TolA modulates the oligomeric status of YbgF in the bacterial periplasm

Krachler, Anne-Marie; Sharma, Amit; Cauldwell, Anna; Papadakos, Grigorios; Kleanthous, Colin

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The trans-envelope Tol complex of Gram-negative bacteria is recruited to
the septation apparatus during cell division where it is involved in
stabilizing the outer membrane. The last gene in the tol operon, ybgF, is
highly conserved, yet does not seem to be required for Tol function.
We have addressed this anomaly by characterizing YbgF from Escherichia coli
and its interaction with TolA, which, based on previous yeast two-hybrid
data, is the only known physical link between YbgF and the Tol system.
We show that the stable YbgF trimer undergoes a marked change in
oligomeric state on binding TolA, forming a one-to-one complex with the
Tol protein. Through a combination of pull-down assays, deletion analysis,
and isothermal titration calorimetry, we map the TolA–YbgF interface to
the C-terminal tetratricopeptide repeat domain of YbgF and 31 residues at
the C-terminal end of TolA domain II (TolA280–313). We show that TolB,
which binds TolA domain III close to the YbgF binding site, has no impact
on the YbgF–TolA association. We also report the crystal structures of the
two component domains of YbgF, the N-terminal coiled coil from E. coli
YbgF, which forms a stable trimer and controls the oligomeric status of
YbgF, and the monomeric tetratricopeptide repeat domain from Xantho-
monas campestris YbgF, which is also able to trimerize. Although the coiled
coil is not directly involved in TolA binding, we demonstrate that the
regular hydrophilic patterning of its otherwise hydrophobic core is a
prerequisite for the TolA-induced oligomeric-state transition of YbgF. We
postulate that rather than YbgF affecting Tol function, it is the change in
YbgF oligomeric status (with an accompanying change in its function) that
likely explains the necessity for tight co-regulation of the ybgF and tol genes
in Gram-negative bacteria.

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Introduction

The Tol–Pal system is a periplasmic protein complex transiently spanning the cell wall of Gram-negative bacteria in a proton motive force-dependent manner that is required for maintaining outer membrane (OM) integrity and correct septation during the late stages of cell division. Consequently, deletion of tol genes or disruption of interactions between Tol proteins leads to loss of OM integrity, which is accompanied by increased susceptibility towards antibiotics, detergents, and killing of pathogenic strains by the host complement system, as well as aberrant cell division and concomitant cell chaining. In addition, the Tol assembly is exploited by bacteriocins and filamentous phages that recruit either TolA or TolB to promote their uptake into the cytoplasm of host cells. Thus, malfunctioning of the Tol system renders cells tolerant to bacteriocins and phage infection.

To ensure co-regulation of the Tol complex components, the proteins are encoded in an operon consisting of seven genes, ybgC, tolQ, tolR, tolA, tolB, pal, and ybgF. The five core components form two subcomplexes, an inner-membrane-bound TolQRA complex and an OM-anchored TolB–Pal complex. TolA, which is thought to span the periplasm, transiently interacts with TolB, and this interaction is mutually exclusive of the TolB interaction with Pal at the OM. Hence, TolB is thought to shuttle between an active (TolA-bound) and an inactive (Pal-bound) state. To date, no evidence for a functional role of ybgC or ybgF has been found. Neither YbgC nor YbgF (both of which are soluble proteins) is required for Tol functionality since deletion strains do not display the characteristic tol phenotype of OM perturbation, leakiness, or colicin tolerance. YbgC, which is the least conserved of the Tol proteins and is expressed in the periplasm, is only present in the subgroup of intracellular parasites such as Chlamydiae (Fig. 1), and possesses thioesterase activity in vitro, but how this is related to the role of the Tol assembly remains to be elucidated.

This leaves the question why these genes are retained within the tol operon. The only evidence of a functional link between YbgF and the Tol system was provided by yeast two-hybrid screening, which identified a putative interaction between YbgF and TolA. However, this link remains to be validated. The aim of the present study was to characterize YbgF alone and in complex with TolA in more detail to shed light on the possible reason for their co-regulation. Here, we verify the interaction between TolA and YbgF in vitro and demonstrate that YbgF is a highly elongated protein that undergoes a dramatic change in oligomeric state on binding TolA. We also identify the interacting regions of the two proteins. We further report the X-ray structures of the two constituent domains of YbgF and describe their roles in TolA complex formation. Our findings suggest that TolA modulates the function of YbgF by controlling its oligomeric state.

Results

Interaction with TolA in vitro disrupts the quaternary structure of YbgF

Based on a yeast two-hybrid study testing pairwise interactions between members of the tol operon, a potential interaction between TolA and YbgF was identified by Walburger et al. We set out to investigate this protein–protein interaction using a variety of biophysical methods. YbgF is a 25-kDa protein that forms a stable trimer in solution, as shown by chemical cross-linking, size-exclusion chromatography–multi-angle laser light scattering (SEC-MALLS), and analytical ultracentrifugation (AUC). Sedimentation velocity experiments have also shown the protein to be an elongated molecule with a frictional ratio of 1.95 ± 0.08 (data not shown). The interaction between YbgF and TolA was first investigated using SEC-MALLS. TolA74–421, corresponding to the two soluble periplasmic domains of TolA, TolAII–III, and excluding its transmembrane domain, eluted as a monomer in SEC-MALLS experiments (theoretical monomer mass, 36.5 kDa; detected mass, 32.8 ± 0.7 kDa; Fig. 2a) while YbgF eluted as a trimer as previously reported. When YbgF and TolA74–421 were mixed at high concentration (~100 μM), they formed a stable complex. Surprisingly, however, the molar mass of the complex corresponded to that of a TolA74–421 / YbgF heterodimer (theoretical mass, 61.9 kDa; detected mass, 57.5 ± 1.2 kDa; Fig. 2a). Thus, these experiments not only verified the proposed interaction between YbgF and TolA in vitro but also demonstrated that binding to TolA disrupts the otherwise stable YbgF homotrimer.

