Isolation and Characterization of VceC Gain-of-Function Mutants That Can Function with the AcrAB Multiple-Drug-Resistant Efflux Pump of *Escherichia coli*

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VceC is the outer membrane component of the major facilitator (MF) VceAB-VceC multiple-drug-resistant (MDR) efflux pump of *Vibrio cholerae*. TolC is the outer membrane component of the resistance-nodulation-division AcrAB-TolC efflux pump of *Escherichia coli*. Although these proteins share little amino acid sequence identity, their crystal structures can be readily superimposed upon one another. In this study, we have asked if TolC and VceC are interchangeable for the functioning of the AcrAB and VceAB pumps. We have found that TolC can replace VceC to form a functional VceAB-TolC MDR pump, but VceC cannot replace TolC to form a functional AcrAB-VceC pump. However, we have been able to isolate gain-of-function (gof) VceC mutants which can functionally interface with AcrAB. These mutations map to four different amino acids located at the periplasmic tip of VceC. Chemical cross-linkage experiments indicate that both wild-type and gof mutant VceC can physically interact with the AcrAB complex, suggesting that these gof mutations are not affecting the recruitment of VceC to the AcrAB complex but rather its ability to functionally interface with the AcrAB pump.

Multiple-drug-resistant (MDR) efflux pumps contribute to the intrinsic and acquired antibiotic resistance in bacteria and significantly impact on the growing problem of emerging drug-resistant pathogens (21, 22, 28–32, 43, 44). The purpose of these pumps is to remove deleterious agents, including antibiotics, from the cell, thus lowering their intracellular accumulation and effectivly increasing the antibiotic dose needed for therapeutic intervention. In gram-negative bacteria, these efflux pumps are usually tripartite in architecture, composed of three distinct classes of proteins: (i) cytoplasmic membrane translocase proteins (CMTs), which often utilize proton-motive force as an energy source and act as proton antiporters; (ii) outer membrane channel or efflux proteins (OEPs), through which substrates traverse the periplasm and outer membrane; and (iii) periplasmic accessory or membrane fusion proteins (MFPs), which are often tethered to the cytoplasmic membrane and act to consolidate the CMTs and OEPs to form an active MDR efflux complex (22, 25). Unlike most transporters, MDR pumps often have broad overlapping substrate specificities, including a variety of antibiotics. These efflux systems can be amplified in resistant cells (20, 24, 30) and can shift or expand their substrate profiles with mutation (14, 34). MDR extrusion pumps in conjunction with other antibiotic resistance mechanisms result in highly resistant bacteria, making them a major threat to antibiotic therapy. Understanding the structure, assembly, and mechanism(s) of action of these efflux systems will be paramount to the design and development of new drugs which can either inactivate or circumvent their action.

Well-studied examples of gram-negative MDR efflux pumps include the *Escherichia coli* AcrAB-TolC and the *Pseudomonas aeruginosa* MexAB-OprM pumps (31, 39, 45, 46). Chemical cross-linkage studies have indicated that all three components of the AcrAB-TolC pump interact with each other (16, 40, 41), and crystal structures for OEPs (TolC [19], OprM [3], and VceC [11]), for an MFP (MexA [15]), and for a CMT (AcrB [27]) have been solved. This information has led to several models on how these pump components interact to form a functional efflux pump complex (2, 4, 9–12, 15, 18, 19, 27, 41). However, detailed interactions between these different components, their stoichiometry, and the means by which they remove substrates from the cell are just beginning to be deciphered.

Recently, the crystal structure of VceC, an OEP for the *Vibrio cholerae* VceAB pump (7, 43), has been solved (11), and its architecture has been shown to be very similar to that of TolC (19) and OprM (3). In fact, despite a very low degree of amino acid sequence identity, these three OEP structures can be readily superimposed upon one another (11). However, regardless of the similarities in these OEP structures, it has been suggested that the VceA-VceB-VceC pump may differ substantially from the AcrA-AcrB-TolC pump in details of the protein-protein interaction and subunit stoichiometry (11).

