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SUPPLEMENTARY-RESULT
REPLICATION

A negative charge at position D^{+5} of Motif A is critical for function of the major facilitator superfamily multidrug/H^+antiporter MdtM

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Abstract

The phenomenon of antimicrobial resistance represents a major public health risk. The activity of integral membrane transporter proteins contributes to antimicrobial resistance in pathogenic bacteria and proton gradient-driven multidrug efflux representatives of the major facilitator superfamily (MFS) of secondary transporters are the dominant antimicrobial efflux proteins in *Escherichia coli*. In many, but not all, of the characterized MFS multidrug transporters, an aspartic acid residue at position D^{+5} of the conserved signature Motif A is essential for transport activity. The present work extends those studies to the *E. coli* MFS multidrug/H^+ antiporter MdtM and used a combination of mutagenesis, expression studies, antimicrobial resistance assays, and transport activity measurements to reveal that a negatively charged residue at position D^{+5} is critical for MdtM transport function.

Key words: active transport; antibiotic resistance; antimicrobial resistance; membrane protein; membrane transporter

1. Introduction

Energy-dependent transport of cytotoxic compounds out of cells is a contributor to the phenomenon of multidrug resistance, one of the most pressing global health challenges of the 21st century (Piddock, 2006). In the model organism *Escherichia coli*, representatives of the ubiquitous major facilitator superfamily (MFS) of secondary active transporters are the dominant multidrug efflux proteins. Electrochemical gradient-driven multidrug efflux representatives of the MFS are typically single polypeptides of 12 or 14 membrane-spanning α-helices organized in two pseudosymmetry-related domains linked by a cytoplasmic loop region. A central cavity nested between the domains serves as both the substrate binding site and translocation pathway (Law et al., 2008). A characteristic of most MFS transporters is their possession of a highly conserved signature motif, Motif A ($Gx_3D^{+5}RxGR^{+9}R$), located on the cytoplasmic loop that links membrane-spanning helices 2 and 3, and which contains the most conserved acidic residue in the MFS, D^{+5}, alongside three conserved basic Arg residues (Supplementary Figure S1). In the bacterial MFS multidrug transporters LmrP, TetL, and YajR, a negative charge at the D^{+5} position of Motif A is necessary for transport activity (Jiang et al., 2013; Masureel et al., 2014; Yamaguchi et al., 1992). The current study extends these findings to the MFS multidrug/H^+ antiporter MdtM (Holdsworth & Law, 2012) and validates the functional necessity of a negative charge at the +5 position in Motif A of many MFS multidrug efflux proteins.

2. Objectives

That a highly conserved Asp residue in Motif A is essential for the activity of many, but not all, MFS multidrug efflux proteins prompts further questions. First, is Asp at position D^{+5} irreplaceable in the *E. coli* MFS multidrug/H^+ antiporter MdtM? Second, is the effect of D^{+5} mutation dependent on the charge of the substrate? The objectives of this study were to systematically address these questions by testing the effects of mutation of the MdtM D^{+5} residue (Asp73) on: (a) the drug resistance phenotype of *E. coli* that overexpressed mutant transporter from plasmid by determination of IC_{50} for the neutral antibiotic chloramphenicol (Cm) and cytotoxic cationic compound tetraphenylphosphonium (TPP^+) and (b) the transport activity of mutant protein in inverted *E. coli* vesicles.

3. Methods

3.1 Bacterial strains, plasmids, and site-directed mutagenesis

The *E. coli* strains used for antimicrobial resistance assays and for production of inverted vesicles are described in detail in a previous publication (Alegre et al., 2016). MdtM Asp73 mutations were constructed using a method described before (Alegre et al., 2016) in a template harboring a His_{10} tag to enable clear detection and comparison of expression levels. The fidelity of each mutant construct was verified by DNA sequence analysis.

3.2 Antimicrobial resistance assays

The IC_{50} values of chloramphenicol and TPP^+ were determined using a plate-based microtiter assay described previously (Alegre et al., 2016).

3.3 Transport assays

Assays of chloramphenicol/H^+ and TPP^+/H^+ antiport were conducted by measuring the fluorescence quenching/dequenching of the pH-sensitive indicator acridine orange upon addition of substrate to energized inverted membrane vesicles generated from *E. coli* that overproduced recombinant MdtM or negative control GlpT as described before (Paul et al., 2014).

3.4 Western blots

Detection of His-tagged MdtM was performed using a method described previously (Alegre et al., 2016).

3.5 Statistical analysis

Analysis of IC_{50} values was performed using an unpaired *t*-test. Results were considered statistically significant if $p < .05$.

