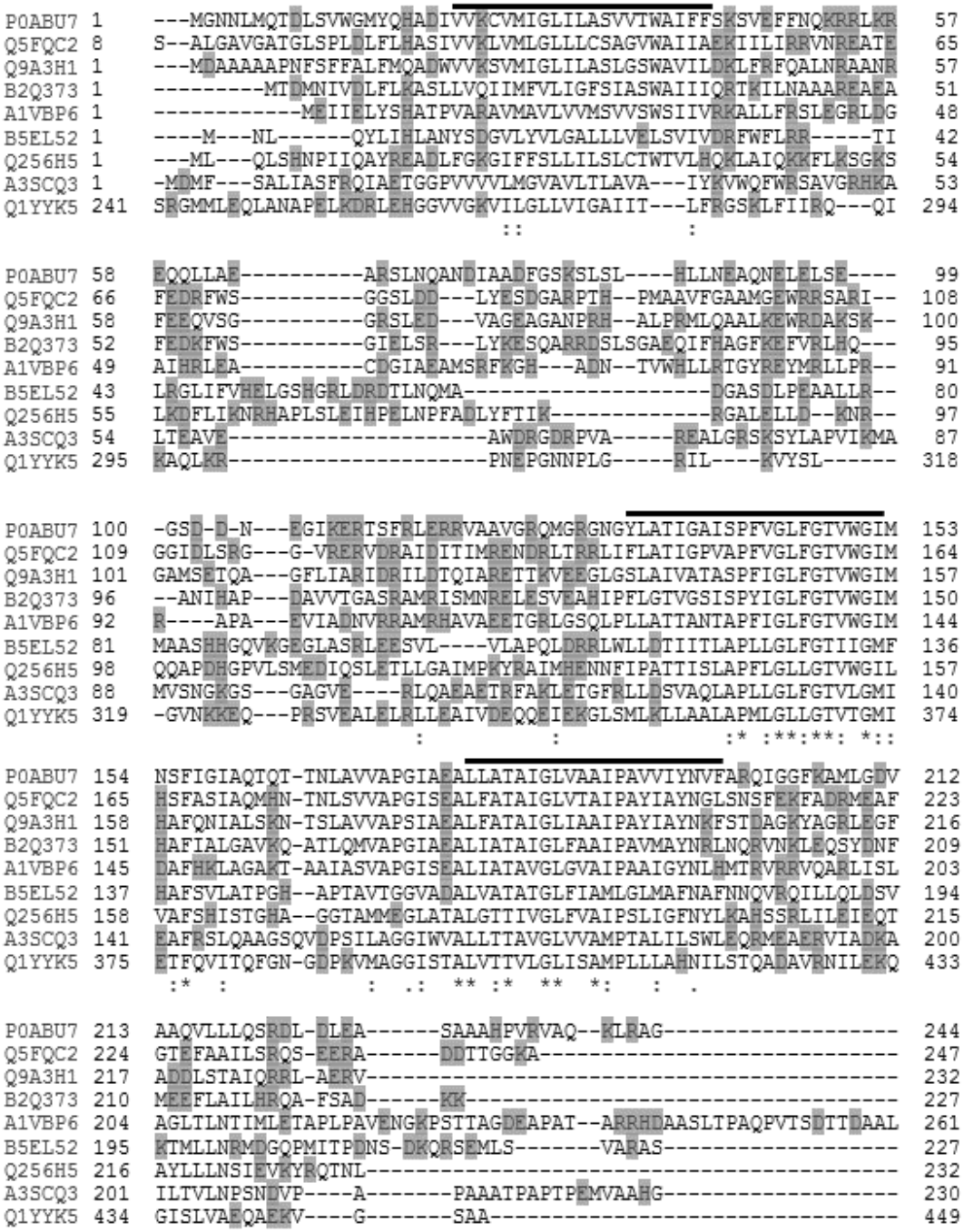


FIG. S1. *Alignment of distantly related ExbB sequences*. Distantly related ExbB sequences were aligned using clustal omega multiple sequence alignment program (http://www.ebi.ac.uk/Tools/msa/clustalo/). Charged residues are highlighted in grey.

TOPCONS-predicted TMDs are indicated by a black bar above the sequence: TMD1 (22-42), TMD2 (132-152), TMD3 (178-198). Names of distantly related species, their corresponding accession numbers, and expect scores relative to ExbB from *E.coli* K12 are: *Escherichia coli* (P0ABU7, e $^{-130}$), *Gluconobacter oxydans* (Q5FQC2, 2e $^{-28}$), *Caulobacter crescentus* (Q9A3H1, 9e $^{-29}$), *Providencia stuartii* (B2Q373, 9e $^{-21}$), *Desulfovibrio vulgaris* (A1VBP6, 1e $^{-20}$), *Acidithiobacillus ferrooxidans* (B5EL52, 4e $^{-7}$), *Chlamydophila felis* (Q256H5, 4e $^{-7}$), *Sulfitobacter sp. EE-36* (A3SCQ3, 3e $^{-7}$), *Photobacterium profundum* (Q1YYK5, 4e $^{-7}$).

