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Characterization of putative multidrug resistance transporters of the major facilitator-superfamily expressed in *Salmonella* Typhi

Aqsa Shaheen [a, b, c], Fouzia Ismat [a], Mazhar Iqbal [a, b], Abdul Haque [e], Rita De Zorzi [c, d], Osman Mirza [f], Thomas Walz [c, d], Moazur Rahman [a, b, c, *]

[a] *Drug Discovery and Structural Biology Group, Health Biotechnology Division, National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan*
[b] *Pakistan Institute of Engineering and Applied Sciences, Islamabad, Pakistan*
[c] *Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA*
[d] *Howard Hughes Medical Institute, Harvard Medical School, Boston, MA 02115, USA*
[e] *The University of Faisalabad, Faisalabad, Pakistan*
[f] *Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark*

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ABSTRACT

Multidrug resistance mediated by efflux pumps is a well-known phenomenon in infectious bacteria. Although much work has been carried out to characterize multidrug efflux pumps in Gram-negative and Gram-positive bacteria, such information is still lacking for many deadly pathogens. The aim of this study was to gain insight into the substrate specificity of previously uncharacterized transporters of *Salmonella* Typhi to identify their role in the development of multidrug resistance. *S.* Typhi genes encoding putative members of the major facilitator superfamily were cloned and expressed in the drug-hypersensitive *Escherichia coli* strain KAM42, and tested for transport of 25 antibacterial compounds, including representative antibiotics of various classes, antiseptics, dyes and detergents. Of the 15 tested putative transporters, STY0901, STY2458 and STY4874 exhibited a drug-resistance phenotype. Among these, STY4874 conferred resistance to at least ten of the tested antimicrobials: ciprofloxacin, norfloxacin, levofloxacin, kanamycin, streptomycin, gentamycin, nalidixic acid, chloramphenicol, ethidium bromide, and acriflavine, including fluoroquinolone antibiotics, which were drugs of choice to treat *S.* Typhi infections. Cell-based functional studies using ethidium bromide and acriflavine showed that STY4874 functions as a H^{+}-dependent exporter. These results suggest that STY4874 may be an important drug target, which can now be tested by studying the susceptibility of a STY4874-deficient *S.* Typhi strain to antimicrobials.

1. Introduction

Typhoid fever is a major health problem in the developing countries and is caused by *Salmoella enterica* serovar Typhi, which exclusively infects humans [1,2]. In 2000, an estimated 21.1 million cases of typhoid fever resulted in approximately 200,000 deaths. Over 90% of this morbidity and mortality occurred in Asia [3], which might be due to inadequate sanitary measures and unhygienic conditions, self medication and misuse of anti-typhoid antibiotics that resulted in the emergence of multidrug-resistant strains of *S.* Typhi [4]. The first antibiotic-resistant *S.* Typhi strain was reported in 1950s, and isolates exhibiting multidrug resistance (MDR) to all first-line antibiotics (chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole) emerged in the 1980s [5,6]. MDR isolates of *S.* Typhi were also found to have reduced susceptibility to the fluoroquinolone class of antibiotics, the antibiotics of choice for the treatment of enteric fever [7–9].

Bacteria have evolved different mechanisms of drug resistance that include enzymatic inactivation of drugs, mutational alteration of drug targets, and resistance mediated by efflux pumps [10]. Efflux-based drug resistance may be attained either by an increase

* Corresponding author. Drug Discovery and Structural Biology group, Health Biotechnology Division, National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan. Tel.: +92 41 2651475; fax: +92 41 2651472.
E-mail addresses: moazur@yahoo.com, moaz@nibge.org (M. Rahman).

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in the expression of an efflux pump protein or by a mutation in a transporter that makes it more efficient for the drug. Such resistance lowers the concentration of antimicrobials in the cell, and bacteria become less vulnerable to them [11]. Efflux pumps may be specific for a substrate or may transport a range of structurally dissimilar compounds. The latter can be associated with MDR and are of clinical significance due to their importance for drug design.

