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A Mutation in the Amino Terminus of a Hybrid TrpC-TonB Protein Relieves Overproduction Lethality and Results in Cytoplasmic Accumulation

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We have developed a selection for mutations in a *trpC-tonB* gene fusion that takes advantage of the properties of the plasmid-encoded TrpC-TonB hybrid protein. The TrpC-TonB hybrid protein consists of amino acids 1 through 25 of the normally cytoplasmic protein, TrpC, fused to amino acids 12 through 239 of TonB. It is expressed from the *trp* promoter and is regulated by the *trpR* gene and the presence or absence of tryptophan. Under repressing conditions in the presence of tryptophan, the *trpC-tonB* gene can restore φ80 sensitivity to a *tonB* deletion mutant, which indicates that TrpC-TonB can be exported and is functional. High-level expression of TrpC-TonB protein in the absence of tryptophan results in virtually immediate cessation of growth for strains carrying the *trpC-tonB* plasmid. By selecting for survivors of the induced growth inhibition (overproduction lethality), we have isolated a variety of mutations. Many of the mutations decrease expression of the TrpC-TonB protein, as expected. In addition, three independently isolated mutants expressing normal levels of TrpC-TonB protein result in a Gly→Asp substitution within the hydrophobic amino terminus of TonB. The mutant proteins are designated TrpC-TonBG26D. The mutations are suppressed by *prlA* alleles, known to suppress export (signal sequence) mutations. TrpC-TonB proteins carrying the Gly→Asp substitution accumulate in the cytoplasm. We conclude that the Gly→Asp substitution is an export mutation. TrpC-TonBG26D protein has been purified and used to raise polyclonal antibodies that specifically recognize both TrpC-TonB protein and wild-type TonB protein.

A significant number of *Escherichia coli* proteins are known to be exported from the cytoplasm to the cell envelope. However, mutations that block export have been isolated in relatively few of these proteins (summarized in reference 4). The small number of proteins for which export mutations are available undoubtedly reflects the difficulty of designing selections for such mutants. In the case of mutations created by in vitro mutagenesis, the small number may also reflect the difficulty of predicting which mutations will result in export defects (10).

One method that has been used successfully to select export mutations in both MalE protein (maltose-binding protein) and LamB protein (the bacteriophage λ receptor) employs genetic fusions of large amino-terminal fragments of the exported proteins to carboxy-terminal, enzymatically active fragments of the cytoplasmic protein β-galactosidase (2, 8). When expression of the fusion proteins is induced by addition of maltose, the fusion proteins appear to jam export sites and prevent proper localization of essential envelope proteins, leading to the phenomenon known as overproduction lethality. Mutations that decrease expression or prevent export of the fusion proteins relieve overproduction lethality.

The *tonB* gene product is a membrane-associated, periplasmically exposed, protein required for transport of iron-bearing siderophores and vitamin B_{12}. It is also required for bacteriophage φ80 and T1 infection and for the action of the B-group colicins. The chemical half-life of exported TonB is 10 min at 42°C (20). Since the half-life of TonB and its function appear to be related (13), we reasoned that cytoplasmically localized, nonfunctional TonB protein might be more stable than exported, functional TonB and thus easier to purify.

This paper describes a selection resulting in mutations in a hybrid protein consisting of the amino terminus of TrpC, a normally cytoplasmic protein, fused to the carboxy terminus of TonB. The selection takes advantage of the observation that high-level expression of the TrpC-TonB protein (expressed from the *trp* promoter) rapidly inhibits cell growth. Among the survivors of the overproduction lethality are export mutants. The export-defective TrpC-TonB proteins accumulate in the cytoplasm, where they can be easily purified and used for antibody production.

