Functional analysis of the *Escherichia coli* and *Vibrio cholerae* multidrug resistance transporter MdtK

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Introduction

- According to the CDC, < 23,000 people die annually from antibiotic resistant bacterial infections (1).
- MdtK is a transmembrane protein that functions as a multidrug efflux pump with the ability to export several antimicrobial agents including ciprofloxacin, ethidium bromide and doxorubicin (2).
- Residue F285 has been shown to be important for cation binding and export function (3).
- We have recently shown that *E. coli* can develop resistance to the aminoglycoside Kasugamycin (Ksg) through a single phenylalanine to valine mutation in MdtK at the F285 residue. The *E. coli*/Ksg relationship is a novel mechanism that does not rely on an increased MdtK synthesis but rather a gain of function mutation that is predicted to increase the pump's affinity for Ksg, thus empowering greater drug export.
- Given that there are only the wild type and a single mutant available for further analysis, there is a lack of understanding of the contributions of the 18 other possible amino acids at this location and their effect on *E. coli* Ksg resistance.
- Additionally, crystal structures are available for the *Vibrio cholerae* MdtK protein and it is predicted that F285 is a cation binding site.
- The purpose of this study is to generate MdtK proteins containing the most structurally relevant amino acids and determine any changes in the pump's substrate specificity profile.

Hypothesis

We hypothesize that amino acids with a similar structure to valine will result in similar if not raised export of kasugamycin while the amino acids that are closer in structure to phenylalanine will result in a lower export of kasugamycin or in a nonfunctional protein.

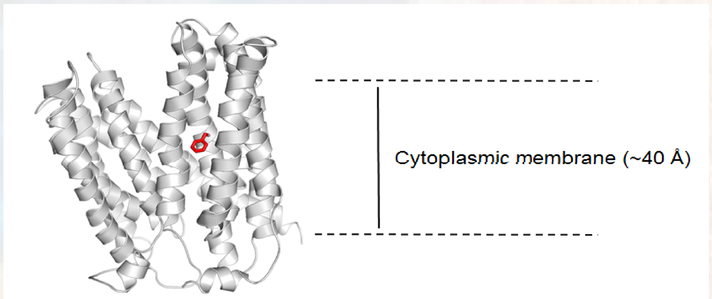

Figure 1. Crystal structure of the multidrug efflux transporter NorM (MdtK) from *Vibrio cholerae*. Ribbon representation (grey) of NorM viewed from the plane of the membrane (upper) and from the cytoplasmic face (lower). The position of a key phenylalanine residue is shown in stick representation (red). The ribbon structure was drawn with PyMOL (Schrödinger, LLC, New York) using coordinates deposited in the Protein Data Bank with accession code 3MKT.

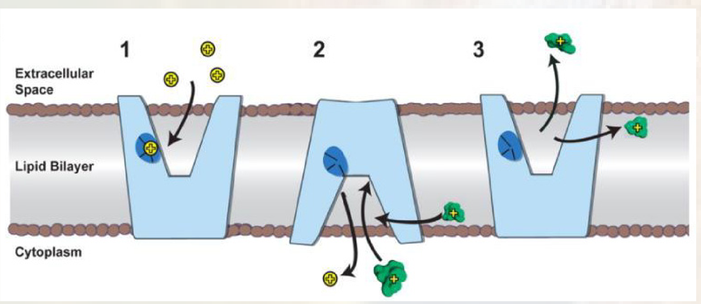

Figure 2. Proposed transport mechanism for MdtK: In the outward-facing conformation, cation (yellow) binds at a conserved site (blue oval; step 1). Cation binding induces structural changes to the inward-facing conformation (step 2), which is competent to bind substrate (organic cation in green) from the inner membrane leaflet or cytoplasm. Substrate binding causes structural changes back to the outward-facing conformation (step 3), allowing export and cation binding. Figure 4 in reference 3 with legend was reproduced with permission from Nature Publishing Group (license # 4160300626985).

