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Functional Relationships between the AcrA Hairpin Tip Region and the TolC Aperture Tip Region for the Formation of the Bacterial Tripartite Efflux Pump AcrAB-TolC▽†‡

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Tripartite efflux pumps found in Gram-negative bacteria are involved in antibiotic resistance and toxic-protein secretion. In this study, we show, using site-directed mutational analyses, that the conserved residues located in the tip region of the α-hairpin of the membrane fusion protein (MFP) AcrA play an essential role in the action of the tripartite efflux pump AcrAB-TolC. In addition, we provide *in vivo* functional data showing that both the length and the amino acid sequence of the α-hairpin of AcrA can be flexible for the formation of a functional AcrAB-TolC pump. Genetic-complementation experiments further indicated functional interrelationships between the AcrA hairpin tip region and the TolC aperture tip region. Our findings may offer a molecular basis for understanding the multidrug resistance of pathogenic bacteria.

The tripartite efflux pumps that are found in Gram-negative bacteria have been implicated in their intrinsic resistance to diverse antibiotics, as well as their secretion of protein toxins (10, 12, 24, 31). The bacterial efflux pump is typically assembled from three essential components: an inner membrane transporter (IMT), an outer membrane factor (OMF), and a periplasmic membrane fusion protein (MFP) (10, 12, 24, 31). The IMT provides energy for transporters, like the resistance nodulation cell division (RND) type and the ATP-binding cassette (ABC) type (18). The OMF connects to the IMT in the periplasm, providing a continuous conduit to the external medium. This conduit uses the central channel, which is opened only when in complex with other components (11, 18). The third essential component of the pump is the MFP, which is an adapter protein for the direct interaction between the IMT and OMF in the periplasm (32). The MFP consists of four linearly arranged domains: the membrane-proximal (MP) domain, the β-barrel domain, the lipoyl domain, and the α-hairpin domain (1, 6, 16, 22, 30). The MFP α-hairpin domain is known to interact with OMF, while the other domains are related to interaction with the IMT (15, 22).

The *Escherichia coli* AcrAB-TolC pump, comprised of RND-type IMT-AcrB, MFP-AcrA, and OMF-TolC, is the major contributor to the multidrug resistance phenotype of the bacteria (7, 8, 25). The AcrAB-TolC pump, together with its homolog, the *Pseudomonas aeruginosa* MexAB-OprM pump (7, 13), has primarily been studied in order to elucidate the molecular mechanisms underlying the actions of the tripartite efflux pumps. Whereas the crystal structures of these proteins have revealed that RND-type IMTs (AcrB and MexB) and OMFs (TolC and OprM) are homotrimeric in their functional states (1, 6, 11, 16, 22, 30), the oligomeric state of MFP remains a topic of debate, despite the presence of crystal structures (3, 5, 17, 18, 22, 27, 30).

MacAB-TolC, which was identified as a macrolide-specific extrusion pump (9), has also been implicated in *E. coli* enterotoxin secretion (29). While MFP-MacA shares high sequence similarity with AcrA and MexA, IMT-MacB is a homodimeric ABC transporter that uses ATP hydrolysis as the driving force (9, 14). MacA forms hexamers, and the funnel-like hexameric structure of MacA is physiologically relevant for the formation of a functional MacAB-TolC pump (30). Although the α-hairpins from AcrA and MacA are commonly involved in the interaction with TolC (30, 32), the interaction mode between AcrA and TolC remains to be elucidated. In this study, we provide experimental evidence showing that the conserved amino acid residues in the AcrA hairpin tip region is important for the action of the AcrAB-TolC efflux pump and is functionally related to the TolC aperture tip region.

MATERIALS AND METHODS

Strains and plasmids. The plasmids and primers used in this study are listed in Table S1 in the supplemental material. BW25113 Δ*acrA* Δ*tolC210*::Tn*10* and BW25113 Δ*acrAB* Δ*tolC210*::Tn*10* were constructed from BW25113 Δ*acrA* and BW25113 Δ*acrAB*, respectively, by P1-mediated transduction using CAG12184 (Δ*tolC210*::Tn*10*) as a donor strain. The construction of the strain with *acrAB* deleted, BW25113 Δ*acrAB*, has been described previously (30).