MATERIALS AND METHODS

Plasmids and bacterial strains. *E. coli* K-12 MO (F^- Str^r), SG932 [F^- *lac*(Am) *trp*(Am) *pho*(Am) *mal*(Am) *rpsL supC*(Ts) *tsx*::Tn*10 lon-100*], MC4100 [*araD139 rpsL150, deoC1 ptsF25 rbsR flbB5301* Δ(*arg-lac*)*U169 relA1 thi*] (5), SE6004 (MC4100 *lamBS60 prlA4*), and RL402 (MC4100 *lamBS60 prlA402*) (7) were the strains used in these studies. Plasmid pWU5 is a pBR322 derivative containing the tryptophan t and t' terminators cloned downstream from the tryptophan promotor (24). To construct pKP925, a 500-base-pair *Eco*RI fragment from pWU5 containing the terminators was excised and replaced by a 925-base-pair *Hae*III fragment from pRZ540 containing part of the *tonB* gene (19). Plasmid pKP925 encodes an in-frame fusion of the amino-terminal 25 amino acids of TrpC to the carboxy-terminal amino acids 12 to 239 of TonB (Fig. 1).

Media. YT agar was used for the general growth of all strains (16). Casamino Acid plates contained 1.5% agar, 0.2% glucose, 0.3% Casamino Acids (Difco Laboratories, Detroit, Mich.), 1× M9 salts (22), 0.12% citrate, 4 μg of thiamine per ml, 1 mM $MgSO_4$, 500 ng of $FeSO_4$ per ml, and

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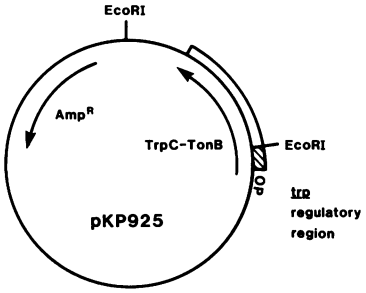

FIG. 1. Map of pKP925. Plasmid pKP925 encodes a *trpC-tonB* fusion protein. OP, *trp* operator and promoter; ▨, *trpC* DNA; □, *tonB* DNA.

100 μg of ampicillin per ml. M9 salts supplemented with 0.4% glucose, 0.2% Casamino Acids, 4 μg of thiamine per ml, and 1 mM $MgSO_4$ were used as the general growth medium for all strains, unless otherwise indicated. Where indicated, tryptophan was added at 40 μg/ml. All strains were grown at 30°C.

Enzymes and biochemicals. In vitro transcription-translation kits, [^{35}S]methionine (~1,000 Ci/mmol), and [α-^{32}P] dGTP (>3,000 Ci/mmol) were purchased from Amersham Corp., Arlington Heights, Ill. Acrylamide (97% pure) and high-pressure liquid chromatography-grade acetone (99.9% pure) were purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis. Sodium dodecyl sulfate (specially pure) was purchased from BDH Chemicals Ltd., Poole, England. Proteinase K was from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Bisacrylamide was purchased from Bio-Rad Laboratories, Richmond, Calif. Hydroxylamine HCl, 3β-indoleacrylic acid, diethylpyrocarbonate, phenylmethylsulfonyl fluoride, and all deoxy- and dideoxynucleotides were purchased from Sigma Chemical Co., St. Louis, Mo. Klenow fragment and *Pst*I were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md. The synthetic oligonucleotide, 5′-CGCAAACAGCAGCAACC-3′, was purchased from the Peptide-Oligonucleotide Synthesis Facility, University of California, San Diego.

Hydroxylamine mutagenesis of pKP925. Plasmid pKP925 was mutagenized with hydroxylamine as described (11). Mutant pKP925 was used to transform *E. coli* MO, and survivors were selected on Casamino Acid plates (which lack tryptophan) supplemented with 100 μg of ampicillin per ml. Approximately 500 candidate mutants arose from each microgram of mutagenized plasmid DNA. Single colonies were purified on plates, cultured overnight in L broth (16) supplemented with ampicillin (100 μg/ml), and used as a source of plasmid DNA (15). Plasmids from 18 colonies were retransformed into strain MO and screened on Casamino Acid plates supplemented with ampicillin to ensure that the mutations were plasmid borne.