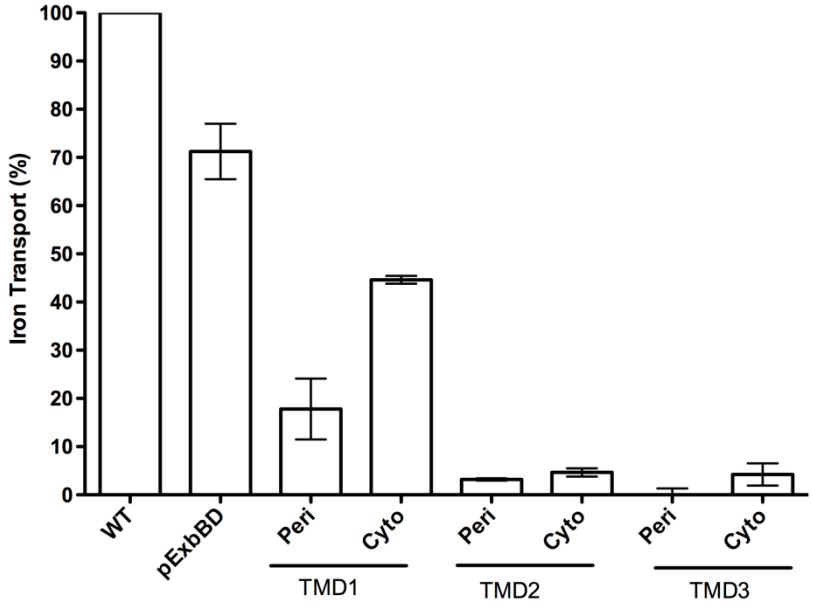


FIG. S2. *Most of the ExbB half-Ala substitutions are dominant.* ExbB half-Ala substitution mutants co-expressed with ExbD were induced at subculture with 0.01% L-arabinose in wild type (WT) strain W3110. All mutants were overexpressed greater than 100-fold as determined by immunoblot (data not shown). Initial rates of [^{55}Fe]-ferrichrome transport were determined from multiple triplicate experiments and normalized to W3110 (100%). Substituted portions and their corresponding plasmid numbers were: pExbBD (pKP660); TMD1 peri (pKP1459); TMD1 cyto (pKP1460); TMD2 peri (pKP1481), TMD2 cyto (pKP1482); TMD3 peri (pKP1483) ; TMD3 cyto (pKP1484).

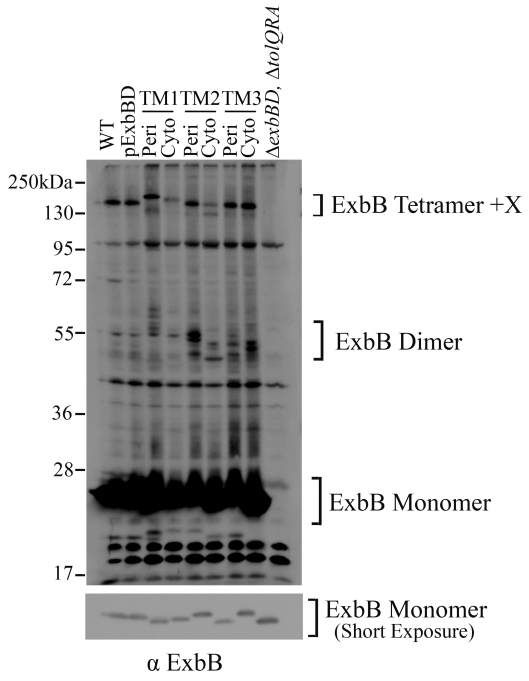
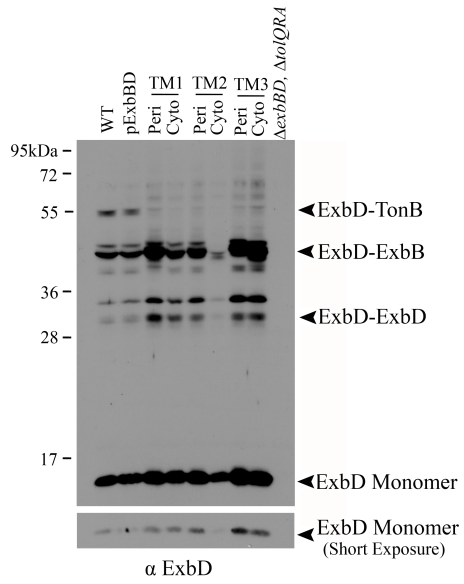


