In Vivo Evidence for TonB Dimerization

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TonB, in complex with ExbB and ExbD, is required for the energy-dependent transport of ferric siderophores across the outer membrane of Escherichia coli, the killing of cells by group B colicins, and infection by phages T1 and ϕ80. To gain insights into the protein complex, TonB dimerization was studied by constructing hybrid proteins from complete TonB (containing amino acids 1 to 239) [TonB(1–239)] and the cytoplasmic fragment of ToxR which, when dimerized, activates the transcription of the cholera toxin gene ctx. ToxR(1–182)-TonB(1–239) activated the transcription of lacZ under the control of the ctx promoter (P<sub>ctx</sub>::lacZ). Replacement of the TonB transmembrane region by the ToxR transmembrane region resulted in the hybrid proteins ToxR(1–210)-TonB(33–239) and ToxR(1–210)-TonB(164–239), of which only the latter activated P<sub>ctx</sub>::lacZ transcription. Dimer formation was reduced but not abolished in a mutant lacking ExbB and ExbD, suggesting that these complex components may influence dimerization but are not strictly required and that the N-terminal cytoplasmic membrane anchor and the C-terminal region are important for dimer formation. The periplasmic TonB fragment, TonB(33–239), inhibits ferrichrome and ferric citrate transport and induction of the ferric citrate transport system. This competition provided a means to positively screen for TonB(33–239) mutants which displayed no inhibition. Single point mutations of inactive fragments selected in this manner were introduced into complete TonB, and the phenotypes of the TonB mutant strains were determined. The mutations located in the C-terminal half of TonB, three of which (Y163C, V188E, and R204C) were obtained separately by site-directed mutagenesis, as was the isolated F230V mutation, were studied in more detail. They displayed different activity levels for various TonB-dependent functions, suggesting function-related specificities which reflect differences in the interactions of TonB with various transporters and receptors.

Active import of substrates, such as siderophores and vitamin B<sub>12</sub>, and sensitivity of cells to certain phages and group B colicins are mediated by outer membrane transport proteins which also serve as receptor proteins. In addition, the required energy is provided by the proton motive force of the cytoplasmic membrane (2), along with an energy-transducing device in the cytoplasmic membrane which is composed of the TonB, ExbB, and ExbD proteins (3, 22, 37). The N terminus of TonB (residues 13 to 32) anchors it to the inner membrane, while most of the protein is located in the periplasm, where it interacts with outer membrane transporters (7, 15, 35, 40). The topography of ExbB resembles that of TonB (23), whereas ExbB spans the cytoplasmic membrane three times, with most of the protein being located in the cytoplasm (24). TonB, ExbB, and ExbD seem to form a complex, but its stoichiometry is unknown. Under iron-replete conditions, the copy number of the TonB, ExbB, and ExbD proteins per cell was found to be approximately 335:2,463:741 (1:7:2) (20); however, these ratios may not reflect the ratios of the proteins in the predicted complex. The TonB, ExbB, and ExbD proteins interact with each other mainly through their membrane-embedded regions (1, 4, 6, 11, 28, 30). Cross-linking with formaldehyde yielded ExbB and ExbD homodimers and homotrimers whose formation did not depend on the presence of TonB (19). Recently, the carboxyl-terminal fragment of TonB from amino acids 164 to 239 [TonB(164–239)] was crystallized and shown to be a dimer (9). In contrast, sedimentation experiments and size exclusion chromatography of the periplasmic TonB(33–239) fragment yielded mostly the monomeric form (34). Therefore, we determined by an in vivo method whether TonB and TonB fragments form monomers, dimers, or multimers and whether dimerization, if it occurs, depends on the presence of ExbBD.

