

## Identification of TonB Homologs in the Family *Enterobacteriaceae* and Evidence for Conservation of TonB-Dependent Energy Transduction Complexes

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**The transport of Fe(III)-siderophore complexes and vitamin B<sub>12</sub> across the outer membrane of *Escherichia coli* requires the TonB-dependent energy transduction system. A set of murine monoclonal antibodies (MAbs) was generated against an *E. coli* TrpC-TonB fusion protein to facilitate structure and function studies. In the present study, the epitopes recognized by these MAbs were mapped, and their distribution in gram-negative organisms was examined. Cross-species reactivity patterns obtained against TonB homologs of known sequence were used to refine epitope mapping, with some epitopes ultimately confirmed by inhibition experiments using synthetic polypeptides. Epitopes recognized by this set of MAbs were conserved in TonB homologs for 9 of 12 species in the family *Enterobacteriaceae* (including *E. coli*), including previously unidentified TonB homologs in *Shigella*, *Citrobacter*, *Proteus*, and *Kluyvera* species. These homologs were also detected by a polyclonal  $\alpha$ -TrpC-TonB serum that additionally recognized the known *Yersinia enterocolitica* TonB homolog and a putative TonB homolog in *Edwardsiella tarda*. These antibody preparations failed to detect the known TonB homologs of either *Pseudomonas putida* or *Haemophilus influenzae* but did identify potential TonB homologs in several other nonenteric gram-negative species. In vivo chemical cross-linking experiments demonstrated that in addition to TonB, auxiliary components of the TonB-dependent energy transduction system are broadly conserved in members of the family *Enterobacteriaceae*, suggesting that the TonB system represents a common system for high-affinity active transport across the gram-negative outer membrane.**

The outer membrane of gram-negative bacteria is a diffusion barrier that excludes a variety of toxic agents from the cell proper. This barrier is circumvented by small hydrophilic nutrients, which can enter the periplasmic space by simple diffusion through nonspecific aqueous channels created by porin proteins, and by certain larger nutrients, which can enter the periplasm by facilitated diffusion through stereospecific pores like LamB. Conversely, a third group of nutrients, Fe(III)-complexed siderophores and vitamin B<sub>12</sub>, are dependent on active transport via high-affinity outer membrane receptors to enter the periplasmic space.

The active transport of Fe(III)-bearing siderophores and vitamin B<sub>12</sub> (as well as certain colicins and bacteriophage that exploit this process) across the outer membrane is complicated by the absence of a local energy source. Free diffusion of protons through porins renders the outer membrane unable to sustain an electrochemical potential sufficient to energize active transport. In addition, periplasmic phosphatases preclude the use of high-energy phosphate carriers as an energy source. Early experiments with  $\phi$ 80 and T5 found that the electrochemical potential of the cytoplasmic membrane was required for irreversible adsorption of these phage, suggesting that the energy for this outer membrane phenomenon originates at the cytoplasmic membrane (19). This observation has been convincingly confirmed by a set of experiments that strongly implicate cytoplasmic membrane proton motive force as the en-

ergy source (6). The transduction of cytoplasmic membrane proton motive force to the outer membrane for transport of Fe(III)-complexed siderophores, vitamin B<sub>12</sub>, group B colicins, and certain bacteriophage is uncoupled in *Escherichia coli* strains with mutations at the *tonB* locus (3, 15, 21, 46). *E. coli* TonB is anchored in the cytoplasmic membrane by an uncleaved leader sequence (45), where it associates with two other cytoplasmic membrane proteins also involved in energy transduction, ExbB and ExbD (2, 18, 19, 22, 54). At least one of these proteins (ExbB) appears to assist TonB function by modulating the conformation of TonB (34) and possibly mediating its recycling (34, 39). In vivo chemical cross-linking studies identify two additional, uncharacterized *E. coli* polypeptides with sizes of approximately 7 and 41 kDa that appear to closely associate with TonB (34, 35, 54). Cumulative evidence suggests that these auxiliary proteins associate with TonB to form an energy transduction complex (42), with the energy transduction event itself appearing to involve direct physical contact between TonB and outer membrane high-affinity receptors (42, 54).

In the present study, antibody probes that we developed for the examination of *E. coli* TonB structure and function were used to screen other bacterial species for the presence of TonB. The availability of sequence data from certain of these species (5, 7, 8, 16, 20, 26, 30) afforded the opportunity to refine the mapping of epitopes identified by these antibodies. The observation that some of the epitopes recognized by these antibodies were broadly conserved allowed the identification of putative TonB homologs in species from which *tonB* had not been previously identified. The identification of additional TonB homologs was supported by the results of in vivo chemical cross-linking studies, which further suggested that the de-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference <sup>b</sup>
<b>Strains</b>		
<i>E. coli</i>		
W3110	F- IN( <i>rrnD-rrnE</i> )1	24
KP1026	W3110 $\lambda$ RS205( <i>tonB168</i> ), <i>tonB::kan</i>	41
KP1032	W3110, <i>tonB::kan</i>	34
KP1082	GM1 $\Delta$ ( <i>trp-tonB opp galU ana</i> )	54
KP1096	W3310 <i>trpB::Tn10 tonB<math>\Delta</math>(66-100)</i>	35
KP1136	W3110 <i>trpB::Tn10 tonB175</i> (Am)	33
KP1150	W3110 <i>trpB::Tn10 tonB162</i> (Am)	33
KP1229	W3110 $\Delta$ ( <i>trp tonB opp galU ana</i> )	2
<i>Actinobacillus pleuropneumoniae</i> serotype 7 <sup>a</sup>		
<i>Aeromonas hydrophila</i>		R. Bonnah
<i>Campylobacter jejuni</i> M275 <sup>a</sup>		ATCC 7965
<i>Citrobacter freundii</i>		M. Konkel
<i>Coxiella burnetii</i> Nine mile RSA493 <sup>a</sup>		ATCC 8090
<i>Edwardsiella tarda</i> CDC1483-59		L. Mallavia
<i>Enterobacter aerogenes</i>		ATCC 15947
<i>Fremyella diplosiphon</i> 33 <sup>a</sup>		ATCC 13048
<i>Haemophilus influenzae</i> DL63 <sup>a</sup>		N. Federspiel
<i>Klebsiella pneumoniae</i> KAY2026		R. Bonnah
<i>Kluyvera ascorbata</i> CDC0648-74		J. Lengeler
<i>Listonella (Vibrio) anguillarum</i> NCMB829		ATCC 33433
<i>Morganella morganii</i> M11		ATCC 14181
<i>Neisseria meningitidis</i> B16B6 <sup>a</sup>		ATCC 25830
<i>Pasteurella haemolytica</i> disease isolate <sup>a</sup>		R. Bonnah
<i>Proteus vulgaris</i>		R. Bonnah
<i>Pseudomonas aeruginosa</i> PAO1		ATCC 13315
<i>Pseudomonas putida</i> WCS358		D. Ohman
<i>Salmonella typhimurium</i> LT2		W. Bitter
<i>Serratia marcescens</i> W225		ATCC 19585
<i>Shigella dysenteriae</i> Newcastle		U. K. Winkler
<i>Yersinia enterocolitica</i> WA		ATCC 13313
<i>Bacillus subtilis</i>		ATCC 27729
<i>Micrococcus lysodekcticus</i>		WSU stock
<i>Staphylococcus aureus</i>		WSU stock
<i>Streptococcus lactis</i>		WSU stock
<b>Plasmids</b>		
pRZ553	ColE1 (Kan <sup>r</sup> ) derivative, <i>tonB::IS1</i> near codon 193	44
pRZ554	ColE1 (Kan <sup>r</sup> ) derivative, <i>tonB::IS1</i> at codon 104	44
pKP287	pJA113 <i>tonB</i> <sup>+</sup>	47
pKP290	pKP287 derivative, <i>tonB::phoA</i> (at codon 207)	47
pKP293	pKP287 derivative, <i>tonB::phoA</i> (at codon 125)	47
pKP291	pKP287 derivative, <i>tonB::phoA</i> (at codon 60)	47
pKP294	pKP287 derivative, <i>tonB::phoA</i> (at codon 32)	47
pBST324	pACYC177 <i>bla::tetR</i>	38
pES3	<i>colB</i> Amp <sup>r</sup>	V. Braun

