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Molecular Characterization of *eutF* Mutants of *Salmonella typhimurium* LT2 Identifies *eutF* Lesions as Partial-Loss-of-Function *tonB* Alleles

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The *eutF* locus of *Salmonella typhimurium* LT2 was identified as a locus necessary for the utilization of ethanolamine as a sole carbon source. Initial models suggested that EutF was involved in either ethanolamine transport or was a transcriptional regulator of an ethanolamine transporter. Phenotypic characterization of *eutF* mutants suggested EutF was somehow involved in 1,2-propanediol, propionate, and succinate utilization. Here we provide evidence that two alleles defining the *eutF* locus, Δ*903* and *eutF1115*, are partial-loss-of-function *tonB* alleles. Both mutations were complemented by plasmids containing a wild-type allele of the *Escherichia coli tonB* gene. Immunoblot analysis using TonB monoclonal antibodies detected a TonB fusion protein in strains carrying *eutF* alleles. Molecular analysis of the Δ*903* allele identified a deletion that resulted in the fusion of the 3′ end of *tonB* with the 3′ end of *trpA*. In-frame translation of the *tonB-trpA* fusion resulted in the final 9 amino acids of TonB being replaced by a 45-amino-acid addition. We isolated a derivative of a strain carrying allele Δ*903* that regained the ability to grow on ethanolamine as a carbon and energy source. The molecular characterization of the mutation that corrected the Eut⁻ phenotype caused by allele Δ*903* showed that the new mutation was a deletion of two nucleotides at the *tonB-trpA* fusion site. This deletion resulted in a frameshift that replaced the 45-amino-acid addition with a 5-amino-acid addition. This change resulted in a TonB protein with sufficient activity to restore growth on ethanolamine and *eut* operon expression to nearly wild-type levels. It was concluded that the observed EutF phenotypes were due to the partial loss of TonB function, which is proposed to result in reduced cobalamin and ferric siderophore transport in an aerobic environment; thus, the *eutF* locus does not exist.

Salmonella typhimurium and *Escherichia coli* can use the nonfermentable amino alcohol ethanolamine as the sole carbon and/or nitrogen source (8, 21). The initial step in the catabolism of ethanolamine involves the cleavage of ethanolamine into acetaldehyde and ammonia by the adenosylcobalamin (AdoCbl)-dependent enzyme ethanolamine ammonia-lyase (5, 7, 8). In addition to the requirement of AdoCbl for the enzymatic degradation of ethanolamine, work with *S. typhimurium* has shown that AdoCbl is also required for the induction of the genetically defined *eut* operon (35, 36, 45). This operon encodes proteins involved in ethanolamine catabolism in this bacterium and *E. coli* (5, 6, 37, 48). The requirement of AdoCbl for both ethanolamine catabolism and *eut* operon expression presents a challenge to these organisms growing aerobically, since *S. typhimurium* can synthesize AdoCbl de novo only under anaerobic conditions and *E. coli* is unable to synthesize the complete coenzyme de novo (20, 24). Both organisms meet this challenge by using transport systems to acquire exogenous complete and incomplete corrinoids under aerobic conditions.

Transport of exogenous cobalamin (Cbl) and other corrinoids from the environment into the cytoplasm of *S. typhimurium* or *E. coli* requires two independently functioning transport systems; the first actively transports Cbl across the outer membrane, while the second transports Cbl across the cytoplasmic membrane (10). Transport across the outer membrane involves BtuB, a high-affinity outer membrane receptor for Cbl, and the TonB-dependent energy-transducing complex consisting of the cytoplasmic membrane proteins TonB, ExbB, ExbD, and other, yet to be identified proteins (4, 18, 32, 46). TonB is anchored in the cytoplasmic membrane and spans the periplasm to interact directly with a number of outer membrane receptors involved in Cbl or ferric siderophore transport (32). The TonB-dependent energy-transducing complex couples electrochemical potential from the cytoplasmic membrane to the active transport of Cbl and ferric siderophores across the outer membrane. In the absence of a functional transport system, aerobically growing cells become starved for iron and respond by hypersecreting siderophores in a futile attempt to access iron. More relevant to ethanolamine utilization, these cells cannot access exogenous Cbl unless Cbl is present in a concentration high enough to overcome the transport defect (4, 34). Transport across the cytoplasmic membrane is carried out by the ABC transport system of BtuB, BtuC, and BtuD and functions independently of the TonB-dependent system (10).

eutF mutants were originally identified by the inability to grow on ethanolamine as a sole source of carbon, and EutF was proposed to play a role in ethanolamine transport or regulation of an ethanolamine transporter (28). Since then, we have also observed other phenotypes associated with *eutF* mutations which included the inability to grow on 1,2-propanediol as a sole carbon source and reduced growth rates on the nonfermentable carbon sources propionate and succinate (30). Here we present evidence that these phenotypes are the result of partial-loss-of-function *tonB* alleles and are not due to a new gene locus.

