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Transport of Vitamin B_{12} in *Escherichia coli*: Cloning of the *btuCD* Region

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The transport of vitamin B_{12} in *Escherichia coli* requires a specific vitamin B_{12} receptor protein in the outer membrane and the *tonB* gene product. In addition, the *btuC* gene, located at min 38 on the genetic map, has been found to influence vitamin B_{12} uptake or utilization. The *btuC* function is required for the growth response to vitamin B_{12} when the outer membrane transport process (*btuB* or *tonB* function) is defective. However, even in a wild-type strain, *btuC* is required for proper transport of vitamin B_{12}. Additional mutations in the vicinity of *btuC* were isolated as *lac* fusions that produced a phenotype similar to that of a *btuC* mutant. The *btuC* region was cloned by selection for complementation of a *btuC* mutation. Complementation testing with plasmids carrying various deletions or transposon Tn*1000* insertions demonstrated that the new mutations defined a separate, independently expressed locus, termed *btuD*. The coding regions for both genes were identified on a 3.4-kilobase *Hin*dIII-*Hin*cII fragment and were 800 to 1,000 base pairs in length. They were separated by a 600- to 800-base-pair region. The gene order in this portion of the chromosome map was found to be *pps-zdh-3*::Tn*10-btuD-btuC-pheS*. Expression of β-galactosidase in the *btuD-lac* fusion-bearing strains, whether proficient or defective in vitamin B_{12} transport, was not regulated by the presence of vitamin B_{12} in the growth medium.

The uptake systems for vitamin B_{12} and numerous iron-siderophore complexes in *Escherichia coli* are unusual in that they employ specific receptor proteins in the outer membrane as an essential component of the high-affinity active transport process (26). The *tonB* product appears to be necessary for the energy-dependent stages of many transport processes mediated by these receptors (21, 32). For example, whereas the uptake of vitamin B_{12} by means of the *btuB*-coded B_{12} receptor is *tonB* dependent, the entry of the E colicins and phage BF23, which use the same receptor, is not (2, 8, 13). In addition to the receptors, other gene products have been implicated in siderophore transport at steps subsequent to receptor binding. Mutations in the *fecB*, *fepB*, *iutB*, and *fhuBCD* loci prevent uptake of ferric citrate, ferric enterochelin, ferric aerobactin, and hydroxamate-type siderophores, respectively, but do not impair formation of the corresponding receptor (15, 20, 26, 27, 30, 31, 33).

Mutations affecting vitamin B_{12} uptake in an identical manner to those blocking iron uptake have not been observed (7). Mutations at *btuC* only slightly impair the growth response of *metE* mutants to vitamin B_{12} in place of methionine (3, 14). The initial rate of vitamin B_{12} transport in a *btuC* mutant strain resembles that in wild-type strains. A steady-state level of vitamin B_{12} accumulation is attained, which differs from the parental situation in that most or all of the accumulated vitamin B_{12} can be chased from the cell upon addition of excess nonradioactive vitamin B_{12} (3). Parental strains retain most of the transported substrate. The vitamin B_{12} accumulated in a *btuC* strain is not converted into the various metabolic derivatives normally found and can be released from the cell by EDTA treatment (28). Thus, *btuC* function is required for proper transport or processing of B_{12} in strains proficient for the outer membrane transport components.

Strains deficient in the components of the outer membrane transport process (altered at *btuB*, *tonB*, or both) can still utilize elevated (5 μM) levels of vitamin B_{12} (3). Receptor-independent utilization of vitamin B_{12} is more effective in strains with altered outer membrane barrier properties (3). In these cases, the growth response to vitamin B_{12} is absolutely dependent on *btuC* function. *btuC* appears to be involved solely in B_{12} utilization, since the *btuC* genotype has no effect on the response to the E colicins or phage BF23 or on the utilization of ferric-siderophore complexes as iron source (unpublished observations).

The genetic analysis of the *btuC* region described in this paper was prompted by the uncertainty of whether this function was involved in the transport of vitamin B_{12} across the cytoplasmic membrane or its intracellular processing. Only three *btuC* alleles were available, and it was unknown whether the dispensability of *btuC* function for the growth response to vitamin B_{12}, when the outer membrane components were intact, reflected only partial loss of function in these mutants. Another impetus for this study concerned the possible role in transport of a vitamin B_{12}-binding protein of approximately 22,000 molecular weight, which was released upon osmotic shock (7, 29).

In this paper, we report the isolation of *lac* fusions near *btuC*, which define another gene involved in B_{12} utilization, termed *btuD*, and the cloning of this region. By generation of deletions between restriction endonuclease cleavage sites and subcloning procedures, the regions able to complement *btuC* and *btuD* mutations were identified and separated. These results were confirmed by the isolation of insertions of the transposon Tn*1000*.

MATERIALS AND METHODS

Media and chemicals. L broth and minimal medium A were as described by Miller (25). Solid media contained 2% agar. Antibiotics were added to the following final concentrations: ampicillin, 25 μg/ml; tetracycline, 20 μg/ml; and kanamycin sulfate, 40 μg/ml. Cyanocobalamin (vitamin B_{12}) was ob-