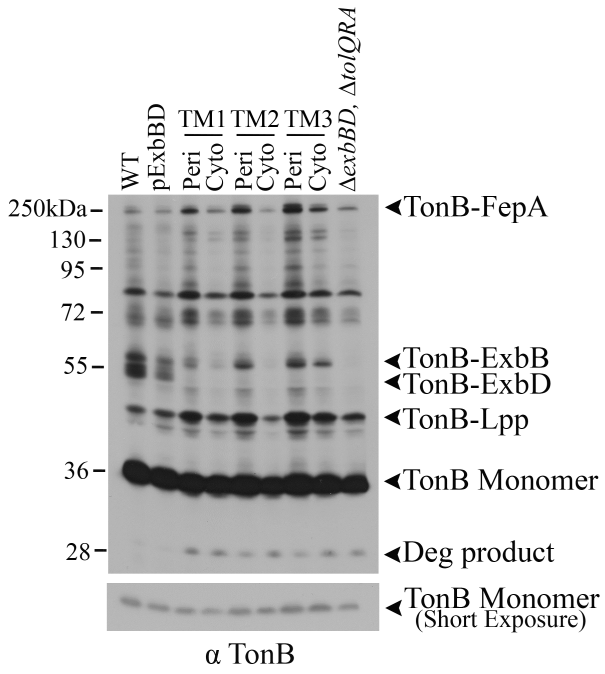
Fig. S3A. *ExbB half-Ala TMDs do not form the TonB-ExbD complex.* ExbB half-Ala mutants and the plasmid encoded (pExbBD) control were expressed to near chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*) and parent strain W3110 served as the wild type (WT) chromosomal control. The corresponding locations of the half-Ala substitutions are indicated above each lane as TMD1, TMD2 or TMD3 and Peri or Cyto. Cultures grown to mid-exponential phase were cross-linked with monomeric formaldehyde and solubilized in LSB at 60°C. Samples were resolved on 11% or 13% SDS-polyacrylamide gels and immunoblotted with (A) anti-ExbB, (B) anti-ExbD or (C) anti-TonB antibodies. Positions and composition of complexes are indicated on the right. Molecular mass standards are indicated on the left. A shorter exposure of the same immunoblot is shown in the lower panel for monomer levels. Corresponding plasmid names and percentages of L-arabinose used to induce chromosomal level expression are as follows: pExbBD (pKP660) 0.0004%; TMD1-peri (pKP1459) 0.0005%; TMD1-cyto

(pKP1460) 0.0005%; TMD2-peri (pKP1481) 0.0005%, TMD2-cyto (pKP1482) 0.0009%; TMD3-peri (pKP1483) 0.0005%; TMD3-cyto (pKP1484) *0.0008%.*



S3B. *ExbB half-Ala TMDs do not form the TonB-ExbD complex.* ExbB half-Ala mutants and the plasmid encoded (pExbBD) control were expressed to near chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*) and parent strain W3110 served as the wild type (WT) chromosomal control. The corresponding locations of the half-Ala substitutions are indicated above each lane as TMD1, TMD2 or TMD3 and Peri or Cyto. Cultures grown to mid-exponential phase were cross-linked with monomeric formaldehyde and solubilized in LSB at 60°C. Samples were resolved on 11% or 13% SDS-polyacrylamide gels and immunoblotted with (A) anti-ExbB, (B) anti-ExbD or (C) anti-TonB antibodies. Positions and composition of complexes are indicated on the right. Molecular mass standards are indicated on the left. A shorter exposure of the same immunoblot is shown in the lower panel for monomer levels. Corresponding plasmid names and percentages of L-arabinose used to induce chromosomal level expression are as follows: pExbBD (pKP660) 0.0004%; TMD1-peri (pKP1459) 0.0005%; TMD1-cyto (pKP1460) 0.0005%;

TMD2-peri (pKP1481) 0.0005%, TMD2-cyto (pKP1482) 0.0009%; TMD3-peri (pKP1483) 0.0005%; TMD3-cyto (pKP1484) *0.0008%.*



S3C. *ExbB half-Ala TMDs do not form the TonB-ExbD complex.* ExbB half-Ala mutants and the plasmid encoded (pExbBD) control were expressed to near chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*) and parent strain W3110 served as the wild type (WT) chromosomal control. The corresponding locations of the half-Ala substitutions are indicated above each lane as TMD1, TMD2 or TMD3 and Peri or Cyto. Cultures grown to mid-exponential phase were cross-linked with monomeric formaldehyde and solubilized in LSB at 60°C. Samples were resolved on 11% or 13% SDS-polyacrylamide gels and immunoblotted with specific (A) anti-ExbB, (B) anti-ExbD or (C) anti-TonB antibodies. Positions and composition of complexes are indicated on the right. Molecular mass standards are indicated on the left. A shorter exposure of the same immunoblot is shown in the lower panel for monomer levels. Corresponding plasmid names and percentages of L-arabinose used to induce chromosomal level expression are as follows: pExbBD (pKP660) 0.0004%; TMD1-peri (pKP1459) 0.0005%; TMD1-cyto (pKP1460)

0.0005%; TMD2-peri (pKP1481) 0.0005%, TMD2-cyto (pKP1482) 0.0009%; TMD3-peri (pKP1483) 0.0005%; TMD3-cyto (pKP1484) *0.0008%.*

