tn*5*
RK4786	RK4793 *ompF*::Tn*5*
RK4787	RK5173 Δ*ompA zcb*::Tn*10*
RK4788	RK4793 Δ*ompA zcb*::Tn*10*
RK4791	RK5173 *ompR151 malP*::Tn*10*
RK4792	RK4793 *ompR151 malP*::Tn*10*
RK5016	RK5173 *btuB argH recA*
RK5046	RK5173 *recA*
RK5437	RK5046 Δ*tonB*/pBJM002 (pACYC184 with 1.7-kb *Hin*dII insert carrying *tonB*⁺)

a *metE* mutant to utilize 5 nM vitamin B_{12} in place of methionine for growth on minimal medium.

Cloning and analysis of plasmid DNA. Phage or plasmid DNA was digested with specified restriction endonucleases, and the resulting fragments were separated by electrophoresis in 0.9% agarose gels in Tris-borate buffer, followed by staining with ethidium bromide (20). Restriction fragments were eluted from agarose gels by freezing in the presence of phenol (4). For cloning, eluted fragments were combined and treated with T4 DNA ligase. For rapid screening, plasmids were isolated by an alkaline sodium dodecyl sulfate method (5). Larger scale preparations involved amplification with chloramphenicol or spectinomycin, lysis of spheroplasts, purification on CsCl gradients, extraction with phenol-$CHCl_3$, and precipitation with ethanol (17).

Isolation of Tn*1000* insertions. Plasmid pBJM003 was introduced by transformation into strain CS1129, carrying F′(Ts114) *lac*⁺ *zzf*::Tn*10*. Log-phase cultures of independent transformants resistant to chloramphenicol (Cml) and tetracycline (Tet) were mated with strain RK5016 (*btuB recA*) for 4 to 12 h at 30°C. Selection was for the transfer of Cml resistance, with counterselection for streptomycin resistance. Transfer of pBJM003 cointegrates occurred at 10^{-4} to 10^{-6} of the frequency of transfer of the Tet resistance of the F plasmid. Transconjugants were tested for response to BF23 and vitamin B_{12} and for the location and orientation of Tn*1000* inserts in pBJM003. These plasmids are identified by their isolation number.

Electrophoresis of proteins. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as described by Lugtenberg et al. (19). Gels were stained with Coomassie brilliant blue and dried between sheets of cellophane. Outer membrane preparations were obtained by differential solubilization in Triton X-100, as described by Hantke (12).

Radioactive labeling was carried out by the basic maxicell procedure of Sancar et al. (28), except that the host strain was RK5016 (*recA*). Strains were UV irradiated, incubated overnight with cycloserine, labeled in minimal medium with 25 μCi of L-[^{35}S]methionine for 60 min. Cells were washed twice and dissolved by boiling in sample buffer. Autoradiographs were made with Kodak XRP film.

Vitamin B_{12} transport. The rate of [^{3}H]vitamin B_{12} uptake was measured as described previously (15) with a range of cell densities to maintain proportionality of uptake rate to cell number over the time period of the assay. Activity is reported as picomoles accumulated per microliter of cell water.

Phage adsorption. The rate of adsorption of phage BF23 was determined by mixing cells in growth medium (2×10^9/ml) or membranes with phage at a multiplicity of infection of 0.1 to 0.2. Portions were removed at intervals, diluted 100-fold in L broth with $CHCl_3$, and then diluted and plated with a lawn of RK5173. The rate constant (k) for adsorption was calculated from the equation $\log(P_0/P_t) = kBt/2.3$, where P is the number of plaques (unadsorbed phage) present at time zero and time t (in minutes) and B is the number of cells.

Colicin sensitivity. Colicin susceptibility was measured in two ways. Colicinogenic strains were inoculated onto an L plate in a streak and allowed to grow overnight. These cells were killed by exposure to $CHCl_3$ vapor and then covered with a layer of 2% agar in L broth containing streptomycin (100 μg/ml). Strains to be tested were streaked perpendicular to the colicinogenic strains. Relative sensitivities were estimated from the sizes of the zones of killing (9). For the second method, 5 μl of serial dilutions of partially purified colicin preparations were spotted onto a lawn of the test strain on L agar, and the highest dilution that gave complete killing was determined.

Chemicals. Radioactive materials were obtained from Amersham Corp. Enzymes were obtained from Bethesda Research Laboratories, Inc., Boehringer-Mannheim Biochemicals, or New England Biolabs. Other chemicals were obtained from Sigma Chemical Co.

