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Two Outer Membrane Transport Systems for Vitamin B_{12} in *Salmonella typhimurium*

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The involvement of an outer membrane transport component for vitamin B_{12} uptake in *Salmonella typhimurium*, analogous to the *btuB* product in *Escherichia coli*, was investigated. Mutants of *S. typhimurium* selected for resistance to bacteriophage BF23 carried mutations at the *btuB* locus (*butB*$_S$) (formerly called *bfe*, at the analogous map position as the *E. coli* homolog) and were defective in high-affinity vitamin B_{12} uptake. The cloned *E. coli btuB* gene (*btuB*$_E$) hybridized to *S. typhimurium* genomic DNA and restored vitamin B_{12} transport activity to *S. typhimurium btuB*$_S$ mutants. An M_r-60,000 protein in the *S. typhimurium* outer membrane was repressed by growth with vitamin B_{12} and was eliminated in a *btuB*$_S$ mutant. The *btuB*$_S$ product thus appears to play the same role in vitamin B_{12} transport by *S. typhimurium* as does the *E. coli btuB*$_E$ product. A second vitamin B_{12} transport system that is not present in *E. coli* was found by cloning a fragment of *S. typhimurium* DNA that complemented *btuB* mutants for vitamin B_{12} utilization. In addition to this plasmid with a 6-kilobase insert of *S. typhimurium* DNA, vitamin B_{12} utilization by *E. coli btuB* strains required the *btuC* and *btuD* products, necessary for transport across the cytoplasmic membrane, but not the *btuE* or *tonB* product. The plasmid conferred low levels of vitamin B_{12}-binding and energy-dependent transport activity but not susceptibility to phage BF23 or utilization of dicyanocobinamide. The cloned *S. typhimurium* DNA encoding this new transport system did not hybridize to the *btuB*$_E$ gene or to *E. coli* chromosomal DNA and therefore does not carry the *S. typhimurium btuB*$_S$ locus. Increased production of an M_r-84,000 polypeptide associated with the outer membrane was seen. The new locus appears to be carried on the large plasmid in most *S. typhimurium* strains. Thus, *S. typhimurium* possesses both high- and low-affinity systems for uptake of cobalamins across the outer membrane.

Escherichia coli possesses a high-affinity active transport system for vitamin B_{12} (CN-Cbl) and other cobalamins. The first stage of this complex transport process is the energy-independent binding to the outer membrane protein BtuB (K_d, 0.5 nM), present at about 200 copies per cell (38). The M_r-66,000 BtuB polypeptide is also the receptor for bacteriophage BF23 and the E colicins (6, 7, 13). Active transport of cobalamins into the periplasmic space by BtuB requires the proton motive force and the *tonB* product (1, 5, 32). Mutants lacking BtuB or TonB require 10^4-higher concentrations of CN-Cbl than do wild-type cells for methionine synthesis (2), which demonstrates the requirement for the outer membrane transport system. Transport of vitamin B_{12} across the cytoplasmic membrane requires the membrane-associated BtuC and BtuD proteins, encoded by the *btuCED* operon (11, 16), and may be driven by phosphate bond energy (32).

The process of vitamin B_{12} transport in *Salmonella typhimurium* is likely to be similar to that in *E. coli*. Rough strains of *S. typhimurium* are susceptible to phage BF23 and, if the *tolAB* region from *E. coli* is present, to the E colicins (18, 30). Resistance to these lethal agents results from mutations in *bfe* (susceptibility to phage BF23 and the E colicins), which is located at the position on the *S. typhimurium* chromosome map (88 min) analogous to that of the *E. coli btuB* gene (*btuB*$_E$) (30). In the latest version of the *S. typhimurium* genetic map (35), the *bfe* locus was renamed *btuB*. We describe here genetic evidence for the role of the *S. typhimurium btuB* (*btuB*$_S$) product in cobalamin transport in *S. typhimurium*. Mutations at *btuB*$_S$ that confer resistance to phage BF23 severely impaired vitamin B_{12} transport ability, and this impairment could be corrected by introduction of the cloned *btuB*$_E$ gene.

Evidence was obtained for the presence of another, minor cobalamin uptake system that is not present in *E. coli* and for the high-affinity *btuB*-dependent system. An *S. typhimurium* DNA fragment that complemented *btuB* mutants for growth with CN-Cbl was cloned. The presence of this plasmid resulted in expression of a new outer membrane protein and conferred low levels of cobalamin binding and transport.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. Bacterial strains used in this study (Table 1) are derivatives of *S. typhimurium* LT2 or *E. coli* K-12. Plasmid pKH3-8 carries the *E. coli btuB* gene on a 2.3-kilobase (kb) insert in the *Bam*HI site of pBR322 (21). Bacteriophage BF23 was from laboratory stock. Generalized transduction in *S. typhimurium* was done with phage P22 HT105/1 *int-201* (from K. E. Sanderson), as described by Roth (34), with phage lysates prepared by the method of Davis et al. (10). For infection with P22, *galE* strains were grown in the presence of 0.05% galactose.