Results

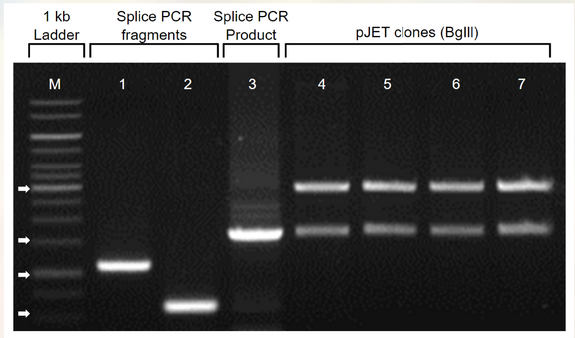

Figure 3. Generation of site directed mutants of *E. coli mdtK*. From top to bottom, the arrows indicate base pair sizes of 3000, 1500, 1000, and 500. Mutant oligonucleotides are incorporated into the smaller splice fragment (lane 2). The two overlapping fragments are then annealed to each other to form a larger splice PCR product (lane 3).

PCR (lanes 1 and 2)
Fifty µL polymerase chain reactions (PCR) were performed. The reaction was ran at 98℃ for 5 minutes, followed by 30 cycles of 98℃ for 1 minute, 57℃ for 30 seconds, and by 72℃ for 1 minute, followed by one cycle of 72℃ for 5 minutes.

Splice overlap extension PCR (lane 3)
PCRs were constructed. The reaction was ran at 98℃ for 5 minutes, followed by 15 cycles of 98℃ for 1 minute, 25℃ for 30 seconds, and 72℃ for 30 seconds, followed by one cycle of 72℃ for 5 minutes.The outside forward and reverse primers and enzyme were added to each tube. Another reaction was ran at 98℃ for 5 minutes, followed by 15 cycles of 98 V for 1 minute, 57℃ for 30 seconds and 72℃ for 1 minute followed by one cycle of 72℃ for 5 minutes.

Cloning of mutant alleles into pJET (lanes 4-7)
The PCR product was purified using the Qiagen PCR purification kit. A 10 µL ligation reaction was performed using 0.5 µL of buffer, 0.5 µL of pJET1 blunt cloning vector, 4.5 µL of PCR insert and 0.5 µL of DNA ligase and incubated for 30 minutes at room temperature. Ligations were transformed into competent *E. coli* DH10B cells and plasmid DNA was purified from the resulting transformants. Plasmids containing the mutant *mdtK* genes were identified by the presence of a 1.7-kb BglII fragment following restriction digestion.

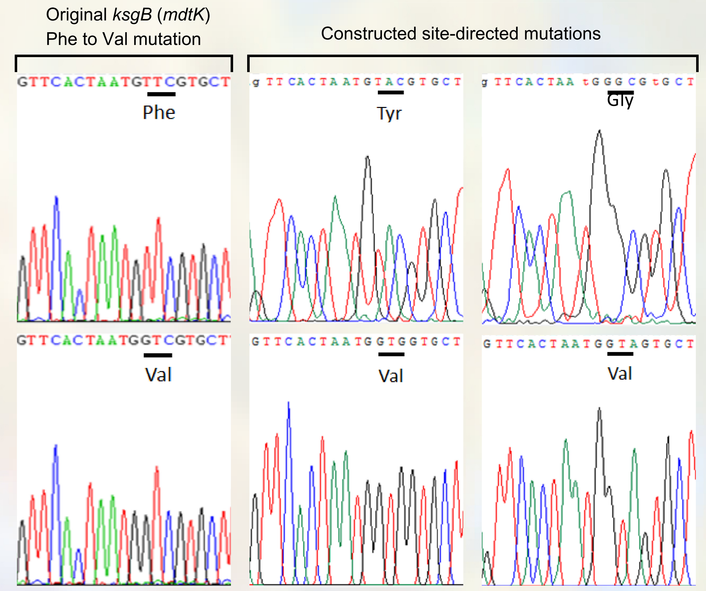

Figure 4. Site-directed mutagenesis of *E. coli* MdtK residue 285. The Phe residue was mutated using splice overlap extension PCR. The resulting amplicons were ligated into plasmid pJET and transformed into *E. coli* DH10B cells. Transformants containing the appropriate 1.7-kb insert were sequenced at the MU DNA Core facility. Shown above are the chromatograms of the region containing the mutated residue. Two new mutations were obtained, together with two different synonymous mutations of the valine mutation that was identified as the *ksgB* mutation.