In vitro expression of TrpC-TonB proteins. Proteins encoded by 18 mutant plasmids were synthesized with [^{35}S]methionine and the Amersham in vitro transcription-translation kit and were analyzed on polyacrylamide gels as described previously (20).

In vivo assay for mutant TrpC-TonB accumulation. Strain SG932 carrying mutant plasmids was grown in supplemented M9 medium with 100 μg of ampicillin per ml and tryptophan at either 40 or 1.5 μg/ml for 18 h at 30°C. Cells grown in the presence of 1.5 μg of tryptophan per ml had exhausted the supply of tryptophan by 18 h. Portions (600 μl) of the cultures diluted to an optical density at 550 nm of 0.4 were placed in an equal volume of high-pressure liquid chromatography-grade acetone and set on ice for a minimum of 30 min. Samples were centrifuged at 4°C for 5 min, the supernatant was discarded, and the pellets were suspended in 50 μl of sample buffer (14). The samples were then heated at 95°C for 3 min, and 40-μl portions were electrophoresed on a sodium dodecyl sulfate (SDS)–13% polyacrylamide gel (0.5-mm spacers) and stained with Coomassie blue dye to visualize the proteins.

DNA sequencing. Purified plasmid DNA was linearized by digestion with *Pst*I and annealed with the synthetic oligonucleotide by heating for 3 min at 95°C and then cooling on ice for 15 min. The dideoxy-chain termination method of DNA sequence analysis (21) was used to sequence the region of the *trpC-tonB* gene which contains the hydrophobic TonB amino terminus. The oligonucleotide corresponds to codons 19 through 24 of *trpC-tonB*.

Effect of *prlA* mutations on strains carrying pKP925 or pKP931. MC4100 (*prlA*$^+$), SE6004 (*prlA4*), and RL402 (*prlA402*) carrying either pKP925 or pKP931 were grown to saturation in supplemented M9 medium with tryptophan and ampicillin at 30°C. Overnight cultures were diluted 1:50 in the same medium and grown until they reached an optical density at 550 nm of 0.5. Cells were stored at 4°C overnight, diluted 1:100, grown to an optical density at 550 nm of 0.3, filtered, washed, suspended in the same medium (except lacking tryptophan and supplemented with 5 μg of 3β-indoleacrylic acid per ml), and incubated at 30°C. Optical density was measured every 30 min until the parent strain (MC4100) reached saturation.

Proteinase K accessibility of TrpC-TonBG26D. SG932 carrying pKP931 was grown in supplemented M9 containing tryptophan and ampicillin overnight at 30°C. Cells were diluted 1:50 and grown in the same medium at 30°C until they reached an optical density at 550 nm of 0.3. Cells were filtered, washed, and suspended in M9 minimal medium (without tryptophan) in the presence of 20 μg of 3β-indoleacrylic acid per ml. After 10 min, 60 μCi of [^{35}S]methionine per ml was added and the cells were incubated for 1 min. Cells were pelleted by centrifugation for 1 min in an Eppendorf 5414 centrifuge at 4°C. They were then converted to spheroplasts and treated with proteinase K as previously described (20).

Purification of TrpC-TonBG26D. *E. coli* SG932 carrying pKP931 was grown for 18 h in minimal medium with limiting tryptophan (1.5 μg/ml). Cells were harvested by centrifugation (10,400 × *g* for 30 min) and suspended in 20 ml of extraction buffer (10 mM potassium phosphate [pH 7.0], 7 mM 2-mercaptoethanol, 1 mM EDTA, 0.4 M NaCl, 0.1 mM NaN_3). Cells were ruptured by three passes through a French press at 18,000 to 20,000 lb/in^2. The resulting lysate was centrifuged at 600 × *g* for 1 h, and the pellet was suspended in sample buffer (14). Analysis of the 600 × *g* pellet and its supernatant for total protein with the Bio-Rad protein assay indicated that only 4% of the total cellular protein fractionated in the pellet. Analysis of the pellet by two-dimensional polyacrylamide gel electrophoresis indicated that there were no other proteins in the pellet with the same apparent molecular weight as that of TrpC-TonBG26D. The solubilized pellet was then electrophoresed on SDS–11% polyacrylamide slab gels (14), and the TrpC-TonBG26D band was cut from the gel. TrpC-TonBG26D was electroeluted from the gel slices overnight into electrophoresis buffer to which 2-mercaptoethanol had been added to a final concentration of 7 mM. The protein was then precipitated in an equal volume of acetone and suspended in phosphate-