We investigated the possibility that this unusual binding stoichiometry was specific to the Escherichia coli system by analyzing the equivalent complexes from Salmonella enterica serovar Typhimurium (90% and 80% similarity to E. coli YbgF and TolA, respectively) and Xanthomonas campestris (70% and 50% similarity to E. coli YbgF and TolA, respectively), both of which showed the same change in oligomeric status for YbgF on binding TolA (data not shown). That this effect is species-independent is emphasized by the fact that the same changes in stoichiometry occur for non-cognate complexes for YbgF and TolA.
E. coli and S. Typhimurium proteins; Fig. 2b shows the non-cognate complex of S. Typhimurium YbgF forming a 1:1 complex with E. coli TolA\textsubscript{74–421}. Salmonella YbgF trimer: theoretical mass, 79.5 kDa; detected mass, 76.2 ± 1.5 kDa; 1:1 complex of E. coli TolA\textsubscript{74–421} with S. Typhimurium YbgF: theoretical mass, 63.0 kDa; detected mass, 60.6 ± 1.2 kDa. These data show that YbgF not only is a trimer in other Gram-negative bacteria but also forms the same 1:1 heterodimeric complex with the inner-membrane protein TolA.

The YbgF TPR domain binds TolA\textsubscript{280–313}

We have shown previously that YbgF consists of two autonomous domains, an N-terminal coiled-coil domain (NTD) and a C-terminal tetratricopeptide repeat (TPR) domain, both of which have been overexpressed and purified.\textsuperscript{16} His-tagged versions of these domains in conjunction with constructs for domains II (TolA\textsubscript{48–310}), III (TolA\textsubscript{314–421}), and II–III (TolA\textsubscript{74–421}) of TolA were used to establish which domains are involved in forming the complex. Preliminary experiments indicated that the YbgF–TolA interaction is not strong enough to withstand multiple washing steps and thus complexes were first cross-linked with formaldehyde prior to pull-down experiments (Fig. 3). Specific complexes could be detected between YbgF-His or TPR-His and TolAII–III or TolAII, demonstrating that the YbgF TPR domain interacts with TolAII and that the YbgF NTD and TolAIII domains are not required for complex formation.

Delineation of the YbgF binding site on TolAII, a long domain that is thought to span the periplasm, was accomplished by deletion analysis coupled with isothermal titration calorimetry (ITC), from which we also characterized the thermodynamics of the TolA–YbgF protein–protein interaction. First, we compared TolAII–III (TolA\textsubscript{74–421}) binding full-length YbgF (Fig. 4a) with its individual domains (Fig. 4), which indicated that full-length YbgF and the TPR domain alone bind TolA with equal but low affinity (\(K_d = 36 ± 10 \text{ µM for TolA}_{74–421}/YbgF\) and 55.8 ± 11.2 µM for TolA\textsubscript{74–421}/TPR domain), while the NTD did not bind, in agreement with the pull-down experiments. The ITC experiments showed the YbgF–TolA interaction to be weakly exothermic and enthalpically driven (\(\Delta H = -16 ± 5\text{ kcal/mol}\) and having a 1:1 binding stoichiometry, consistent with the stoichiometry observed by SEC-MALLS (Table 1).

To further narrow down the YbgF binding site, we truncated TolA from both its N- and C-termini; the secondary structure of all TolA constructs was analyzed using far-UV CD spectroscopy to ensure that the proteins remained folded (data not shown). His-tags were placed at the C-terminus of TolA for N-terminal truncations or at the N-terminus of TolAII for C-terminal truncations, so that they would be distant from the expected binding site and not interfere with the interaction. A total of 12 TolA truncates were constructed in pET21d or pET16b vectors, purified, and tested for YbgF binding using ITC (Fig. 5a; Table 1). Truncation of TolA either from its N-terminus up to Lys\textsubscript{279} or from its C-terminus up to Gly\textsubscript{313} did not affect the interaction with YbgF (a representative ITC trace for TolA\textsubscript{234–421} is shown in Fig. 5b). TolA\textsubscript{254–421}, TolA\textsubscript{107–323}, and TolA\textsubscript{107–301} showed weakened endothermic binding (\(K_d \sim 60–120 \text{ µM}\), which was entropically driven (\(\Delta S \sim 20–30\text{ cal K}^{-1}\text{ mol}^{-1}\)).
mol\(^{-1}\), e.g., TolA\(^{294-421}\) in Fig. 5c, black squares). The decrease in affinity suggested that these constructs contained a truncated YbgF-binding epitope. Deletion of the entire domain II (TolA\(^{314-421}\)) or in-frame deletion of the C-terminal part of domain II (TolA\(^{48-421,421-310}\)) abolished binding (see Fig. 5c, white squares for trace of TolA\(^{314-421}\)), which confirms that the binding site is restricted to this region. Interestingly, a TolA construct covering the region immediately following the glycine stretch at the end of domain I (TolA\(^{45-421}\)) exhibited different thermodynamics of binding despite containing the entire YbgF binding epitope (the reasons for this remain unclear at present). A comprehensive list of thermodynamic parameters for all tested TolA constructs can be found in Table 1. To conclude, the YbgF binding site of TolA is located between residues 280 and 313 in domain II. This implies that the C-terminal part of the TolA coiled-coil region (residues 280–293) and the entire region C-terminal to the coiled coil (residues 294–313) are required for binding the TPR domain of YbgF.