4. Results

Mutation of MdtM Asp73 increases susceptibility of E. coli to Cm and TPP^+. Mutation of MdtM Asp73 to Ala or Asn resulted in a significant ($p < .01$) loss of function efflux phenotype for both Cm and TPP^+ compared to positive control cells that overexpressed wild type (WT) transporter (Figure 1). The conservative Asp73 to Glu mutation of MdtM also caused a significant ($p < .05$) loss of function with respect to Cm resistance (Figure 1a). In contrast, the same conservative mutation had no statistically significant effect on the ability of cells to protect themselves from the cytotoxic effects of TPP^+ (Figure 1b). Comparison of expression levels of WT and mutant MdtM by Western blot analysis revealed the differences in IC_{50} did not arise from differences in expression (Figure 2).

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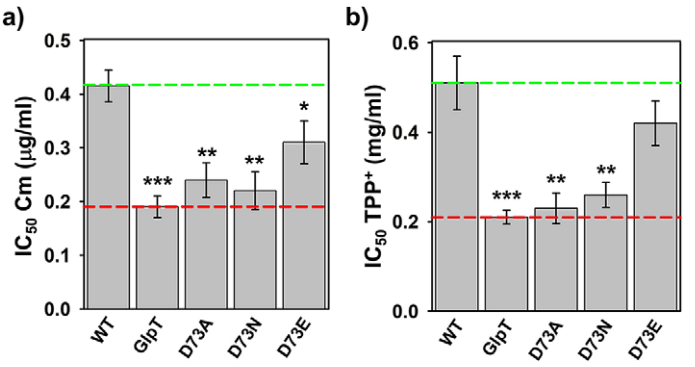

Figure 1. Antimicrobial efflux phenotypes of *Escherichia coli* Δ *mdtM* cells that overexpressed wild type or mutant MdtM, or GlpT negative control, as determined by IC_{50} values. (a) IC_{50} values for chloramphenicol (Cm) as antimicrobial substrate. (b) IC_{50} values for TPP^+ as antimicrobial substrate. In (a) and (b) bars and error bars represent the mean ± standard deviation of eight separate measurements. *, **, and *** denote IC_{50} values that represent a statistically significant $p < .05$, $p < 0.01$, and $p < 0.001$ loss of efflux function, respectively.

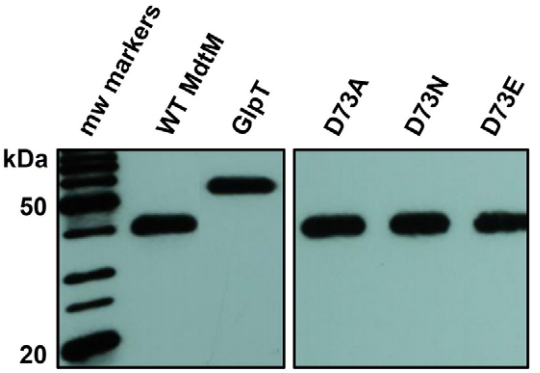

Figure 2. Western blot analysis of expression levels of wild type MdtM, GlpT, and MdtM D73 mutants. Protein was visualized via detection of the C-terminal decahistidine tag. Each lane of the gels was loaded with 80 μg of dodecylmaltoside (DDM) detergent-solubilized total membrane protein.

Nonconservative mutation of MdtM Asp73 causes loss of transport activity. Inverted vesicles generated from *E. coli* cells that overexpressed WT MdtM exhibited clear transport activity for both Cm and TPP^+, as revealed by a dequench of the fluorescence signal of the acridine orange reporter upon addition of substrate (Figure 3). In contrast, addition of substrate to negative control vesicles that harbored GlpT resulted in an insignificant fluorescence dequench, which was due to activity of chromosomally encoded multidrug transporters. When MdtM Asp73 was replaced by Ala or Asn, the transport activity was completely lost, whereas when it was replaced by Glu, transport activity was reduced to between 30 and 50% of the WT level. These results also reveal that the effect(s) of D^{+5} mutation in MdtM is independent of the charge carried by the drug substrate. To ensure the measured transport activity was solely due to the effect of mutation, the expression levels of the transporters in the vesicle membrane were analyzed by Western blot. As shown in Figure 4, the westerns revealed similar levels of expression of each protein.

5. Discussion

The high degree of conservation of negative charge at the D^{+5} position of Motif A of MFS drug efflux proteins suggests an important functional role for the acidic Asp residue. The present study supports this contention in that a negative charge at position D^{+5} of Motif A in MdtM is essential for transport.