RESULTS

Cloning of the *btuB* region. The specialized transducing phage λ d*arg*13 conferred BF23 sensitivity and the ability to utilize vitamin B_{12} in lysogens of a *btuB recA* host. A *Bam*HI digest of this phage DNA was cloned into the *Bam*HI site of plasmid pACYC184. Chloramphenicol-resistant transformants of strain RK5016 (*metE btuB*) were tested for growth on 5 nM vitamin B_{12}. Restriction endonuclease analysis of the plasmid (pBJM003) present in one BtuB⁺ transformant revealed the presence of two *Bam*HI fragments in addition to the vector (Fig. 1). The 9.1-kilobase (kb) fragment was a portion of the bacterial DNA carried on the transducing phage, whereas the 6.5-kb fragment was derived from the λ DNA adjacent to the site of integration of the transducing phage. Another BtuB⁺ plasmid was mapped and also contained two *Bam*HI fragments: the 9.1-kb fragment of bacterial DNA present in pBJM003 and a 7.1-kb fragment from phage λ sequences. Attempts to obtain a plasmid carrying only the 9.1-kb fragment were unsuccessful.

The restriction map of pBJM003 was determined (Fig. 1). The approximate location of *btuB* on the 9.1-kb *Bam*HI fragment was known from the heteroduplex mapping described by Mazaitis et al. (21) and from the transposon insertion studies described below. It was not possible to reduce the size of this plasmid with retention of the BtuB⁺ characteristic by using restriction sites (cloning of the 2.9-kb *Hin*dIII fragment or deletion between the *Pst*I sites); sequence analysis (13) has shown that both of these sites lie within the structural gene.

The *btuB* gene was obtained by partial digestion of pBJM003 with *Sau*3A followed by ligation into the *Bam*HI site of pBR322 and selection for complementation of *btuB* strain RK5016. The restriction maps of nine BtuB⁺ plasmids were determined (Fig. 1). All plasmids contained overlapping inserts that ranged in size from 2.2 to 2.8 kb and were

