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Functional response of the small multidrug resistance protein EmrE to mutations in transmembrane helix 2

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ABSTRACT

Escherichia coli **EmrE is a small multidrug resistance protein encompassing four transmembrane (TM) sequences that oligomerizes to confer resistance to antimicrobials. Here we examined the effects on in vivo protein accumulation and ethidium resistance activity of single residue substitutions at conserved and variable positions in EmrE transmembrane segment 2 (TM2). We found that activity was reduced when conserved residues localized to one TM2 surface were replaced. Our findings suggest that conserved TM2 positions tolerate greater residue diversity than conserved sites in other EmrE TM sequences, potentially reflecting a source of substrate polyspecificity.**

1. Introduction

Widespread use of antimicrobials has resulted in the emergence of nosocomial pathogens such as methicillin resistant *Staphylococcus aureus* (MRSA, [1]), an organism that causes more deaths among U.S. hospital patients than HIV/AIDS and tuberculosis combined [2]. Persistence of MRSA strains is linked to multidrug efflux transporters of the small multidrug resistance (SMR) family [3,4]. Indeed, the first SMR identified, Smr/QacC, was isolated from clinical *S. aureus* resistant to cationic disinfectants and antiseptics (termed biocides) [5]. Half of sequenced bacterial chromosomes, and numerous plasmids and transposons, contain SMR protein genes [6,7] from among three subclasses [suppressor of groEL mutation (SUG); paired SMR (PSMR); and small multidrug protein (SMP)]. SMPs associate with bacteria that are pathogenic and/or sources of nosocomial infection [3–5,8–11]; their maintenance within such microorganisms reflects frequent exposure to biocidal quaternary ammonium compounds (QACs) and other naturally-occurring or man-made toxicants [7].

The *Escherichia coli* SMP EmrE is the functional and structural paradigm of SMRs. EmrE is a 110-residue antiporter of the inner membrane that extrudes substrates in exchange for two protons [12]. EmrE substrates include natural and man-made QACs (betaine, choline [13] and tetraphenylphosphonium (TPP) [14]), herbicides (methyl viologen (MV) [15]), dyes (ethidium (Et) [16,17]), clinical biocides (benzalkonium, acriflavine (Ac) [18]), and aminoglycoside antibiotics (streptomycin, tobramycin [19]). The minimal functional unit of EmrE is a homodimer [20], although higher-order oligomers may exist [21–23]. Structural models at subatomic resolution [24–28] of substrate-bound and apo-forms of the antiparallel dimer are similar [29]: TMs 1–3 of each monomer form a substrate-binding pocket, and the two TM4 helices form a dimerization arm. The plasticity and dynamics of apo-EmrE permit adaptation to substrates via re-arrangements among TMs 1–3 [28,30,31]; TPP binding rigidifies EmrE and changes packing of TM1, tilting of TM2, and alters backbone configurations of TM3 and the adjacent loop connecting it to TM4 [30–32]. TM4–TM4 contacts are less variable than those among TMs 1–3, consistent with the essential role of this helix in dimerization [33–35].

Abbreviations: Ac, acriflavine; EmrE, *Escherichia coli* small multidrug resistance protein EmrE; Et, ethidium; EtBr, ethidium bromide; MRSA, methicillin resistant *Staphylococcus aureus*; MV, methyl viologen; PSMR, paired small multidrug resistance; QAC, quaternary ammonium compound; SMP, small multidrug protein; SMR, small multidrug resistance; SUG, suppressor of *groEL* mutation; TM, transmembrane; TPP, tetraphenylphosphonium

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Available EmrE structures do not specify side chain positions, so protein contacts mediating self-assembly and/or substrate translocation have yet to be precisely defined. Lipid-exposed and protein-facing side chains in the TPP-bound, antiparallel EmrE dimer, delineated using an atomistic model generated from the X-ray structure, matched in vivo activity assessments [36]. For example, Glu14 in TM1 and Gly90/Gly97 in TM4 were found to be protein-facing and required for Et resistance activity [36], consistent with conservation of these residues among all SMR proteins [6], and their respective role(s) in coupling flux of protons and substrates at TM1 [37], or stabilizing TM4–TM4 dimerization [33–35].

Trp substitutions at protein-facing EmrE TM2 sites (Tyr40, Ser43, Phe44, Leu47, and Ala48; [36]) also reduced or eliminated Et resistance activity, reinforcing previously-identified role(s) in activity and/or folding of select TM2 residues [30,36,38–40]. Tyr40, for example, is important, although not fully essential, for catalyzing transport [40], Cys41 forms part of the substrate pathway [38], and Trp45 may aid EmrE insertion into the cytoplasmic membrane [41]. TM2 thus participates in activity and folding of SMR proteins. Here we sought to determine the sensitivity of in vivo EmrE protein accumulation and Et resistance activity to TM2 residue replacements. Using an alignment of 108 SMPs [7], we classified TM2 residues as highly-conserved, semi-conserved, semi-variable, and variable. By replacing residues at various conservation levels in site-directed mutagenesis experiments, we found that in vivo EmrE protein accumulation levels did not universally correlate with residue conservation. However, replacement of each highly-conserved TM2 residue essentially eliminated in vivo resistance to Et. Our results show that key functional residues are within the most highly conserved quartile of TM2 positions.

