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Genetic Analysis of Components Involved in Vitamin B_{12} Uptake in *Escherichia coli*

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The products of three genes are involved in cyanocobalamin (B_{12}) uptake in *Escherichia coli*. *btuB* (formerly *bfe*), located at min 88 on the *Escherichia coli* linkage map, codes for a protein component of the outer membrane which serves as receptor for B_{12}, the E colicins, and bacteriophage BF23. Four phenotypic classes of mutants varying in response to these agents were found to carry mutations that, based on complementation and reversion analyses, reside in the single *btuB* cistron. In one mutant class, ligand binding to the receptor appeared to be normal, but subsequent B_{12} uptake was defective. The level of receptor and rate of uptake were responsive to *btuB* gene dosage. Previous studies showed that the *tonB* product was necessary for energy-dependent B_{12} uptake but not for its binding. Other than those in *tonB*, no mutations that conferred insensitivity to group B colicins affected B_{12} utilization. The requirement for the *btuB* and *tonB* products could be bypassed by elevated levels of B_{12} (>1 μM) or by mutations compromising the integrity of the outer membrane as a permeability barrier. Utilization of elevated B_{12} concentrations in strains lacking the *btuB-tonB* uptake system was dependent on the function of the *btuC* product. This gene was located at 37.7 min on the linkage map, with the order *pps-btuC-pheS*. Strains altered in *btuC* but with an intact *btuB-tonB* system were only slightly impaired in B_{12} utilization, being defective in its accumulation. This defect was manifested as inability to retain B_{12}, such that intracellular label was almost completely lost by exchange or efflux. It is proposed that *btuC* encodes a transport system for B_{12} in the periplasm.

Cyanocobalamin (B_{12}) uptake by *Escherichia coli* is a biphasic process. The rapid, energy-independent initial phase represents binding to a receptor protein in the outer membrane of the cell envelope, and is followed by a slower, energy-dependent phase of B_{12} accumulation in the cell (9, 27, 30). The receptor protein is also involved in specific binding of the E colicins and bacteriophage BF23 (11). There is competition for binding to the cells or receptor among these three classes of ligands, and most mutants selected for resistance to these colicins or phage are deficient in B_{12} binding and transport (18). The binding activities reside in a 60,000-dalton protein present in 200 to 300 copies per cell (25, 30). Little is known about steps of B_{12} uptake subequent to binding to the receptor other than that accumulation is energized by the proton-motive gradient (3). The secondary, but not the initial, phase of uptake is dependent on the function of the *tonB* gene product (2).

Isolation of mutants defective in either phase of B_{12} uptake, by selection for decreased B_{12} utilization, has been described (10). The associated mutations were mapped adjacent to one another at 88 min on the revised linkage map (1). It was proposed (18), based on their different phenotypic properties, that the mutants carried lesions in two separate genetic loci. One class (termed BtuA, now BtuI) had normal binding activities but lacked the secondary phase of uptake. The other class (termed BtuB) had an altered receptor, being resistant to the E colicins and phage BF23 and lacking both phases of B_{12} uptake. The genetic location was very near that of the previously described *bfe* locus, encoding receptor for the E colicins and phage BF23 (5, 11, 16).

We describe here the genetic basis for the various phenotypic manifestations of mutations in *btuB*. From complementation, reversion, and localized comutagenesis analyses, we conclude that all of the phenotypes result from alterations within the same cistron. Although it was originally denoted *bfe*, we will employ the mnemonic *btuB* since it portrays the physiological role of the gene product in B_{12} uptake.

All mutants defective in B_{12} utilization owing

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to mutation at *btuB* or *tonB* were able to utilize higher levels of B_{12}. We define here the *btuC* locus, whose product plays a role in the response to B_{12} by some means independent of the *btuB-tonB* uptake system.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. The strains of *E. coli* K-12 employed are listed in Tables 1 and 2. Isolation of TonB$^-$ mutants and mutants resistant to the E colicins or phage BF23 has been described previously (2, 18). Common procedures for transduction mediated by phage P1 and for mutagenesis with phage Mu-1 or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine were employed (21). Bacteriophages P1*vir*, Mu-1, T4, and T7 are maintained as laboratory stocks. Colicin E3 was prepared and diluted in L broth as decribed previously (17). The minimal growth medium was medium A of Davis and Mingioli (8) supplemented with glucose (0.5%), required amino acids (100 μg/ml), thiamine (1 μg/ml), and B_{12} as specified. Medium in the plates was solidified with 2% agar; overlays contained 0.7% agar. Most antibiotics, detergents, and other chemicals were obtained from Sigma Chemical Co., St. Louis, Mo.

Genotype descriptions follow the recommendations of Bachmann et al. (1). Our phenotypic designations, along with previous designations, are presented in Table 3.

Stable episomes (F' factors) carrying the *argH-rpoB* region of the *E. coli* chromosome were obtained from Hfr strain Ra-2 by the method of Low (20). This Hfr strain was mated with the *recA1* recipient RK4150, with selection for Arg$^+$ clones, about 10% of which carried stable episomes capable of transferring both *argH*$^+$ and *rpoB*$^+$ at high frequency. Episomes carrying particular *btuB* alleles were obtained in a similar manner after transfer of the *btuB* allele into strain Ra-2 by cotransduction with *rpoB*.

