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Research paper

Functional and biochemical characterisation of the *Escherichia coli* major facilitator superfamily multidrug transporter MdtM

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ABSTRACT

Multidrug resistance (MDR) occurs when bacteria simultaneously acquire resistance to a broad spectrum of structurally dissimilar compounds to which they have not previously been exposed. MDR is principally a consequence of the active transport of drugs out of the cell by proteins that are integral membrane transporters. We characterised and purified the putative *Escherichia coli* MDR transporter, MdtM, a 410 amino acid residue protein that belongs to the large and ubiquitous major facilitator superfamily. Functional characterisation of MdtM using growth inhibition and whole cell transport assays revealed its role in intrinsic resistance of *E. coli* cells to the antimicrobials ethidium bromide and chloramphenicol. Site-directed mutagenesis studies implied that the MdtM aspartate 22 residue and the highly conserved arginine at position 108 play a role in proton recognition. MdtM was homologously overexpressed and purified to homogeneity in dodecyl-β-D-maltopyranoside detergent solution and the oligomeric state and stability of the protein in a variety of detergent solutions was investigated using size-exclusion HPLC. Purified MdtM is monomeric and stable in dodecyl-β-D-maltopyranoside solution and binds chloramphenicol with nanomolar affinity in the same detergent. This work provides a firm foundation for structural studies on this class of multidrug transporter protein.

1. Introduction

Drug efflux is a general mechanism that enables bacteria to resist the toxic effects of antimicrobial chemotherapeutics that have penetrated the cell [1]. The efflux process is mediated by expression of integral membrane protein pumps called multidrug resistance (MDR) transporters and individual transporters can catalyse active extrusion of a myriad of structurally and chemically disparate compounds from the bacterial cell [2]. The substrate promiscuity displayed by many of these efflux proteins means that expression of merely a single type can confer resistance to multiple classes of antibiotics typically used to treat human and animal infections [3].

Representatives of MDR transporter families are found in all bacterial genomes, underlining their fundamental role in survival. The preponderance of MDR genes, and their persistence in the absence of a selective pressure by antibiotics [4], has raised questions as to the true physiological role of the proteins they encode [5]. Studies of null mutants [6] and the resistance phenotypes of *Escherichia coli* overexpressing MDRs from multicopy plasmid [7] identified putative MDR transporters belonging to five families; the major facilitator superfamily (MFS), the ATP-binding cassette (ABC), resistance-nodulation-division (RND), small multidrug resistance (SMR) and multidrug and toxic compound (MATE) families. Although the single-component transporters account for the majority of MDR transporters in *E. coli* [7], the major intrinsic resistance of this bacterium is imparted by the RND AcrAB–TolC tripartite efflux system [8,9], suggesting only a minor role for the other MDR transporters. There is now convincing evidence that drug efflux by *E. coli* is a dual process in which the single-component MFS, SMR and MATE family MDRs function to deplete cytotoxins in the cytoplasm to sub-lethal levels by actively transporting them into the periplasm from where the AcrAB–TolC system can pump them into the extracellular milieu [9]. Furthermore, the substrate polyspecificity displayed by single-component MFS MDR transporters [3,10] enables an overlapping functionality

Abbreviations: BCA, bicinchoninic acid; BSA, bovine serum albumin; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; DDM, dodecyl-β-D-maltopyranoside; DHA1, drug/H^+ antiporter family 1; DM, decylmaltoside; EtBr, ethidium bromide; FC, Fos-choline; MDR, multidrug resistance; MATE, multidrug and toxic compound extrusion; MFS, major facilitator superfamily; NG, nonylglucoside; NM, nonylmaltoside; OG, octylglucoside; PMSF, phenylmethanesulfonyl fluoride; RND, resistance-nodulation-division; SE-HPLC, size-exclusion-high performance liquid chromatography; SMR, small multidrug resistance; TMS, transmembrane segment.

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that provides redundancy with respect to drug efflux and allows transporters to substitute for one another.

Of the 37 genes reported to encode putative MDR transporters in *E. coli*, eighteen belong to the MFS, making this the dominant group [7]. MFS proteins are secondary active transporters that drive substrate translocation by exploiting the free energy stored in ion or solute gradients. Mechanistically, they can function as either uniporters, symporters or antiporters; bacterial MDR transporters of the superfamily are generally drug/H^+ antiporters that use the electrochemical potential across the cell membrane to drive antibiotics and other cytotoxic compounds out of the cell [11]. Six families within the embrace of the MFS contain polyspecific MDR transporters: of these six, the most frequently occurring in bacteria are those of the 12- or 14-transmembrane segment (TMS) drug/H^+ antiporter families (DHA1 and DHA2, respectively) [12].

The structures of five 12-TMS MFS proteins have been resolved to date [13–17], and one of these, EmrD from *E. coli*, is a multidrug transporter [16]. Regrettably, EmrD is poorly characterised, which makes it challenging to relate its structure to its function, and any detailed interpretation of a structural basis for its substrate promiscuity is precluded by the fact that no substrate molecule is resolved in the structure. Nevertheless, the aforementioned structures have provided valuable general insight into the architecture and mechanism of the 12-TMS MFS transporters. Despite the lessons learnt from structural studies of the MFS, specific aspects of MFS MDR transporter function remain obscure: fundamental questions pertaining to kinetic and structural mechanisms, the structural basis of substrate polyspecificity and recognition, the role of conserved residues, and how proton transport is coupled to drug efflux all still require answers [18]. Biochemical, genetic and homology modelling studies of MdfA - the prototypical MDR transporter of the MFS [19] – have made inroads towards unravelling some of the complexities of multidrug efflux [20–26] and have provided a basis for answering some of these questions.