<sup>a</sup> Species was provided as either an LSB-solubilized sample or in the case of *Fremyella diplosiphon* as frozen cells.

<sup>b</sup> ATCC, American Type Culture Collection; WSU, Washington State University.

gree of conservation and range of distribution of TonB were shared by the auxiliary proteins that participate in TonB-dependent energy transduction.

## MATERIALS AND METHODS

**Materials.** The reagents used in this study were purchased from Sigma (St. Louis, Mo.), with the following exceptions. Media components were purchased from Difco Laboratories (Detroit, Mich.). Enhanced chemiluminescence (ECL) immunoblot kits were purchased from Dupont NEN (Boston, Mass.). Horseradish peroxidase-conjugated sheep  $\alpha$ -mouse and donkey  $\alpha$ -rabbit immunoglobulins were purchased from Amersham, Inc. (Arlington Heights, Ill.). Immobilin P was purchased from Millipore Corp. (Bedford, Mass.). Polypeptides were synthesized by Research Genetics, Inc. (Huntsville, Ala.). Acrylamide was purchased from Fisher Biotechnology (Pittsburg, Pa.), and bisacrylamide was purchased

from Bio-Rad Laboratories (Richmond, Calif.). Sodium dodecyl sulfate (SDS) was purchased from Schwartz/Mann (Cleveland, Ohio). Formaldehyde was purchased from J. T. Baker, Inc. (Phillipsburg, N.J.). 5a,6-anhydrotetracycline (AT) was kindly provided by Kevin Bertrand. Colicin B was prepared as previously described from pES3-bearing strains (35). Both polyclonal rabbit  $\alpha$ -TonB serum and murine monoclonal antibodies (MAb) were generated against gel-purified TrpC-TonB fusion protein (56) as described previously (53, 54). All MAbs were obtained as ascites preparations from female BALB/c mice.

**Strains and plasmids.** The strains and plasmids used in this study are described in Table 1. All *E. coli* strains were K-12 derivatives. Strains KP1136 and KP1150 contain spontaneous amber mutations, originally derived in MC4100 and then transduced by P1vir to the suppressor-free W3110 background (52). These strains will be described elsewhere (33). KP1229 is a  $\Delta$ (*trp-tonB-opp-galU-ana*) derivative of W3110 (2). For *Salmonella*, *Klebsiella*, *Enterobacter*, *Serratia*, and *Yersinia* species and *Pseudomonas putida*, the species and strains from which *tonB* sequences were previously determined were used. For the other species examined, the neotype strain was used, except for *Listonella (Vibrio) anguillarum*

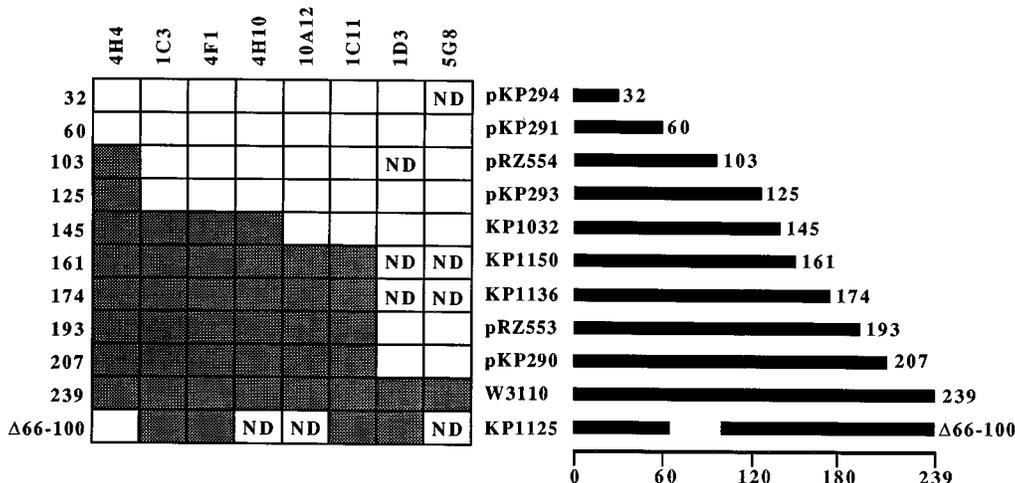


FIG. 1. Localization of epitopes recognized by *E. coli* TonB-specific MAbs. The results of immunoblot analysis of SDS-11% PAGE-resolved lysates of *E. coli* strains and plasmids encoding either wild-type TonB, TonB truncations, or an internal deletion are summarized in the matrix on the left of the strain and plasmid identification. All MAbs were reacted as 1:5,000 dilutions of murine ascites except 1D3 and 5G8, which were diluted 1:1,000. Filled boxes represent a positive score, reflecting the detection of a band that migrated at an apparent mass consistent with the predicted mass of the designated *tonB* product. Negative results are indicated by unfilled boxes. Strain and MAb combinations not examined are indicated by ND. The individual MAbs tested are identified at the top of the matrix, while the length (in amino acid residues) of each carboxy-terminal-truncated TonB is indicated on the left and diagrammatically represented on the right. W3110 represents full-length, wild-type TonB; Δ66-100 indicates TonB with an internal deletion of residues 66 to 100 (35).

and the gram-positive species, which previously existed as laboratory stocks, and for those species acquired as prepared samples (Table 1).