**TolB/TolA complexation does not influence YbgF binding to TolA**

The complex that TolAIII forms with the periplasmic protein TolB is required for OM stability and colicin uptake.\(^{11,17}\) The relative proximity of the YbgF- and TolB-binding epitopes on TolA (residues 280–313 and \(\sim360–421\), respectively) led us to investigate the possibility of coupling between them. We therefore performed ITC experiments where 2.2 mM YbgF was titrated into a pre-mixed 80 \(\mu M\) equimolar solution of TolB\(^{22-430}\) and TolA\(^{24-421}\) (domains II and III). (It should be noted that TolB exhibits poor solubility beyond 100 \(\mu M\), which limited the concentration range that could be accessed in these experiments.) Given that the \(K\_d\)
for the TolA/TolB complex is ~40 μM, approximately 50% of TolB binding sites on TolA would be occupied at this concentration. Although complete saturation of the TolB/TolA complex could not be achieved, it was sufficient to assess its impact on YbgF binding. We found that the binding affinity of the YbgF–TolA complex was essentially unchanged in the presence of TolB (K_d = 48 and 33 μM in the presence and absence of TolB, respectively; Table 1). We conclude that, under the conditions used, there is no evidence of coupling between the YbgF- and TolB-binding epitopes on TolA.

The N-terminal trimeric coiled-coil domain defines the oligomeric status of YbgF

Cross-linking has previously shown E. coli YbgF NTD to form trimers.16 This was confirmed in the present work using SEC-MALLS, which showed that the NTD is a stable trimer in solution (theoretical monomer mass, 9.5 kDa; detected mass, 27.9 ± 1.8 kDa; Fig. 6a). To gain insight into the mechanism of trimerization, we solved the structure of the NTD. Crystals were obtained for YbgF27–142, and data sets were collected for both native and K2Au(CN2)-derivatized crystals, the latter being used for single-wavelength anomalous dispersion phasing (see Table 2 for data collection and refinement statistics). The native structure was solved to a resolution of 1.8 Å and showed good density for YbgF residues 36–103 (Fig. 6b). The asymmetric unit contained six chains, which were arranged to form 2 three-stranded, parallel coiled-coil bundles. Figure 6c shows the arrangement of helices for one trimer. The structures of Au-derivatized and native YbgF NTD were in good agreement and superimposable with a root-mean-square deviation (rmsd) of 0.3 Å; further analysis was therefore based on the native structure only. All 68 residues resolved in the structure formed a parallel coiled coil based on 10 heptad repeats, starting from Ser35 (position a1). In agreement with previously characterized coiled-coil structures, 70% of the heptad a and d positions are occupied by apolar residues.18 The pattern of apolar residues forming a hydrophobic core that stabilizes the coiled-coil conformation is broken by the presence of Ser35/49, His52, Asn66/80, and Gln91, which means that alternate heptad repeats, starting with the first repeat, contain at least one polar residue. For repeats 1, 3, 5, and 7, water molecules that are clearly visible in the structure hydrate the polar core residues (e.g., Fig. 6c).
6b). Buried polar residues have been shown to be involved in maintenance of specific stoichiometries of coiled coils, with their substitution often leading to loss of a specific oligomerization state.\textsuperscript{18,19} In the case of YbgF, these buried polar residues also aid in the dissociation of YbgF when it binds TolA (see below). A feature distinguishing most trimeric, parallel coiled coils from dimers or higher-order oligomers is the presence of a specific “trigger motif”\textsuperscript{18,19} also present in the coiled-coil domain of YbgF (Fig. 6d and e). The YbgF trigger motif consists of Arg41 (position $g$ in the first heptad repeat), which engages

![Image](https://example.com/image)

**Fig. 4.** YbgF NTD is dispensable for the interaction with TolA. (a) 1.6 mM YbgF-His, (b) 1.3 mM TPR-His (black squares), or 1.5 mM NTD-His (white squares) in 20 mM Tris–HCl, pH 7.5, and 600 mM NaCl was titrated into 75 $\mu$M TolA$^{74-421}$ at 25 °C. See Table 1 for details of thermodynamic parameters.

### Table 1. ITC analysis of TolA binding YbgF

<table>
<thead>
<tr>
<th>Syringe injectant</th>
<th>Protein in cell</th>
<th>$T^\circ$ (°C)</th>
<th>$N^a$</th>
<th>$K_d$ (μM)</th>
<th>$\Delta H$ (kcal mol$^{-1}$)</th>
<th>$\Delta S$ (cal K$^{-1}$ mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YbgF TolA$^{48-421}$-His$^6$</td>
<td>25</td>
<td>0.14±0.03</td>
<td>37±2</td>
<td>−75.5±15.0</td>
<td>−233±51</td>
<td></td>
</tr>
<tr>
<td>YbgF TolA$^{74-421}$-His$^6$</td>
<td>25</td>
<td>0.84±0.06</td>
<td>33±4</td>
<td>−5.0±0.5</td>
<td>3.6±2.3</td>
<td></td>
</tr>
<tr>
<td>YbgF TolA$^{107-421}$-His$^6$</td>
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<td>0.95±0.02</td>
<td>45±1</td>
<td>−9.0±0.2</td>
<td>−10±5</td>
<td></td>
</tr>
<tr>
<td>YbgF TolA$^{121-421}$-His$^6$</td>
<td>25</td>
<td>1.04±0.02</td>
<td>42±4</td>
<td>−13.3±2.5</td>
<td>24±8</td>
<td></td>
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<tr>
<td>YbgF TolA$^{234-421}$-His$^6$</td>
<td>25</td>
<td>0.92±0.05</td>
<td>37±4</td>
<td>−14.2±3.8</td>
<td>−28±12</td>
<td></td>
</tr>
<tr>
<td>YbgF TolA$^{280-421}$-His$^6$</td>
<td>25</td>
<td>0.93±0.04</td>
<td>7±0</td>
<td>−17.4±0.1</td>
<td>−34.9</td>
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<tr>
<td>YbgF TolA$^{294-421}$-His$^6$</td>
<td>25</td>
<td>1$^b$</td>
<td>62±8</td>
<td>3.4±0.8</td>
<td>30±4</td>
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<tr>
<td>YbgF TolA$^{314-421}$-His$^6$</td>
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<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>YbgF His$^4$-TolA$^{107-293}$</td>
<td>25</td>
<td>1$^b$</td>
<td>67±5</td>
<td>2.6±0.8</td>
<td>28±7</td>
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<tr>
<td>YbgF His$^4$-TolA$^{107-313}$</td>
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<td>1.05±0.04</td>
<td>31±2</td>
<td>−9.6±0.5</td>
<td>−12±3</td>
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</tr>
<tr>
<td>YbgF TPR TolA$^{48-421}$-His$^6$</td>
<td>25</td>
<td>0.85±0.12</td>
<td>55.8±11.2</td>
<td>−21.7±11.6</td>
<td>−44.4±29.9</td>
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</tr>
<tr>
<td>YbgF NTD TolA$^{74-421}$-His$^6$</td>
<td>25</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>YbgF S49L H52I TolA$^{74-421}$-His$^6$</td>
<td>25</td>
<td>3.03±0.05</td>
<td>50.0±0.1</td>
<td>−9.8±0.2</td>
<td>−13.6</td>
<td></td>
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<tr>
<td>YbgF TolB</td>
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<tr>
<td>YbgF TolA$^{74-421}$-TolB$^c$</td>
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<td>0.73±0.08</td>
<td>47.8±5.3</td>
<td>−6.7±0.8</td>
<td>−2.6±1.5</td>
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</table>