In addition, we isolated TonB point mutants to localize functionally important sites outside the cytoplasmic membrane domain and to determine whether a lack of activity is related to TonB homodimerization. We took advantage of a previous study in which it was shown that TonB fragments which were lacking the cytoplasmic membrane anchor were transported into the periplasm via the FecA signal sequence. These fragments inhibited the transport of ferrichrome by the FhuA transporter and of ferric citrate by the FecA transporter. FecA-dependent transcriptional induction of the fec<sub>ABCDE</sub> ferric citrate transport genes, infection by phage ϕ80, and cell killing by colicin M; ϕage ϕ80 and colicin M both bind to FhuA (21). In the present study, this inhibition by TonB fragments was used to select for mutated TonB fragments which no longer inhibited TonB-related functions. Random mutagenesis of the gene encoding the periplasmic TonB fragment resulted in point mutants that could grow again on ferric citrate as a sole iron source. The point mutations were cloned into the complete low-copy-number <i>tonB</i> gene, and the mutants were tested for their ability to support the transport of ferric citrate and...
ferrichrome and the sensitivity of cells to phage ϕ80 and colicin M.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *Escherichia coli* strains and plasmids used in this study are listed in Table 1.

<table>
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<tr>
<th>Strain or plasmid</th>
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For cloning of wild-type tonB in the low-copy-number vector pHS576, PCR-amplified tonB of plasmid pCG752 was cloned into pGEM-T-Easy, which was then digested with *XmaI* and ligated into the *EcoRI*/*PstI* digested vector pCG752. The *PstI* overhang was digested by treatment with the Klenow enzyme before ligation.

Plasmid pAS1 was constructed by removing the "tonB-cat" fragment of plasmid pMFTC (21) by restriction digestion with *BamHI* and *SalI*. This fragment was replaced by a 14-bp *BamHI* polylinker of vector pCSTon30. For cloning of wild-type *tonB* in the low-copy-number vector pHS576, PCR-amplified *tonB* of plasmid pCG752 was cloned into pGEM-T-Easy, which was then digested with *XmaI* and ligated into the *EcoRI*/*PstI* digested vector pCG752. The *PstI* overhang was digested by treatment with the Klenow enzyme before ligation.

Plasmid pAS2 was constructed by cleavage of plasmid pACYC184 with *EcoRI*/*SacI* and ligation with the *EcoRI*/*PstI* digested vector pCG752. For cloning of wild-type *tonB* in the low-copy-number vector pHS576, PCR-amplified *tonB* of plasmid pCG752 was cloned into pGEM-T-Easy, which was then digested with *XmaI* and ligated into the *EcoRI*/*PstI* digested vector pCG752. The *PstI* overhang was digested by treatment with the Klenow enzyme before ligation.

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pASTM163, pASTM188, pASTM204, and pASTM230, respectively, into SprA/HindIII-digested vector pAS2.

For the construction of plasmid pAS3, the fecABDCE fragment was amplified by PCR from chromosomal DNA of strain AB2847, digested with BamHISaiI, and cloned into BamHI/SaiI-digested vector pT7-7. The resulting plasmid, unless

as the original PCR product was then digested with SalI/HindIII and ligated.

For the construction of plasmids pASAToxRToxR163C, pASAToxRToxR188E, pASAToxRToxR1204C, and pASAToxRToxR1204B, PCR products of plasmids pASTM163, pASTM188, pASTM204, and pASTM230, respectively, were cleaved with PstI/Xhol and cloned into PstI/Xhol-digested plasmid pFRTRTMC. Plasmid pMFTC was used as the source for wild-type toxR fragments, unless indicated otherwise. All plasmid constructs were confirmed by DNA sequencing.

Construction of strains. To construct the esbBD knockout mutant ASA23, chromosomal DNA of E. coli AB2847 was amplified by PCR such that a fragment resulting which, by use of a NotI/SalI fragment, could be cloned into NotI/SalI-digested shuttle vector pWSK30. The NotI/SalI insert of pWSK30 was cloned into NotI/SalI-digested suicide vector pCAR109, resulting in plasmid pASAcexbBDdel. Conjugation with donor S17-1 agrbApASAexbBDdel and recipient FHK12 was done as described by Heinrichs and Poole (18). Selection plates contained ampicillin and kanamycin. To remove the suicide vector, the recipient FHK12 was done as described by Heinrichs and Poole (18). Selection plates contained ampicillin and kanamycin. To remove the suicide vector, the colonies without conjugants were plated on TY plates containing ampicillin and 10% sucrose.

