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Identification of Functionally Important TonB-ExbD Periplasmic Domain Interactions *In Vivo*

Anne A. Ollis* and Kathleen Postle

Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, Pennsylvania, USA

In Gram-negative bacteria, the cytoplasmic membrane proton-motive force energizes the active transport of TonB-dependent ligands through outer membrane TonB-gated transporters. In *Escherichia coli*, cytoplasmic membrane proteins ExbB and ExbD couple the proton-motive force to conformational changes in TonB, which are hypothesized to form the basis of energy transduction through direct contact with the transporters. While the role of ExbB is not well understood, contact between periplasmic domains of TonB and ExbD is required, with the conformational response of TonB to presence or absence of proton motive force being modulated through ExbD. A region (residues 92 to 121) within the ExbD periplasmic domain was previously identified as being important for TonB interaction. Here, the specific sites of periplasmic domain interactions between that region and the TonB carboxy terminus were identified by examining 270 combinations of 45 TonB and 6 ExbD individual cysteine substitutions for disulfide-linked heterodimer formation. ExbD residues A92C, K97C, and T109C interacted with multiple TonB substitutions in four regions of the TonB carboxy terminus. Two regions were on each side of the TonB residues known to interact with the TonB box of TonB-gated transporters, suggesting that ExbD positions TonB for correct interaction at that site. A third region contained a functionally important glycine residue, and the fourth region involved a highly conserved predicted amphipathic helix. Three ExbD substitutions, F103C, L115C, and T121C, were nonreactive with any TonB cysteine substitutions. ExbD D25, a candidate to be on a proton translocation pathway, was important to support efficient TonB-ExbD heterodimerization at these specific regions.

The TonB system of Gram-negative bacteria couples the proton-motive force (PMF) of the cytoplasmic membrane (CM) to energize active transport across the outer membrane (OM) through TonB-gated transporters (recently reviewed in references 9, 25, 26, and 36). Multiple specific, high-affinity transporters bind ligands, including iron-chelating siderophores, vitamin B_{12}, heme, maltodextrin, sucrose, and nickel, for subsequent active transport across the OM (1, 6, 11, 34, 48, 49). In *Escherichia coli* K-12, at least 7 OM transporters are TonB dependent (36).

The TonB system includes three integral CM proteins, TonB, ExbB, and ExbD. While the stoichiometry of the potentially heterooligomeric complex of these proteins is unknown, TonB, ExbB, and ExbD are present in a cellular ratio of 1:7:2 (18). TonB (239 residues) has a transmembrane domain (TMD) signal anchor from residues 12 to 32 and a carboxy-terminal periplasmic domain from residues 33 to 239 (17, 21, 24, 45). ExbD (141 residues) has an identical topology, with TMD residues 23 to 43, followed by a 98-residue periplasmic domain (22). ExbB (244 residues) contains three TMDs and adopts an N-out, C-in topology. In contrast to TonB and ExbD, the majority of residues in soluble domains of ExbB are exposed to the cytoplasm (23).

TonB is the physical connection from the CM PMF energy source to the unenergized OM, spanning the periplasm to make direct contact with TonB-gated transporters *in vivo* (4, 37, 52). ExbB and ExbD appear to couple the proton-motive force to TonB energization, which can be detected *in vivo* as an ExbB-dependent formaldehyde cross-link between the periplasmic domains of TonB and ExbD (38).

The TonB TMD anchors TonB in the CM throughout its energy transduction cycle (15). The TonB TMD lacks residues for direct response to the PMF, but it is important for interaction with ExbB (21, 28, 31, 53). The periplasmic carboxy terminus is the region through which TonB contacts both TonB-gated transporters and the ExbD periplasmic domain (4, 38). The TonB periplasmic domain is unusual in its high tolerance to internal deletions or residue substitutions. Internal deletions within the region of residues 33 to 149 retain detectable activity, even with as many as 47 residues (103 to 149) deleted, suggesting that the conformation of the domain immediately following the transmembrane domain is not essential for TonB function (32, 44, 50). Between residues 150 and 239, only 7 residues are functionally important. Substitutions at these residues exhibit idiosyncratic phenotypes depending on the residue being substituted and the transporter-specific assay being used. While no single residue is essential for TonB function, the simultaneous replacement of any two of the seven residues with alanine inactivates TonB, a synergistic phenotype, suggesting that they all interact at some point (13, 14, 44). Because the majority of TonB is predicted to have a disordered structure (28), the overall conformation of the TonB periplasmic domain is potentially defined by its association with another protein. Based on its topology, ExbD is a logical candidate.

The TonB periplasmic domain assumes multiple conformations *in vivo*. Five different TonB cysteine substitutions in the extreme carboxy terminus (residues 150 to 239) each appear to exhibit three conformations of disulfide-linked homodimers on nonreducing SDS-polyacrylamide gels. These TonB triplet ho-

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Address correspondence to Kathleen Postle, postle@psu.edu.

* Present address: Anne A. Ollis, School of Chemical and Biomolecular Engineering, Cornell University, Ithaca, New York, USA.

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TABLE 1 Strains and partial list of plasmids used in this study

Strain or plasmid	Description	Reference
Strains		
W3110	F⁻ IN(*rrnD-rrnE*)*1*	19
KP1509	W3110, Δ*exbD*, Δ*tonB*::*kan*, Δ*tolQR*	38
Plasmids		
pKP945	TonB C18G, A150C	38
pKP1000	ExbD A92C	38
pKP1005	ExbD K97C	40
pKP1011	ExbD F103C	40
pKP1017	ExbD T109C	40
pKP1023	ExbD L115C	40
pKP1029	ExbD T121C	40
pKP1049	ExbD D25N, A92C	38
pKP1050	ExbD D25N, K97C	40
pKP1051	ExbD D25N, F103C	40
pKP1052	ExbD D25N, T109C	40
pKP1053	ExbD D25N, L115C	40
pKP1082	ExbD D25N, T121C	40

