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AcrA suppressor alterations reverse the drug hypersensitivity phenotype of a TolC mutant by inducing TolC aperture opening

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Summary

In *Escherichia coli*, the TolC–AcrAB complex forms a major antibiotic efflux system with broad substrate specificity. During the complex assembly, the periplasmic helices and bottom turns of TolC are thought to interact with a hairpin helix of AcrA and hairpin loops of AcrB respectively. In the present study we show that a four-residue substitution in TolC's turn 1, which connects outer helices 3 and 4 proximal to TolC's periplasmic aperture, confers antibiotic hypersensitivity without affecting TolC-mediated phage or colicin infection. However, despite the null-like drug sensitivity phenotype, chemical cross-linking analysis revealed no apparent defects in the ability of the mutant TolC protein to physically interact with AcrA and AcrB. A role for TolC turn 1 residues in the functional assembly of the tripartite efflux pump complex was uncovered through isolating suppressor mutations of the mutant TolC protein that mapped within *acrA* and by utilizing a labile AcrA protein. The data showed that AcrA-mediated suppression of antibiotic sensitivity was achieved by dilating the TolC aperture/channel in an AcrB-dependent manner. The results underscore the importance of the periplasmic turn 1 of TolC in the functional assembly of the tripartite efflux complex and AcrA in transitioning TolC from its closed to open state.

Introduction

The outer membrane protein TolC, together with the periplasmic adapter protein AcrA and the inner membrane pump protein AcrB, forms the major antibiotic efflux system in *Escherichia coli* (Fralick, 1996). High resolution structures of all three proteins have been solved (Koronakis *et al.*, 2000; Murakami *et al.*, 2002; Yu *et al.*, 2003; Mikolosko *et al.*, 2006; Murakami *et al.*, 2006; Seeger *et al.*, 2006; Das *et al.*, 2007; Sennhauser *et al.*, 2007; Bavro *et al.*, 2008; Drew *et al.*, 2008), and several models have been proposed that envisage the mechanism by which drugs are expelled by the TolC–AcrAB complex (Koronakis *et al.*, 2000; Andersen *et al.*, 2002; Koronakis, 2003; Eswaran *et al.*, 2004; Fernández-recio *et al.*, 2004; Tamura *et al.*, 2005; Lobedanz *et al.*, 2007; Bavro *et al.*, 2008; Misra and Bavro, 2009; Pos, 2009). These studies have suggested that the transitioning of TolC's periplasmic aperture from its resting closed state to a transport-active open state is a key event during drug extrusion. For this to occur, TolC's periplasmic helices guarding the tunnel entrance are proposed to undergo considerable realignments in order to open the aperture in an iris-like fashion (Koronakis *et al.*, 2000). Experiments have identified crucial hydrogen bonds and salt

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bridges in the proximity of TolC's aperture that are important for keeping the aperture in a closed state, presumably by constraining the periplasmic helices (Andersen *et al.*, 2002). Independent verification of the involvement of one of the key residues, R367, in aperture closing came from the isolation of a TolC mutant in which a R367H substitution kept TolC's aperture in a constitutively open state and caused a hypersensitivity phenotype (Augustus *et al.*, 2004). Recent structural resolution of a TolC mutant in its partial open-state confirmed *in vivo* data and earlier predictions and showed realignment of TolC's inner helices relative to outer helices in transition to an open aperture (Bavro *et al.*, 2008). While structural and mutational data have been helpful in identifying key TolC residues involved in controlling aperture diameter, precisely how TolC's outer and inner helices undergo realignments to facilitate aperture widening and what triggers this transition remain poorly understood.

Physical interactions between any two components of the TolC–AcrAB complex in the absence of the third component have been reported (Husain *et al.*, 2004; Tikhonova and Zgurskaya, 2004; Touze *et al.*, 2004; Tamura *et al.*, 2005). However, it is reasonable to expect that TolC's aperture opening under normal circumstances is accomplished only in the presence of the two other components and when their interacting surfaces are properly juxtaposed (Bavro *et al.*, 2008; Misra and Bavro, 2009). Consistent with this notion that proper interfacing between the three components of the efflux complex is required, expression of efflux components from heterologous bacterial sources does not often lead to the functional assembly of an efflux-competent complex (Bokma *et al.*, 2006; Stegmeier *et al.*, 2006; Vediyappan *et al.*, 2006; Krishnamoorthy *et al.*, 2008) despite the fact that the heterologous components can physically interact, as shown by chemical cross-linking analysis (Bokma *et al.*, 2006; Stegmeier *et al.*, 2006; Vediyappan *et al.*, 2006). However, gain-of-function compensatory mutations can be obtained that make heterologous complexes partially functional, presumably by establishing quasi-normal interfacing between heterologous partners (Bokma *et al.*, 2006; Vediyappan *et al.*, 2006; Krishnamoorthy *et al.*, 2008). On the other hand, functional interactions among native partners can be disrupted by loss-of-function missense mutations in one component and restored by compensatory alterations in the other interacting component (Gerken and Misra, 2004). These compensatory alterations often identify residues or regions of proteins that directly or indirectly influence interfacing between the interacting partners.

Since proper interfacing between TolC, AcrA and AcrB is required for the assembly of an active efflux complex, it is important to identify residues critical for the complex assembly. Based on systematic cysteine-mediated cross-linking analysis, it has been proposed that the exposed residues along TolC's α-helices H3 and H7 located below the equatorial domain most likely interact with residues of the α-helical hairpin domain of AcrA (Lobedanz *et al.*, 2007). Further support for this notion came from mapping of the gain-of-function suppressor alteration of either an efflux-defective TolC mutant (Gerken and Misra, 2004; this study) or a non-functional heterologous TolC-AcrA-MexB pump (Krishnamoorthy *et al.*, 2008) in the α-helical region of AcrA. Conversely, suppressor alterations of a non-functional heterologous VceC-AcrAB complex were found in the α-helical domain of VceC (Vediyappan *et al.*, 2006). Similarly, modelling and experimental data have indicated that the tip of AcrB's hairpin domain docks with the bottom of TolC's α-helical domain (Fernández-recio *et al.*, 2004; Tamura *et al.*, 2005; Bavro *et al.*, 2008). More specifically, based on analyses of *in vivo* cross-linking between non-native cysteine residues placed at the two proposed interacting regions of AcrB and TolC, it has been concluded that residues of the AcrB hairpin loop are in close proximity to residues of the TolC turn regions between the inner helices H7 and H8 and the outer helices H3 and H4 (Tamura *et al.*, 2005).

In this study, we investigated the role of four residues located in the turn region between the outer helices H3 and H4 of TolC in efflux function. Simultaneous alterations of four turn residues from $_{147}$GLVA$_{150}$ to AGSG obliterated efflux activity without affecting cross-

linkable interactions between TolC and AcrA or TolC and AcrB. Characterization of antibiotic resistant revertants revealed compensatory changes in AcrA, which induced widening of the TolC aperture. This indicated that the inability of the mutant TolC protein to properly dock with AcrB prevented it from undergoing an AcrAB-mediated transition from the closed to open state. However, compensatory alterations in AcrA could overcome this block by inducing opening of the TolC aperture in an AcrB-dependent manner.

Results

Mutational analysis of the periplasmic turn 1 residues of TolC

Based on the AcrB structure, Murakami *et al.* (2002) proposed that hairpins protruding from the top end of AcrB intermesh with the α-helix-turn-α-helix structure extending from the bottom of TolC to form a contiguous drug expulsion passage crossing the two membranes. Experimental evidence for this proposal came from demonstrating that certain cysteine-modified residues of the TolC turns could form disulphide bonds with the corresponding cysteine-modified residues of the AcrB loops *in vivo* (Tamura *et al.*, 2005). In this study we investigated what roles in pump assembly and drug efflux the TolC turn 1 residues $_{147}$GLVA$_{150}$, present between the static H3 and H4 helices play, through two different genetic approaches involving alanine scanning and localized frameshift mutagenesis (Fig. 1A).

Of three alanine mutants, $_{147}$ALVA$_{150}$, $_{147}$GAAA$_{150}$ and $_{147}$AAAA$_{150}$, only the $_{147}$AAAA$_{150}$ variant displayed a modest drug sensitivity phenotype (Fig. 1A and B). Alanine mutagenesis also revealed that G147 is important for TolC folding/stability, but not for antibiotic efflux (Fig. 1B). This is consistent with our previous observation (Vakharia *et al.*, 2001), showing that a G147D substitution lowers TolC levels without affecting its efflux-associated activity (Fig. 1C). The modest drug sensitivity of the $_{147}$AAAA$_{150}$ variant (Fig. 1B) is unlikely due to a disruption of the side-chain-mediated interactions, as the wild type TolC turn 1 residues either lack a side-chain (G) or have neutral side-chains (LVA). Interestingly, the $_{147}$GAAA$_{150}$ variant, which had 20% higher TolC levels than the $_{147}$AAAA$_{150}$ variant, displayed negligible drug sensitivity (Fig. 1B). On the other hand, the $_{147}$ALVA$_{150}$ and $_{147}$AAAA$_{150}$ have identical protein levels, yet the former displays no novobiocin sensitivity, while the latter is moderately sensitive (Fig. 1B). Thus the significance of G147A in efflux function becomes apparent only when the $_{148}$LV$_{149}$ residues are simultaneously substituted with alanine. Together, these data suggest that a reduced overall hydrophobicity of the side-chains coupled with lower TolC levels causes the modest hypersensitivity phenotype of the $_{147}$AAAA$_{150}$ variant.

In the second mutagenesis approach, we introduced a −2 frameshift mutation by deleting the last two nucleotides of the G147 codon, thereby disrupting the TolC reading frame downstream of the V146 codon (Fig. 1A). This mutant displayed a null phenotype owing to the premature appearance of a stop codon 25 codons downstream from the site of the −2 frameshift mutation. We then either restored the reading frame past the codon 150 to create a $_{147}$AGSG$_{150}$ variant or sought spontaneous revertants of the −2 frameshift mutant to gauge the ‘structural flexibility’ of the turn 1 residues and those present in the vicinity of turn 1, in the drug efflux function. However, repeated efforts yielded revertants in which the original reading frame was restored, indicating the extremely narrow sequence flexibility of the affected region to synthesize an efflux-proficient TolC protein.

We further investigated the $_{147}$AGSG$_{150}$ variant, which had half the TolC level relative to wild type but displayed a null-like hypersensitivity drug phenotype (Fig. 1C). Because the importance of G147 in TolC’s folding/stability has already been demonstrated (see above), we therefore changed A147 of $_{147}$AGSG$_{150}$ back to the wild type G residue to see if elevated TolC levels can reduce or abolish the drug sensitivity phenotype of this mutant. Although the TolC

levels in the resulting $_{147}$GGSG$_{150}$ variant rose nominally, this was accompanied by a significant reduction in the drug sensitivity phenotype (Fig. 1C). A second change of G148 back to the wild type L residue ($_{147}$GLSG$_{150}$) restored almost wild type TolC levels and the mutant no longer displayed drug sensitivity (Fig. 1C). It is worth noting that the $_{147}$DLVA$_{150}$ variant, which we originally isolated among TolC mutants defective in hemolysin secretion (Vakharia *et al.*, 2001), produces almost same TolC levels as the $_{147}$AGSG$_{150}$ mutant, and yet the $_{147}$DLVA$_{150}$ variant displays no drug sensitivity while the $_{147}$AGSG$_{150}$ mutant is hypersensitive. Thus reduced TolC levels alone do not contribute to the drug hypersensitivity phenotype of the $_{147}$AGSG$_{150}$ mutant. The introduction of a small polar serine residue at position 148 or 149 in the wild type turn 1 sequence neither affected the protein level nor produced a drug sensitivity phenotype (Fig. 1C). Together, these results indicated that the $_{147}$GL$_{148}$ residues of turn 1 are the most critical for protein's folding/stability and its efflux-associated activity.

Detailed characterization of the TolC turn 1 mutant

We focused on the $_{147}$AGSG$_{150}$ variant because its strong, null-like hypersensitivity phenotype is best suited for dissecting the cause of its defects and shed some light on the role of the TolC turn 1 region in efflux function. We examined various TolC activities through employing seven different agents that require TolC for either entry into (TLS phage and colicin E1) or exit from (novobiocin, erythromycin, CCCP and HlyA) the cell. We also used vancomycin, which we have found to be a useful *in vivo* probe in ascertaining the open or closed state of TolC's periplasmic aperture (Augustus *et al.*, 2004).