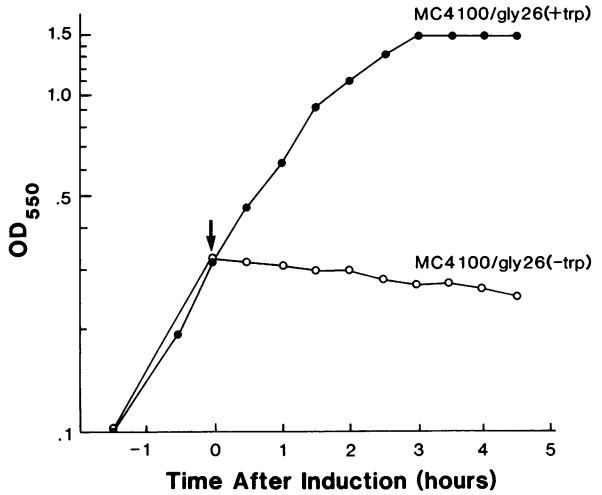

FIG. 2. Growth of MC4100-pKP925 in the presence or absence of tryptophan. The arrow indicates the point at which the cells were filtered, washed, and suspended in the same medium (+trp [●]) or medium lacking tryptophan and including 3β-indoleacrylic acid (−trp [○]). OD_{550}, Optical density at 550 nm.

buffered saline. The concentration of TrpC-TonBG26D was determined by BCA assay (Pierce Chemical Company, Rockford, Ill.). Samples of the purified TrpC-TonBG26D were sent to Bethyl Laboratories (Montgomery, Tex.) for the production of rabbit antiserum.

RESULTS

Isolation of mutations in the *trpC-tonB* gene fusion. Cloning of a 925-base-pair *Hae*III fragment with *Eco*RI ends from the *tonB* gene into plasmid pWU5 at the *Eco*RI site resulted in the in-frame fusion of codons 1 through 25 of *trpC* with codons 12 through 239 of *tonB* (plasmid pKP925 [Fig. 1; 19]). Expression of the hybrid protein is under *trp* operon control: *trp* promoter-operator, *trpR*, and tryptophan.

Plasmid pKP925 has an interesting phenotype in that it can complement a *tonB* deletion with respect to ϕ80 sensitivity, indicating that the TrpC-TonB protein is, to some extent, exported and functional. This result is not totally surprising, however, since the predicted TrpC-TonB hybrid protein includes most of the hydrophobic amino terminus of TonB (amino acids 13 to 32 [18, 20]). We have previously demonstrated, by using a Tn*phoA* insertion, that approximately the first 41 amino acids of TonB are sufficient to export alkaline phosphatase (20). Perhaps more surprisingly, removal of tryptophan from the growth medium of a prototrophic strain carrying pKP925 resulted in a virtually immediate cessation of growth (Fig. 2). We suspected that the growth cessation was due either to events associated with export of the hybrid protein or to events subsequent to its export such as aberrant TonB-like function. It, therefore, should be possible to isolate mutations which can no longer export the TrpC-TonB protein by selecting for mutants in pKP925 which survive the induced growth inhibition (overproduction lethality). Plasmid pKP925 was mutagenized in vitro with hydroxylamine and transformed into strain MO. Mutant plasmids were selected by plating the transformation mixture onto glucose minimal plates supplemented with Casamino Acids and ampicillin.

In vitro transcription-translation of mutant plasmids. Purified plasmids were initially screened in vitro for the ability to direct synthesis of normal levels of full-length 38-kilodalton TrpC-TonB protein (Fig. 3). This screen resulted in candidates pKP926, pKP929, pKP930, pKP931, pKP932, and pKP933. Other plasmids directed synthesis of lower levels of TrpC-TonB (pKP927, pKP928, pKP935, and pKP936) or, in one case, normal levels of truncated TrpC-TonB (pKP934).