MDR efflux pumps constitute about 10% of the total number of transporters expressed by an organism, showing that these proteins perform critical functions [12]. Based on sequence similarities, MDR efflux pumps fall into five major families: the ATP-binding cassette (ABC) family, the multidrug and toxic compound exporters (MATE), the small multidrug resistance (SMR) family (part of the much larger drug/metabolite transporter superfamily), the resistance-nodulation-division proteins (RND), and the major facilitator superfamily (MFS) [13].

The MFS transporters represent the largest group of secondary active transporters, constituting 25% of all known membrane transport proteins in prokaryotes [14]. Although a number of MDR pumps of the MFS type from several bacteria have been characterized, including Bmr and Blt of *Bacillus subtilis*, MdfA and EmrD of *Escherichia coli* [15], LmrP of *Lactobacillus lactis* [16], NorA, QacA and LmrS of *Staphylococcus aureus* [17–19], KmrA of *Klebsiella pneumonia* [20], EmrAB, MdfA and MdtK from *S. Typhimurium* [21], SmfY from *Serrartia marcescens* [22] and VmrA of *Vibrio parahaemolytcus* [23], the transport characteristics of many putative and poorly characterized MFS-type MDR pumps have not yet been studied. To date, only one structure of an MFS-type MDR transporter, that of EmrD from *E. coli*, is available, and only in its apo state. Since EmrD is functionally not well characterized and since the structure shows no substrate molecule, the EmrD structure provided only limited information with regard to the molecular mechanisms that underlie drug export [24]. Such studies are of critical importance for the development of inhibitors of MDR transporters that can be used to combat antimicrobial resistance developed by pathogenic bacteria.

Although the genome of *S.* Typhi was sequenced in 2001 [25] and despite widespread *S.* Typhi infections in the developing world, none of the transporters encoded in the *S.* Typhi genome, including MFS-type MDR transporters, have yet been characterized. In the present study, we have therefore selected 16 putative MFS members from *S.* Typhi for functional characterization. The genes were cloned into a plasmid under the control of an isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible promoter, expressed in the drug-hypersensitive *E. coli* strain KAM42, which lacks the major multidrug efflux system AcrB-TolC and the MATE transporter YdhE [26], and the cells were assayed for their resistance to a set of 25 antimicrobials to characterize the substrate specificities of the expressed transporters. The functional cloning strategy used here to elucidate the antimicrobial efflux potential of putative MFS-type MDR transporters of *S.* Typhi using *E. coli* as a non-pathogenic host can be easily extended to other kinds of transporters from other pathogenic bacteria. Among the selected transporters, expression of STY4874, a homolog of the *E. coli* MdtM (or YjiO) transporter, conferred resistance to ten different antimicrobial compounds. STY4874 thus qualifies as an MDR efflux pump, and its physiological relevance can now be determined by studying the susceptibility of *S.* Typhi deficient in STY4874 to various antibiotics.

2. Methods

2.1. Cloning and expression of putative MFS-type MDR transporters

Genomic DNA for 16 putative MFS-type MDR transporters was isolated from *S. enterica* serovar Typhi strain NIBGE-AS1, cloned into an expression vector with C-terminal oligohistidine tag, and expressed in *E. coli* strain KAM42 as detailed in Supplementary Information.

2.2. Determination of the minimum inhibitory concentration (MIC)

The MIC of various compounds was determined following the guidelines of the Clinical and Laboratory Standards Institute (http://www.clsi.org/) as described in Supplementary Information.

2.3. Efflux assays

Acriflavine and ethidium bromide efflux assays were performed as described before [19,23] with the minor modifications described in Supplementary Information.