Inhibition were tested for resistance to albomycin, colicin M, and phage 29 as described by Miller (32) and Giacomini et al. (14). The periplasmic dimerizing portion of ToxR can be replaced by a dimerizing periplasmic protein such as alkaline phosphatase which, as a ToxR-PhoA hybrid protein, activates the transcription of lacZ.

The cytoplasmic membrane anchor, were fused to ToxR(1–210)-MalE(27–210)-PhoA(27–210) was isolated and sequenced.

RESULTS

TonB dimers. To examine TonB dimer formation in vivo, a bacterial two-hybrid system was used. ToxR of Vibrio cholerae spans the cytoplasmic membrane and as a dimer acts as a transcriptional activator of the cholera toxin gene ctx (10, 33). The periplasmic dimering portion of ToxR can be replaced by a dimerizing periplasmic protein such as alkaline phosphatase which, as a ToxR-PhoA hybrid protein, activates the transcription of lacZ. The periplasmic dimerizing portion of ToxR was selected on NB plates supplemented with 1 mM IPTG and 1 mM sodium citrate. ASA23 cultures were grown as described before but without IPTG. β-Galactosidase activity was determined as described by Miller (32) and Giacomini et al. (14). Transport assays. Quantitative transport of [55Fe3+] and ferrichrome and sodium citrate concentrations on NB-D-thiogalactopyranoside (IPTG), and chloramphenicol. Colonies that grow on the plates, were plated, and DNA was isolated and sequenced.

Phenotype assays. All phenotype assays were performed with freshly transformed E. coli K-12 strains AB2847 and H2300. The sensitivity of cells to FlhA ligands (phages T1, T5, and 480; colicin M; microcin 325; and albomycin) was tested by spotting 4 μl of various dilutions (see Table 3) on TY agar plates overlaid with 3 ml of TY soft agar that contained 0.1 ml of a TY overnight culture of the strain to be tested.

Growth promotion was tested by placing filter paper disks containing 10 μl of various ferrichrome and sodium citrate concentrations on NB–250 μM dipyridyl agar plates overlaid with 3 ml of NB soft agar that contained 0.1 ml of an NB overnight culture of the strain to be tested. The diameter and the growth density around the filter paper disks were determined after overnight incubation. The assays were performed at least three times, and the results varied less than 10%.

Induction of the ctx promoter-lacZ fusion (Pctx::lacZ). Fusion proteins

were selected on NB plates supplemented with 250 μM dipyridyl, 1 mM sodium citrate, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and chloramphenicol. Colonies that grow on the plates, were plated, and DNA was isolated and sequenced.

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table 2. β-Galactosidase activity of E. coli strains in response to

ToxB dimers. To examine TonB dimer formation in vivo, a bacterial two-hybrid system was used. ToxR of Vibrio cholerae spans the cytoplasmic membrane and as a dimer acts as a transcriptional activator of the cholera toxin genectx

β-Galactosidase activity in Miller units (%) in the following E. coli strains and ligated.

Transport assays. Quantitative transport of [55Fe3+] and ferrichrome and sodium citrate, 1 mM dipyridyl, 1 mM sodium citrate, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and chloramphenicol. Colonies that grow on the plates, were plated, and DNA was isolated and sequenced.

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dimers, whereas TonB(164–239) has a high propensity for dimer formation.

The above experiments might have failed to demonstrate TonB dimer formation if the transmembrane region of TonB were important for dimerization. Therefore, complete TonB was fused to the cytoplasmic portion of ToxR which is represented by residues 1 to 182, which are followed by the transmembrane region spanning residues 183 to 198 in the native protein. ToxR(1–182)-TonB(1–239) activated P_cro::lacZ transcription (Table 2). This experiment showed that the transmembrane region was important for the dimer formation of full-length TonB. Whether dimer formation depended on the membrane region was important for the dimer formation of transcription (Table 2). This experiment showed that the trans-

182)-TonB(1 protein. ToxR(1–210)-TonB(33–239) fragment was

affected P_cro::lacZ transcription was tested with an E. coli FHK12 transformant which contained plasmid-carried exbB exbD (pASA27) in addition to chromosomally carried exbB exbD. The overexpression of exbB exbD did not greatly influence dimer formation by ToxR(1–182)-TonB(1–239) or ToxR(1–210)-TonB(164–239), nor did it alter the lack of dimer formation by ToxR(1–210)-TonB(33–239) (Table 2).