Media and growth conditions. Bacteria were grown aerobically at 37°C in L medium or in minimal medium A containing 0.4% glucose supplemented with required amino acids (100 μg/ml) and vitamin B_{12} at the indicated concentrations (28). Utilization of vitamin B_{12} by *metA* strains was tested in the presence of 0.01% DL-homocysteine thiolactone hydrochloride. Final concentrations of antibiotics were 25

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TABLE 1. *S. typhimurium* and *E. coli* strains used

Strain	Genotype	Source or reference
S. typhimurium LT2		
LB5010	*metA22 metE551 ilv-452 leu-3121 trpC2 xyl-404 galE856 hsdL6 hsdSA29 hsdSB121 rpsL120* H1-b H2-e,n,x *fla-66* nml(−) Fel-2(−)	K. E. Sanderson
ST422	*recA1 metA22 metE551 trpC2 ilv-452 flaA66 rpsL120 xyl-404 hsdT6 hsdSA29 galE496* H1-b nml H2-e,n,x(Fels2)	K. E. Sanderson
RK7405	ST422 *btuB*	This study
TR2299	*hisC527 metE338 pur-847 galE542*	J. R. Roth
TT137	*argH1823*::Tn*10*	K. E. Sanderson
RK7425	LB5010 *argH1823*::Tn*10*	P22(TT137)→LB5010
χ3300	Wild type, pStLT100$^+$	17; R. Curtiss III
χ3344	χ3300, but pStLT100$^-$	17; R. Curtiss III
E. coli K-12		
LE392	*hsdR514* (r_K^- m_K^-) *supE44 supF58 lacY galK2 galT22 metB1 trpR55* λ$^-$	N. E. Murray
MC4100	(*argF-lac*)U169 *araD139 relA1 rpsL150 flbB5301 deoC1 tonA21 thi*	8
RAM105	MC4100 Δ*ompF80 zeiO6*::Tn*10 ompC119*	27
RK4379	MC4100 *non gyrA219 ton*$^+$ *metE70*	20
RK4793	RK4379 *btuB493*	20
RK5016	RK4379 *btuB461 argH1 recA56*	20
RK5041	RK4379 *recA56*	
RK5048	RK4379 *recA56* Δ*tonB499*	
RK6006	RK4379 *recA56 btuC456 zdh-600*::Tn*5*	12
RK6532	MC4100 Δ*metE71 btuD467*::*plac*Mu50 *btuB491*	12
RK6555	RK4379 Δ*btuD471 zdh-3*::Tn*10*	12
RK6686	RK4379 *btuC480*::Tn*1000 btuB493 zdh-3*::Tn*10*	11
RK7817	RK4379 Δ*btuE501,502 zdh-3*::Tn*10*	
RK7822	RK4793 Δ*btuE501,502 zdh-3*::Tn*10*	

and 20 μg/ml for ampicillin (sodium salt) and tetracycline, respectively.

Chemicals. Carbonyl cyanide *m*-chlorophenylhydrazone, purified maltodextrins (maltotriose, maltotetraose, and maltopentaose), and vitamin B_{12} and its related compounds were obtained from Sigma Chemical Co. (St. Louis, Mo.). Cobalt-57-labeled cyanocobalamin (CN[^{57}Co]Cbl; 20.3 Ci/mmol), [α-^{32}P]dCTP (3,000 Ci/mmol), and [^{35}S]methionine (>800 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, Ill.).

DNA extractions. Chromosomal DNA was prepared as described by Silhavy et al. (36). Plasmids were isolated from cultures amplified with chloramphenicol or spectinomycin and purified by CsCl-ethidium bromide equilibrium centrifugation (27, 36). For rapid screening, plasmid DNA was extracted with alkaline sodium dodecyl sulfate (4). DNA concentrations were determined fluorometrically by the method of Labarca and Paigen (24).

Recombinant DNA methods. Plasmid DNA was introduced into bacterial cells by calcium chloride-mediated transformation (27). The method of Hanahan (19) was used for transformation of ligation products into recipient cells. Because of the low efficiency of transformation into *S. typhimurium* strains, ligation mixtures were first amplified in *E. coli* LE392 (r^- m^+).

Digestions with restriction endonucleases and ligations with T4 DNA ligase were carried out as recommended by the manufacturers (Bethesda Research Laboratories, Inc., Gaithersburg, Md., and New England BioLabs, Inc., Beverly, Mass.). Restriction fragments were separated by electrophoresis on agarose gels with Tris-borate-EDTA buffer and stained with ethidium bromide (27). DNA fragments were extracted from agarose gels by freezing in the presence of phenol (3). Linear pBR322 DNA was dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) by the procedure of Maniatis et al. (27) and purified by electrophoresis on 0.7% agarose.

Isolation of *bfe* mutants of *S. typhimurium*. Spontaneous mutants of *galE* strain ST422 resistant to phage BF23 were selected by incubating 10^8 cells and 4×10^{10} PFU of BF23 at 37°C for 10 min. Washed cells were spread on L-agar plates, and survivors were tested for response to phage BF23 by cross-streaking.

Cloning of *S. typhimurium* genomic DNA in pBR322. *S. typhimurium* TR2299 DNA was partially digested to a size range of 5 to 10 kb with *Sau*3A (27). These fragments were extracted with phenol-chloroform (1:1, vol/vol) and chloroform and precipitated with ethanol. Approximately 1 μg of genomic DNA fragments was ligated with 2 μg of pBR322 that had been digested with *Bam*HI and treated with alkaline phosphatase. The ligation mixture was transformed into *E. coli* LE392 and spread on ampicillin-containing L-medium plates. A total of 4,000 Amp^r colonies were pooled and stored at −70°C. Whenever cells were selected for growth with CN-Cbl, tests were made to ensure that reversion of the *metE* marker had not occurred.