3. Results

3.1. Selection of putative MFS-type MDR transporters for investigation

The TransportDB database (http://www.membranetransport.org/) contains many putative and partially characterized MFS-type MDR transporters for *S.* Typhi. However, a previous study demonstrated that many predicted MFS-type MDR transporters in *E. coli* did not confer resistance to any of the tested antimicrobials [27]. We therefore decided to functionally characterize the putative MFS-type MDR pumps in *S.* Typhi. Sequence analysis revealed that, except for STY2603 and STY4230, the selected putative MFS-type MDR transporters have less than 20% sequence identity (Fig. 1A). Their transmembrane helices were predicted using the hidden Markov topology predictor TMHMM (Supplementary Table 1) [28], and their primary sequences were analyzed for motifs conserved in MFS proteins as described [29] (Supplementary Table 2).

3.2. Strategy for the generation of expression vectors for the putative MFS-type MDR pumps

It has been shown that a membrane protein expresses at a much higher level in *E. coli* when an oligohistidine tag is fused to an N or C terminus that is located in the cytoplasm than when it is fused to a terminus that is located in the periplasm [30]. We therefore used the program TMHMM [28] to predict the location of the N and C termini of the putative MFS-type MDR transporters (Supplementary Table 1). Whenever possible, we also used knowledge for the localization of the termini of *E. coli* homologs [31,32] to predict the location of the termini of our target proteins. Since these analyses suggested that the C termini of all our selected targets are located in the cytoplasm, we decided to express them with a C-terminal oligohistidine tag.

Expression vectors were constructed as described in Methodology. The genes encoding putative MFS-type MDR transporters were cloned into the pTTQ18-based expression vector pMR4, in which multiple cloning sites are flanked by the strong hybrid *trp-lac* (*tac*) promoter and the *rrnB* transcription terminator [33]. The pMR4 vector also carries the *lacI^q* allele of the *lac* repressor gene, which ensures repression of the *tac* promoter in the absence of IPTG. A derivative of the pTTQ18 expression vector allowed expression of membrane proteins in *E. coli* up to ~25% of the total inner membrane protein content [30,34]. Expression vectors constructed in this study were annotated according to the name of the genes in the membrane transport database (http://www.membranetransport.org/other_family.php?fFID=MFS&oOID=styp1) (Supplementary Table 1).