Point mutations in inhibitory periplasmic TonB fragments restore TonB-dependent growth on ferric citrate. The inhibition of chromosomally encoded wild-type TonB activity by plasmid-carried periplasmic TonB fragments provides a means to positively select for TonB mutant fragments which are no longer inhibitory. Among the isolated mutant fragments should be mutations which impair TonB function when cloned into complete TonB. Furthermore, if TonB fragments compete with the binding of TonB to outer membrane transport proteins, then the mutated fragments may not restore the various outer membrane transport systems to the same degree and the mutations cloned in TonB may not affect all TonB-related activities in the same way. By this means, distinct interactions between TonB and currently unknown transporters may be identified.

TonB(33–239) fused to the signal sequence of FecA was secreted into the periplasm and inhibited all tested FecA- and FhuA-related functions. fecA::tonB was located on plasmid pMFTC under lac control, a scenario which allowed transcription to be induced by IPTG (21). The tonB fragment was randomly mutagenized by PCR and recloned into pMFTC by replacement of the wild-type TonB fragment or into plasmid pAS1, which contains a convenient BamHI/NcoI polynolinker. E. coli AB2847, which does not synthesize a siderophore, was transformed with plasmids carrying the mutagenized tonB fragments, and the transformants were spread on NB agar plates supplemented with 250 μM dipyridyl to restrict the available iron and 1 mM sodium citrate, which complexes a portion of the iron and makes it available to AB2847. The transcription of fecA::

'tonB was induced with 1 mM IPTG. Under these conditions, the wild-type TonB(33–239) fragment inhibited growth.

Plasmids were isolated from colonies which appeared on the selection plates, used to confirm the phenotype by retransformation of the host strain, and sequenced. Thirty mutants which synthesized TonB fragments in amounts similar to that of the wild-type TonB fragment, as revealed by Western blotting (data not shown), were isolated. Nine mutants did not synthesize TonB fragments and, for this reason, did not interfere with wild-type TonB activity. They probably contained nonsense mutations. The tonB gene fragments (tonB) of mutants that expressed TonB fragments were sequenced. Ten mutants contained a single mutation, and the rest contained two or more mutations. Most of the mutations were clustered in the C-terminal third of TonB, as shown for the single mutations in Fig. 1. Further growth tests revealed that the mutated TonB fragments affected growth on ferric citrate and ferrichrome to different extents.

Four single TonB mutant fragments with strong phenotypes are described further here. These include 'TonB(Y163C), which contained an additional silent mutation, 'TonB(V188E), and 'TonB(R204C), all of which were isolated in the PCR experiments, and TonB(F230V), which was not selected by the fragment screening procedure but was constructed by site-directed mutagenesis as described previously (M. Anton, A. Bruske, and K. J. Heller, 3rd Int. Symp. Iron Transport, Storage Metab., poster, 1992). 'TonB(Y163C) and 'TonB(F230V) did not inhibit chromosomally encoded wild-type TonB of AB2847 in the transport of ferric citrate, since the transformants grew even at the lowest citrate concentration, whereas transformants containing 'TonB(V188E) and 'TonB(R204C) could grow only at 10 μM citrate. A lack of growth around the filter paper loaded with 1 mM citrate was not caused by a lack of fec transport gene transcription, since chromosomally carried fecB-lacZ of E. coli ZI418 was transcribed in the presence of all mutated TonB fragments.