Southern hybridization. Nucleic acid hybridization was detected by a procedure similar to that of Southern (37). DNA fragments separated by electrophoresis in agarose gels were transferred to nylon membranes by capillary blot and hybridized to probes that were labeled with [α-^{32}P]dCTP by nick translation (by using the kit from Bethesda Research Laboratories) or the random-primed DNA-labeling system of Boehringer Mannheim. Conditions for transfer and hybridization were as recommended in the GeneScreen manual (Dupont, NEN Research Products, Boston, Mass.).

Analysis of outer membrane proteins. Cells from an overnight culture were harvested and suspended in 10 mM sodium phosphate (pH 7.0). The cells were disrupted by two passages through a French pressure cell at 20,000 lb/in^2. The cell lysate was centrifuged at 4,500 × *g* for 10 min at 4°C, and

the cell envelope fraction was isolated by centrifugation at 100,000 × *g* for 40 min at 4°C. The cytoplasmic membrane was removed by differential solubilization with sodium lauryl sarcosinate (14). The outer membrane was sedimented by centrifugation at 100,000 × *g* for 40 min at 4°C. Protein content was determined by the procedure of Lowry et al. (25), with bovine serum albumin as the standard. Proteins were resolved by electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate (26) and visualized by silver staining (31).

Vitamin B_{12} binding and transport assays. Binding and uptake of CN[^{57}Co]Cbl by *E. coli* and *S. typhimurium* cells were measured as described elsewhere (30; C. R. Rioux and R. J. Kadner, Mol. Gen. Genet., in press). Inhibitors were present during the 5-min incubation before addition of substrate. Results are expressed as picomoles of vitamin B_{12} bound or accumulated per 10^9 cells.

RESULTS

Role of *Salmonella btuB* product in vitamin B_{12} transport. Vitamin B_{12} (CN-Cbl) utilization can be assessed in *metE* mutants of *E. coli* or *S. typhimurium*, which lack the cobalamin-independent homocysteine methyltransferase and hence exhibit a growth requirement for methionine or vitamin B_{12} (9). *S. typhimurium metE* strain ST422, like the *E. coli metE* strain RK4379, grew well at CN-Cbl concentrations as low as 0.5 nM.

The role of the *S. typhimurium btuB* product in cobalamin transport was determined by the isolation of mutants resistant to phage BF23. The rough *S. typhimurium* strain ST422 was susceptible to phage BF23, as expected (18). Plaques on ST422 were substantially smaller than those on *E. coli* hosts, even if the phage had been propagated on ST422. Plaque formation was blocked by 5 μM CN-Cbl. Spontaneous mutants of strain ST422 resistant to phage BF23 were impaired to some degree in growth on minimal medium with 5 nM CN-Cbl, although all grew normally with 5 μM CN-Cbl, as occurs with *E. coli btuB* mutants (2). Some mutants showed no growth with 5 nM CN-Cbl, whereas others grew at a greatly reduced rate. The mutant strain that was used for further study, RK7405, did not grow with 5 nM CN-Cbl but did grow with 50 nM CN-Cbl, in contrast to the *E. coli btuB* mutant RK5016, which showed comparable growth only with 5 μM CN-Cbl.

Uptake of CN[^{57}Co]Cbl by these strains was measured (Fig. 1). Both parental strains, *E. coli* RK4379 and *S. typhimurium* ST422, exhibited similar rates of energy-dependent transport. Accumulated substrate was not released from the cells after a chase with excess nonradioactive CN-Cbl, and energy-depleted cells of both strains bound approximately 200 molecules of CN-Cbl per cell. In contrast, mutant strain RK7405 displayed no significant binding or transport of CN-Cbl.

The impairment of CN-Cbl uptake in strain RK7405 was corrected by the introduction of the $btuB_E$ gene. All of five transformants carrying plasmid pKH3-8 were susceptible to phage BF23 and grew normally with 5 nM CN-Cbl. The level of CN-Cbl binding to a pKH3-8-bearing transformant (10.7 pmol/10^9 cells) was about 30 times higher than the level of binding to the wild-type parent ST422 and at least 200 times higher than that of RK7405. Although binding capacity was greatly amplified, the rate of vitamin B_{12} accumulation and the size of the internal pool of nonexchangeable substrate were about the same as in strain ST422 (Fig. 1), which suggested that the rates of cobalamin transport into the cytoplasm were similar in the two strains. Marginal amplification of the transport rate across the cytoplasmic membrane in response to cloned *btuB* is also seen in *E. coli* (21). Thus, strain RK7405 carries a mutation that eliminates CN-Cbl binding and transport and that can be complemented by plasmid-borne $btuB^+$.

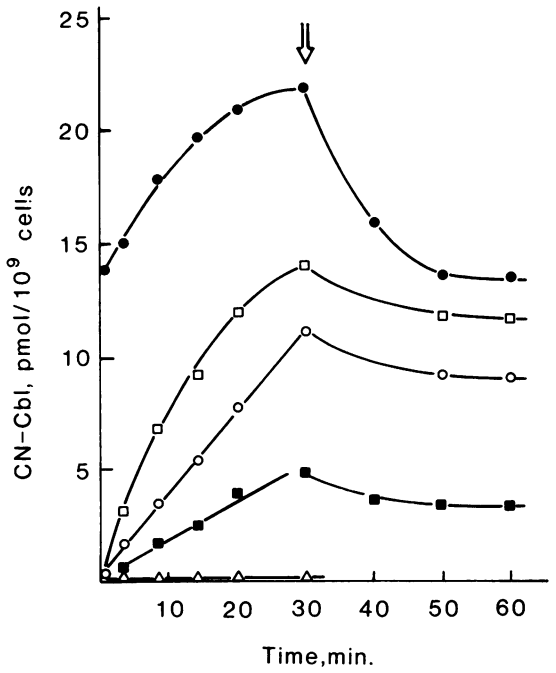

FIG. 1. Transport of CN[^{57}Co]Cbl in strains of *E. coli* and *S. typhimurium*. Cells were grown in minimal medium A with glucose and required amino acids. The strains were *E. coli* RK4379 (○), *S. typhimurium* ST422 (□), and RK7405 carrying no plasmid (△), pCRR10 (■), or pKH3-8 (●). CN[^{57}Co]Cbl was added at 10 nM. At 30 min (⇩), 2 μM nonradioactive CN-Cbl was added.