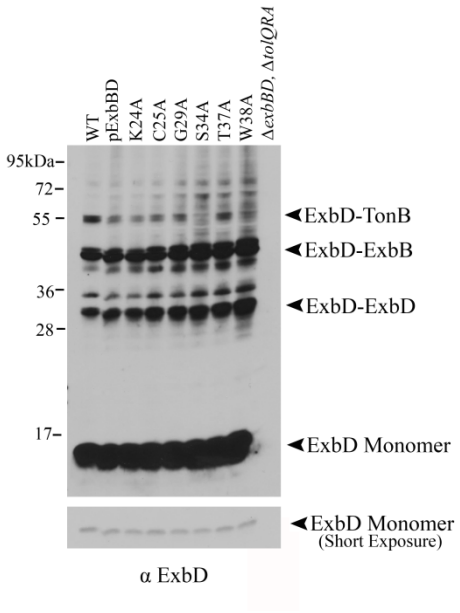


FIG. S4A. *Formaldehyde crosslinking profile of ExbB Ala-substituted mutants using anti-ExbD antibody*. ExbB Ala substituted TMD mutants were expressed at chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*). W3110 served as the wild type (WT) chromosomal control. ExbB TMD mutants are indicated above each lane. Cultures were grown to mid-exponential phase, crosslinked with monomeric formaldehyde and solubilized in LSB at 60°C. Samples were resolved on 13% SDS-polyacrylamide gels and immunoblotted with anti-ExbD antibody. Positions and composition of complexes are indicated on the right. Molecular mass standards are indicated on the left. A shorter exposure of the same immunoblot is shown in the lower panel for monomer levels. (A) TMD1 ExbB Ala substitutions. (B) ExbB TMD2 Ala substitutions (C) ExbB TMD3 Ala substitutions. Aberrant migration of the ExbD-ExbB complex of ExbB E176A and ExbB P190A corresponds to the faster migration of the ExbB monomer (refer to Fig. 6)

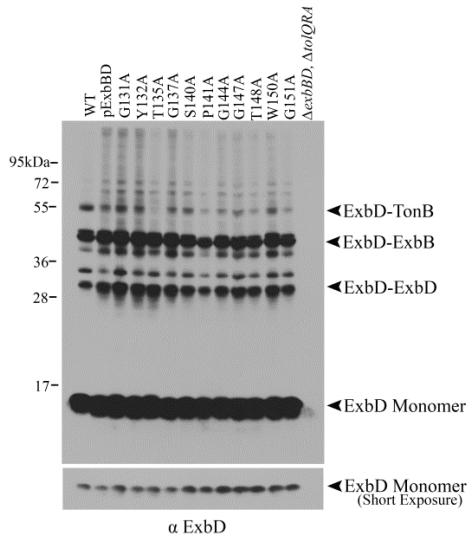


FIG. S4B. *Formaldehyde crosslinking profile of ExbB Ala substituted mutants using anti-ExbD antibody.* ExbB Ala substituted TMD mutants were expressed at chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*). W3110 served as the wild type (WT) chromosomal control. ExbB TMD mutants are indicated above each lane. Cultures were grown to mid-exponential phase, crosslinked with monomeric formaldehyde and solubilized in LSB at 60°C. Samples were resolved on 13% SDS-polyacrylamide gels and immunoblotted with anti-ExbD antibody. Positions and composition of complexes are indicated on the right. Molecular mass standards are indicated on the left. A shorter exposure of the same immunoblot is shown in the lower panel for monomer levels. (A) TMD1 ExbB Ala substitutions. (B) ExbB TMD2 Ala substitutions (C) ExbB TMD3 Ala substitutions. Aberrant migration of the ExbD-ExbB complex of ExbB E176A and ExbB P190A corresponds to the faster migration of the ExbB monomer (refer to Fig. 6)

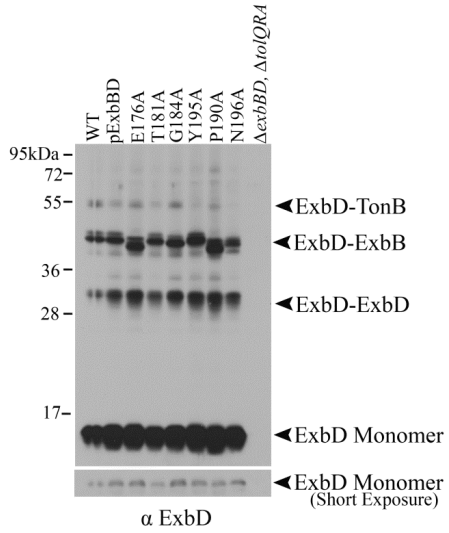


FIG. S4C. *Formaldehyde crosslinking profile of ExbB Ala substituted mutants using anti-ExbD antibody.* ExbB Ala substituted TMD mutants were expressed at chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*). W3110 served as the wild type (WT) chromosomal control. ExbB TMD mutants are indicated above each lane. Cultures were grown to mid-exponential phase, crosslinked with monomeric formaldehyde and solubilized in LSB at 60°C. Samples were resolved on 13% SDS-polyacrylamide gels and immunoblotted with anti-ExbD antibody. Positions and composition of complexes are indicated on the right. Molecular mass standards are indicated on the left. A shorter exposure of the same immunoblot is shown in the lower panel for monomer levels. (A) TMD1 ExbB Ala substitutions. (B) ExbB TMD2 Ala substitutions (C) ExbB TMD3 Ala

substitutions. Aberrant migration of the ExbD-ExbB complex of ExbB E176A and ExbB P190A corresponds to the faster migration of the ExbB monomer (refer to Fig. 6)