Transport assays. For measurement of B_{12} uptake, growing cells were immediately mixed with cyanocobalamin labeled either with ^{57}Co (20.2 Ci/mmol; 5.0 nM, final concentration; Amersham/Searle, Arling-

TABLE 1. *Bacterial strains employed*

Strain	Genotype	Source/reference
KL96	Hfr *thi-1 rel-1*	J. Miller
Ra-2	Hfr *supE42 mal-28*	J. Miller
RK4101	F$^-$ *metE argH1 proC lysA leu tonA rpsL rpoB tsx*	18
RK4102	As RK4101 but *arg*$^+$ *btuB451*	18
RK4103	Aa RK4101 but *arg*$^+$ *btuB452*	18
RK4113	As RK4101 but *rpoB*$^+$	17
RK4117	As RK4113 but *non*	17
RK4118	As RK4117 but *arg*$^+$ *btuB451*	Transduction from RK4102
RK4119	As RK4117 but *arg*$^+$ *btuB454*	Transduction from RK4121
RK4120	As RK4101 but *lys*$^+$ *recA thyA*	From KL162 (*thyA recA*)
RK4121	As RK4101 but *btuB454*	Phage BF23-resistant
RK4122	As RK4101 but *btuB*::Mu	Mu-1 insertion in *btuB*
RK4123	As RK4113 but *btuB458 rpoB*	
RK4124	As RK4113 but *btuB459 rpoB*	
KBT101	F$^-$ *metE proC lysA leu trp purE tonA rpsL tsx btuC456*	10
KBT103	As KBT101 but *btuC457*	10
RK4136	F$^-$ *metE argE pyrE60 rpsL btuC455*	Derived from RK1034 (19)
RK4137	As RK4136 but *btuC*$^+$	
RK4138	As RK4137 but *arg*$^+$ *btuB452*	Transduction from RK4103
RK4139	As RK4136 but *arg*$^+$ *btuB452*	
RK4140	As RK4137 but *arg*$^+$ *butB454*	Transduction from RK4121
RK4141	As RK4136 but *arg*$^+$ *btuB454*	
RK4142	As RK4137 but *tonB2*	
RK4143	As RK4136 but *tonB2*	
RK4144	As RK4137 but *tonB15*	
RK4145	As RK4136 but *tonB15*	
RK4150	As RK4113 but *lys*$^+$ *recA1*	RK4113 × KL162
RK4152	As RK4150 but *rpoB btuB451*	RK4113 + P1 (RK4102), then × KL162
RK4154	As RK4150 but *rpoB butB452*	RK4113 + P1 (RK4103), then × KL162
RK4156	As RK4150 but *rpoB btuB454*	RK4113 × P1 (RK4121), then × KL162
RK4158	As RK4150 but *rpoB btuB458*	RK4113 + P1 (RK4123), then × KL162
RK4151	RK4150/F' *argH*$^+$ *btuB*$^+$ *rpoB*$^+$	
RK4196	F$^-$ *metE pyrE60 rpsL btuC*$^+$	From RK4137
RK4197	As RK4196 but *btuC455*	From RK4136
BW183	F$^-$ *pabB3 pps-2 pheS11 nadB30*	B. Weiss (29)
RK4527	As KBT101 but *btuB* (*tonB-trp*)Δ	Resistance to BF23 and φ80
RK4528	As KBT103 but *btuB* (*tonB-trp*)Δ	As above
RK4529	As RK4527 but *btuC*$^+$ *pps-2 pheS11*	RK4527 + P1 (BW183)
KL162	Hfr *thi thyA recA1*	K. B. Low

TABLE 2. *Merodiploid strains used*

Recipient *btuB* allele	Strain with no episome	Strain with *btuB* allele on F′ *arg*⁺ *rpoB*⁺			
		btuB⁺	*btuB451*	*btuB452*	*btuB454*
btuB⁺	RK4150	RK4151	RK4162	RK4181	RK4186
btuB451	RK4152	RK4153	RK4163	RK4182	RK4187
btuB452	RK4154	RK4155	RK4164	RK4183	RK4188
btuB454	RK4156	RK4157	RK4165	RK4184	RK4189
btuB458	RK4158	RK4159	RK4166	RK4185	RK4190

TABLE 3. *Properties of various btuB mutants*

Strain	Relevant phenotype	Response[a] to:		B_{12} binding[b]	Growth[c] on minimal agar plates with:						
		ColE3	BF23		Methionine (100 μg/ml)	B_{12} (M)					
						5×10^{-6}	5×10^{-7}	5×10^{-8}	5×10^{-9}	5×10^{-10}	5×10^{-11}
RK4101	Btu⁺	S	S	1.0	+++	+++	+++	+++	+++	+++	+++
RK4102	BtuI (*btuB451*, BtuA⁻)	S	S	1.0	+++	+++	+				
RK4103	BtuII (*btuB452*, BtuB⁻)	R	PR[d]	0[d]	+++	+++	+++	+++	++	+	
RK4121	BtuIII (*btuB454*, Bfe⁻)	R	R	0	+++	+++	+				
RK4122	BtuIII (Mu)[e]	R	R	0	+++	+++	+				
RK4123	BtuIV	S	S	0	+++	+++	+				
RK4124	BtuIV										

[a] S, Sensitive; R, resistant, PR, partially resistant.

[b] Relative binding (approximate) of labeled B_{12} to cells treated with NaF and NaN_3. Binding of strain RK4101 set at 1.0.

