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Identification of the BtuCED Polypeptides and Evidence for Their Role in Vitamin B_{12} Transport in *Escherichia coli*

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Passage of vitamin B_{12} across the outer and cytoplasmic membranes of *Escherichia coli* occurs in two steps, each involving independent transport systems. Since the vitamin accumulated in *btuC* or *btuD* mutants is readily released from the cell by chase or osmotic shock and does not undergo the usual metabolic conversions, the products of these genes might participate in transport across the cytoplasmic membrane. Mutations in *btuC* and *btuD* are complemented by recombinant plasmids carrying a 3,410-base-pair *Hin*dIII-*Hin*cII DNA fragment. Transposon Tn*1000* mutagenesis and subcloning defined the location of these two genes and showed that they are separated by approximately 800 base pairs. The polypeptides elicited by this fragment and its derivatives were identified by using a maxicell system. The apparent molecular weight of the *btuC* product was approximately 26,000, that of the *btuD* product was 29,000. Both polypeptides were associated with the cell membrane. Transposon insertions in the region between *btuC* and *btuD*, as well as those in the two genes, conferred a deficiency in vitamin B_{12} utilization and transport when they were crossed onto the chromosome. This region, termed *btuE*, encoded a 22,000-M_r polypeptide and lesser amounts of a 20,000-M_r species. A portion of the BtuE protein was released from maxicells by osmotic shock or spheroplast formation. The relative production of BtuE and BtuD in response to plasmids carrying transposon insertions suggested that the three genes are arranged in an operon in the order *btuC-btuE-btuD* and that internal promoters exist since polarity was incomplete. Substantial elevation of transport activity was engendered by plasmids carrying the intact *btu* region, but not when any of the *btu* genes was disrupted. The *btuCED* region thus may encode a transport system for passage of vitamin B_{12} across the cytoplasmic membrane. This system bears similarities to periplasmic binding protein-dependent transport systems, although the putative periplasmic component is not required for its function.

Several genetic loci in *Escherichia coli* are required for the high-affinity and irreversible uptake of vitamin B_{12} (cyanocobalamin) across the outer and cytoplasmic membranes (4). The *btuB* gene encodes the 66,000-M_r outer membrane receptor protein for cobalamin and cobinamide, the E colicins, and bacteriophage BF23 (6, 9, 15, 18). The *tonB* product is necessary for the energy-dependent transfer of cobalamin from the receptor into the periplasm (3, 25). This protein is also required for siderophore-mediated iron uptake (reviewed in reference 22). These iron uptake systems also employ specific receptors in the outer membrane as well as other gene products that mediate transport steps after receptor binding (10, 24, 29).

The *btuCD* locus, located next to *himA* at min 37.4 on the *E. coli* chromosome map (2), is required for normal vitamin B_{12} transport. Mutants defective in *btuC* do show energy-dependent uptake of vitamin B_{12}, but to a lower steady-state level than that achieved by *btuC*$^+$ cells (4). The substrate accumulated in these mutants is not appreciably converted into the usual intracellular metabolic species and is chased from the cell by the addition of excess nonradioactive substrate or EDTA or by osmotic shock (25). Reynolds et al. (25) concluded from these results that *btuC* mutants are capable of energy-dependent accumulation of vitamin B_{12} into the periplasm, but are defective in translocation of substrate into the cytoplasm.

Mutations in *btuD* confer a less extreme deficiency in vitamin B_{12} utilization than do *btuC* mutations, although transport was strongly impaired (8). The *btuCD* region was cloned from the chromosome as a 3.4-kb *Hin*dIII-*Hin*cII fragment. Multicopy plasmids carrying this fragment complemented the available *btuC* and *btuD* mutations and conferred elevated rates of vitamin B_{12} uptake. The coding regions for these two genes were roughly defined from the effect of transposon Tn*1000* insertions on complementation properties of derivative plasmids. These studies indicated that *btuC* and *btuD* are expressed independently and are separated by approximately 800 base pairs, which is shown here to contain the *btuE* gene.

We describe here the identification of the polypeptides encoded by this region expressed in a maxicell system. The gene-polypeptide assignment was determined from the coding capacity of the Tn*1000* insertion mutations. Fractionation of maxicells suggested that BtuC and BtuD are membrane associated and that BtuE is periplasmic. Finally, the transposon insertion mutations were crossed onto the bacterial chromosome to allow a demonstration of the role of each coding region in vitamin B_{12} transport activity. The 17-kilodalton product of the gene adjacent to *btuCED* is not involved in vitamin B_{12} transport or regulation.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this study are listed in Table 1. The construction of the plasmids and the isolation, restriction mapping, and complementation properties of the transposon Tn*1000* insertions

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TABLE 1. Strains of *E. coli*

