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A New Class of Cobalamin Transport Mutants (*btuF*) Provides Genetic Evidence for a Periplasmic Binding Protein in *Salmonella typhimurium*

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No periplasmic binding protein has been demonstrated for the ATP-binding cassette (ABC)-type cobalamin transporter BtuCD. New mutations (*btuF*) are described that affect inner-membrane transport. The BtuF protein has a signal sequence and resembles the periplasmic binding proteins of several other ABC transporters.

Cobalamin is actively transported by *Salmonella typhimurium* and *Escherichia coli* (8, 24). The BtuB protein (in concert with the TonB protein) transports cobalamin across the outer membrane. The *btuCED* operon encodes two membrane proteins (BtuC and BtuD) that provide transport across the inner membrane (4, 5, 17, 22). The inner-membrane BtuCD system is an ATP-binding cassette (ABC) or "traffic ATPase" transporter (2, 7), a type that generally includes a periplasmic binding protein in addition to membrane-spanning components. None of the *E. coli* transport mutants is defective for a periplasmic binding protein. Direct assays of osmotic shock fluid revealed a protein able to bind cobalamin (8, 15, 27, 28), but no role in transport has been demonstrated (14, 22). It was suggested that the outer-membrane transport system (BtuB-TonB) concentrates cobalamin in the periplasm sufficiently to reduce the need for a binding protein (7, 8, 22). We describe a new class of *Salmonella* cobalamin transport mutations that may eliminate a periplasmic vitamin B_{12} binding protein.

New transport mutants. Under aerobic conditions, a *metE* mutant, which, under aerobic conditions, requires methionine unless exogenous vitamin B_{12} is provided (24) at a minimum concentration of about 0.1 nM. Known cobalamin transport mutants (*btuB* or *btuCED* mutants) require a higher level of exogenous cobalamin (Table 1). The *btuF* insertion mutants described here were isolated in the *metE* deletion strain, TT15696. Their phenotype resembled that of previously known *btuC*D mutants (Table 1). The *btuF* gene maps near 5 min of the *S. typhimurium* chromosome (Fig. 1), far from *btuB* and *btuCED*.

Epistasis tests show that BtuF and BtuCD act together. The transport defect of a *btuF btuCED* double mutant was indistinguishable from that of either single mutant, suggesting that the BtuF and BtuCD proteins contribute to the same function (Table 1). That is, the residual transport ability in each mutant type does not require the function encoded by the other gene. In contrast, the combination of a *btuF* and a *btuB* mutation causes a much more severe defect than either single mutation. This synergistic effect is also seen for a *btuCD btuB* double mutant. This suggests that BtuB acts in both *btuCD* and *btuF* single mutants to provide the residual transport seen. The BtuF protein does not seem to be needed for outer-membrane transport but appears to act with BtuCD to provide inner-membrane transport.

Repression of the *cob* operon in *btuF* insertion mutants. Transcription of the *cob* operon is induced by propanediol (6, 23) and repressed by adenosylcobalamin (Ado–B_{12}) (1). Repression by exogenous CN–B_{12} requires transport and internal adenosylation (13, 24). If the *btuF* mutation impairs cobalamin transport, it should also impair repression of the *cob* operon by exogenous CN–B_{12}.

In wild-type strains, the *cob* operon is repressed by 0.1 μM CN–B_{12}. Strains with a *btuF* (or *btuCED*) mutation required a 10-fold-higher CN–B_{12} concentration for repression. Strains with a *btuB* mutation were not fully repressed even by 1 mM CN–B_{12}. These results were obtained with derivatives of strain TT20707, which carries a *cobD24*::MudJ insertion (forming a *cob-lac* operon fusion) and a deletion mutation (*cobR4*) which renders transcription independent of propanediol but still subject to repression by Ado–B_{12} (3, 10).

Transport assays. Assays of cyanocobalamin (CN-Cbl) transport were initiated by addition of ^{57}Co-labeled CN–B_{12}. After 50 min of incubation, an excess of unlabeled CN–B_{12} was added to stop uptake and allow observation of loss of previously assimilated cobalamin from cells. The *btuF* mutants were indistinguishable from *btuC* mutants (Fig. 2). Both mutant types initially took up vitamin B_{12} (12.7 nM) as well as wild-type cells, suggesting no defect in outer-membrane transport; this initial transport is eliminated by a *btuB* mutation. Both *btuF* and *btuC* mutants accumulated less CN-Cbl at steady state, suggesting that import is ultimately opposed by leakage of periplasmic CN-Cbl back out through the outer membrane. In wild-type cells, unlabeled CN–B_{12} stopped the accumulation of vitamin B_{12}, but in *btuF* and *btuC* mutants, addition led to loss of labeled vitamin B_{12} by diffusion out of the periplasm. Four other *btuF* mutants behaved essentially like those in Fig. 2. In all cases, *btuF* mutations appear to block inner-membrane transport.