In this report we have examined the interchangeability of TolC and VceC with the AcrAB and VceAB pumps. We have found that while TolC can functionally replace VceC of the VceAB-VceC MDR efflux pump, VceC does not function with the AcrAB pump. We have, however, been able to select VceC gain-of-function (gof) mutations, which enables the mutant VceC to function with the AcrAB pump in *E. coli*. These substitution mutations map to four different amino acids positioned at the periplasmic tip of VceC, in alpha helixes 3, 4, and 8, where it may interact with AcrB. Finally, chemical cross-linkage experiments have indicated that both wild-type and gof mutant VceC can physically interact with the AcrAB complex.
TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant characteristic(s) | Reference |
|-------------------|-----------------------------|-----------|
| LBB1149           | W4573 (wild type)           | 13        |
| LBB1135           | N453 acrAB derivative of W4573 | 13       |
| LBB1136           | tolC derivative of LBB1135  | 13        |
| LBB1175           | tolC::Tn10 derivative of LBB1149 | 13   |

| Plasmids         | Relevant characteristic(s) | Reference |
|------------------|----------------------------|-----------|
| pVC2             | pBR322 carrying vceCAB      | 7         |
| pVC4             | pBR322 carrying vceC        | 7         |
| pVC91            | pACYC184 carrying vceC      | 43        |
| pVC91-A209V       | pVC91 with gain-of-function mutation A209V | This study |
| pVC91-L219R       | pVC91 with gain-of-function mutation L219R | This study |
| pVC91-L219F       | pVC91 with gain-of-function mutation L219F | This study |
| pVC91-Q236R       | pVC91 with gain-of-function mutation Q236R | This study |
| pVC91-V445E       | pVC91 with gain-of-function mutation V445E | This study |
| pTolC            | tolC                        | 13        |

No inhibitors, including novobiocin (NOV), could replace VceC in the VceAB-VceC pump (7). Deoxycholate (DOC) is a substrate for both VceAB-VceC and AcrAB-TolC pumps (29, 37). On the other hand, the antibiotic novobiocin (NOV), which is a substrate for the AcrAB-TolC pump (13, 29, 37), is not a substrate for the VceAB-VceC pump (7). Deoxycholate (DOC) is a substrate for both VceAB-VceC and AcrAB-TolC pumps (7, 13, 29, 37). The differences in substrate specificity between these two MDR efflux pumps allow us to distinguish between their functioning in the same cell. To determine if TolC could replace VceC in the VceAB-VceC pump, we placed a plasmid carrying the vceCAB genes (pVC91) (7) into an acrAB mutant (LBB1135) (13) and examined its sensitivity to CCCP, DOC, and NOV. The results are presented in Table 2 and indicate that TolC can replace VceC in the functioning of the VceAB pump (i.e., in the removal of CCCP and DOC but not NOV).

VceC cannot replace TolC for resistance to CCCP, DOC, or NOV. To determine if VceC can replace TolC in the functioning of MDR efflux pumps which remove CCCP, DOC, or NOV, we transformed a tolC mutant (LBB1175) (13) with a plasmid carrying vceC (pVC91) (43) and examined the transformant’s sensitivity to these inhibitors. As can be seen from Table 3, the absence of VceC does not increase resistance to these inhibitors or to any other inhibitors we have examined, including nalidixic acid, erythromycin, acriflavine, sodium dodecyl sulfate, and bile salts, in a tolC mutant (data not shown).