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MATERIALS AND METHODS

Bacteria, media, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Carbon source utilization was tested on no-carbon medium E (NCE) (9, 49) supplemented with 1 mM $MgSO_4$, 0.3 mM

TABLE 1. *S. typhimurium* LT2 strains and plasmids used

Strain or plasmid	Genotype	Source or reference
S. typhimurium strains		
TR6583[a]	*metE205 ara-9*	K. Sanderson via J. R. Roth
Derivatives of TR6583		
JE1291	*trpC3484*::Tn*10d*(Tc)[b]	28
JE1418	Δ*903*	28
JE1684	*eutE18*::Mud1-8	Lab collection
JE1685	*eutE18*::Mud1-8 Δ*903*	28
JE1690	*tonB251* derivative of JE1291	28
JE2123	Δ*1235 zde-6396*::Tn*10d*(Tc) Δ*903*	
JE4163	*trpC3480*::Tn*10d* (Cm^r) Δ*903*	
JE4386	Δ*1235*	
JE4387	*eutE18*::Mud1-8 Δ*1235*	
Plasmids		
pRZ526	$tonB^+$ (*E. coli* $tonB^+$) (Km^r)	33
pRZ531	$tonB^+$ (*E. coli* $tonB^+$) (Km^r)	33
pKP292	*tetp/o-tonB-phoA* (*E. coli* $tonB^+$) (Ap^r)	38
pBST324	Tet repressor for regulation of *tonB* from pKP292 (Km^r)	38
pTONB1	$tonB^+$ (*E. coli* $tonB^+$) (Cm^r)	
pYCIBC	*tonB* (*E. coli* $yciB^+$ $yciC^+$) (Cm^r)	

[a] Formerly strain SA2979.
[b] Abbreviation for Tn*10*Δ*16*Δ*17*.

methionine, 0.1 mM tryptophan, 15 nM cyanocobalamin (CNCbl), and, as a carbon source, 30 mM ethanolamine, 1,2-propanediol, propionate, succinate, or glycerol. Concentrations were the same regardless of whether growth was tested on solid or liquid medium. The final concentrations of antibiotics in complex medium were 20 (tetracycline), 50 (kanamycin), 20 (chloramphenicol), and 100 (ampicillin) μg/ml. In NCE medium, the final concentrations used were 10, 100, 10, and 50 μg/ml, respectively. Growth of cultures at 37°C was monitored with a Spectronic 20D spectrophotometer (Milton Roy Co., Rochester, N.Y.) at 650 nm. CAS medium was a gift from Michelle R. Rondon. All experiments were done under aerobic conditions.

Genetic techniques. (i) Transductions. All transductional crosses were performed with mutant phage P22 HT*105 int-201* (42, 43), and all phage manipulations were performed as previously described (9).

(ii) Isolation of allele Δ*1235*. The derivative of strain JE1418 capable of growing on ethanolamine as a carbon and energy source was isolated by plating JE1418 on NCE minimal medium supplemented with ethanolamine as a carbon source. Portions of 10 independent cultures (~10^9 cells) were plated individually. After incubation for 3 days, we recovered a single colony with a reversion rate estimated to be 10^{-9}. The mutant strain JE4386 was reconstructed by growing phage P22 on the revertant, using the phage lysate as the donor to transduce strain JE1418 to Eut^+, and then further characterized. A Tn*10*Δ *16*Δ*17* element (50) [referred to as Tn*10d*(Tc)] located near Δ*1235* was isolated by genetic means as described elsewhere (12). This transposon [*zde-6396*::Tn*10d*(Tc)] was ca. 30% cotransducible with Δ*1235* by phage P22.

Recombinant DNA techniques. (i) Plasmid constructions. To make pTONB1, pRZ526 was digested to completion with *Hpa*I and the ~4.9-kb fragment containing the original insert was gel purified by the QIAquick gel extraction protocol as instructed by the manufacturer (Qiagen Inc., Valencia, Calif.). This fragment was digested to completion with *Bgl*II; the ca. 3,100-bp fragment containing *tonB*, *yciC*, *yciB*, and *yciA* was gel purified by QIAquick gel extraction and cloned into the *Bam*HI/*Hin*cII site of vector pSU19, resulting in plasmid pTONB1.