TABLE 2 Plasmids expressing TonB C18G with Cys substitutions

Plasmid from[a]:			
Reference 44			Reference 13
pKP947 (G152C)	pKP597 (G174C)	pKP609 (P198C)	pKP508 (F180C)
pKP949 (R154C)	pKP598 (V176C)	pKP643 (G218C)	pKP469 (N200C)
pKP951 (L156C)	pKP601 (V178C)	pKP617 (P220C)	pKP415 (F202C)
pKP953 (R158C)	pKP604 (V182C)	pKP619 (S222C)	pKP418 (R204C)
pKP588 (Q160C)	pKP610 (P184C)	pKP623 (I224C)	pKP463 (V206C)
pKP587 (Q162C)	pKP612 (G186C)	pKP625 (V226C)	pKP416 (N208C)
pKP585 (P164C)	pKP614 (V188C)	pKP627 (I228C)	pKP466 (M210C)
pKP589 (R166C)	pKP638 (N190C)	pKP629 (I232C)	pKP471 (R212C)
pKP593 (Q168C)	pKP639 (Q192C)	pKP631 (G234C)	pKP473 (R214C)
pKP591 (L170C)	pKP641 (L194C)	pKP634 (T236C)	pKP475 (E216C)
pKP600 (I172C)	pKP607 (A196C)	pKP636 (I238C)	pKP510 (F230C)

[a] The Cys substitutions are listed in parentheses.

modimers form through substitutions at four aromatic residues, F202, W213, Y215, and F230, and nonaromatic residue G186 (44). The TonB conformations represented by the triplet homodimers are dependent on the presence of ExbB and ExbD and a wild-type TonB transmembrane domain (13). These *in vivo* TonB homodimers represent unique conformations compared to the homodimeric TonB observed in the crystal structure of a $TonB_{165-239}$ carboxy-terminal fragment that lacks input from the TonB transmembrane domain, ExbB, and perhaps most importantly, ExbD (7, 44).

ExbD also forms homodimers *in vivo* through its periplasmic domain, with specific interactions identified between residues 92 through 121 (40). The ExbD periplasmic domain appears to function as a homodimer, because ExbD I102C, which dimerizes completely, is still fully active. While the nuclear magnetic resonance (NMR) structure of the monomeric ExbD periplasmic domain shows ExbD residues 92 to 121 as part of a region of defined tertiary structure, conformational changes in that structure are required to account for all of the sites of homodimerization observed *in vivo* (12, 40).

ExbD TMD D25 is an essential residue, and the D25N substitution renders ExbD inactive (2). Cognate aspartate residues in the TolR and MotB transmembrane domains are proposed to participate in proton translocation (5, 57). ExbD D25N prevents the conformational response of both TonB and ExbD to proton-motive force as well as *in vivo* formaldehyde cross-linking through their periplasmic domains (38, 39). Three stages in the energization of TonB through its relationship with the ExbD periplasmic domain have been recently identified. The analysis of the effects of ExbD mutants such as D25N on ExbD and TonB conformation demonstrated that the ExbD transmembrane domain responds to changes in the proton-motive force, mediating changes in the ExbD periplasmic domain that are then transmitted to the TonB carboxy terminus (39). ExbD D25 is also important for an apparent transition of localized regions of the ExbD periplasmic domain from specific sites of homodimerization to heterodimeric interaction with the TonB periplasmic domain *in vivo* (40).

Here, we mapped specific disulfide-linked interactions between a subdomain of the ExbD periplasmic domain and the extreme carboxy terminus of TonB *in vivo*. Multiple significant interactions with TonB Cys substitutions were observed for 3 of the 6 ExbD Cys substitutions examined, with the 3 remaining being nonreactive. Interactions clustered in 4 important regions of the TonB carboxy terminus. In the presence of an ExbD D25N TMD mutation, all interactions were absent or significantly reduced.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Tables 1 and 2.

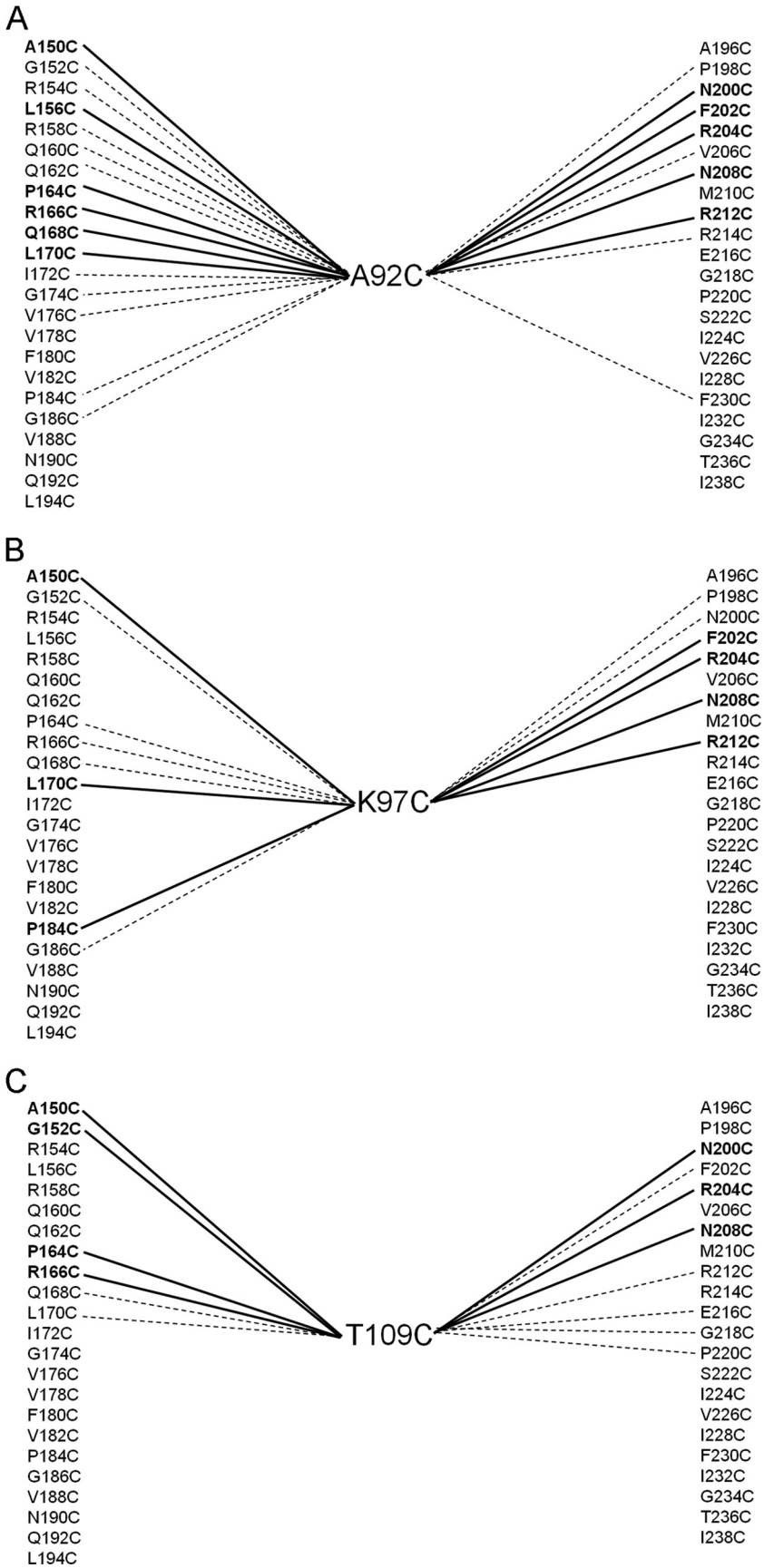
Media and culture conditions. Luria-Bertani (LB) and tryptone (T) broth were prepared as previously described (35). Liquid cultures and agar plates were supplemented with 34 μg ml^{-1} chloramphenicol, 100 μg ml^{-1} ampicillin, and plasmid-specific levels of sodium propionate and L-arabinose (percent wt/vol) as needed for the expression of ExbD and TonB proteins from plasmids. Cultures were grown with aeration at 37°C.