Genetic mapping. To show whether the mutations that conferred BF23 resistance were at *btuB*, the genetic linkage of these mutations to *argH* was demonstrated by P22-mediated transduction. Seven independent BF23-resistant mutants, which displayed a wide range of growth impairment on CN-Cbl, were used as donors into strain RK7425 (*argH1823*::Tn*10 metE galE*), with selection for Arg^+ transductants. For all seven strains, the phenotypes of BF23 resistance and impaired CN-Cbl utilization were 52 to 68% linked to *argH*, within the range previously described for linkage of *arg* and *bfe* (18). All BF23-sensitive transductants displayed wild-type utilization of CN-Cbl. The BF23-resistant transductants showed the same growth response with 5 nM CN-Cbl as did each original mutant. Therefore, different mutations at $btuB_S$ affect CN-Cbl utilization to varying degrees.

Revertants of strain RK7405 were selected for growth with 5 nM CN-Cbl. Some revertants were susceptible to phage BF23. Unexpectedly, other revertants remained resistant to BF23, which suggested either that second-site mutations in *btuB* could restore cobalamin transport but not phage binding or that mutations in another gene could confer increased cobalamin transport in the $btuB_S$ strain. Thus, although both *S. typhimurium* and *E. coli* required the *btuB* product for high-affinity CN-Cbl transport, there were differences between the two strains. *S. typhimurium btuB* mutants were able to utilize lower concentrations of CN-Cbl than could the corresponding *E. coli* mutants, and some revertants of

TABLE 2. Growth response of *btuB* strains of *E. coli* and *S. typhimurium* to various corrinoids

Host strain	Plasmid	Relative colony size in presence of[a]:			
		CN-Cbl	Adenosyl-cobalamin	Methylco-balamin	Dicyanoco-binamide
RK5016	pBR322	−	−	−	−
RK5016	pKH3-8	++++	++++	++++	++++
RK5016	pCRR10	+	+	+	−
RK7405	pBR322	−	−	−	−
RK7405	pKH3-8	++++	++++	++++	++++
RK7405	pCRR10	+++	+++	++	+

[a] Host strains carrying the indicated plasmids were streaked on minimal agar medium with glucose, required amino acids, and ampicillin. Growth response is given as the time required for the strain to form single colonies of the same size on the indicated concentration of CN-Cbl as the strain formed in 15 h on the same medium with methionine: ++++, 15 h; +++, 24 h; ++, 36 h; +, 72 h; −, no or very slight growth within 72 h.

RK7405 that regained CN-Cbl utilization were still BF23 resistant. One possible explanation for these results is that a second cobalamin-transport system operates in *S. typhimurium*.

Cloning of *Salmonella* sequences that complement *bfe*. Evidence for the existence of a second cobalamin transport system came from the isolation of an *S. typhimurium* DNA fragment able to complement the growth defect of *E. coli btuB* strains. Random 5- to 10-kb *Sau*3A fragments of TR2299 DNA were cloned into the *Bam*HI site of pBR322. The resultant library was introduced into *E. coli* LE392 for amplification and then into RK5016. About 4,000 ampicillin-resistant colonies were replica plated onto minimal medium with 5 nM vitamin B_{12}. All of the 35 colonies that arose within 3 days were still resistant to BF23. Plasmids isolated from each strain were introduced into RK5016 and RK7405. Only one recombinant plasmid, pCRR10, complemented both strains for growth on 5 nM CN-Cbl. Growth promotion by pCRR10 was weak, and transformants carrying this plasmid required several days to reach the same colony size that the host strains transformed with pKH3-8 reached in 15 h.

Strains carrying pCRR10 grew well on MacConkey agar, which suggested that cobalamin entry was not the result of deranged outer membrane barrier function, which can bypass the requirement for *btuB* (2). Furthermore, the presence of pCRR10 did not allow the *lamB ompF* RAM105 mutant to grow on maltotriose or higher maltodextrins, as might be expected if the plasmid led to production of a new porin (29).

The growth responses to cobalamin derivatives of *btuB* strains RK7405 and RK5016 harboring plasmid pBR322, pKH3-8, or pCRR10 were examined (Table 2). Both host strains carrying pBR322 showed slight growth after 3 days in the presence of 5 nM CN-Cbl, adenosylcobalamin, methylcobalamin, or dicyanocobinamide. Rapid growth on all four compounds occurred when either host carried pKH3-8. Differences were seen when pCRR10 was present. *E. coli* RK5016(pCRR10) required 66 to 72 h to form standard-sized colonies (same size as colonies formed at 15 h with methionine) with the cobalamins, and no growth was seen with dicyanocobinamide. *S. typhimurium* RK7405(pCRR10) responded better to the three cobalamins, forming standard-sized colonies in 36 to 48 h. It showed only slight response to dicyanocobinamide. This difference in response to dicyanocobalamin, which lacks the axial 2,3-dimethylbenzimidazole moiety present in the cobalamins, suggested that the specificity of uptake conferred by pCRR10 was different from that conferred by pKH3-8.