Strain	Genotype
SE5000	F⁻ *araD* Δ(*argF-lac*)*205 flbB ptsF relA rpsL deoC recA*
RK4373	*araD139* Δ(*argF-lac*)*U169 relA1 rpsL150 flbB5301 deoC1 thi non gyrA metE70*
RK4446	RK4373 Δ(*tonB-trp*) *btuB btuC456 recA zdh-600*::Tn*5*
RK5720.5	*trpA46 lysA xyl glyS polA1 argH metE zdh-3*::Tn*10*
RK6563	RK4373 Δ(*btuD-zdh3*)*478*
RK6680	RK4373 *btuE480*::Tn*1000* Kan *zdh-3*::Tn*10*
RK6683	RK4373 *btuC480*::Tn*1000 recA*
RK6686	RK4373 *btuC480*::Tn*1000* Δ*btuB zdh-3*::Tn*10*
RK6687	RK4373 *btuC480*::Tn*1000 zdh-3*::Tn*10*
RK6688	RK4373 *btuD481*::Tn*1000* Δ*btuB zdh-3*::Tn*10*
RK6689	RK4373 *btuD481*::Tn*1000 zdh-3*::Tn*10*
RK6690	RK4373 *btuE482*::Tn*1000* Δ*btuB zdh-3*::Tn*10*
RK6691	RK4373 *btuE482*::Tn*1000 zdh-3*::Tn*10*

were previously described (8). The material carried on the plasmids and the location of the Tn*1000* insertions are described in the legend to Fig. 1. Plasmid pLCD31 uses pACYC184 as a vector, and the other plasmids employ pBR322.

The neomycin and kanamycin resistance determinant, *neo*, was inserted into the Tn*1000* insertion 23 in *btuE* by replacement of the *Xho*I fragment in Tn*1000* with the appropriate *Xho*I fragment from transposon Tn*5*; this generated the *btuE*::Tn*1000* allele. Plasmid constructions employed conditions for restriction endonucleases and T4 DNA ligase as recommended by their manufacturers (20).

Growth media and materials. The minimal salts media were A of Davis and Mingioli and M9; the rich medium was L broth (21). All plasmid-bearing strains were grown in the presence of the appropriate antibiotic at 25 μg/ml.

[^{35}S]methionine was purchased from New England Nuclear Corp. Other chemicals were from Sigma Chemical Co.

Maxicell labeling. The basic protocol of Sancar et al. (26) was employed for the radioactive labeling of plasmid-coded proteins. Various *recA* host strains were used, with little difference in the results. Cells were grown in M9 salts medium supplemented with 0.4% glucose, thiamine (1 μg/ml), methionine and required amino acids (100 μg/ml), 10 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.5% Casamino Acids (Difco Laboratories), and the appropriate antibiotic. Logarithmically growing cells at 2×10^8/ml were irradiated in 5-ml portions for 16 s by a germicidal lamp at 360 μW/cm^2. Irradiated cells were incubated in the dark for 1 h at 37°C and then overnight after the addition of cycloserine to 200 μg/ml. Maxicells were then washed twice with M9 salts medium and suspended in labeling medium (growth medium minus methionine and Casamino Acids). After incubation for 1 h to deplete the methionine pools, [^{35}S]methionine was added to 25 to 30 μC/ml. After incubation for 30 to 60 min at 37°C, the labeled maxicells were washed twice by centrifugation and suspension in 10 mM Tris hydrochloride (pH 7.4) before solubilization or fractionation and analysis by polyacrylamide gel electrophoresis. The amounts of sample applied corresponded to equivalent amounts of cell extract. In most cases, expression of the *btu* genes was low, so that relatively large volumes of extract had to be applied, with consequent overexposure of labeled vector polypeptides and their degradation products.

Proteins were electrophoresed in 11.5 or 14% polyacrylamide gels with a modified Laemmli Tris-glycine hydrochloride buffer system (19). Resolved gels were stained with Coomassie blue, fixed, and dried on Whatmann 3MM filter paper. Distribution of radioactive polypeptides was determined by autoradiography at −70°C for 1 to 7 days with Kodak XRP film and Cronex enhancers.

Fractionation of maxicells. For osmotic shock, washed maxicells in 0.2 volume of 10 mM Tris hydrochloride (pH 7.0)–20% sucrose were incubated for 10 min at room temperature, collected by centrifugation at 4°C, and quickly suspended in an equal volume of cold water. Released proteins were precipitated with 5% trichloroacetic acid and then boiled in electrophoresis sample buffer.

Spheroplasts were prepared as described by Osborn et al. (23). Proteins released after lysozyme treatment were collected by precipitation by trichloroacetic acid. Spheroplasts were lysed by brief sonication, and the cellular membranes were separated from cytoplasmic constituents by centrifugation in an SW50.1 rotor for 2 h at 150,000 × *g*. The

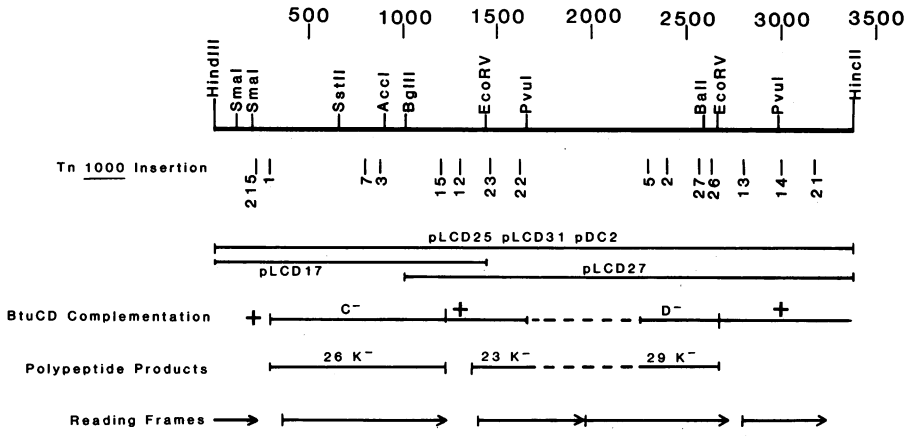

FIG. 1. Restriction map of the 3.4-kilobase *btuCED* region. The coordinates at the top of the figure are in base pairs from the *Hin*dIII site. The approximate locations of the Tn*1000* insertions are designated by their isolation number. In the text, the plasmid carrying insertion 1, for example, is described as pLCD31-1. The material carried on plasmid subclones is indicated, as are the location of the genes defined by complementation ability (8) or polypeptide production in maxicell systems. The bottom line shows the location of the open reading frames deduced from the nucleotide sequence (11).