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

Strain	Genotype	Reference or source
CH923	F⁻ *trpA46 recA441 xyl glyS polA1 argH*	(16)
CS1129	*ompR151 lac recA*/F'*ts*114 *lac*⁺ *zzf*::Tn*10*	C. Schnaitman
MC4100	Δ(*argF-lac*) *U169 araD139 relA1 rpsL150 flb-5301 deoC1 tonA21 thi*	(10)
RK4446	MC4100 *non gyrA tonA*⁺ Δ*metE* Δ(*tonB-trp*) *btuB btuC456 zdh-600*::Tn*5 recA*	
RK4954	MC4100 *non gyrA ton*⁺ *zib-636*::Tn*10*	(22)
RK5000	MC4100 *recA*	
RK5173	MC4100 *non gyrA ton*⁺ *metE70*	
RK5419	RK5173 · λp1(209)[btuB-lacZ] · λd*arg*13 [BtuB⁺]	
RK6006	RK4446 *btuB*⁺	
RK6007	RK6006 *btuC*⁺	
RK6036	MC4100 Δ*metE* Δ*btuB*	
RK6048	RK5173 *zdh-1*::Tn*10*	
RK6049	RK6048 *btuC456*	
RK6063	RK4446 *rec*⁺ *btuC*⁺ *zdh-3*::Tn*10*	
RK6210	*metE pro lysA trp btuB451 btuC456 pheS11 zdh-3*::Tn*10*	
RK6213	RK5173 *zdh-1*::Tn*10*	
RK6215	RK5173 *zdh-1*::Tn*10 btuC456*	
RK6216	RK5173 *zdh-3*::Tn*10*	
RK6218	RK5173 *zdh-3*::Tn*10 btuC456*	
RK6531	RK6036 *btuD467*::λ*plac*Mu50 *zdh-3*::Tn*10*	
RK6532	RK6036 *btuD467*::λ*plac*Mu50	
RK6533	RK6036 *btuD468*::λ*plac*Mu50 *zdh-3*::Tn*10*	
RK6534	RK6036 *btuD468*::λ*plac*Mu50	
RK6535	RK6036 *btuD469*::λ*plac*Mu50 *zdh-3*::Tn*10*	
RK6536	RK6036 *btuD469*::λ*plac*Mu50	
RK6537	RK6036 *btuD470*::λ*plac*Mu50 *zdh-3*::Tn*10*	
RK6538	RK6036 *btuD470*::λ*plac*Mu50	
RK6543	RK5173 *btuD467*::λ*plac*Mu50 *zdh-3*::Tn*10* λ540[btuCD⁺]	
RK6544	RK5173 *btuD468*::λ*plac*Mu50 *zdh-3*::Tn*10* λ540[btuCD⁺]	
RK6545	RK5173 *btuD469*::λ*plac*Mu50 *zdh-3*::Tn*10* λ540[btuCD⁺]	
RK6546	RK5173 *btuD470*::λ*place*Mu50 *zdh-3*::Tn*10* λ540[btuCD⁺]	
RK6551	RK6534 Δ*btuD475*	
RK6552	RK6538 Δ*btuD476*	
RK6553	RK6538 Δ*btuD477*	
RK6554	RK6531 Δ(*zdh-3-btuD*)*478*	
RK6555	RK5173 Δ*btuD471 zdh-3*::Tn*10*	

tained from Sigma Chemical Co. The chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Bachem, Inc.) was prepared as a 2.0% stock in dimethyl sulfoxide (Fisher Scientific Co.) and used at a final concentration of 25 μg/ml.

Bacterial strains and phage. All bacterial strains employed in these experiments are listed in Table 1 and are derivatives of *E. coli* K-12. Bacteriophage P1 (laboratory stock) was used for transductions. The hybrid phages λ*plac*Mu50 and λpMu507 used for the construction of the *btuD-lacZ* operon fusions were a gift of E. Bremer and are described below. The *Hin*dIII cloning vector λNM540 was provided by M. Smith. Transposon Tn*10* insertions near *btuC* (*zdh-1* and *zdh-3*) were kindly provided by D. G. Fraenkel (12).

Construction of *btuD-lacZ* operon fusions. A derivative of bacteriophage λ, λ*plac*Mu50, was used to isolate the *btuD-lacZ* operon fusion by transposition of the phage into the *btuD* gene (5) (Fig. 1). This phage contains both ends of bacteriophage Mu and its *c*I and A genes and is able to carry out random transposition similar to intact Mu phage. The Mu *s* end sequences are positioned next to the sequence encoding the N-terminal portion of the *lacZ* structural gene in a manner that allows transcription of *lac* from external promoters. Stable *lacZ* operon fusions can be generated in a single step whenever the hybrid phage integrates in the correct orientation. In the resultant operon fusion, *lacZ* expression is determined by the transcriptional signals of the target gene (5). For the construction of *btuD* fusions, Lac⁺ λ*plac*Mu50 lysogens of strain RK6063 were selected after infection of 1 ml of an overnight L-broth culture with 2×10^9 λ*plac*Mu50 and λpMu507 hybrid phage. Phage λpMu507 was used as a helper phage to increase the frequency of transposition of the λ*plac*Mu50 phage. Infection was carried out for 20 min at 37°C, and the suspension was centrifuged and washed three times with L broth. Samples of the cell suspension in L broth were plated on minimal medium containing lactose and methionine. Approximately 15,000 Lac⁺ lysogens were pooled, and a P1 phage lysate was prepared on the pool. The lysate was used to transduce strain RK6036 to tetracycline resistance, with screening on minimal medium plates containing glucose, tetracycline, and the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside for the acquisition of Lac⁺ character and λ immunity.

Transposon insertion mutagenesis. Insertion mutagenesis with Tn*1000* (the γδ segment of the F plasmid) was performed by the method of Guyer (17), with several modifications. Plasmids pLCD31 and pLCD32 (derivatives of pACYC184 carrying the 3.4-kilobase (kb) *Hin*dIII-*Hin*dII fragment in the *Hin*dIII site) were introduced into strain CS1129 by transformation, with selection for chlorampheni-

col resistance. These transformants were used as donors in mating experiments with strain RK5000 (*recA btu*$^+$). Overnight cultures of both strains were diluted in L broth, and the cultures were grown to the early logarithmic phase. Equal volumes of both cultures were mixed and incubated for 8 h at 30°C without aeration. Portions of the mixture were plated on L broth with nalidixic acid, which selects against the donor, and chloramphenicol, to select for transfer of the *btuCD* plasmid to the recipient. The transconjugants were