E. coli AB2847 synthesizing the 'TonB(V188E) and 'TonB (F230V) fragments grew at 1 mM ferrichrome, whereas the other two transformants showed no growth. Sensitivities to colicin M and phage δ80 were tested as additional FhuA-related activities. The sensitivity of the transformants to colicin M was restored to the level of E. coli AB2847 carrying vector pMalP2 (titer, 10^6) except for the 'TonB(R204C) transformant, which yielded a turbid growth inhibition zone at a 10^-3 dilution of colicin M solution. Sensitivity to phage δ80 was also restored to the wild-type level (titer, 10^6) in E. coli AB2847 synthesizing 'TonB(Y163C) and E. coli AB2847 synthesizing 'TonB(V188E), whereas E. coli AB2847 synthesizing 'TonB (F230V) formed turbid plaques at the 10^-9 dilution of the phage suspension and E. coli AB2847 synthesizing 'TonB (R204C) still inhibited wild-type TonB (a very turbid inhibition zone at a 10^-3 dilution of the phage suspension).

Insertion of the point mutations into complete TonB yields TonB derivatives that fail to support growth on ferric citrate. The four single mutations described above were cloned by DNA fragment exchange into the low-copy-number plasmid pAS2, which carries the complete tonB gene. E. coli H2300 ΔtonB was transformed with the mutant plasmids, and growth promotion around filter paper disks loaded with 1 and 10 mM solutions of ferric citrate was tested with iron-limited NB–250 μM dipyridyl plates. The tonB mutants did not grow on ferric citrate (Table 3). To examine whether the failure to grow on ferric citrate was caused by a lack of fec transport gene transcription, E. coli AS418 fecB-lacZ tonB was transformed with the mutated tonB plasmids, and β-galactosidase activity was measured after 3 h of growth with and without ferric citrate in
NB. TonB(F230V) supported no induction, TonB(Y163C) supported a low level of induction, TonB(V188E) supported an intermediate level of induction, and TonB(R204C) supported a high induction level (Table 3). It thus appears that less TonB activity is required for induction than for transport of ferric citrate.

All of the TonB mutants, except for E. coli H2300 ΔtonB expressing TonB(F230V), supported growth around filter paper disks loaded with 1 mM ferrichrome. Since a 1 mM concentration of ferrichrome may be too high for this assay, testing was repeated with 0.01, 0.03, 0.1, and 0.3 mM ferrichrome. E. coli H2300 ΔtonB expressing TonB(R204C) and E. coli H2300 ΔtonB expressing TonB(Y163C) grew around disks impregnated with 0.01 mM ferrichrome, whereas the growth of E. coli H2300 ΔtonB expressing TonB(V188E) and E. coli H2300 ΔtonB expressing TonB(F230V) around these disks was barely evident (data not shown).

Transport of ferric citrate and ferrichrome into TonB mutant cells. The semiquantitative growth promotion data were further substantiated by quantitative transport data. The transport of [55Fe3+]-citrate and [55Fe3+]-ferrichrome was determined with E. coli H2300 ΔtonB (Table 4). TonB(F230V) was not able to transport ferric citrate, whereas TonB(R204C), TonB(V188E), and TonB(Y163C) supported ferric citrate transport; the level of transport correlated with the degree of induction, as determined by β-galactosidase activity expressed by the fecB-lacZ reporter gene (Table 3). Since the lack of induction mediated by TonB(F230V) (Table 3) could have caused a lack of transport, the E. coli CO93 ΔfecIRABCDE tonB mutant was transformed with plasmid pAS3, which carried the fecABCDE transport genes on the pT7-7 medium-copy-number plasmid. The synthesis of FecABCDE proteins from this plasmid without induction is sufficient to support ferric citrate transport. E. coli CO93(pAS3) transported ferric citrate. Transformants carrying mutated tonB on pAST230, pAST163, or pAST188 displayed 10 to 19% the transport rates of wild-type tonB transformants, indicating that the TonB mutations affected both transport and induction by FecA, both of which were most strongly reduced for the TonB(F230V) mutant.