chloroform shock method was as described by Ames et al. (1).

Crossing Tn*1000* insertions onto the chromosome. To obtain strains with chromosomal *btu*::Tn*1000* insertions, the approach of Greener and Hill (13) and Gutterson and Koshland (14) was employed. Plasmid pACYC184 derivatives carrying the *btuCED* fragment with representative Tn*1000* insertions were introduced by transformation into the *metE polA* strain RK5720.5 by selection for chloramphenicol resistance. The *polA* defect prevents plasmid replication, and maintenance of drug resistance requires integration of the plasmid into the chromosome, thereby generating a tandem duplication of the *btu* genes flanking the plasmid. Purified chloramphenicol-resistant transformants were grown for 2 days in L broth and then plated on L agar plates to give about 300 colonies per plate. Replica plating onto L and L-chloramphenicol plates revealed that 0.1 to 10% of the colonies were chloramphenicol susceptible as a result of homologous recombination between the tandemly duplicated *btuCED* sequences. Some of these recombination events will restore the wild-type sequence, whereas others will retain the transposon insertion. The Btu phenotype of the drug-susceptible variants was determined by the growth response on 0.5 nM vitamin B_{12} and by transport assays. In most cases, the presence of the Tn*1000* insertion in the *btu* region was verified by Southern hybridization analysis of *Eco*RV-digested chromosomal DNA, probed with labeled pLCD25 DNA (data not shown) (20).

Transport assays. The uptake of [^{3}H]cyanocobalamin was measured as previously described (25). Transport is expressed as picomoles of vitamin B_{12} accumulated per 10^9 cells.

RESULTS

Identification of BtuCED polypeptides. Figure 1 presents the restriction map of the *btuCED* region carried on plasmid pLCD25 and its derivatives, along with the approximate location of transposon Tn*1000* insertions and their complementation properties. Proteins encoded by pLCD25 and several of its subclones were synthesized in maxicells (Fig. 2). The major products of the vector pBR322 (lane a) were the tetracycline and ampicillin resistance proteins, which migrated with mobilities of 34K and 30K. Minor amounts of smaller polypeptides were apparent. Plasmid pLCD25 (BtuC$^+$ D$^+$ E$^+$) (lane b) elicited synthesis of the β-lactamase species but not the tetracycline resistance protein. The three major insert-specified polypeptides had mobilities of 29K, 26K, and 22K. Lesser amounts of 20K and 17K polypeptides were seen in some experiments.

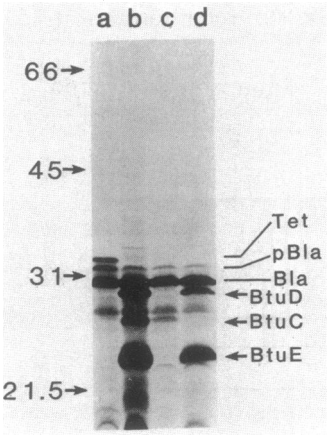

FIG. 2. Autoradiogram of ^{35}S-polypeptides synthesized in maxicells by plasmid pLCD25 and several derivatives. The host strain was SE5000, and the plasmids were as follows: a, pBR322; b, pLCD25; c, pLCD17; d, pLCD27. The mobilities of molecular weight standards are on the left, and the location of the plasmid-coded polypeptides on the right. Molecular masses are in kilodaltons; Tet, tetracycline resistance protein; pBla, the precursor of β-lactamase; Bla, β-lactamase.

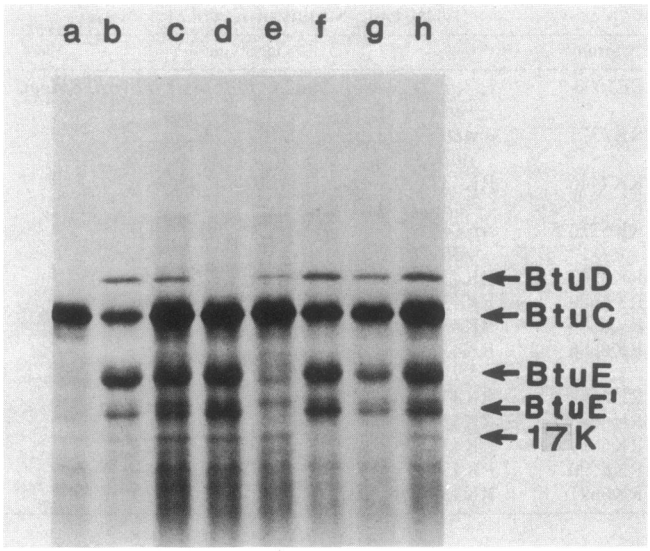

FIG. 3. Autoradiogram of ^{35}S-polypeptides produced by maxicells carrying plasmid pLCD31 and derivatives carrying transposon Tn*1000* insertions. The host strain was SE5000 and the plasmids were as follows: a, pACYC184; b, pLCD31 (C$^+$ E$^+$ D$^+$); c, pLCD31-3 (C$^-$ E$^+$ D$^+$); d, pLCD31-27 (C$^+$ E$^+$ D$^-$); e, pLCD31-22 (C$^+$ E$^-$ D$^+$); f, pLCD31-13 (17K$^-$); g, pLCD31-14 (17K$^-$); h, pLCD31-21. Vector-encoded CAT migrated at the same location as BtuC.