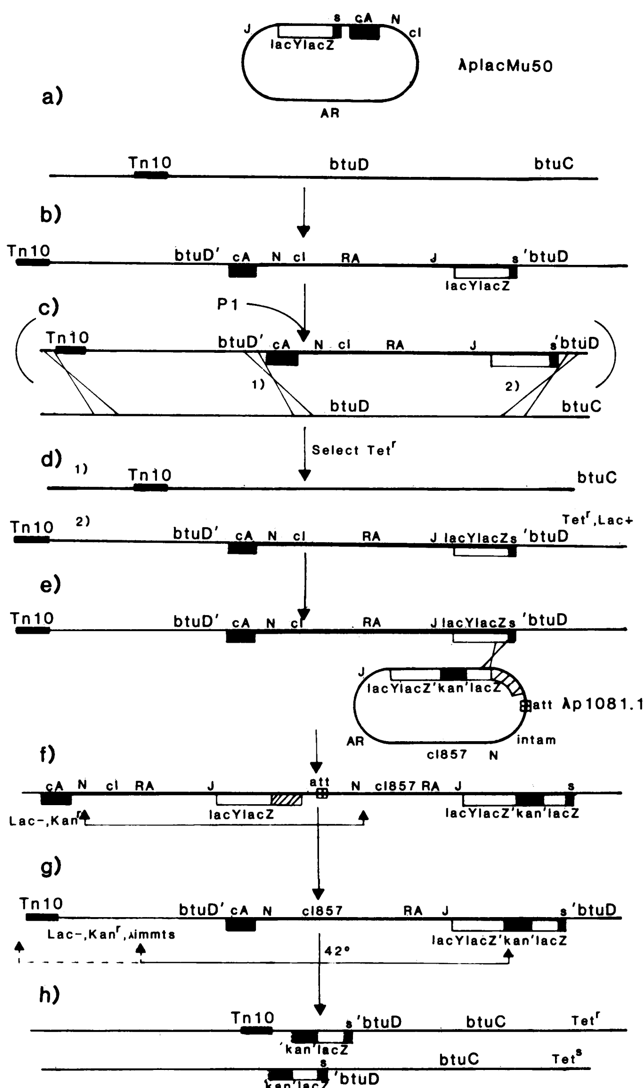

FIG. 1. Construction of *btuD-lac* operon fusions and generation of deletions. Some of the Lac$^+$ strains generated by transposition of λp*lac*Mu50 into the chromosome of a strain with a Tn*10* insertion near *btuC* (a, b) have an integrated prophage that can be transduced by phage P1 along with the tetracycline resistance of Tn*10* (c). A few tetracycline-resistant transductants were Lac$^+$, and a few of these exhibited a Btu$^-$ phenotype (d). Lysogenization of Lac$^+$ Btu$^-$ strains with phage λp1081.1 (e) was obtained by selection for kanamycin-resistant colonies that were Lac$^-$ (f). The tandem dilysogen was unstable and gave rise to temperature-sensitive variants by homologous recombination within the prophages (g). Selection for temperature resistance yielded deletion strains (h). Most of them retained kanamycin resistance. Some were tetracycline susceptible, whereas others retained the tetracycline resistance of the Tn*10*.

purified, and the plasmid DNA was analyzed by restriction digestions.

Restriction endonuclease digestions and ligations. DNA was digested with restriction endonucleases under conditions suggested by the manufacturer (Bethesda Research Laboratories). Products were analyzed on 0.8% agarose gels, and the sizes of the fragments were determined by comparison to λ DNA digested with *Hin*dIII. Ligations were performed as described by Berman et al. (5).

Cloning procedures. Chromosomal DNA was isolated from strain RK4954 and digested to completion with the restriction endonuclease *Pst*I. With T4 DNA ligase, DNA fragments were cloned into the unique *Pst*I site of plasmid pBR322 (24). Hybrid plasmids containing a fragment of DNA carrying the *btuC* gene were isolated in strain RK4446 (*metE btuB tonB btuC recA*) by selection for resistance to tetracycline and ability to grow on 5 μM B_{12}. Isolates were purified on selective medium. Plasmids were isolated by a miniprocedure employing alkaline sodium dodecyl sulfate extraction (6) and digested with restriction endonucleases. Transformations were carried out by the $CaCl_2$ procedure of Dagert and Ehrlich (11).

Southern blot analysis. Preparation of gels and transfer assembly were performed as described by Berman et al. (5). Hybridizations were carried in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–50% formamide at 42°C. DNA used for probes was isolated as restriction fragments from agarose gels as described by Benson (4) and then nick translated with the Bethesda Research Laboratories nick translation kit as suggested by the manufacturer.

β-Galactosidase assays. β-Galactosidase production in strains carrying fusions to *lacZ* was assayed by monitoring change in optical density at 420 nm of *o*-nitrophenyl-β-galactopyranoside in Z buffer (25). Cells were grown to the early logarithmic phase in medium A, washed three times, and disrupted in a French pressure cell. The specific activity of β-galactosidase was expressed as change in optical density at 420 nm per minute per 10^{12} cells.

Vitamin B_{12} transport assays. Assays of vitamin B_{12} transport were modified from the procedure of Kadner and Bassford (19). Cells were grown in medium A to the early logarithmic phase, and ^{3}H-labeled vitamin B_{12} was added to a final concentration of 34 nM (0.23 μCi/ml). At indicated times, 0.2 ml of cells was removed, delivered directly onto a membrane filter (0.45-μm pore size; Millipore Corp.), immediately washed with 5 ml of medium A, and then dried and counted. Retention of transported vitamin B_{12} was assayed by running a duplicate reaction and adding an excess of unlabeled vitamin B_{12} (0.1 μM, final concentration) at 20 min after the addition of the labeled substrate and removing samples at 5-min intervals thereafter. Correction was made for the binding of labeled substrate to filters in the absence of cells. Uptake is expressed in picomoles per microliter of cell water.

RESULTS

Isolation of *btuD-lacZ* fusions. The *btuC* region is located at min 37.5 on the *E. coli* linkage map, between *pheS* and the Tn*10* insertions *zdh-1* and *zdh-3* (1, 3, 12). Mutations resulting from fusion to *lacZ* and located in the *btuC* region were obtained by using phage λp*lac*Mu50, which forms stable *lac* fusions in a single step (9) (Fig. 1). A P1 phage lysate prepared on a pool of lactose-utilizing lysogens was used to transduce strain RK6036 (*metE btuB*) to tetracycline resistance, thereby selecting for recombinants that acquired the

TABLE 2. Representative transduction crosses to determine linkage of *btuD* fusions and deletions to *zdh*::Tn*10* and *btuC*