To make pYCIBC, pTONB1 was digested to completion with *Sna*B1 and *Sma*I. Digested DNA was religated, and resulting plasmids were screened for the loss of the *Sna*B1/*Sma*I fragment containing *tonB* and *yciA*. The resulting plasmid, pYCIBC, contained *yciB* and *yciC* cloned in the direction of the *lac* promoter of pSU19.

(ii) PCR amplification of *tonB* from TR6583 and JE1291. *tonB* was PCR amplified from chromosomal DNA of boiled whole cells of TR6583 and JE1291. An 860-bp amplified product was generated by using the following primers in a standard PCR mixture: 5′*tonB* (5′ TTCAGCTCTGGTTTTTCA 3′, corresponding to bases 82 to 99 of the published *tonB* sequence [16]) and 3′*tonB* (5′ TCCGACGGTAAACCTCGC 3′; corresponding to bases 941 to 924 of the published *tonB* sequence [16]). The amplification profile was as follows: 94°C for 5 min; 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min; 72°C for 10 min; 4°C for 12 h. All primers used in this study were obtained from Integrated DNA Technology, Inc. (Coralville, Iowa).

(iii) PCR amplification of *tonB* from strain JE1418 (Δ*903*). *tonB* was amplified from strain JE1418 chromosomal DNA by using boiled whole cells as the template. The universal Tn*10* primer (2) was used in conjunction with the 5′*tonB* primer described above to amplify the DNA between *trpC3480*::Tn*10d*(Cm^r) to the 5′ end of *tonB*. The amplification profile was as follows: 94°C for 5 min; 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 3 min; 72°C for 10 min; 4°C for 12 h.

(iv) PCR amplification of *tonB* from strain JE2123 (Δ*1235*). *tonB* was amplified from JE2123 chromosomal DNA by using boiled whole cells as the template. The *trpA* primer 5′ AGCTTAAAGAGTACCATGCC 3′ (corresponding to bp 665 to 685 of the *trpA* sequence [26]) was used in amplifications with the 5′*tonB* primer described above to amplify from the *trpA* coding region, across the deletion, to the 5′ end of *tonB*. The amplification protocol was the same as for the universal Tn*10* and 5′*tonB* primers discussed above.

(v) DNA sequencing of PCR products. All PCR products used for sequencing were purified by using a QIAquick gel extraction kit as instructed by the manufacturer. All sequencing was done by nonradioactive sequencing at the Nucleic Acid and Protein Facility at the University of Wisconsin Biotechnology Center. Primers used for sequencing include those described above in addition to the primer *tonB* DEL (5′ GCATCGGCGACCAGCAAG 3′, corresponding to bases 538 to 555 of the *S. typhimurium tonB* sequence [16]).

Biochemical procedures. (i) β-Galactosidase assays. β-Galactosidase activity assays were performed by a modification of the method of Miller (25) as described elsewhere (14).

(ii) Immunoblot analysis of TonB. Immunoblot analysis of TonB was done as previously described (23), with minor modifications. Briefly, cells were grown in NCE medium supplemented with glycerol (30 mM), $MgSO_4$ (1 mM), methionine (0.3 mM), and tryptophan 0.1 mM, in the presence or absence of $FeSO_4$ (45 μM). Cells were grown to A_{650} of 0.5 in 5 ml of NCE medium. A 1-ml sample of culture was removed, diluted with 0.5 ml of 15% trichloroacetic acid, and incubated on ice for 30 min. The acid-precipitated material was pelleted, washed once with 1.0 M Tris-Cl (pH 8.0) at 25°C, resuspended in 50 μl of 2× sample buffer, and boiled for 5 min. Samples were then resolved by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS-PAGE) (22) and transferred to an Immobilon-P membrane (Millipore, Bedford, Mass.) by means of a Mini Trans-Blot apparatus (Bio-Rad, Richmond, Calif.) at 100 V for 1 h according to the manufacturer's specifications. Immunoblot analyses were performed according to the Phototope-horseradish peroxidase Western blot detection kit (New England Biolabs, Inc., Beverly, Mass.) protocol, with minor modifications. Membranes were blocked at 25°C overnight, and 1× Tris-buffered saline–0.1% Tween 20–5% dry milk was used for all incubations. Mouse monoclonal antibody 4H4 (a gift from Kathleen Postle) was used at a 1:5,000 dilution. The secondary antibody was horseradish peroxidase-conjugated donkey anti-mouse immunoglobulin G (a gift from Heidi Goodrich-Blair) diluted 1:20,000. X-ray film (XAR-50; Kodak) was used to detect signal.