All titrations were conducted in 150 mM NaCl and 20 mM Tris–HCl, pH 7.5, at 25 °C. NA, no measurable interaction.

* Stoichiometry of binding ($N$), dissociation constant ($K_d$), enthalpy change ($\Delta H$), and entropy change ($\Delta S$) upon binding are presented for each titration pair. The values represent means of experiments performed at least in duplicate±absolute errors.

* Stoichiometry was constrained to 1 to allow fitting.

* Equimolar mixture of TolA and TolB at 80 μM.
in hydrogen bonds and ionic interactions with Thr43 (b') and Glu46 (c', both in the consecutive repeat). Val42 (a') and Leu45 (d') form a well-packed hydrophobic core that is further stabilized by hydrophobic contacts between Leu45 and the apolar part of Glu46 (Fig. 6e). However, the main function

![Diagram](image-url)
Fig. 6. X-ray structure of the trimeric YbgF N-terminal coiled-coil domain from *E. coli* at 1.8 Å. (a) SEC-MALLS analysis shows trimerization of YbgF NTD (detected mass, 27.9 ± 1.8 kDa; calculated mass, 28.5 kDa). (b) Part of the YbgF NTD electron density map showing His52 from strands A, B, and C and a bound water molecule in the core contoured at 1 σ. (c) Side and top views of the trimeric coiled-coil bundle of YbgF NTD (individual strands shown in red, green, and blue, respectively, N-termini at the top). (d) Coiled-coil trigger motif within the YbgF NTD. Ionic interactions between Arg41 N\(^e\)/N\(^g\) and both O\(^e\)\(^1,2\) of Glu46 as well as hydrogen bonds between Arg41 N\(^d\) and Thr43 O\(^1\) of Glu46 O\(^1,2\) stabilize the coiled coil. (e) Val42 and Leu45 from all three strands form a hydrophobic core, which is well shielded by the apolar part of Arg41 and Glu46 side chains.
of this trigger motif is not to stabilize the coiled coil but to determine its stoichiometry. The network of ionic and hydrogen-bonding interactions established between the polar residues within the motif could not be formed in a coiled-coil dimer due to distance restraints. Equally, the formation of quaternary or higher-order bundles would compromise contacts between polar residues or those between hydrophobic core residues and apolar side chains of $g$ and $e'$ residues, or both.\textsuperscript{18} Taken together, these observations underline our earlier finding that the presence of the YbgF NTD restricts the oligomeric state of YbgF to a trimer.\textsuperscript{16}

Crystalline structure of the YbgF C-terminal domain reveals a monomeric TPR

Attempts to crystallize the TPR domain of \textit{E. coli} YbgF failed presumably due to its high lysine content (12\%) and resulting high solubility. Consequently, TPR domains from YbgF homologues