Linkage	Cross	Donor strain	Recipient strain	Relevant genotype		No. of recombinants with donor *btu* allele/no. Tc^r
				Donor	Recipient	
btuD-lac to *zdh-3*::Tn*10*						
	1	RK6531	RK5173	*zdh-3*::Tn*10 btuD-lac467*	+	37/75
	2	RK6533	RK5173	*zdh-3*::Tn*10 btuD-lac468*	+	45/100
	3	RK6535	RK5173	*zdh-3*::Tn*10 btuD-lac469*	+	6/25
	4	RK6537	RK5173	*zdh-3*::Tn*10 btuD-lac470*	+	30/100
	5	RK6217	RK5173	*zdh-3*::Tn*10 btuC456*	+	112/124
	6	RK6213	RK6532	*zdh-1*::Tn*10*	Φ(*btuD-lac*)*467*	184/196
	7	RK6216	RK6532	*zdh-3*::Tn*10*	Φ(*btuD-lac*)*467*	95/100
	8	RK6213	RK6534	*zdh-1*::Tn*10*	Φ(*btuD-lac*)*468*	171/190
	9	RK6216	RK6534	*zdh-3*::Tn*10*	Φ(*btuD-lac*)*468*	96/100
	10	RK6213	RK6536	*zdh-1*::Tn*10*	Φ(*btuD-lac*)*469*	173/194
	11	RK6216	RK6536	*zdh-3*::Tn*10*	Φ(*btuD-lac*)*496*	96/100
	12	RK6213	RK6538	*zdh-1*::Tn*10*	Φ(*btuD-lac*)*470*	167/195
	13	RK6216	RK6538	*zdh-3*::Tn*10*	Φ(*btuD-lac*)*470*	96/100
	14	RK6215	RK5173	*zdh-1*::Tn*10 btuC456*	+	147/201
	15	RK6216		*zdh-3*::Tn*10*	*btuC456*	88/96
Δ*btuD* to *zdh-1* and *zdh-3*						
	16	RK6547	RK6036	*zdh-3*::Tn*10* Δ*btuD471*	+	82/82
	17	RK6548	RK6036	*zdh-3*::Tn*10* Δ*btuD472*	+	112/114
	18	RK6549	RK6036	*zdh-3*::Tn*10* Δ*btuD473*	+	101/103
	19	RK6550	RK6036	*zdh-3*::Tn*10* Δ*btuD474*	+	199/199
	20	RK6213	RK6552	*zdh-1*::Tn*10*	Δ*btuD476*	91/100
	21	RK6215	RK6552	*zdh-1*::Tn*10 btuC456*	Δ*btuD476*	96/99
	22	RK6216	RK6552	*zdh-3*::Tn*10*	Δ*btuD476*	93/97
	23	RK6218	RK6552	*zdh-3*::Tn*10 btuC456*	Δ*btuD476*	98/100
	24	RK6213	RK6554	*zdh-1*::Tn*10*	Δ(*zdh3-btuD*)*478*	94/100
	25	RK6215	RK6554	*zdh-1*::Tn*10 btuC456*	Δ(*zdh3-btuD*)*478*	95/98
	26	RK6216	RK6554	*zdh-3*::Tn*10*	Δ(*zdh3-btuD*)*478*	100/100
	27	RD6218	RK6554	*zdh-3*::Tn*10 btuC456*	Δ(*zdh3-btuD*)*478*	95/100

btuC region from the donor. Of approximately 5,000 tetracycline-resistant recombinants screened, 30 were blue on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside medium (Lac^+); of these, 4 were unable to utilize 5 μM B_{12}.

To determine that the Lac^+ phenotype and the defect in B_{12} utilization resulted from the same genetic event, P1 phage lysates of each of the four Btu^- Lac^+ strains were used to transduce strain RK5173 to tetracycline resistance (Table 2, crosses 1 to 4). Less than half (30 to 50%) of the recombinants that acquired *zdh-3*::Tn*10* were Lac^+ Btu^- and λ immune. In no recombinants were these characters separated.

When selection was made for the Lac^+ character, all of the recombinants were Btu^- and roughly half were tetracycline resistant. When some Lac^+ Btu^- tetracycline-susceptible recombinants were transduced with donor lysates prepared on btu^+ strains carrying either *zdh-1*::Tn*10* or *zdh-3*::Tn*10*, approximately 90 and 96%, respectively, of the tetracycline-resistant recombinants were Lac^- Btu^+ and λ sensitive (Table 2, crosses 6 to 13). The linkages of the Tn*10* insertions to *btuC* were 71 and 90%, respectively (Table 2, crosses 5, 14, and 15). The closer linkage to the Tn*10* insertions when the four mutations were present in the recipient than when in the donor was consistent with the insertion of λ*plac*Mu50 into a gene located in the *btuC* region and involved in vitamin B_{12} utilization.

Generation of deletions. The presence of λ DNA associated with the *lac* fusions facilitated the isolation of deletions. The cI^+ prophages in the *btuC* region were replaced with λ1081.1 by homologous recombination (Fig. 1) (5). This phage carries *c*I857 and *lacZ*::Tn*5*, so that temperature-sensitive lysogens can be obtained by selection for kanamycin-resistant strains that are Lac^-. In this way thermoinducible lysogens were obtained for all four fusions with and without the adjacent *zdh-3*::Tn*10* insertion. Selection for temperature resistance should yield deletions removing λ and flanking chromosomal sequences (5).

Temperature-resistant variants were obtained at low frequency (less than 10^{-8}). All were λ sensitive, but most remained kanamycin resistant, suggesting that the deletions extended only in one direction, away from the promoter for the gene into which insertion had occurred (Fig. 1). In transduction crosses, kanamycin resistance was 100% linked to the Btu^- phenotype (data not shown). In several temperature-resistant variants that retained *zdh-3*::Tn*10*, tetracycline resistance and the Btu^- phenotype were very closely linked (98 to 100% cotransduction), in contrast to the 90% linkage between this transposon and *btuC* (Table 2, crosses 16 to 19). Some of the deletions appeared to remove the adjacent *zdh-3*::Tn*10* insertion, since the strains became tetracycline susceptible. The temperature-resistant derivatives that had become tetracycline susceptible were used as recipients to examine the extent of the deletion (Fig. 2). When a *zdh-3*::Tn*10* $btuC^+$ strain was used as a donor (Table 2, cross 26; compare with cross 22), all of 100 tetracycline-resistant recombinants were Btu^+. When the more distant *zdh-1*::Tn*10* insertion (70% cotransduction with *btuC*) was in the donor strain, a few (6 of 100) tetracycline-resistant recombinants remained Btu^- (Table 2, cross 24). These results showed that the events resulting in formation of temperature-resistant, tetracycline-susceptible derivatives from the *zdh-3*::Tn*10* fusion-bearing strain were deletions

extending to or beyond the site of the *zdh-3*::Tn*10* insertion, but not as as far as the site of the *zdh-1*::Tn*10* insertion.

To determine whether the deletions removed *btuC*, transduction crosses between a donor carrying *zdh-3*::Tn*10* *btuC* and various deletion strains were performed (Fig. 2b). If the deletion included the site of the *btuC* mutation, all tetracycline-resistant transductants must be Btu^-. If the deletion did not cover the site of the *btuC* mutation, recombination could occur between the endpoint of the deletion and the *btuC* mutation to give rise to Btu^+ transductants. In all crosses (Table 2, crosses 21, 23, 25, and 27), approximately 5% of the tetracycline-resistant recombinants were Btu^+. Thus, the deletions did not cover the site of the *btuC* mutation in the donor strain. This result and those presented below demonstrated that the fusions were in a gene distinct from *btuC*. This gene is termed *btuD* and, on the basis of the mapping and deletion results, lies in the gene order *zdh-3-btuD-btuC-pheS* (Fig. 3).