[c] Colony size relative to that of strain RK4101 on methionine after 48 h of growth on minimal medium with the indicated supplements.

[d] According to Bradbeer et al. (4), BtuII⁻ mutants average 0.5 receptor per cell.

[e] P1 transduction mapping showed that the Mu-1 prophage was inserted into the *btuB* gene and was responsible for the BtuIII⁻ phenotype.

ton Heights, Ill.) or with ^3H (4.5 Ci/mmol; 31.7 nM, final concentration; Amersham/Searle). Assays were usually carried out at 21°C; similar results were obtained at 37°C. At the indicated times, a 0.20-ml portion of the uptake mixture was transferred to the center of a membrane filter (0.45-μm-pore size; Millipore Corp., Bedford, Mass.), filtered, washed with 5 ml of medium A at room temperature, immediately removed from the filtration apparatus, and air dried. Radioactivity retained on the filters was measured in a scintillation counter with toluene-Omnifluor (New England Nuclear Corp.) and was corrected for label bound in the absence of cells. B_{12} binding was measured with cells poisoned by incubation with 100 mM NaF and 10 mM NaN_3 for 10 min before substrate addition.

RESULTS

B_{12} utilization in mutants resistant to colicin E3 and phage BF23. Mutation at *metE*, which codes a B_{12}-independent homocysteine transmethylase, results in a block in methionine synthesis that can be bypassed by a B_{12}-dependent methylase (*metH* product). This alternative requirement for methionine or B_{12} allows detection and selection of mutations affecting B_{12} uptake and utilization. Mutants obtained in a MetE⁻ strain by selection for decreased utilization of B_{12} as an alternative methionine source fell into two classes (10). One class exhibited no detectable binding or transport of labeled B_{12} and was partially resistant to the E colicins and phage BF23; however, their utilization of B_{12} was almost as efficient as that in the parental strain. To test the generality of this behavior, we measured growth on B_{12} of a large number of mutants selected directly for resistance to colicin E3 or phage BF23. The majority of the phage BF23-resistant derivatives of strain RK4101 (*metE*) were unable to utilize 5 nM B_{12}; these were termed BtuIII (Table 2). The BtuII class (ca. 10%) grew on 5 nM B_{12} but at less than the wild-type rate; they showed partial sensitivity to phage BF23, as do the previously described BtuB mutants (18). The remaining mutants were mucoid and utilized B_{12} efficiently; these were discarded.

Colicin E3-resistant mutants showed similar behavior, with the addition of a fourth class of nonmucoid strains capable of normal growth at 5 nM B_{12}. Sensitivity of this latter class to phage BF23 allowed their designation as colicin-tolerant mutants (17).

All mutants resistant to colicin E3 or phage BF23, although deficient in utilization of 5 nM B_{12}, responded at least partially to 500 nM B_{12}

and were identical to the wild type at 5 μM (Table 3). Even mutants induced by insertion of phage Mu-1 into *btuB* responded to elevated B_{12} concentrations. Except for mucoid and tolerant strains, none of over 2,000 mutants (whether spontaneous or mutagen induced and whether selected for resistance to colicin E3 or phage BF23) utilized 5 nM B_{12} normally or failed to utilize 5 μM B_{12}. A strain exhibiting the BtuI phenotype (formerly BtuA), which lacks the secondary phase of uptake and is unable to utilize 5 nM B_{12} (10), responded normally to 5 μM.

Localized mutagenesis in the *rpoB* region. Since the defect in BtuI mutants was located near mutations in BtuII strains (18), the possible presence of several cistrons involved with B_{12} uptake was investigated by employment of the comutagenesis procedure described by Oeschger and Berlyn (22). Strain RK4113 was treated with nitrosoguanidine, and rifampin-resistant mutants were selected and scored for defects in B_{12} utilization. Several were unable to respond to B_{12} at any concentration. Their normal B_{12} uptake and growth in the presence of B_{12} plus homocysteine indicated that they were defective in one of the methionine biosynthetic enzymes whose structural genes are near *rpoB*. Both BtuII and BtuIII mutants were obtained (46 of 4,000). None with a BtuI phenotype was found.

Two mutants exhibited unusual behavior (BtuIV). They lacked both phases of B_{12} uptake but remained sensitive to the E colicins and phage BF23. Even though there was little or no detectable binding of B_{12}, it still protected these cells against all three E colicins at least as well as in a Btu^+ strain.

Complementation analysis with merodiploid strains. All mutations conferring the BtuI, II, III, or IV phenotype were located between *argH* and *rpoB* (38 to 72% cotransduction with *argH*). To investigate the number of cistrons involved in B_{12} uptake, merodiploid strains were constructed combining representative alleles that confer the five Btu phenotypes.

Whenever one allele in the merodiploid strains was $btuB^+$, the strain was sensitive to colicin E3 and phage BF23 and grew normally at 5 and 0.5 nM B_{12} (Table 4). In no case did a mutant allele interfere with expression of the $btuB^+$ allele. In merodiploid strains combining mutant alleles, sensitivity to colicin E3 and phage BF23 was dominant to resistance and, as shown by Buxton (5), there was no complementation with respect to sensitivity. Similarly, utilization of 5 nM B_{12} was dominant to lack of response, and no combination of mutant alleles demonstrated complementation with respect to B_{12} utilization.