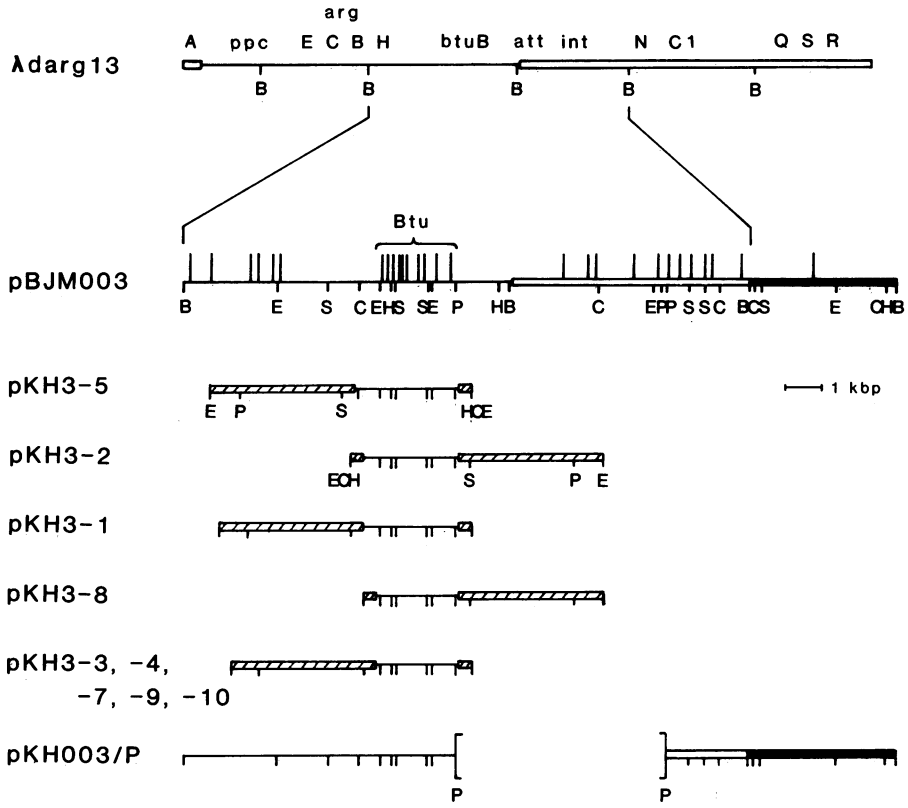

FIG. 1. Cloning of the *btuB* region and description of the plasmids employed in this study. The top line shows the approximate location of the bacterial and viral genes, in relation to the *Bam*HI cleavage sites, on the specialized transducing phage λ d*argECBH13*. Bacterial DNA is represented by a single line, and phage DNA is represented by a double line. The plasmids derived from λ darg13 are portrayed below the phage and are oriented relative to pBJM003. The vertical bars above the map of pBJM003 designate the location of Tn*1000* insertions in that plasmid. Those insertions enclosed by the bracket designated Btu are unable to complement a *btuB* recipient for growth on 5 nM vitamin B_{12}. Insertions outside the bracket remained Btu^+. Below each map are shown the location of cleavage sites for the following enzymes: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I. The solid regions represent pACYC184, and the striped regions are pBR322. The pKH3 series of plasmids were generated by insertion of pBJM003 DNA that was partially digested with *Sau*3A into the *Bam*HI site of pBR322, followed by selection for Btu^+. Plasmid pKH003/P was generated by digestion of pBJM003 with *Pst*I. Kbp, kilobase pair.

present in both orientations in the vector. Relative to the orientation of the restriction map shown in Fig. 1, all nine inserts ended at the same *Sau*3A site on the right side, located 100 base pairs from the *Pst*I site. There were three different left-end joints, one just before an *Eco*RI site (pKH3-3, -4, -7, -8, -9, and -10), one just past the *Cla*I site (pKH3-1 and -2), and one before the *Cla*I site (pKH3-5). This narrow size distribution of *Sau*3A fragments yielding a $BtuB^+$ phenotype suggested that disruption of the sequences flanking *btuB* prevented maintenance of that plasmid.

Isolation of Tn*1000* insertions. To define the regions on plasmid pBJM003 affecting expression of the vitamin B_{12} receptor, insertions of transposon Tn*1000* (the γδ segment of the F plasmid) were obtained by selection for conjugal transfer of pBJM003-F′ *lac* cointegrates. Potential insertions were characterized by restriction mapping and the ability to restore vitamin B_{12} utilization or BF23 sensitivity to a *btuB recA* recipient (Fig. 1). All insertions that prevented complementation for growth on vitamin B_{12} were located in the 2-kb segment between the *Eco*RI and *Pst*I sites that was defined as the *btuB* region in the subcloning experiments described above. All of those insertions which lay between the *Hin*dIII and *Pst*I sites, i.e., within the *btuB* coding sequence, prevented those plasmids from conferring BF23 sensitivity. In contrast, two insertions, located approximately 100 and 300 base pairs to the left of the *Hin*dIII site, outside the coding region for the receptor, still conferred phage sensitivity. Although these insertions prevented utilization of 5 nM vitamin B_{12} for growth, vitamin B_{12} was still effective at inhibiting BF23 infection, indicating that the receptor produced in these mutants was still able to bind vitamin B_{12}. It is likely that these insertions disrupted the promoter or other regulatory regions, leading to reduced expression of the receptor. This suggests that the direction of transcription is rightward, from the *Hin*dIII site toward the *Pst*I site.

All Tn*1000* insertions located outside the 2-kb *btuB* region conferred a $BtuB^+$ phenotype identical to that of the parental plasmid. However, the distribution of these insertions was unusual. They were randomly distributed throughout the insert, except that none were found in the 2-kb region to the left of *btuB* or the 3-kb region to its right. As in the subcloning studies, this result suggests that disruption of the sequences flanking *btuB* prevents maintenance of those plasmids.

Production of receptor protein. The effect of various BtuB plasmids on the outer membrane protein profile and on the production of labeled polypeptides in maxicells was determined. The $BtuB^+$ plasmids L23D (pBJM003 with a Tn*1000* insertion outside *btuB*), pKH3-3, pKH3-8, and pBJM003