Phenotype of *btuD* mutants. When resident in a *metE tonB btuB* strain, the *btuD* fusions and deletions conferred a less severe impairment of vitamin B_{12} utilization than did a *btuC* mutation. Deficiency at *btuC* resulted in complete inability to utilize vitamin B_{12} in place of methionine. The presence of the *btuD* fusions and deletions in this genetic background resulted in markedly decreased growth with 5 μM B_{12} after 18 h, but full growth after 48 h. Growth on minimal medium with methionine appeared normal.

In strains wild type at *btuB* and *tonB*, the presence of the *btuC* mutation prevented growth on 0.5 nM vitamin B_{12} and resulted in reduced colony size on 5 nM vitamin B_{12}. Strains carrying *btuD* fusions or deletions grew normally on 5 nMvitamin B_{12} and showed reduced growth on 0.5 nM vitamin B_{12}. This difference in growth phenotype supported the view that the deletions left *btuC* intact.

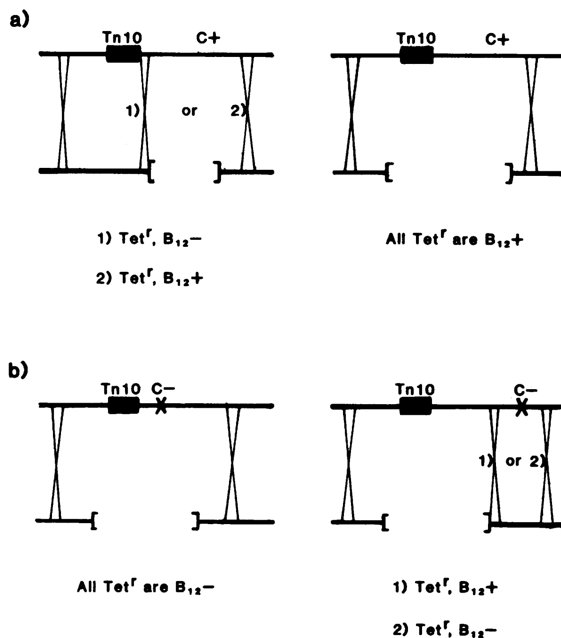

FIG. 2. Diagram of the genetic crosses to determine the endpoints of the Δ*btuD* deletions with respect to adjacent Tn*10* insertions and *btuC*. (a) Alternative locations of a deletion in the recipient, represented by the bracket in the lower line, with respect to the *zdh*::Tn*10* insertions. (b) Results expected if the deletion does or does not overlap the site of the *btuC* mutation in the donor chromosome. The results, presented in the text and Table 2, showed that the deletions did not extend past *zdh-1*::Tn*10* on the left or *btuC456* on the right. The letter C represents the *btuC* locus.

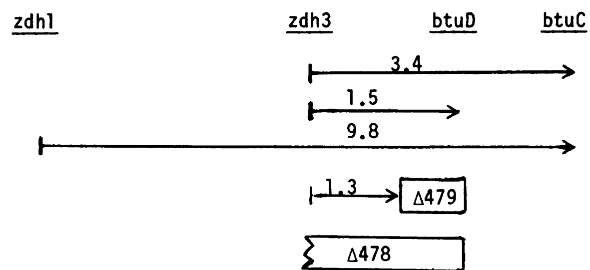

FIG. 3. Distances between markers, judged from transduction frequencies. The distances (in kb) between the markers depicted in this graphic were estimated by conversion of cotransduction frequencies into distances using the formula of Wu (33). The values are approximate because of the small number of recombinants examined and the high cotransduction frequencies. However, subcloning and nucleotide sequence information places the distance between *btuC* and *btuD* at 1.6 to 2.4 kb.

The vitamin B_{12} uptake properties of these strains were determined (Fig. 4). Both the *btuC* and *btuD* mutations had a similar effect on transport activity. The initial phases of uptake were similar to that in the parental strain. In both mutant strains, a steady-state level of vitamin B_{12} accumulation lower than that in the parental strain was reached. Most of the accumulated substrate was released from the mutant strains upon addition of excess nonradioactive vitamin B_{12}. In the parental strain, a larger proportion of the accumulated label was retained under these conditions.

Cloning of the *btuC* region. To investigate the genetic organization of the *btuC* region, its molecular cloning was undertaken. A library of *Pst*I-digested chromosomal DNA from strain RK4954 was constructed in pBR322 and was introduced into strain RK4446 (*metE tonB btuB btuC recA*). Tetracycline-resistant transformants were screened for growth on minimal medium with 5 μM B_{12}. Two $btuC^+$

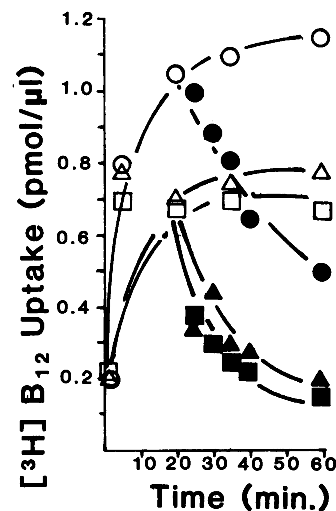

FIG. 4. Effect of mutations in *btuC* and *btuD* on B_{12} uptake activity. The uptake of strains RK6048 (btu^+) (●, ○), RK6049 (*btuC456*) (■, □), and RK6555 (*btuD461*) (▲, △) were measured with (●, ■, ▲) or without (○, □, △) the addition at 20 min of nonradioactive vitamin B_{12} to 10^{-7} M.