\begin{table}[ht]
\centering
\caption{Data collection and refinement statistics for Au-derivatized and native \textit{E. coli} YbgF NTD}
\begin{tabular}{llll}
\hline
\textbf{Data collection} & NTD (Au derivative) & NTD (native) \\
\hline
Wavelength (Å) & 1.0395 & 0.97 \\
Space group & \textit{P}2,2,2, & \textit{P}2,2,2, \textit{P}2,2,2, \\
Cell dimensions & \textit{a}, \textit{b}, \textit{c} (Å) & 39.7, 41.4, 237.7 & 39.4, 40.9, 237.9 \\
& $\beta$, $\gamma$ (°) & 90.0, 90.0, 90.0 & 90.0, 90.0, 90.0 \\
Resolution (Å) & 50.0–2.48 & 50.0–1.82 \\
$R_{\text{merge}}$\textsuperscript{a,b} & (2.52–2.48) & (1.85–1.82) \\
$I/\sigma(I)$ & 50.1 (23.7) & 37.8 (3.0) \\
Total number of reflections & 367,867 & 682,032 \\
Number of unique reflections & 26,790 & 35,623 \\
Completeness (%)\textsuperscript{a} & 100 (100) & 99.9 (99.6) \\
Redundancy\textsuperscript{a} & 7.8 (7.5) & 6.7 (5.0) \\
\hline
\textbf{Refinement} & & & \\
Resolution (Å)\textsuperscript{a} & 39.61–2.48 & 39.4–1.82 \\
Number of reflections & 19,420 & 33,802 \\
$R_{\text{work}}/R_{\text{free}}$ & 21.2/27.5 & 22.5/27.5 \\
\hline
\textbf{Number of atoms} & & & \\
Protein & Chain A & 539 & 542 \\
& Chain B & 555 & 544 \\
& Chain C & 543 & 543 \\
& Chain D & 548 & 540 \\
& Chain E & 555 & 541 \\
& Chain F & 511 & 514 \\
& Glycerol & 12 & 0 \\
& Acetate & 8 & 4 \\
& Ethylene glycol & 4 & 0 \\
& Dicyanoaurate (I) complex & 5 & 0 \\
Sodium & 1 & 1 \\
Gold (Au) & 2 & 0 \\
Water & 94 & 83 \\
\hline
\textbf{B-factor (Å$^2$)} & & & \\
Chain A & 21.9 & 32.6 \\
Chain B & 19.1 & 30.2 \\
Chain C & 17.6 & 28.5 \\
Chain D & 26.6 & 34.6 \\
Chain E & 21.0 & 31.8 \\
Chain F & 19.4 & 29.7 \\
Water & 20.4 & 31.8 \\
\hline
\textbf{rmsd}\textsuperscript{c} & & & \\
Bonds (Å) & 0.016 & 0.022 \\
Angles (°) & 1.53 & 1.88 \\
\hline
\textbf{Ramachandran plot (%)} & & & \\
Favored region & 99.7 & 100 \\
Allowed region & 0.3 & 0 \\
Disallowed region & 0 & 0 \\
\hline
\textsuperscript{a} Limits and values for the outer resolution bin are given in parentheses. \\
\textsuperscript{b} $R_{\text{merge}} = \sum \sum |I_{ij} - I_{ij}| / \sum \sum I_{ij}$. \\
\textsuperscript{c} rmsd given from ideal values.
\end{tabular}
\end{table}

\begin{table}[ht]
\centering
\caption{Data collection and refinement statistics for \textit{X. campestris} YbgF TPR domain}
\begin{tabular}{llll}
\hline
\textbf{Data collection} & & & \\
Wavelength (Å) & 0.9795 & & \\
Space group & \textit{P}2,1 & & \\
Cell dimensions & $a$, $b$, $c$ (Å) & 86.4, 37.1, 90.6 & \\
& $\beta$, $\gamma$ (°) & 90.0, 117.14, 90.0 & \\
Resolution (Å)\textsuperscript{a} & 40.32–1.57 (1.66–1.57) & & \\
$R_{\text{merge}}$\textsuperscript{a,b} & 0.106 (0.588) & & \\
$I/\sigma(I)$ & 5.6 (1.8) & & \\
Total number of reflections & 261,010 & & \\
Number of unique reflections & 71,985 & & \\
Completeness (%)\textsuperscript{a} & 99.9 (99.9) & & \\
Redundancy\textsuperscript{a} & 3.6 (3.5) & & \\
\hline
\textbf{Refinement} & & & \\
Resolution (Å)\textsuperscript{a} & 18.75–1.57 & & \\
Number of reflections & 68,305 & & \\
$R_{\text{work}}/R_{\text{free}}$ & 19.2/22.2 & & \\
\hline
\textbf{Number of atoms} & & & \\
Protein & Chain A & 983 & \\
& Chain B & 1014 & \\
& Chain C & 992 & \\
& Water & 158 & \\
\hline
\textbf{B-factor (Å$^2$)} & & & \\
Protein & Chain A & 21.1 & \\
& Chain B & 19.0 & \\
& Chain C & 17.27 & \\
& Water & 27.15 & \\
\hline
\textbf{rmsd}\textsuperscript{c} & & & \\
Bonds (Å) & 0.034 & 0.029 \\
Angles (°) & 2.56 & 2.86 \\
\hline
\textbf{Ramachandran plot (%)} & & & \\
Favored region & 98.9 & 100 \\
Allowed region & 1.1 & 0 \\
Disallowed region & 0 & 0 \\
\hline
\textsuperscript{a} Limits and values for the outer resolution bin are given in parentheses. \\
\textsuperscript{b} $R_{\text{merge}} = \sum \sum |I_{ij} - I_{ij}| / \sum \sum I_{ij}$. \\
\textsuperscript{c} rmsd given from ideal values.
\end{tabular}
\end{table}
Fig. 7. X-ray structure of the YbgF C-terminal TPR domain from *X. campestris* at 1.7 Å. (a) Details of the YbgF TPR electron density map depicting Leu29, Tyr30, and Pro31 contoured at 1σ. (b) The chain contains three consecutive helix–turn–helix TPR motifs colored red, blue, and green, respectively (helices corresponding to the same motif are marked α and β) and a C-terminal capping helix (orange). The *X. campestris* YbgF TPR domain contains three residue insertions between TPR1/2 and TPR2/3, leading to extension of the loop regions and α2/α3 helices (gray). (c) The TPR fold of YbgF TPR (blue) is similar to that of CTPR3 (green, 1na0; rmsd = 1.28 Å) and hSGT1 (red, 2vyi; rmsd = 2.58 Å). (d) AUC sedimentation velocity experiment of *X. campestris* YbgF TPR domain. At a concentration of 100 μM, ~20% of the material is trimeric with the rest being monomeric (sedimentation coefficients = 1.12 S and 1.57 S for monomer and trimer, respectively). Chemical cross-linking with dithiobis(succinimidyl propionate) also indicates the presence of oligomeric species (inset).
featuring lower lysine content were purified and screened. Needle-shaped crystals diffracting to 1.57 Å were obtained using the YbgF TPR domain from X. campestris (4% lysine content), and its structure was solved by molecular replacement using CTTPR3 as template (see Table 3 for data collection and refinement statistics). The asymmetric unit contained three molecules, all with well-defined density (Fig. 7a). The three TPR motifs detected at sequence level are represented by three consecutive helix–turn–helix motifs in the structure while the last 14 residues at the C-terminus form a capping helix (Fig. 7b). The X. campestris YbgF TPR sequence contains three-residue insertions between TPR motifs 1/2 and 2/3, respectively. This leads to an extension of the loop regions between these motifs and the helices z2 and z3 by a quarter turn and half a turn, respectively. The overall fold, however, is well conserved between the YbgF TPR and previously characterized TPR domains (Fig. 7c).