Vitamin B_{12} transport in response to pCRR10. CN-Cbl transport activity of *btuB* strain RK7405 carrying pCRR10 was lower than that of *bfe*$^+$ strain ST422 but considerably higher than that of RK7405 with the vector (Fig. 1). Variability in the CN-Cbl transport activity of pCRR10-bearing cells was seen from day to day, perhaps as a result of variation in plasmid copy number. Addition of excess nonradioactive CN-Cbl after 30 min of uptake caused little release of label, which suggested that uptake into the cytoplasm had occurred.

The level of CN-Cbl binding to energy-poisoned RK7405(pCRR10) cells (50 to 100 molecules per cell) was about 50% of the amount bound to wild-type ST422 (230 molecules per cell) and was substantially higher than the very low amount bound to RK7405(pBR322) (<15 molecules per cell). In comparison, the amount of CN-Cbl bound by RK7405(pKH3-8) cells was about 30 times higher than the wild-type level of binding. CN-Cbl binding and transport in RK7405(pCRR10) were not altered by growth in the presence of 10 μM vitamin B_{12}, whereas binding was repressed 15- to 20-fold in the parental strains and in RK7405(pKH3-8).

The substrate specificity and affinity of the pCRR10-directed transport system were investigated. No saturation of CN[^{57}Co]Cbl binding was seen even at substrate concentrations of 200 nM (data not shown). Binding of 20 nM CN[^{57}Co]Cbl to strain RK7405(pKH3-8) was reduced six- to ninefold in the presence of a ninefold molar excess of nonradioactive CN-Cbl, methylcobalamin, adenosylcobalamin, or dicyanocobinamide, as expected for their high-affinity binding to BtuB. In contrast, the amount of label from 100 nM CN[^{57}Co]Cbl bound to RK7405(pCRR10) was not decreased at all by a ninefold excess of these competitors. These results indicate that the substrate-binding activity of the pCRR10-specified system was low affinity (K_d, >100 nM) as well as low capacity.

The pCRR10-specified CN-Cbl transport activity was energy dependent. Uptake in both ST422 and RK7405 (pCRR10) was inhibited >90% in cells preincubated for 5 min with the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (50 μM) and 65 to 75% in cells exposed to 10 mM sodium arsenate. This response to energy poisons was similar to that of *E. coli* and is consistent with the dependence of transport across the outer membrane on the proton motive force and of transport across the cytoplasmic membrane on phosphate bond energy (32).

Genetic requirements for CN-Cbl transport. Growth is a more sensitive index of low levels of vitamin B_{12} transport than are transport assays. The growth behavior of *E. coli* strains mutant for various components of the vitamin B_{12} transport system and carrying pCRR10 was compared with growth when the strains carried pBR322 or pKH3-8. The *btu*$^+$ strain RK5041, with an intact uptake system, grew well with 5 nM CN-Cbl with each of the plasmids (Table 3).

E. coli strains with an intact outer membrane transport system (*btuB*$^+$ *tonB*$^+$) are only slightly impaired in their utilization of CN-Cbl when they are defective at *btuC* or *btuD* (12). The decreased growth with 5 nM CN-Cbl of host strains RK6006 (*btuC*) and RK6555 (*btuD*) was corrected by the presence of plasmid pKH3-8 (Table 3). Surprisingly, these *btuC* and *btuD* mutant strains did not grow at all with 5 nM CN-Cbl when plasmid pCRR10 was present, and their growth with 5 μM CN-Cbl was substantially slower. Strain RK7817, carrying a nonpolar deletion of *btuE*, grew well with 5 nM vitamin B_{12} in the presence of all plasmids. Thus,

TABLE 3. Growth on vitamin B_{12} of *btu* and *tonB* strains carrying various plasmids

Host strain[a]	CN-Cbl (nM)	Relative colony size of strain carrying plasmid[b]:		
		pBR322	pKH3-8	pCRR10
RK5041 (*btu*$^+$)	5	++++	++++	++++
RK7405 (*btuB*$_S$)	5	−	++++	+++
RK6006 (*btuC*)	5	++	++++	−
	5,000	++++	++++	++
RK6555 (*btuD*)	5	+++	++++	−
RK7817 (*btuE*)	5	++++	++++	++++
RK5048 (*tonB*)	50	−	−	++
RK4793 (*btuB*)	50	−	+++	++
RK6686 (*btuB btuC*)	5,000	−	++++	−
RK6532 (*btuB btuD*)	5,000	−	++++	−
RK7822 (*btuB btuE*)	50	−	+++	++

[a] All strains except RK7405 are *E. coli*.

[b] All cobamides were present at 5 nM. Growth is described as the time required to form single colonies of the same size as those formed on methionine after 16 h: ++++, 24 h; +++, 36 h; ++, 48 h; +, 72 h; −, slight growth in 72 h.