The 1.4-kilobase *Hin*dIII-*Eco*RV insert in pLCD17 complements *btuC* but not *btuD* (8). This plasmid elicited synthesis of β-lactamase and of the 26K polypeptide. The 2.4-kilobase *Bgl*II-*Hin*cII fragment carried on pLCD27 complements *btuD* but not *btuC*. The insert-encoded products of this plasmid were the 29K, 22K, 20K, and 17K polypeptides (Fig. 2, lanes c and d).

The identity of these polypeptides was established by examining the effect of transposon Tn*1000* insertions in plasmid pLCD31, which was pACYC184 carrying the 3.4-kilobase *Hin*dIII-*Hin*cII fragment (8). This plasmid elicited synthesis of the 29K and 22K polypeptides and low amounts of the 20K and 17K polypeptides (Fig. 3, lane b). The production of the 26K polypeptide was obscured by its migration with the vector-encoded chloramphenicol acetyltransferase (CAT).

The coding capacities of several transposon insertions were correlated with their physical location and complementation properties (Fig. 1). Insertions 3 (Fig. 3, lane c), 1, 7, and 15 did not complement *btuC* and encoded all of the polypeptides. The loss of 26K BtuC was seen on some gels as the absence of a band running slightly above CAT; on other gels, it was seen as the absence of the band in membrane fractions. These insertions also reduced the amounts of the other insert-specific products relative to CAT.

Insertions in *btuD* (isolates 27 [Fig. 3, lane d], 2, 5, and 26]

TABLE 2. Growth phenotype and complementation properties of *btu* mutants[a]

Host (phenotype) with indicated genotype[b]	Growth with plasmid				
	Haploid	pLCD31	pLCD31-1	pLCD31-23	pLCD31-2
btuB$^+$					
RK4373 (Btu$^+$)	+	+	+	+	+
RK6687 (BtuC$^-$)	−	+	−	+	+
RK6689 (BtuD$^-$)	*	+	+	+	*
RK6691 (BtuE$^-$)	*	+	+	*	−
btuB					
RK4379	+	+	+	+	+
RK6686 (BtuC$^-$)	−	+	−	+	+
RK6688 (BtuD$^-$)	*	+	+	+	*
RK6690 (BtuE$^-$)	*	+	+	±	*

[a] Growth is relative to that on the same medium with methionine: +, approximately the same colony size; ±, colonies less than 50% in diameter; −, no detectable growth. *, No growth on minimal plates with the indicated concentration of vitamin B_{12} in place of methionine after 18 h of incubation, but normal growth after 48 h.

[b] *btuB*$^+$ strains were grown with 0.5 nM vitamin B_{12}. *btuB* strains were grown with 5 μM vitamin B_{12}. *btuB* strains only responded to concentrations of vitamin B_{12} of 2 μM or higher.

eliminated synthesis of the 29K polypeptide and had no apparent effect on the level of any of the other products. Insertions 22 (Fig. 3, lane e) and 23, lying between *btuC* and *btuD*, eliminated production of the 22K and 20K polypeptides and reduced the amount of the 29K product. These insertions defined the *btuE* gene. Insertions 13 and 14, to the right of *btuD*, blocked production of the 17K polypeptide (Fig. 3, lanes f and g).

Chromosomal Tn*1000* insertions. Several of the Tn*1000* insertions (isolates 1, 2, and 23, which eliminated synthesis of the 26K BtuC, 29K BtuD, and 22K and 20K BtuE polypeptides, respectively) were crossed onto the chromosome to examine their effect on vitamin B_{12} utilization in the haploid state. This was accomplished by integration through homologous recombination of the insertion-bearing plasmids into the chromosomal *btuC* region in a *polA* strain. The integrated plasmid was lost at a high frequency (in the range of 1%). Most of the chloramphenicol-susceptible derivatives from all crosses exhibited wild-type behavior in growth and vitamin B_{12} transport assays. A few isolates were defective in both assays and were found by Southern hybridization tests to contain an insertion in the appropriate region of the host chromosome. Transduction of that chromosomal region into other hosts was accomplished by selection for inheritance of the adjacent *zdh-3*::Tn*10* marker (8).