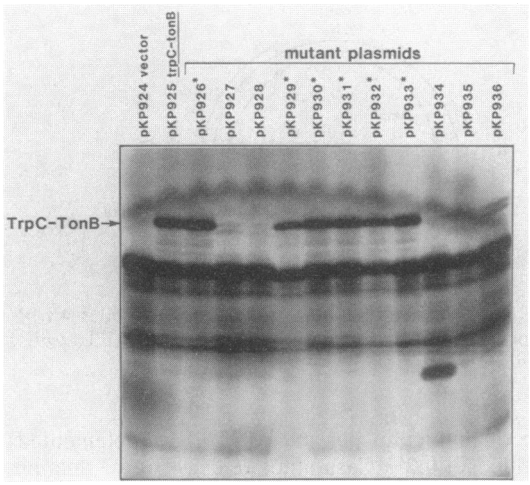

FIG. 3. In vitro analysis of [^{35}S]methionine-labeled TrpC-TonB protein encoded by pKP925 and 11 of 18 of its mutant derivatives. Plasmids designated by an asterisk were chosen for further study.

Accumulation of mutant TrpC-TonB proteins in vivo. Wild-type exported TonB has a short chemical half-life in vivo (20) that appears to be related to its function (13). We speculated that, if export mutants did exist among the six plasmids to arise from the in vitro screen, they might result in stabilization of TrpC-TonB by virtue of its localization to the cytoplasm. Proteins from *lon* strains carrying the six candidate plasmids grown in the presence or absence of tryptophan were analyzed by polyacrylamide gel electrophoresis

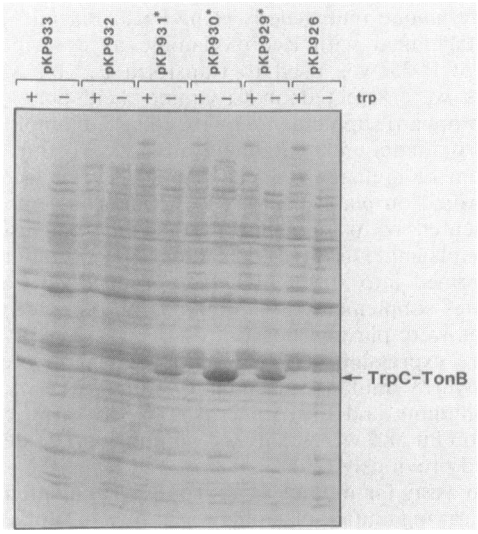

FIG. 4. In vivo analysis of total cellular protein encoded by mutant plasmids in SG932 (*lon*). Bacteria were grown in the presence of high (+) or limiting (−) levels of tryptophan as described in Materials and Methods. Plasmids designated by an asterisk were chosen for further study.

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TrpC-TonB  MetGluThrValLeuAlaLysIleValAlaAspLysAlaIleLeuValGluAlaArgLys
TonB

TrpC-TonB  GlnGlnGlnProLeuGluPheProGlySerVal ProThrLeuLeuSerValCysIleHis
TonB       MetThrLeuAspLeuProArgArgPheProTrp ProThrLeuLeuSerValCysIleHis

                    Asp
TrpC-TonB  GlyAlaValValAlaGlyLeuLeuTyrThrSerValHisGlnValIleGluLeuProAla
TonB       GlyAlaValValAlaGlyLeuLeuTyrThrSerValHisGlnValIleGluLeuProAla
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FIG. 5. Comparison of the amino-terminal region of the deduced TrpC-TonB and the TonB amino acid sequences. The position of the Gly→Asp mutation found in pKP929, pKP930, and pKP931 is indicated. The amino acids amino terminal to the space in the sequence reflect differences between TrpC-TonB and TonB. The amino acids carboxy terminal to the space are identical in both proteins. The hydrophobic amino-terminal region common to both proteins is indicated by a closed bar.