***In vivo* disulfide cross-linking.** Saturated LB cultures grown overnight were subcultured 1:100 in T broth. Equivalent ODmLs (total amounts of cells; obtained by multiplying the A_{550} of the culture by the volume of culture used) of mid-exponential-phase cultures were harvested and precipitated by the addition of an equal volume of 20% trichloroacetic acid (TCA). Cell pellets were solubilized in nonreducing Laemmli sample buffer (27) containing 50 mM iodoacetamide as previously described (13). Samples were resolved on nonreducing 11 and 13% SDS-polyacrylamide gels and immunoblotted with TonB-specific monoclonal antibodies (29) or ExbD-specific polyclonal antibodies (18). Disulfide-linked complexes still formed when samples were not TCA precipitated (data not shown). Mapping classifications of strong or weak interactions were determined by the comparison of at least duplicate cross-linkings to the intensity of representative strong (ExbD A92C-TonB P164C) and weak (ExbD A92C-TonB Q162C) complexes on TonB-specific immunoblots. Strong or weak complex formation was determined after the comparison of band intensities across the full set of cross-linkings. Complexes observed only after the longest exposures were not mapped in this study, as these were more likely to represent transitory or chance interactions.

RESULTS

Three of 6 ExbD Cys substitutions interact significantly with the TonB carboxy terminus *in vivo*. Recently, 70 Cys substitutions in the extreme TonB carboxy terminus were characterized, completing a set of 90 single substitutions from TonB A150C through TonB Q239C. From those studies, no single residues were found to be essential for TonB activity (13, 14, 44). This provided a large pool for studies of TonB interactions through *in vivo* disulfide cross-linking.

In an accompanying paper, we found that A92 initiates a 30-


A
A150C
G152C
R154C
L156C
R158C
Q160C
Q162C
P164C
R166C
Q168C
L170C
I172C
G174C
V176C
V178C
F180C
V182C
P184C
G186C
V188C
N190C
Q192C
L194C
A92C
A196C
P198C
N200C
F202C
R204C
V206C
N208C
M210C
R212C
R214C
E216C
G218C
P220C
S222C
I224C
V226C
I228C
F230C
I232C
G234C
T236C
I238C
B
A150C
G152C
R154C
L156C
R158C
Q160C
Q162C
P164C
R166C
Q168C
L170C
I172C
G174C
V176C
V178C
F180C
V182C
P184C
G186C
V188C
N190C
Q192C
L194C
K97C
A196C
P198C
N200C
F202C
R204C
V206C
N208C
M210C
R212C
R214C
E216C
G218C
P220C
S222C
I224C
V226C
I228C
F230C
I232C
G234C
T236C
I238C
C
A150C
G152C
R154C
L156C
R158C
Q160C
Q162C
P164C
R166C
Q168C
L170C
I172C
G174C
V176C
V178C
F180C
V182C
P184C
G186C
V188C
N190C
Q192C
L194C
T109C
A196C
P198C
N200C
F202C
R204C
V206C
N208C
M210C
R212C
R214C
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I232C
G234C
T236C
I238C