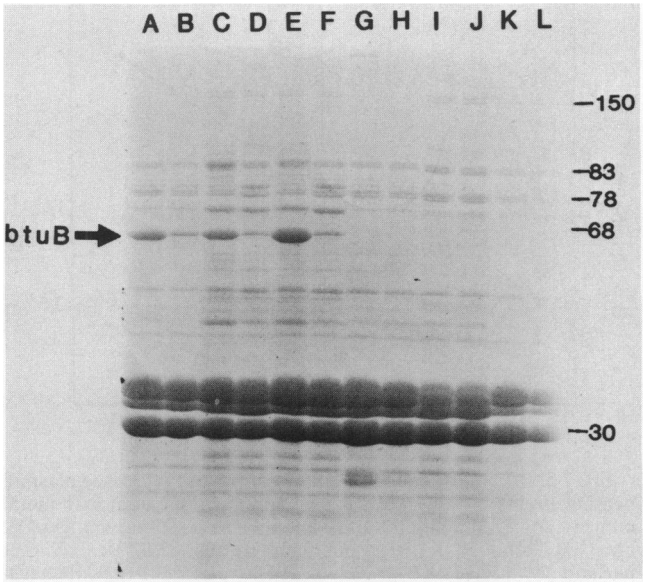

FIG. 2. Outer membrane proteins of strains with BtuB plasmids. All strains were RK5016 carrying the indicated plasmids and were grown in minimal medium with methionine (MET) or 5 μM vitamin B_{12} (B_{12}). Lane A, pBJM003-L23D::Tn*1000*, MET; lane B, pBJM003-L23D::Tn*1000*, B_{12}; lane C, pKH3-3, MET; lane D, pKH3-3, B_{12}; lane E, pKH3-8, MET; lane F, pKH3-8, B_{12}; lane G, pBJM003-M57C (*btuB*::Tn*1000*), MET; lane H, pBJM003-L1B (*btuB*::Tn*1000*), MET; lane I, pBJM003-N43D (*btuB*::Tn*1000*), MET; lane J, pBJM003-L34D (*btuB*::Tn*1000*), MET; lane K, pKH003/P, B_{12}; lane L, pKH003/P, MET. Outer membranes were prepared as the material that was insoluble in Triton X-100.

(data not shown) caused marked elevation in production of an outer membrane polypeptide with a molecular weight of 66,000 (Fig. 2, lanes A, C, E). In all strains, the level of this polypeptide was reduced when the cells were grown in medium containing 5 μM vitamin B_{12} (Fig. 2, lanes B, D, and F). Densitometer scans of the BtuB region of the gel in Fig. 2 allowed quantitation of the amount of receptor protein relative to the adjacent polypeptide bands. The decrease in BtuB resulting from vitamin B_{12} repression was a factor of 3.4 for plasmids L23D and pKH3-3 and a factor of 8.0 for plasmid pKH3-8. Repression by vitamin B_{12} was seen even with plasmids carrying the smallest *btuB*$^+$ insert and with the insert in either orientation, indicating that *btuB* and its control region is carried intact on this insert. This polypeptide was missing from the outer membrane of cells carrying plasmids with Tn*1000* insertions in *btuB* (Fig. 2, lanes G, H, I, and J).

The polypeptides encoded by these plasmids were identified in a maxicell system (Fig. 3). The polypeptide with a molecular weight of 66,000 was specifically labeled in maxicells carrying the BtuB$^+$ plasmid pKH3-8 (lane B). Plasmid pBJM003 and its derivatives carrying Tn*1000* insertions encoded several additional polypeptides, reflecting the larger size of their insert. The polypeptide with a molecular weight of 66,000 was encoded by pBJM003 (data not shown) but was missing when the plasmid carried Tn*1000* insertions in *btuB* (lanes D, E, and F). No truncated polypeptides were seen with [^{35}S]methionine labeling. Trace amounts of the BtuB polypeptide (not visible in Fig. 3) were synthesized in response to plasmids with Tn*1000* insertions in the *btuB* control regions (plasmids N91D and L1B; lanes G, H). Thus, the BtuB$^+$ plasmids carry intact the gene for the outer membrane vitamin B_{12} receptor.

Plasmid pKH003/P was generated by deletion of the region between the *Pst*I sites in pBJM003 (Fig. 1). This plasmid conferred BF23 sensitivity but not vitamin B_{12} utilization. In addition, vitamin B_{12} did not protect cells against killing by BF23, suggesting that the vitamin B_{12}-binding site was affected. Outer membranes of cells carrying this plasmid contained, at most, trace amounts of a polypeptide, with a mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis near that of BtuB (Fig. 2, lanes K and L). In maxicells, this plasmid encodes a polypeptide with a slightly higher molecular weight than that of the wild-type receptor (Fig. 3, lanes A and B; Fig. 4, lanes B and C). The lanes for the deletion plasmid were overloaded with sample to allow visualization of the lowered relative amounts of the altered BtuB polypeptide. There was a small amount of a polypeptide with the same mobility as the wild-type receptor. The fact that deletion from the *Pst*I site led to formation of a product with altered electrophoretic mobility showed that the *Pst*I site lies within *btuB*. These results provide a preliminary indication that the carboxyl terminus of BtuB might be involved in both vitamin B_{12} binding and the processing of this protein during export to the outer membrane.