Bioinformatic Analysis

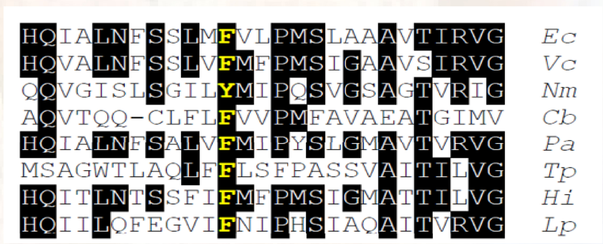

Figure 5. The Phe (F) residue is highly conserved in MdtK proteins. The F285 region of MdtK proteins from eight Gram-negative pathogens were aligned. Amino acid residues that are identical in 4 or more representatives are highlighted with a black background. The F285 residue (Y in *Neisseria meningitidis*) is shown in yellow. *Ec: Escherichia coli, Vc: Vibrio cholerae, Nm: Neisseria meningitidis, Cb: Coxiella burnetii, Pa: Pseudomonas aeruginosa, Tp: Treponema pallidum, Hi: Haemophilus influenzae, Lp: Legionella pneumophila.*

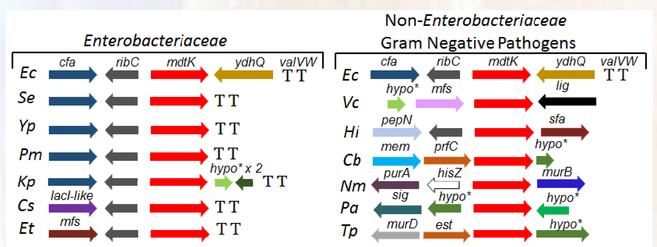

Figure 6. The genomic environment of *mdtK* in Gram negative pathogens. *all genes labeled hypo represent different genes encoding hypothetical proteins with unknown function. T is representative of tRNA genes. *Ec: Escherichia coli, Se: Salmonella enteritidis, Yp: Yersinia pestis, Pm: Pseudomonas mirabilis, Kp: Klebsiella pneumoniae, Cs: Cronobacter sakazakii, Et: Edwardsiella tarda, Vc: Vibrio cholerae, Hi: Haemophilus influenzae, Cb: Coxiella burnetii, Nm: Neisseria meningitidis, Pa: Pseudomonas aeruginosa, Tp: Treponema pallidum.*

Results: To date, there have been no published comparative analysis on the genetic synteny of *mdtK* in *Enterobacteriaceae,* or more distantly related Gram-negative taxa. Through a BLAST bioinformatic analysis (4,5) of the genetic environment surrounding the *mdtK* gene, it was found that *cfa, ribC,* and two tRNA genes neighboring *mdtK* in *Enterobacteriaceae* species are highly conserved while the genes surrounding *mdtK* in other, non-*Enterobacteriaceae* Gram negative species are not conserved, with the exception of *ribC* found in *Haemophilus influenzae* . It was also found that in *Enterobacteriaceae*, *mdtK* is monocistronic (based on gene orientation) while in non-*Enterobacteriaceae* Gram negative species *mdtK* was found to be either monocistronic (e.g. *Haemophilus influenzae*) or had the potential to be polycistronic (e.g. *Coxiella burnetii*).

Conclusions

A bioinformatic analysis was performed which showed that while the genetic environment is conserved in many *Enterobacteriaceae* species, it is not conserved in other non-*Enterobacteriaceae* Gram negative species. Additionally, novel mutants of both *E. coli* and *V. cholerae* (data not shown for the latter) were constructed using molecular genetic techniques which will provide the basis for future structure/function research of the *mdtK* gene.

Future Directions

This investigation will continue with verified plasmids being transformed into *E. coli* with a *mdtK* knockout mutation from the Keio collection (6). A minimum inhibitory concentration (MIC) determination will then be run on the transformed cells to determine the effect the mutation has on the ability of the bacteria to resist antibiotics. This procedure allows for the direct testing of our hypothesis as well as obtaining a better understanding of the differences between species pertaining to a gain or loss of function mutation of a cation binding site (3).

References

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Acknowledgements

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