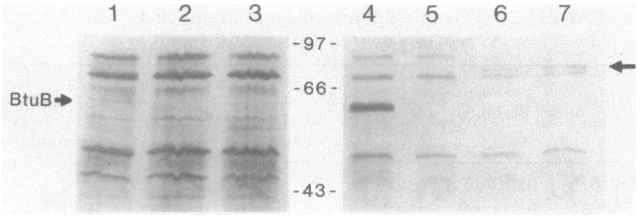

FIG. 2. Identification of outer membrane proteins involved in CN-Cbl transport. Outer membrane polypeptides were isolated by sarcosyl extraction, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and silver stained. For lanes 1 to 3, 30 μg of protein was loaded; for lanes 4 to 7, 5 μg of protein was loaded. Lanes contained material from ST422 (lane 1), ST422 grown in the presence of 10 μM CN-Cbl (lane 2), RK7405 (lane 3), RK7405 (pKH3-8) (lane 4), RK7405(pKH3-8) grown in the presence of 10 μM CN-Cbl (lane 5), RK7405(pCRR10) (lane 6), and RK7405(pCRR10) grown in the presence of 10 μM CN-Cbl (lane 7). The mobilities of molecular weight standards (in kilodaltons) are indicated in the central lane. On the left and right are indicated the locations of BtuB and the 84-kilodalton polypeptide associated with the presence of pCRR10, respectively.

the presence of plasmid pCRR10 interferes with the *btuCD*-independent uptake of CN-Cbl mediated by the BtuB-TonB system.

The response of strains defective in both the outer membrane and the cytoplasmic membrane transport steps was examined in the same manner (Table 3). With plasmid pBR322, double mutants defective in *btuB* and either *btuC* or *btuD* (RK6532 and RK6686) were totally unable to respond to CN-Cbl. This defect was corrected by the *btuB*$^+$ plasmid pKH3-8 but not at all by pCRR10. The inability of *tonB* strain RK5048 to grow with 50 nM CN-Cbl was substantially corrected by pCRR10 but not by pKH3-8. These results showed that CN-Cbl uptake in response to pCRR10 required the BtuC and BtuD cytoplasmic membrane transport components but not BtuB, BtuE, or TonB function. Conversely, growth on CN-Cbl in cells carrying pKH3-8 required TonB but not BtuC or BtuD function.

Effect of pCRR10 on outer membrane protein composition. Since the genetic studies suggested that plasmid pCRR10 might encode a new outer membrane transport system for cobalamins, the effect of this plasmid on outer membrane protein composition was investigated (Fig. 2). Strain ST422 produced low amounts of an M_r-60,000 polypeptide, which was absent when the strain was grown with CN-Cbl. It was also absent in the *btuB*$_S$ mutant RK7405, but large amounts of a polypeptide with the same mobility were produced in strain RK7405 carrying pKH3-8. Synthesis of BtuB$_E$ in *S. typhimurium* was strongly repressed by growth with CN-Cbl. The only apparent difference in outer membrane protein composition of strain RK7405 carrying pCRR10 was the presence of a polypeptide with an apparent molecular weight of 84,000. Synthesis of this polypeptide was not affected by growth in the presence of CN-Cbl. Thus, the presence of pCRR10 resulted in increased production of a high-molecular-weight outer membrane polypeptide that was not BtuB.

pKH3-8 and pCRR10 carry different DNA segments. Southern hybridization analysis was used to detect the presence of sequences homologous to the inserts in pKH3-8 and pCRR10. Genomic DNA from *E. coli* and *S. typhimurium* was probed with the 1.8-kb *Hin*dIII-*Pst*I fragment from pKH3-8 carrying most of the *E. coli btuB* coding region (Fig. 3A). The sizes of the homologous fragments derived from *E. coli* DNA were in agreement with the nucleotide sequence of *btuB*$_E$ (20) and its flanking genes and with the restriction map of Kohara et al. (23). The *btuB*$_E$ probe hybridized to *S. typhimurium* DNA, although hybridization was weaker and the number and locations of restriction sites within the *btuB*$_S$ region were different. For example, *Eco*RI, *Hin*dIII, and *Sal*I cut within *btuB*$_E$ but not within the corresponding region of *S. typhimurium*. Conversely, *Ava*I cut twice in *btuB*$_S$ but not in *btuB*$_E$. Thus, *S. typhimurium* contains a region weakly homologous to *btuB*.

Plasmid pCRR10 carries a 6-kb insert. A 6.9-kb *Nhe*I-*Sph*I fragment from pCRR10 carrying the entire insert was used as a hybridization probe with genomic DNA from the same strains (Fig. 3B). Strong hybridization to *S. typhimurium* DNA was seen. The number and locations of restriction sites were substantially different from those seen in the *btuB* regions. For example, there were at least five *Pst*I and four *Pvu*II sites in the region homologous to pCRR10 but no more than one site for either enzyme in *btuB*. No significant hybridization of the insert in pCRR10 was seen to *E. coli* genomic DNA (Fig. 3B) or to the insert in pKH3-8 (not shown).

Since pCRR10 did not hybridize to the *E. coli* chromosome, we examined whether the insert might be derived from the large, 100-kb plasmid carried by most *S. typhimurium* strains (22). Southern hybridization analysis using the two probes described above was carried out on DNA from *S. typhimurium* strain χ3344, cured of the plasmid, and its isogenic parent χ3300 (17) (Fig. 4). All of the strains tested showed the expected hybridization to the *btuB*$_E$ probe. All of the plasmid-bearing *S. typhimurium* strains displayed similar hybridization to the pCRR10 probe, whereas neither χ3344 nor *E. coli* DNA showed any hybridization to this probe. It is therefore likely that the insert in pCRR10 is not of chromosomal origin but is derived from the large virulence-associated plasmid.