**Culture conditions.** *Actinobacillus pleuropneumoniae*, *Campylobacter jejuni*, *Coxiella burnetii*, *Haemophilus influenzae*, *Neisseria meningitidis*, and *Pasteurella haemolytica* were provided as Laemmli sample buffer-solubilized samples (32); *Fremyella diplosiphon* was provided as frozen cells. *Yersinia enterocolitica* was grown on brain heart infusion-based medium. *Listonella anguillarum* was grown on marine broth-based medium. All other strains and species were maintained on Luria-Bertani (LB) agar and grown in LB broth with aeration. All species were grown at 37°C except *Pseudomonas putida* and *Aeromonas hydrophila*, grown at 30°C; *L. anguillarum*, grown at 22°C; and *Y. enterocolitica*, grown at both 37°C and 22°C. For epitope mapping, plasmid-bearing *tonB* strains were grown with aeration at 37°C in LB supplemented with either 50 µg of kanamycin ml<sup>-1</sup> or 50 µg of kanamycin ml<sup>-1</sup>, 100 µg of ampicillin ml<sup>-1</sup>, and 0.2 µg of AT ml<sup>-1</sup> (for induction of TonB-PhoA expression) as appropriate.

**Epitope mapping.** Epitopes recognized by individual MAbs were localized by scoring immunoblot reactivity against *E. coli* TonB truncations and an internal (residues 66 to 100) deletion. For strains KP1026 and KP1032, the *tonB* gene is interrupted by a *kan* cassette insertion, such that only the first 145 codons of TonB are expressed (34, 41). Strains KP1150 and KP1136 harbor *tonB* genes with amber mutations, such that only the first 161 (KP1150) and 174 (KP1136) residues are translated (these products are unstable, with some detected protein occurring as an approximately 140-residue degradation product [33]). Plasmids pRZ554 and pRZ553 carry *tonB* genes with IS1 insertions and produce carboxyl-terminally-truncated TonB products with lengths of 103 (33) and approximately 193 residues, respectively (44). Plasmids pKP294, pKP291, pKP293, and pKP290 carry *tonB::phoA* fusions and express fusion products containing the first 32, 60, 125, and 207 residues of TonB, respectively (47). These fusions are under control of the *tetA* promoter and are repressed in the presence of pBST324 (which encodes the repressor protein TetR from pBR322 [38]). In this study, *tonB::phoA* fusions were induced with AT. For mapping, all strains were grown at 37°C with aeration in LB (and supplements as appropriate) to an  $A_{550}$  of 0.5 (determined with a Spectronic 20 spectrophotometer; path length, 1.5 cm), precipitated at 4°C with an equal volume of 10% trichloroacetic acid, pelleted, washed once with 50 mM Tris-Cl (pH 8.0), and then solubilized in Laemmli sample buffer at 97°C for 5 min. All samples were solubilized in a volume of Laemmli sample buffer such that 1 µl contained cells at an  $A_{550}$  of 0.001 (1.0  $A_{550}$  unit is equivalent to 0.5 ml of culture at an  $A_{550}$  of 0.5). Aliquots of 5 to 20 µl were resolved on SDS-11% polyacrylamide gels (32), electrotransferred to Immobilon P membranes, immunoblotted, and visualized by ECL as previously described (54).

**Cross-species reactivity.** Bacteria were grown under the conditions described above in 5 ml of the appropriate medium to an  $A_{550}$  of 0.5, precipitated with trichloroacetic acid, washed, and solubilized as described above. Samples representing 0.1  $A_{550}$  equivalent (10 µl) were resolved on SDS-11% polyacrylamide gels, visualized by Coomassie blue staining, and compared. On the basis of these results, sample volumes were adjusted where necessary such that subsequent gels (except for cross-linking experiments) were loaded with a total amount of protein equivalent to that present in 0.05 *E. coli*  $A_{550}$  unit. Following SDS-polyacrylamide gel electrophoresis (PAGE), samples were electrotransferred to Immo-

bilon P and processed for ECL as described above. Serum containing polyclonal α-TonB and ascites preparations of MAbs 1D3 and 5G8 were used at a dilution of 1:1,000; all other MAbs (as ascites) and secondary horseradish peroxidase conjugates were used at a dilution of 1:5,000.

**In vivo chemical cross-linking.** Species expressing polypeptides putatively identified as TonB homologs and specifically detected by the MAb 4H4 were grown in LB to an  $A_{550}$  of 0.5 (except *Proteus vulgaris*, grown to an  $A_{550}$  of 1.0) and chemically cross-linked with 1.0% (wt/vol) formaldehyde at 22°C for 15 min as previously described (54), except that samples were solubilized at 60°C (rather than 37°C) prior to electrophoresis. Samples equivalent to 0.2 *E. coli*  $A_{550}$  unit (0.4  $A_{550}$  unit for the *Proteus* sample) were resolved on SDS-11% polyacrylamide gels and processed as described above.

**Epitope identification.** Predicted polypeptide sequences of known TonB homologs in the family *Enterobacteriaceae* were aligned (11), and the regions corresponding to the portion of *E. coli* TonB identified as containing the epitope for a given MAb were compared. Since all MAbs examined reacted with SDS-denatured *E. coli* TonB, the criteria used for the identification of a putative epitope were the presence of a continuous stretch of six or more residues conserved in all species detected by a given MAb and the absence of that motif from all species that did not react with the MAb. For five MAbs, these criteria were not met (see Results); however, in some of those cases, regions could be excluded as potential epitope sites on the basis of sequence identity between species with differential reactivity for a given MAb. For three MAbs, these criteria were met (see Results), providing putative identification of epitopes. Potential epitopes were evaluated by examining the ability of synthetic polypeptides to block MAb binding to *E. coli* TonB in immunoblots. Briefly, *E. coli* W3110 samples representing 0.025  $A_{550}$  unit (2.5 µl) were resolved on SDS-11% polyacrylamide gels and electroblotted onto Immobilon P membranes, which were then cut into strips containing individual lanes. Single strips were then processed for ECL as described above, except that for the 1°-antibody-adsorption incubation ascites dilutions ranging from 1:10,000 to 1:50,000 were used and various concentrations of synthetic polypeptides were included as potential competitive substrates for antibody binding.

## RESULTS

**Mapping of *E. coli* TonB epitopes detected by MAbs.** Regions of *E. coli* TonB essential for the binding of individual MAbs were identified by immunoblot analysis of lysates from a set of strains and plasmids encoding partial TonB molecules (Table 1). The results of these analyses are summarized in Fig. 1. Because all reactivities were determined with samples denatured by heat, β-mercaptoethanol, and SDS, it was likely that in each case a linear epitope, rather than a conformational epitope, was recognized by the antibody.

