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Evidence that the C-terminal region is involved in the stability and functionality of OprM in *E. coli*

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ABSTRACT

In order to understand the specificity of interactions between the components of multidrug-resistant (MDR) efflux pumps and how they are recruited/assembled, we analyzed the effect of C-terminal truncation, deletion, and peptide swapping on the stability and functionality of OprM in *Escherichia coli*. The efflux activity of OprM was not affected by removing up to 19 amino acid residues from the C-terminus, while depletion of more than 20 residues or disruption the $_{463}$LGGG$_{466}$ motif diminished both the stability and activity of OprM. The replacement of the OprM C-terminus 23 residues with the corresponding part of TolC or VceC did not affect the stability and the functionality of OprM. Therefore, it is confirmed that the C-terminal $_{463}$LGGG$_{466}$ motif is one of the crucial components for the stability of OprM and for the functionality of the OprM-VceAB chimeric pump in *E.coli*. The results also indicate that one residue substitution on the hairpin domain of the membrane fusion protein (MFP) VceA could suppress the null like mutations on the C-terminal modified OprM. This finding will be the direct genetic evidence that the C-terminal domain of outer efflux protein (OEP) is involved in the functional assembly of OEP-MFP.

1. Introduction

Multi-drug resistant (MDR) efflux pumps in bacteria can severely diminish the effects of many antibiotics. Bacteria use these pumps to remove not only the antibiotics but also some detergents and organic solvents, thus reducing their overall concentration in the cell (Zgurskaya and Nikaido, 2000). Furthermore, an expansion in their substrate profile as well as an increase in their expression can be brought by mutation (Gerken and Misra, 2004; Vediyappan et al., 2006; Nehme and Poole, 2007). Typically, this proton driven MDR efflux pump drug exportation is comprised of three proteins. The first component being identified is the outer membrane channel or efflux proteins (OEP) (Paulsen et al., 1997). Its periplasmic section may be near to or in-contact with the second component of the pump, and the cytoplasmic membrane translocase proteins (CMT) (Vediyappan et al., 2006). The OEP and CMT form a continuous channel to export antimicrobials through the periplasmic space and outer membrane. Besides its drug specificity, CMT has also been recognized as a proton antiporter that provides energy for drug exporting (Vediyappan et al., 2006). The third member, membrane fusion proteins (MFP), is believed to anchor to the cytoplasmic membrane and acts as an adaptor to bring OEP and CMT together (Poole, 2004). Although the crystal structures of all three components of the RND (Resistance-Nodulation-Division) pump (Marger and Saier, 1993): AcrAB-TolC, MexAB-OprM, and VceC of MFS (*M*ajor-*F*acilitator-*S*uperfamily) pump VceAB-VceC have been solved (Koronakis et al., 2000; Elkins and Nikaido, 2003; Akama et al., 2004a,b; Federici et al., 2005a; Mikolosko et al., 2006; Sennhauser et al., 2009), the details by which the three proteins interact to form the functional pumps are only now beginning to be understood. In addition, the majority of the functional assembly studies are focused on the bottom alpha helices domain in which the interaction between OEP-CMT and OEP-MFP may occur (Bavro et al., 2008; Janganan et al., 2011; Tikhonova et al., 2011), few studies have been conducted to uncover the importance of the flexible C-terminus of OEPs.

Previously, our *gain-of-function* (*gof*) study showed that efflux activities of the chimeric pump, which comprised VceAB (CMT-MFP

Abbreviations: MDR, multidrug-resistant; *gof*, gain-of-function; OEP, outer efflux protein; CMT, cytoplasmic membrane translocase; MFP, membrane fusion proteins; RND, Resistance-Nodulation-Division; MFS, Major-Facilitator-Superfamily; Tet, tetracycline; Cap, chloramphenicol; Cb, carbenicillin; DOC, dexycholate; CCCP, cyanide carbonyl m-chlorophenylhydrazon; NOV, novobiocin; PVDF, polyvinylidene difluoride; OprM$^{22/14}$, 22 amino acids on the C-terminus of OprM were replaced by 14 residues from the phagemid (Poole et al. 1993).