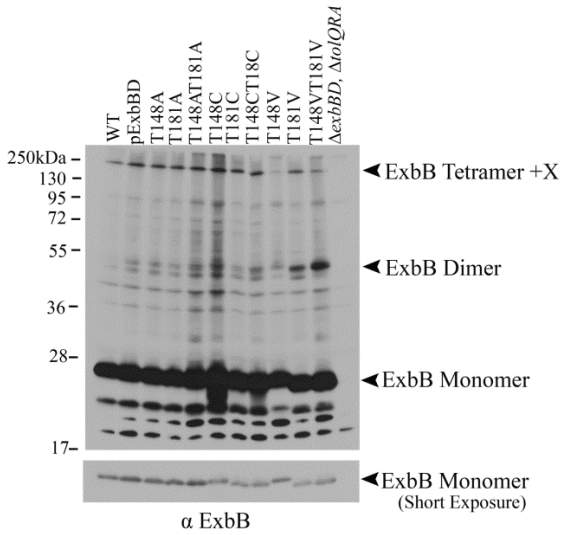


FIG. S5A. *Formaldehyde crosslinking profile of ExbB T148 and T181 substitutions.* Substituted TMD mutants were expressed at chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*) and parent strain W3110 (WT) served as the wild type chromosomal control. Plasmids expressing ExbB substitutions are indicated above each lane. Cultures were grown to mid-exponential phase and crosslinked with monomeric formaldehyde then solubilized in LSB at 60°C. Samples were resolved on two 13% and one 11% SDS-polyacrylamide gels then immunoblotted with (A) anti-ExbB (B) anti-ExbD (C) anti-TonB antibodies. Positions and composition of complexes are indicated on the right. Molecular mass standards are indicated on the left. A shorter exposure of the same immunoblot is shown in the lower panel for monomer levels.

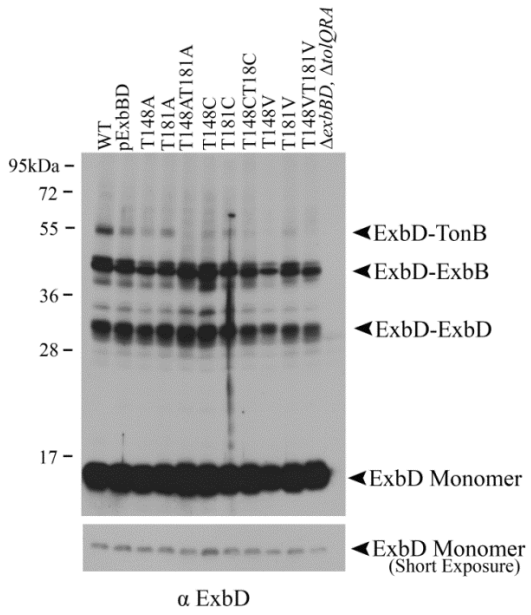


FIG. S5B. *Formaldehyde crosslinking profile of ExbB T148 and T181 substitutions.* Substituted TMD mutants were expressed at chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*) and parent strain W3110 (WT) served as the wild type chromosomal control. Plasmids expressing ExbB substitutions are indicated above each lane. Cultures were grown to mid-exponential phase and crosslinked with monomeric formaldehyde then solubilized in LSB at 60°C. Samples were resolved on two 13% and one 11% SDS-polyacrylamide gels then immunoblotted with (A) anti-ExbB (B) anti-ExbD (C) anti-TonB antibodies. Positions and composition of complexes are indicated on the right. Molecular mass standards are indicated on the left. A shorter exposure of the same immunoblot is shown in the lower panel for monomer levels.

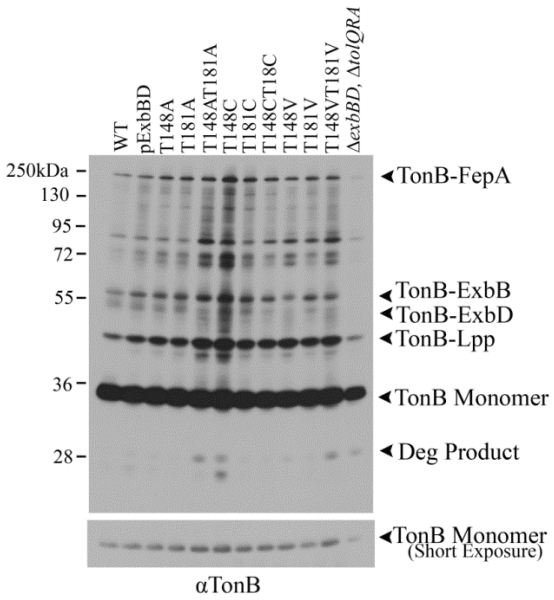


FIG. S5C. *Formaldehyde crosslinking profile of ExbB T148 and T181 substitutions.* Substituted TMD mutants were expressed at chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*) and parent strain W3110 (WT) served as the wild type chromosomal control. Plasmids expressing ExbB substitutions are indicated above each lane. Cultures were grown to mid-exponential phase and crosslinked with monomeric formaldehyde then solubilized in LSB at 60°C. Samples were resolved on two 13% and one 11% SDS-polyacrylamide gels then immunoblotted with (A) anti-ExbB (B) anti-ExbD (C) anti-TonB antibodies. Positions and composition of complexes are indicated on the right. Molecular mass standards are indicated on the left. A shorter exposure of the same immunoblot is shown in the lower panel for monomer levels.