(Fig. 4). In the absence of tryptophan, strains carrying plasmids pKP929, pKP930, and pKP931 clearly accumulate a 38-kilodalton protein with the same mobility as TrpC-TonB protein synthesized in vitro. This same protein is not detectable when strains carrying those plasmids are grown in the presence of tryptophan. TrpC-TonB protein does not accumulate under either condition in strains carrying plasmid pKP926, pKP932, or pKP933.

DNA sequence of putative export mutations. The DNA sequences corresponding to codons 31 to 87 of the six mutant *trpC-tonB* genes were determined (Fig. 5). Plasmids pKP929, pKP930, and pKP931 all contain a mutation of a glycine codon to an aspartate codon (GGT→GAT) at position 46 of the TrpC-TonB open reading frame. Since this corresponds to position 26 in the deduced TonB amino acid sequence (18, 20), we refer to the mutant protein as a TrpC-TonBG26D. No mutations were detected in the corresponding regions of plasmid pKP926, pKP932, or pKP933.

Effect of *prlA* alleles. To determine whether the Gly→Asp mutation was an export mutation, we attempted to suppress it with *prlA* alleles known to suppress signal sequence mutations. Efficient *prlA* suppression of the Gly→Asp mutation would be characterized by growth cessation in the absence of tryptophan similar to that observed for strains carrying pKP925. Figure 6 shows that growth of the *prlA*$^+$ strain carrying pKP931 (TrpC-TonBG26D) is unaffected by a shift to medium without tryptophan. In contrast, growth of the *prlA402* strain carrying pKP931 virtually parallels the growth cessation of the *prlA*$^+$ strain carrying pKP925 (TrpC-TonB). The *prlA4* allele appears to have an intermediate effect.

Proteinase K accessibility of TrpC-TonBG26D. In order to determine if the accumulated TrpC-TonBG26D protein was cytoplasmically localized, proteinase K accessibility experiments were performed. A *lon* strain carrying plasmid pKP931 was shifted to medium without tryptophan and pulse-labeled with [^{35}S]methionine. A portion of the labeled cells was converted to spheroplasts and either lysed by osmotic shock or left untreated. Half of each of the three fractions (whole cells, spheroplasts, and lysed spheroplasts) was treated with proteinase K. Results indicated that the TrpC-TonBG26D protein is sensitive to proteinase K only in lysed spheroplasts and is thus cytoplasmically localized (Fig. 7). These results also demonstrate that the in vitro- and in vivo- synthesized TrpC-TonB proteins have identical mobilities.

Purification of TrpC-TonBG26D protein. The TrpC-TonBG26D protein accumulated to a high degree in strains

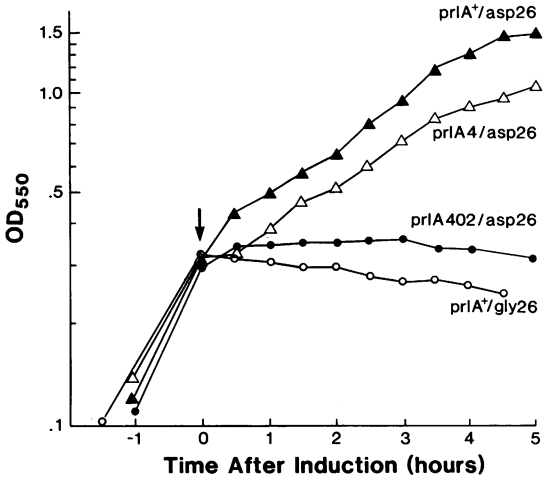

FIG. 6. Effect of *prlA* alleles on growth of strains carrying TrpC-TonB or TrpC-TonBG26D. The arrow indicates the time at which cultures were shifted to medium without tryptophan and with 3β-indoleacrylic acid. Triangles and closed circles, pKP931 (encoding TrpC-TonBG26D); open circles, pKP925 curve shown in Fig. 2. OD_{550}, Optical density at 550 nm.