RESULTS

eutF mutants were identified by the inability to grow on ethanolamine as source of carbon (28). Two of the mutants,

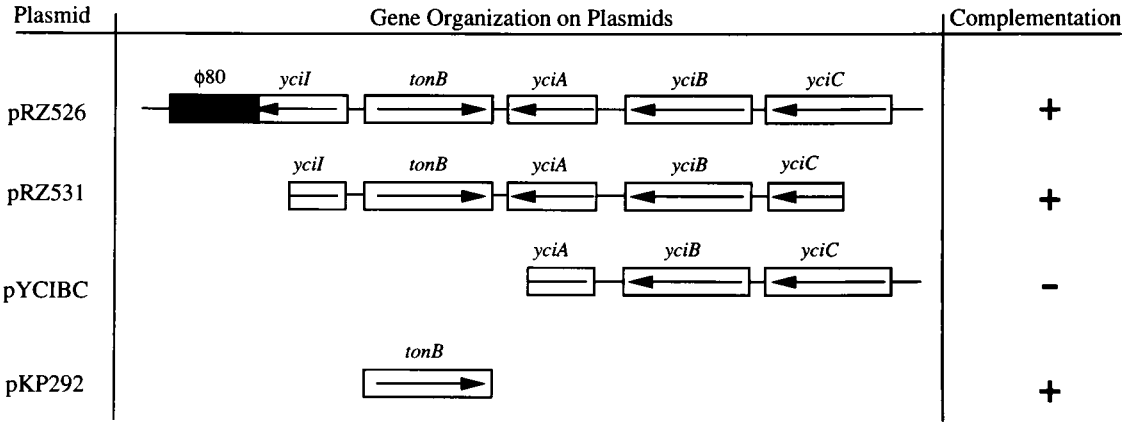

FIG. 1. Gene organization of plasmids tested for complementation. All represented reading frames are based on the *E. coli* sequence (6). Complementation is scored as growth (+) or no growth (−) on NCE plates supplemented with ethanolamine (30 mM), Met (0.3 mM), Trp (0.1 mM), $MgSO_4$ (1 mM), and CNCbl (15 nM).

one putative point mutant (strain JE1690) and a deletion mutant (strain JE1418), are characterized in this study.

The strain carrying allele *Δ903* (JE1418) was reported to be able to use ethanolamine as a sole nitrogen source but not as a sole carbon source, suggesting the *eutF* locus was only partially affected by this mutation. Characterization of strain JE1418 revealed that the *eutF* locus mapped between the *trp* operon and *tonB*. Strain JE1418 was found to be a tryptophan auxotroph, thus defining one end of the deletion somewhere within the *trp* operon. The other end of the deletion was determined based on the following tests for TonB protein function. First, strain JE1418 was sensitive to infection by bacteriophage ES18, which requires a functional TonB protein for infection (47). Second, strain JE1418 could use CNCbl or AdoCbl for the synthesis of methionine at a concentration of 15 nM. *tonB* mutants require micromolar concentrations of Cbl to overcome the transport defect (4). The putative point mutant showed TonB function as defined by these two criteria (data not shown). Further phenotypic analysis of the *eutF* locus determined that *eutF* mutants were unable to grow on 1,2-propanediol as the sole carbon source and had reduced growth rates on the nonfermentable carbon sources propionate and succinate (30).

Complementation of *eutF* mutants. Attempts to isolate a complementing clone from an *S. typhimurium* library were unsuccessful. However, complementation of the EutF phenotypes was achieved with a plasmid carrying an ~4,900-bp fragment containing the *E. coli tonB* locus and surrounding loci. This plasmid, pRZ526, also contained a fragment of bacteriophage ϕ80 DNA (33). Plasmid pRZ526 complemented strains JE1418 and JE1690 for growth on ethanolamine, suggesting that pRZ526 contained the *eutF* locus (Fig. 1). This finding suggested that the *eutF* locus was complemented by one (or more) of five possible *E. coli* genes or possibly by a ϕ80 gene. To narrow the possibilities, complementation was tested with plasmid pRZ531 (Fig. 1). pRZ531 complemented both JE1418 and JE1690, suggesting *eutF* was not *yciA*, *yciC*, or ϕ80 DNA. Previous minicell analysis of protein expression from pRZ531 determined that only two proteins were synthesized from this cloned region of *E. coli* DNA (33). The apparent molecular weights of the products, based on SDS-PAGE analysis, suggested these proteins were TonB and YciB. To address whether *yciB* was *eutF*, *yciB* and *yciC* were subcloned from pRZ526 resulting in plasmid pYCIBC (Fig. 1). Plasmid pYCIBC failed to complement strain JE1418 or JE1690, suggesting that *yciB* was not *eutF*. These results supported the previous finding that *yciC* was not involved in the complementation of EutF phenotypes.