PCR-amplified fragments encoding AcrA and TolC were digested with NdeI/

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XbaI and ligated into the same sites in pKAN6B to produce pAcrA1 and pTolC1, respectively. Two DNA fragments containing the 5′ untranslated region (UTR) and the coding region of AcrAB were amplified using PCR, digested with either NotI/DraIII or DraIII/XbaI, and ligated into NotI/XbaI sites in pKAN6B to produce pAcrAB1. Plasmid pAcrA2 was constructed by subcloning the NotI and XbaI fragments containing the coding region for AcrA from pAcrA1into the same sites in pPM30. Plasmids pAcrA1-R128D, -L132D, and -S139D were constructed using the overlap extension PCR method. Plasmids pAcrA-MacA hybrids 1, 2, 3, 4, and 5 for the chimeric AcrAs were constructed as follows. DNA fragments encoding the regions of AcrA (residues 1 to 120 for hybrids 1 and 3, residues 1 to 119 for hybrids 2 and 4, and residues 1 to 96 for hybrid 5), MacA (residues 124 to 147 for hybrids 1, 2, 3, and 4; residues 93 to 184 for hybrid 5), and AcrA (residues 145 to 397 for hybrids 1 and 2, residues 146 to 397 for hybrids 3 and 4, and residues 175 to 397 for hybrid 5) were separately amplified, and the three resulting PCR fragments were recombined using an overlap extension PCR method. The resulting PCR DNA was digested with NdeI/XbaI and ligated into the same sites in pKAN6B. Plasmids pPRO-AcrA-R128D, -L132D, and -S139D, which expressed mutant AcrA proteins, were constructed by ligating the NcoI- and XhoI-digested PCR DNA fragments amplified from pAcrA1 containing the corresponding mutations using overlap extension PCR into the same restriction enzyme sites in the pPROEX-HTA vector (Invitrogen). pAcrA2-derived plasmids for expression of AcrA cysteine variants were constructed by subcloning PCR DNA into the NotI and XbaI sites of pAcrA2. The PCR DNA was amplified using overlap extension PCR with primers listed in Table S1 in the supplemental material.

Overexpression and purification of recombinant proteins. The expression, purification, and generation of the substitution mutants of *E. coli* AcrA were followed using the same procedure as for *Actinobacillus actinomycetemcomitans* MacA (20).

Measurement of the MIC. Overnight cultures grown in LB medium containing appropriate antibiotics (100 μg of kanamycin, 100 μg of ampicillin, and/or 5 μg of tetracycline per ml) were diluted 1 to 100 in the same medium. At an optical density at 600 nm (OD_{600}) of 0.1, the expression of proteins was induced by adding 0.1% arabinose and/or 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the cultures, and they were incubated for 2 more hours. Approximately 10^4 of the induced cells were then added to the same medium containing 0.1% arabinose and/or 1 mM IPTG and novobiocin, erythromycin, or vancomycin at increasing concentrations. The cultures were grown for an additional 16 to 20 h, and the lowest concentration of novobiocin, erythromycin, or vancomycin that completely inhibited growth was designated the MIC.

Isolation of TolC variants that complement AcrA-R128D. To introduce random mutations into the TolC aperture end regions from both hairpins (145 to 155 and 363 to 373), each of the target regions was amplified and recombined using an overlap extension PCR method. The final PCR fragment was digested with NdeI/XbaI and ligated into the same sites in pTolC1, which resulted in pTolC1-M. Primers tolC-M1 and tolC-M2 were prepared by regulating the doping ratio (95:5) at the targeted TolC regions (residues 145 to 155 and 363 to 373) for random mutagenesis.

The pTolC1-M plasmid was transformed into BW25113 Δ*acrA* Δ*tolC210*::Tn*10* cells expressing nonfunctional AcrA mutants from pAcrA2-R128D, and the transformed cells were selected on LB agar medium containing 3 μg of novobiocin per ml, a concentration at which cells expressing AcrA-R128D do not grow.