All MAbs recognized full-length (239-residue) TonB (indi-

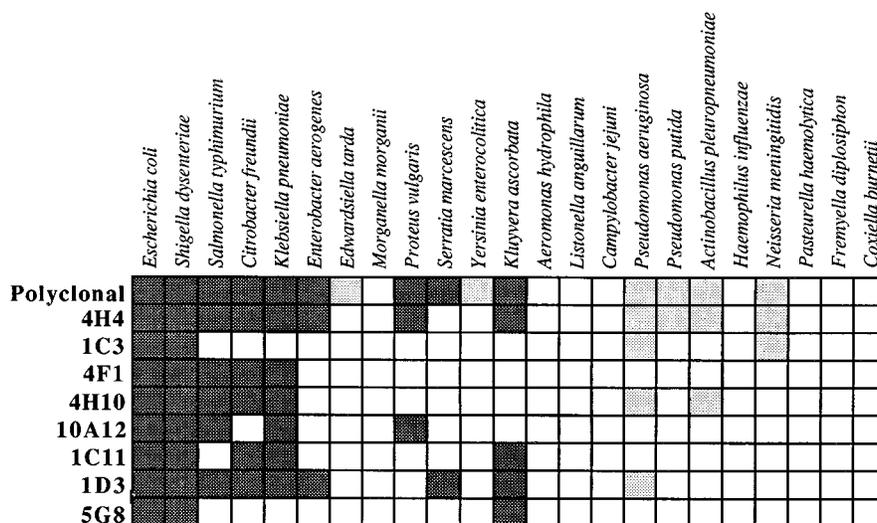


FIG. 2. Interspecies reactivity of  $\alpha$ -TonB polyclonal serum and TonB-specific MAbs. The results of immunoblot analysis of SDS-11% PAGE-resolved lysates representing 23 gram-negative organisms (indicated above the matrix) with either polyclonal serum or MAbs (indicated on the right). Filled boxes represent positive scores, reflecting the specific detection of a band that met two criteria: (i) migration at an apparent mass within the range of predicted masses for known *tonB* products and (ii) confirmation by detection at the same apparent mass with a second MAb known to recognize a distinct epitope. Stippled boxes indicate instances where either only one of the two criteria for a positive result was fulfilled or multiple bands were identified. Empty boxes represent negative scores. All MAbs were reacted as 1:5,000 dilutions of murine ascites except 1D3 and 5G8, which were diluted 1:1,000. The polyclonal serum was used at a dilution of 1:1,000. Results obtained with *Y. enterocolitica* lysates were identical for organisms grown at either 22° or 37°C.

cated by filled boxes), whereas no MAb recognized TonB-PhoA fusion proteins encoding either the first 32 or the first 60 residues of TonB (indicated by empty boxes), despite the fact that residues 12 to 239 were contained in the original TrpC-TonB fusion protein used as the immunogen. However, the MAbs could be differentiated by reactivity to the remaining partial TonB molecules, allowing for localization of regions involved in binding. For example, MAb 4H4 recognized a partial TonB protein containing only the first 103 residues, indicating that all amino acid residues necessary for binding occurred before residue 104. This MAb did not react with a TonB-PhoA fusion protein containing only the first 60 residues of TonB, indicating that at least some residues critical to the 4H4 epitope occurred carboxy terminal to TonB residue 60, but because epitopes consist of multiple residues, these data did not exclude the possibility that a portion of the 4H4 epitope occurred amino terminal to residue 60. Because all epitopes considered are assumed to be linear, this mapping strategy allowed localization of epitopes to regions of TonB for which the carboxyl-end boundary was clearly defined but for which the amino-end boundary was not absolute. Thus, for three MAbs (1C3, 4F1, and 4H10), at least some residues critical for binding occurred after residue 125 and the entire epitope occurred prior to residue 146; for two MAbs (10A12 and 1C11), at least some residues critical for binding occurred after residue 145 and the entire epitope occurred prior to residue 162; and for two MAbs (1D3 and 5G8), at least some residues critical for binding occurred after residue 207 and the entire epitope occurred prior to the carboxyl terminus (Fig. 1). An internal deletion of TonB residues 66 to 100 (35) and the inability of MAb 4H4 to detect this polypeptide indicated that the 60 to 103 region contained the entire 4H4 epitope. Unfortunately, additional internal deletions corresponding to the other regions where epitopes were localized were unavailable. The  $\alpha$ -TonB reactivities of two additional MAb preparations (4G10 and 1A10) were mapped as described above (to regions 60 to 103 and 103 to 125, respectively); however, the 36-kDa

TonB band was not the major polypeptide detected by either preparation (data not shown). It is possible that these MAbs were not effective immunoblotting reagents because of either weak affinity or partial denaturation of the epitope recognized. These preparations were not considered further.

**Cross-species reactivity of  $\alpha$ -TonB antibodies.** The interspecies distribution of epitopes recognized by antibodies generated against *E. coli* TrpC-TonB was evaluated by immunoblot analysis of SDS-PAGE-resolved bacterial lysates. As expected, reactivity to lysates from several gram-positive genera (*Bacillus*, *Micrococcus*, *Staphylococcus*, and *Streptococcus*) was not observed with any antibody preparation (data not shown). The reactivity patterns of polyclonal and MAb preparations to lysates of gram-negative organisms are summarized in Fig. 2. The broadest range of reactivity was obtained with the polyclonal antiserum. In previous studies, this preparation, generated against a gel-purified TrpC-TonB fusion product, specifically identified TonB-PhoA hybrid proteins (53) and could immunoprecipitate TonB protein (55). At the titer used here, this serum detected several *E. coli* proteins in addition to TonB (Fig. 3A). All bands detected in *E. coli* were also present in *E. coli*  $\Delta$ trpC strains (data not shown), thus the source of the additional reactivity was unclear. Putative TonB homologs were recognized as specific bands of 33 to 40 kDa in lysates of 10 of the 11 *Enterobacteriaceae* species, including bands migrating at  $\sim$ 40 kDa in *Edwardsiella tarda* and *Y. enterocolitica* not detected by any other preparation (Fig. 3A). This polyclonal antiserum failed to detect potential TonB-like bands in *Morganella morganii* or any of the nonenteric organisms examined, except *Neisseria* and *Pseudomonas* species (see below).