Vitamin B_{12} transport. The vitamin B_{12} uptake activity of cells carrying BtuB plasmids and grown in the absence or presence of 5 nM vitamin B_{12} was determined (Fig. 5). The presence of plasmid pKH3-5 resulted in nearly a fivefold elevation in the rate of energy-dependent uptake in both normal and repressed cultures, relative to that of a haploid *btuB*$^+$ strain. There was a 20-fold elevation in vitamin B_{12} binding to isolated outer membranes (C. Bradbeer, personal communication). Uptake activities of both the haploid and plasmid-bearing strains were repressed 80 to 90% by growth in 5 nM vitamin B_{12}.

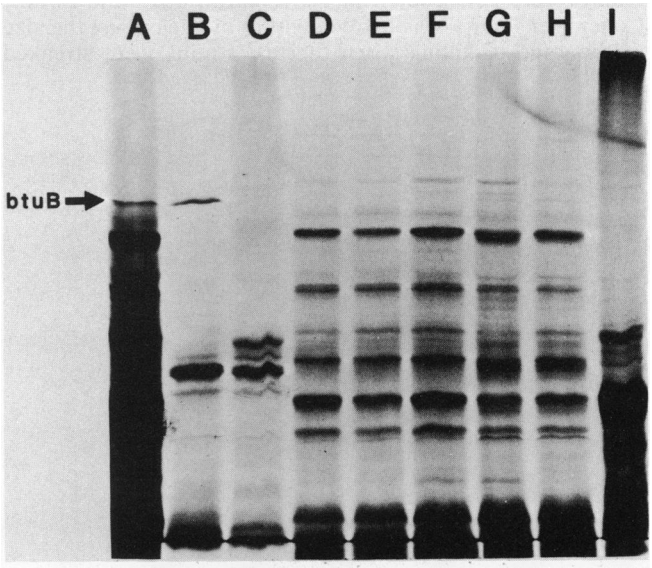

FIG. 3. Plasmid-directed synthesis of polypeptides in a maxicell system. Strains were derivatives of RK5016 carrying the following plasmids: lane A, pKH003/P; lane B, pKH3-8; lane C, pBR322; lane D, pBJM003-L34D (*btuB*::Tn*1000*); lane E, pBJM003-N43D (*btuB*::Tn*1000*); lane F, pBJM003-L96D (*btuB*::Tn*1000*); lane G, pBJM003-N91D (*btuB*::Tn*1000*); lane H, pBJM003-L1B (*btuB*::Tn*1000*); lane I, pACYC184. Maxicells were labeled with [^{135}S]methionine. Only a portion of the autoradiograph is shown.

A deletion of most of *btuB* was constructed by the removal of the *Hin*dIII fragment of pKH3-5, resulting in a plasmid that carried only the *btuB* 5′ control region and the first five amino acids of the putative signal peptide (13). It was anticipated that multiple copies of the control region might alter the regulatory behavior of the chromosomal *btuB*$^+$ allele. In fact, the presence of this plasmid had no significant effect on the rate of vitamin B_{12} uptake or on its repressibility by vitamin B_{12} (Fig. 5).

Since the amplification in vitamin B_{12} uptake activity was not strictly proportional to gene dosage, the possibility that the TonB function might be limiting was examined. Strain RK5437 contains a plasmid carrying the *tonB* gene cloned, as a 1.7-kb *Hin*dII fragment, in pACYC177. Relative to a haploid strain, this strain had normal levels of vitamin B_{12} binding activity (data not shown), but there was a 60 to 70% reduction in the vitamin B_{12} uptake rate in either normal or repressed cultures (Fig. 5). When the cloned *btuB*$^+$ gene was also present on a compatible high-copy-number plasmid (pKH3-5), cells grown in minimal medium with methionine exhibited vitamin B_{12} uptake rates that were equal to those in strains with the BtuB$^+$ plasmid alone. Thus, it appears that the haploid level of the TonB function is sufficient to allow the increased vitamin B_{12} uptake that occurs upon amplification of the vitamin B_{12} receptor. When the strain with both plasmids was grown in 5 nM vitamin B_{12} to repress receptor synthesis, vitamin B_{12} uptake activity was only 40% of that in the strain carrying only the BtuB$^+$ plasmid. These results indicate that overproduction of the *tonB* protein results in reduced TonB function, which can be relieved by overproduction of at least this *tonB*-dependent receptor.