With regard to novobiocin, erythromycin and CCCP, the TolC turn 1 $_{147}$AGSG$_{150}$ mutant behaved just like a TolC null mutant (Table 1). Similarly, the mutant TolC protein failed to secrete active hemolysin, an observation consistent with our previous report where we showed that a TolC mutant with a G147D alteration in the turn 1 region was impaired in hemolysin secretion (Vakharia *et al.*, 2001). In contrast to these defects, the TolC $_{147}$AGSG$_{150}$ mutant behaved just like the wild type strain with respect to TLS phage and colicin E1 infections, reflecting that its cell surface properties and hence its insertion into the outer membrane were not affected. Therefore, reduced levels of the TolC turn 1 $_{147}$AGSG$_{150}$ mutant is likely due to defects in earlier steps of its assembly, leading to the degradation of roughly half of the newly synthesized nascent polypeptide chains. Finally, the TolC turn 1 $_{147}$AGSG$_{150}$ mutant did not display any sensitivity towards vancomycin (Table 1), showing that the hypersensitivity towards novobiocin, erythromycin and CCCP is due to the loss of efflux activity and not to a gross breach in the outer membrane permeability barrier caused by mis-insertion of the mutant protein, or a constitutively open TolC aperture. The TolC$_{147AGSG150}$ mutant is therefore a first of its kind, being completely impaired in its export activities but fully functional in its import activities.

To assess whether the drug hypersensitivity phenotype was due to a loss of interaction with other members of the TolC–AcrAB complex, *in vivo* cross-linking was carried out using DSP as described in Fig. 2A. The analysis failed to uncover any obvious defects in the mutant TolC protein's ability to simultaneously pull down AcrA and AcrB in a DSP-dependent manner (Fig. 2A). Because TolC is thought to share a greater surface area with AcrA than with AcrB, it is possible that TolC–AcrA interactions are perturbed in the TolC turn 1 $_{147}$AGSG$_{150}$ mutant. To test this possibility, we used SPDP, which facilitates amine-to-sulfhydryl cross-links. As TolC does not contain any cysteine residues, we introduced a Q142C substitution that has proven to be useful in probing TolC–AcrA interactions (Lobedanz *et al.*, 2007). As with a 6xHis tag, the presence of the Q142C substitution did not alter the phenotype of wild type or the TolC turn 1 $_{147}$AGSG$_{150}$ mutant protein (data not shown). Both wild type and the TolC turn 1 mutant efficiently pulled down AcrA regardless of whether DSP or SPDP was used as

a cross-linker (Fig. 2B and C), thus showing no obvious defects in the ability of the mutant TolC protein to 'physically' interact with AcrA.

Suppressors of the TolC turn 1 $_{147}$AGSG$_{150}$ mutant

Because cross-linking analysis failed to reveal any gross defects in the TolC turn 1 $_{147}$AGSG$_{150}$ mutant's ability to interact with AcrA or AcrB, we resorted to reversion analysis in an attempt to identify suppressor mutations that could provide insight into the reason for the mutant TolC's defect. Antibiotic resistant revertants were isolated by simultaneously utilizing two antibiotics, novobiocin and erythromycin, in the selection medium to avoid mutations that alter the cellular targets of these antibiotics. Approximately 5×10^8 cells from 24 independent cultures were plated onto selection medium and incubated at 37°C for 20 h. Nineteen cultures produced a total of 55 spontaneous antibiotic resistant mutants at a frequency of about 10^{-8}, indicating the prevalence of missense mutations. To test whether the mutations in these isolates mapped within the plasmid DNA from which the mutant *tolC* gene was expressed, the plasmids were extracted and transformed into a Δ*tolC* strain and transformants were tested for their ability to grow on medium supplemented with novobiocin and erythromycin. In no cases did the mutations conferring antibiotic resistance move with the plasmid, showing that they all resided on the chromosome. We then carried out P1 transductional mapping using the selectable marker Tn*10*-Tc^r, which is linked to the *acrAB* genes (40% co-transducible by P1 phage). For all 55 revertants, mutations conferring antibiotic resistance moved with the *acrAB*-linked Tn*10* marker at the expected frequency, indicating that the mutations mapped in or near the *acrAB* genes.

Twenty-four of the 55 revertants, representing multiple independent isolates of each distinct phenotype, were analysed. Nucleotide sequence analysis of PCR-amplified *acrAB* DNA templates obtained from these 24 mutants revealed five different missense mutations within the *acrA* gene, resulting in a single amino acid substitution in the mature AcrA sequence: an A135T substitution was obtained once, while S83G, T111P, T153P and L252R substitutions were found in seven, three, eight and five isolates, respectively. These alterations were localized in the α-helical hairpin (S83G, T111P and A135T), lipoyl (T153P) or β-barrel (L252R) domain of AcrA (Fig. 3A). Interestingly, despite affecting some of the same domains of AcrA, the five AcrA suppressor alterations isolated in this study affected different AcrA residues than 10 previous AcrA suppressors isolated against an assembly-defective TolC protein (Fig. 3B).

As expected, all five AcrA suppressors reduced the hypersensitivity phenotype of the TolC turn 1 $_{147}$AGSG$_{150}$ mutant (Table 2). Note that an AcrA suppressor-mediated reduction in CCCP sensitivity is consistent with our data (Table S2) and that of Colmer *et al.* (1998) showing that AcrAB confers resistance against CCCP independent of EmrAB. Accordingly, AcrA$_{L252R}$, the strongest of the five AcrA suppressors, reduces the CCCP sensitivity of TolC$_{147AGSG150}$ independent of EmrAB (data not shown). Western blot analysis data showed that suppression was achieved without significantly elevating the mutant TolC protein level (Table S3). We also examined levels of AcrA mutants and found that only the mutant carrying a T111P substitution had somewhat reduced AcrA levels, regardless of whether it was expressed in a wild type or TolC turn 1 $_{147}$AGSG$_{150}$ mutant background (Table S3). Thus the reduced AcrA$_{T111P}$ level is not caused by an aberrant interaction between AcrA$_{T111P}$ and the mutant TolC protein. Rather, it appears that the T111P substitution affects AcrA's conformation and stability independent of TolC. The isolation of TolC turn 1 $_{147}$AGSG$_{150}$ suppressor mutations in *acrA* suggested either a defective functional interaction between the TolC mutant protein and wild type AcrA or that the AcrA suppressors actually mend a possible aberrant interaction between the mutant TolC protein and AcrB.

Mechanism of suppression

It has been proposed that AcrA plays an active role in transducing conformational energy from drug/proton-bound AcrB to TolC to help open TolC's channel (Fernández-recio *et al.*, 2004; Murakami *et al.*, 2006; Seeger *et al.*, 2006; Bavro *et al.*, 2008; Misra and Bavro, 2009). Accordingly, once AcrB is proton/drug bound, con-formational signals received by the AcrB-proximal β-barrel and lipoyl domains of AcrA are relayed, via AcrA's α-helical hairpin domain, to the TolC helices guarding the aperture. This would allow for a stable engagement between the AcrA hairpin helices and intra-protomer grooves of the TolC helices, leading to full dilation of the TolC aperture and successful drug extrusion. We considered the possibility that the AcrA suppressors isolated in this study represent constitutively 'activated' AcrA forms, which the wild type protein normally assumes only transiently upon receiving conformational signals from AcrB. If the AcrA suppressors are indeed constitutively activated, do they then also transform TolC into a constitutively open state? To test this possibility, we resorted to the vancomycin sensitivity assay which we have shown to be an effective way to monitor the open or closed state of the TolC aperture/channel *in vivo* (Augustus *et al.*, 2004; Bavro *et al.*, 2008).

Remarkably, four of the five AcrA suppressors isolated against $TolC_{147AGSG150}$ significantly increased sensitivity to vancomycin in a $TolC_{147AGSG150}$-specific manner (Figs 4 and 6), indicating that their mode of suppression involves constitutively dilating the mutant TolC aperture/tunnel. The remaining suppressor, with a T111P substitution, produced a modest (11%) increase in vancomycin sensitivity despite efficiently reducing novobiocin sensitivity. It is worth noting that $AcrA_{T111P}$ is present at approximately 60% that of wild type AcrA level, so a modest increase in vancomycin sensitivity in the case of $AcrA_{T111P}$ could be in part due to its reduced level compared with other AcrA suppressors (Table S3). To see whether suppressor AcrA-mediated vancomycin sensitivity is dependent on AcrB, we expressed one of the suppressor AcrA mutants, $AcrA_{S83G}$, from a plasmid in Δ*acrAacrB*$^+$ and Δ*acrAB* backgrounds. Vancomycin sensitivity was observed only in an Δ*acrAacrB*$^+$ background expressing $TolC_{147AGSG150}$ (Fig. 5A), indicating that the presumed activated state of AcrA induces opening of the mutant TolC aperture/channel in an AcrB-dependent manner. Finally, we asked whether the presence of an efflux-active AcrB protein is required for the observed vancomycin sensitivity. For this we introduced a D407A substitution in AcrB that abolishes AcrB's pump activity by disrupting the proton translocation pathway of AcrB (Guan and Nakae, 2001;Murakami *et al.*, 2002). Interestingly, $AcrA_{S83G}$-mediated vancomycin sensitivity was observed even in a nonfunctional $AcrB_{D407A}$ background (Fig. 5B). This supports the notion that the AcrA suppressor proteins have adopted a constitutively activated state, thus requiring AcrB only as a scaffold to induce opening of $TolC_{147AGSG150}$ aperture.

Do alterations that constitutively open the TolC aperture/channel reverse the drug sensitivity phenotype of $TolC_{147AGSG150}$?

The AcrA suppressor data suggest that the efflux function is partially restored in part by inducing opening of the mutant TolC aperture/channel. This prompted us to ask whether an alteration in TolC that constitutively induces TolC aperture/channel opening will also reduce the drug sensitivity phenotype of $TolC_{147AGSG150}$. To test this, we used two different TolC alterations, R367E and R390E, which constitutively transform the TolC aperture/channel into an open state. R367E destabilizes the network of ionic interactions at the entrance of TolC channel (Andersen *et al.*, 2002), while R390E is thought to affect the aperture/channel by influencing the supercoiling of the coiled coils (Bavro *et al.*, 2008). Alterations affecting R367 and R390 were originally obtained among drug sensitive (leaky) TolC mutants (Augustus *et al.*, 2004).

The presence of R367E or R390E in an otherwise wild type TolC backbone elevated sensitivity to novobiocin and vancomycin, confirming the leaky nature of these mutant TolC channels, with R367E conferring a leakier phenotype than R390E (Fig. 6A). When these two alterations were independently introduced into a $TolC_{147AGSG150}$ mutant, R367E failed to reduce the novobiocin sensitivity of $TolC_{147AGSG150}$, while R390E did (Fig. 6A). Interestingly, in both cases a significant reduction in vancomycin sensitivity was observed. Western blot analysis revealed that the presence of R367E or R390E in the $TolC_{147AGSG150}$ backbone significantly lowered the mutant TolC levels (Fig. 6A), which would at least in part explain the reduced vancomycin sensitivity. However, despite diminished protein levels, the reduced novobiocin sensitivity of the $TolC_{147AGSG150, R390E}$ double mutant compared with the $TolC_{147AGSG150}$ single mutant indicated that forced opening of the TolC aperture/channel can indeed improve the efflux function of $TolC_{147AGSG150}$. The inability of R367E to reduce the novobiocin sensitivity of $TolC_{147AGSG150}$ is likely due to a negative effect on the mutant protein's stability and the acute leaky nature of the TolC aperture/channel (Fig. 6A).

Combined effects of AcrA suppressors and leaky alterations on $TolC_{147AGSG150}$

Vancomycin sensitivity data in Fig. 4 indicated that AcrA suppressors reduce the novobiocin sensitivity of the $TolC_{147AGSG150}$ mutant in part by inducing TolC aperture/channel opening. This hypothesis was further corroborated by data showing that the presence of R390E, which only moderately opens the TolC aperture/channel, also reduces the novobiocin sensitivity phenotype of $TolC_{147AGSG150}$. However, manifestation of a synthetic phenotype, i.e. reduced protein level, when $_{147}AGSG_{150}$ was combined with R390E (Fig. 6A) made it difficult to determine whether the AcrA suppressors and R390E acted via the same mechanism, i.e. by opening the TolC aperture/channel, to reduce the novobiocin sensitivity of $TolC_{147AGSG150}$. Nevertheless, to see whether the two mutations would act synergistically or antagonistically when combined, we introduced the AcrA suppressor alterations in a $TolC_{147AGSG150, R390E}$ background. We also analysed the effect of AcrA suppressors on $TolC_{147AGSG150, R367E}$, which display a null-like hypersensitivity phenotype towards novobiocin (Fig. 6A).