The TPR subunits did not share any significant interface with adjacent molecules in the asymmetric unit or with symmetry-related molecules; using the PISA server, the largest interface was determined to cover 550 Å², which is much less than normally observed for a stable protein–protein interaction. Hence, the structure reflects the architecture of a YbgF TPR monomer. This is consistent with AUC sedimentation velocity experiments with X. campestris TPR domain, which showed that only ~20% of the protein is present as a trimer, the majority being monomeric (Fig. 7d). This is in contrast to the previously described CTTPR3Y3 protein, where a consensus monomeric CTPR protein was engineered to oligomerize by incorporating tyrosine–for-aspartate residues in loops identified as key for oligomerization of the YbgF TPR. TPR oligomerization requires displacement of the capping helix, which ordinarily forms hydrophobic contacts with the β helix, thereby blocking the surface required for intersubunit associations. The greater hydrophilicity of the consensus CTTPR3Y capping helix favors the oligomeric over the monomeric state. In contrast, the greater hydrophobicity of the YbgF TPR capping helix enables good contact with the β helix and thus favors the monomeric state, as seen in the crystal structure presented here.

**Patterning of hydrophilic versus hydrophobic coiled-coil core residues enables the oligomeric-state transition of YbgF**

As described above, the N-terminal coiled-coil domain of YbgF is not required for TolA binding but the trimer must nevertheless dissociate to form the heterodimeric complex with TolA. We hypothesized that the regular hydrophilic patterning of its core residues primes this part of YbgF for the oligomeric-state transition the protein undergoes on binding TolA. To test this hypothesis, we designed a mutant featuring a stabilized coiled-coil region. Two consecutive core positions occupied by hydrophilic residues (Ser49 and His52, Fig. 8a) were mutated to Leu and Ile, respectively, which represent the most frequently observed residues at these heptad positions. In other coiled-coil structures, these residues normally form a well-packed hydrophobic core (as modeled in Fig. 8b) that stabilizes the coiled-coil fold significantly. The double mutant (YbgF Ser49Leu His52Ile) was purified, and its secondary structure and thermal stability were analyzed using CD spectroscopy. As predicted, the double mutation did not alter the overall fold of YbgF, but the thermal stability of the resulting coiled coil was significantly increased; whereas wild-type YbgF shows a single thermal denaturation transition (Tm) at 70 ± 1 °C, the double mutant unfolded in two transitions, with Tm values at 67 ± 1 and 79 ± 1 °C, respectively. The influence of the stabilized coiled coil on TolA binding was analyzed subsequently by ITC and SEC-MALLS.

ITC experiments indicated that increasing the stability of the coiled coil did not affect TolA binding significantly—the affinity of the interaction was only slightly lower than that measured for wild-type YbgF (Kd of 50 μM versus 36 μM, Fig. 8c). The stoichiometry of binding, however, changed from 1:1 to 1:3 for TolA:YbgF as demonstrated by both ITC and SEC-MALLS experiments (Fig. 8d). SEC-MALLS showed that the YbgF Ser49Leu His52Ile mutant was trimeric (theoretical mass of the trimer, 79.5 kDa; detected mass, 81 ± 1.8 kDa) and TolA74–313 was monomeric (theoretical mass, 22.8 kDa; detected mass, 23.6 ± 1.4 kDa). The complex, however, showed a molecular mass of 98.8 ± 6.6 kDa, corresponding to a YbgF Ser49Leu His52Ile trimer bound to a TolA monomer (theoretical mass, 102.3 kDa). Hence, stoichiometric TolA binding, but not binding affinity per se, relies on disruption of the YbgF coiled coil, which, in turn, is dependent on the presence of destabilizing core residues at key positions within the NTD.

**Discussion**

Characterization of YbgF has been neglected despite two decades of research on the Tol complex due to the lack of a tol phenotype upon deletion of its gene. Its strong conservation in the tol operon of most Gram-negative organisms, however, argues for the importance of co-regulation of YbgF with the other members of the Tol complex. The present report details the structures of the individual domains of YbgF, explains how it interacts with the inner-membrane protein TolA, and points to a possible explanation for its retention within the Tol system.
YbgF is an elongated trimer composed of an N-terminal trimeric coiled coil and a monomeric C-terminal TPR domain. The two domains are separated by a flexible linker that is readily cleaved by proteases; in limited proteolysis experiments using papain, for example, the intervening sequence of ~30 amino acids is degraded to leave the individual domains (data not shown). Based on the two structures reported here, a model of intact YbgF is shown in Fig. 9a. Although the crystal structure of the TPR domain of *X. campestris* is monomeric, the TPRs of YbgFs clearly have a propensity to trimerize. Moreover, the NTD juxtaposes the three TPR domains in close proximity to each other, which would further promote their trimerization. Hence, the TPR domains shown in Fig. 8.