CD. The concentration of each protein was 0.5 mg/ml in 20 mM Tris buffer (pH 8.0) containing 150 mM NaCl and 2-mercaptoethanol. The circular dichroism (CD) spectrum was recorded on a Jasco J-715 spectropolarimeter. Spectra were collected from 340 to 200 nm at an interval of 1 nm, with 3 accumulations.

RESULTS

The conserved residues at the α-helical tip region are crucial for AcrA function. Previous studies showed that the residues at the α-helical tip region of MacA, which are conserved among all MFPs, play essential roles both in the drug efflux function of the MacAB-TolC pump and in binding to TolC *in vitro* (28, 30). Since the functionally important residues tested in MacA are also conserved in the RND-type IMT-associated MFPs (Fig. 1), we performed a similar mutagenesis experiment for AcrA in *E. coli*. A single amino acid substitution of a strictly conserved residue, Arg128, Leu132, or Ser139, in the tip region of AcrA abolished the resistance of *E. coli* to antibiotics (Table 1). The results indicate that the conserved amino acid residues of the α-helical tip region of MFPs is functionally important, regardless of the subtypes of MFPs. Normal cellular expression and structural integrities of the mutant AcrA proteins were confirmed by Western blot analysis and circular-dichroism spectroscopy (see Fig. S1 and S2 in the supplemental material).

Both the length and the amino acid sequence of the α-hairpin of AcrA are flexible for the formation of a functional complex. To investigate whether the length and the amino acid sequence of the α-hairpin of MFPs affect the formation of functional efflux pumps, we constructed chimeric AcrAs, whose α-hairpins were partially or completely replaced with the *E. coli* MacA hairpin. AcrA and MacA are commonly associated with TolC in *E. coli*, while AcrA and MacA are coupled with distinct types of IMT. The α-hairpin of MFP is linked to the cognate OMF, and the other domains are engaged with the IMT (15, 21, 22, 30). There are structural features of MacA similar to those of AcrA and MexA that have been proposed (30). The MacA α-hairpin is longer than those of AcrA and MexA by 7 (1 heptad) and 14 (2 heptads) residues, respectively (Fig. 1).

The first chimeric AcrA (Ec AcrA-MacA hybrid 1) has only the α-hairpin turn region (residues 124 to 147) of MacA instead of the matched AcrA α-hairpin turn region (residues 121 to 144). The second chimeric AcrA (Ec AcrA-MacA hybrid 5) has the complete MacA hairpin (residues 93 to 184), which is longer than that of AcrA (residues 97 to 174) by 14 amino acids (Fig. 2). Because the coiled-coil heptad rule is considered at the joint of the two α-hairpins, the chimeric protein should show the conformation of the continuous coiled-coil α-hairpin, like wild-type AcrA. As negative controls, chimeric AcrA constructs (Ec AcrA-MacA hybrids 2, 3, and 4), otherwise identical to Ec AcrA-MacA hybrid 1 and containing an incomplete AcrA-MacA hairpin that lacks 1 or 2 amino acids, were used (Fig. 2). These chimeric AcrAs disobey the coiled-coil heptad rule due to the missing amino acid in the α-hairpin turn region.

These chimeric AcrAs were tested for their *in vivo* function. As shown in Table 2, two chimeric AcrAs (Ec AcrA MacA hybrids 1 and 5) that contain partly or completely replaced MacA α-hairpins fully complemented the function of the wild-type AcrA. In contrast, the *in vivo* functions of Ec AcrA MacA hybrids 2, 3, and 4, which were used as negative controls for the coiled-coil heptad rule, were abolished (Table 2). These results show that the length and the amino acid sequence of the α-hairpin of MFPs are flexible for the formation of a functional complex.