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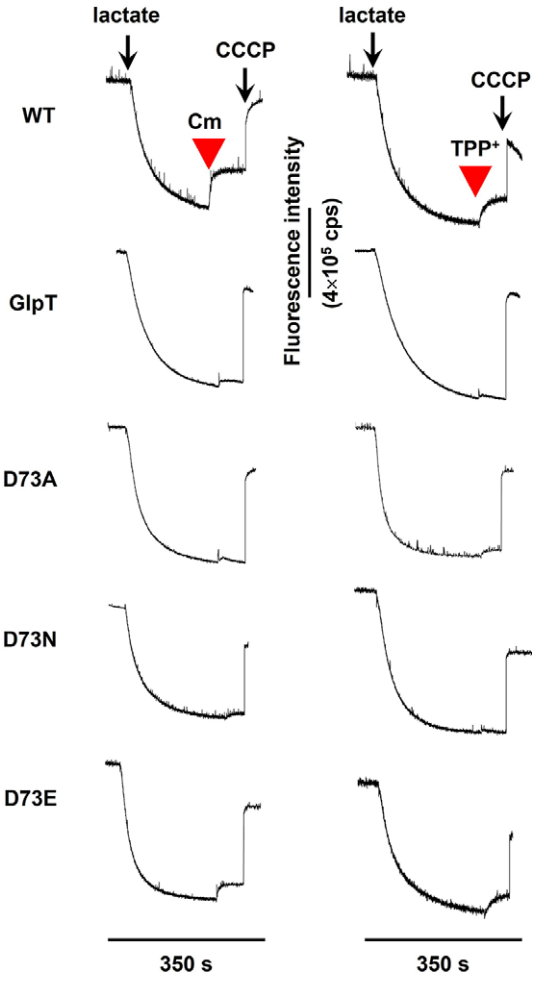

Figure 3. MdtM-dependent TPP^+/H^+ and chloramphenicol (Cm)/H^+ exchange in inverted vesicles. Transport measurements were performed by monitoring the fluorescence quench/dequench of acridine orange upon addition of antimicrobial substrate to inverted vesicles prepared from *Escherichia coli* TO114 cells that overproduced recombinant wild type or mutant MdtM or, as a control, GlpT. Respiration-dependent generation of ΔpH (acid inside) was established by addition of 2 mM Tris-D-L-lactate as indicated and once the fluorescence quench of acridine orange reached a steady state, substrate was added. Addition of 100 μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) at the time indicated dissipated ΔpH and abolished transport. The traces are representative of experiments performed in triplicate on at least two separate preparations of inverted vesicles.

This observation is consistent with those made on the MFS tetracycline efflux protein TetL, in which replacement of the D^{+5} residue by Asn resulted in complete loss of transport (Yamaguchi et al., 1992). In the lactococcal multidrug efflux protein LmrP, the D^{+5} residue, D68, is critical for transport of lipophilic cations (Mazurkiewicz et al., 2002). However, the results of these and the current study contrast with those obtained from a study of MdfA, a close *E. coli* homologue of MdtM, in which replacement of the equivalent Asp to an uncharged residue did not impact transport activity (Sigal et al., 2006). This highlights the subtle differences that exist between even closely related MFS multidrug efflux proteins.

It was proposed that the Motif A D^{+5} residue of YajR multidrug efflux transporter forms part of a charge-relay triad that functions in proton transfer, and in stabilization of the outward facing conformation of the transporter via inter-domain interactions (Jiang et al., 2013). In LmrP, the D^{+5} residue is proposed to act as a "master conformational switch" while also contributing to both proton transfer and

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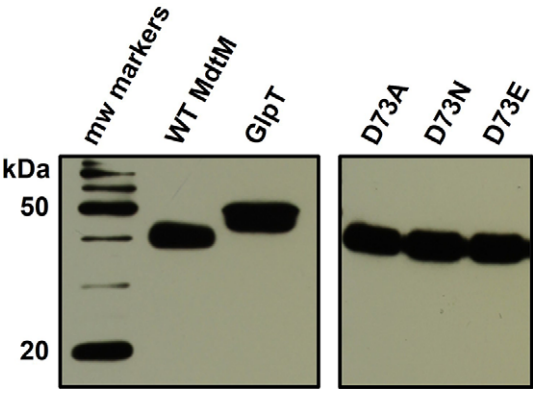

Figure 4. Western blot analysis of expression levels of wild type MdtM, GlpT, and MdtM D73 mutant protein from inverted vesicle membrane. Protein was visualized via detection of the C-terminal decahistidine tag. Each of the gel lanes was loaded with 80 μg of protein.

membrane lipid–protein interactions (Masureel et al., 2014). It is conceivable that the acidic Asp73 residue of MdtM is also involved in similar interactions and functions.

6. Conclusions

The current study reveals that although the conserved acidic Asp73 residue of *E. coli* MdtM is not irreplaceable, a negative charge at this position is essential for the drug efflux activity of the transporter.