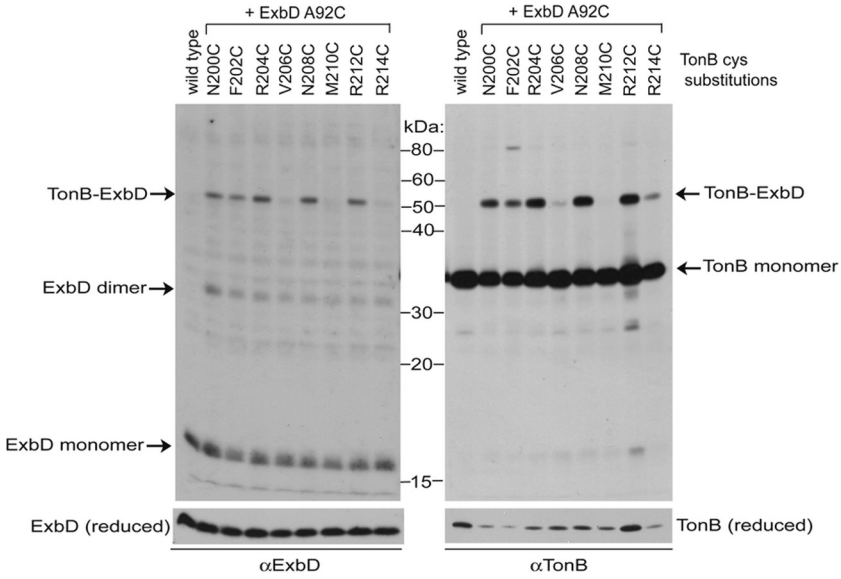

FIG 2 Strong and weak interactions are supported by ExbD A92C and TonB substitutions between N200C and R214C. A strain expressing wild-type ExbD and TonB (W3110) or a Δ*exbD*, Δ*tonB*, Δ*tolQR* strain (KP1509) coexpressing ExbD A92C (pKP1000) with TonB C18G, N200C (pKP469), F202C (pKP415), R204C (pKP418), V206C (pKP463), N208C (pKP416), M210C (pKP466), R212C (pKP471), or R214C (pKP473) was processed in nonreducing sample buffer containing iodoacetamide as described in Materials and Methods. Samples were resolved on a 13% nonreducing SDS-polyacrylamide gel and immunoblotted with ExbD-specific polyclonal antibodies (left) or TonB-specific monoclonal antibodies (right). Samples from the same cultures were processed in reducing sample buffer containing β-mercaptoethanol, resolved on 11 or 13% SDS-polyacrylamide gels, and immunoblotted with TonB-specific monoclonal or ExbD-specific polyclonal antibodies (lower immunoblots). The combinations of substitutions specific to each lane are indicated across the top. Positions of nonreducing molecular mass standards are indicated in the center. Positions of the ExbD or TonB monomers and disulfide-linked complexes are indicated on the left and right, respectively.

residue region of ExbD, from 92 to 121, that is especially important in supporting ExbD protein-protein interactions (40a). Individual Cys substitutions were previously constructed spanning these ExbD residues, and all 30 ExbD Cys substitutions fully supported TonB activity (40). Previous work showed that ExbD A92C is trapped in a disulfide-linked heterodimer when coexpressed with TonB A150C (38). In addition to ExbD A92C, ExbD E95C through D99C, K108C, T109C, Y112C, and E113C also formed significant heterodimers with TonB A150C (40). Here, we extended our study of specific periplasmic ExbD-TonB interactions to a more comprehensive scan of the extreme carboxy terminus of TonB (every other residue from 150 to 239).

To examine specific TonB-ExbD interactions *in vivo*, all plasmids expressing TonB Cys substitutions (pACYC *ori*, Cam^r) in this study were compatible with plasmids expressing the ExbD Cys substitutions (pBR322 *ori*, Amp^r). The introduced Cys in each protein was the only site of potential disulfide cross-linking, as ExbD has no native cysteine residues, and all TonB Cys substitutions were constructed on a Cys-less TonB (C18G) (13). *tonB* and *exbD* expression were under the control of the P_{BAD} and P_{prpB} promoters, respectively, allowing the control of expression of both TonB and ExbD substitutions at native levels of each respective protein as assayed under reducing conditions. Iodoacetamide was also present in the sample buffer to alkylate free sulfhydryl groups and prevent disulfide linkage from occurring after cell lysis.

ExbD A92C and every even-numbered TonB Cys substitution from A150C to I238C were coexpressed in a Δ*exbD*, Δ*tonB*, Δ*tolQR* strain and analyzed for spontaneous disulfide-linked heterodimer formation on nonreducing SDS-polyacrylamide gels. ExbD- and TonB-specific immunoblots showed the previously observed complex at approximately 52 kDa for ExbD A92C with TonB A150C and significant complexes, designated here as strong interactions, of the same apparent molecular mass for TonB Cys substitutions at 156, 164, 166, 168, 170, 200, 202, 204, 208, and 212. Those interactions are summarized in Fig. 1A by solid lines. A number of weaker interactions were also observed (Fig. 1A, dashed lines). Any complexes that formed less efficiently than those in the weak classification were not considered in this study. Immunoblots of ExbD A92C in combination with TonB N200C through R214C are shown as examples of strong (R212C), weak (R214C), and undetectable interactions (M210C) (Fig. 2). Subsequent examples show only the anti-TonB immunoblot.

Results with ExbD A92C suggested that specific interactions between TonB and ExbD span the extreme carboxy terminus of TonB. To extend these studies, TonB interactions with ExbD K97C, F103C, T109C, L115C, and T121C, which were arbitrarily

FIG 1 Mapping specific sites of TonB-ExbD periplasmic domain interactions supported by a functional ExbD TMD. ExbD and TonB cysteine substitutions examined and their abilities to form disulfide cross-linked heterodimers are depicted. TonB Cys substitutions are listed on the left and right, and the ExbD Cys substitution with which each was coexpressed is listed in the center. Solid lines indicate a relatively strong interaction. TonB substitutions supporting strong interactions are highlighted in boldface. Dashed lines indicate a relatively weak or inefficient interaction. (A to C) Interactions supported by ExbD Cys substitutions with a wild-type ExbD TMD. Interactions supported by ExbD D25N were either below the weak interaction level or, for the majority, nonexistent. ExbD F103C, L115C, and T121C were designated nonreactors, because no interactions were detected between them and any TonB Cys substitutions.