The presence of the five AcrA suppressor alterations in $TolC_{147AGSG150, R367E}$ (Fig. 6B) and $TolC_{147AGSG150, R390E}$ (Fig. 6C) backgrounds significantly increased the mutant TolC levels, which rose from 18 to 200%, indicating stabilization of the complex. However, unlike the $TolC_{147AGSG150}$ background where the five AcrA suppressors increased the vancomycin sensitivity to varying degrees (Fig. 4) without significantly altering the mutant TolC levels (Table S3), in the $TolC_{147AGSG150, R367E}$ and $TolC_{147AGSG150, R390E}$ backgrounds the AcrA suppressor-mediated increases in the vancomycin sensitivity were very similar (Fig. 6B and C). This indicated that the increased vancomycin sensitivity seen in the TolC double mutant backgrounds is primarily due to the AcrA suppressor-mediated stabilization of the mutant TolC proteins and not due to the unique effects of individual AcrA suppressors on the TolC aperture/channel. In other words, this increase in vancomycin sensitivity is not due to the additive effects of the TolC open state alterations and the action of the AcrA suppressors on the TolC aperture/channel. As observed in the $TolC_{147AGSG150}$ background, all AcrA suppressors reduced the novobiocin sensitivity of the two TolC double mutants (Fig. 6B and C).

Suppression specificity

We have previously described an assembly-defective TolC protein ($TolC_{P246R, S350C}$) and its antibiotic resistant AcrA suppressors (Gerken and Misra, 2004). As mentioned above, these suppressors affected different AcrA residues than those isolated here against $TolC_{147AGSG150}$ (Fig. 3). So we asked whether these 10 different AcrA suppressors isolated against $TolC_{P246R, S350C}$ could reverse the antibiotic sensitivity defect of the $TolC_{147AGSG150}$ mutant, and if they do, whether their mode of suppression also involves constitutive dilation of the TolC aperture/channel, resulting in vancomycin sensitivity. Six of

the 10 AcrA suppressors isolated against $TolC_{P246R,\ S350C}$ failed to reverse the novobiocin sensitivity of $TolC_{147AGSG150}$, thus showing suppression specificity (Fig. 7). The remaining four AcrA suppressors modestly reduced the novobiocin sensitivity of $TolC_{147AGSG150}$ but conferred only a very small increase in vancomycin sensitivity (less than 8% of wild type AcrA; Fig. 7). In contrast, the majority of AcrA suppressors (four out of five) isolated against $TolC_{147AGSG150}$ elevated vancomycin sensitivity from 28 to 62% over the wild type AcrA background (Fig. 4). Thus in four cases where cross-suppression was observed, the mechanism of suppression appears to be complex stabilization rather than constitutive dilation of the TolC aperture/channel.

Next we carried out a reciprocal analysis and tested whether the AcrA suppressors isolated against $TolC_{147AGSG150}$ are specific to this TolC protein or if they also suppress the assembly-defective $TolC_{P246R,\ S350C}$ protein (Gerken and Misra, 2004). The hypersensitivity phenotype of this TolC mutant was partially reversed by the AcrA suppressors obtained in this study (Fig. 8). Curiously, all the AcrA suppressors also elevated $TolC_{P246R,}$ S350C levels from around two- to greater than 10-fold, reflecting stabilized interactions with the mutant TolC protein (Fig. 8). Moreover, there appeared to be good correlation between the AcrA suppressors' ability to stabilize the mutant TolC protein and reduce antibiotic sensitivity (Fig. 8). Interestingly, however, none of the five AcrA suppressors significantly increased vancomycin sensitivity (Fig. 8), indicating that their mode of suppression in the case of $TolC_{P246R,\ S350C}$ involved complex stabilization rather than constitutively dilating the TolC aperture/channel.

Together, these data showed that while there were some clear cases of suppression specificity, when cross-suppression was observed the mechanism of suppression appears to be complex stabilization and not TolC aperture/channel opening, which was unique to only AcrA suppressors isolated against $TolC_{147AGSG150}$.

Re-probing of TolC–AcrA interactions using a labile AcrA protein

The isolation of suppressor mutations of the TolC turn 1 $_{147}AGSG_{150}$ mutant in *acrA* suggested that the mutant TolC protein is unable to make productive interactions with AcrA. Alternatively, such defects may lie between the mutant TolC protein and AcrB but changes in AcrA can overcome them. Because we could not ascertain any obvious defects in the mutant TolC protein's ability to interact with AcrA or AcrB from chemical cross-linking analyses (Fig. 2), we resorted to a second and perhaps more sensitive approach that indirectly probes *in vivo* interactions between TolC, AcrA and AcrB. We have previously shown that the *in vivo* stability of a mutant AcrA protein ($AcrA_{L222Q}$) is highly dependent on its ability to properly interact with TolC; without TolC, $AcrA_{L222Q}$ is rapidly degraded by a periplasmic protease DegP (Gerken and Misra, 2004). We also noted that the stability of $AcrA_{L222Q}$ is dependent on AcrB, although to a lesser extent than it is on TolC (Fig. S1). TolC–AcrAB interactions were probed through utilizing a genetic background expressing $AcrA_{L222Q}$ from the chromosome and various TolC turn 1 mutants from a plasmid replicon. As expected, in the absence of TolC, $AcrA_{L222Q}$ was rapidly degraded (Fig. 9A). Expressions of TolC turn 1 mutants carrying single and double substitutions stabilized $AcrA_{L222Q}$ just as effectively as the wild type TolC protein, indicating normal interactions. In contrast, TolC turn 1 mutants with triple ($_{147}GGSG_{150}$) and quadruple ($_{147}AGSG_{150}$) substitutions showed intermediate or no protection, respectively, of $AcrA_{L222Q}$ (Fig. 9A). Coincidently, only these two TolC mutants displayed a drug-sensitive phenotype in the presence of wild type AcrA (Fig. 1C), thus further corroborating the notion of an impaired interaction with AcrA or aberrant TolC–AcrAB complex assembly. Figure 9B shows data from a control experiment demonstrating that wild type AcrA is an intrinsically stable protein regardless of the status of the TolC protein. Thus $AcrA_{L222Q}$ stability data presented here more closely mirror the efflux phenotype and functional tripartite complex assembly status than the *in vivo* chemical cross-linking data.

Discussion

The outward movement of the inner helices H7 and H8 is thought to be crucial for the opening of TolC's periplasmic aperture. This movement is most likely initiated when TolC is properly interfaced with AcrA and AcrB. Recent genetic and biochemical data have begun to shed light on the potential interacting regions of TolC, AcrA and AcrB (Elkins and Nikaido, 2003; Gerken and Misra, 2004; Touze *et al.*, 2004; Bokma *et al.*, 2006; Stegmeier *et al.*, 2006; Vediyappan *et al.*, 2006; Lobedanz *et al.*, 2007; Krishnamoorthy *et al.*, 2008; Symmons *et al.*, 2009). From these studies, it has become apparent that the grooves between the inner and outer helices H7/H8/H3 of TolC juxtapose the N-terminal α-helix 1 of AcrA.

The available data suggest the involvement of TolC's periplasmic turns 1 and 2, located between the bottom of outer helices H3 and H4 and inner helices H7 and H8, respectively, in docking with AcrB (Tamura *et al.*, 2005). In this study, we examined the role of TolC periplasmic turn 1 residues in antibiotic efflux through site-directed mutagenesis and isolating extragenic suppressors of a turn 1 TolC mutant. As the residues of turn 1 are thought to be in close proximity to AcrB hairpin loop residues (Tamura *et al.*, 2005), alterations in the turn region encompassing these residues are expected to produce a drug sensitivity phenotype by altering TolC–AcrB interactions. Indeed it was found that simultaneous replacement of the four turn 1 residues $_{147}$GLVA$_{150}$ with the small and neutral residues, AGSG, obliterated TolC's efflux function. The effect was less pronounced when $_{147}$GLVA$_{150}$ were replaced by $_{147}$AAAA$_{150}$, while single or double alterations affecting the four wild type residues produced no efflux defects. These results, coupled with the fact that the four wild type turn residues either lack a side-chain (G147) or have inert side-chains ($_{148}$LVA$_{150}$), suggest that backbone interactions and/or the overall hydrophobicity/packing of the turn 1 region are probably critical for TolC's interaction with other pump components.

Despite a null-like drug hypersensitivity phenotype, the TolC turn 1 $_{147}$AGSG$_{150}$ mutant could be efficiently cross-linked to AcrA and AcrB *in vivo*, indicating that the physical interactions between the three proteins are not grossly defective. Therefore, the defect must stem from either weaker or non-functional interactions between them. Genetic analysis involving *in vivo* stabilization of a labile AcrA protein, AcrA$_{L222Q}$, provided important clues concerning the roles of TolC's turn 1 region and AcrA in pump assembly. Stability of AcrA$_{L222Q}$ is strongly dependent on TolC and weakly on AcrB (Gerken and Misra, 2004; this study). Just as we found in a strain lacking TolC, the TolC turn 1 $_{147}$AGSG$_{150}$ mutant failed to stabilize AcrA$_{L222Q}$, indicating its inability to maintain stable interactions with AcrA$_{L222Q}$. Importantly, there was remarkable correlation between the various TolC mutants' abilities to stabilize AcrA$_{L222Q}$ and their efflux function. The single and double alterations in TolC's turn 1 neither affected TolC's efflux function nor the protein's ability to stabilize AcrA$_{L222Q}$. On the other hand, the triple ($_{147}$GGSG$_{150}$) and quadruple ($_{147}$AGSG$_{150}$) alterations in TolC's turn 1 led to either partial (triple) or complete (quadruple) loss of efflux function and the proteins' ability to fully stabilize AcrA$_{L222Q}$. These results indicate that the TolC turn 1 residues play a role in the functional complex assembly. It is important to emphasize that chemical cross-linking does not distinguish weak or non-functional interactions from those that are normal and functional (Bokma *et al.*, 2006; Stegmeier *et al.*, 2006; Vediyappan *et al.*, 2006; this study). Consistent with this, Touze *et al.* (2004) showed efficient cross-linking between TolC and AcrB in the absence of AcrA, but found no stable interactions between TolC and AcrB by isothermal calorimetry. In contrast to cross-linking analyses, the labile AcrA$_{L222Q}$ and TolC$_{P246R, S350C}$ proteins used here provide a sensitive means of monitoring the existence of functional TolC–AcrAB tripartite complexes *in vivo*. It is interesting to note that the observed instability of AcrA$_{L222Q}$ in the absence of TolC is similar to that occurring naturally for wild type HlyD, AcrA's counterpart of the TolC-HlyBD hemolysin secretion complex (Pimenta *et al.*, 1999).

The drug hypersensitivity phenotype of the TolC turn 1 $_{147}$AGSG$_{150}$ mutant provided the opportunity to isolate antibiotic resistant revertants whose characterization was expected to help understand the basis of the mutant TolC's defect. Characterization of these revertants revealed five different compensatory alterations in the AcrA protein. This was somewhat unexpected because TolC's turn 1 is projected to be in close vicinity to one of AcrB's hairpin loops, which was later confirmed by spontaneous disulfide bond formation between cysteine residues introduced in these regions of TolC and AcrB (Tamura *et al.*, 2005). Instead, the isolation of compensatory alterations in AcrA initially suggested a possible defect in the mutant TolC protein's ability to properly interface with AcrA, thus precluding TolC's tunnel aperture from undergoing the transition from its resting closed state to an efflux-competent open state.

If TolC's periplasmic turn 1 region is in close proximity to AcrB, then why were extragenic suppressors of the TolC turn 1 mutant repeatedly isolated in *acrA* and not in *acrB*? Also, it was surprising that we failed to obtain any intragenic *tolC* mutations. Our inability to isolate suppressor mutations in *tolC* could be due to the fact that restoration of the antibiotic resistant phenotype may require multiple substitutions within the turn 1 or adjacent region – an extremely rare event. Similarly, we cannot rule out the possibility that the primary defect of the TolC mutant stems from its aberrant interaction with AcrB, but alterations in AcrA can partially restore these interactions. However, as TolC and AcrB are expected to share a much smaller overlapping surface area between them than with AcrA (Symmons *et al.*, 2009), a much smaller number of potential sites in AcrB are expected to yield a suppressor phenotype, compared with those in AcrA.