grown in the absence of tryptophan and appeared to form inclusion bodies as the cell growth approached saturation (data not shown). We were able to substantially purify the TrpC-TonBG26D protein from these cells by centrifugation of French-pressed cells at 600 × *g* (Fig. 8). This procedure removed 95% of the contaminating protein. The resultant pellet was solubilized in SDS and electrophoresed on SDS-polyacrylamide gels, and gel slices containing TrpC-TonBG26D were electroeluted. This TrpC-TonBG26D protein was approximately 99% pure by silver stain criteria and was used to prepare antiserum. The resultant antiserum specifically recognizes and precipitates both TrpC-TonB and wild-type TonB from an in vitro transcription-translation reaction (Fig. 9).

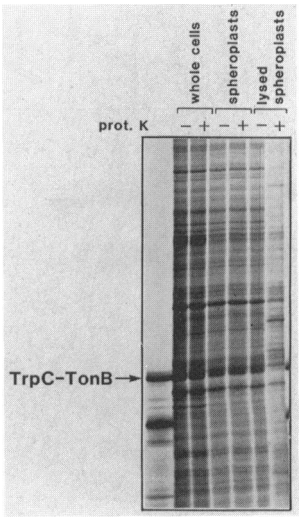

FIG. 7. Proteinase K accessibility of TrpC-TonBG26D encoded by pKP931. An autoradiogram of treated (+) or untreated (−) [^{35}S]methionine-labeled total cellular protein is shown. The leftmost lane represents an in vitro transcription-translation of pKP931.

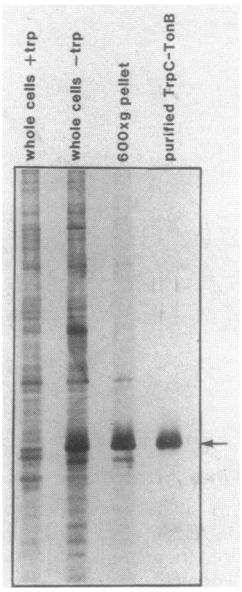

FIG. 8. Purification of TrpC-TonBG26D by differential centrifugation and SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. The gel shown is silver stained.

DISCUSSION

We have developed a selection for mutations in a *trpC-tonB* gene fusion. The selection relies on the use of a multicopy plasmid, pKP925, which encodes a hybrid protein consisting of amino acids 1 through 25 of the cytoplasmic protein, TrpC, fused to amino acids 12 through 239 of TonB. The TrpC-TonB protein is under *trp* operon control, such that its expression is repressed in the presence of tryptophan and derepressed in its absence. Removal of tryptophan from the growth medium of strains carrying pKP925 results in an immediate cessation of growth in the strains. By selecting for survivors of the overproduction lethality, three independent isolates of the same putative export mutation, a glycine→aspartate codon change, were discovered within the TonB portion of the protein. Because these mutations occur at a position corresponding to amino acid 26 of the deduced TonB amino acid sequence, the relevant mutant hybrid proteins are called TrpC-TonBG26D. The subsequent results pertain to the TrpC-TonBG26D protein encoded by plasmid pKP931.

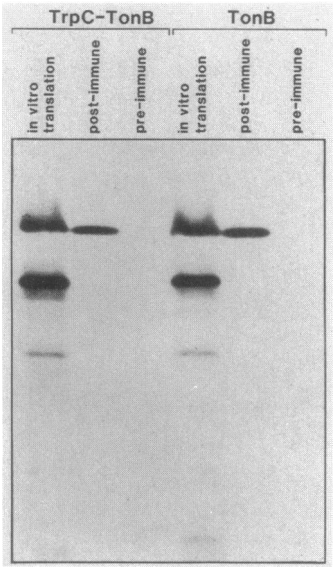

FIG. 9. Immunoprecipitation of TrpC-TonB and TonB from an in vitro transcription-translation with rabbit antisera raised against the purified TrpC-TonBG26D shown in Fig. 8.