Isolation of gain-of-function (gof) mutants. E. coli tolC null mutants are hypersensitive to detergents and bile salts and cannot grow on an LB agar plate containing 0.05% DOC (DOC plates) (13). In search of vceC gof mutations which would enable VceC to functionally replace TolC, we selected

### RESULTS

**TolC can replace VceC in the functioning of the VceAB pump.** The protonophore cyanide carbonyl m-chlorophenyl-hydrazine (CCCP) is a substrate of the VceAB-VceC MDR efflux pump when expressed in an E. coli tolC mutant (7). It is not a substrate for the major E. coli MDR pump, AcrAB-TolC (29, 37). On the other hand, the antibiotic novobiocin (NOV), which is a substrate for the AcrAB-TolC pump (13, 29, 37), is not a substrate for the VceAB-VceC pump (7). Deoxycholate (DOC) is a substrate for both VceAB-VceC and AcrAB-TolC pumps (7, 13, 29, 37). The differences in substrate specificity between these two MDR efflux pumps allow us to distinguish between their functioning in the same cell. To determine if TolC could replace VceC in the VceAB-VceC pump, we placed a plasmid carrying the vceCAB genes (pVC91) (7) into an acrAB mutant (LBB1135) (13) and examined its sensitivity to CCCP, DOC, and NOV. The results are presented in Table 2 and indicate that TolC can replace VceC in the functioning of the VceAB pump (i.e., in the removal of CCCP and DOC but not NOV).

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for DOC resistance by plating a tolc deletion mutant, carrying pVC91, on DOC plates. Using this selection scheme, we were able to isolate DOC-resistant mutants. Plasmids from these mutants were transformed back into a tolc deletion mutant to determine if the gof phenotype cosegregated with pVC91. In all cases it did. Thus far, we have sequenced 15 independently isolated vceC gof mutations. All are point mutations which map to one of four different codons in vceC (Table 4). It should be pointed out that all of the vceC gof mutant OEPs are still functional with the VceAB pump, as determined by sensitivity to CCCP and DOC in a tolC, acrAB background (data not shown). We have also found that the relative amount of VceC produced in cells carrying the vceC gof mutations (pVC91-gof) was very similar to that produced from the same cells carrying wild-type vceC (pVC91) (Fig. 1).

Evidence that VceC gof mutant proteins function with the AcrAB MDR efflux pump. To determine if the VceC gof mutants are utilizing the AcrAB pump for DOC resistance, we have examined a tolc acrAB strain (LBB1136) (13) carrying the vceC gof alleles for sensitivity to DOC, NOV, and CCCP. We have also examined a tolc acrB strain to determine if AcrD might be involved, since it utilizes AcrA and TolC for function (2). In both cases the presence of the vceC gof alleles did not alter the sensitivity of these strains to DOC, NOV, or CCCP (data not shown). We have also determined that the vceC gof mutations could not complement a tolc null mutant with respect to colicin E1 tolerance, hemolysin secretion, and sensitivity to U3 phage (data not shown), suggesting that these VceC gof mutants cannot replace TolC for all of its functions and appear to be specific for the functioning of an MDR efflux pump (e.g., the AcrAB pump). Finally, we have determined the MICs for a tolc mutant (LBB1175) carrying the vceC gof alleles for DOC, NOV, and CCCP (Table 5). It is apparent from these results that the VceC gof mutants increase the resistance of LBB1175 to DOC and NOV, although not to the same extent as does TolC. Furthermore, the VceC gof mutants do not provide resistance to CCCP, which is what one would expect if the mutant VceCs were functioning with the AcrAB pump.

Location of gof mutations in the crystal structure of VceC. According to the crystal structure of VceC (11), three of the gof substitutions map to alpha helix H4 (Q236R), one maps to alpha helix H3 (A209V, L219R, and L219F), one maps to alpha helix H8 (V445E). An anti-ATPase β-subunit (8) was used to detect ATPase as an internal loading standard (lower panel).