DISCUSSION

S. typhimurium can utilize exogenous CN-Cbl as cofactor for methionine biosynthesis, ethanolamine catabolism, and formation of the modified base, queuine, present in some tRNAs (15, 33, 39). Given their close similarities, *E. coli* and *S. typhimurium* are likely to have similar cobalamin transport systems. The *btuB*$_S$ gene encodes the surface receptor

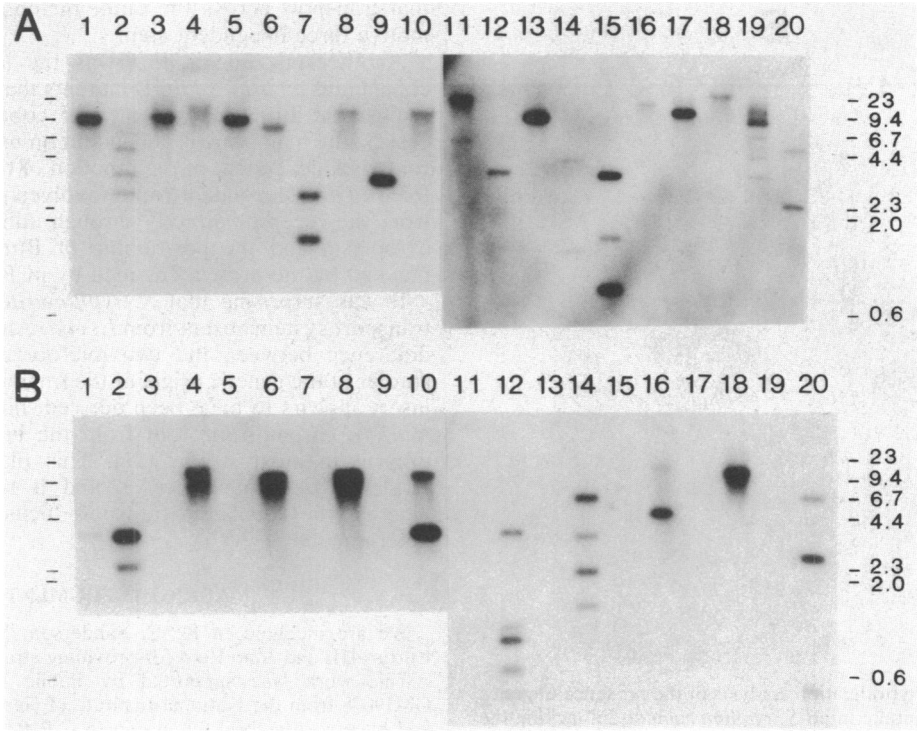

FIG. 3. Southern hybridization analysis of genes involved in CN-Cbl transport. Onto a nylon membrane was transferred DNA from *E. coli* RK4379 (odd-numbered lanes) or *S. typhimurium* ST422 (even-numbered lanes) that was digested with *Ava*I (lanes 1 and 2), *Bam*HI (lanes 3 and 4), *Cla*I (lanes 5 and 6), *Eco*RI (lanes 7 and 8), *Hin*dIII (lanes 9 and 10), *Pst*I (lanes 11 and 12), *Pvu*II (lanes 13 and 14), *Sal*I (lanes 15 and 16), *Sph*I (lanes 17 and 18), or *Hin*cII (lanes 19 and 20). The membranes were hybridized to the ^{32}P-labeled 1.8-kb *Hin*dIII-*Pst*I fragment from pKH3-8, carrying most of the *E. coli btuB* gene (panel A), or to the 6.9-kb *Nhe*I-*Sph*I fragment from plasmid pCRR10 (panel B). Markers indicate the mobilities of λ *Hin*dIII fragments with lengths of 23, 9.4, 6.7, 4.4, 2.3, 2.0, and 0.56 kb. The weak signal in lane 1 of panel B did not appear in any other lanes or in other experiments.

for phage BF23 and the E colicins and is located in the genetic location analogous to that of the $btuB_E$ gene. Both genes were originally called *bfe* before their role in vitamin B_{12} uptake was known, and the mnemonic of the *S. typhimurium bfe* gene was recently changed to *btuB* (35). The presence in *S. typhimurium* of a DNA sequence homologous to $btuB_E$ was shown by Southern hybridization analysis. BF23-resistant mutants of *S. typhimurium* carried mutations at or near $btuB_S$ and were defective in high-affinity uptake of CN-Cbl. The deficiency was corrected by introduction of the cloned $btuB_E$ gene. Silver-stained electropherograms of outer membrane proteins revealed the presence in *S. typhimurium* of an M_r-60,000 polypeptide whose production was repressed by growth with CN-Cbl and eliminated in a *btuB* mutant. These results show that the *S. typhimurium btuB* product plays a major role in the high-affinity transport of cobalamins across the outer membrane.

S. typhimurium possesses a second system for passage of cobalamins across the outer membrane. The first indication of the presence of this minor activity was the observation that BF23-resistant mutants of *S. typhimurium* were able to utilize lower concentrations of CN-Cbl than could the corresponding *E. coli* mutant. Numerous revertants of this strain selected for growth on low levels of CN-Cbl remained BF23 resistant. Most BF23-resistant mutants of *E. coli* show complete loss of receptor function, whereas the analogous mutants of *S. typhimurium* showed a wide range of impairment of CN-Cbl utilization. A possible explanation for this difference is that the $BtuB_S$ protein is a poorer receptor for BF23 than is $BtuB_E$, as suggested by the smaller plaque sizes in *S. typhimurium*. Missense mutations in $btuB_S$ that only somewhat perturb receptor function may be sufficient to confer phage resistance while retaining transport function. Presumably, in *E. coli*, $BtuB_E$ is such an effective receptor that it must be completely eliminated by mutation to allow phage resistance. However, the *btuB* mutation in RK7405 resulted in apparent absence of the BtuB polypeptide.