Uptake of [^{57}Co]B_{12} by merodiploid strains confirmed the failure to observe complementation with respect to B_{12} utilization (Table 4; Fig. 1). Both binding and transport were approximately doubled in strain RK4151 (Btu^+/Btu^+) relative to that in strain RK4150 (Btu^+). B_{12} binding to energy-poisoned cells of the diploid strain indicated the presence of 540 receptors per cell, compared with 220 receptors per cell of the haploid strain. These values agreed with the number of receptors calculated from the relative rate of phage BF23 adsorption (4).

Haploid levels of B_{12} binding and transport were observed when the episome carrying $btuB^+$ was present in 4 independent BtuII and 12 BtuIII recipients. (The contribution to B_{12} binding and transport by the episome-coded allele was usually in excess of haploid levels, reflecting the presence of more than one plasmid copy per chromosome in logarithmically growing cells [13].) Strain RK4163 (BtuI/BtuI) exhibited a twofold increase in B_{12} binding but totally lacked the secondary phase of uptake (Fig. 2). As with the growth responses, there was no complementation of B_{12} uptake when the allele conferring BtuI was combined with any of 15 alleles conferring BtuII, III, or IV behavior. No other combination of mutant alleles, tested with over 20 independent isolates, allowed higher levels of B_{12} uptake than were present in the parental haploid strains.

Bypass of B_{12} receptor. The marked decrease in B_{12} uptake upon loss of *btuB*-coded receptor activity signified that the outer membrane represented a considerable barrier for B_{12}. Mutants in which the barrier function of the outer membrane has been compromised might respond more efficiently to B_{12} in the absence of receptor. Many classes of mutants, including some phage T4-resistant strains, are said to possess "leaky" outer membranes. This defect in barrier function is manifested as increased sensitivity to certain antibiotics and detergents (12, 26).

A considerable number of phage T4-resistant derivatives of strain RK4121 (BtuIII) grew well at 50 nM B_{12} (Table 5). They remained resistant to phage BF23 and did not utilize lower levels of B_{12}, as could Btu^+ strains. Response to B_{12} was a stable property, but the cells yielded mucoid colonies and, typical of "membrane-leaky" mutants, were sensitive to deoxycholate and unable to grow on MacConkey agar. Detergent-resistant revertants had lost the ability to utilize 50 nM B_{12}. Identical behavior was observed with phage T4-resistant mutants of strain RK4102 (BtuI).

B_{12}-utilizing revertants. Since mutants with defective outer membranes could utilize B_{12} fairly efficiently even in the absence of re-

TABLE 4. *Properties of strains diploid for the btuB locus*

Strain	Relevant phenotype		Response[a] to:			Relative B_{12}[b]	
	Exogenote	Endogenote	ColE3	BF23	B_{12}[c] (nM)	Binding	Transport
RK4150		Btu$^+$	S	S	0.5	1.0	1.0
RK4151	Btu$^+$	Btu$^+$	S	S	0.5	2	2
RK4153	Btu$^+$	BtuI	S	S	0.5	2	1
RK4155	Btu$^+$	BtuII	S	S	0.5	1	1
RK4157	Btu$^+$	BtuIII	S	S	0.5	1	1
RK4159	Btu$^+$	BtuIV	S	S	0.5	1	1
RK4152		BtuI	S	S	5,000	1	0
RK4162	BtuI	Btu$^+$	S	S	0.5	2	1
RK4163	BtuI	BtuI	S	S	5,000	2	0
RK4164	BtuI	BtuII	S	S	50	1	0
RK4165	BtuI	BtuIII	S	S	5,000	1	0
RK4166	BtuI	BtuIV	S	S	5,000	1	0
RK4154		BtuII	R	PR	50	0	0
RK4181	BtuII	Btu$^+$	S	S	5	1	1
RK4182	BtuII	BtuI	S	S	50	1	0
RK4183	BtuII	BtuII	R	PR	50	0	0
RK4184	BtuII	BtuIII	R	PR	50	0	0
RK4185	BtuII	BtuIV	S	S	50	0	0
RK4156		BtuIII	R	R	5,000	0	0
RK4186	BtuIII	Btu$^+$	S	S	5	1	1
RK4187	BtuIII	BtuI	S	S	5,000	1	0
RK4188	BtuIII	BtuII	R	PR	50	0	0
RK4189	BtuIII	BtuIII	R	R	5,000	0	0
RK4190	BtuIII	BtuIV	S	S	5,000	0	0
RK4158		BtuIV	S	S	5,000	0	0

[a] S, Sensitive; R, resistant; PR, partially resistant.

[b] Approximate relative B_{12} binding and initial rate of B_{12} uptake. Values for strain RK4150 were set at 1.0.