The genetic data pointing to the importance of TolC's turn 1 region and AcrA in pump assembly are consistent with a recently proposed multi-step pump assembly model (Bavro *et al.*, 2008). According to this model, AcrB docking of TolC results in a partial opening of TolC's aperture, causing a deepening of the intra-protomer grooves of the TolC helices (Fig. 10; steps 1 and 2). AcrA engages with TolC through these surface grooves, further extending the TolC helices and achieving full dilation and stabilization of the TolC aperture/channel (Fig. 10; steps 3–5). We suspect that the TolC turn 1 $_{147}$AGSG$_{150}$ mutant is unable to properly dock with AcrB's periplasmic crown (Fig. 10; step 1), thus not allowing the proper engagement of AcrA's hairpin helices with the TolC helices to achieve full dilation of the TolC aperture/channel. The compensatory changes in AcrA must allow the mutant TolC protein to re-engage with the complex in a manner which partially restores efflux activity.

The vancomycin sensitivity data provided mechanistic clues. Normally, *E. coli* strains are insensitive to vancomycin except for mutants with defective outer membrane biogenesis (Vuong *et al.*, 2008) or expressing TolC variants with a constitutively open aperture/channel (Augustus *et al.*, 2004; Bavro *et al.*, 2008). This TolC mutant-mediated vancomycin sensitivity is independent of AcrA and AcrB (Bavro *et al.*, 2008). The five AcrA suppressors isolated in this study elevated vancomycin sensitivity in a mutant TolC$_{147AGSG150}$-specific manner. Using one of the AcrA suppressors isolated in this study, AcrA$_{S83G}$, we showed that the mutant AcrA-mediated increase in vancomycin sensitivity is dependent on AcrB. Thus, AcrA alterations achieve suppression by inducing opening of the TolC aperture/channel only in the context of the tripartite pump complex. We believe that the AcrA suppressors isolated here transform AcrA into an 'activated' state, which the wild type AcrA protein transiently adopts upon receiving conformational signals from AcrB during the normal course of pump assembly and drug efflux (Fig. 10; steps 3–5). Consistent with the notion that the AcrA suppressors have adopted an 'activated' state, we found that AcrA$_{S83G}$, one of suppressors we tested, increases the vancomycin sensitivity even in the presence of AcrB$_{D407A}$, which is completely disabled in efflux function due to a disruption in its ability to translocate protons (Guan and Nakae, 2001; Murakami *et al.*, 2002). Therefore, AcrA suppressors appear to employ AcrB only as a scaffold to interact with TolC and induce its aperture/channel opening. An independent

verification of our assertion that the mechanism of suppression of the hypersensitivity phenotype of $TolC_{147AGSG150}$ involves its aperture/channel opening comes from the finding that the introduction of an alteration, R390E, which moderately widens the TolC aperture/channel, also reduces the novobiocin sensitivity of $TolC_{147AGSG150}$.

The five suppressor alterations isolated in this study affect residues located in three separate domains of the AcrA protein (Mikolosko *et al.*, 2006). The S83, T111 and A135 residues are located in the α-helical hairpin domain; L252 in the β-barrel domain; and T153 in the lipoyl domain sandwiched between the α-helical hairpin and β-barrel domains (Fig. 2). Recent cysteine-mediated cross-linking analysis showed that exposed residues of the N-terminal α-helix 1 of AcrA make contacts with TolC (Lobedanz *et al.*, 2007). Therefore, substitutions at S83 and T111 are likely to directly influence AcrA's conformation in a manner which facilitates interactions with the mutant TolC protein without adversely affecting interactions with wild type TolC. As A135 is present in AcrA's C-terminal α-helix 2, which is thought to be positioned away from TolC's helices, changes at this site may influence the TolC–AcrA interface indirectly.

The remaining two suppressor alterations, T153P and L252R, affect AcrA residues present in the lipoly and β-barrel domains, respectively, which are positioned distal from TolC's periplasmic helices (Fig. 3). The drastic nature of the T153P and L252R changes suggests that they may broadly impact the folding of their respective domains and thus produce long-range conformational changes that influence the TolC-proximal α-helical domain of AcrA. New asymmetric AcrB structures revealed no significant conformational changes in AcrB at the TolC–AcrB interface during the drug capture and expulsion cycle (Murakami *et al.*, 2006;Seeger *et al.*, 2006). In contrast, significant conformational changes are observed in AcrB's external clefts at the predicted AcrB–AcrA interface. Based on this, it has been speculated that conformational changes in AcrB, triggered by proton/drug binding, are transduced to TolC via long-range conformational changes in AcrA to cause the opening of the TolC channel (Murakami *et al.*, 2006;Seeger *et al.*, 2006). Normally, such AcrB-induced conformational changes in wild type AcrA would be sufficient to trigger the opening of the wild type TolC aperture/channel. However, in the case of the TolC turn 1 mutant, a further conformational nudge, generated by suppressor alterations in AcrA, is needed to achieve mutant TolC aperture/channel opening.

Even though the five AcrA suppressors isolated here continue to function normally with wild type TolC and are even able to suppress a different TolC mutant ($TolC_{P246R, S350C}$), vancomycin sensitivity data indicate that the mechanism of suppression may be different between the two TolC mutants. In the case of $TolC_{P246R, S350C}$, where the protein's stability is a major concern, suppression appears to be achieved through protein/complex stabilization. On the other hand, in the case of $TolC_{147AGSG150}$, where the tripartite complex appears to be stable but non-functional, presumably due to defective TolC–AcrB interface, the suppression mechanism employed involves stimulating TolC aperture/channel opening. The vancomycin data shown here provide experimental validation to the hypothesis of AcrB-assisted, AcrA-mediated dilation of the TolC aperture/channel (Fernández-recio *et al.*, 2004; Murakami *et al.*, 2006; Seeger *et al.*, 2006; Bavro *et al.*, 2008).

Experimental procedures

Strains, culture conditions and chemicals

All the strains and plasmids used in this study are listed in Table 3. Luria broth (LB) and LB agar (LBA) media were prepared as described by Silhavy *et al.* (1984). When required, ampicillin (50 μg ml^{-1}), chloramphenicol (12.5 μg ml^{-1}), kanamycin (25 μg ml^{-1}), tetracycline (10 μg ml^{-1}), isopropyl-β-D-thiogalactopyranoside (IPTG; 0.4 mM), L-arabinose (0.2%) was

added to bacterial cultures. ECF substrate was purchased from Amersham Pharmacia Biotechnologies. All other chemicals were of analytical grade.

DNA manipulations—The *acrA* gene was cloned into pACYC184 (Chang and Cohen, 1978) and pBAD33 (Guzman *et al.*, 1995) plasmid vectors. *acrA* sequence from the chromosome was amplified using a forward primer: 5′-AGATCTCATGA ACAATCC GACTTGTC-3′ and a reverse primer, 5′-GTCTTAACGGATCCTGTTTAAGTTAAG-3′ (underlined sequence indicate the *Bsp*HI and *Bam*HI cut sites, respectively) and ligated into appropriately digested pACYC184. a*crA* from this clone was expressed from its native promoter. To clone *acrA* into pBAD33, *acrA* sequence was amplified using a forward primer: 5′-GCAGGTACCGGACACTCGAGGTTTACATATG-3′ and a reverse primer: 5′-GCTCTAGAAGCTTAGTGAT GGTGATGGTGATGAGACTTGGACTGTTCAGGCTGACG-3′ (underlined sequence indicates *Kpn*I and *Hin*dIII restriction sites respectively). Expression of a*crA* from this clone was controlled by an arabinose-inducible pBAD promoter. Deletion of the chromosomal *tolC*, *acrA* and *acrB* genes was described previously (Augustus *et al.*, 2004) and was carried out the method described by Datsenko and Wanner (2000). The *acrB* locus from an $acrA_{L222Q}$ $acrB^{+}$ strain was replaced by a kanamycin resistance (Km^{r}) gene amplified from the pKD4 plasmid. Gene deletion was confirmed by PCR and DNA sequence analyses. Plasmid transformation and P1 transduction were performed according to the standard laboratory protocols.

In order to modify the TolC turn 1 sequence, two-step site-directed mutagenesis was carried out to first delete the last two bases of the 147th codon creating a frameshift, followed by the insertion of GC after the last base of the 150th codon, thus restoring the original frame, but altering $_{147}GLVA_{150}$ residues to AGSG. Site-directed mutagenesis was carried out by using the Quick Change Site Directed mutagenesis kit (Stratagene) according to manufacturer's instructions. Primers used in site directed mutagenesis are listed in Table S1.

Western blots

Whole-cell extracts were analysed by mini sodium dodecyl sulfate (SDS)-polyacrylamide (11%) gel electrophoresis (PAGE) and transferred onto Immobilon-P polyvinylidenedifluoride membranes (Millipore). Membrane blots were blocked overnight in 5% (wt/vol) non-dairy cream. After blocking, membranes were incubated for 1.5 h in primary antibodies raised against TolC-MBP and/or $AcrA_{6His}$, followed for two 15 min washes and 1 h incubation in secondary antibody (goat anti-rabbit alkaline phosphatase or horseradish peroxidase-conjugated IgG). Detection of hybridized proteins bands was carried out using ECF (when using alkaline phosphatase) or immunostar HRP substrate (when using horseradish peroxidase). Proteins bands were visualized with Molecular Dynamics Storm Imager or Bio-Rad Molecular Imager ChemiDoc XRS System.

Antibiotic sensitivity assays

Sensitivity to antibiotics was analysed by placing pre-soaked antibiotic disks (Becton Dickinson) on bacterial lawn grow on LBA. Typically, zones of inhibition were measured after 8 h of incubation at 37°C, except in case of vancomycin where zones were measured after 16 h of incubation. All antibiotic sensitivity assays were carried in triplicates.

Minimum inhibitory concentrations (MICs) were determined by measuring growth of bacterial cultures on media containing different concentrations of various inhibitors. Approximately 1×10^6 cells, mixed with twofold serial dilutions of inhibitors in microtiter plates, were incubated at 37°C for 18 h. OD_{600} was measured by a microtitre plate reader (Molecular Devices $VERSA_{max}$), and values were plotted against inhibitor concentrations. MIC values were

extrapolated from linear regressions obtained from OD_{600}/concentration plots. Growth was measured from two independent cultures and in duplicates.

In vivo *cross-linking*

Amine-reactive dithiobis (succinimidylpropionate) (DSP) and hetero-bi functional, amine- and sulfyhydryl-reactive (*N*-succinimidyl 3-[2-pyridyldithio]-propionate) (SPDP) were used to carry out *in vivo* cross-linking of proteins. His-tagged variants of wild type TolC and TolC turn 1 quadruple mutant were expressed from a pTrc99A plasmid vector and were used as baits to pull down AcrA and AcrB proteins expressed from a pACYC clone. Cross-linking was carried out essentially as described previously (Thanabalu *et al.*, 1998; Husain *et al.*, 2004; Vuong *et al.*, 2008).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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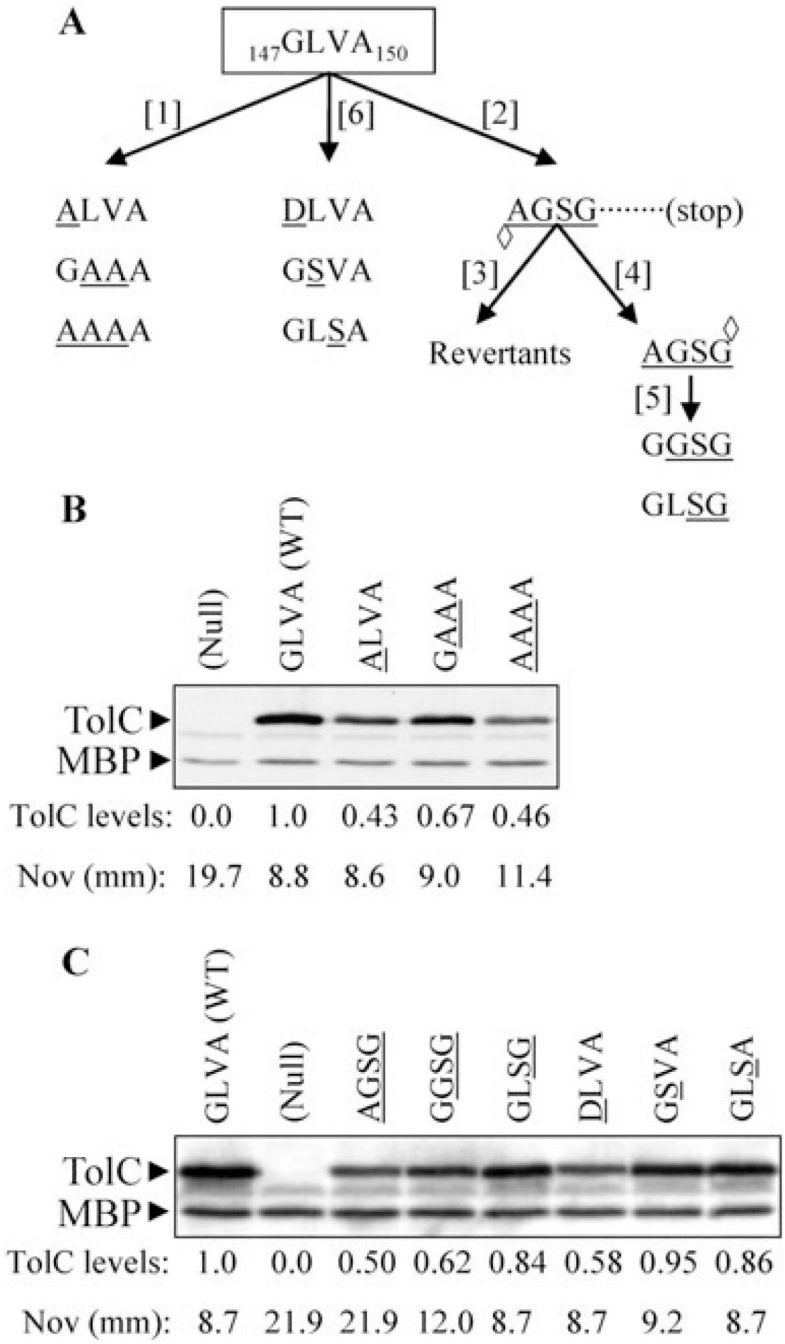