Strains carrying the *btuC480*::Tn*1000* mutation (insertion 1) exhibited the typical BtuC growth phenotype (4, 8) (Table 2). In *btuB* or *tonB* hosts, this mutation completely eliminated the growth response to any concentration of vitamin B_{12}. In a *btuB*$^+$ *tonB*$^+$ host, this mutation allowed growth on 50 nM vitamin B_{12} but not on 5 nM vitamin B_{12}. By comparison, *btuC*$^+$ strains grew well with 50 pM vitamin B_{12} in place of methionine. The *btuD481*::Tn*1000* insertion conferred the phenotype seen previously with *btuD* mutants. In a *btuB* or *tonB* host, growth on 5 μM vitamin B_{12} did occur, but only after several days. In a *btuB*$^+$ host, growth was impaired with 0.5 or 5 nM vitamin B_{12} but was normal with 5 μM vitamin B_{12}.

Three *btuE*::Tn*1000* insertions, lying between *btuC* and *btuD* and blocking synthesis of the 22K and 20K products, conferred a phenotype like that of the *btuD* strains, namely, delayed response to 5 μM vitamin B_{12} in *btuB* strains and reduced growth on 0.5 nM vitamin B_{12} in *btuB*$^+$ strains.

The ability of pLCD31 derivatives carrying the wild-type region or insertion 1, 2, or 23 to complement the growth deficiencies of strains with the chromosomal insertions was tested (Table 2). The *btuC480*::Tn*1000* mutation was complemented fully by all plasmids except the one with the insertion in *btuC*. The *btuD481*::Tn*1000* insertion was complemented by all plasmids except the one with the insertion in *btuD*. The impaired growth response of two *btuE*::Tn*1000* mutants was fully corrected by plasmids carrying the intact region or an insertion in *btuC*. The defect was partially overcome by the plasmid with insertion 23 in *btuE*, but not at

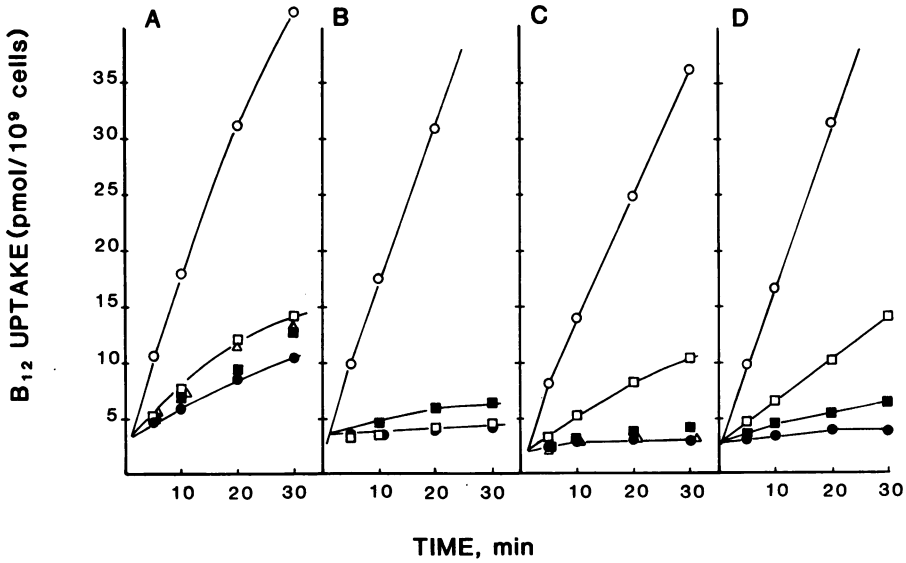

FIG. 4. Transport of vitamin B_{12} in strains carrying *btu* mutations on the chromosome or plasmid. The four host strains were as follows: A, RK4373 (Btu$^+$); B, RK6687 (BtuC$^-$); C, RK6691 (BtuE$^-$); D, RK6689 (BtuD$^-$). The plasmids present in these strains are indicated by the following symbols: pACYC184 (△), pLCD31 (○), pLCD31-1 (□), pLCD31-2 (●), pLCD31-23 (■).

all by the plasmid with the *btuD* insertion. Thus, at least part of the phenotype of the *btuE* mutations appeared to result from their polar effects on the expression of *btuD*.

Function of BtuCED in transport. Vitamin B_{12} transport was measured in strains carrying the plasmid-borne and chromosomal *btu*::Tn*1000* insertions. Uptake in a *btu*$^+$ host (Fig. 4A) was elevated at least fivefold when plasmid pLCD31 was present. However, transport activity was at or below the haploid value when plasmids with any *btu* insertion were resident. Plasmid pLCD31-2 (BtuD$^-$) caused a substantial decrease in transport activity.

Strains with insertions in the chromosomal *btuC*, *-D*, or *-E* genes displayed the reduced but energy-dependent uptake previously described for *btuC* mutants (4, 25). The accumulated vitamin in these mutants was released upon the addition of excess nonradioactive substrate, in contrast to its complete retention in the *btu*$^+$ host (data not shown). Plasmid pLCD31 engendered elevated rates of uptake in all three hosts. Transport in the *btuC* host (Fig. 4B) was not restored to the haploid level by any of the insertion-bearing plasmids. Transport in cells with the *btuD* or *btuE* insertions was restored almost to the haploid level by pLCD31-1 (BtuC$^-$), but not at all by plasmids with insertions in either *btuE* or *btuD* (Fig. 4C and D). Thus, disruption of any one of the three *btu* genes prevents the amplification of transport activity. There was a consistent finding that plasmids encoding BtuC but not BtuD were inhibitory for transport, and that complementation was inefficient, perhaps as a result of polarity.