Response to the E colicins and BF23. *E. coli* mutants defective in the production of major outer membrane proteins exhibit decreased susceptibility to colicins (25). The effect of overproduction of the vitamin B_{12} receptor on colicin tolerance was examined in isogenetic strains deficient in specific major outer membrane proteins (Table 2). The strains were constructed in pairs, both *btuB*$^+$ and Δ*btuB*. Colicin sensitivity was assayed either by measuring the size of the zone of killing when the test strains were streaked perpendicular to colicinogenic strains or by spotting serial dilutions of colicin preparations onto a lawn of the test strains and determining the last dilution to produce killing.

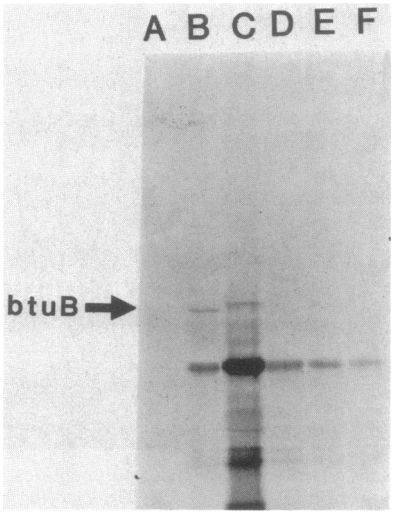

FIG. 4. Synthesis of [^{35}S]methionine-labeled polypeptides in maxicell system with plasmids pBR322 (lane A), pBJM003 (lane B), pKH003/P (lane C), pBJM003-N91D (lane D), pBJM003-L1B (lane E), and pBJM003-M57C (lane F). Only a portion of the autoradiograph is shown.

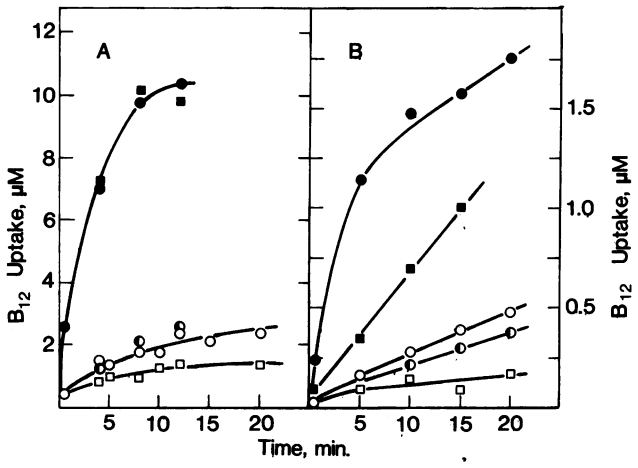

FIG. 5. Uptake of vitamin B_{12} into normal and repressed plasmid-bearing strains. Strains were grown in minimal medium with methionine (A) or 5 nM vitamin B_{12} (B) and then assayed for uptake of 3.5 nM [^{3}H]vitamin B_{12}. The strains were RK5046 (*btu*$^+$) carrying no plasmid (○), pKH3-5 (●), pKH35-H1 (◐), pBJM002 (pTonB$^+$) (□), or both pKH3-5 (pBtuB$^+$) and pBJM002 (pTonB$^+$) (■). Plasmid pKH35-H1 is pKH3-5-deleted between the *Hin*dIII sites. The strains with the pTonB$^+$ plasmid were also *tonB* on the chromosome; otherwise they were isogenic to RK5046. Uptake is in picomoles of vitamin B_{12} accumulated per microliter of cell water (i.e., micromolar).

The presence of pKH3-5 caused no change in the response to colicins in the *btuB*$^+$ strain RK5173. The Δ*btuB* strain RK4793 was fully resistant to the E colicins and had greatly decreased sensitivity to colicin A (7, 9, 25). Plasmid pKH3-5 restored almost full sensitivity to all of these colicins. In this and the other Δ*btuB*/p*btuB*$^+$ strains, numerous resistant colonies arose in the zone of killing, even on plates containing ampicillin to select for maintenance of the plasmid.