These results led to the hypothesis that complementation of the *eutF* phenotype by pRZ526 and pRZ531 was due to *tonB*. To test this hypothesis, a plasmid containing only *tonB* (pKP292) was introduced in strains JE1418 and JE1690. Plasmid pKP292 complemented all *eutF* phenotypes described above (Fig. 1). Complementation of the growth defects on ethanolamine, 1,2-propanediol, propionate, and succinate could be due either to complementation of *tonB* mutations in strains JE1418 and JE1690 or to multicopy suppression of a mutation that affects TonB function.

***eutF* alleles result in a TonB phenotype.** To show that strains JE1418 and JE1690 contained lesions that affected *tonB* function, we tested both strains for the hypersecretion of siderophores. Disruption of TonB function causes a hypersecretion of siderophores due to iron starvation, and the presence of the secreted siderophores can be indirectly detected by the use of CAS medium (44). Both strains (JE1418 and JE1690) grown on CAS medium resulted in large orange-yellow zones around the colonies, diagnostic of siderophore hypersecretion. *tonB*$^+$ control strains TR6583 and JE1291 showed no significant zones (data not shown).

Taken together, the complementation of EutF phenotypes by wild-type *tonB*$^+$ and siderophore hypersecretion by strains JE1418 and JE1690 strongly suggested *eutF* and *tonB* were allelic.

Altered TonB proteins in *eutF* mutants. To analyze directly whether TonB was altered in *eutF* mutants, immunoblotting using an anti-TonB monoclonal antibody was performed to detect chromosomally expressed TonB protein in wild-type and mutant strains (Fig. 2).

The monoclonal antibody used in these studies, 4H4, was specific for the region near amino acids 60 to 103 of the *E. coli* TonB (23). This antibody cross-reacts with the *S. typhimurium* TonB, and it is assumed the same epitope of TonB is recognized. In the two wild-type *tonB*$^+$ strains TR6583 and JE1291, an approximately 40-kDa protein, corresponding to the electrophoretic behavior of TonB in an SDS-PAGE system (16, 23), was detected. The two mutant strains tested, however, also contained an aberrant TonB protein of approximately 45 kDa in addition to a series of bands with molecular masses of ca. 25 kDa (Fig. 2). These bands were not present in the *tonB*$^+$ control lanes, which suggested that strains JE1418 and JE1690

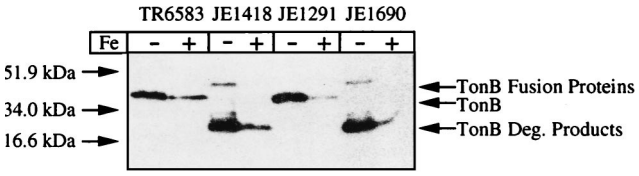

FIG. 2. Immunoblot analysis of TonB protein in strains TR6583 (*tonB*$^+$), JE1418 (Δ*903*), JE1291 (*tonB*$^+$), and JE1690 (*eutF1115*). The amount of material loaded in each lane was the equivalent of 0.1 A_{650} of whole cells. Proteins were separated by SDS-PAGE. Samples were grown in the presence or absence of 45 μM $FeSO_4$. Deg., degradation.

synthesized a TonB fusion protein that was unstable, with the 25-kDa protein being a stable degradation product of the 45-kDa fusion protein.

These results suggested that the deletion present in strain JE1418 generated a TonB fusion protein. Interestingly, strain JE1690, which was isolated during hydroxylamine mutagenesis experiments aimed at isolating point mutations in *eutF*, also contained a TonB fusion protein. The molecular characterization of both mutants is discussed below.

Iron-dependent regulation of *tonB* expression in *eutF* mutants. TonB protein levels in *S. typhimurium* and *E. coli* are reduced in the presence of high concentrations of iron due to Fur regulation of *tonB* expression (31, 51). To ensure that iron regulation of *tonB* was still functional, we grew strains JE1418 and JE1690, along with the wild-type controls, in the presence or absence of 45 μM $FeSO_4$ and detected TonB by immunoblotting as discussed above. Since we routinely use NCE medium without iron supplementation, all strains were expected to express TonB at some increased level to scavenge residual iron in the medium, as seen in studies of *tonB* regulation in *E. coli* (51). Upon addition of $FeSO_4$ to the medium, and assuming that the promoter region of *tonB* is intact, there should be a reduction in the level of TonB. All strains showed iron regulation of TonB levels (Fig. 2), consistent with the promoter region of *tonB* being unaffected in both *eutF* mutants.