[c] Approximate minimum B_{12} concentration required for normal growth after 48 h as compared with that for strain RK4101 on methionine.

ceptor, this might provide a useful "positive" selection for mutants with altered permeability. Derivatives of strains RK4102 (BtuI) and RK4121 (BtuIII) were selected for the ability to grow on 5 nM B_{12}. Some derivatives gave large, nonmucoid colonies and represented revertants to either MetE$^+$ or BtuB$^+$. Further incubation of selection plates for 4 to 5 days revealed large numbers of mucoid colonies (10^{-4} to 10^{-5} per cell). The appearance of mucoid revertants was circumvented by selection in strains carrying a *non* mutation, which blocks capsule formation (24). *non* strains (whether Btu$^+$, BtuI, or BtuIII) could utilize lower concentrations of B_{12} than their *non*$^+$ counterparts (Table 4). The capsule present in *non*$^+$ strains may limit access of B_{12} to the cell surface. Nonmucoid, Met$^-$ B_{12} utilizers were selected from *non* strains RK4118 (BtuI) and RK4119 (BtuIII) at 50 nM B_{12}. Those derived from the BtuIII strain were still totally resistant to colicin E3 and phage BF23. As expected, B_{12} utilization was considerably more efficient than that of the parents, although less so than in Btu$^+$ strains. However, most responders obtained by direct selection were quite unstable. Growth on media supplemented with methionine or even 5 μM B_{12} for one passage resulted in appearance of variants no longer able to utilize 5 nM B_{12}. Further genetic analysis of this response was hampered by the marked instability.

Alteration of the permeability properties of the outer membrane was measured by response of representative B_{12} utilizers to various antibiotics and detergents. Several strains were spread on plates with 5 nM B_{12}, and sterile paper disks containing test substances were placed on the lawn. As determined from diameters of zones of inhibition after overnight incubation, the B_{12}-responder mutants tested were considerably more sensitive than their parents to neomycin, kanamycin, mitomycin C, penicillin G, and nalidixic acid. They were slightly more sensitive to spectinomycin and chloramphenicol and, in con-

trast to the insensitivity of their parents, were quite sensitive to erythromycin, Triton X-100, and sodium dodecyl sulfate. Analogous second-site revertants of TonB$^-$ mutants show identical behavior (2). Neither phage T4-resistant nor directly selected B_{12} utilizers exhibited appreciable B_{12} uptake under the usual assay conditions.

Group B colicin-insensitive mutants. Over 100 mutants insensitive to various group B colicins (B, D, G, Ia, M, and V [7]) were isolated from strain RK4126. Only those classified as TonB (insensitive to all group B colicins and to phage ϕ80) were defective in utilization of 50 or 0.5 nM B_{12}. All strains responded well at 5 μM B_{12}.

Low-affinity utilization of B_{12}. Strains lacking either the *btuB* or *tonB* function still utilized B_{12}, although at least 10^4-fold higher concentrations were necessary for comparable growth. Several strains were fortuitously found to lack low-affinity utilization. Strain RK4136 was a laboratory strain unrelated to the other strains used in this study. This MetE$^-$ strain utilized 5 nM B_{12} normally and was sensitive to the E colicins and phage BF23. Recombinants inheriting *btuB* mutations from BtuI, II, or III mutants (by cotransduction with *argH*), were unable to utilize B_{12} even at a concentration of 500 μM. *btuB* recombinants in this genetic background also did not give rise to the B_{12}-utilizing

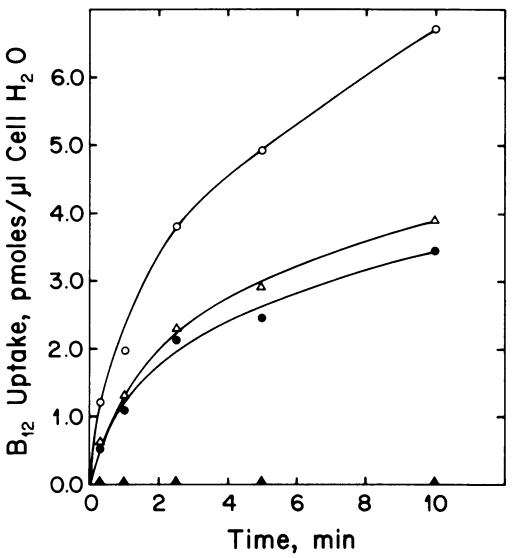

FIG. 1. *B_{12} uptake in haploid and merodiploid strains. Uptake of [^{57}Co]B_{12} in growing cells of strains RK4150 (Btu$^+$; ●), RK4151 (Btu$^+$/Btu$^+$; ○), RK4156 (BtuIII$^-$; ▲), and RK4157 (Btu$^+$/BtuIII$^-$; △).*

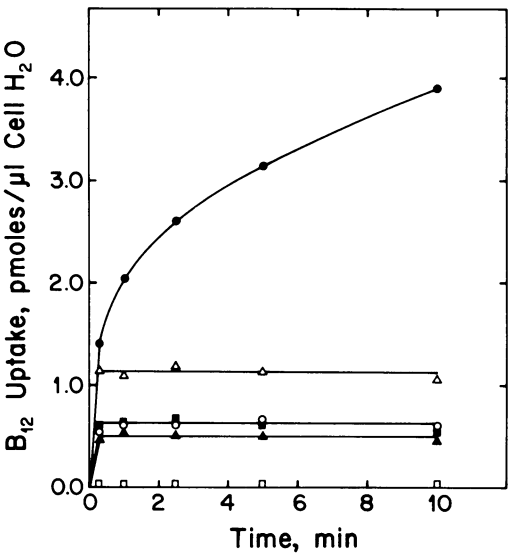

FIG. 2. *B_{12} uptake in haploid and merodiploid strains. Uptake of [^{57}Co]B_{12} in growing cells of strains RK4152 (BtuI$^-$; ▲), RK4154 (BtuII$^-$; □), RK4162 (Btu$^-$/Btu$^+$; ●), RK4163 (BtuI$^-$/BtuI$^-$; △), RK4164 (BtuI$^-$/BtuII$^-$; ■), and RK4165 (BtuI$^-$/BtuIII$^-$; ○).*