Function of the 17K polypeptide and regulation of transport. Production of the minor 17K polypeptide was elicited by all of the pLCD31 derivatives except those with insertions 13 and 14, to the right of *btuD* (Fig. 3). The following results indicated that this polypeptide is not involved in vitamin B_{12} transport or its regulation.

Plasmids carrying insertions in *btuC*, *-E*, or *-D* do not cause increased uptake in a wild-type host. Plasmids with insertions 13, 14, and 21 all elicited elevated uptake rates to the same degree as the parental plasmid, showing that the insertions did not affect a gene necessary for the amplification of transport function (Fig. 5).

The Δ(*zdh-3-btuD*)*478* deletion extends rightward at least 1.5 kilobases from *btuD* to the site of the *zdh-3*::Tn*10* insertion and removes the coding region for the 17K protein (8). The growth deficiency of this strain was corrected by plasmid pLCD31 and the derivatives with insertions 13, 14, and 21. These plasmids all conferred elevated rates of transport in this host (Fig. 5C). Plasmid pLCD31-2 (BtuD$^-$) did not restore the growth response or transport activity to this strain, indicating that *btuD* was the only gene removed by the deletion that is involved in vitamin B_{12} uptake.

In attempts to cross insertions 13, 14, and 21 onto the chromosome, none of the chloramphenicol-susceptible variants exhibited a defective phenotype with respect to vitamin B_{12} utilization. Although we did not verify that any of these variants carried the insertion on the chromosome, this result was consistent with the other findings indicating the lack of a role of this polypeptide in vitamin B_{12} transport.

Repression of vitamin B_{12} transport by growth with vitamin B_{12} was markedly reduced in *btuE*::Tn*1000* or *btuD* strains (Fig. 5). The presence in the *btuE* host of pLCD31 or its derivatives with insertions in *btuC*, *btuD*, or the coding region for the 17K protein restored complete repressibility. The presence of pLCD31-23 (BtuE$^-$) resulted in partial repressibility, suggesting that the BtuE protein is not essential for transport if BtuD is provided. In the Δ(*btuD-zdh-3*) host lacking BtuD and the 17K protein, full repressibility of transport activity required the production of BtuE and BtuD, but not the 17K protein (Fig. 5D).

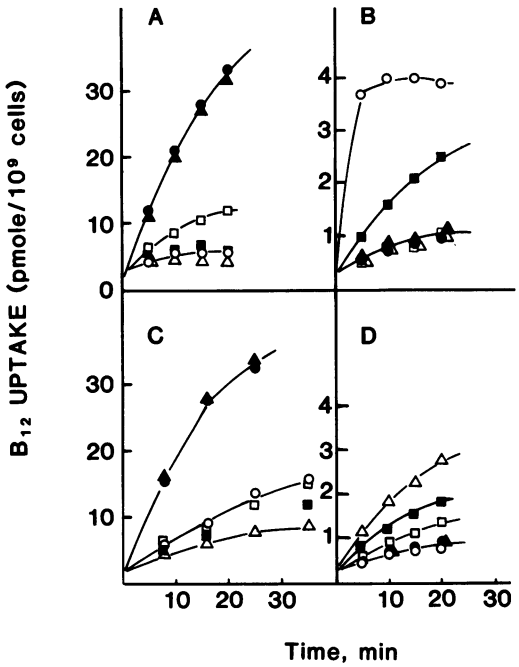

FIG. 5. Repression of vitamin B_{12} transport. Vitamin B_{12} transport was measured in strain RK6680 (*btuE*::Tn*1000*) (A and B) and strain RK6563 [Δ(*btuD-zdh-3*)] (C and D). Cells were grown in minimal medium without (A, C) or with (B, D) 5 nM vitamin B_{12}. The symbols indicate the plasmids resident in the strains as follows: haploid (○), pLCD31 (●), pLCD31-1 (□), pLCD31-23 (■), pLCD31-2 (△), pLCD31-13 (▲). Strains with plasmids pLCD31-14 and pLCD31-21 gave results identical to those with pLCD31-13.

Cellular localization. Fractionation of labeled maxicells into periplasmic, soluble, and membrane fractions was performed to obtain information about the possible cellular location of the Btu polypeptides. Results obtained with derivatives of pLCD31 are shown in Fig. 6A; similar results were seen with pBR322 derivatives. Cold osmotic shock caused release of a fraction of the 22K BtuE polypeptide (lanes d, h, and l); the remainder was recovered in the soluble fraction (lanes e, i, and m). The 26K BtuC polypeptide was found exclusively in the membrane-containing fraction when maxicells carrying pBR322 derivatives were used to avoid the presence of CAT (data not shown). The 29K BtuD polypeptide was found predominantly in the membrane fraction (lanes f, j, and n), with variable amounts in the soluble fraction.