The deletion of *ompC*, encoding one of the two porins normally present in *E. coli* K-12, had no effect on susceptibility to the colicins tested or on the expression of *btuB* on the plasmid. Loss of the OmpF porin by either *ompF*::Tn*5* or the polar *ompR101* mutations did affect the response to some colicins. These strains were fully resistant to colicins A, K, and L and had decreased sensitivity to colicin E3. The resistance to colicin A was partially reversed by the presence of the BtuB$^+$ plasmid; this reversal was much more prominent in the *ompR* strains than in the *ompF*::Tn*5* strains. The plasmid did not affect the tolerance to colicin E3 seen in these strains. Loss of OmpA (in Δ*ompA* strains) did not affect susceptibility to colicins A or E but did result in resistance to colicins K and L. All of the strains remained equally sensitive to colicin E1. The insensitivity to colicins K and L of the *ompF*, *ompA*, or *ompR* strains was not affected by the presence of the BtuB$^+$ plasmid. Thus, the tolerance to colicins A and E3 of *ompF* or *ompR* strains can be at least partially overcome by amplified production of BtuB protein.

The rates of adsorption of phage BF23 to whole cells of these strains were determined. The rate constant for adsorption was three times higher in strains carrying pKH3-5 than in their haploid parents ($k = 3 \times 10^{-9}$ cell^{-1} min^{-1} for RK4793 [*btuB*]/pKH3-5 and 9.5×10^{-10} for RK5173 [*btuB*$^+$]).

TABLE 2. Effect of host genotype and *btuB* plasmid on colicin susceptibility

Strain	Genotype		Plasmid pKH3-5	Response to the following colicins[a]:					
	omp	*btuB*		E1	E2	E3[b]	A[b]	K	L
RK5173	+	+	−	S	S	S (4×10^5)	S (2×10^6)	S	S
			+	S	S	S (4×10^5)	S (2×10^6)	S	S
RK4793	+	Δ	−	R	R	R (<1)	r (6×10^2)	S	S
			+	S	S	S (8×10^4)	S (2×10^6)	S	S
RK4783	Δ*ompC*	+	−	S	S	S	S	S	S
			+	S	S	S	S	S	S
RK4784	Δ*ompC*	Δ	−	R	R	R	r	S	S
			+	S	S	S	S	S	S
RK4785	*ompF*::Tn5	+	−	S	S	r (8×10^4)	R (<5)	r	R
			+	S	S	P (8×10^4)	r (6×10^2)	r	R
RK4786	*ompF*::Tn5	Δ	−	R	R	R (<1)	R (<5)	r	R
			+	r	r	P (1×10^4)	r (6×10^2)	r	R
RK4787	Δ*ompA*	+	−	S	S	S (4×10^5)		R	R
			+	S	S	S (8×10^4)	S (2×10^6)	R	R
RK4788	Δ*ompA*	Δ	−	R	R	R (<5)	r (6×10^2)	R	R
			+	S	P	S (8×10^4)	S (2×10^6)	R	R
RK4791	*ompR151*	+	−	S	P	P (8×10^4)	R (25)	R	R
			+	S	P	P (8×10^4)	P (8×10^4)	R	R
RK4792	*ompR151*	Δ	−	R	R	R (<1)	R 5	R	R
			+	S	P	P (1×10^4)	P (1×10^4)	R	R

[a] Colicin susceptibility was determined by measurement of the size of the inhibition zone when the strain was streaked perpendicular to the indicated colicinogenic strains. The results are presented as S, inhibition zone at least 50% that of RK5173; P, zone was 10 to 40% that of RK5173; r, some killing in immediate vicinity of colicin-producing strain; R, no detectable killing. Many resistant variants were observed for the plasmid-bearing Δ*btuB* strains.

[b] Serial dilutions of colicin A or E3 were spotted onto a lawn of the test strain. The titer (in parentheses) is the reciprocal of the last dilution giving complete killing.

The same threefold increment was seen in pairs of strains carrying the *ompF*, *ompA*, or *ompR* mutations described above.

DISCUSSION

This and the accompanying paper (13) describe the structure and expression of the *btuB* gene, which encodes the minor outer membrane protein responsible for the initial steps of entry of vitamin B_{12}, phage BF23, and the E colicins. There is considerable evidence that the cloned gene is *btuB*. The plasmid was derived from a transducing phage known to carry chromsomal loci adjacent to *btuB*. Both the phage and the Btu^+ plasmids derived from it complemented all *btuB* functions. The cloned fragment encoded an outer membrane protein with a molecular weight of 66,000 which was missing if the *btuB* region was disrupted by insertion of transposon Tn*1000*. Production of this polypeptide was repressed by growth in the presence of vitamin B_{12}. The high copy number of the plasmids was associated with elevated adsorption of phage BF23 and increased binding and transport of vitamin B_{12}. As described in the accompanying paper (13), the composition of this polypeptide determined from the DNA sequence agreed very well with that determined by amino acid analysis of the purified receptor (14).