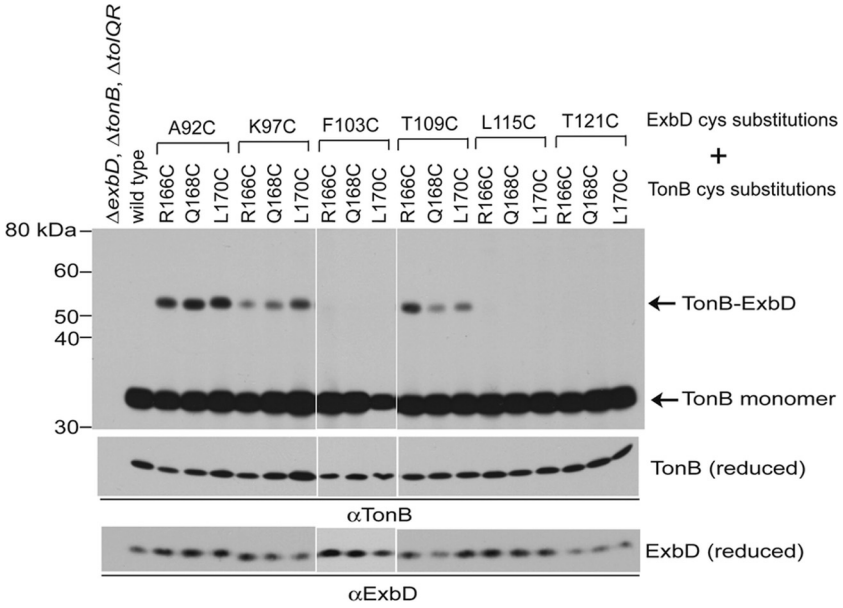

FIG 3 ExbD A92C, K97C, and T109C but not F103C, L115C, and T121C form significant complexes with TonB Cys substitutions. A strain expressing wild-type ExbD and TonB (W3110) or a Δ*exbD*, Δ*tonB*, Δ*tolQR* strain (KP1509) coexpressing ExbD A92C (pKP1000), K97C (pkP1005), F103C (pKP1011), T109C (pKP1017), L115C (pKP1023), or T121C (pKP1029) with TonB C18G, R166C (pKP589), Q168C (pKP593), or L170C (pKP591) was processed in nonreducing sample buffer containing iodoacetamide as described in Materials and Methods. Samples were resolved on an 11% nonreducing SDS-polyacrylamide gel and immunoblotted with TonB-specific monoclonal antibodies. Samples from the same cultures were processed in reducing sample buffer containing β-mercaptoethanol, resolved on 11 or 13% SDS-polyacrylamide gels, and immunoblotted with TonB-specific monoclonal or ExbD-specific polyclonal antibodies (lower immunoblots). The division of the immunoblot indicates that ExbD F103C combinations came from a separate gel and immunoblot. All other combinations came from the same gel and immunoblot. The combinations of substitutions specific to each lane are indicated across the top. Positions of nonreducing molecular mass standards are indicated on the left. Positions of the ExbD or TonB monomers and disulfide-linked complexes are indicated on the right.

chosen to span the 30-residue ExbD subdomain, were examined. This study, therefore, included 3 previously identified ExbD interactive sites and 3 ExbD sites through which interaction with TonB was not previously detected (40). Like ExbD A92C, ExbD K97C and T109C exhibited strong and weak interactions with a number of TonB Cys substitutions that clustered in similar regions (Fig. 1B and C, solid lines). TonB substitution sites of strong interaction with ExbD K97C were at 150, 170, 184, 202, 204, 208, and 212. Strong TonB interactions with ExbD T109C occurred with TonB residue substitutions at 150, 152, 164, 166, 200, 204, and 208. Three ExbD Cys substitutions at F103C, L115C, and T121C were designated nonreactors, because no interactions were detected between them and any of the examined TonB Cys substitutions. Degrees of interactions among ExbD A92C, K97C, F103C, T109C, L115C, or T121C with TonB R166C, Q168C, or L170C are shown as examples (Fig. 3).

A D25N mutation in the ExbD TMD significantly reduces TonB-ExbD disulfide-linked heterodimer formation. Since the disulfide-linked heterodimers in this study formed spontaneously, the effect of protonophores on complex formation could not be meaningfully examined. Either the disulfide-linked complexes would be preexisting in cells when the Cys substitutions were expressed at steady-state levels, or the induction of expression in the presence of protonophores would prevent the export of newly synthesized TonB and ExbD Cys substitutions from the cytoplasm. Because PMF-dependent interaction between ExbD and TonB periplasmic domains and the conformational response of ExbD to the PMF are prevented by a D25N mutation, we used D25N here to mimic the effects of PMF collapse (38–40). The same 270 TonB-ExbD Cys substitution combinations were examined with a D25N mutation in the TMD of all ExbD Cys substitutions. Disulfide-linked dimer formation was significantly reduced in the presence of the D25N TMD mutation for all combinations (examples are shown in Fig. 4). Of the few heterodimers detected with ExbD D25N Cys substitutions, all levels were below those designated weak interactions, with the caveat that for much darker exposures, some complexes were evident at a low level. The most intense complexes detected in the presence of the D25N mutation, such as ExbD A92C or T109C cross-linked with TonB P164C, were at least 4 times less intense than the strong classification based on dilutions (Fig. 4A and C). Complexes with D25N were also observed as 2 to 4 times less intense than weak complexes, such as ExbD A92C cross-linked with TonB Q162C or ExbD K97C cross-linked with P164C (Fig. 4A and B). For most combinations carrying the D25N substitution, e.g., ExbD A92C with TonB Q160C (Fig. 4A), no complexes were observed. No new heterodimers between the regions of ExbD and TonB examined in this study were detected in the presence of the D25N mutation. Similarly to results here with ExbD D25N-TonB interactions, the treatment of flagellated cells with the protonophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP) prior to bis(maleimido) ethane (BMOE) cross-linking through introduced Cys substitutions significantly reduces, but does not entirely eliminate, heterodimeric interactions between the ExbD paralogue MotB and flagellar motor protein FlgI compared to when PMF is present (20).