![FIG. 1. Western blot analysis of VceC from cells (LBB1175) expressing different vceC gof alleles (lanes 2 to 6). Exponentially grown cells were collected by centrifugation, adjusted to equal cell numbers as determined by absorbance at an optical density of 600 nm, reduced and boiled with sample buffer, and separated on SDS-PAGE. Proteins were transferred to a PVDF membrane and immunoblotted using anti-VceC polyclonal antibody (upper panel) as described in Materials and Methods. Lanes: 1, pVC91 (wild-type VceC); 2, pVC91-A209V; 3, pVC91-L219R; 4, pVC91-L219F; 5, pVC91-Q236R; and 6, pVC91-V445E. An anti-ATPase β-subunit (8) was used to detect ATPase as an internal loading standard (lower panel).](http://jb.asm.org/)

### Table 3. VceC does not function with AcrAB

| Strain       | Relevant genotype | Sensitivity (mm)†  |
|--------------|-------------------|--------------------|
|              |                   | CCCP | NOV | DOC |
| LBB1149      | Parent            | 17   | 15  | 8   |
| LBB1175      | tolc              | 37   | 31  | 25  |
| LBB1175/pVC91| tolC vceC⁺         | 37   | 30  | 26  |
| LBB1175/pTolC| tolC⁺             | 18   | 18  | 8   |
| LBB1175/pVC2 | tolC vceC⁺ vceA⁺ vceB⁺ | 10   | 31  | 0   |

† Sensitivity to indicated inhibitors was determined as described for Table 2. The sensitivity values are the averages of three or more experiments rounded off to the nearest whole number.

### Table 4. Gain-of-function (gof) mutants of VceC

| No. of mutants | Mutation | Location on: |
|---------------|----------|--------------|
| 5             | GCG > GTG | A209V (H3)   |
| 2             | CTT > TTT | L219R (H3)   |
| 3             | CTT > CTT | L219R (H3)   |
| 4             | CAA > GAA | Q236R (H4)   |
| 1             | GTA > GAA | V445E (H8)   |

### Table 5. Sensitivities of vceC gof mutants to inhibitors

| Strain       | Relevant genotype | DOC | CCCP | NOV |
|--------------|-------------------|-----|------|-----|
| LBB1175      | tolc              | 78  | 2.5  | 1   |
| LBB1175/pVC91| vceC              | 78  | 2.5  | 1   |
| LBB1175/pVC91-A209V| vceC A209V | 1,250 | 2.5  | 4   |
| LBB1175/pVC91-L219R| vceC L219R | 1,250 | 2.5  | 6   |
| LBB1175/pVC19-L219F| vceC L219F | 1,250 | 2.5  | 6   |
| LBB1175/pVC91-Q236R| vceC Q236R | 938  | 2.5  | 4   |
| LBB1175/pVC91-V445E| vceC V445E | 938  | 2.5  | 4   |
| LBB1175/pTolC| tolC⁺           | >10,000 | >20 | >40 |
The architecture of the OEPs whose crystal structure has been solved (ToIC, OprM, and VceC) has substantially from the AcrAB-TolC pump with respect to the substantial differences in sequence identity (39, 44). However, even these OEPs show some similarities, even though their amino acid sequence identity is quite low (3, 11, 19). In each case the homotrimeric proteins make up a long “cannon-shaped” structure consisting of a 40-Å-long β-barrel, which passes through the outer membrane and a 100-Å-long α-helical barrel which projects into the periplasm and is closed at its periplasmic end (19). Based on this structure and the crystal structures of AcrB (27) and MexA (15), an AcrA homologue, and with the evidence that TolC could be cross-linked independently to either AcrA or AcrB (16, 40, 41), models have been proposed which attempt to explain the assembly and function of MDR pumps (2, 4, 9–12, 15, 18, 19, 27, 41). In such models, the periplasmic ends of a trimeric AcrB and trimeric TolC are envisioned to dock in such a manner as to form a continuous channel which crosses the periplasm and spans the outer membrane. The periplasmic contact between the CMT and OEP has been suggested to involve the TolC/EmrE triad of efflux pump (30). During the assembly of the MDR pump, a periplasmic end of the OEP must open in order for the pump to function. This transition to the open state has been likened to an “iris-like” realignment of the entrance helices (4, 19). This opening of the OEP is thought to occur through conformational changes in TolC via its interaction(s) with either CMT or MFP (19). The observation that a “locked” TolC (i.e., by introducing intermonomer disulfide bridges at the narrowest point of the TolC entrance constriction) can still be recruited by the HylBD complex suggests that the opening step can be decoupled from recruitment and assembly steps (4). Obviously, the opening of the OEP is a key step in the functioning of an efflux pump.