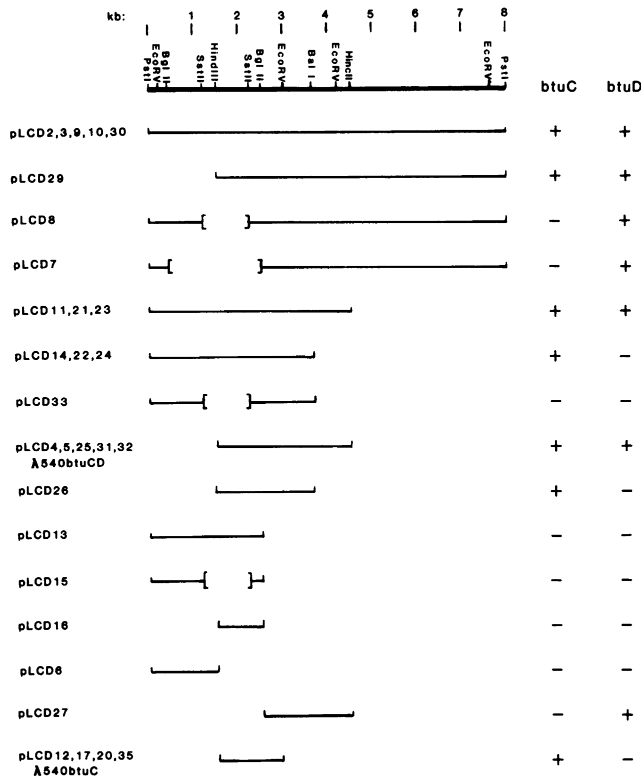

FIG. 5. Physical maps and genetic activity of the DNA fragment complementing *btuC* and *btuD* mutations and its derivatives. The top line presents the restriction map of the 8-kb *PstI* fragment. The outer lines show the material carried in the inserts of the indicated plasmids, which were derived by subcloning the indicated restriction fragments into the corresponding sites of appropriate vectors. Deletions were generated by digestion with the appropriate restriction enzyme and self-ligation. The plasmid sequences are not indicated. The ability of each plasmid to complement *recA btuC* or *btuD* recipients for growth with vitamin B_{12} is indicated on the right.

transformants contained plasmids that carried a common 8-kb *PstI* fragment, whose restriction endonuclease cleavage sites were mapped. As diagrammed in Fig. 5, a variety of deletions between restriction sites and subclones carrying portions of the 8-kb *PstI* insert were constructed. The details of the subcloning of some of the restriction fragments are not shown, but involved the addition of restriction site linkers and cloning into the polylinker region of plasmid pUC9, so that the fragments contained *HindIII* or *EcoRI* sites at their ends. This allowed cloning into the appropriate sites of pBR322. Several of the inserts were also cloned into the phage vector, λNEM540.

The complementation properties of the plasmids described in Fig. 5 were determined by introducing them by transformation into *recA btuB* derivatives of *btuC* or *btuD* strains. Transformants were tested for growth on 5 μM B_{12}. Plasmid pLCD2, carrying the 8-kb *PstI* fragment, complemented all *btuC* and *btuD* mutations, including Δ*btuD* deletions. Deletion of the 1.5-kb *PstI*-*HindIII* fragment in pLCD29 did not affect complementing activity. Deletion of either the *SstII* fragment (pLCD8) or the larger overlapping *BglII* fragment (pLCD7) abolished *btuC*-complementing activity, but with retention of *btuD*-complementing activity. The smallest fragment capable of complementing both *btuC* and *btuD* was the 3.4-kb *HindIII*-*HincII* fragment (pLCD25 and others). Within this 3.4-kb fragment (Fig. 5), *btuC* must lie to the left side, since the 1.4-kb *HindIII*-*EcoRV* fragment (pLCD12 and others) complemented *btuC*, whereas the 1.0-kb *HindIII*-*BglII* fragment (pLCD16) did not. The *btuD* gene lies on the right side of the 3.4-kb fragment, since the 2.4-kb *BglII*-*HincII* fragment (pLCD27) complemented *btuD*, whereas the 2.2-kb *HindIII*-*BalI* fragment (pLCD26) did not. Note that *btuC* and *btuD* were expressed even when the appropriate fragments were cloned separately in either orientation in the vector, suggesting that they comprise separate transcription units.

Chromosomal origin of the cloned genes. The approach of Greener and Hill (16) was employed to show that the cloned genes were derived from the *btuC* region of the chromosome. Plasmid pUC9 and its derivative, pLCD4, carrying the 3.4-kb *HindIII*-*HincII* fragment, were introduced into strain RK5721. This strain carries the *polA* mutation, which prevents autonomous replication of ColE1-like plasmids (23). Selection for maintenance of the plasmids' ampicillin resistance determinant requires integration of the plasmid into the chromosome, preferentially by homologous recombination at the site on the chromosome from which the cloned DNA originated. Genetic crosses were conducted to reveal the location of the integrated drug resistance determinant and thereby the origin of the cloned fragment. Phage P1 lysates of ampicillin-resistant transformants were used to infect strain RK6210 (*pheS11 zdh-3*::Tn*10*). Ampicillin-resistant transductants were tested for their response to tetracycline and to *p*-fluorophenylalanine, specified by the *pheS11* allele present in the recipient strain. When the donor strain carried the vector pUC9, all of the ampicillin-resistant trans ductants remained resistant to tetracycline and *p*-fluorophenylalanine. When the donor strain carried plasmid pLCD4, 35% of the ampicillin-resistant transductants were susceptible to tetracycline and 55% were susceptible to p-fluorophenylalanine. These results showed that plasmid pLCD4 had integrated into the *btuCD* region of the chromosome and hence must carry DNA derived from that region.

Physical location of the *btuC* and *btuD* mutations. The location of *btuC* on the 3.4-kb *HindIII*-*HincII* fragment was determined by marker rescue experiments testing the ability of plasmids carrying portions of the 3.4-kb insert to yield *btuC*$^{+}$ recombinants when introduced into a strain carrying the *btuC456* allele. Plasmid pLCD16, carrying the 1.0-kb *HindIII*-*BglII* fragment, did give rise to *btuC*$^{+}$ recombinants. In contrast, neither plasmid pLCD27, carrying the 2.4-kb *BglII*-*HincII* fragment, nor plasmid pLCD33, carrying the region from the leftward *PstI* terminus to the *BalI* site but deleted between the *SstII* sites, gave any *btuC*$^{+}$ recombinants, even after prolonged incubation of the selection plates. These results placed the location of the *btuC456* mutation in the 660-base-pair (bp) region between the *HindIII* site and the rightward *SstII* site.