Highly specifically complementing mutations were found at the aperture end of TolC. The 3 residues (Arg128, Leu132, and Ser139) of AcrA play an essential role in the function of the tripartite pump. To examine whether the α-helical tip region of AcrA is functionally related to the TolC aperture, we designed experiments to select for TolC variants that can complement the nonfunctional AcrA mutant bearing R128D. Random mutations were introduced into the DNA segments encoding the TolC aperture end regions from both hairpins (amino acid residues 145 to 155 and 363 to 373) and were ligated into pTolC1. The resulting ligation products were transformed into *E. coli* cells with *acrA* and *tolC* deleted (BW25113 Δ*acrA* Δ*tolC210*::Tn*10*) harboring pAcrA2-R128D. The transfor-

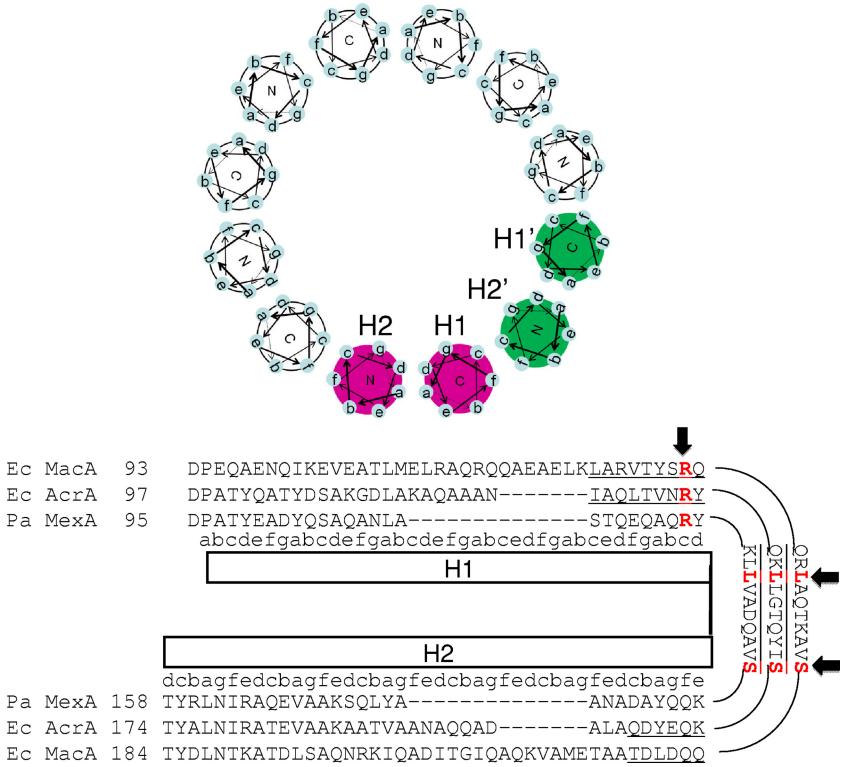

FIG. 1. Sequence comparison of α-hairpins with antiparallel coiled-coil structures. (Top) A lateral section of the α-helical barrel is shown in the helical-wheel representation. The helical wheels of six double-stranded antiparallel coiled coils with residues from one helix are labeled a to g (19). Note that the "lock-and-hole" interactions occur between c and f residues from the adjacent α-helix of the neighboring protomer, while the typical "lock-and-hole" interactions occur between a and d residues from two α-helices (H1 and H2) in a protomer. (Bottom) Sequence alignment of α-hairpins from four MFPs. The corresponding heptad positions are shown below or above the sequence. The strictly conserved residues in the tip region are indicated by the arrows. The target amino acid sequences for construction of Ec AcrA-MacA hybrid 1 (Fig. 2B) are underlined. Note that the differences in the lengths of α-hairpins are in multiples of seven. In the sequence alignment, Ec stands for *E. coli* and Pa for *P. aeruginosa*.

mants were selected for novobiocin resistance at a concentration (3 μg/ml) that does not allow survival of the parental *E. coli* cells expressing AcrA-R128D and wild-type TolC. Nine out of about 10^4 transformants showed a gain of ability to grow in the presence of 3 μg/ml novobiocin. The sequencing analysis showed that these complementing clones contained 1- to 4-amino-acid substitutions in the mutagenized region of the TolC coding region (Table 3).