2. Materials and methods

2.1. Reagents

The EmrE-Myc/His plasmid [37] was a kind gift from Prof. Shimon Schuldiner, Hebrew University of Jerusalem. *E. coli* BL21(DE3) cells and anti-His (C-term)-HRP conjugate antibodies were from New England Biolabs. Ampicillin was purchased from BioShop. ECL Plus reagents were purchased from GE Healthcare. GelCode Blue protein stain and BCA reagents were from Thermo Fisher Scientific. Amido Black total protein stain and ethidium bromide (EtBr) solution were from Sigma Aldrich. All SDS–PAGE gels and molecular weight standards were purchased from Life Technologies.

2.2. Bioinformatics

The amino acid sequence of EmrE was aligned with 107 other sequences previously-identified as SMP [7] using the Clustal O Web server (v. 1.2.1). Care was taken to exclude sequences of SUGs, and of PSMRs previously identified to segregate with SMPs (e.g. EbrA/EbrB, YvaE/YvaD, and YdgE/YdgF [7]). The resulting multiple sequence alignment (Supplementary file 1) was submitted to the Protein Variability Server (http://imed.med.ucm.es/PVS/, [42]), and default parameters used to determine the value of Shannon entropy (H) at each alignment position. Shannon entropy is a sensitive means to estimate the diversity of a multiple sequence alignment (reviewed in [42]), where values of H at a given position range from 0 (one residue present at a given alignment position) to 4.39 (all 20 residues equally represented, including gaps). Lower H values thus correspond to higher residue conservation and vice versa.

Cut-offs that define conserved vs. variable sequence positions have not been unequivocally defined, although it has been suggested based on analyses of immunoglobulins that positions with $H \leqslant 2.0$ or $H \leqslant 1.0$ should be considered semi-conserved or highly-conserved, respectively [43]. Here, the minimum, 25th percentile, median, 75th percentile, and maximum of the group of H values among all alignment positions encompassing EmrE, and among those corresponding to EmrE TM2 (Table 1) were calculated and used to define conservation levels as follows: highly-conserved, $H \leqslant$ 25th percentile; semi-conserved, 25th percentile $< H \leqslant$ median; semi-variable, median $< H \leqslant$ 75th percentile; and variable, $H >$ 75th percentile. EmrE TM sequence start and end sites followed [39]: TM1, residues 4–21; TM2, residues 34–52; TM3, residues 57–81; TM4, residues 85–105.

2.3. Mutagenesis

The QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used to construct EmrE point mutants in the EmrE-Myc/His plasmid, and each mutant was verified by DNA sequencing at The Centre for Applied Genomics at the Hospital for Sick Children.

2.4. Ethidium growth assay

Growth of *E. coli* expressing WT and mutant EmrE proteins in the presence of Et was assayed on 96-well microplates. *E. coli* BL21(DE3) cells were transformed with EmrE-Myc/His plasmid carrying wild-type (WT) EmrE, EmrE TM2 point mutants, or with pUC19 (negative control). Isolated colonies of fresh transformants were grown overnight in 5 mL LB containing 100 μg/mL ampicillin at 37 °C. Each overnight culture was standardized by dilution to an OD600 = 0.5 in LB and then serially diluted from 10^{-1} to 10^{-7}. An equal volume of each serial dilution was added to plate wells and mixed with an equal volume of LB to final concentrations of 100 μg/mL ampicillin and 100 μg/mL EtBr. Each growth assay plate contained at least one serial dilution of WT and of negative control. Plates were covered with air-permeable rayon plate seals and incubated at 37 °C for 20 h, after which cell growth density in each well was measured by OD600, using uninoculated medium as a reference. Here, Et resistance is conferred by leaky T7 RNA polymerase-mediated expression of EmrE from the EmrE-Myc/His plasmid rather than by induced overexpression, thereby avoiding toxic levels of EmrE [44].

Plots of OD600 at 20 h vs. dilution factor were generated for WT, mutants, and negative control, and the area under each growth-dilution curve (AUC) determined. Growth relative to WT was calculated as:

Table 1
Descriptive statistics of whole-protein and TM2 sequence variability in aligned SMPs.

Statistic	Shannon entropy (H)[a]	
	EmrE	TM2
Minimum	0.152	0.750
25th Percentile	1.38	1.75
Median	1.94	2.10
75th Percentile	2.56	2.55
Maximum	3.42	2.72

[a] H values range from 0 (one residue present at a given alignment position) to 4.39 (all 20 residues equally represented, including gaps). Lower H values thus correspond to higher residue conservation and vice versa. H values among all positions in the multiple sequence alignment of SMPs that encompass the complete EmrE protein (residues 1–110), and those corresponding to EmrE TM2 (residues 34–52), were normally distributed (N = 110 and N = 19, respectively, $P > 0.10$ in both cases). Quartile values (25th percentile, median, and 75th percentile) were used to define conservation levels (Section 2). Note that H values of eleven alignment positions were lower than the TM2 minimum (Supplementary file 1), corresponding to EmrE residues in TM1 (Tyr7 and Glu14), TM3 (Tyr60, Ala61, Trp63, Gly65, and Gly67), TM4 (Gly90), and in loops (Gly27 and Pro55).