Fig. 1. Mutagenesis of the TolC turn 1 residues and characterization of various TolC turn 1 mutants

A. The wild type TolC turn 1 residues $_{147}$G LVA$_{150}$ were substituted with alanine [1] or subjected to −2 frameshift [2] mutagenesis. The TolC frame was subsequently restored either through reversion analysis [3] or by +2 frameshift site-directed mutagenesis [4]. Open diamonds point to the sites of frameshift mutations. The two residues of the resulting frameshift mutant [4] were further altered by site-directed mutagenesis [5]. Individual alterations of the wild type turn 1 residues were made by site-directed mutagenesis [6]. All mutant residues are underlined. Western blot analysis to determine levels of various TolC turn 1 alanine (B) and frameshift-derived (C) mutants (amino acid substitutions in mutants are underlined). Protein extracts from approximately 5×10^7 cells grown overnight at 37°C were analysed by SDS-

PAGE and electro-transferred onto PVDF membranes. Membranes were blotted with primary antibodies against TolC-MBP (maltose binding protein). MBP was used as a gel loading control. Protein levels were quantified with Quantity One software (Bio-Rad). Wild type TolC level was taken as 1 and other values were adjusted relative to wild type TolC. Zones of inhibition around a pre-soaked novobiocin disk (30 μg) are shown in millimeters (mm). Average inhibition zones recorded from three independent assays are shown, with zones varying no greater than 10%.

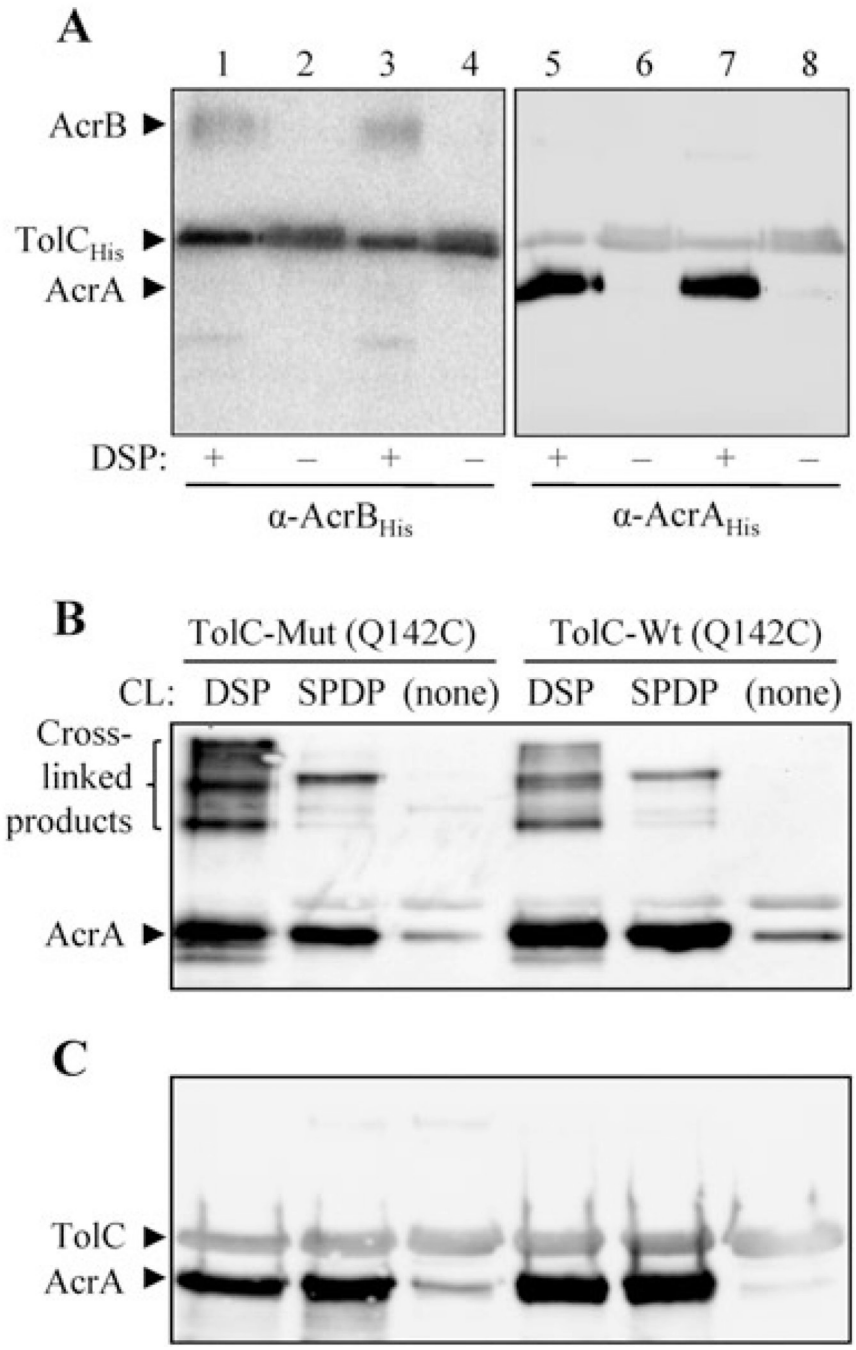

Fig. 2. ***In vivo*** **cross-linking analysis to probe TolC–AcrAB and TolC–AcrA interactions**

A. To analyse TolC–AcrAB interactions, freshly grown bacterial cultures were incubated with or without DSP. TolC$_{His}$ was purified through a Ni^{2+} affinity column, and AcrB and AcrA from two identical sets of eluates were probed by Western analysis using AcrB$_{His}$ or AcrA$_{His}$ antibodies. These antibodies also recognize TolC due to the presence of a C-terminal 6xHis tag in TolC. Note that the wild type (lanes 1, 2, 5 and 6) and mutant TolC$_{147AGSG150}$ (lanes 3, 4, 7 and 8) proteins can pull down AcrB and AcrA only in the presence of DSP cross-linker. All protein samples were boiled in sample buffer containing β-mercaptoethanol prior to gel electrophoresis. B and C. To analyse TolC–AcrA interaction, a Q142C substitution was introduced into both wild type (TolC-Wt) and the TolC turn 1 $_{147}$AGSG$_{150}$ mutant (TolC-Mut)

to facilitate SPDP-mediated cross-linking. Both wild type and mutant TolC proteins contain a 6xHis tag at the C-terminal end of the protein for affinity purification. TolC from cultures incubated with DSP, SPDP, or no cross-linker (CL) was affinity purified through Ni^{2+} affinity columns, AcrA and TolC from two identical sets of eluates were detected through Western blots using $AcrA_{His}$ antibodies, which also recognize $TolC_{His}$. Prior to gel electrophoresis, protein samples were boiled in sample buffer either without (B) or with (C) β-mercaptoethanol.

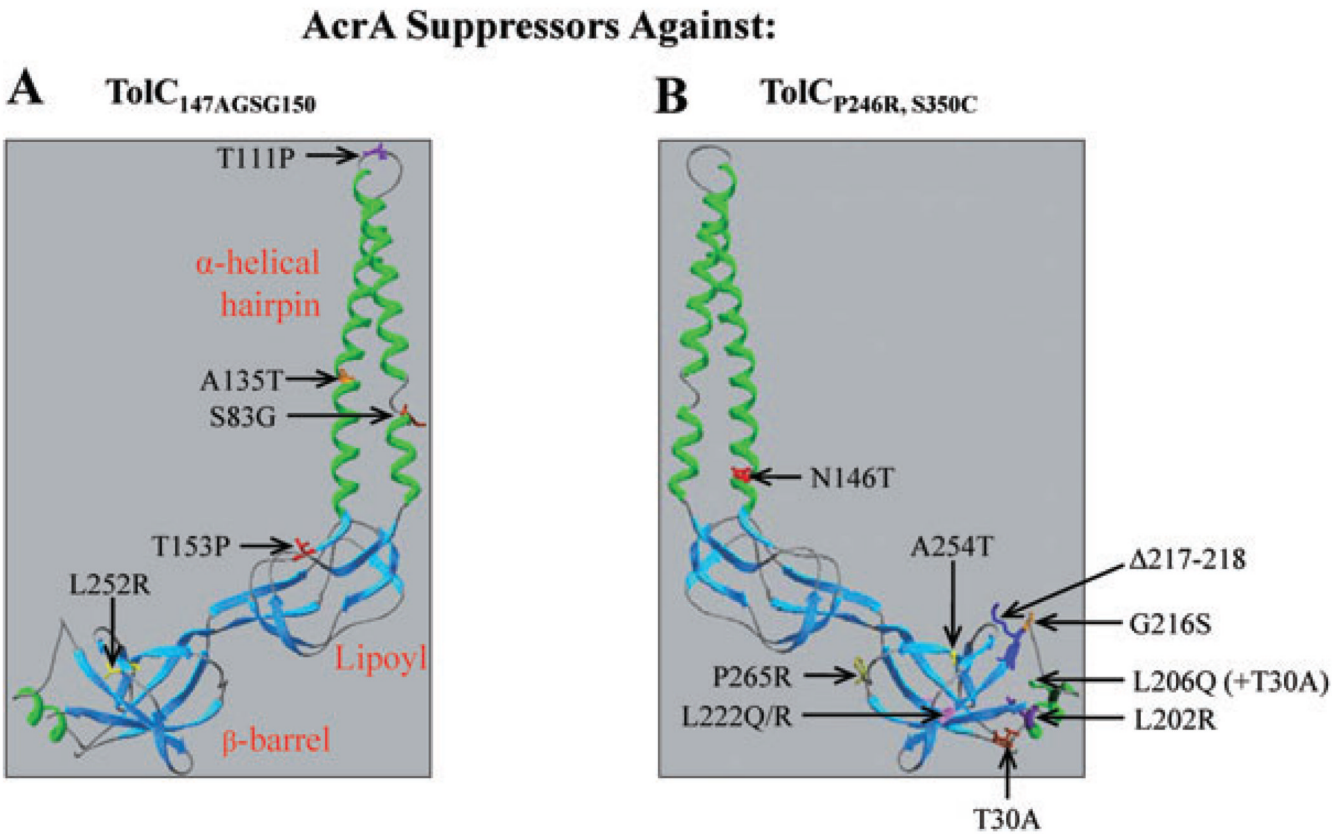

Fig. 3.

A cartoon showing X-ray structures of AcrA (2F1M). Locations of five AcrA substitutions obtained in this study and those obtained previously (Gerken and Misra, 2004) are shown in A and B respectively. AcrA residue numbering corresponds to that of the mature protein.