Supplementary Materials. To view supplementary material for this article, please visit http://dx.doi.org/10.1017/exp.2022.1.

Data availability statement. Data used for this article are available from the corresponding author on reasonable request.

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Conflict of interest. The author declares none.

Authorship contributions. C.J.L. conceived and performed the work, interpreted the data, prepared the figures, and wrote the manuscript.

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Peer Reviews

Reviewing editor: Dr. Steve Meaney

Technological University Dublin - Dublin City Center Campus, School of Biological and Health Sciences, College of Sciences and Health, Dublin, Dublin, Ireland

This article has been accepted because it is deemed to be scientifically sound, has the correct controls, has appropriate methodology and is statistically valid, and has been sent for additional statistical evaluation and met required revisions.

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Review 1: A negative charge at position D^{+5} of Motif A is critical for function of the major facilitator superfamily multidrug/H^+ antiporter MdtM

Reviewer: Tewfik Soulimane

Date of review: 23 September 2021

Conflict of interest statement. Reviewer declares none

Comments to the Author: Through site-directed mutagenesis, this paper nicely demonstrates that the conserved acidic Asp 73 residue of E. coli MdtM is not irreplaceable, however, a negatively charged amino acid such as glutamic acid is essential for the drug efflux activity of the transporter at this position. This has been well documented by combined experimental results of expression studies, antimicrobial resistance assays and transport activity measurements of MdtM mutants.

Excellent work, clear and concise manuscript which will be of great interest to scientific community working on antimicrobial resistance, but also on membrane transport and membrane proteins.

Score Card

Presentation

5.0 /5

Is the article written in clear and proper English? (30%)	5/5
Is the data presented in the most useful manner? (40%)	5/5
Does the paper cite relevant and related articles appropriately? (30%)	5/5

Context

5.0 /5

Does the title suitably represent the article? (25%)	5/5
Does the abstract correctly embody the content of the article? (25%)	5/5
Does the introduction give appropriate context? (25%)	5/5
Is the objective of the experiment clearly defined? (25%)	5/5

Analysis

Does the discussion adequately interpret the results presented? (40%)	5/5
Is the conclusion consistent with the results and discussion? (40%)	5/5
Are the limitations of the experiment as well as the contributions of the experiment clearly outlined? (20%)	5/5

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Review 2: A negative charge at position D^{+5} of Motif A is critical for function of the major facilitator superfamily multidrug/H^+ antiporter MdtM

Reviewer: Dr. Esben M. Quistgaard

Date of review: 17 December 2021

Conflict of interest statement. I declare that I have no conflicts of interest

Comments to the Author: A hallmark of MFS transporters is the presence of the so-called motif A, which features a highly conserved aspartic residue that in many but not all cases has been shown to be important for transport activity.

Here, Law tests the effect on activity of mutating this aspartic residue in the E. coli multidrug/H+ MFS antiporter MdtM, using both an antibiotic resistance assay and a vesicle-based transport assay.

Both assays show that the aspartic residue is important, and emphasize the role of the charge, since a mutation to glutamate is less detrimental than mutations to either alanine or asparagine.

I have only a few comments:

1) Line 82: Change 'vesicles generated E. coli' to 'vesicles generated from E. coli'

2) Fig. 2 shows a control experiment for the assay reported in Fig. 1. Similarly, Fig. 4 shows a control experiment for the assay reported in Fig. 3. I think it would work better to fuse figures 1+2 and fuse figures 3+4. I will let it be up to the author and editor to evaluate if these proposed changes are needed.

3) Jiang et al. 2013 is cited for proposing that motif A functions in proton transfer and stabilizing the outward facing conformation. Indeed the Jiang et al. paper (and other MFS structure papers) strongly suggests that motif A functions in orchestrating conformational changes (i.e. in 'gating') by stabilizing the outward facing conformation. However, I believe that the authors do not propose a role for motif A in proton transfer.

Score Card

Presentation

4.6 /5

Is the article written in clear and proper English? (30%)	5/5
Is the data presented in the most useful manner? (40%)	4/5
Does the paper cite relevant and related articles appropriately? (30%)	5/5

Context

5.0 /5

Does the title suitably represent the article? (25%)	5/5
Does the abstract correctly embody the content of the article? (25%)	5/5
Does the introduction give appropriate context? (25%)	5/5
Is the objective of the experiment clearly defined? (25%)	5/5

Analysis

Does the discussion adequately interpret the results presented? (40%)	5/5
Is the conclusion consistent with the results and discussion? (40%)	5/5
Are the limitations of the experiment as well as the contributions of he experiment clearly outlined? (20%)	5/5

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