The isolated *tolC* clones greatly restored the novobiocin resistance of the cells expressing the nonfunctional AcrA2-R128D (MIC values from 0.78 to 12.5 μg/ml). In addition, most of the TolC mutants (eight out of nine) did not complement AcrA-L132D or AcrA-S139D as well as they did AcrA2-R128D. The isolated TolC mutants appeared to be impaired in forming the functional drug efflux pump when coexpressed with wild-type AcrA in the cells. The MIC values of those cells with coexpressed TolC mutants and wild-type AcrA ranged from 1.56 to 6.25 μg/ml, while the MIC of the cells that coexpressed wild-type AcrA and TolC proteins was 25 μg/ml. These results showed the specificity of functional complementation of AcrA2-R128D by the isolated TolC mutants. In the complementing TolC mutants, residues Val149, Ile151, I369, Val370, and Leu373 were also involved in restoring the functions of the nonfunctional AcrA mutants. These residues are located in the

TABLE 1. Effects of amino acid substitutions for the conserved residues in the AcrA hairpin tip region on the *in vivo* function of the AcrAB-TolC pump[a]

Antibiotic	MIC (μg/ml)[b]				
	pKAN6B	pAcrA1 (WT [His])	pAcrA1-R128D (R128D [His])	pArcA1-L132D (L132D [His])	pAcrA1-S139D (S139D [His])
Novobiocin	6.25	100	6.25	6.25	6.25
Erythromycin	3.13	50	3.13	3.13	3.13

[a] Wild-type or AcrA mutants were overexpressed in *E. coli* strain BW25113 Δ*acrA*, and the degree of resistance to novobiocin and erythromycin was measured. The AcrA protein expression is shown in Fig. S1 in the supplemental material. To confirm the structural integrity of the mutant AcrAs, the recombinant versions of mutant AcrA proteins were applied to circular-dichroism spectroscopy (see Fig. S2 in the supplemental material).

[b] In the BW25113 Δ*acrA* background with the indicated plasmids and substitutions. WT, wild type. The antibiotic concentrations used to measure MICs were 0, 0.78, 1.56, 3.13, 6.25, 12.50, 25.00, 50.00, and 100.00 μg/ml.

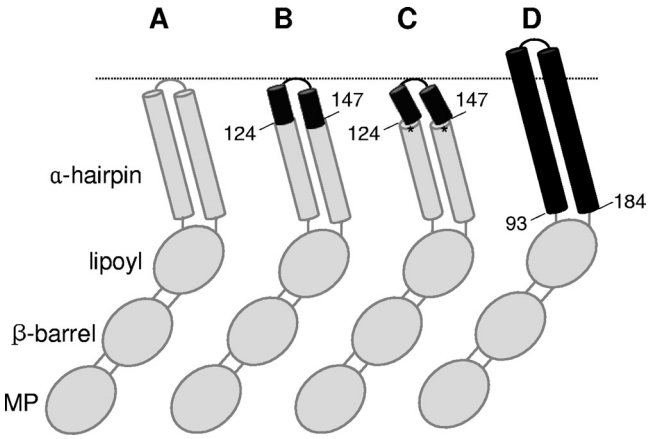

FIG. 2. Schematic drawings for the chimeric AcrA constructs used in Table 2. (A) Structure of wild-type AcrA. (B) Ec AcrA-MacA hybrid 1. Only the turn region of the α-hairpin of AcrA was replaced with the corresponding region of MacA (residues 124 to 147) (black). (C) Ec AcrA-MacA hybrids 2, 3, and 4. The AcrA α-hairpin was incompletely replaced with the MacA α-hairpin. Only 1 amino acid (residue 120 or 145 of AcrA) (*) is missing, compared with Ec AcrA MacA hybrid 1. (D) Ec AcrA-MacA hybrid 5. The whole α-hairpin of AcrA was replaced with the MacA α-hairpin (residues 93 to 184) (black). Note that the α-hairpin of the chimeric AcrA protein is longer than that of wild-type AcrA because of the longer α-hairpin of MacA.

conserved turn region, which makes a small hydrophobic pocket at the aperture end of TolC (11).