The overall spectrum of cross-species reactivity exhibited by the MAbs was similar to that obtained with the polyclonal serum, with the broadest individual ranges occurring for 1D3 (data not shown) and 4H4 (Fig. 3B). The specificity of 4H4 for the TonB protein of *E. coli* has been previously established (2, 27, 34, 35, 54) and was demonstrated here by the presence of a single band migrating at 36 kDa in wild-type *E. coli* but

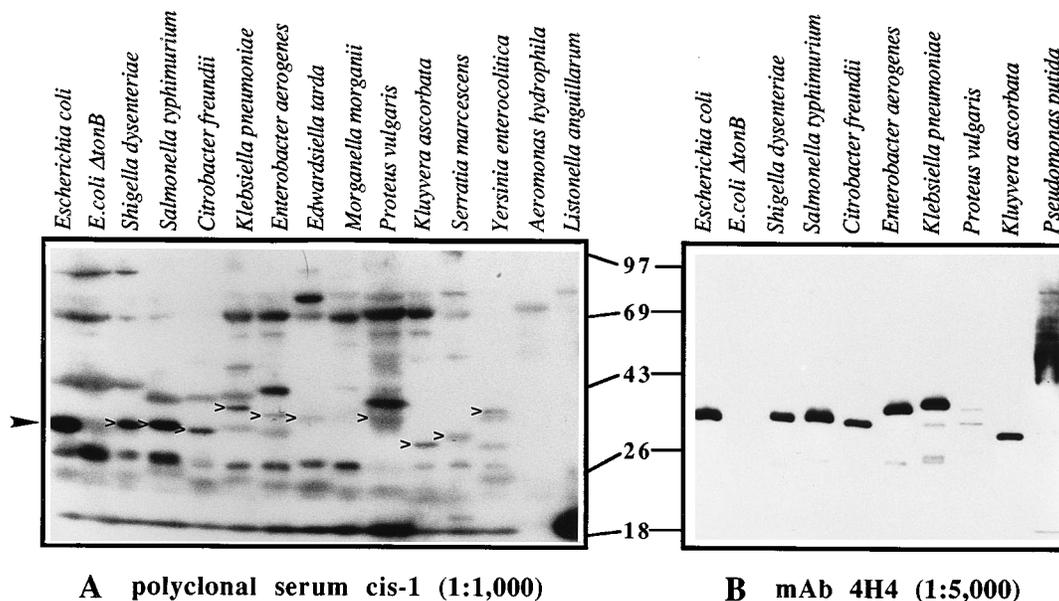


FIG. 3. Immunoblots of SDS-11% PAGE-resolved cell lysates. (A) Reactivity pattern of polyclonal  $\alpha$ -TonB antiserum. Not all species examined with this preparation are presented. The *Y. enterocolitica* sample presented is a lysate of cells grown at 37°C and was indistinguishable from an identically prepared lysate of cells grown at 22°C (data not shown). *E. coli* TonB is indicated by the arrowhead on the left. Putative TonB homologs are identified by carets in the panel. (B) Reactivity pattern of MAb 4H4. This summary immunoblot presents all the 4H4-reactive TonB homologs identified, a negative control lysate (*E. coli*  $\Delta$ tonB), and a *Pseudomonas putida* lysate. Molecular mass standards (in kilodaltons) are indicated between the panels.

absent from a  $\Delta$ tonB strain (Fig. 3B). On prolonged exposure other, faint bands were noted for both the wild-type and the *tonB* deletion strain, indicating the presence of weak, nonspecific cross-reactivity (data not shown). Specific bands were also obtained with 4H4 when reacted with the lysates of several other species; for *Salmonella typhimurium*, *Enterobacter aerogenes*, and *Klebsiella pneumoniae*, the apparent molecular masses of these detected proteins were consistent with both the reported values of in vitro-translated products from *tonB* clones (7, 8, 20) and with the bands obtained by using the other antibodies reactive to these lysates (summarized in Fig. 2). Conversely, 4H4 did not detect bands consistent with the product of the cloned and sequenced *Pseudomonas putida tonB* gene (5); rather, numerous diffuse bands were obtained in the range of 45 to 85 kDa (Fig. 3B). Specific bands were also not obtained when 4H4 was used to probe lysates of two other species from which the *tonB* gene has been characterized (*Serratia marcescens* and *Y. enterocolitica*) (data not shown); however, in other blots, both the polyclonal antiserum (Fig. 3A) and the MAb 1D3 (data not shown) detected a protein that migrated with the reported characteristics of *Serratia marcescens* TonB (16).

Reaction with 4H4 also produced bands with apparent molecular masses consistent with a TonB homolog from lysates of four species in the family *Enterobacteriaceae* for which *tonB* has not been characterized (Fig. 3B). Specific bands were obtained from *Shigella dysenteriae*, *Citrobacter freundii*, and *Kluyvera ascorbata* lysates, while two weaker bands were evident with the *Proteus vulgaris* lysate. Bands detected by either polyclonal antiserum or other MAbs reactive with lysates from these species (summarized in Fig. 2) had identical apparent molecular masses (data not shown). Reactivity of MAb 4H4 was also noted with lysates from several species outside the family *Enterobacteriaceae*. MAb 4H4 detected two bands in *Neisseria meningitidis* lysates, with migrations suggestive of TonB: a 38- to 40-kDa band that was also detected by the MAb 1C3 and a

36-kDa band not detected by any other preparation. MAb 4H4 similarly detected two bands in *Actinobacillus pleuropneumoniae* lysates: a 40-kDa band that was also detected by the polyclonal serum and a 28-kDa band also detected by the polyclonal serum and a 28-kDa band also detected by MAb 4H10 but, interestingly, not by the polyclonal preparation. Broad cross-reactivity was apparent when MAb 4H4 was reacted with the *Pseudomonas aeruginosa* lysate, although two distinct, intense bands were present at 40 and 44 kDa (Fig. 4). Specific bands with the same apparent masses were also obtained with polyclonal antiserum (again with apparent cross-reactivity [data not shown]) and with several MAbs (Fig. 2 and 4), while one MAb (1C11) was uniquely specific for a 70-kDa *Pseudomonas aeruginosa* protein, presumably not TonB (Fig. 4). Other instances of bands with apparent molecular masses not consistent with known TonB proteins occurred with *Campylobacter jejuni* and *Fremyella diplosiphon* lysates, for which multiple signals of higher apparent molecular mass were obtained with several MAbs (data not shown), and with *Actinobacillus pleuropneumoniae*, for which a weak signal migrating at ~28 kDa was obtained with both 4H10 and 4H4 (data not shown).

**Refinement of epitope maps.** Correlation of initial epitope mapping (Fig. 1), individual MAb cross-species reactivity patterns (Fig. 2), and the predicted polypeptide sequences of known TonB homologs (7, 8, 16, 20, 30) allowed us to make predictions about the precise locations of some epitopes (Fig. 5). For example, initial experiments suggested that the epitope identified by MAb 4H4 occurred between residues 60 and 103 of *E. coli* TonB (Fig. 1). Comparison of this region among *Enterobacteriaceae* species (Fig. 5A) revealed two distinct regions (*E. coli* residues 66 to 73 and 79 to 84) conserved in this region only by those species detected by 4H4. Similar comparisons (Fig. 5B) suggested that MAbs 4F1 and 4H10 recognized epitopes contained within the region defined by *E. coli* residues 120 to 127.