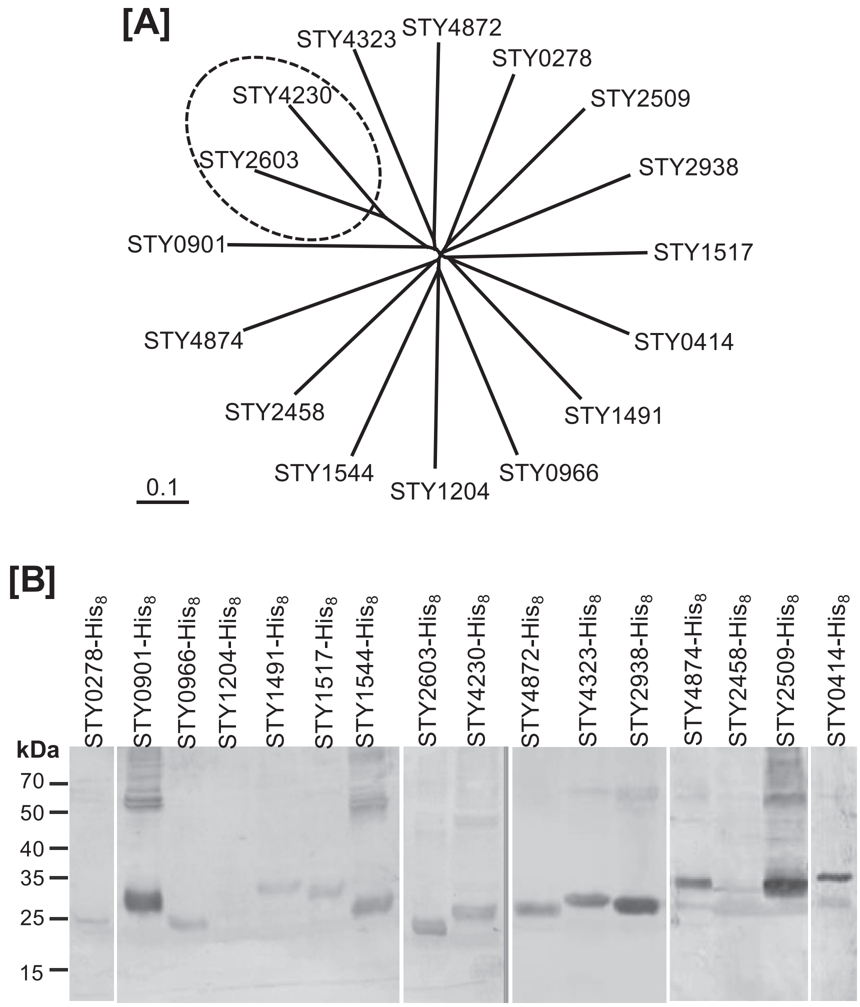

Fig. 1. The selected putative MFS-type MDR transporters of *S.* Typhi and their expression in *E. coli*. (A) Phylogenetic tree of the selected transporters. With the exception of STY4230 and STY2630 (circled), which share a sequence identity of 41%, all other proteins share a sequence identity of less than 20%. The sequence alignment was done with the program ClustalX, version 1.83, and the tree was drawn using the program TreeView. The scale bar indicates an evolutionary distance of 0.1 amino acid substitutions per site. (B) Western blot of the selected putative MFS-type MDR transporters expressed in *E. coli*. All proteins expressed in *E. coli* strain KAM42, except for STY1204, which did not express under all tested conditions.

3.3. Expression of the putative S. Typhi MFS-type MDR transporters in E. coli

Test expression of the selected *S.* Typhi MFS homologs in *E. coli* host strain KAM42 showed that all proteins expressed to a detectable level, with the exception of STY1204, which failed to express under all tested conditions and was therefore dropped from further investigation. A Western blot of all the expressed proteins is shown in Fig. 1B. The faster migration of the proteins on SDS-PAGE than expected from their amino acid sequences (Supplementary Table 1) likely reflects their highly hydrophobic nature [34].

3.4. Anitmicrobial profiling of the putative MFS-type MDR transporters

The minimum inhibitory concentration (MIC) assay was used to assess the substrate specificity of the putative MFS-type MDR transporters. Expression constructs (Supplementary Table 1) and vector-only control (pMR4) were transformed into the drug-hypersensitive *E. coli* strain KAM42, which lacks the major multidrug efflux system AcrB-TolC and an MDR pump, YdhE [35], and the MIC was determined for a set of 25 different compounds that included various classes of antibiotics, antiseptics, fluorescent dyes and detergents (Table 1).

The results revealed that cells expressing STY0901 were two times more resistant to benzalkonium chloride than control cells, indicating that STY0901 transports this compound. Expression of STY2458, a homolog of the *E. coli* bicyclomycin transporter (Bcr), increased the resistance of the cells to doxycyline, tetracycline and kanamycin by a factor of two. STY2458 expression initially also appeared to result in a two-fold resistance to methyl viologen, but this finding was not reproducible. Expression of STY4874, a homolog of the *E. coli* MDR transporter MdtM (or YjiO), increased the MICs for ten of the tested compounds belonging to different classes of antibiotics and dyes. These are the fluoroquinolone antibiotics ciprofloxacin (four-fold), norfloxacin (four-fold) and levofloxacin (two-fold), the aminoglycoside antibiotics kanamycin (four-fold), streptomycin (four-fold) and gentamycin (two-fold), the quinolone antibiotic nalidixic acid (two-fold), the amphenicol antibiotic chloramphenicol (two-fold), and the fluorescent dyes ethidium bromide (four-fold) and acriflavine (two-fold). These results demonstrate that STY4874 is a true MDR transporter that is most effective against antibiotics of the fluoroquinolone and aminoglycoside classes.

Table 1
Minimum inhibitory concentration (MIC) of various antimicrobials for *E. coli* KAM42 cells expressing putative MFS-type MDR transporters.