Three procedures for release of periplasmic polypeptides all caused partial release of BtuE. The β-lactamase produced in maxicells carrying pBR322 was extensively released upon spheroplast formation, but only slightly by chloroform shock (Fig. 6B, lanes a through e). BtuE was partly released from maxicells carrying pLCD25 by chloroform shock, without release of other labeled polypeptides (lanes g and h). Partial release of BtuE was also seen upon spheroplast formation by lysozyme-EDTA treatment (lanes i and j) or cold osmotic shock (lanes k and l). These procedures resulted in extensive release of β-lactamase, slight release of BtuD, and no release of BtuC. Thus, the BtuE polypeptide made in maxicells may have a periplasmic location, but it does not behave in

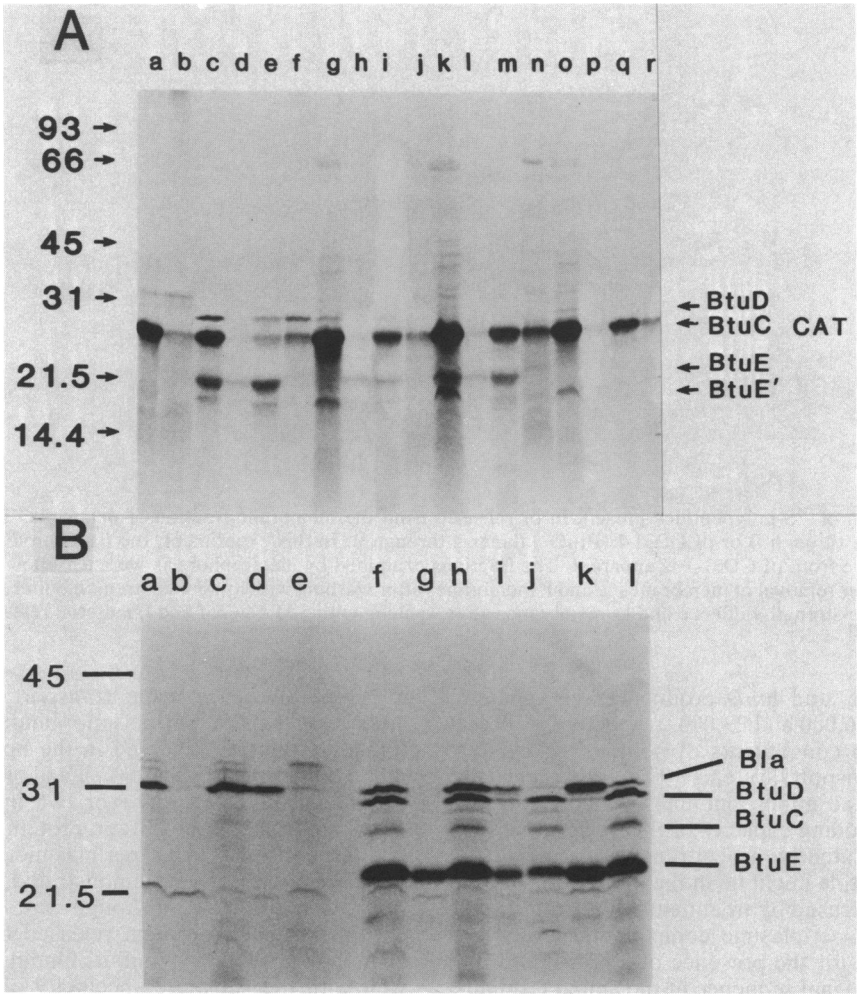

FIG. 6. Fractionation of maxicells carrying *btuCED* polypeptides. (A) Autoradiogram of ^{35}S-polypeptides synthesized in maxicells by plasmid pLCD31 and derivatives carrying Tn*1000* insertions. The host strain was RK4446, and the plasmids were as follows: a and b, pACYC184; c through f, pLCD31; g through j, pLCD31-1 (BtuC$^-$); k through n, pLCD31-2 (BtuD$^-$); o through r, pLCD31-23 (BtuE$^-$). The cells fractions represented in each lane are as follows: a, c, g, k, and o, whole cells; d, h, l, and p, polypeptides released by osmotic shock; e, i, m, and q, soluble polypeptides after disruption of maxicells; b, f, j, n, and r, membrane-associated polypeptides. (B) ^{35}S-polypeptides produced in maxicells of strain SE5000 carrying pBR322 (lanes a through e) or pLCD25 (lanes f through l). The cell fractions are as follows: a and f, total maxicells; b and g, material released by chloroform shock; c and h, material retained after chloroform shock; d and i, material released upon lysozyme-EDTA-sucrose treatment; e and j, material retained after lysozyme-EDTA-sucrose treatment; k, material released upon cold osmotic shock; l, material retained after cold osmotic shock. The mobilities of the molecular weight standards are indicated on the left, and the locations of the respective gene products are shown on the right: BtuD, 29K; BtuC, 26K; BtuE, 22K; BtuE', 20K; Bla, β-lactamase.

fractionation studies in the same manner as known periplasmic proteins.

Membrane attachment of BtuD. The association of the 26K BtuC and 29K BtuD polypeptides with the membrane fraction was examined by washing membranes from labeled maxicells either with low-ionic-strength buffer or with 5 M NaCl (27) (Fig. 7). All of BtuC remained particulate, but an appreciable amount of BtuD was released during washing, suggesting a peripheral membrane association (lanes c through f). Both proteins were released upon solubilization of the cytoplasmic membrane with Triton X-100 (data not shown).

To investigate whether the association of BtuD with the membrane required the presence of BtuC, the maxicells were prepared in strain RK6683 (*btuC480*::Tn*1000 recA*) carrying either plasmid pLCD31 (C$^+$ E$^+$ D$^+$) or pLCD31-1 (C::Tn*1000* E$^+$ D$^+$). In the latter strain, no BtuC should be present. In both strains, equivalent proportions of BtuD were recovered in the particulate fraction, and the amount removed by washing was comparable (lanes i through l). These results suggested that BtuC was not necessary for the peripheral membrane binding of BtuD. However, there was considerable retention of the soluble CAT and BtuE proteins in these membrane fractions.