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of *Vibrio cholerae*) and OprM$^{22/14}$ (OEP of *Pseudomonas aeruginosa*), could be restored by a single amino acid substitution on VceA (Bai et al., 2010). The results suggested that the amino acid on the hairpin domain of VceA plays important roles in stabilizing the C-terminal modified OprM and promoting the functional assembly of the chimeric pump. Interestingly, we also found that OprM$^{22/14}$ (Poole et al., 1993) carries an alternate C-terminal domain when compared with the wild type OprM (NCBI gi|3184189|). The altered C-terminus of OprM prevents it from interacting with VceA and thus forming a functional pump with the VceAB complex. In addition, the depletion of the OprM C-terminal $_{463}$LGGGW$_{466}$ motif causes the diminishment of the OprM protein (Li and Poole, 2001). However, the means by which the motif depletion impacts the stability and functionality of OprM remain unknown.

Therefore, the speculation is that the C-terminus of the OprM may play a role in the functional assembly of a MDR efflux pump. Attempts to understand the importance of the C-terminus in affecting the stability and functionality of OprM are addressed in this study.

2. Materials and methods

2.1. Bacterial strains, plasmids, and media

Bacterial strains and plasmids are listed in Table 1. *Escherichia coli* strains were routinely grown in LB broth (Luria and Burrous, 1957) in a gyratory shaking water bath (New Brunswick) at 250 rpm and 37 °C unless otherwise specified. Tetracyclines (Tet), Chloramphenicol (Cap), and Carbenicillin (Cb) were used at 12.5 μg/ml, 30 μg/ml, and 100 μg/ml, respectively.

2.2. Drug sensitivity determination

Cells were grown overnight in LB medium at 37 °C. 0.1 ml of the overnight culture was added to 3 ml of LB soft agar (0.75%) and poured over LB agar plate. An 8-mm disk containing either 20 μl of Dexycholate (DOC) (10%), Cyanide Carbonyl m-Chlorophenylhydrazon (CCCP) (8 mM) or Novobiocin (NOV) (20 mg/ml) was placed on an *E. coli* lawn and incubated overnight at 37 °C. The diameters of the zones of inhibition were measured in millimeters, the average of the three measurements was recorded and used to determine the relative sensitivities of the cell. NOV is a substrate of the AcrAB and MexAB system, but it is not a substrate of VceAB. Thus, NOV is used as a negative control to make sure that OprM or its derivatives is not crossreact with the AcrAB system in *E. coli*. DOC is the common substrate of both AcrAB and VceAB, while CCCP is the substrate of VceAB but not the AcrAB system. Therefore, three compounds could be used to distinguish the functionality of the specific pumps.

Table 1
List of bacterial strains and plasmids.

Name	Genotype	Reference
E. coli		
LBB1600	*tolC* derivitive of LBB1274	Lab collection
Plasmid		
pLACO-6His	pLAC11::*opr*M carry 6 His-tagged at C-terminus	This study
pLACO^{474}	pLAC11::*opr*M with Δ11 residues	This study
pLACO^{468}	pLAC11::*opr*M with Δ17 residues	This study
pLACO^{466}	pLAC11::*opr*M with Δ19 residues	This study
pLACO^{465}	pLAC11::*opr*M with Δ20 residues	This study
pLACO^{464}	pLAC11::*opr*M with Δ21 residues	This study
pLACO^{463}	pLAC11::*opr*M with Δ22 residues	This study
pLACO^{462}	pLAC11::*opr*M with Δ23 residues	This study
pLACO$^{\Delta L}$	pLAC11::*opr*M with Δ_{463}L	This study
pLACO$^{\Delta G}$	pLAC11::*opr*M with Δ_{464}G	This study
pLACO$^{\Delta LG}$	pLAC11::*opr*M with Δ_{463}LG$_{465}$	This study
pLACO$^{\Delta GGG}$	pLAC11::*opr*M with Δ_{464}GGG$_{466}$	This study
pLACO$^{\Delta LGGG}$	pLAC11::*opr*M with Δ_{463}LGGG$_{466}$	This study
pLACO$^{22/14}$	pLAC11::*opr*M$^{22/14}$	Lab collection
pLACOT	pLAC11::*opr*M::*tol*C C-terminus	This study
pLACOC	pLAC11::*opr*M::*vce*C C-terminus	This study
pVC41	pACYC184::*vceAvceB*	(Colmer et al., 1998)
pVC41^{D155Y}	pACYC184::*vceA*^{D155Y}*vceB*	This study
pRSP19	pRK415::*mecAmexB*	(Srikumar et al., 1998)
pRSP08	pRK415::*opr*M$^{22/14}$	(Srikumar et al., 1998)
pRSP08^{466}	pRK415::*opr*M$^{22/14}$ with Δ11 residues	This study
pRSP08$^{W/G}$	pRK415::*opr*M$^{22/14}$ W-464 replaced by G	This study