Revelations that MDR transporters also contribute to virulence of pathogenic bacteria underline their clinical significance and provide impetus to gain a more intimate understanding of their structures and mechanisms [27]. We therefore decided to functionally and biochemically characterise the gene product of the *E. coli* multidrug transporter MdtM. MdtM (formerly termed YjiO) is a putative 12-TMS drug/H^+ antiporter that shares 41% sequence identity with MdfA [19]. Although the drug resistance phenotype of the *mdtM* gene has been reported [7] and implicated in protecting bacterial cells from the effects of several types of antibiotic, the antiparasitic drug tinidazole, and the antimicrobials ethidium bromide (EtBr), acriflavine, tetraphenylphosphonium (TPP), crystal violet and 5-nitro-2-furaldehyde [7,9,10,19], the gene product has not previously been isolated or characterised. We show that our construct protects *E. coli* cells from the effects of the cationic antimicrobial ethidium bromide (EtBr) and neutral antibiotic chloramphenicol when overexpressed from multicopy plasmid, and that a negatively charged aspartate residue located in putative TM1 is important for transport function. Moreover, we demonstrate that MdtM can compensate for loss of MdfA, further highlighting the functional versatility of the MFS multidrug transporters and providing further evidence for compensatory backup as an effective survival mechanism in *E. coli*.

Purification of MdtM permitted the first biochemical investigation of this hitherto uncharacterised transporter, and we provide evidence to show the purified protein is stable and monomeric in dodecylmaltoside (DDM) detergent solution and retains substrate-binding activity in the same detergent. This work represents a critical step towards structural studies of a novel transporter belonging to an important and clinically relevant class of antibiotic resistance proteins.

2. Materials and methods

All growth media, antibiotics and chemicals were purchased from Sigma–Aldrich (Poole, Dorset, UK) unless stated otherwise.

2.1. Bioinformatics

Prediction of the transmembrane helices was done by inputting the primary sequence of MdtM from *E. coli* K-12 (UniProtKB P39386) into TMHMM [28]. Sequence alignments were performed using ClustalW2 with default settings [29].

2.2. Cloning, plasmids and bacterial strains

The 1230 bp coding region of the *mdtM* open reading frame was amplified from *E. coli* K-12 genomic DNA by PCR using forward and reverse primers designed according to the EcoGene DNA sequence (EG12576). The primers introduced an *Nco*1 site at the 5′ end of the gene and an *Eco*R1 site at the 3′ end to enable ligation into a modified pBAD/*Myc*-His A expression vector (Invitrogen) to give a construct encoding the 410 amino acid residues of MdtM with a C-terminal *myc*-epitope and a hexahistidine tag to facilitate purification of the protein. Use of the *Nco*1 restriction site introduced a Pro→Gly mutation at the second amino acid position of the transporter. A thrombin-specific proteolysis site permitted cleavage of the *myc*-His tag. The resulting plasmid was denoted pMdtM, and places *mdtM* expression under tight control of the *araBAD* promoter [30]. The D22A and R108K MdtM mutants were constructed using the QuikChange Lightning site-directed mutagenesis kit (Stratagene) with pMdtM as the template; these plasmids are termed pMdtM-D22A and pMdtM-R108K, respectively. The fidelity of all constructs was verified by DNA sequence analysis.

E. coli TOP10 (Invitrogen) was used as the host for cloning procedures. *E. coli* BW25113 [31] and its single-deletion Δ*mdtM* and Δ*mdfA* mutants were obtained from the *E. coli* Keio Knockout Collection [32] and used in assays that tested resistance to ethidium bromide and chloramphenicol. *E. coli* UTL2 [33] was used for the whole cell ethidium bromide transport assays. *E. coli* LMG194 [30] was used for overproduction of MdtM for protein purification.

2.3. Resistance to antimicrobials

Resistance assays were based on those described previously [9]. The resistance of wild-type (WT) and Δ*mdtM* single-deletion mutant *E. coli* BW25113 cells to various concentrations of ethidium bromide or chloramphenicol was determined in both solid and liquid media. To test the contribution of MdtM to resistance, assays were performed with WT and single-deletion deletion mutant BW25113 cells transformed with multicopy pMdtM. The effect of the D22A and R108K mutations was tested by transforming BW25113 cells with multicopy pMdtM-D22A or pMdtM-R108K. Empty pBAD/*Myc*-His A (henceforth referred to simply as pBAD) was used as the control plasmid. The effect of complementing Δ*mdfA* single-deletion mutant *E. coli* BW25113 cells with pMdtM on resistance to ethidium bromide was also assayed using solid and liquid media as described below.

For solid media plate assays, liquid cultures of *E. coli* BW25113 WT cells were grown at 37 °C in LB broth to an OD_{600} of 1.0. Single-deletion mutants were grown in the same medium supplemented with 30 μg ml^{-1} kanamycin. For cells harbouring plasmidic MdtM or pBAD vector the medium was supplemented with 100 μg ml^{-1} carbenicillin for selection. 4 μl aliquots from a series of logarithmic dilutions (covering the range of 10^{-2}–10^{-5}) of each culture were spotted onto LB agar plates containing 0.002% L-arabinose and the indicated concentration of antimicrobial compound. The inoculated