Analysis of the Tn*1000* insertions did not allow determination of the direction of transcription of *btuB* because no truncated polypeptides were observed. Since the mature polypeptide contains only two methionines (13), truncated polypeptides would be difficult to detect by the method employed. Results in this report led to the postulation of a rightward direction of transcription. Deletion at the *Pst*I site on the right end of the fragment led to formation of a nearly normal-sized polypeptide with altered binding properties. Insertions at the left end of the fragment appeared to produce a normal receptor, but in greatly decreased amounts, as if the left end harbored the transcription initiation site. The sequencing results confirmed this proposal (13).

The cloning experiments pointed to a requirement for the integrity of the sequences flanking *btuB*. No Tn*1000* insertions were recovered in the 2 kb to the left and the 3 kb to the right of *btuB*, although they were randomly distributed throughout the *btuB* gene and elsewhere on the plasmid. Perhaps disruption of these regions, either by transposon insertion or by subcloning, alters gene expression from the plasmid or host such that these plasmids cannot be maintained or cells carrying them do not survive.

The nature of the regulation of *btuB* by repression is not understood, and no regulatory gene has been identified. Repression still occurred in cells carrying multiple copies of the intact *btuB* gene or the 5′ regulatory region alone. This fact suggests that the putative repressor is produced in appreciable amounts and is not titrated out by multiple copies of its target. It was noted that the repression ratios were different for the two orientations of the smallest $BtuB^+$ fragment in pBR322. The repressed level of expression from both plasmids was the same, and it was the derepressed level that was two to three times higher in pKH3-8. The insert in this plasmid is oriented so that transcription of *btuB* could initiate from the *tet* promoter of the vector as well as the *btuB* promoter.

The reason that mutants lacking certain outer membrane proteins are tolerant to certain group A colicins remains unclear. The availability of the cloned *btuB* gene may prove to be useful in studies of this phenomenon. Some possibilities are that the major outer membrane protein, the loss of which results in tolerance, serves as a colicin receptor or

plays a necessary role in a step of colicin uptake subsequent to the binding of colicin to the *btuB* protein. Perhaps the major outer membrane protein serves in a more indirect manner to maintain the receptor in an orientation or location necessary for colicin entry. The presence of elevated levels of vitamin B_{12} receptor may circumvent the need for the outer membrane protein in the latter case. The experiments reported here, indicating decreased tolerance in strains with elevated levels of receptor, favor this view and will be extended.

Another observation made here and worthy of continued investigation concerns whether all of the receptors are functional. Densitometric analysis of stained electropherograms of outer membrane proteins showed that the amount of vitamin B_{12} receptor was elevated 20- to 30-fold over the haploid level of 200 to 300 molecules per cell. Preliminary studies indicate that the increase in BF23-inactivating activity of outer membrane preparations of plasmid-bearing cells was also in this range. However, phage adsorption and vitamin B_{12} transport in whole cells were only amplified three to sevenfold. A similar apparent crypticity of phage receptors has been reported for the case of T5 binding to cells with the cloned *fhuA* (*tonA*) gene (22). It remains to be seen whether this phenomenon reflects improper insertion of the receptors to the membrane or their interaction (e.g., aggregation) such that only a few receptors are active or exposed at the surface.

Perhaps the most intriguing observation concerned the properties of the altered receptor encoded by the plasmid deleted from the *Pst*I site within the coding region. The C-terminal 12 amino acids of the wild-type receptor should be affected by this deletion (13), based on the sequence of *btuB* and the λ DNA in which the other endpoint occurs. However, the sequence around the deletion site has not yet been determined, so the following statements must be considered tentative. In maxicells, an altered polypeptide was produced in decreased amounts relative to the cloned $btuB^+$ gene, perhaps as a result of the increased lability of mRNA owing to the loss of the normal termination sites. However, the decrease in amount of this polypeptide in the outer membrane was far more drastic than the decrease in production seen in maxicells. Perhaps the altered polypeptide is unstable in the membrane or its export has been hampered. It is very suggestive that the polypeptide seen in maxicells has a mobility expected of a protein approximately 2000 daltons larger than the wild-type mature receptor and that traces of a molecule with the mobility of the wild-type protein are seen, as if the efficiency of removal of the signal sequence is impaired. It will be determined whether the higher-molecular-weight species is the unprocessed form retaining the leader peptide. If this is the case, this protein would join β-lactamase as a secreted protein in which the intact carboxyl terminus appears to be necessary for proper processing and export (18).

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