To characterize the basis of complementation of AcrA-R128D by the isolated TolC variants, we tested the states of the TolC apertures of the TolC variants by measuring the degrees of sensitivity of cells that coexpressed the isolated TolC variants and wild-type AcrA to a large hydrophilic antibiotic, vancomycin. This antibiotic has been used to examine the influx of antibiotics through TolC tunnels that are in a constitutively open state because it cannot penetrate the intact outer membrane and is not a substrate of the AcrAB-TolC pump (2, 4). It has been reported that alterations in the TolC aperture region could lead to a constitutively open TolC aperture and resulted in the increased influx of antibiotics (2). Expression of most of the isolated TolC variants exerted moderately increased sensitivities to vancomycin (MIC range, from 150 to 225), while expression of a TolC variant containing R367S and T368S exhibited a degree of resistance to vancomycin similar to that of wild-type TolC. The results indicate that vancomycin-sensitive TolC mutants are likely to be leaky mutants whose antibiotic efflux functions are complemented by AcrA-R128D (Table 3; see Fig. S4 in the supplemental material). It is therefore possible that the vancomycin-sensitive compensatory TolC mutants isolated in this study induce partial opening of the TolC aperture to overcome the defect of the AcrA hairpin tip region mutant, which fails to induce wild-type TolC aperture opening.

With our results, we were able to obtain complementing TolC mutants that restored the functions of the critical residues at the tip of AcrA without changing the residues on the outer surface region of TolC. These results indicate that the TolC aperture end region is functionally related to the AcrA α-hairpin tip region.

DISCUSSION

In the present study, we demonstrated that the hairpin tip region of AcrA plays an important role in the action of the AcrAB-TolC pump and that the length of the α-hairpin is somewhat flexible in its function. Replacing the whole α-hairpin of AcrA with a longer MacA (Fig. 2 and Table 2), or a shorter *P. aeruginosa* MexA hairpin (21), did not alter the efficiency of the chimeric AcrA function, despite large changes in the length and the sequence of the hairpin region. In contrast, a single substitution mutation in the hairpin tip region of MFPs completely abolished binding to TolC *in vitro* (28) and resistance to antibiotics *in vivo* (Table 1). Furthermore, genetic-complementation experiments showed that the AcrA α-hairpin tip region is functionally related to the TolC aperture tip region (Table 3). The identified compensatory mutations in the TolC aperture were highly specific to AcrA-R128D; therefore, it is unlikely that functional complementation by TolC mutants results from alterations in the TolC aperture that lead to opening or weakening of TolC closure and allow drug efflux.

Functional interrelationships between the AcrA α-hairpin tip region and the TolC aperture tip region can explain the structural and functional conservation of the α-hairpin tip region of MFP homologs for the formation of the functional AcrAB-TolC pump, regardless of variation in the length of the α-hairpin. The cogwheel structure is not changed in the shorter or longer hairpins of other MFPs. The conserved residues in the cogwheel of the α-hairpin tip region are not changed by replacing the α-hairpins. The importance of the AcrA α-hairpin tip region for binding to TolC has been shown by *in vivo* chemical-cross-linking experiments that utilized AcrA variants containing a single cysteine at the targeted residue (15). However, our results showing functional interrelationships between the AcrA α-hairpin tip region and the TolC aperture tip region cannot be easily explained by the currently prevailing model, in which the AcrA molecules wrap outside the TolC α-barrel using the α-hairpins (3, 15, 22, 23). For this reason, we attempted to perform chemical-cross-linking experiments that have been used by other research groups to detect interactions between the components of the AcrAB-TolC efflux pump (15, 26). First, we constructed all of the Cys-mutated forms of AcrA that showed interactions with wild-type TolC (15). However, inconsistent with the results that were previously reported (15), all of these AcrA cysteine variants resulted in decreased func-

TABLE 2. *In vivo* effects of *E. coli* AcrA chimeric proteins[a]