TonB(R204C) and TonB(Y163C) transported [55Fe3+] ferrichrome, but TonB(V188E) was transport inactive, as was TonB(F230V). The latter results correlate with the results of

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**TABLE 3. Growth of E. coli H2300 ΔtonB transformants with ferric citrate or ferrichrome as a sole iron source and fecB-lacZ induction of tonB point mutants**

<table>
<thead>
<tr>
<th>Expressed protein</th>
<th>Ferric citrate at:</th>
<th>ferric citrate at:</th>
<th>fecB-lacZ transcription</th>
<th>Sensitivity to:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 mM</td>
<td>10 mM</td>
<td>NB</td>
<td>NB + citrate</td>
</tr>
<tr>
<td>Wild type</td>
<td>9</td>
<td>17</td>
<td>28</td>
<td>49</td>
</tr>
<tr>
<td>No TonB</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>TonB(Y163C)</td>
<td>—</td>
<td>8</td>
<td>30</td>
<td>19</td>
</tr>
<tr>
<td>TonB(V188E)</td>
<td>—</td>
<td>11</td>
<td>29</td>
<td>23</td>
</tr>
<tr>
<td>TonB(R204C)</td>
<td>18</td>
<td>29</td>
<td>18</td>
<td>429</td>
</tr>
<tr>
<td>TonB(F230V)</td>
<td>—</td>
<td>—</td>
<td>27</td>
<td>48</td>
</tr>
</tbody>
</table>

* Growth was measured as the diameter in millimeters of the growth zone around 6-mm filter paper disks which had been saturated with 10 μl of the indicated solutions. Values in italic type show weak growth; those in bold type show weaker growth; —, no growth.

* fecB-lacZ transcription was determined with E. coli AS418 fecB-lacZ tonB transformants that expressed the listed TonB proteins. Values are reported in Miller units.

The results of the sensitivity assays are presented as the last of 10-fold dilution series that resulted in a clear zone of lysis. R, resistance to the tested ligand.
The competition experiments with the TonB fragments, in which TonB(V188E) and TonB(F230V) no longer inhibited wild-type TonB function. The latter mutations thus abolished the interference of the TonB fragments with the interaction of TonB with outer membrane transporters and also inactivated full-length TonB.

The TonB point mutant proteins remain dimerization competent. The full-length TonB mutant proteins were fused to ToxR(1–182), and the activation of P_{ctx}:lacZ transcription by the ToxR dimer was determined. The TonB mutant proteins yielded high β-galactosidase activity, which exceeded the activity of wild-type TonB (Table 2). The same result was obtained with the exbB exbD deletion mutant (Table 2).

### DISCUSSION

The studies described here provide evidence that TonB can form homodimers and that dimer formation is not dependent on but is influenced by the cosynthesis of ExbB and ExbD. The evidence is based on the TonB-mediated dimer formation of cytoplasmic ToxR, which activates P_{ctx}:lacZ transcription only in the dimeric form. This finding does not necessarily predict the presence of TonB dimers in the complex with ExbB and ExbD but demonstrates the self-association of TonB. It is possible that TonB multimers exist in the complex and that a rather large complex is formed in association with ExbB and ExbD, for which an association with TonB and the existence of homodimers and homo trimers have been demonstrated (19).

The specificity of TonB dimer formation was shown by the requirement for the TonB N terminus in the cytoplasmic membrane, which could not be replaced by the transmembrane region of ToxR. Spontaneous disulfide cross-linked TonB dimers were also formed in vivo when single cysteine residues were introduced at position 160 of TonB (7, 8, 36). This result suggests that the ToxR approach did not yield artifacts. Rather, it supports the in vivo relevance of the crystal data, which demonstrated a dimeric TonB(164–239) fragment (9). This finding was particularly evident with the exbB exbD deletion mutant, in which the mutant proteins showed a transcription level twofold higher than that of wild-type TonB. It is possible that during the reaction cycle, TonB dissociates into monomers; if so, the affinity of the mutant proteins may be too great for TonB polypeptide separation, thus trapping it in an inactive dimer.

Both the N-proximal and the C-proximal regions of TonB mediate the dimerization of TonB. The dimerization of only complete TonB was reduced in the exbBD deletion mutant, consistent with an interaction of the N-proximal region with ExbB (28).