The identities of these epitopes were tested by including



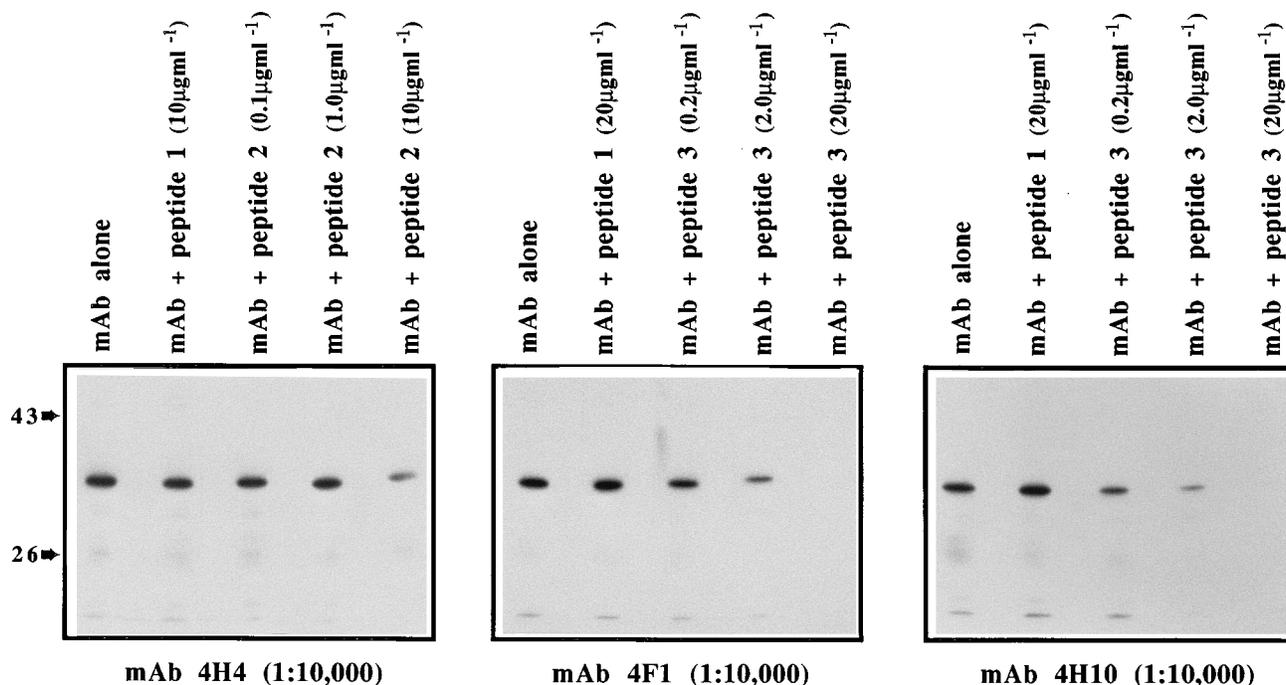


FIG. 6. Inhibition of MAb reactivity by synthetic peptides corresponding to regions containing putative MAb epitopes. *E. coli* W3110 lysates equivalent to 0.025  $A_{550}$  unit were resolved by SDS-11% PAGE and electrotransferred onto Immobilon P membranes. Following transfer, membranes were cut into strips and processed as previously described (54), except for the addition of synthetic peptide during the  $1^{\circ}$ -antibody-adsorption phase. Samples in each panel were resolved on the same gel. The MAb and the concentration used are indicated below each panel, with the probing regimen used for individual membrane strips indicated above each panel. Molecular mass standards (in kilodaltons) are indicated on the left.

teractions (37) between TonB and two proteins known to function in energy transduction (ExbB and FepA) and between TonB and two polypeptides that at present are known only from cross-linking (34, 35, 54). On the basis of the motility of complexes containing the latter two polypeptides in SDS-polyacrylamide gels, these polypeptides are referred to as 7- and 41-kDa proteins; however, because cross-linking potentially emphasizes the contribution of molecular shape to the migration of polypeptides in such matrices, these mass assignments should be considered tentative. The broad interspecies reactivity of MAb 4H4 (Fig. 2) provided the opportunity to identify similar interactions in other species. For each species examined, *in vivo* formaldehyde cross-linking resulted in a set of TonB-containing complexes comparable to the 43-kDa (TonB plus 7-kDa protein), 59-kDa (TonB plus ExbB), and 77-kDa (TonB plus 41-kDa protein) complexes generated in *E. coli* (Fig. 7). The complexes did not form in the absence of formaldehyde (Fig. 7). In each case, the apparent mass of a TonB homolog-containing complex was displaced from that of its *E. coli* counterpart an amount equivalent to the apparent mass difference between the homolog TonB monomer and *E. coli* TonB. Accurate apparent mass estimates were not possible for larger (>150-kDa) complexes, the presence, number, and migration of which varied among species. Because the 195-kDa complex in *E. coli* results from TonB associations with the outer membrane high-affinity receptor FepA, it may be that this variation among the high-molecular-mass cross-linked complexes resulted from the cross-linking of alternative receptors in other species. The absence of detectable high-molecular-mass complexes in some species may reflect the fact that all strains were grown under conditions where iron was not limiting, a condition under which detection of the TonB-FepA complex is greatly decreased (48).

It was necessary to grow *Proteus vulgaris* to a higher density ( $A_{550}$  of 1.0 versus 0.5) to detect TonB at levels sufficient for cross-linking. Under those circumstances, a set of complexes analogous to those generated in other species and consistent with a 40-kDa TonB homolog monomer was observed (Fig. 7). Cross-linking studies with *Pseudomonas aeruginosa* were inconclusive because the MAbs that detected the 40- and 44-kDa polypeptides either generated very high backgrounds with this species (4H4) or did not produce signals intense enough to distinguish complexes (Fig. 4). *Serratia marcescens* was not examined, again because the single MAb reactive with this TonB homolog (1D3) did not produce a signal-to-noise ratio sufficient to identify TonB homolog complexes (data not shown).

## DISCUSSION

The complications that an outer membrane imposes on the acquisition of iron in oxidizing environments are resolved in *E. coli* by TonB-dependent high-affinity transport systems (recently reviewed in references 29 and 42). Given that iron acquisition across the outer membrane is a problem faced by all gram-negative bacteria, one might predict that they would solve the problem in a way similar to *E. coli*. This is indeed the case: genes encoding homologs of *E. coli* TonB have been cloned and sequenced from *Salmonella typhimurium* (20), *Klebsiella pneumoniae* (7), *Enterobacter aerogenes* (8), *Serratia marcescens* (16), *Y. enterocolitica* (29), *H. influenzae* (26), and *Pseudomonas putida* (5). With the exception of the *Pseudomonas putida tonB* gene, each homolog can at least partially complement *E. coli tonB* strains when provided *in trans*. From other, less direct evidence, the presence of TonB-like molecules in additional gram-negative species can be inferred.