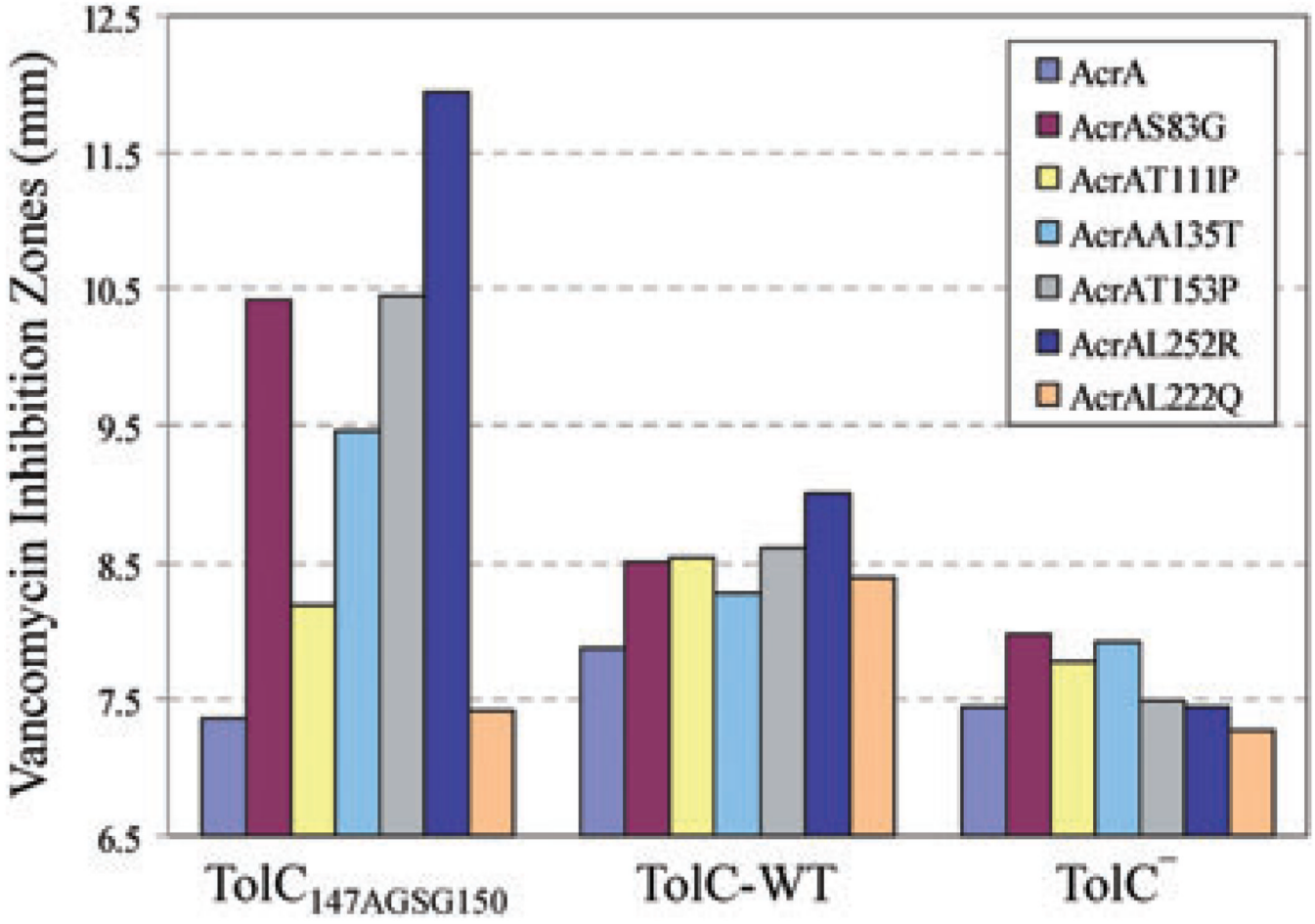

Fig. 4.

AcrA suppressor alterations induce opening of the mutant TolC aperture. Vancomycin sensitivity, which reflects an open state of the TolC aperture, was tested in wild type and suppressor AcrA strains expressing $TolC_{147AGSG150}$, wild type TolC (TolC-WT), or no TolC ($TolC^{-}$). A strain expressing $AcrA_{L222Q}$, which does not suppress $TolC_{147AGSG150}$, was included as a control. Zones of inhibition around disks soaked with vancomycin (75 μg) were measured after incubating plates for 16 h at 37°C. Average inhibition zones recorded from three independent assays are shown, with zones varying no greater than 10%.

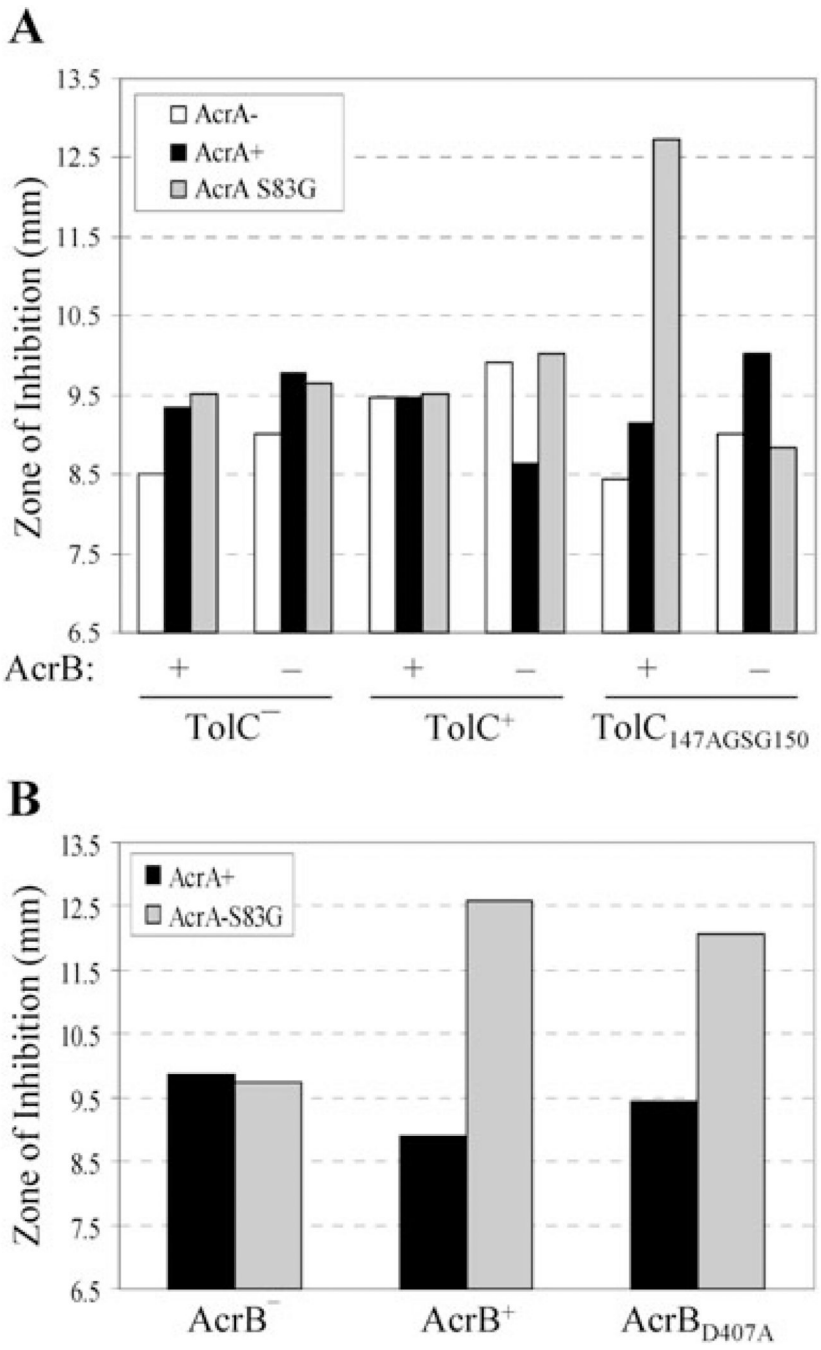

Fig. 5. AcrB is required for AcrA suppressor-induced opening of the mutant TolC aperture

A. Vancomycin sensitivity was determined, as described in Fig. 4 legend, from 18 different genetic backgrounds shown in the graph.

B. An active AcrB protein is not required for the $AcrA_{S83G}$-mediated vancomycin sensitivity. Average inhibition zone diameters were plotted from three independent experiments, with zones varying no greater than 10%.

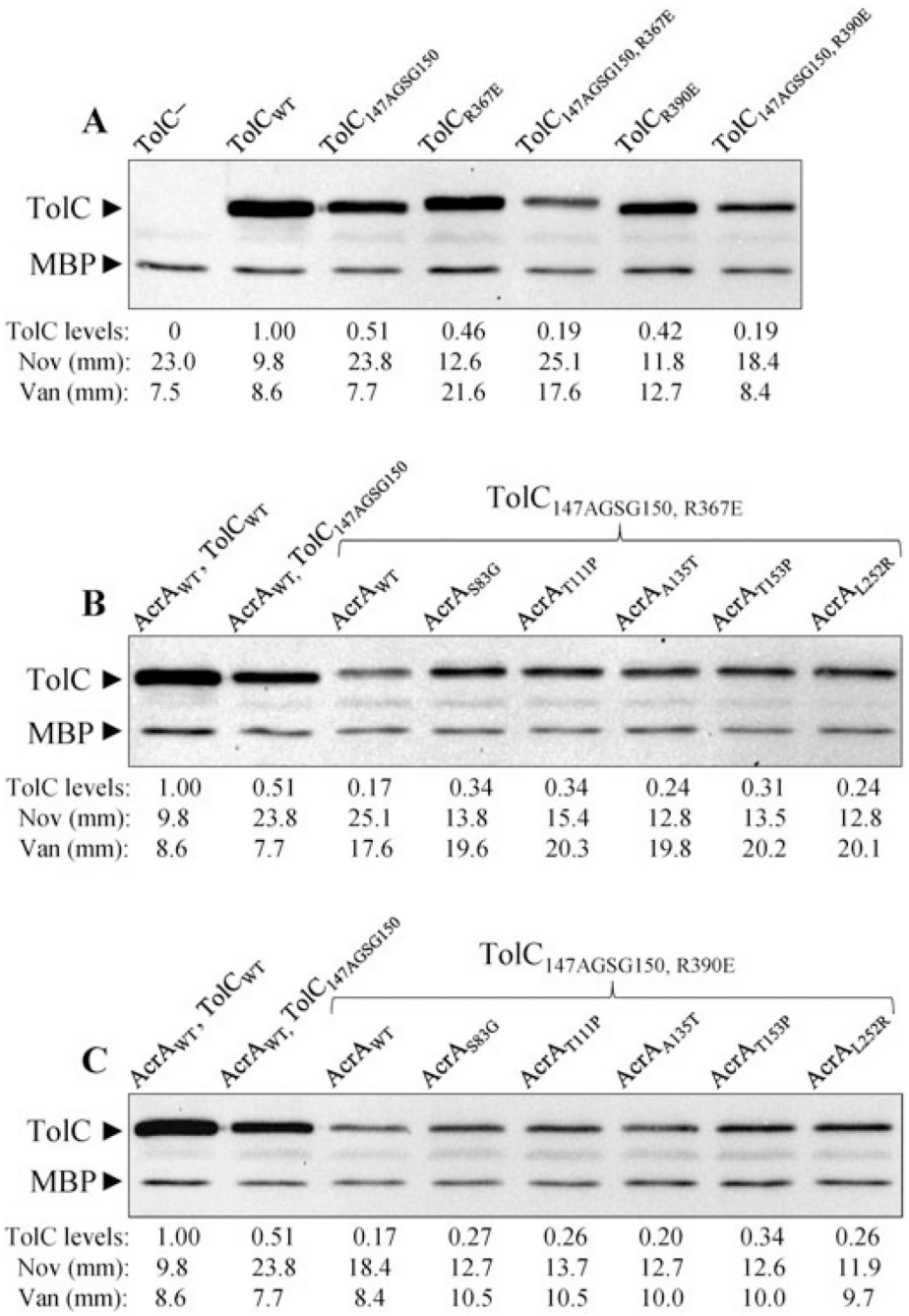

Fig. 6. Combined effects of TolC alterations, which influence the TolC aperture/channel, and various AcrA suppressors on TolC$_{147AGSG150}$

A. Effects of R367E and R390E that increase the TolC aperture/channel opening on TolC levels and vancomycin and novobiocin sensitivities.