The analysis of our data was initially complicated by the confusion in the literature regarding the orientation of the *tonB* gene in *S. typhimurium*. The *S. typhimurium* genetic maps published after 1988 all show *tonB* expression in the same direction as the *trp* operon (39, 40). However, the original publication discussing the orientation of *tonB* (19) and the *S. typhimurium* genetic map published in 1988 (41) showed *tonB* and the *trp* operon as convergently transcribed. The results shown here are consistent with the proposal for convergent transcription (discussed further below). Therefore, the TonB fusion protein detected in JE1418 was concluded to be the result of a fusion to the carboxy terminus of TonB.

Physical characterization of *tonB* in strains JE1418 and JE1690. To show that *tonB* was *eutF*, we attempted amplification and sequencing of chromosomal *tonB* alleles in strains JE1418 and 1690. Primers flanking *tonB* allowed amplification and sequencing of the complete coding region of *tonB*. For both of the *tonB*$^+$ strains TR6583 and JE1291, an 860-bp product was amplified as expected from the published sequence (16) (data not shown). The product from TR6583 was sequenced, and the results were consistent with the amplified product being *tonB* (data not shown). In a control experiment, the *cobU* gene of *S. typhimurium* was amplified in a parallel reaction and always resulted in successful amplification from both wild-type and mutant DNAs (data not shown).

(i) DNA sequence analysis of the *tonB* allele in strain JE1418. Reactions using DNA from strain JE1418 failed to produce an amplified *tonB* product. These results supported the hypothesis that *tonB* was affected by the Δ*903* allele in this strain. To characterize the Δ*903* allele in strain JE1418, the transposition-deficient element *trpC3480*::Tn*10d*(Cm) (>90% cotransducible with allele Δ*903*) was recombined into the chromosome of JE1418, with retention of the Δ*903* mutation (strain JE4163). The DNA between the insertion and the 5′ end of *tonB* was amplified by using a primer specific for the end of the insertion and the 5′*tonB* primer. The amplification product (ca. 3,000 bp) was sequenced by using the transposon primer. DNA sequence information determined the *trpC* sequence on one side of the PCR product, while the 5′*tonB* primer determined the *tonB* sequence as expected (data not shown). Primer walking from the *tonB* side of the PCR product identified the deletion site of strain JE4163 (Fig. 3A). Sequence data demonstrated that the fusion of *tonB* with *trpA* resulted in the removal of the last nine codons of *tonB*. In-frame translation across the *tonB-trpA* junction predicted the removal of the final 9 amino acids of TonB and the addition of 45 amino acids after amino acid 231 of wild-type TonB (Fig. 3B). These data confirmed that *tonB* was disrupted in strains carrying the Δ*903* allele and that the orientation of *tonB* transcription was toward the *trp* operon as originally proposed (19).

Interestingly, Anton and Heller previously constructed C-terminally altered TonB proteins from *E. coli* in which the final 8 amino acids of TonB were replaced with either 19 or 2 amino acids (3). In that study, the TonB fusion proteins were also rapidly degraded to an approximately 29-kDa degradation product likely similar to that seen in this study (Fig. 2). Surprisingly, the TonB fusions in the previous study did not affect TonB function, based on ϕ80 infectivity or growth response to ferrichrome or vitamin B_{12} (3). Therefore, we believe that the phenotype of JE1418 is not due to the rapid degradation of the TonB fusion protein but instead is due to the addition of 45 amino acids to the C-terminus causing a partial-loss-of-function TonB fusion protein.

(ii) DNA sequence analysis of the *tonB* allele in strain JE1690. Surprisingly, *tonB* could not be amplified from the putative point mutant, strain JE1690, which suggested that the inability to amplify *tonB* from JE1690 was probably due to the deletion of one of the *tonB* primer sites. We hypothesized that the 3′ end of *tonB* in this strain was affected since the 5′*tonB* primer was designed to hybridize immediately downstream of the −10 region, and hence a deletion that removed this site would abolish *tonB* expression.

Results from Western blot analysis discussed above confirmed that *tonB* expression was regulated by iron (Fig. 2). The inability to amplify *tonB* from strain JE1690 was an unexpected result since strain JE1690 was isolated during an experiment aimed at isolating hydroxylamine-generated point mutants (28). Although unusual, it is not unprecedented to generate deletions with this procedure (27). Regardless of how this deletion arose, PCR amplification and immunoblot analysis clearly showed that strain JE1690 carried a deletion that affected the *tonB* gene. It is also clear that the lesion in strain JE1690 is not the same as the Δ*903* allele in JE1418, since the two strains are phenotypically distinct (28).