The location of the *btuD* gene could not be determined by marker rescue crosses because of the leaky phenotype of *btuD* fusion or deletion strains on minimal medium with vitamin B_{12}. However, the approximate location of the sites of the insertions could be determined by Southern hybridization analysis (data not shown). Chromosomal DNA from *btuD-lacZ* fusion-bearing strains and the parental strain was digested with *EcoRV*, separated by agarose gel electrophoresis, transferred to nitrocellulose filters, and probed with the ^{32}P-labeled, 3.4-kb *HindIII*-*HincII* fragment. Three *EcoRV* fragments from the parental strain hybridized to the 3.4-kb probe, as expected from the restriction map in Fig. 5. The fusion-bearing strains showed hybridization to the 2.9-kb and 3.2-kb bands, representing the *EcoRV* fragments on the left and right site of the region, respectively. However,

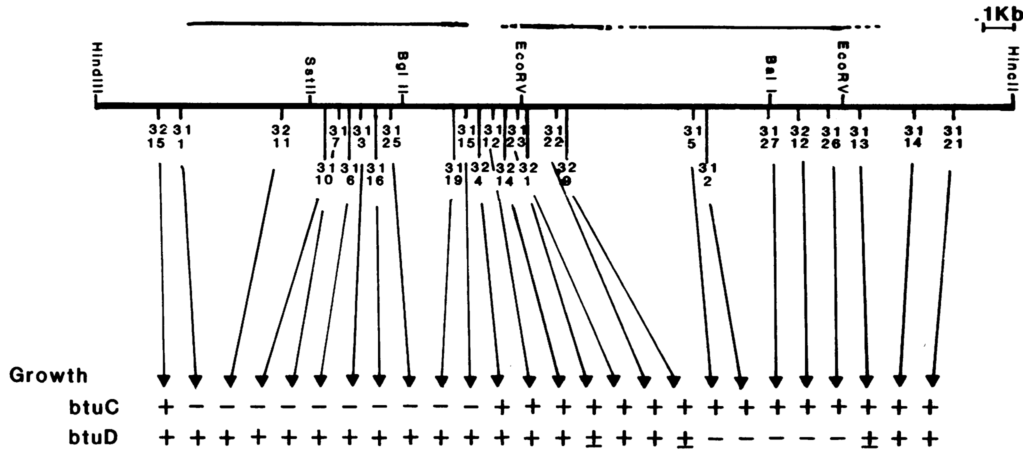

FIG. 6. Complementation properties of plasmids carrying Tn*1000* insertions. The thick line presents the restriction map of the 3.4-kb *Hin*dIII-*Hin*cII fragment, and the location of Tn*1000* insertions in plasmid pLCD31 or pLCD32 are indicated. The arrow from each insertion indicates the complementation activity of that plasmid in a *btuC* or *btuD* host. The lines above the restriction map designate the approximate locations of the *btuC* and *btuD* genes and the extent of the region between these genes in which insertions did not affect complementing activity.

hybridization to the central 1.4-kb *Eco*RV fragment was disrupted and replaced by hybridization to fragments of different sizes in the different insertions.

DNA from strains carrying *btuD* deletions was analyzed in the same manner. Hybridization to the 2.9-kb *Eco*RV fragment, which contains *btuC*, still occurred. The 1.4-kb and 3.2-kb *Eco*RV fragments no longer hybridized and were replaced by other fragments of various sizes. These results were in agreement with the complementation behavior and showed that the *btuC* and *btuD* genes were separated by the *Eco*RV site at the 3.0-kb coordinate defined in Fig. 5.

Transposon insertions. More precise localization of th-e*btuC*- and *btuD*-coding regions was allowed by the isolation of insertions of transposon Tn*1000* into plasmids pLCD31 and pLCD32, carrying the 3.4-kb *Hin*dIII-*Hin*cII fragment in opposite orientation. The location and orientation of each insertion were determined by appropriate restriction endonuclease digestions. The complementation properties of each plasmid were determined and agreed well with the subcloning results (Fig. 6). Insertions causing loss of *btuC*-complementing activity defined a contiguous region on the left side of the *Hin*dIII-*Hin*cII fragment. This region ex-

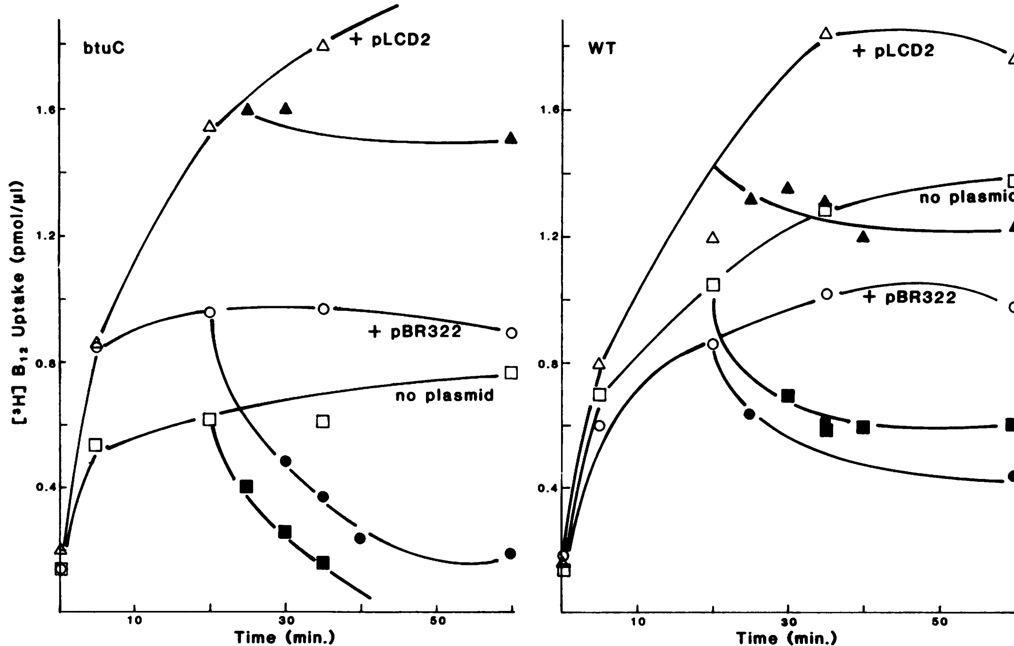

FIG. 7. Uptake of vitamin B_{12} in strains carrying the *btuCD*$^+$ plasmid pLCD2. The recipient strains were RK6049 (*btuC*) and RK6048 (*btu*$^+$). They carried no plasmid (■, □), the vector pBR322 (●, ○), or pLCD2 (▲, △). The uptake and retention of labeled vitamin B_{12} were determined. The amount of radioactive substrate retained was measured with (■, ●, ▲) or without (□, ○, △) the addition of nonradioactive vitamin B_{12} to 10^{-7} M.