B and C. Effects of various AcrA suppressors on TolC$_{147AGSG150, R367E}$ (B) and TolC$_{147AGSG150, R390E}$ (C) levels and novobiocin and vancomycin sensitivities. Proteins were detected by Western blots as described in Fig. 1 legend. Antibiotic disk sensitivity assays were carried out as described in Figs 1 and 4 legends. Average inhibition zone diameters were plotted from two independent experiments, with zones varying no greater than 10%.

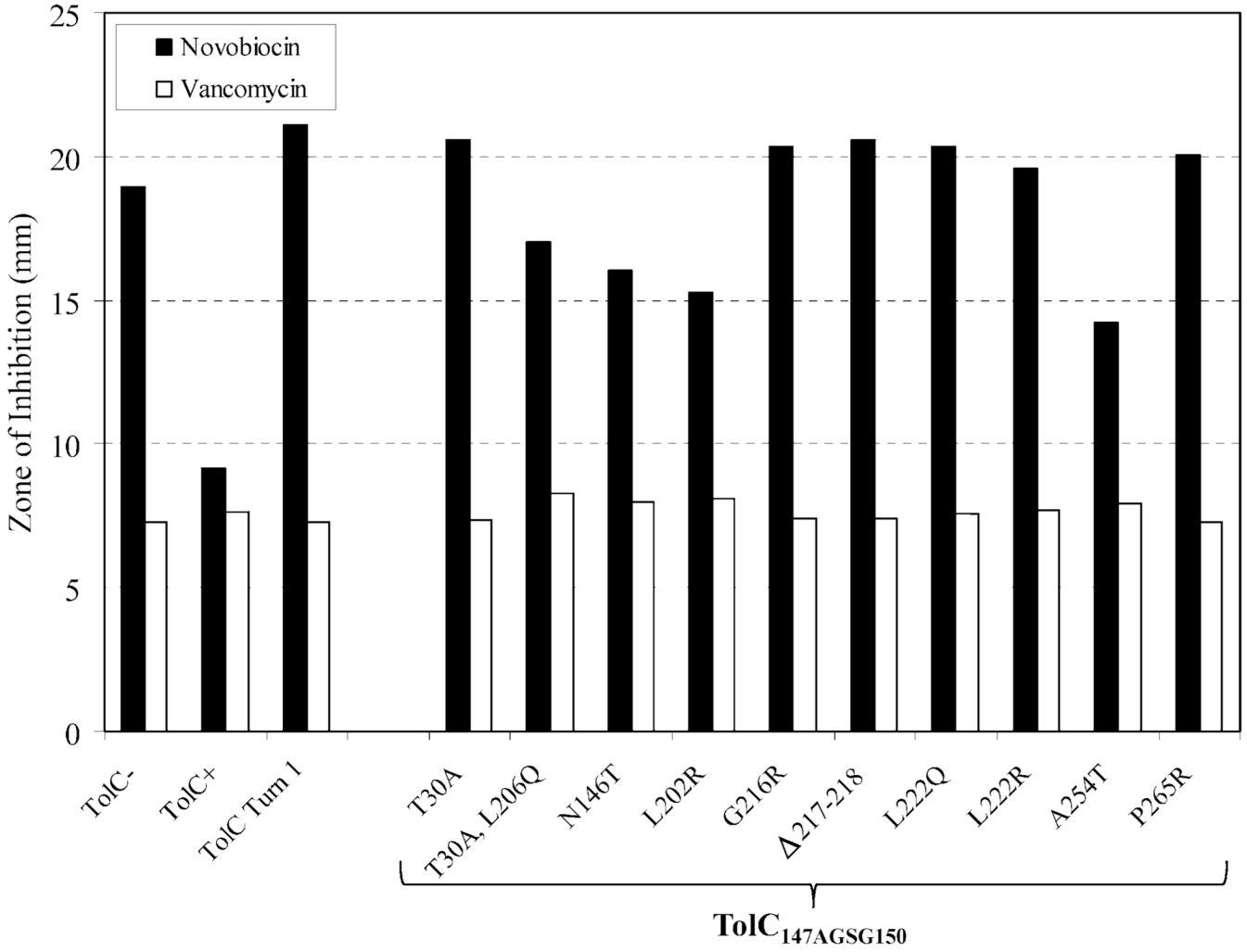

Fig. 7.

Effects of different AcrA suppressors on efflux function and TolC aperture were assessed by measuring novobiocin and vancomycin sensitivities, respectively, in a background expressing TolC turn 1 mutant ($TolC_{147AGSG150}$). Inhibition zones were also measured in $AcrA^+$ control strains expressing no TolC ($TolC^-$), wild type TolC (TolC-WT) and TolC turn 1 mutant ($TolC_{147AGSG150}$). Zones of inhibition around disks soaked with novobiocin (30 μg) and vancomycin (75 μg) were measured after incubating plates for 16 h at 37°C. Average inhibition zone diameters from three independent experiments were plotted, with zones varying no greater than 10%.

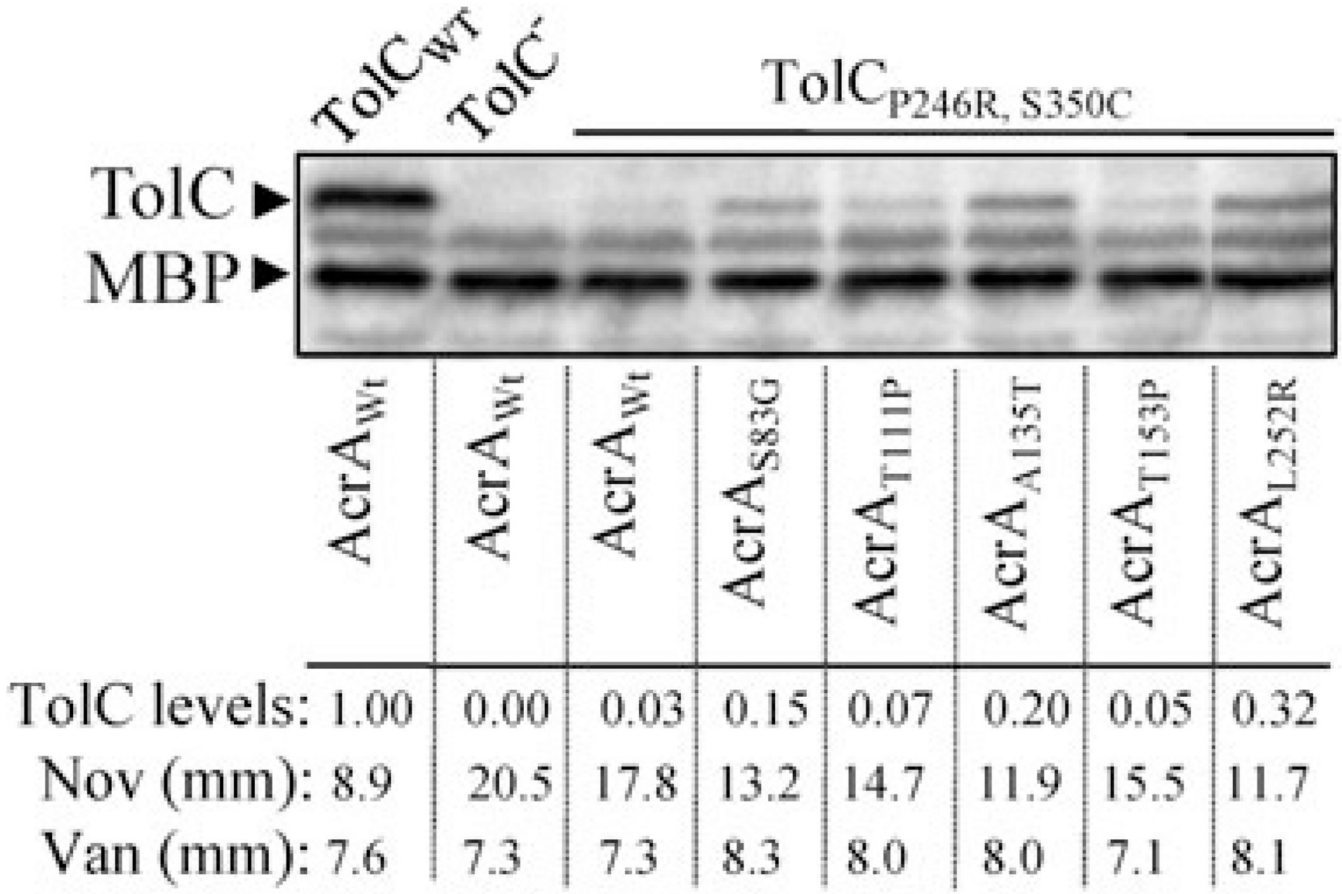

Fig. 8.

Effects of wild type and suppressor AcrA variants on TolC$_{P246R, S350C}$. Protein extracts, obtained from cultures grown overnight at 37°C, were used to detect TolC and MBP, a gel loading control, by Western blots using TolC-MBP antibodies. TolC was expressed from a plasmid replicon under the control of an IPTG-inducible pTrc promoter; *acrA* alleles were expressed from their chromosomal locations. Wild type TolC levels were taken as 1 and levels of TolC$_{P246R, S350C}$ in various AcrA backgrounds were calculated relative the wild type TolC levels. Zones of inhibition around pre-soaked novobiocin (30 μg) and vancomycin (75 μg) disks are also shown in millimeters (mm).

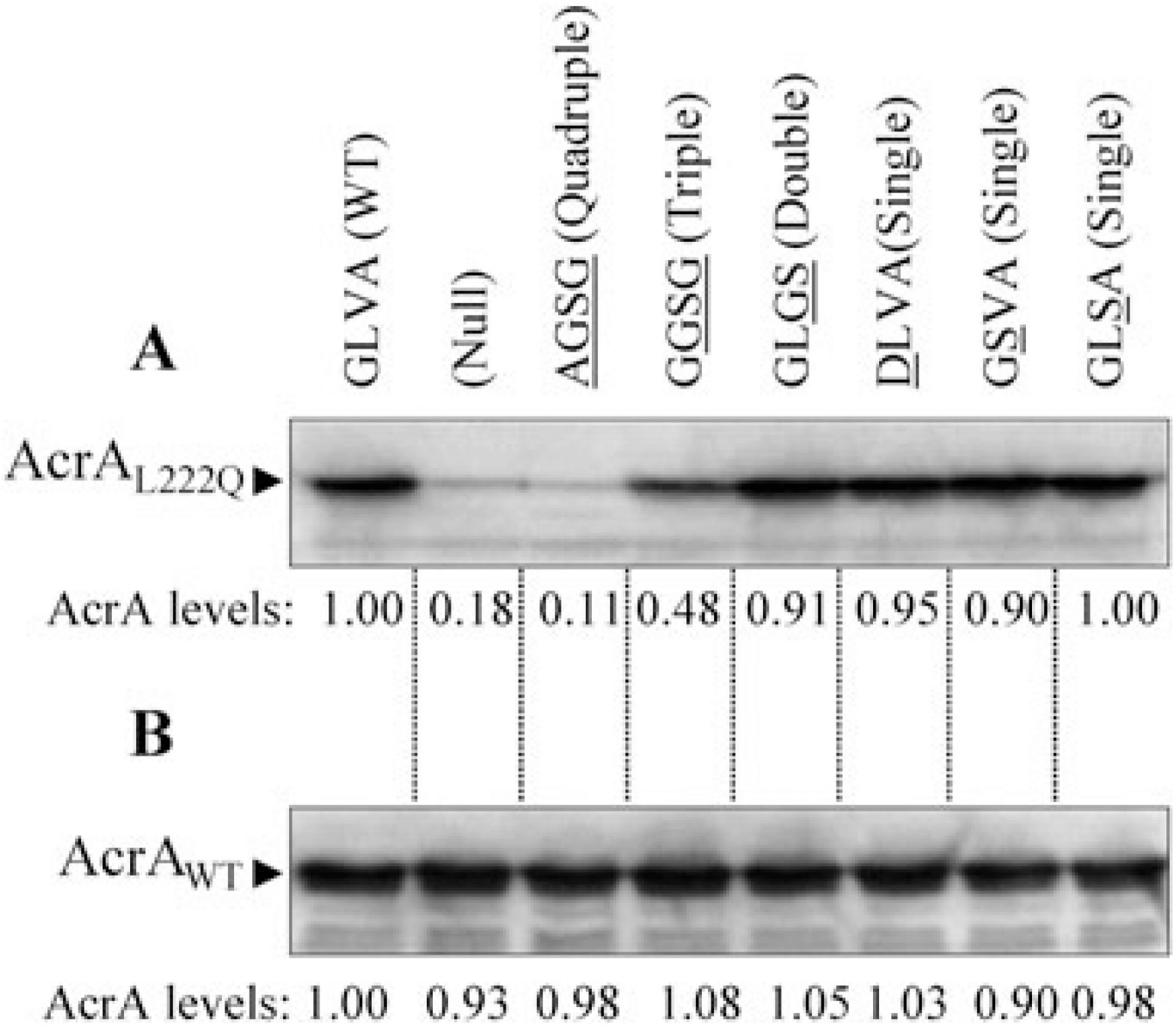

Fig. 9.