Isolation and characterization of a mutation that restores growth of strain JE1418 on ethanolamine. Before determining that *tonB* was disrupted in strain JE1418, we isolated a derivative of it capable of growing on ethanolamine as a sole carbon source. This revertant strain displayed *eut* operon expression to ca. 60% of the level measured in a wild-type strain in the presence of ethanolamine and Cbl (Table 2). A Tn*10d*(Tc) insertion linked to the mutation causing this reversion was isolated and found to be ca. 30% cotransducible with the Δ*903*

TABLE 2. Effects of allele Δ*1235* on *eut* operon expression and growth on ethanolamine

Relevant genotype	Addition	Sp act[a] (U/A_{650})	Growth on EA[b]
tonB$^+$	None	4	NT[c]
	EA/Cbl[d]	189	+
Δ*903*	None	3	NT
	EA/Cbl	16	−
Δ*1235*	None	6	NT
	EA/Cbl	113	+

[a] Specific activity of β-galactosidase from the *lacZ* gene fused to the *eut* operon.
[b] Growth on ethanolamine (EA) as a sole carbon source in a derivative of the strain listed with a wild-type *eut* operon. The medium used was NCE supplemented with tryptophan and $MgCl_2$.
[c] NT, not tested.
[d] EA/Cbl, ethanolamine (20 mM) plus Cbl (15 nM).

allele by phage P22. This result raised the possibility that the mutation causing the reversion was in *tonB*.

We used PCR amplification and sequencing to characterize the reversion mutation. A *trpA* primer was designed and used in combination with the 5′*tonB* primer to amplify *tonB* from strain JE2123 (Δ*1235*). Analysis of DNA sequence data determined that the revertant strain carried a deletion of two bases at the junction site of *tonB* and *trpA* (Fig. 3A). This deletion (Δ*1235*) resulted in a frameshift in the coding region of the *tonB* allele generated by Δ*903*. The net result was a replacement of the 45-amino-acid addition seen in strain with the Δ*903* allele by 5 amino acids in strain JE2123 (Fig. 3B). The restored ability of strain JE2123 to grow on ethanolamine, and the near-wild-type *eut* operon expression observed in this strain clearly showed that the observed EutF phenotypes were due to mutations in *tonB* and not to the deletion of an unidentified gene.

DISCUSSION

Data presented herein show that the mutations initially defining the *eutF* locus are partial-loss-of-function *tonB* alleles. This conclusion is based on the following findings: (i) mutations in the *eutF* locus can be complemented by a plasmid containing only the wild-type allele of the *tonB* gene from *E. coli*; (ii) *eutF* mutant strains hypersecrete siderophores, consistent with lesions in *tonB*; (iii) immunoblot analyses of TonB from both mutant strains suggest the synthesis of an unstable TonB fusion protein; (iv) sequencing of *tonB* from a *eutF* mutant, JE1418 (Δ*903*), showed that the mutation resulted in the replacement of the final 9 amino acids of TonB with 45 amino acids from a *tonB-trpA* gene fusion; and (v) a 2-bp deletion (Δ*1235*) in the coding region of the *tonB-trpA* gene fusion of strain JE1418 removed the bulky addition to the carboxy terminus of TonB, resulting in a partially functional TonB protein.

Why were *eutF* mutants not identified as mutants partially defective in *tonB* function? The initial characterization of *eutF* mutants originally eliminated *tonB* from consideration for two reasons. First, *eutF* strains were still sensitive to infection by bacteriophage ES18, which requires a functional *tonB* locus for infection (47). Second, both strains were able to grow in the presence of CNCbl or AdoCbl at a concentration of 15 nM. *tonB* mutants require micromolar concentrations of Cbl to overcome the TonB transport mutation (4). We now believe that the original phenotypic screen for *eutF* alleles led to the isolation of partial-loss-of-function *tonB* alleles. Strain JE1418 was isolated in a screen for deletions of *cobA*, a gene encoding the adenosyltransferase enzyme involved in AdoCbl biosynthesis. *cobA* mutants do not grow on ethanolamine as a sole carbon source because (i) ethanolamine ammonia-lyase requires AdoCbl as a coenzyme and (ii) *cobA* mutants cannot synthesize AdoCbl (15). Strain JE1418 did not grow on ethanolamine but was found to be wild type for *cobA*, which suggested that an alternative locus was responsible for the phenotype; this locus was named *eutF* (28).