The existence of the low-activity system was demonstrated by molecular cloning studies. The DNA fragment carried in pCRR10 complemented $BtuB^-$ strains, although it conferred a substantially lower level of CN-Cbl uptake than did the cloned $btuB_E$ gene. This fragment does not encode $BtuB_S$, and no clones carrying $btuB_S$ were obtained in numerous attempts. In retrospect, we were similarly unable to clone the $btuB_E$ gene in this manner (21).

The presence of pCRR10 conferred lower levels of CN-Cbl binding and transport than did the *btuB* gene. It did not confer susceptibility to phage BF23 or the E colicins, and the DNA insert did not hybridize to the *btuB* gene. The new transport system allowed entry of cobalamins but not of cobinamide, which suggests that it differs in substrate specificity from BtuB. Cobalamin binding was of low affinity and showed no saturation even at 100 nM CN-Cbl. The low affinity and low transport activity can account for the failure to detect this transport system in haploid cells. This new system appears to mediate transport only across the outer membrane, since cobalamin uptake still requires all of the components involved in passage across the cytoplasmic membrane. It is likely that the new outer membrane protein present in cells carrying pCRR10 is necessary for CN-Cbl

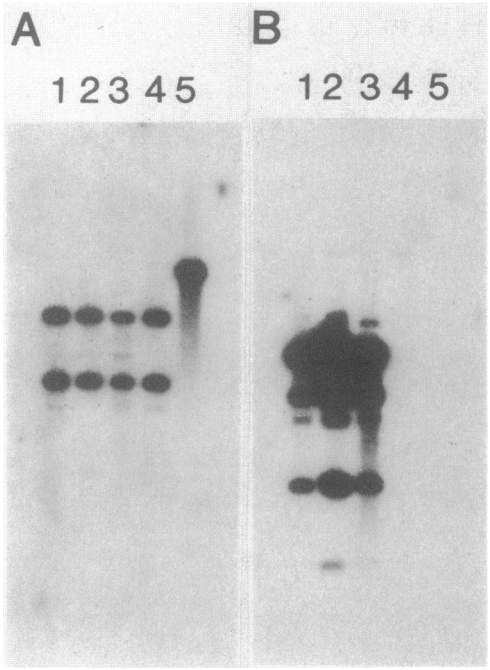

FIG. 4. Southern hybridization analysis of the presence of genes involved in CN-Cbl uptake in an *S. typhimurium* strain lacking the 100-kb plasmid. Both panels present hybridization to *Ava*I-digested genomic DNA from RK7401 (lane 1), ST422 (lane 2), χ3300 (lane 3), χ3344 (lane 4), and *E. coli* RK4379 (lane 5). The ^{32}P-labeled probes were the 1.8-kb *Hin*dIII-*Pst*I fragment from pKH3-8 (panel A) or the 6.9-kb *Nhe*I-*Sph*I fragment from plasmid pCRR10 (panel B).

transport, but the requirement for additional plasmid-coded proteins remains to be determined.

The low affinity and capacity for CN-Cbl suggests that some other nutrient may be the primary substrate of this transport system and that cobalamins enter by being poor analogs of this substrate. A likely substrate could be some iron siderophore, since the uptake of iron siderophores requires an outer membrane transport component. Production of the M_r-84,000 polypeptide was not repressed by growth with excess iron, and plasmid pCRR10 did not improve growth on iron-poor medium of *E. coli ent* or *ent fepA* mutants defective in enterochelin biosynthesis and transport (C. Rioux, unpublished data). However, these results do not prove that the outer membrane protein encoded by pCRR10 is not involved in iron uptake, since other *Salmonella* genes not carried on pCRR10 may be responsible for synthesis of the siderophore substrate for the new system.

All known ferrisiderophore uptake systems require *tonB* function, but the cobalamin uptake catalyzed by pCRR10 was independent of *tonB*. It is possible that the *E. coli* TonB protein is unable to function with outer membrane transport proteins from *S. typhimurium*. Since pCRR10 did not confer markedly better cobalamin uptake in *S. typhimurium* than in *E. coli*, it is unlikely that the new transport system is *tonB* dependent. The poor response to cobinamide and maltodextrins suggests that the pCRR10-encoded outer membrane protein does not act as a porin to allow nonspecific passage of cobalamins. Although this transport system conferred only a low level of CN-Cbl uptake, its activity was strongly inhibited by energy poisons. Since CN-Cbl uptake appears to involve multiple serial systems, it must not be concluded that transport across the outer membrane was the proton motive force-dependent step.

Another puzzling finding was the lack of response to cobalamins by *btuC* or *btuD* mutants that carried pCRR10. It seems that BtuB allows entry of cobalamin by two processes; the major one is dependent on *btuCD*, and the minor route is independent of the function of these genes. Perhaps the *btuCD*-independent route involves passage of substrate from the receptor directly through adhesion sites into the cytoplasm and the positioning of BtuB at these sites is blocked by the protein encoded by pCRR10.

It was surprising that *S. typhimurium* would possess a transport system absent from *E. coli*. An explanation for this difference between the two microorganisms comes from studies of the genetic origin of the fragment in pCRR10. This insert appears to have been derived, not from the *S. typhimurium* chromosome, but from the large cryptic plasmid present in most strains (22). This plasmid carries some virulence determinants (17), and it remains to be seen whether the cobalamin transport locus is related to these determinants.

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