The Gly→Asp mutation can be characterized as an export mutation by several criteria. (i) The nature and location of the substitution within the TrpC-TonB coding region is characteristic of *E. coli* signal sequence mutations (for a summary, see reference 4). The mutation occurs within a region of TrpC-TonB corresponding to the hydrophobic amino terminus of TonB (amino acids 13 through 32 [Fig. 5]), which has already been suggested to function in export (20). The mutation changes an uncharged amino acid to a charged amino acid. (ii) The Gly→Asp mutation can be suppressed by *prlA* alleles known to suppress a wide range of signal sequence mutations (6). *prlA* suppressors restore the overproduction lethality phenotype to the TrpC-TonBG26D protein. Consistent with other studies, *prlA402* is a stronger suppressor of TrpC-TonBG26D than *prlA4* (1). The *prlA* alleles do not suppress (i.e., restore overproduction lethality to) any of the other mutant plasmids isolated in our selection (data not shown). (iii) The majority of TrpC-TonBG26D protein is cytoplasmically localized, although TonB protein itself is known to be an exported protein (20).

TrpC-TonB protein must be exported to some extent, since it is phenotypically TonB$^+$. Plasmids encoding the TrpC-TonBG26D protein are also TonB$^+$ by the criterion of ϕ80 sensitivity. This is not surprising, however, since many signal sequence mutations are not 100% defective (23). It does emphasize, however, the nonquantitative aspects of the ϕ80 infectivity assay.

Our selection for mutations in TrpC-TonB appears to be similar in some respects to genetic selections which take advantage of the Mal^s phenotype of specific *malE-lacZ* and *lamB-lacZ* gene fusions. Both selections rely on attempted export of highly expressed, abnormal fusion proteins. There are differences in the two selections, however, which suggest that the basis of TrpC-TonB overproduction lethality may differ from that observed for MalE-LacZ or LamB-LacZ proteins. In the case of the MalE and LamB fusion proteins, overproduction lethality is caused by jamming of export sites (3, 12). The growth effects of overproduction lethality are seen 2 to 3 h after induction by maltose, at which time the cells lyse (3). In the case of the TrpC-TonB protein, the growth inhibitory effect of overproduction is immediate, much like effects observed for overproduction of gpI of bacteriophage f1 and for a deletion mutation of *lpp* (9, 17). In those cases, growth cessation results from rapid depolarization of the cytoplasmic membrane. It is important to note that induction of wild-type TonB protein synthesized from the *trp* promoter on a plasmid analagous to pKP925 does not affect cell growth (data not shown). Thus, overproduction lethality almost certainly resides in the unique character of the TrpC-TonB protein. We are currently investigating the nature of TrpC-TonB overproduction lethality.

A variety of other mutations were obtained from this selection. Mutations which generally reduce expression, such as promoter down or ribosome-binding site mutations, were eliminated from consideration by an in vitro transcription-translation assay. A plasmid which encoded normal levels of a TrpC-TonB nonsense fragment was also detected by this assay. The most puzzling mutants were those which

showed normal levels of expression in vitro but failed to accumulate TrpC-TonB in vivo under inducing conditions (the absence of tryptophan). These mutations are not suppressed by *prlA*, nor did we detect lowered yields when these plasmids were purified. Further characterization of these mutant proteins may provide insight into the basis of TrpC-TonB overproduction lethality.

The ultimate goal of this project was to purify mutant TrpC-TonB and obtain polyclonal antibodies directed against TonB protein. The isolation of an export mutation which results in cytoplasmic localization and accumulation of TrpC-TonBG26D allowed us to circumvent the problems posed by the short chemical half-life of wild-type TonB. The accumulated TrpC-TonBG26D was readily purified by differential centrifugation and SDS-polyacrylamide gel electrophoresis. The antibodies raised against TrpC-TonBG26D can sensitively detect TonB protein as well as TrpC-TonB. The anti-TrpC-TonBG26D antiserum will be a valuable tool in future experiments to determine the nature of TrpC-TonB overproduction lethality and in our continuing investigation of TonB structure and function.

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