The strain used in our laboratory to study cobalamin biosynthesis is TR6583, which contains a *metE205* mutation that disrupts the Cbl-independent methionine synthase gene. Strain TR6583, therefore, is dependent on exogenous methionine or must use the Cbl-dependent methionine synthase, which requires the addition of exogenous Cbl in an aerobic environment. All strains in this and the original *eutF* study are TR6583 derivatives, and exogenous Cbl was added in all media for complementation of the *metE205* allele. In the phenotypic screen that isolated strain JE1418, a positive control was used to screen out unwanted auxotrophs. This positive control was growth on minimal medium containing glucose as a carbon source, plus 15 nM Cbl to complement the *metE205* mutation. Therefore, the screen required a functional Cbl transport system for growth on the positive control plate. This growth requirement would therefore eliminate all *tonB* mutations that completely abolished TonB function. Unexpectedly, however, this would allow partial-loss-of-function *tonB* mutants to grow as long as enough Cbl was transported for the methionine requirements. The amount of Cbl needed to meet the methionine requirement is approximately 25-fold less than the amount required for growth on ethanolamine (13). Therefore,

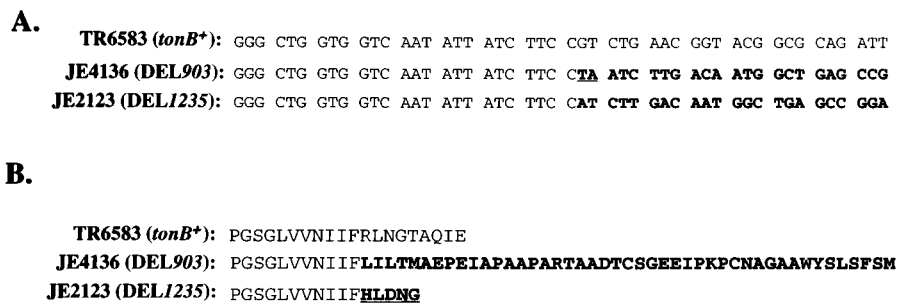

FIG. 3. Partial nucleotide and predicted amino acid sequences of *tonB* genes from TR6583, JE4163, and JE2123. (A) Nucleotide sequence flanking the *tonB-trpA* fusion site. The TR6583 sequence corresponds to bases 790 to 837 of wild-type *tonB* (16). Boldface bases correspond to bases from *trpA*; boldface underlined bases correspond to bases deleted in a strain carrying allele Δ*1235*. (B) Predicted amino acid sequence of TonB. The starting proline residue corresponds to amino acid 221 of the published sequence (16). Boldface residues are predicted amino acids from in-frame translation of *tonB* from the Δ*903* allele; boldface underlined residues are predicted amino acids from in-frame translation of the Δ*1235* allele. DEL*903* and DEL*1235* are referred to as Δ*903* and Δ*1235*, respectively, throughout the text.

it was possible to isolate *tonB* mutants that meet the Cbl requirement for methionine synthesis but do not meet the increased demand for Cbl for growth on ethanolamine. The sensitivity to ES18 infection would still occur because of the much higher sensitivity of phage infection as a TonB function assay (1). This would explain the isolation of strain JE1418, and we believe that a similar type of selection was the reason for the isolation of strain JE1690.

Explaining EutF phenotypes in terms of partially functional TonB proteins. Based on the conclusion that *eutF* mutants were actually partial-loss-of-function *tonB* alleles, the various carbon source utilization phenotypes can be explained in the following way. As described above, ethanolamine utilization requires Cbl for breakdown of ethanolamine and the induction of the genetically defined *eut* operon (35, 36, 45). Recent molecular characterization of a portion of the putative *eut* operon identified a proposed ethanolamine permease (48). Based on the findings presented here, the expression of this permease is likely to be dependent on the genetically defined regulator *eutR* (35). Therefore, a strain unable to access exogenous Cbl would not fully express the ethanolamine permease and would exhibit decreased ethanolamine transport. The combination of reduced Cbl acquisition and decreased ethanolamine transport would result in decreased expression of *eutR*-controlled genes and reduced growth rates on ethanolamine. This phenotype is exactly what was observed in *eutF* mutants (28).

The first enzyme involved in 1,2-propanediol degradation, diol dehydratase, also requires AdoCbl as a coenzyme. Aerobically growing *S. typhimurium*, therefore, requires exogenous Cbl for growth on 1,2-propanediol. Partial-loss-of-function *tonB* alleles would be expected to have growth defects on 1,2-propanediol, again as seen for the *eutF* alleles.

The final two phenotypes, reduced growth on the nonfermentable carbon sources succinate and propionate, are more difficult to explain. Growth on succinate or propionate does not require Cbl; however, mutations in *fur* have been shown to affect growth on succinate as a sole carbon source (17). Fur is a regulatory protein involved in controlling expression of a wide number of genes involved in iron acquisition (11). It is assumed that *tonB* mutations will decrease iron levels in the cell and lead to phenotypes similar to that of a *fur* mutant. The observed phenotypes may be caused by disruption of the Fur regulon, or, alternatively, growth on nonfermentable carbon sources may be more sensitive to iron levels in the cell due to the requirement of iron for oxidative phosphorylation.

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