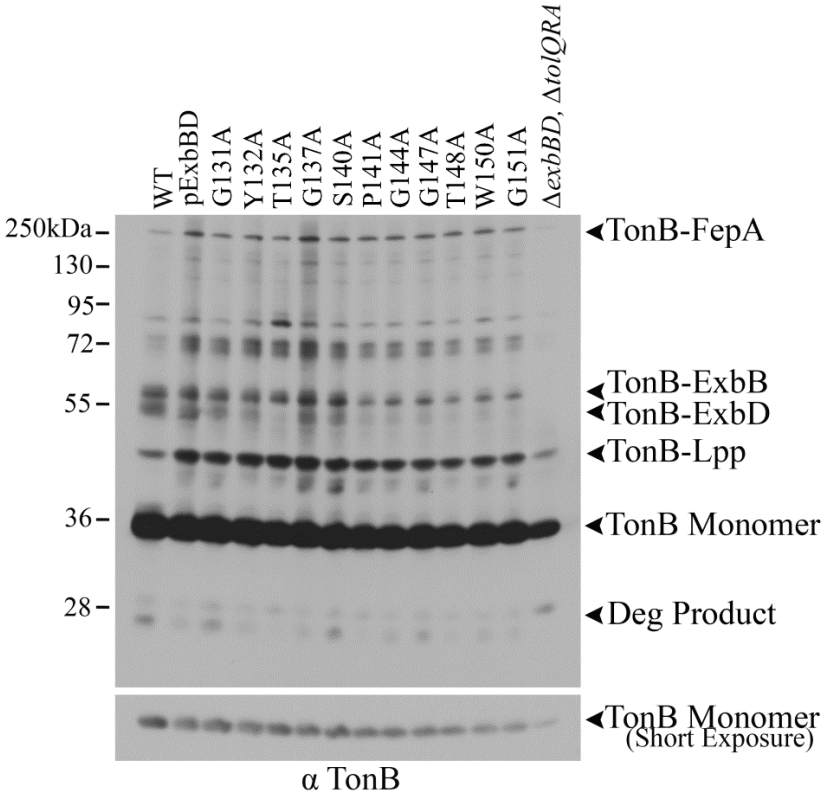


FIG. S6A. *Formaldehyde crosslinking profile of ExbB Ala substituted mutants using anti-TonB antibody.* ExbB Ala substituted TMD mutants were expressed at chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*) and parent strain W3110 (WT) served as the wild type chromosomal control. ExbB TMD mutants are indicated above each lane. Cultures grown to mid-exponential phase were crosslinked with monomeric formaldehyde and solubilized in LSB at 60°C. Samples were resolved on 11% SDS-polyacrylamide gels and immunoblotted with anti-TonB antibody. Positions and composition of complexes are indicated on the right. Molecular mass standards are indicated on the left. A shorter exposure of the same immunoblot is shown in the lower panel for monomer levels. (A) ExbB TMD2 Ala substitutions (B) ExbB TMD3 Ala substitutions.

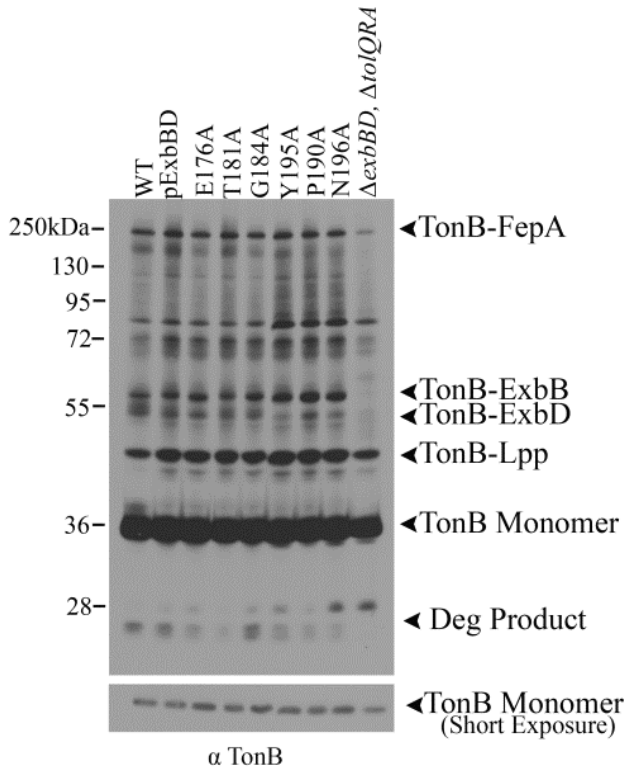


FIG. S6B. *Formaldehyde crosslinking profile of ExbB Ala substituted mutants using anti-TonB antibody.* ExbB Ala substituted TMD mutants were expressed at chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*) and parent strain W3110 (WT) served as the wild type chromosomal control. ExbB TMD mutants are indicated above each lane. Cultures grown to mid-exponential phase were crosslinked with monomeric formaldehyde and solubilized in LSB at 60°C. Samples were resolved on 11% SDS-polyacrylamide gels and immunoblotted with anti-TonB antibody. Positions and composition of complexes are indicated on the right. Molecular mass standards are indicated on the left. A shorter exposure of the same immunoblot is shown in the lower

panel for monomer levels. (A) ExbB TMD2 Ala substitutions (B) ExbB TMD3 Ala substitutions.