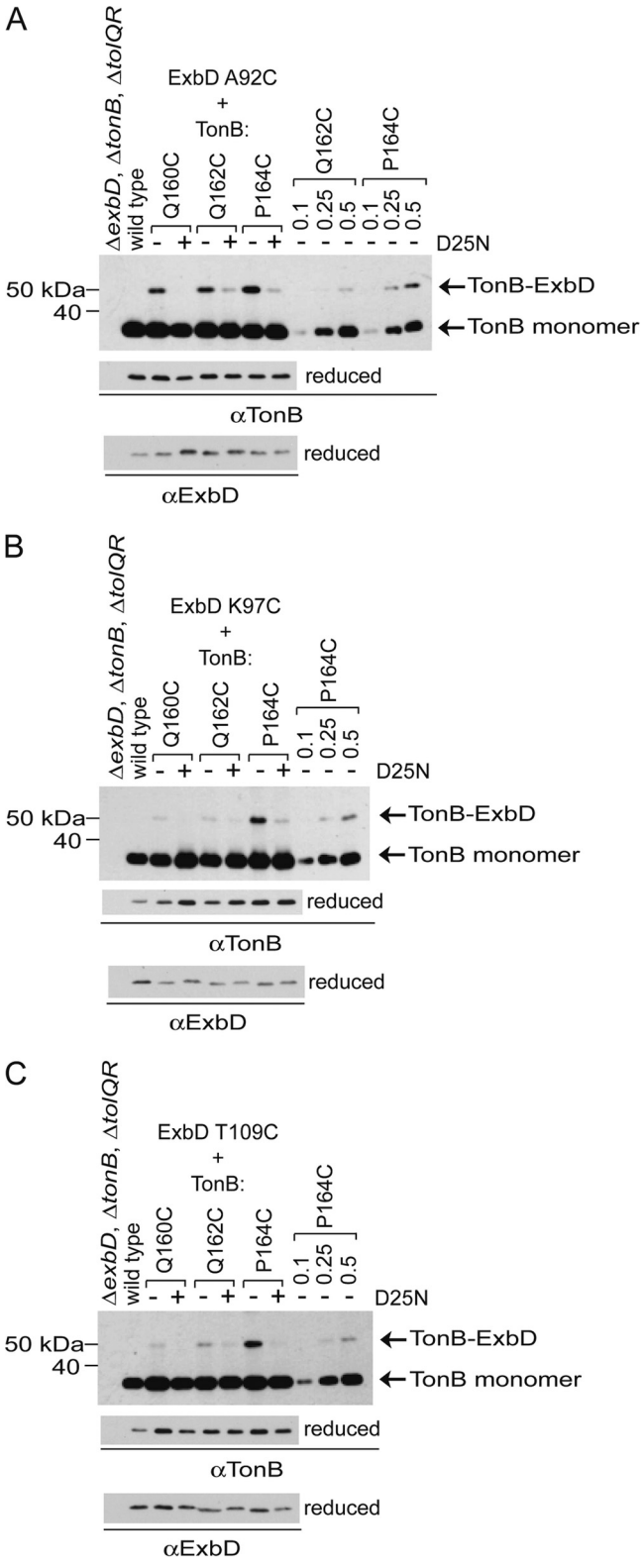

FIG 4 D25N TMD mutation in ExbD significantly reduces TonB-ExbD disulfide-linked interactions. A strain expressing wild-type ExbD and TonB (W3110) or Δ*exbD*, Δ*tonB*, Δ*tolQR* strains (KP1509) coexpressing ExbD A92C (pKP1000) and D25N, A92C (pKP1049) (A), K97C (pKP1005) and D25N, K97C (pKP1050) (B), or T109C (pKP1017) and D25N, T109C (pKP1052) (C) with TonB C18G, Q160C (pKP588), Q162C (pKP587), or P164C (pKP585) was processed in nonreducing sample buffer containing iodoacetamide as described in Materials and Methods. Samples were resolved on 11% nonreducing SDS-polyacrylamide gels and immunoblotted with TonB-specific monoclonal antibodies. Samples from the same cultures were processed in reducing sample buffer containing β-mercaptoethanol, resolved on 11 or 13% SDS-polyacrylamide gels, and immunoblotted with TonB-specific monoclonal or ExbD-specific polyclonal antibodies (lower immunoblots labeled reduced). Lanes on the right are dilutions of the indicated wild-type TMD disulfide-cross-linked samples for comparison to respective D25N cross-links. The combinations of substitutions specific to each lane are indicated across the top. A minus or plus indicates the absence or presence, respectively, of the D25N mutation in the TMD of the ExbD Cys substitution. Positions of nonreducing molecular mass standards are indicated on the left. Positions of the ExbD or TonB monomers and disulfide-linked complexes are indicated on the right.

DISCUSSION

Recent results from our laboratory have demonstrated that ExbD forms both homodimers and TonB-ExbD heterodimers *in vivo* with nearly the same carboxy-terminal periplasmic ExbD residues involved in both interactions (40). Three stages in the energization of TonB have been identified based on the effects of ExbD mutations L132Q in the periplasmic domain and D25N in the transmembrane domain (39). ExbD L132 is required for an initial correct PMF-independent assembly between TonB and ExbD that subsequently leads to the formation of a PMF-dependent conformation detectable by formaldehyde cross-linking through the TonB and ExbD periplasmic domains (38). ExbD D25 is required for the PMF-dependent interaction. Specific residues important in mediating heterodimeric interactions between the TonB and ExbD periplasmic domains, however, were largely unknown.

Here, we examined combinations of TonB and ExbD periplasmic domain Cys substitutions for the spontaneous formation of disulfide-linked heterodimers and the importance of ExbD D25 in complex formation. The targeted regions in this study were the TonB extreme carboxy terminus (residues 150 to 239), where the only functionally important TonB periplasmic domain residues are located (44), and a 30-residue region of the ExbD periplasmic domain (residues 92 to 121) that is important in supporting protein-protein interactions of ExbD (40a). A significant number of TonB Cys substitutions were trapped in spontaneous disulfide-linked interactions through 3 of the 6 ExbD Cys substitutions examined, with the other 3 showing essentially no reactivity. These were designated nonreactors. When all sites of heterodimer formation were compared, 4 regions of ExbD-TonB interactions were apparent based on TonB sites where at least one ExbD Cys substitution exhibited strong interaction or at least 2 exhibited weak interactions.

ExbD contacts four regions of the extreme TonB carboxy terminus. The first and second regions contacted by ExbD included TonB A150C and G152C and TonB P164C, R166C, Q168C, and L170C, respectively. Both of these regions are located toward the carboxy-terminal end of a large region of the TonB periplasmic domain (residues 33 to 169), which is predicted to be disordered, and residues 103 to 151, which are disordered in the TonB solution structure (28, 43). The predicted unstructured nature of this region may facilitate conformational changes that permit the interaction of ExbD at multiple sites. The close interaction of ExbD with this unstructured region would support a previously proposed role for ExbD in the potential disorder-to-order transitions of this region of TonB (54).