Although TonB(164–239) forms a dimer in vivo when fused to ToxR(1–210) and in vitro in the crystal structure (9), there was no evidence of dimer formation by TonR(1–210)-TonB(33–239). It thus seems that a region between residues 33 and 164 prevents dimer formation. The inhibition of dimer formation is abolished, however, in complete TonB, in which the N-terminal region spans the cytoplasmic membrane. Either the transmembrane region also forms a dimer and adds to the dimer formation of complete TonB or complete TonB assumes a conformation different from that of TonB(33–239) and prevents the inhibition of dimer formation by a region between residues 33 and 164. Results similar to these were obtained with the TolA protein, which is structurally and functionally similar to TonB in the TolAQR system. The C-terminal fragment of TolA, called TolAIII, consisting of the last 132 residues of the 421-residue protein, forms a dimeric crystal structure very similar to that of TonB(164–239), but solution X-ray scattering indicates a monomeric structure for the TolII-TolIII fragment, which encompasses the entire periplasmic TolA structure and is equivalent to TonB(33–239). In addition, TolAIII has been fused to the N1 domain of the minor coat gene 3 protein (g3p) of phage M13 via a flexible linker. TolA is required for infection of E. coli by M13 and, during this process, TolIII replaces the N2 domain of g3p. However, the crystal structure of the N1-TolIII hybrid protein contains a TolIII monomer with the same fold as that in the TolAIII dimer (31). In this case, g3p prevents dimer formation by TolIII.

Previously, Howard et al. provided evidence that the inhibition of wild-type TonB activity by the overexpressed TonB(33–239) fragment was most likely caused by competition for binding to FecA and FhuA (21) and not by competition with the
binding of TonB to ExbB and ExbD, the overexpression of FhuA but not the overexpression of ExbBD reversed the inhibition. In this study, we showed that ExbBD did not strongly influence dimer formation. Thus, although TonB(33–239) did not form dimers and, as argued above, seems to prevent dimer formation, it does not appear likely that it inhibits activity by interfering with TonB dimer formation.

A spectrum of phenotypes was displayed by the TonB C-terminal missense mutants. For example, TonB(V188E) displayed 41% ferric citrate induction compared to wild-type TonB and 24% wild-type ferric citrate transport activity but only 2% ferrichrome transport activity. Conversely, TonB (Y163C) showed low fecB transcription (17%) and low ferric citrate transport (14%) but rather high ferrichrome transport (52%). Distinct effects of the TonB mutations were also observed with respect to sensitivities to colicin M and phage ø80, in that the TonB(Y163C) and TonB(V188E) mutants were colicin M resistant but fully phage sensitive. It should also be noted that in contrast to the low activity of TonB(Y163C) in ferric citrate induction and transport, this mutant fully supported growth on vitamin B₁₂ and transport of vitamin B₁₂ (7, 8).

Differential effects of TonB mutations on colicin and phage sensitivities were observed previously. Infection by phage ø80 and, even more clearly, by phage T1 still took place in certain TonB mutants which had lost other TonB-dependent activities. For example, mutants expressing TonB(G186D), obtained by bisulfite mutagenesis, showed strong reductions in most TonB-related activities, except for sensitivity to phage T1, which was unaffected (44). TonB(G186S) conferred full sensitivity to phages T1 and ø80, conferred a 10-fold reduction in sensitivity to colicins B and M, strongly impaired growth on ferrichrome, and resulted in no growth on the fungal siderophore coprogen. TonB(G174R,V178I) conferred resistance to colicin Iα but sensitivity to colicin B, whereas Serratia marcescens TonB conferred sensitivity to colicin B but resistance to colicin Iα in an E. coli tonB mutant (13, 43). Some of the TonB mutants, when combined with certain FhuA point mutants, increased or decreased sensitivity to phage T5 up to 1,000-fold, although T5 does not require TonB to infect cells (25). These data make it clear that interactions of TonB with outer membrane transporters and presumably also with colicins show protein-specific features. The mutations are also located in the C-terminal half of TonB, like the Tyr215 amber mutation which, when suppressed by tRNAs resulting in nine amino acid substitutions, resulted in distinct phenotypes with regard to sensitivities to colicins B, Iα, and M and phage ø80 (29).

In summary, the data presented here suggest that TonB forms homodimers and contains both N-proximal and C-proximal dimerization domains. In addition, the mutations in the TonB C-proximal region described here reduce TonB activity but not by preventing dimerization.

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