**Fig. 8.** Stabilization of the YbgF coiled-coil domain blocks TolA-mediated disruption of the YbgF trimer. (a) The third heptad repeat of the YbgF coiled-coil domain contains two consecutive hydrophilic core residues, Ser49 and His52, involved in a water-mediated hydrogen-bonding network. (b) Model showing replacement of these residues by Leu and Ile, respectively. (c) ITC analysis of TolA–YbgF binding. Wild-type YbgF (black squares) and S49L H52I double mutant (red squares) bind TolA<sub>74–421</sub>-His<sub>6</sub> with comparable affinities (K<sub>d</sub> = 36 μM versus 50 μM) but different stoichiometries (1:1 versus 1:3 TolA:YbgF). (d) SEC-MALLS analysis of YbgF S49L H52I (green, detected mass, 81 ± 1.8 kDa; calculated mass, 79.5 kDa), TolA<sub>74–313</sub> (red, detected mass, 23.6 ± 1.4 kDa; calculated mass, 25.4 kDa), and TolA–YbgF complex (blue, detected mass, 98.8 ± 6.6 kDa; calculated mass, 104.9 kDa).
TolA binding disrupts trimeric YbgF to form a heterodimer with weak affinity ($K_d \approx 40 \mu M$). Our attempts to estimate the YbgF copy number in cell lysates or periplasmic extracts were hampered by the poor specificity of our anti-YbgF antibody. Previous work, however, has estimated that the copy numbers of other Tol components range from $\sim 800$ copies for TolA to $10,000$–$30,000$ copies in the case of TolB and Pal,\textsuperscript{25–27} which translates to protein concentrations in the range of $20$–$500 \mu M$. Since ybgF is transcriptionally co-regulated with tolB and pal, and hence may have similar protein levels, this is indirect evidence that significant amounts of the TolA–YbgF complex would be present \textit{in vivo}. This, however, has yet to be demonstrated.

The complex, which likely forms close to the OM in the periplasm, involves the TPR domain of YbgF and a short stretch of amino acid sequence at the C-terminal end of TolAII close to the junction with domain III. While dissociation of the YbgF trimer is clearly a consequence of TolAII binding, our results suggest that this plays little if any role in the thermodynamics of binding. Two pieces of evidence support this view. First, the thermodynamic signature of TolAII binding either intact YbgF or the isolated TPR domain is basically the same by ITC (Table 1). Second, the thermodynamics of the TolAII–YbgF interaction are unchanged even if dissociation of the NTD is blocked through stabilizing mutations in the hydrophobic core of the coiled coil (although the actual YbgF binding species are different between the wild-type and the S49L/H52I YbgF). In these experiments, we observe that a heterodimeric complex is no longer able to form. Instead, a single TolAII binds a single YbgF trimer, demonstrating that dissociation of the complex is no longer possible. These latter experiments point to long-range steric/electrostatic clashes with the Tol protein as the most likely mechanism by which dissociation of the WT-YbgF coiled coil occurs on binding of its TPR to TolAII. These long-range effects are sufficient to drive dissociation of an NTD coiled coil destabilized by hydrophilic core residues but insufficient to cause dissociation of a coiled coil with a more stable and better packed hydrophobic core.

In summary, our data highlight how the affinity of the YbgF–TolA complex has been finely tuned to enable a stable heterodimeric complex to form at the expense of the trimer structure of YbgF. We hypothesize that the strong conservation of ybgF in the context of the tol operon is not due to a role in Tol activity but rather to guarantee tight regulation of the population of heterodimeric and trimeric YbgF. Since the role of YbgF in the cell has yet to be identified, we can only speculate as to the functional consequences of this elaborate regulatory mechanism. An involvement in Tol activity is ruled out based on our finding that YbgF does not influence the interaction between TolA and TolB, which has been shown to be crucial for Tol function, as well as the previously reported lack of a tol phenotype upon ybgF deletion.\textsuperscript{11,12} Deletion of pal, which acts as an off-switch for the Tol system, also shows a distinct phenotype from other tol deletions since it is not colicin tolerant.\textsuperscript{11} We excluded the possibility that YbgF and Pal have similar functions for two reasons: First, if YbgF was acting as an off-switch, it would be expected to be functionally redundant with Pal, yet a ybgF deletion does not show the same OM instability phenotype as a pal deletion strain. Second, we have observed that overexpression of YbgF does not alter the cell’s sensitivity to colicins (data not shown). This also rules out YbgF as an off-switch for the Tol system at least from the standpoint of Tol-mediated colicin translocation.

Two possible models in which either monomeric YbgF bound to TolA is the functional unit or trimeric YbgF is the functional form can be envisaged (Fig. 9). Depending on which form is active, YbgF is either limited to or excluded from areas containing the Tol complex. Since the Tol complex is recruited to the mid-cell during the late stages of cell division where it is necessary for septation and/or tethering of the cell wall layers during invagination, it is conceivable that YbgF’s activity is also tied in with this process.

Fig. 9a have been modeled as a trimer, the structure based on the CTPR3Y3 protein, a TPR domain induced to oligomerize by the introduction of YbgF residues that favor intersubunit self association.\textsuperscript{16}
Materials and Methods

Cloning and mutagenesis of plasmids

The cloning and purification of YbgF and its individual domains were described elsewhere.\textsuperscript{12,16} Constructs for the expression of YbgF, YbgF NTD, and YbgF TPR domain and TolA containing a C-terminal His\textsubscript{6}-tag as well as tagless TolA were generated by amplifying the corresponding sequences from MG1655 genomic DNA and cloning into the NcoI-XhoI sites of plasmid pET16b. YbgF-His S49L H52I was generated by two sequential site-directed mutagenesis steps using pET21d containing YbgF-His as initial template.