In this study, we have shown that E. coli ToIC can function with the V. cholerae VceAB efflux pump but that VceC cannot function with the E. coli AcrAB efflux pump. We were, however, able to isolate VceC gof mutants which enabled this OEP to functionally interface with the AcrAB pump, albeit not as effectively as ToIC (Table 5). These results demonstrate that VceC can replace ToIC for the functioning of the AcrAB pump and suggest that the VceAB-VceC pump may not differ substantially from the AcrAB-ToIC pump with respect to the assembly of the OEP with the pump complex. All of the isolated vceC gof mutations were single-base-pair substitutions (transitions and transversions) that mapped to the β-barrel (distal) end, and the amino acid substitutions. The protomer is aligned vertically. The tation of a single protomer of VceC indicating the location of the gof from the Protein Data Bank website (http://www.rcsb.org/pdb/), and the crystal structure of VceC was downloaded 3760 VEDIYAPPAN ET AL. J. BACTERIOL.
four different codons, resulting in five different amino acid substitutions. These substitutions appear to be specific and are located at positions near the periplasmic tip of VceC in the H3, H4, and H8 alpha helices. An analogous region of TolC has been proposed to interact with AcrB, such that the six hairpins at the periplasmic end of the AcrB trimer could dock with the six α-helix-turn-α-helix structures at the base of the TolC trimer (18, 19). We have compared the amino acid substitutions of the vceC gof mutations with the corresponding amino acid residues of TolC (Table 4) as determined by superposition of VceC on TolC (11). It can be seen that none of the gof substitutions resulted in the same amino acid as that found in TolC. In fact, for the most part, the substitutions were of a different class than the corresponding TolC residue. We also found no difference in the functionality of VceC containing a single substitution versus VceC containing four gof substitutions, as determined by DOC, NOV, and CCCP sensitivity (data not shown). These results, and the findings that VceC gof mutations do not affect their ability to functionally interact with the VceAB pump and wild-type VceC can be cross-linked to the AcrAB pump, suggest that the gof substitutions may not define amino acids directly involved in the physical docking of VceC to the AcrAB complex. A more likely explanation would be that these substitutions change the structural aspects of VceC such that it can functionally interface with the AcrAB pump. This could involve interactions between or within protomers. Interestingly, one of the gof mutations targets an acidic (Glu) residue, similar to that found at the same location in TolC (D374). It has been hypothesized that this amino acid and A449, which become exposed to the solvent upon the opening of the VceC channel, may interact with VceA in the assembly of the VceAB-VceC pump (11). However, as stated above, this change does not appreciably affect its ability to function with VceAB.

Models for the assembly of MDR efflux pumps have suggested that the functional docking of an OEP with its corresponding pump involves a two-step process: (i) the physical docking of the OEP with its corresponding CMT-MFP pump and (ii) the opening of the OEP channel (9, 12, 18, 19, 27). Our results and those of Andersen et al. (4) support that view and suggest that these two steps can be genetically separated. Hence, an intriguing, albeit speculative, explanation for our results would be that the difference between wild-type VceC and the VceC gof proteins is the ability of the mutant VceC to undergo the transition to the open state when in association with the AcrAB complex (or persist in an open or partially open state), whereas the wild-type VceC cannot. Deciphering the differences in the interaction(s) between VceC and VceC gof proteins with the AcrAB complex may provide important insights into the functional assembly and specificity of OEPs with their CMT-MFP pump counterparts.

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