TABLE 5. *Growth on B_{12} of mutants with altered outer membrane permeability*

Strai	Relevant phenotype	Growth[a] on minimal agar plates with:							
		Methionine (100 µg/ml)	B_{12} (M)						
			5×10^{-6}	5×10^{-7}	5×10^{-8}	5×10^{-9}	5×10^{-10}	5×10^{-11}	5×10^{-12}
RK4101	But$^+$	+++	+++	+++	+++	+++	+++	+	
RK4121	BtuIII	+++	+++	+					
RK4121-1	BtuIII Tfr[b]	+++	+++	+++	+++	+			
RK4117	Non Btu$^+$	+++	+++	+++	+++	+++	+++	++	+
RK4118	Non BtuI	+++	+++	+++	+				
RK4118-1	Non BtuI B_{12}^{+c}	+++	+++	+++	+++	+++	+		
RK4119	Non BtuIII	+++	+++	+++	+				
RK4119-1	Non BtuIII B_{12}^{+c}	+++	+++	+++	+++	+++	+		

[a] Colony size after 48 h of growth relative to that of strain RK4101 on methionine.
[b] Representative of several spontaneous phage T4-resistant isolates which responded to 50 nM B_{12}.
[c] Growth of these B_{12}-utilizing strains on methionine resulted in loss of their ability to grow on 5 nM B_{12}.

revertants described above; only Met^+ or Btu^+ revertants were recovered. Identical behavior was exhibited by two mutants, KBT101 and KBT103, that had previously been described as being altered in some aspect of B_{12} transport or utilization (10). The missing locus allowing utilization of high levels of B_{12} in strains lacking the *btuB-tonB* system was designated *btuC*.

Genetic location of *btuC*. Inheritance of *btuC* alleles was scored by testing for utilization of 5 μM B_{12} in strains also altered in *btuB* or *tonB*. Conjugal crosses with several Hfr strains localized the lesion between *his* and *trp*. Interrupted matings with Hfr strain KL96 revealed that the ability to utilize 5 μM, but not 5 nM, B_{12} entered approximately 6 min after his^+ and 10 min before trp^+, indicating a location on the chromosome represented by 38 min on the recalibrated linkage map (1).

The order of several genes in this region of the chromosome has been determined (28, 29). The location of *btuC* with respect to some of these markers was determined by phage P1-mediated transduction. The *btuC* recipient strains, carrying mutations in *metE, btuB,* and *tonB*, were unable to utilize B_{12}. Phage P1 propagated on strain BW183 (*pheS pps pabB*) donated the ability to utilize 5 μM B_{12}. Of the $MetE^-$ $BtuC^+$ recombinants, approximately 90% had inherited the donor *pheS* marker, 64% had inherited the donor *pps* marker, and none had inherited the donor *pabB* marker (Table 6). A recombinant carrying mutations in *pheS* and *pps* (as well as in *metE, btuB,* and *tonB*) was transduced to pps^+ (growth on lactate as the sole carbon source) with P1 phage propagated on any of three strains that exhibited the $BtuC^-$ phenotype. Identity of the minority class of recombinants and cotransduction frequencies were consistent only with the gene order: *pps-btuC-pheS* (Table 5). *btuC* was placed at 37.7 min on the linkage map, approximately 0.16 min from *pps* and 0.08 min from *pheS*. Identical locations were deduced from crosses with all three *btuC* alleles or with a *pheS*(Ts)*5* allele as the selective marker.

Growth properties of $BtuC^-$ mutants. B_{12} utilization was determined in isogenic strains differing at *btuC* and carrying other mutations affecting B_{12} uptake (Table 7). Both *btuB* (I, II, or III) *btuC* and *tonB btuC* double mutants were unable to utilize B_{12}, even at 0.5 mM. Mutation at *btuC* did not affect the response to a number of antibiotics and detergents. Although the *btuC* mutation did not affect growth of Btu^+ $TonB^+$ strains on 5 nM B_{12}, utilization of lower concentrations was impaired. A $btuC^+$ strain achieved

TABLE 6. *Localization of btuC by phage P1-mediated transduction*[a]

Donor strain	Recipient strain	Selected marker	No. analyzed	Recombinant class	No.	%
BW183 (*pheS pps pabB*)	RK4527 (*btuC456*)	$btuC^+$	42	*pheS*	39	92
				pps	27	64
				pabB	0	
BW183	RK4528 (*btuC457*)	$btuC^+$	91	*pheS*	82	90
				pps	58	64
				pabB	0	
RK4527 (*btuC456*)	RK4529 (*pps pheS*)	pps^+	800	$pheS^+$ *btuC*	554	69.3
				$pheS^+$ $btuC^+$	8	1.0
				pheS btuC	74	9.3
				pheS $btuC^+$	164	20.5
RK4528 (*btuC457*)	RK4529	pps^+	699	$pheS^+$ *btuC*	472	67.4
				$pheS^+$ $btuC^+$	10	1.4
				pheS btuC	69	9.9
				pheS $btuC^+$	148	21.1
RK4136 (*btuC455*)	RK4529	pps^+	946	$pheS^+$ *btuC*	627	66.3
				$pheS^+$ $btuC^+$	11	1.2
				pheS btuC	101	10.7
				pheS $btuC^+$	207	21.9