Effects of TolC on AcrA$_{L222Q}$ and wild type AcrA levels. AcrA levels from approximately 5 × 10^7 cells grown overnight at 37°C were determined by Western blot analysis using antibodies against AcrA$_{6His}$. AcrA$_{L222Q}$ (A) and wild type AcrA (B) levels from the wild type TolC background were taken as 1 and other values were adjusted relative to it.

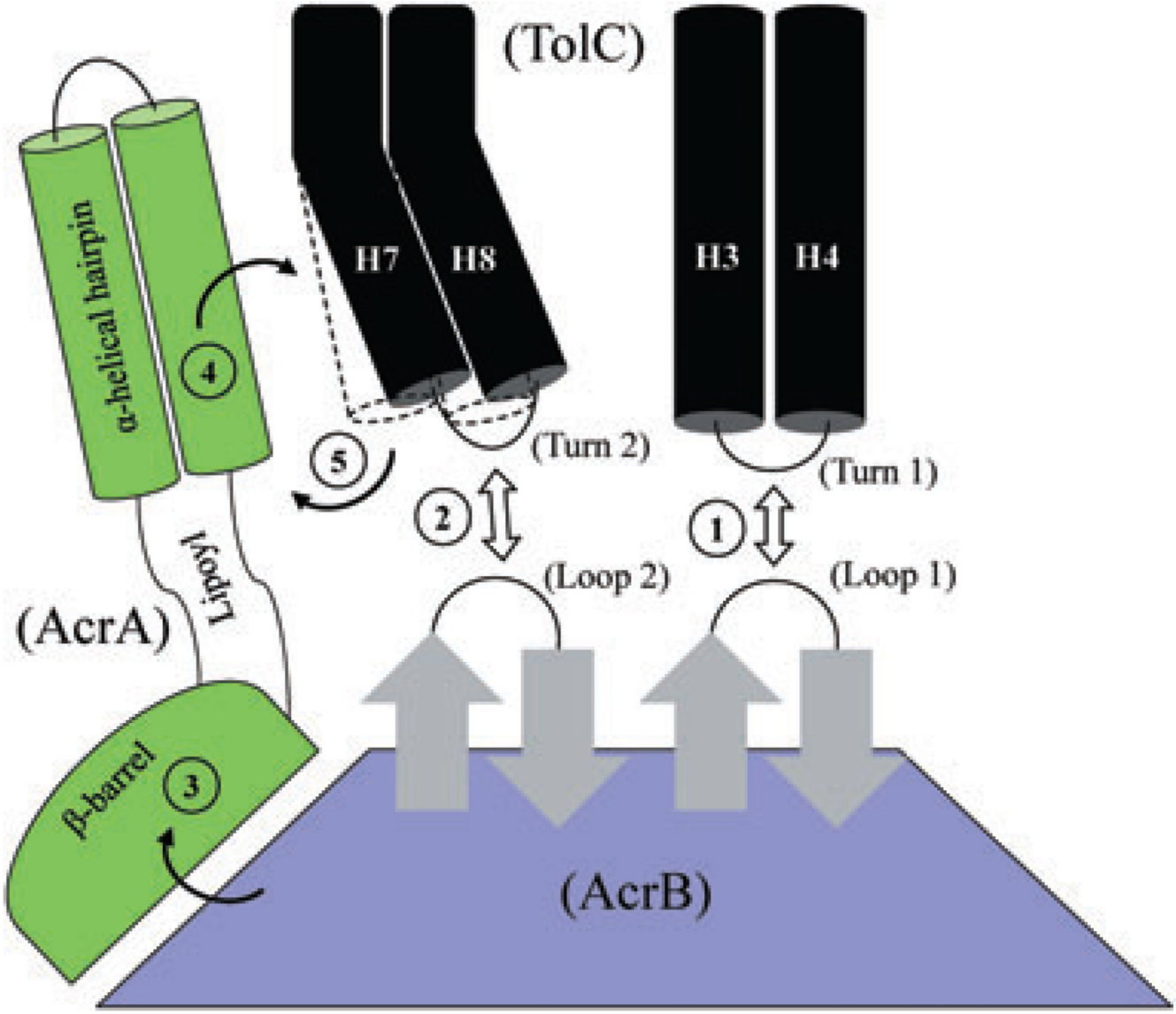

Fig. 10.

A cartoon depicting possible stepwise interactions between TolC, AcrA and AcrB, leading to full dilation of the TolC aperture/channel. Static (H3/H4) and mobile (H7/H8) paired helices guarding the TolC aperture are shown as black cylinders. In the resting stage, the TolC aperture is kept closed by intra- and inter-subunit ionic bridges. The three domains of AcrA and only the top half of AcrB, including its TolC-docking domain, are shown. It is envisaged that initial interactions between AcrB hairpin loops and TolC turns (steps 1 and 2) can trigger partial opening of the TolC aperture; however, these bilateral interactions are not sufficient to fully dilate the TolC aperture. Binding of substrates and protons induce conformational changes in AcrB that are then transduced via AcrA's β-barrel and lipoyl domains (step 3) to its TolC-proximal α-helical hairpin domain. Direct interactions between the conformationally induced α-helical hairpin domain of AcrA and intra-protomer grooves formed between H3/H4 and H7/H8 pairs (step 4) allow for outward extension of the TolC helices (outlined by dashed lines), leading to full dilation of the TolC aperture (step 5). The model is based on proposals by Fernández-Recio *et al.* (2004), Murakami *et al.* (2006), Seeger *et al.* (2006) and Bavro *et al.*

(2008). The AcrA suppressors obtained in this study dilate the TolC aperture in an AcrB-dependent manner but without receiving its conformational energy generated by the drug/proton translocation cycle.

Table 1

Sensitivity of wild type, null and mutant TolC proteins to various inhibitors.

	Sensitivity to inhibitors[a]		
Inhibitors	**$TolC_{WT}$**	**$TolC^{-}$**	**$TolC_{Q}$[b]**
Novobiocin	15.80	1.02	1.01
Erythromycin	61.60	1.90	1.82
CCCP	11.20	1.13	0.97
Vancomycin	(8.38)	(6.60)	(6.60)
HlyA	+	–	–
TLS	1	$>10^{-6}$	1
E1	1	2^{-12}	1

[a] Numbers for novobiocin, erythromycin and CCCP represent minimum inhibitory concentrations. For vancomycin, zones of inhibition in mm are shown in parenthesis. A 10 μl of solution containing 75 μg of vancomycin was spotted on paper disks of 6.5 mm diameter. Average inhibition zones recorded from three independent assays are shown, with diameter varying no greater than 10%. Plus and minus signs indicate presence (+) or absence (−) of hemolytic zones on blood agar medium. Sensitivity to TLS phage is measured as efficiency of plaguing. Colicin E1 sensitivity data report inhibition zones after spotting 10 μl of twofold serial dilutions of colicin E1 stock on an agar plate overlayed with bacterial cultures.

[b] $TolC_{Q}$ denotes TolC turn 1 $_{147}$AGSG$_{150}$ quadruple mutant.

Table 2

Sensitivity of wild type, mutant TolC and mutant TolC containing AcrA suppressors to various inhibitors.

	Sensitivity to inhibitors[b]			
TolC and AcrA proteins[a]	**Novobiocin**	**Erythromycin**	**CCCP**	**HlyA**
$TolC_{WT}$ $AcrA_{WT}$	15.80	61.60	11.20	+
$TolC_{Q}$ $AcrA_{WT}$	1.00	1.82	0.97	−
$TolC_{Q}$ $AcrA_{S83G}$	2.00	7.15	1.05	−
$TolC_{Q}$ $AcrA_{T\ 111P}$	3.60	24.60	2.50	−
$TolC_{Q}$ $AcrA_{A135T}$	3.80	13.50	1.20	−
$TolC_{Q}$ $AcrA_{T153P}$	7.70	13.70	2.70	−
$TolC_{Q}$ $AcrA_{L252R}$	14.50	31.10	4.20	−

[a] $TolC_{Q}$ denotes TolC turn 1 $_{147}$AGSG$_{150}$ quadruple mutant.

[b] Numbers for novobiocin, erythromycin and CCCP represent minimum inhibitory concentrations. Plus and minus signs indicate presence (+) or absence (−) of hemolytic zones on blood agar medium. For hemolysin sensitivity tests, strains shown in the table were transformed with a plasmid expressing the entire hemolysin operon.

Table 3

Bacterial strains and plasmids used in this study.

Strain/plasmid	Characteristics	Reference or source
Strains		
MC4100	F*araD139* Δ(*argF-lac*)*U139 rpsL 150 flbB5301 ptsF25 deoC1 thi-1 rbsR relA*	Casadaban, 1976
RAM1129	MC4100 Δ*tolC*::Km^r	Augustus *et al.*, 2004
RAM1130	MC4100 Δ*tolC*::Cm^r	Augustus *et al.*, 2004
RAM1292	MC4100 Δ*ara714*	Werner and Misra, 2005
RAM1330	RAM1292 Δ*tolC*::Km^r	Masi *et al.*, 2007
RAM1181	RAM1292 Δ*acrA*-scar	Gerken and Misra, 2004
RAM1182	RAM1181 Δ*tolC*::Km^r	Gerken and Misra, 2004
RAM1197	RAM1292 Δ*acrAB*-scar	Gerken and Misra, 2004
RAM1198	RAM1197 Δ*tolC*::Km^r	Gerken and Misra, 2004
RAM1335	MC4100 Δ*ara714* Δ*tolC*-scar	Husain *et al.*, 2004
RAM1350	RAM1130 Δ*tolC*::Cm^r *acrA* (L222Q) *zba*::Tn*10*–10.5	Gerken and Misra, 2004
RAM1351	RAM1130 Δ*tolC*::Cm^r *acrA* (L222Q) Δ*acrB*::Km^r *zba*::Tn*10*–10.5	This study
RAM1418	RAM1335 Δ*acrA*::Km^r	This study
RAM1419	RAM1335 Δ*acrAB*::Km^r	This study
Plasmids		
pTrc99A	Ap^r; expression vector	Pharmacia
pTrc-*tolC* (*Nco*I clone)	Ap^r; expresses wild type TolC	Vakharia *et al.*, 2001
pTrc-*tolC* (*Nco*I clone)	Ap^r; expresses $TolCP_{246R,\ S350C}$	Gerken and Misra, 2004
pTrc-*tolC* (*Bsp*HI clone)	Ap^r; expresses wild type TolC	Augustus *et al.*, 2004
pTrc-*tolC* (*Bsp*HI clone)	Ap^r; expresses $TolC_{147AGSG150}$	This study
pTrc-*tolC* (6His)	Ap^r; expresses $TolC_{6His}$	Husain *et al.*, 2004
pTrc-*tolC* (6His)	Ap^r; expresses $TolC_{147AGSG150,\ 6His}$	This study
pTrc-*tolC* (6His)	Ap^r; expresses $TolC_{Q142C,\ 6His}$	This study
pTrc-*tolC* (6His)	Ap^r; expresses $Tol_{CQ142C,\ 147AGSG150,\ 6His}$	This study
pCP20	Ap^r, Cm^r *ts* replicon, thermal induction of FLP synthesis	Datsenko and Wanner, 2000
pKD46	Ap^r; Lambda-red recombinase	Datsenko and Wanner, 2000
pKD4	Km^r	Datsenko and Wanner, 2000
pACYC184	Tc^r, Cm^r; cloning vector	Chang and Cohen, 1978
pSF4000-*hlyCABD* $^+$	Cm^r, expresses Haemolysin proteins	Welch *et al.*, 1981
pACYC184-*acrA*	Cm^r; expresses wild type AcrA	This study
pACYC184-*acrAB*	Cm^r; expresses wild type AcrAB	Husain *et al.*, 2004
pBAD33	Cm^r; expression vector	Guzman *et al.*, 1995
pBAD33-*acrA*	Cm^r; expresses AcrA	This study