The first and second regions are also found on either side of a

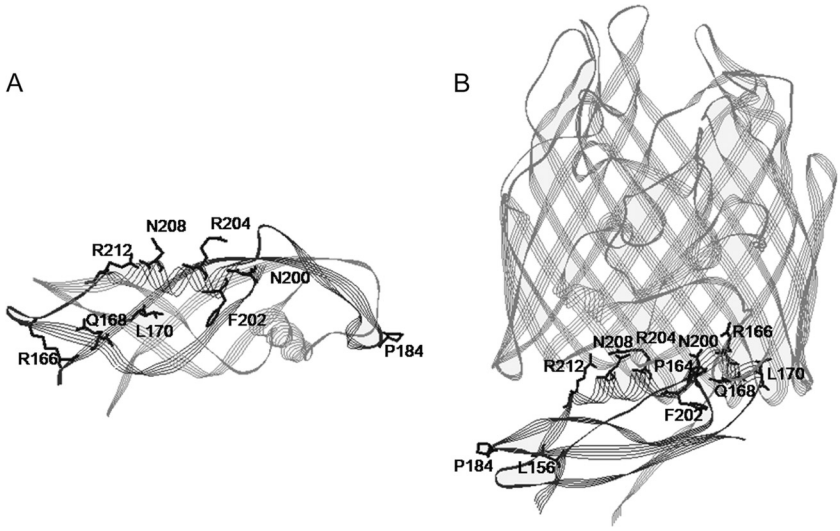

FIG 5 Sites of *in vivo* disulfide-linked heterodimer formation mapped on two TonB periplasmic domain fragment crystal structures. Side chains depicted in black show sites where TonB Cys substitutions formed strong disulfide-linked heterodimers with at least 1 ExbD Cys substitution. Two representative TonB structures, homodimeric $TonB_{165-239}$ (Protein Data Bank [PDB] code 1ihr) (A) and BtuB-$TonB_{153-239}$ (PDB code 2gsk) (B), are shown. Interactive sites A150, G152, and L156 (for 1ihr only) are not part of the structures and could not be mapped. The image was generated using Swiss-PdbViewer (http://www.expasy.org/spdbv/ [16]).

region of TonB, residues 159 to 164, that makes direct *in vivo* contact with the conserved amino-terminal TonB boxes of the OM TonB-gated transporters BtuB and FecA (3, 37). This result, that TonB interacted with ExbD at sites unique but adjacent to interactions with transporter TonB boxes, suggested that ExbD could interact simultaneously with TonB that is bound to the TonB box or even direct bound TonB to the TonB box. Perhaps ExbD "primes" TonB for proper TonB box interaction by stabilizing a specific conformation of the periplasmic domain, similar to the priming of pilus subunits, for subsequent donor strand exchange by a periplasmic chaperone in the chaperone-usher pathway (47).

The third region of ExbD-TonB interaction was TonB P184C and G186C, which exhibited primarily weak interactions with 2 of the 3 reactive ExbD Cys substitutions, A92C and K97C. G186 is 1 of the 7 residues, and the only nonaromatic residue, in the TonB periplasmic domain that is functionally important. The high conservation of G186 compared to the other functionally important residues led to the proposal that it has a fundamental mechanistic role, as opposed to being a site of direct transporter recognition by TonB, as the other aromatic residues are hypothesized to be (44). It may be that interaction with this region is important for ExbD to catalyze TonB conformational changes.

The fourth region was characterized by strong interactions of the three reactive ExbD Cys substitutions with TonB N200C, F202C, R204C, N208C, and R212C. This region is found within a predicted amphipathic helix (residues 199 to 216) of TonB, one of the most highly conserved TonB features across Gram-negative bacteria, although its functional significance is unknown (8). Of the even-numbered TonB residues exhibiting strong ExbD interactions, N200, R204, N208, and R212 mapped to one face of this helix in the crystal structures of TonB periplasmic domain fragments (Fig. 5 shows two examples). The exception was F202, which maps to the opposite face of the helix. In the monomeric TonB NMR structure (42) or crystal structures of TonB in complex with BtuB or FhuA (41, 51), N200 and F202 are not part of the helix but are in an adjacent loop (Fig. 5B, for example). The alignment of strong interactive sites almost exclusively to one face of the predicted helix suggests that it is partially solvent exposed at some point in the energy transduction cycle. Strong interactions between ExbD A92C or K97C with these TonB residues on one face of this helix and with TonB F202C on the opposite face suggested that ExbD interacted with multiple conformations of TonB. F202 is a buried residue in both dimeric TonB structures and is inaccessible to interaction with ExbD, suggesting that this helix is a dynamic structural element.

A model for the role of PMF in ExbD-TonB interactions. The overall lack of ExbD D25N interaction with the TonB extreme carboxy terminus (residues 150 to 239) provided a rationale for the proteinase K resistance of the amino-terminal 1 to ~156 residues of TonB and the concomitant proteinase K sensitivity of residues ~157 to 239 that occurs in the presence of ExbD D25N (39). Perhaps the PMF-independent interaction between TonB and ExbD involves TonB residues 1 to 156, with contact expanding to include the entire TonB carboxy terminus as PMF is utilized. The low level of detection of some heterodimers with ExbD D25N Cys substitutions suggested that D25 was important for either the formation of the complexes, such that they formed inefficiently in the presence of D25N, or for maintaining ExbD-TonB periplasmic domains in the cross-linkable conformations. Strong interactions in the presence of D25 may reflect the conformational changes due to the response to PMF (39).