[a] *pps* — 0.16 min — *btuC* — 0.08 min — *pheS*; 0.23 min

TABLE 7. *Growth of btuC strains with* B_{12}

Strain	Relevant genotype	Growth[a] on minimal agar plates with: Methionine (100 μg/ml)	B_{12} (M) 5×10^{-6}	5×10^{-7}	5×10^{-8}	5×10^{-9}	5×10^{-10}	5×10^{-11}
RK4136	*btuC455*	+++	+++	+++	+++	+++	+	
RK4137	*btuC*$^+$	+++	+++	+++	+++	+++	+++	+
RK4138	*btuB452*	+++	+++	+++	+++	++	+	
RK4139	*btuB452 btuC*	+++	(−)[b]					
RK4140	*btuB454*	+++	+++	+				
RK4141	*btuB454 btuC*	+++	(−)[b]					
RK4142	*tonB451*	+++	+++	++	+			
RK4143	*tonB451 btuC*	+++	(−)[b]					
RK4144	*tonB452*	+++	+++	+++	+++	++	+	
RK4145	*tonB452 btuC*	+++	(−)[b]					

[a] Colony size after 48 h relative to that of strain RK4137 on methionine.
[b] These strains did not respond to a B_{12} concentration of 5×10^{-4} M.

half-maximal growth rate with 0.24 nM B_{12}, whereas a *btuC* strain required 4 nM B_{12} for comparable growth. Both strains had the same growth rate on 30 nM B_{12} or methionine (65 min).

When *btuC*$^+$ strain RK4196 was grown overnight on 50 nM B_{12} and then was washed and suspended in minimal growth medium lacking a methionine source, it underwent almost five doublings before methionine limitation occurred. Under identical conditions, *btuC* strain RK4197 underwent only one doubling. Cells of either strain grown overnight with methionine increased by less than 50%. This suggested that BtuC$^-$ mutants were impaired either in accumulation or retention of B_{12}.

B_{12} uptake in *btuC* strains. Binding and uptake of B_{12} were measured in strains differing at *btuC*. Amounts of B_{12} bound to energy-poisoned cells were essentially identical. Energy-dependent B_{12} uptake clearly differed (Fig. 3). Initial rates of uptake were fairly similar, but a marked disparity was apparent at later times in the uptake process. B_{12} was extensively accumulated by the *btuC*$^+$ strain, whereas uptake reached a plateau in *btuC* strains.

Exchange and chase experiments indicated that the defect might not be at the influx stage but in retention of substrate. Addition of excess unlabeled B_{12} after a period of substrate accumulation resulted in loss of the label (Fig. 3). A *btuC*$^+$ strain rapidly lost up to half of the accumulated label with a half-time of 3.5 to 4.5 min; remaining label was lost very slowly. In a *btuC* strain, most of the label (>80%) was lost at the rapid rate. These results were obtained with [^{57}Co]- or [^{3}H]B_{12}. DiGirolamo and Bradbeer (unpublished data) had also noted a rapid loss of substrate from strains KBT101 and KBT103, which were shown here to carry mutations in *btuC*.

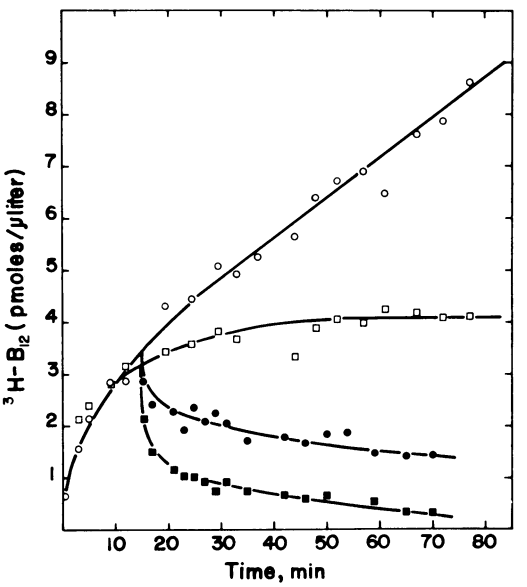

FIG. 3. *B_{12} uptake and exodus in btuC*$^+$ *and btuC strains. At zero time, [^{3}H]B_{12} was added to growing cells of RK4196 (btuC*$^+$*; ○, ●) or RK4197 (btuC457; □, ■). To a duplicate uptake mixture, unlabeled B_{12} was added to 560 nM (●, ■) at 15 min.*

Addition of unlabeled B_{12} was not required to observe deficient retention in *btuC* strains. Strains RK4196 (*btuC*$^+$) and RK4197 (*btuC*) were allowed to accumulate labeled B_{12} for 10 min. The cells were then collected on filters and washed with 5 or 40 ml of medium A. Relative to cells washed with 5 ml, BtuC$^+$ cells washed with 40 ml lost 1 to 5% of the total label. In contrast, BtuC$^-$ cells lost 20 to 35%.

Transport of maltose and of a number of amino acids was unaffected by the *btuC* mutation (data not shown).