2.2.1. DNA manipulations

PCR cloning of wildtype oprM: To clone *opr*M (pLACO), PAO1 genomic DNA sample (2 μl, 25 ng) was used as template, forward and reverse primers were: 5′-ATGGAATTC ATGAAACGGTCCTTC-CTTTCC-3′ and 5′-CGAAGCTTTCAATGGTGATGGTGATGATG-AGCCTGG GGATCTTC-3′ (0.5 μl, 10 μM). The template and primers were mixed with a PCR master mix (Promega), and the reaction mixture was then subjected to a standardized thermal cycle: initial 2 min denaturation step at 94 °C, 30 cycles of 30 s at 94 °C, 1 min at 55 °C, 1 min 50 s at 68 °C, and 10 min at 68 °C. The PCR product was tested on a 0.8% agarose gel and purified with QIAEX II Gel Extraction Kit (Qiagen). The DNA fragments were inserted into pLAC11 (Warren et al., 2000) at the *EcoR*I-*Hind*III (NewEngland Biolab) restriction sites.

To construct chimeric OprMs (pLACOT, pLACOC): According to the results of structure alignment (Federici et al., 2005a), amino acid Leu at OprM^{463}, VceC^{433} and TolC^{428} are identical and located on the same structural sites, while the peptides downstream showed great diversity on both sequences and structures. Therefore, we chose Leu (L) at OprM^{463}, VceC^{433} and TolC^{428} as the joint point to construct the chimeric OprMs. To construct the OprM^{463} plus 9 amino acids from the C-terminus of VceC by PCR, pLACO DNA was used as a template (25 ng), forward primer: 5′-ATGGAATTCATGAAACGGTCCTTCCTTTCC-3′, 10 μM reverse primers carrying the C-terminal sequence of *vce*C: 5′-TATTAAGCTT *TTAAGATTCTGTTG- TTTCAAAACCG CCGCC* TAGGGCCTTGTA CAGGTTGA-3′ (10 μM), were mixed with the PCR master mix (Promega). The reaction mixture was subjected to an initial 2 min at 94 °C, 30 cycles of 30 s at 94 °C, 1 min at 60 °C, 1 min 50 s at 68 °C, and 10 min at 68 °C. To construct the OprM^{463} with 67 residues from the C-terminus of TolC, we employed the 'Two-Step Overlap' PCR technique: **Step 1**: A total of 1389 bps of *opr*M^{463} were amplified from pLACO by using the PCR primers: forward: 5′-ATGGAATTCATGAAACG-GTCCTTC-CTTTCC-3′ (0.5 μl, 10 μM) and reverse carrying 23 bps from *tol*C: 5′*-AGATCCTGCTCGTTCAACGT-ACC* GAGGGCCTTG TACAGGTTGA-3′ (0.5 μl, 10 μM). A total of 204 bps coding for the TolC C-terminal (67 residues) were amplified from pLACT by using the PCR primers: forward carrying 20 bps from *opr*M^{463}: 5′*-TCAACC TGTACAAGGC-CCTC* GGTACGTTGAACGAGCAG-3′ (0.5 μl, 10 μM) and reverse: 5′-AAGCTTTCAGTTA CGGAAAGGGTTATGACCG-3′ (0.5 μl, 10 μM), respectively. The thermal cycles of this PCR were similar to the ones described above, except that the amplification time for the *tol*C fragment was reduced to 20 s. The PCR products were tested on a 0.8% agarose gel and purified with a QIAEX II Gel Extraction Kit (Qiagen). Two DNA fragments were mixed at a 1:1 ratio, and the final concentration of DNA mixture was adjusted to 12.5 ng/μl. **Step 2**: To connect the two fragments, 25 μl of PCR reaction mixture contained the mixed template (2 μl, 25 ng total) from **Step 1**: PCR master mix (2X, 12.5 μl), and dH_2O (10.5 μl). In the absence of primers, the mixture was subjected to an initial 2 min denaturation step at 94 °C, 12 cycles of 30 s at 94 °C, 1 min at 60 °C, 2 min at 68 °C,, and 10 min at 68 °C. After being chilled on ice, the reaction was