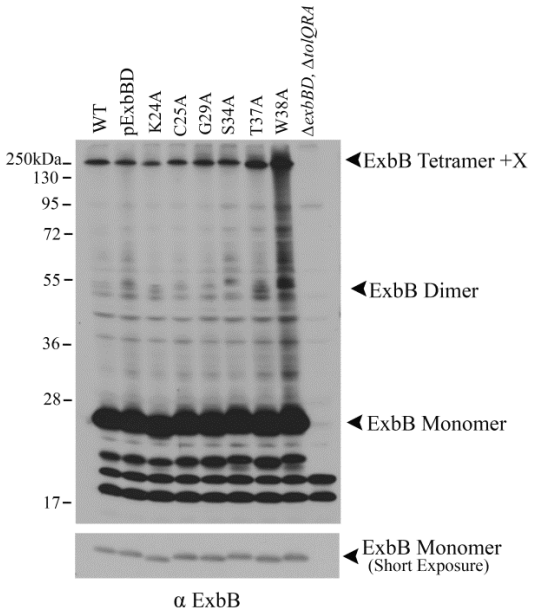


FIG. S7A. *Formaldehyde crosslinking profile of ExbB Ala substituted mutants using anti-ExbB antibody.* ExbB Ala substituted TMD mutants were expressed at chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*) and W3110 (WT) served as the wild type chromosomal control. ExbB TMD mutants are indicated above each lane. Cultures were grown to mid-exponential phase, crosslinked with monomeric formaldehyde and solubilized in LSB at 60°C. Samples were resolved on 13% SDS-polyacrylamide gels and immunoblotted with anti-ExbB antibody. Positions and composition of complexes are indicated on the right. Molecular mass standards are indicated on the left. A shorter exposure of the same immunoblot is shown in the lower panel for monomer levels. (A) TMD1 ExbB Ala substitutions. (B) ExbB TMD2 Ala substitutions.

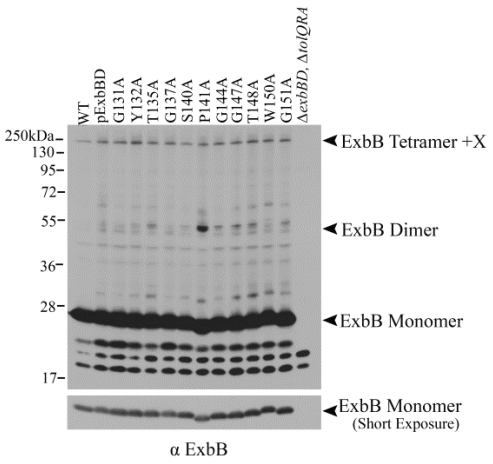


FIG. S7B. *Formaldehyde crosslinking profile of ExbB Ala substituted mutants using anti-ExbB antibody.* ExbB Ala substituted TMD mutants were expressed at chromosomal levels in strain RA1017 (Δ*exbBD* Δ*tolQRA*) and W3110 (WT) served as the wild type chromosomal control. ExbB TMD mutants are indicated above each lane. Cultures were grown to mid-exponential phase, crosslinked with monomeric formaldehyde and solubilized in LSB at 60°C. Samples were resolved on 13% SDS-polyacrylamide gels and immunoblotted with anti-ExbB antibody. Positions and composition of complexes are indicated on the right. Molecular mass standards are indicated on the left. A shorter exposure of the same immunoblot is shown in the lower panel for monomer levels. (A) TMD1 ExbB Ala substitutions. (B) ExbB TMD2 Ala substitutions.

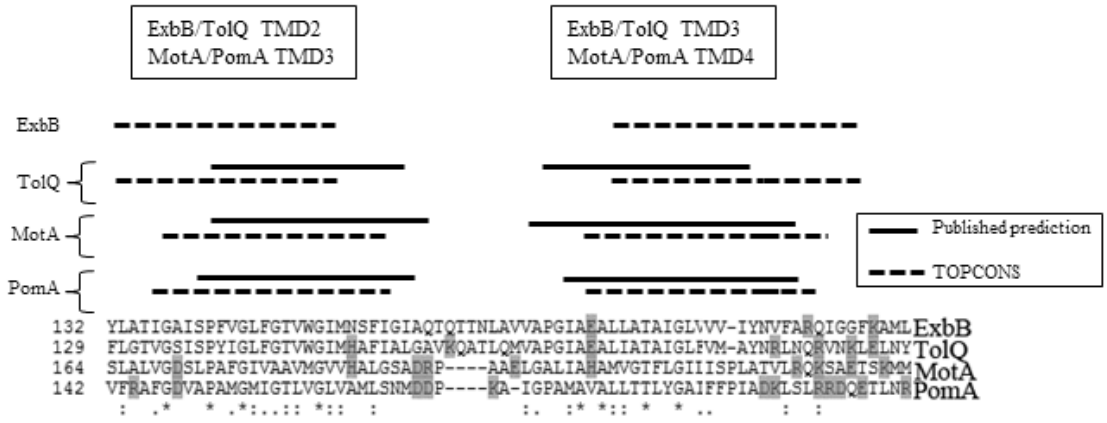


Fig S8. *TMD predictions of ExbB/TolQ TMD2/3 and MotA/PomA TMD3/4.* Sequence aligment of the last two TMD of ExbB,TolQ, MotA and PomA is shown. In sold lines above the alignment are the TMD predictions for ExbB presented in this study and the published TMD predictions for TolQ, MotA and PomA (3-5). The dashed line shows the TOPCONS predicted TMDs. The sequences were aligned using clustal omega multiple sequence alignment program (http://www.ebi.ac.uk/Tools/msa/clustalo/). Charged residues are highlighted in grey. Predicted ExbB TMDs are indicated by a black bar above the sequence. ExbB (YP_491200.1), TolQ (YP_489017.1) and MotA (YP_490152.1) sequences are from *Escherichia coli* K12 strain W3110. The PomA sequence is from *Vibrio alginolyticus* (BAA20284.1). Accession numbers are shown in parenthesis.