Sequencing. The DNA sequence of the *btuF* gene was determined following PCR amplification of sequences between genetically characterized insertion mutations as indicated in the genetic map (Fig. 1). Methods and primers used are described elsewhere (18). All sequenced *btuF* insertion mutations lie within a 801-nucleotide open reading frame (ORF) near the *hemL* gene. Their positions are *btuF23*::Tn*10*dTc (bp 97),

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TABLE 1. Effect of *btu* mutations on minimum level of CN–B_{12} required to support methionine synthesis

Strain	Relevant genotype[a]	Ability to grow on minimal medium containing the indicated supplement[b]									
		Nothing	Met	CN–B_{12} (Concn)							
				0.1 nM	1 nM	10 nM	100 nM	1 μM	10 μM	100 μM	500 mM
LT2	WT	+	+	+	+	+	+	+	+	+	+
TT15696	*metE*	−	+	+	+	+	+	+	+	+	+
TT20702	*btuB12*::Tn*10*dCm	−	+	−	−	−	−	±	+	+	+
TT20703	*btuCED9*::Tn*10*dCm	−	+	−	±	+	+	+	+	+	+
TT20698	*btuF21*::Tn*10*dTc	−	+	−	±	+	+	+	+	+	+
TT20704	*btuF21*::Tn*10*dTc *butCED9*::Tn*10*dCm	−	+	−	±	+	+	+	+	+	+
TT20706	*btuF21*::Tn*10*dTc *btuB12*::Tn*10*dCm	−	+	−	−	−	−	−	−	−	+
TT20705	*btuB20*::Tn*10*dTc *btuCED9*::Tn*10*dCm	−	+	−	−	−	−	−	−	−	+

[a] All strains except LT2 carry a *metE* deletion mutation (Del1077) which renders methionine synthesis dependent on the B_{12}-dependent MetH enzyme. Three other *btuF* mutations (TT20699 through TT20701) showed the same phenotype as strain TT20698. WT, wild type.

[b] All tests were performed with cells grown overnight in E-glucose, methionine, and histidine. Cells were diluted in NaCl, and approximately 10 to 100 cells (in 5 μl) were dropped onto an E-glucose–histidine plate with the indicated concentration of CN–B_{12} or methionine (Met). Growth was scored as the appearance of colonies after 3 days.

btuF22::Tn*10*dTc (bp 400), and btuF79::Tn*10*dCm (bp 477). The inferred BtuF sequence includes a highly probable signal sequence with a cleavage site at amino acid 22 (score, 0.97; maximum, 1), a feature expected of a periplasmic binding protein. The BtuF amino acid sequence shows a very strong similarity to the *yadT* ORF of *E. coli*. The sequence resembles those of three known ABC-type, periplasmic binding proteins. One transports hemin across in the inner membrane of *Yersinia enterocolitica* and *Yersinia pestis* (16, 26). Another (FecB of *E. coli*) transports citrate-iron chelates (25), and the third transports ferrisiderophores (CbrA of *Erwinia chrysanthemi*) (21).

Regulation of expression. Expression of *btuF* is not regulated, based on tests using a MudJ insertion mutation (*btuF80*::MudJ), which fuses transcription of the inserted *lac* operon to the *btuF* gene. A strain with this fusion (TT20711) was grown on glucose, on glycerol, and on ethanolamine; the fused *lacZ* gene produced about 45 U of β-galactosidase under all conditions. Addition of CN–B_{12} (5 nM or 5 μM) had no