DISCUSSION

At least four different phenotypic classes of mutants with altered B_{12} uptake carry lesions in the *argH-rpoB* region. Several lines of evidence suggest that, contrary to an earlier proposal (18), all responses are manifestations of alterations in a single cistron, *btuB*. Lack of complementation between BtuI and other Btu$^-$ mutants is inconsistent with their representing alterations of separate cistrons. Subunit interference or polarity effects have not been eliminated, but are unlikely to be responsible for the absence of complementation, in light of the inability of BtuI to complement any of a number of Btu$^-$ mutants. Less than half of phage BF23-resistant mutants are suppressible by *supD* (2). In addition, the parental strain in these studies carries low-level suppressor activity. BtuII mutants have some synthesis of receptor which could have complemented BtuI mutants that ostensibly are altered in the promoter-distal locus. Second, BtuI mutants responded to high levels of B_{12} and gave rise to similar B_{12}-utilizing revertants defective in outer membrane function as did receptor-deficient mutants. This suggested that the structure altered in the BtuI mutant is not necessary for B_{12} uptake once the barrier of the outer membrane has been passed. Third, no additional BtuI mutants were obtained in a comutagenesis procedure that yielded numerous BtuII, III, and IV mutants.

The BtuI phenotype may result from an alteration of receptor protein such that its ligand-binding sites are unaffected, but interaction with a subsequent component for uptake, possibly *tonB* product, is defective. Attempts to find alterations in *tonB* among B_{12}-utilizing revertants of BtuI were hampered by the frequent appearance of variants with generalized permeability alterations. Verification of this model must await chemical comparison of receptors from wild-type and mutant strains.

As proposed by Bradbeer et al. (4), the BtuII phenotype can be explained by decreased production of normal receptor. Fine-structure mapping of *btuB* might be useful in distinguishing whether BtuII results from promoter mutations or structural alterations reducing the rate of insertion of this protein into the membrane. The nature of the BtuIV phenotype (sensitivity to colicin E3 and phage BF23, but no transport or binding of B_{12}) is under investigation. A possibly analogous situation has been observed for phage λ receptor, in which receptor still adsorbs phage normally, but can no longer facilitate permeation of maltose across the outer membrane (M. Schwartz, personal communication).

Both binding and transport of B_{12} were affected by *btuB* gene dosage. Thus, the outer membrane can readily accept additional B_{12} receptors in functional form, and the small number of receptors per cell must not result from the presence of limited numbers of insertion sites. In contrast, Datta et al. (6) have shown that the level of a major outer membrane protein (II* or 3a) was not elevated in a diploid strain. Dependence of B_{12} uptake rate on the number of receptors suggests the level of receptor is limiting and subsequent components are in excess.

Existence of a *btuB-tonB*-independent B_{12} uptake system is obvious from the utilization of high levels of B_{12} in mutants carrying deletions or phage Mu-1 insertions in these genes. Moderate disruption of the barrier properties of the outer membrane enhanced B_{12} utilization by this low-affinity system. The *btuC* product is a component of this system, since mutants altered in this gene totally lack *btuB-tonB*-independent uptake or utilization. Since strains altered only in *btuC* are not seriously impaired in B_{12} utilization, this product is probably not necessary for the function of the *btuB-tonB* uptake system, for metabolism of B_{12} to coenzyme forms, or for formation of other intermediates in methionine biosysthesis. Derangement of the outer membrane did not bypass the defect in BtuC$^-$. The results point to the existence of a *btuC*-coded B_{12} uptake system in the cytoplasmic membrane, independent of the *btuB-tonB* uptake system and acting on substrate in the periplasm. The apparent lower efficiency of this system and the lack of appreciable uptake of labeled B_{12} by *btuB* or *tonB* mutants underscore the low rate of B_{12} permeation across the outer membrane in absence of receptor.

The observed effect of *btuC* alteration on B_{12} uptake can be accounted for with several ad hoc assumptions. Assume that B_{12} accumulation represents a summation of influx and efflux steps and that influx via the *btuB-tonB* system acts only on extracellular substrate, but that efflux via this system releases substrate both to the periplasm and the extracellular space. Then, the lack of a system able to take up periplasmic substrate would be manifested as a lower steady-state level and more extensive loss by exchange, both of which are observed. Kinetic parameters of low-affinity uptake cannot be measured in cells owing to lack of knowledge concerning the periplasmic concentration of substrate and the rate of B_{12} diffusion through nonspecific pores in the outer membrane.

Several points concerning the genetic location of *btuC* are noteworthy. *btuC* is almost exactly 180° across the chromosome map from *btuB*, which might point to their physiological or genetic relatedness (31). Both genes are linked to

a number of other cistrons involved with vitamin synthesis or transport. Almost half of the known operons coding some step of vitamin metabolism are located in the two 5-min segments flanking *btuB* and *btuC* (17 loci between 85 and 90 min, and 5 loci between 35 and 40 min [1]).

The substrate specificity of low-affinity B_{12} uptake from the periplasm is unknown. It is difficult to propose the selective advantage for the existence of two independent systems specific for a substrate that is neither synthesized nor required. It has been proposed that ferric-enterochelin can be transported from the periplasm without participation of specific receptor in the outer membrane or *tonB* product (14), although both proteins are necessary for efficient uptake from the medium (15, 23). Possible involvement of *btuC* product in iron uptake is under study. One possibly important point from the properties of $BtuC^-$ strains is that B_{12} uptake from the periplasm, mediated by *btuC* product, does not appear to be a part of receptor-mediated uptake via the *btuB-tonB* system.

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