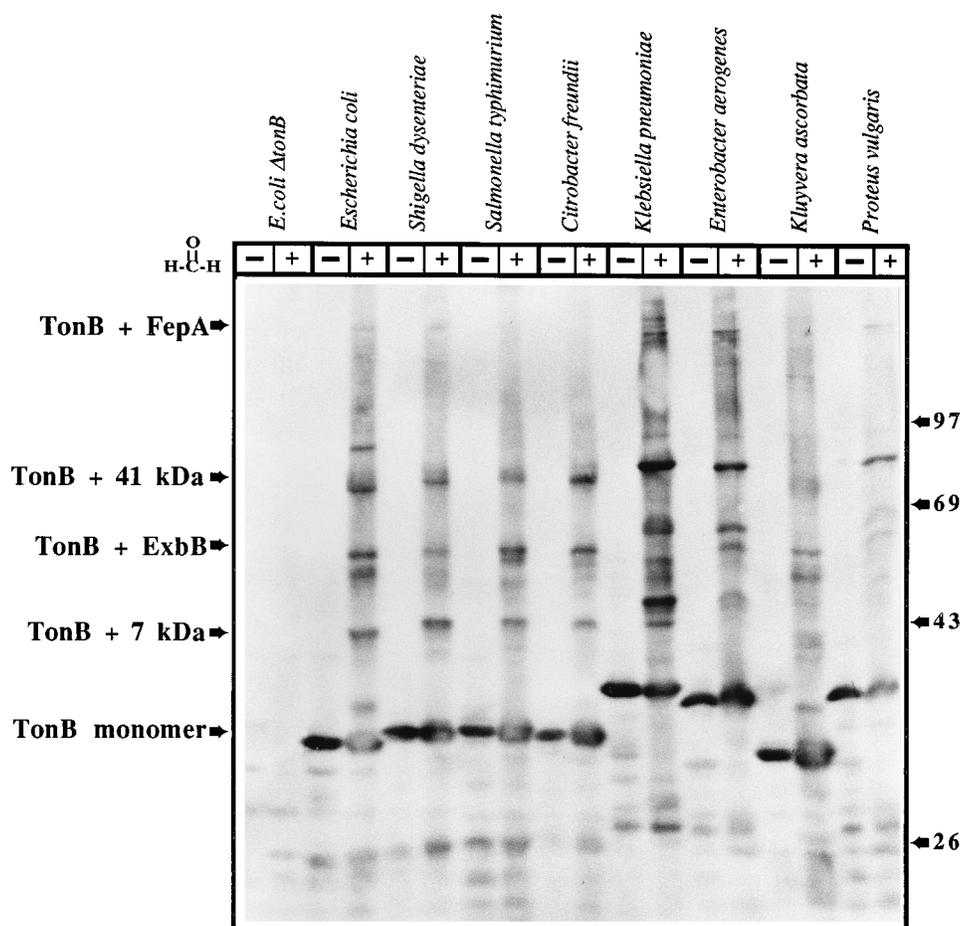


FIG. 7. In vivo formaldehyde cross-linking of TonB homologs. Immunoblots of SDS-11% PAGE-resolved lysates from cells grown in LB to mid-log phase (except *Proteus vulgaris*, grown to late log phase), treated with or without formaldehyde (indicated above the panel as + or -, respectively, and probed with MAb 4H4 ascites (diluted 1:5,000). Only species bearing a TonB homolog detected by MAb 4H4 (Fig. 3B) are shown. *E. coli ΔtonB* samples are included as negative controls. Apparent molecular masses are indicated on the right, with *E. coli* TonB and TonB-containing complexes identified on the left. Molecular mass assignments for uncharacterized partners (7 and 41 kDa) are approximations based solely on the migration characteristics of the complex.

MAbs generated against FepA, the TonB-specific enterochelin receptor of *E. coli*, recognize similar proteins in other gram-negative enteric species and in the more distantly related genera *Aeromonas*, *Haemophilus*, *Neisseria*, *Pseudomonas*, and *Vibrio* (49). In *E. coli*, the TonB-dependent high-affinity outer membrane receptors share a common amino-terminal motif (36) termed the TonB box (51). Mutations at this site block transport function but can be weakly suppressed by mutations that map to *tonB* (4, 23, 50). High-affinity outer membrane receptors with amino-terminal TonB boxes have been identified in other gram-negative species, including (but not limited to) *Vibrio cholerae* (17), *Haemophilus ducreyi* (13), *Neisseria meningitidis* (40), and *Neisseria gonorrhoeae* (10).

In the present study, a polyclonal antiserum prepared against an *E. coli* TrpC-TonB fusion protein detected TonB-like polypeptides in 10 of 11 members of the family *Enterobacteriaceae* examined (Fig. 2). Eight of these polypeptides were detected by one or more MAbs. Four of these polypeptides were from species with a *tonB* gene of known sequence (*Salmonella typhimurium*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, and *Serratia marcescens*); for these species, the polypeptide that we detected migrated with apparent molecular masses consistent with previous reports. The other four were from species for which a *tonB* homolog has yet to be

described (*Shigella dysenteriae*, *Citrobacter freundii*, *Proteus vulgaris*, and *Kluyvera ascorbata*); for these species, polypeptides with migration patterns similar to those of known TonB homologs (33 to 40 kDa) were identified by two or more MAbs that recognized discrete epitopes. Potential TonB-like polypeptides from *Edwardsiella tarda* and *Y. enterocolitica* were detected only by the polyclonal antibody. Specific antibody reactivity against species outside of the family *Enterobacteriaceae* was limited. The only strong indications of TonB-like polypeptides in this group were seen for *Pseudomonas aeruginosa*, *Neisseria meningitidis*, and *Actinobacillus pleuropneumoniae*, for which multiple bands with migration characteristics consistent with a TonB homolog were identified by several antibody preparations. Which, if any, of these bands represent TonB homologs remains unclear.