DISCUSSION

Results presented here identified the three polypeptides encoded by the *btuCED* region and showed that they are involved in vitamin B_{12} transport across the cytoplasmic membrane. The gene boundaries identified from the effect of Tn*1000* insertions on complementation properties and polypeptide production agreed well with the location of the open reading frames seen in the nucleotide sequence of the region

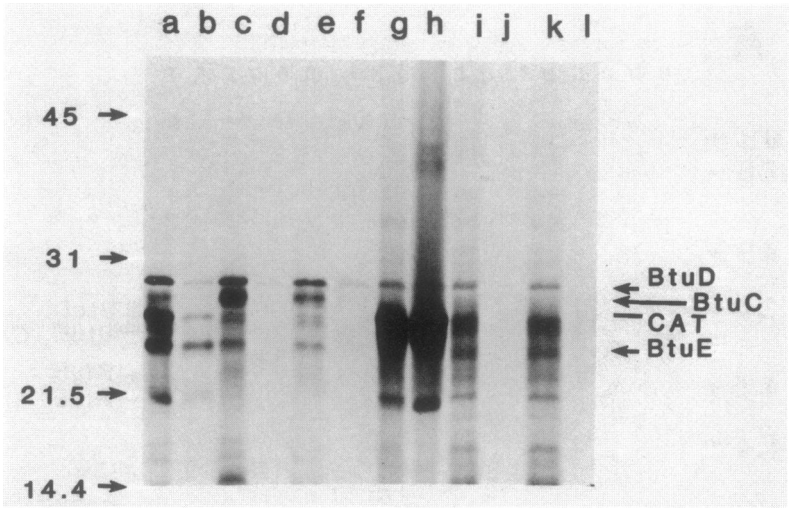

FIG. 7. Autoradiogram of ^{35}S-polypeptides present in or released from the membrane fraction of maxicells. The strains were RK6683 carrying pLCD31 (lanes a through f) or pLCD31-1 (BtuC$^-$) (lanes g through l). In this experiment, the BtuC product migrated more slowly than CAT, and its absence from pLCD31-1 is apparent. The fractions examined by electrophoresis were as follows: a and g, whole cells; b and h, soluble fraction after removal of membranes; c and i, membranes after washing with low-ionic-strength buffer; d and j, material released by washing with low-ionic-strength buffer; e and k, membranes after washing with 5 M NaCl; f and l, material released by washing with 5 M NaCl.

(Fig. 1) (11). The *btuC* and *btuD* products have apparent molecular weights of 26,000 and 29,000, respectively. These proteins are similar to constituents of periplasmic binding protein-dependent transport systems. The *btuE* gene encodes a 22K species and minor amounts of a 20K species. Owing to the limited coding capacity of this region, the two forms are probably related by a posttranslational modification. The 22K polypeptide might be in the periplasm, since a proportion of it was released by treatments thought to cause preferential release of periplasmic components. However, there was no evidence for the presence of a precursor, and there was no obvious signal sequence at the amino terminus of the predicted BtuE polypeptide.

The requirement for the 22K polypeptide in vitamin B_{12} transport could not be unambiguously determined. Insertions in *btuE* prevented the elevation of transport when present on multicopy plasmids and impaired vitamin transport and growth response when present on the chromosome. However, the failure of the *btuD*::Tn*1000* plasmid to complement these phenotypes indicated that one effect of the *btuE* mutation was to cause a polar decrease in *btuD* expression. Evidence for the role of the *btuE* product in transport is currently being sought by generation of nonpolar deletions.

Sequence analysis showed that the three *btu* genes are transcribed in the same direction, left to right in Fig. 1 or counterclockwise relative to the genetic map (2). The complementation and subcloning experiments showed that *btuC* and *btuD* can be expressed independently in either orientation with respect to the vector. However, the results with maxicells, albeit difficult to quantitate, suggested that insertions in *btuC* resulted in decreased production of BtuE and BtuD, and that insertions in *btuE* decreased production of BtuD. These polar effects were only partial, suggesting that all three genes are transcribed from a promoter before *btuC* and that internal promoters are also present to allow independent transcription of *btuE* and *btuD*. Expression of genes for other outer membrane-dependent transport systems also exhibits incomplete polarity (10).

We propose that the three *btuCED* polypeptides comprise a cytoplasmic membrane transport system that may be analogous to the periplasmic binding protein-dependent transport systems involved in the uptake of histidine (16), maltose (7, 12), phosphate (27), and oligopeptides (17). All of these systems employ one or two integral membrane proteins, a peripheral membrane protein having putative ATP-binding domains, and a periplasmic substrate-binding protein. We have not yet demonstrated that the BtuE protein binds vitamin B_{12}, although the existence of a 22K cobalamin-binding protein released by osmotic shock has been described (5, 28), and its binding properties correlated well with the substrate specificity of the overall transport process (5). BtuE does not appear to be essential for transport activity. It is possible that the operation of the energy-dependent outer membrane transport system generating high concentrations of the vitamin in the periplasm circumvents the requirement for a periplasmic binding protein.

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