TABLE 3. Regulation of β-galactosidase production by *btuD-lacZ* and *btuB-lacZ* operon fusions

Strain	Relevant genotype	1 μM vitamin B_{12} in growth medium	β-Galactosidase activity (U)
RK6543	Φ(*btuD-lac*)*467*	−	92
		+	89
RK6544	Φ(*btuD-lac*)*468*	−	96
		+	94
RK6545	Φ(*btuD-lac*)*469*	−	94
		+	110
RK6546	Φ(*btuD-lac*)*470*	−	111
		+	104
RK5419	Φ(*btuB-lac*)	−	42
		+	11

tended from approximately 300 bp from the *Hin*dIII end to approximately 150 bp to the left of the central *Eco*RV site.Insertions on either side of this region (e.g., insertions 32-15 and 32-4) did not impair *btuC*-complementing activity. These results confirmed that the *btuC* region comprises 800 to 1,000 bp in the region between the *Hin*dIII and *Eco*RV sites.

Insertions resulting in the loss of *btuD*-complementing activity defined a contiguous region on the right side of the fragment. The boundaries for this gene could not be precisely stated, but the maximum size was 1,200 bp. Insertions on either side of the *btuD* region did not impair complementation activity, confirming the view that *btuC* and *btuD* are independent transcription units.

Insertions between *btuC* and *btuD* allowed the expression of both genetic activities. This region could be as large as 600 to 800 bp and thus could encode another protein.

Effect of *btuCD* plasmids on B_{12} uptake. Vitamin B_{12} transport activity in strains carrying the $btuCD^+$ plasmid pLCD2 was determined (Fig. 7). The presence of this plasmid resulted in elevated uptake rates in both $btuC^+$ and *btuC* strains, relative to these strains with or without the vector plasmid pBR322. Addition of nonradioactive vitamin B_{12} resulted in extensive loss of label in the *btuC* mutant. This chase did not occur when this strain carried plasmid pLCD2. Thus, this plasmid was able to complement a *btuC* mutant both for ability to retain B_{12} and with amplification of uptake activity.

Regulation of *btuD* expression. The expression of the *btuB*-coded receptor protein is subject to repression by growth with vitamin B_{12} (18). To examine whether *btuD* expression was also regulated in this manner, β-galactosidase production by strains carrying the four *btuD-lac* operon fusions was determined. These strains were made lysogenic for phage λ540$btuCD^+$, which carries the 3.4-kb *btuCD* region, to restore normal vitamin B_{12} transport activity. All four *btuD-lac* fusion-bearing strains, grown without or with 1 μM vitamin B_{12}, exhibited approximately the same levels of β-galactosidase (near 100 U) (Table 3). By contrast, the β-galactosidase activity of a strain carrying a *btuB-lacZ* operon fusion was in this range, but was repressed 3.4-fold by growth with 1 μM vitamin B_{12}. Thus, the expression of *btuD* appears not to be regulated by vitamin B_{12} levels in the growth medium.

DISCUSSION

This paper provides an initial characterization of genes in the *btuC* region which are involved in the transport or utilization of vitamin B_{12}. Mutant isolation and complementation tests with subclones and Tn*1000* insertions showed that at least two genes located in this region contribute to this function. The complementation analysis involving transformation of *btuC* or *btuD* mutant strains with various plasmids allowed the physical localization of the coding region for these genes on a 3.4-kb DNA segment. The two genes were expressed independently, and transposon insertions in one did not impair the expression of the other. This result showed that *btuC* and *btuD* are not part of the same transcription unit. The basic complementation results have been confirmed by using DNA fragments cloned into a λ vector and maintained at single copy, showing that the observed complementation behavior was not an aberration resulting from high dosage of these genes.

Strains carrying either the 8-kb *Pst*I fragment or the 3.4-kb *Hin*dIII-*Hin*cII fragment in multicopy plasmids exhibited elevated rates of vitamin B_{12} uptake. Retention of accumulated substrate was observed even when the host carried the *btuC* mutation. This result suggested that the cloned 3.4-kb fragment carries all of the genes in this vicinity that contribute to the uptake activity. The increase in the rate of transport was not proportional to the increase in gene copy number. However, the transport process is complex, involving in addition the outer membrane receptor and the *tonB* product. The site of the rate-limiting step is not known, but is probably not the transport across the cytoplasmic membrane. These results do not rule out a role for the products of *btuC* or *btuD* or both in the metabolic conversions of vitamin B_{12}, rather than its transport across the cytoplasmic membrane. Perhaps elucidation of the cellular location of these polypeptides might be informative.

The *btuD* mutations isolated in this study resulted from insertion of the transposable element λp*lac*Mu50 (9), which allows single-step formation of *lac* fusions to the target gene. Fusions in the *btuC* region were selected on the basis of the cotransduction of their Lac^+ character with a Tn*10* insertion near *btuC*. The fusions obtained in this manner could be transduced into other strains without serious problems from induction of the prophage or further transposition. The four fusions obtained were all to the *btuD* gene. Despite the fact that insertion of the λ prophage or deletions from the site of the prophage probably result in complete loss of this gene function, the phenotype of these mutants with respect to growth with B_{12} was markedly less severe than the phenotype of *btuC* mutants. This indicates that the *btuD* product is not essential for B_{12} utilization, even in strains lacking the outer membrane transport system. When assayed by uptake of labeled vitamin B_{12}, both *btuC* and *btuD* mutants have essentially identical transport properties. The growth response to vitamin B_{12} is a more sensitive assay than is uptake of labeled substrate, since uptake of as few as 25 molecules per cell per generation is sufficient to allow full growth (14).

Some of the deletions generated from the fusion strain retained kanamycin resistance, but had lost λ sequences and the adjacent *zdh-3* insertion. The simplest explanation is that the direction of transcription of *btuD* is toward *zdh-3* because the kanamycin resistance determinant lies between the *btuD* promoter and the λ sequences. Since these deletions did not remove *btuC*, *btuC* must lie on the other side of *btuD* from *zdh-3*. Hence the gene order in this region has to be *zdh-3-btuD-btuC-pheS*.

Recent observations (unpublished) showed that three polypeptides are encoded in the *btuC* region, showing that the region between *btuC* and *btuD* does encode a protein. It

is not possible to prove yet that the product of this middle gene is also involved in B_{12} uptake, since chromosomal mutations have not yet been obtained in this gene. Attempts are being made to cross the Tn*1000* insertions in this gene onto the chromosome to examine the B_{12} growth phenotype of these mutants.

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