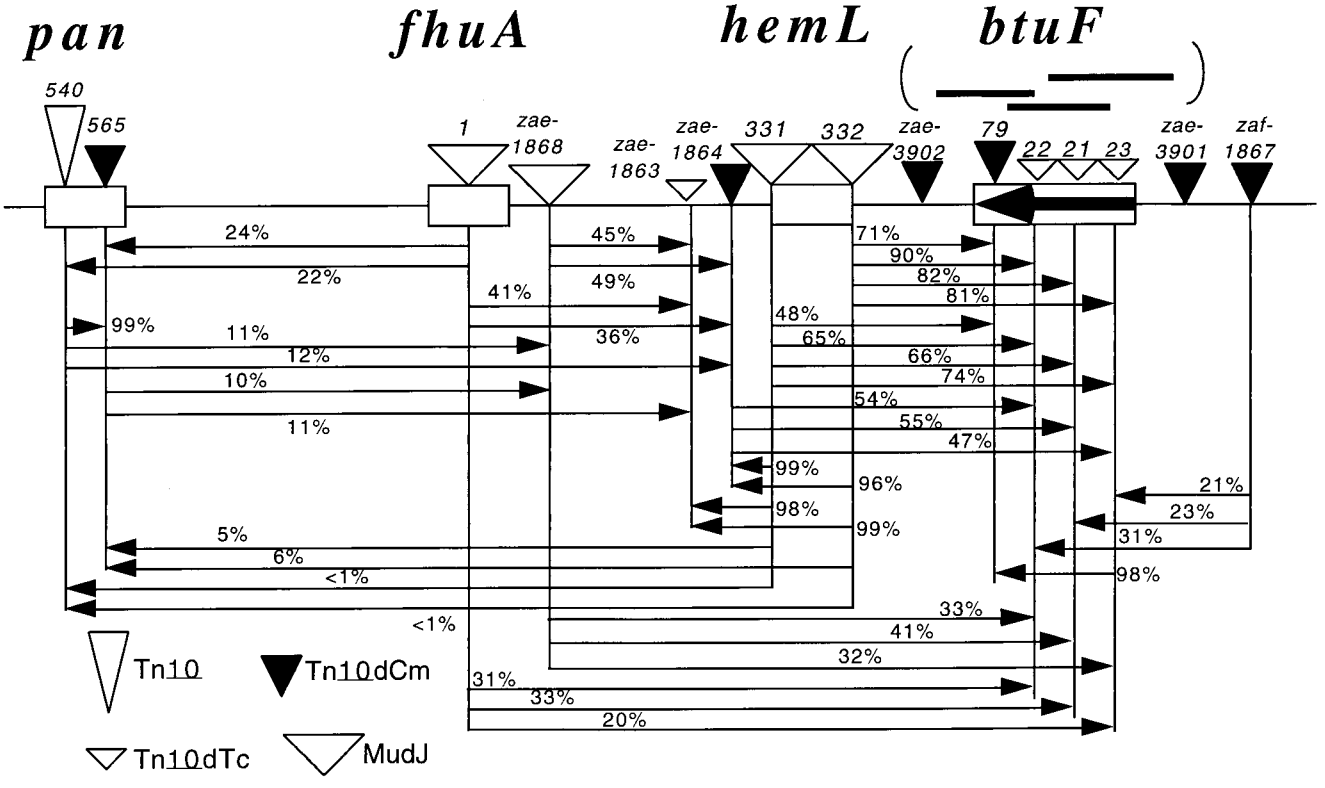

FIG. 1. Genetic map of the *btuF* region. This region is at min 5 of the latest *Salmonella* map; the homologous region of the *E. coli* at min 3.7 includes the *yadT* gene, which is homologous to the *btuF* gene of *Salmonella*. Linkages are presented as percent cotransduction in P22-mediated transduction crosses. The arrowheads point to the selective donor marker used in the cross. Transduction methods have been described previously (12).

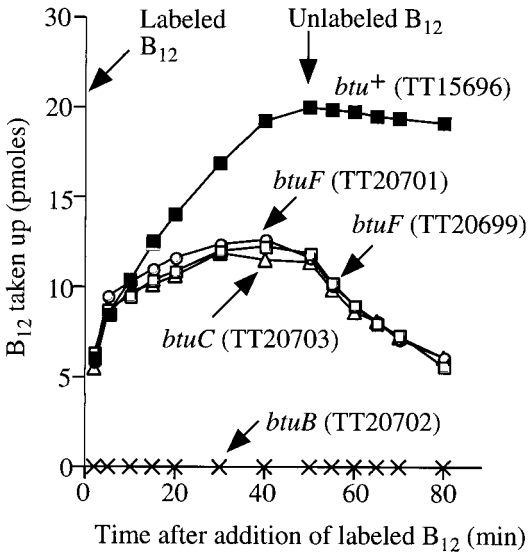

FIG. 2. Transport of CN-Cbl by *btu* mutants. Cells were grown to mid-log phase (optical density at 660 nm [OD_{660}], 0.4 to 0.8) in the Davis-Mingioli minimal medium (11). Harvested cells were suspended to a final OD_{660} of 6 in 0.1 M potassium phosphate buffer (pH 6.6) containing 20 μM calcium nitrate and 1% glucose. These suspensions were stored on ice and assayed within 3 h of harvest. Transport was assayed by the method of Bradbeer and Woodrow (9). Each 15-ml reaction mixture was 0.1 M potassium phosphate buffer (pH 6.6) containing final concentrations of 20 μM calcium nitrate, 1% glucose, and sufficient cells to give an OD_{660} of 0.2. After 5 min of incubation at 37°C, the reaction was started by adding ^{57}Co-labeled CN-Cbl to a final concentration of 12.67 nM. Samples were removed at intervals and filtered (0.45-μm-pore-size Millipore filter HAWP) to collect cells. After 50 min of uptake, unlabeled CN-Cbl was added (final concentration, 5 μM) and sampling was continued. All filters were washed twice with 10 ml of 0.1 M lithium chloride, air dried, placed in Beckman Ready Safe scintillation fluid, and counted in a Beckman LS6500 liquid scintillation counter. Results are expressed as picomoles of CN-Cbl taken up per milliliter of cell suspension with an OD_{660} of 0.6.

effect on transcription during growth on any of these three carbon sources. This is interesting in light of the fact that the *btuA* gene is controlled (by Ado-B_{12}) (19, 20), while the *btuCED* operon is not (22).

In summary, we infer that the BtuF protein acts as a periplasmic binding protein with BtuCD to transport cobalamins across the inner membrane. Such a protein is expected for a transporter of this type but was not found among previously characterized *E. coli* cobalamin transport mutants.

Nucleotide sequence accession number. The *Salmonella btuF* sequence is GenBank accession no. AF096877.

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