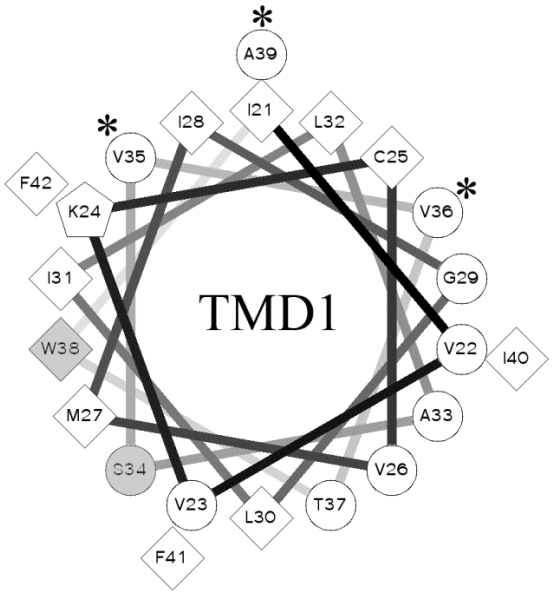


FIG. S9A. *Helical Wheel projection of ExbB TMDs.* ExbB TMD α-helices are projected as helical wheels from periplasm to cytoplasm, using the helical wheel projection program: (http://rzlab.ucr.edu/scripts/wheel/wheel.cgi). Functionally important residues are marked in gray. Hydrophilic residues are presented as circles, hydrophobic as diamonds, negatively and positively charged residues as triangles and pentagons respectively. (A) TMD1. The location of the TonB TMD ExbB suppressors, V35E, V36D and A39E, are indicated by asterisks (1, 2). (B) TMD2. Conserved Gly residues are indicated by asterisks. (C) TMD3.

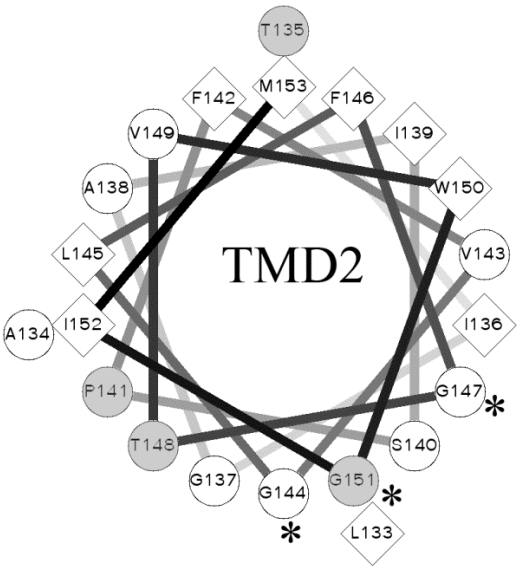


FIG. S9B. *Helical Wheel projection of ExbB TMDs.* ExbB TMD α-helices are projected as helical wheels from periplasm to cytoplasm, using the helical wheel projection program: (http://rzlab.ucr.edu/scripts/wheel/wheel.cgi). Functionally important residues are marked in gray. Hydrophilic residues are presented as circles, hydrophobic as diamonds, negatively and positively charged residues as triangles and pentagons respectively. (A) TMD1. The location of the TonB TMD ExbB suppressors, V35E, V36D and A39E, are indicated by asterisks (1, 2). (B) TMD2. Conserved Gly residues are indicated by asterisks. (C) TMD3.

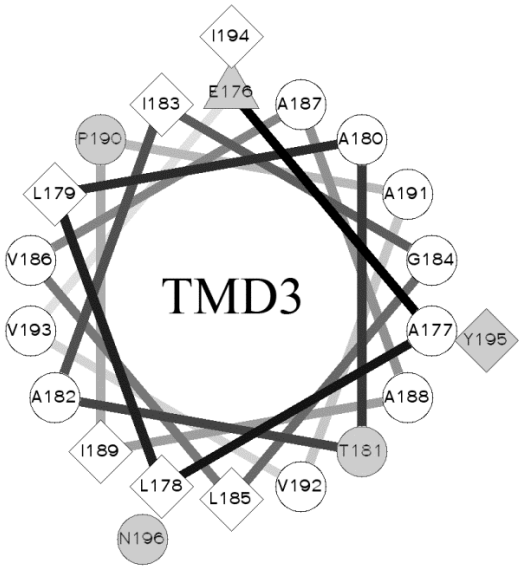


FIG. S9C. *Helical Wheel projection of ExbB TMDs.* ExbB TMD α-helices are projected as helical wheels from periplasm to cytoplasm, using the helical wheel projection program: (http://rzlab.ucr.edu/scripts/wheel/wheel.cgi). Functionally important residues are marked in gray. Hydrophilic residues are presented as circles, hydrophobic as diamonds, negatively and positively charged residues as triangles and pentagons respectively. (A) TMD1. The location of the TonB TMD ExbB suppressors, V35E, V36D and A39E, are indicated by asterisks (1, 2). (B) TMD2. Conserved Gly residues are indicated by asterisks. (C) TMD3.

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