The apparent limitation of antibody reactivity to TonB homologs within the family *Enterobacteriaceae* is consistent with the available sequence data. Within the family, TonB homologs are very similar, sharing overall sequence identities of 50 to 85% (7, 8, 16, 20, 30, 43). Conversely, *Pseudomonas putida* and *Haemophilus influenzae* TonB homologs are only 28 and 31% identical, respectively, to *E. coli* TonB (5, 26). Alignments of predicted polypeptide sequences of TonB homologs identify several regions where sequence identities score signif-

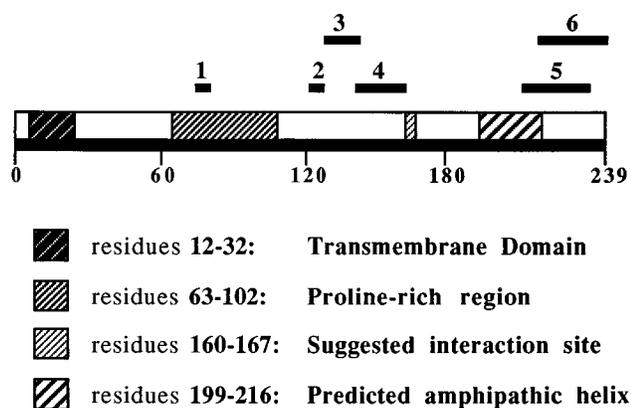


FIG. 8. Spatial relationships of epitopes detected by  $\alpha$ -TonB MAbs with structural and functional features of *E. coli* TonB. TonB is represented by the bar, with individual features indicated by the crosshatched boxes identified in the key. Relative positions are indicated by the scale, in number of amino acid residues. Regions containing specific epitopes are indicated by the solid lines above TonB and identified by number as follows: 1, MAb 4H4 epitope; 2, MAb 4F1 and 4H10 epitopes; 3, MAb 1C3 epitope; 4, MAb 10A12 and 1C11 epitopes; 5, MAb 1D3 epitope; and 6, MAb 5G8 epitope.

icantly higher than do the overall sequences. These regions and their spatial relationships to the MAb epitopes identified in this study are summarized in Fig. 8.

The first region is the TonB transmembrane domain, which is highly conserved among the *Enterobacteriaceae*, and contains four invariant residues (Ser-16, His-20, Leu-27, and Ser-31) believed to comprise one face of the transmembrane  $\alpha$ -helix (30, 42). This face appears to be involved in TonB-ExbB interactions (34). Outside of the family *Enterobacteriaceae*, the transmembrane domain appears to have diverged. For *Pseudomonas putida*, only three of the four residues on this face are conserved (Ser-16 is replaced by Val), and the overall identity of the transmembrane domain to *E. coli* TonB is significantly less than that seen for enteric species (5, 42). Initial examination of the alignment of predicted *H. influenzae* and *E. coli* TonB polypeptide sequences found no identity in this region (27); however, reexamination of a putative *H. influenzae* transmembrane domain identified by a hydrophobicity profile (31) found the first two of the four residues comprising the invariant in enteric species to be conserved, although the overall degree of identity of the region relative to the transmembrane domains of enteric species remained low (33). None of the MAb epitopes mapped in this study localized to this domain (Fig. 8).

A second region with a high degree of sequence identity is the proline-rich region of TonB, present in all homologs identified thus far. Evidence suggests that, while not essential for function, this region increases TonB efficiency by physically extending the reach of the molecule, enhancing its ability to interact with the outer membrane (35). In enteric species, this region occurs as sets of Glu-Pro and Lys-Pro repeats, separated by a short spacer. In *H. influenzae*, this region is similar, but less regular (26), while in *Pseudomonas putida* the region remains rich in prolyl and charged residues, but a repeat periodicity is absent (5, 42). The epitope recognized by MAb 4H4 mapped to this area.

A third, short region corresponding to *E. coli* residues 163 to 167 appears to represent the most highly conserved motif in TonB (33). This motif occurs as Tyr-Pro-X-Arg-Ala in all species except *Pseudomonas putida*, where Arg is replaced by Asp. This motif is adjacent to the *E. coli* site (Gln-160) involved in

the suppression of TonB box mutations described above (4, 23, 50). Interestingly, the Gln-160 residue is itself not conserved. None of the MAb epitopes mapped in this study localized to this domain (Fig. 8).

The fourth region with a high degree of sequence identity is a motif near the carboxyl terminus with characteristics predictive of an amphipathic helix (29, 35). This region is conserved in all enteric species for which the *tonB* sequence is available, but is absent from *Pseudomonas putida* and *H. influenzae* (33, 35, 42). The epitope recognized by MAb 1D3 mapped to a region that overlapped this area.

In summary, the two MAbs with the broadest range of reactivity (4H4 and 1D3 [Fig. 2]) recognized epitopes within or potentially overlapping regions with a high degree of sequence identity (Fig. 8), whereas the other six MAbs recognized epitopes in regions with less sequence identity. For the TonB homologs identified in this study from species for which the sequence has yet to be determined, all six of these epitopes were conserved in *Shigella dysenteriae*, three were conserved in *Citrobacter freundii*, and one was conserved in both *Kluyvera ascorbata* and *Proteus vulgaris*. These results are consistent with the apparent relative phylogenetic distances of these species from *E. coli* (1).

In vivo chemical cross-linking reveals the association of *E. coli* TonB with other proteins including the outer membrane receptor FepA and three additional proteins: ExbB, an  $\sim$ 41-kDa polypeptide, and an  $\sim$ 7-kDa polypeptide (54). Analogous complexes containing TonB homologs were generated when species with a 4H4-detectable TonB homolog were cross-linked and examined (Fig. 7). The presence of these complexes has two implications. First, it provides convincing evidence that the polypeptides identified in uncross-linked samples were TonB homologs. Second, it strongly suggested that the ability of TonB to associate with a set of proteins was conserved, as were the proteins with which it associated. One interpretation is that these proteins are components of the energy transduction complex (42). For ExbB, a role in energy transduction has been clearly established (34, 54). ExbB homologs have not been as widely characterized as TonB homologs; however, comparison of the available sequences from *E. coli* (12), *Pseudomonas putida* (5), and *H. influenzae* (25), and a partial sequence from *Salmonella typhimurium* (39) reveals a significant degree of sequence identity (28, 33), particularly in the putative first transmembrane domain, through which interactions with TonB appear to occur (34). The roles of the  $\sim$ 41- and  $\sim$ 7-kDa polypeptides remain unknown; however, the apparent conservation of their interactions with TonB homologs strengthens the argument that they are auxiliary components in the TonB-dependent energy transduction complex (42, 54).

TonB homologs in the family *Enterobacteriaceae* differ from those homologs identified in other species. As discussed above, their overall degrees of sequence identity to *Pseudomonas* and *Haemophilus* TonB are low, with divergence evident even in regions known to be important for function. The distinction is also present at the genetic level; *tonB* occurs as the third gene of potential operons, which include *exbB* and *exbD* in *Pseudomonas putida* (5) and *H. influenzae* (25, 26), but is physically separated from the *exb* operon on the chromosomes of enteric species. Despite their dissimilarity, the presence of TonB homologs in organisms as diverse as *E. coli* and *H. influenzae* suggests that TonB-dependent transport systems represent a common adaptation by which gram-negative organisms acquire iron. We suspect that a TonB system is necessary for all gram-negative organisms which dwell in the presence of oxygen, although it may not be required for parasites such as *Legionella*

*pneumophila* (9) and *Francisella tularensis* (14), which have evolved to exploit the iron-gathering machinery of their hosts.

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