Protein expression and purification

All constructs were purified from \textit{E. coli} BL21 DE3 grown to mid-log phase at 37 °C after induction with 1 mM IPTG for 4 h. Tagless YbgF, TolA constructs, and TolB were purified as described previously.\textsuperscript{11,16} For the purification of His-tagged proteins, cells were harvested by centrifugation (12 min, 5470 \(g\)), and MgCl\(_2\) were added to concentrations of 1 mM, 2 mg/ml, and 5 mM, respectively, and the suspension was sonicated on ice. After addition of 33 \(\mu\)g/ml DNase I and incubation on ice for 30 min, the solution was centrifuged (30 min, 8000 \(g\), 4 °C) and the supernatant was loaded onto a 10-ml column containing HisBind resin charged with 50 mM NiSO\(_4\) and equilibrated with binding buffer. Bound proteins were eluted using a linear gradient of 5–500 mM imidazole over 10 column volumes and dialyzed against gel-filtration buffer (50 mM Tris–HCl, pH 7.5, and 250 mM NaCl). Dialyzed fractions were further purified by gel-filtration on a Superdex S75 HL 26/60 column (GE Healthcare) at a flow rate of 0.5 ml/min. A mass spectrometry analysis was used to verify the masses and purity of all proteins.

Cloning and data collection for the YbgF N-terminal domain

Crystals used for data collection were grown in 0.1 M sodium acetate, pH 5.0, 8% (w/v) polyethylene glycol 4000, and 7.5% dioxane at a protein concentration of 10 mg/ml using the hanging drop method and a drop size of 2 \(\mu\)l. Crystals were obtained at 20 °C after 1 day. For phasing, crystals were derivatized using heavy metal soaking. K\(_2\)Au(CN\(_2\))-derivatized crystal to find the peak wavelength for the LIII absorption edge of Au. The raw data were then processed with the program CHOOCH to obtain the anomalous scattering factors, \(f\) and \(f^*\). The output was then used to tune the X-ray beam to the wavelength corresponding to the peak of the absorption edge (\(\lambda = 1.0395\) Å). Subsequently, data were collected at the inflection point and remote high-energy wavelengths. The highly redundant data set collected at the peak wavelength was processed using HKL2000. The space group after indexing was \(P2_1\bar{2}1_2\), with cell dimensions of \(a=b=40\) Å, \(c=240\) Å. The cell dimensions were similar to those obtained for the native data set. Merged data obtained after HKL2000 were processed using the SHELEXC/D/E pipeline to detect anomalous signal. A good anomalous signal was obtained, and the coordinates of one Au atom with full occupancy could be found. Further, ARP/wARP was used to calculate the phases for the rest of the structure. The model was refined using REFMAC5, interspersed with visualization and manual model building in Coot.

Crystalization and data collection for the YbgF TPR domain

Crystals for the YbgF TPR domain from \textit{X. campestris} were grown in 10 mM ZnCl\(_2\), 0.1 M Heps, pH 7.0, 20% (w/v) polyethylene glycol 6000, and 10% (v/v) glycerol at a protein concentration of 10 mg/ml using the hanging drop method and a drop size of 2 \(\mu\)l. To set up drops, we mixed protein and reservoir solution 1:1, and crystals were obtained at 20 °C after 2 days. Data were collected at the Diamond Light Source, beamline I02, and initial data processing was carried out using imosfilm. Reflections were indexed in space group \(P2_12_1\) with unit cell dimensions of 86.4 Å, 37.12 Å, and 90.6 Å. After integration with imosfilm, data were scaled using SCALA. Cell content analysis showed the presence of 3 molecules/asymmetric unit. The structure was solved by molecular replacement using the program BALBES from the CCP4i suite and the structure of CTPR3 (1na0)\textsuperscript{28} as search model. The initial solution obtained after molecular replacement was subjected to model building using ARP/wARP from the CCP4i suite and several cycles of rigid-body and restrained refinement.

Size-exclusion chromatography–multi-angle laser light scattering

Proteins and protein complexes were prepared at a concentration of 100 \(\mu\)M in 20 mM Tris–HCl, pH 7.5, and 150 mM NaCl; pre-incubated at 22 °C for at least 30 min; and centrifuged at 12,000 \(g\) for 2 min prior to analysis. Samples were separated on a Superdex \(50\) \(16/100\) GL column (GE Healthcare) at a flow rate of 0.5 ml/min. A Wyatt Dawn Heleos-II MALLS instrument and an Optilab Rex Refractive index detector were used for detection at 25 °C. For data collection and analysis, the Astra software (Wyatt) was used.

Analytical ultracentrifugation

Sedimentation velocity experiments were carried out in a Beckman Optima XL/1 analytical ultracentrifuge using 12-
mm-path-length cells in an AN-60Ti rotor. TPR domain from *X. campestris* YbgF was prepared at a concentration of 100 μM in 20 mM Tris–HCl, pH 7.5, and 150 mM NaCl. The experiment was run at 20 °C and at a speed of 45,000 rpm, measuring absorbance at 285 nm every minute over the course of the experiment. Data were analyzed using SEDFIT software. Buffer viscosity and partial specific volume of the TPR domain, which were required as input for SEDFIT, were calculated using the program SEDNTERP.29

**Isothermal titration calorimetry**

Solutions of 1–2.2 mM YbgF, TPR domain, or NTD were titrated into 50–100 μM of TolA (TolB was applied at 80 μM where present) using a VP-ITC and an iTC200 isothermal titration calorimeter (Microcal). All the titrations were conducted in 20 mM Tris–HCl, pH 7.5, and 150 mM NaCl at 25 °C. Samples were injected in 28 increments of 10 μl with a spacing of 300 s between consecutive injections and at a stirring speed of 309 rpm in the VP-ITC. A set of 19 injections at 2 μl or a set of 39 injections at 1 μl with a spacing of 150 s and a stirring speed of 1000 rpm was applied in the case of the iTC200. All traces shown were obtained by subtracting heats of dilution, which were determined by titrating identical solutions of YbgF, TPR domain, or NTD as used for the experiment into buffer and fitting of the experimental data to a single-site model using the Origin 8 software.

**Accession numbers**

Final coordinates for the *E. coli* YbgF native and derivatized NTD were deposited in the Protein Data Bank (accession numbers 2xdj and 2wz7, respectively). Coordinates for the *X. campestris* YbgF TPR domain were deposited in the Protein Data Bank (accession number 2xev).

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**References**