Approximately 40% of wild-type TonB fractionates with the OM following sucrose density gradient fractionation (30, 33). Because TonB remains associated with the CM during its energy transduction cycle, this fraction appears to represent TonB that is so tightly bound to the OM that it can be pulled out of its association with ExbB/ExbD during the fractionation process (15, 33). The formation of TonB triplet homodimers through the extreme carboxy terminus can preclude this interaction of TonB with the

OM, with disulfide-linked complexes detected only with CM fractions. It is hypothesized that these TonB homodimeric interfaces must normally be reorganized before the TonB periplasmic domain can interact with OM proteins (13). The formation of TonB-ExbD heterodimers through sites common to TonB homodimer formation, such as G186C and F202C, further supported the idea that the TonB interfaces represented by the triplet homodimers are not permanent. Because significant TonB-ExbD interactions in this study required ExbD TMD residue D25, these heterodimeric interactions appeared to occur in response to PMF. These data were consistent with previous observations that a formaldehyde-specific cross-link between TonB and ExbD periplasmic domains requires the PMF. Taken together, these studies extended our data on the ExbD-directed remodeling of the TonB periplasmic domain (39, 40) to involve a network of specific D25-promoted ExbD-TonB interactions. This work further suggested that one role of these interactions is to resolve TonB homodimeric interactions to free sites for interaction with OM TonB-gated transporters.

Implications from solved structures of ExbD and TonB carboxy termini. The solved structures of the TonB carboxy terminus are all similar, with two main conformational variations observed, whether the TonB fragment is monomeric or in complex (41, 51, 55). The specific sites of TonB-ExbD interactions observed *in vivo* mapped primarily along one side of these structures, and here, two representative structures are shown (Fig. 5). In the $TonB_{165-239}$ homodimer, most sites exhibiting strong interactions with ExbD were exposed (Fig. 5A). This differs from $TonB_{153-239}$ crystallized in complex with the OM transporter BtuB, where the general region involved in interaction with ExbD interfaced with the periplasmic face of the transporter (Fig. 5B). TonB residues forming interactions with BtuB *in vitro* spanned the regions from 158 to 172, 199 to 213, and 225 to 233 (analyzed using the program Monster [46]; data not shown). TonB R166, L170, N200, and R204, which all exhibited strong interactions with ExbD *in vivo*, are involved in specific interactions with the β-barrel of BtuB *in vitro*. R166, Q168, and L170 also exhibit interactions with the plug domain of BtuB. It is unknown if these specific TonB-BtuB interactions occur *in vivo*, but this might identify some common TonB interactive sites that are shared with both ExbD- and TonB-gated transporters. It is known that the TonB box is not the only site through which TonB interacts with the transporters *in vivo*, although specific sites remain to be determined (10). Two TonB regions, from 158 to 163 and 226 to 233, exhibit extensive *in vitro* interactions with residues of the BtuB TonB box (residues 6 to 12) in the cocrystal structure (51). It is notable that neither of these regions exhibited strong *in vivo* interactions with ExbD. Additionally, TonB P184 and G186 exhibited interactions specific to ExbD *in vivo*. While unique sites of interaction may relate to functions specific to the individual partner proteins, TonB sites common to interaction with both proteins may have mechanistic implications. The sequestering of interaction sites by one partner may be important in the relay of signals or directing a sequence of interactions between the multiple protein partners in the TonB system.

An important finding of this work is that the same three ExbD residues were involved in all interactions with the TonB carboxy terminus, with the remaining three ExbD Cys residues exhibiting no interactions. When the three sites of significant ExbD interactions with the TonB carboxy terminus were mapped on the ExbD periplasmic domain NMR structure, A92 and K97 were located at one end of the structure with T109 at the opposite end, suggesting the possibility of multiple interfaces of interaction of the ExbD periplasmic domain with TonB (Fig. 6). The nonreactors F103, L115, and T121 were each positioned closer to the middle of the structure. Although the periplasmic domains of TonB and ExbD clearly interact *in vivo*, no significant interactions are observed *in vitro*, either at pH 3, which allowed the solution of the monomeric ExbD NMR structure, or pH 7, which approximates the pH of the periplasm (12, 56). The TonB carboxy-terminal fragment used in those studies (residues 103 to 239) assumes a conformation nearly identical to that of the monomeric unit of $TonB_{153-239}$ crystallized in complex with BtuB (43, 51) (Fig. 5B). Thus, these observed conformations do not appear to represent the interactive states of the ExbD and TonB periplasmic domains *in vitro* or *in vivo* (12, 38). Nevertheless, elements of the structures, such as the TonB amphipathic helix, may mediate important interactions for energy transduction *in vivo*. Localized secondary structural elements could provide interactive surfaces, but perhaps the stable tertiary structures observed *in vitro* lack the proper context for these elements that would normally allow functional interactions to occur.

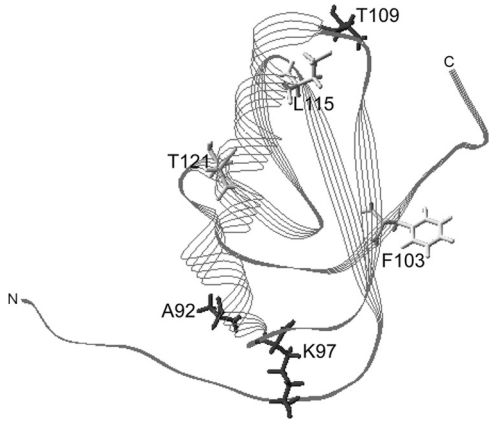

FIG 6 ExbD Cys substitutions showing strong interactions with TonB Cys substitutions *in vivo* map to opposite ends of the ExbD periplasmic domain NMR structure. The locations of the six ExbD Cys substitutions examined in this study, with side chains of the native residue shown, are mapped on the ExbD periplasmic domain NMR structure (PDB code 2pfu). The image was generated using Swiss-PdbViewer (http://www.expasy.org/spdbv/ [16]). Black side chains indicate sites of significant spontaneous ExbD-TonB heterodimer formation. Gray side chains indicate sites of no significant spontaneous heterodimer formation. C and N indicate the carboxy and amino terminus of the domain, respectively.

This work provided a first extensive view of specific and apparently PMF-promoted interactions between TonB and ExbD periplasmic domains *in vivo*. By identifying TonB regions involved in interactions with both ExbD- and TonB-gated transporters, these studies supported a role for ExbD in regulating TonB conformation and suggested a model where ExbD presents specific conformations of TonB for interactions with the transporters.

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