Antibiotic	MIC (μg/ml)[b]						
	pKAN6B	pAcrA1	pAcrA-MacA hybrid				
			1	2	3	4	5
Novobiocin	6.25	100.00	100.00	6.25	6.25	6.25	100.00

[a] The effects of the *E. coli* AcrA chimeric protein, whose hairpin tip region was replaced with that of *E. coli* MacA, are shown. The effects of overexpressing the wild-type AcrA (C-terminally hexahistidine tagged; pAcrA1) or the chimeric AcrA-MacA hybrid (C-terminally hexahistidine tagged; pAcrA-MacA hybrid) on the resistance of *E. coli* strain BW25113 Δ*acrA* to novobiocin were measured as MICs. The level of AcrA protein expressed is shown in Fig. S3 in the supplemental material.

[b] In the BW25113 Δ*acrA* background with the indicated plasmids. The novobiocin concentrations used to measure MICs were 0, 0.78, 1.56, 3.13, 6.25, 12.50, 25.00, 50.00, and 100.00 μg/ml.

TABLE 3. TolC variants complementing the nonfunctional AcrA mutants[a]

TolC protein[c]	Vancomycin[d] MIC (μg/ml) (AcrA-wt[b])	Novobiocin[e] MIC (μg/ml)			
		AcrA-wt[b]	AcrA-R128D	Acr-L132D	AcrA-S139D
TolC[−][f]	500	0.78	0.78	0.78	0.78
TolC-wt	300	25.00	1.56	1.56	1.56
TolC-N145I/I151L/L373S	175	3.13	12.50	1.56	0.78
TolC-V149E/V370A	200	6.25	12.50	3.13	0.78
TolC-V149E/V372L/L373V	175	1.56	12.50	3.13	0.78
TolC-V149L/V364I/I369L/V370G	225	6.25	12.50	3.13	12.50
TolC-V149L/V370A	225	6.25	12.50	3.13	12.50
TolC-V149L/V370D	175	6.25	12.50	6.25	0.78
TolC-V164 M/R367S/V370I/A418V	150	6.25	12.50	1.56	0.78
TolC-R367S/T368A	300	6.25	6.25	1.56	1.56
TolC-V370F	200	6.25	12.5	1.56	0.78

[a] *E. coli* strain *BW25113 ΔacrA Δtolc210*::Tn*10* was used to select TolC variants that were capable of complementing the nonfunctional AcrA variants. Clones resistant to novobiocin at 3 μg/ml were selected. The levels of wild-type AcrA and TolC variant protein expressed are shown in Fig. S4 in the supplemental material.
[b] AcrA-wt and AcrA variants were expressed from pAcrA2.
[c] TolC-wt and TolC variants were expressed from pTolC1.
[d] An increment of 25 μg of vancomycin/ml between 0 and 550 μg of vancomycin/ml was used to measure MICs.
[e] 0, 0.78, 1.56, 3.13, 6.25, 12.50, and 25.00 μg of novobiocin/ml were used to measure MICs.
[f] An empty vector, pKAN6B, was used.

tion of the AcrAB-TolC efflux pump, as indicated by increased sensitivity to novobiocin (see Fig. S5 and Table S2 in the supplemental material). While it is conceivable that partially functional AcrA cysteine variants can still be used for detecting interactions between AcrA and TolC, we could not use this method, since we thought that introduction of an additional cysteine mutation to the nonfunctional AcrA-R128D would further demolish the function of the AcrAB-TolC efflux pump and would consequently produce experimental results that are hard to interpret. We believe that no other methods are currently available that allow us to detect direct interactions between AcrA and TolC. Considering the overwhelming amount of experimental and theoretical data that support the currently prevailing model, one attractive explanation for our results is that functional interactions between the AcrA α-hairpin tip region and the TolC aperture tip region are required at a certain stage of the assembly process of an efflux-ready complex.

While further structural and functional studies on the binary and ternary complexes are required to envisage the fully assembled complex, we have provided experimental results implicating functional interactions between the AcrA α-hairpin tip region and the TolC aperture tip region in the assembly of the functional tripartite efflux pump.

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