Antibiotics/Biocides/Detergents/Dyes		MIC (μg/mL)															
		Putative transporter gene															
		pMR4-STY0278	pMR4-STY0414	pMR4-STY0901	pMR4-STY0966	pMR4-STY1491	pMR4-STY1517	pMR4-STY1544	pMR4-STY2458	pMR4-STY2509	pMR4-STY2603	pMR4-STY2938	pMR4-STY4230	pMR4-STY4323	pMR4-STY4872	pMR4-STY4874	pMR4 (control)
Antibiotics	Ciprofloxacin	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.008	0.002
	Levofloxacin	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.008	0.004
	Nalidixic acid	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1	0.5
	Norfloxacin	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.125	0.03
	Doxycycline	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.25	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125
	Tetracycline	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.5	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
	Kanamycin	1	1	1	1	1	1	1	2	1	1	1	1	1	1	4	1
	Streptomycin	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	2	0.5
	Gentamycin	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.5	0.25
	Chloramphenicol	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1.0	0.5
Biocide	Benzalkonium chloride	4	4	8	4	4	4	4	4	4	4	4	4	4	4	4	4
Dyes	Acriflavine	4	4	4	4	4	4	4	4	4	4	4	4	4	4	8	4
	Ethidium bromide	8	8	8	8	8	8	8	8	8	8	8	8	8	8	32	8

All putative MFS-type MDR transporter constructs were transformed into drug-hypersensitive *E. coli* strain KAM42 (*ΔacrB, ΔtolC ΔydhE*) [35] and expressed under the control of the *tac* promoter. LB agar plates were supplemented with 0.01 mM IPTG to induce expression and 50 μg/ml Ap^r to ensure plasmid maintenance. The test compounds were added with two-fold serial dilution, and the MIC was defined as the lowest concentration of the test compound that prevented visible growth of bacteria on the LB agar plate. Among the tested compounds, following compounds did not show resistance against any putative transporter; methyl viologen, cetyl trimethyl ammonium bromide, deoxycholate, sodium dodecyl sulfate, rhodamine 6 g, trimethoprim, amoxicillin, rifampicin, cefixime, cefotaxime, neomycin, erythromycin.

Expression of all the other proteins did not confer resistance to any of the tested compounds. Possible reasons for this are that these proteins may have different physiological role(s), for example in the colonization and persistence of bacteria in the host [11], or they transport substrates different from those tested in this study. Further studies will be required to establish the biological function of these MFS proteins.

3.5. Real-time measurement of ethidium bromide and acriflavine transport by STY4874

To ascertain that the increased resistance to antimicrobials of cells expressing STY4874 seen in the cell-based MIC assay was due to STY4874-mediated export of the tested compounds rather than other factors, such as, for example, a decreased permeability of the *E. coli* outer membrane for these compounds, we decided to directly measure the export of ethidium bromide and acriflavine by STY4874 (Table 1). These two dyes intercalate with DNA, which changes their fluorescence characteristics. Binding to DNA enhances the fluorescence of ethidium bromide [19], whereas DNA binding reduces the fluorescence of acriflavine [23]. These characteristics allow real-time monitoring of the intracellular dye concentration in live cells without the need of sampling, cell disruption or antibiotic quantification [36].

Efflux of ethidium bromide and acriflavine was measured for *E. coli* KAM42 cells harboring either the pMR4-STY4874 expression vector or, as negative control, the empty pMR4 vector. The cells were loaded with ethidium bromide or acriflavine, and fluorescence was monitored at 595 nm and 535 nm, respectively. After 50–100 s, the cells were energized by addition of glucose to generate an electrochemical gradient across the cell membrane through aerobic metabolism. In the case of ethidium bromide, glucose addition to cells expressing STY4874 resulted in a decrease in fluorescence intensity compared to control cells, showing that ethidium bromide was actively extruded from the cells (Fig. 2A). In contrast, the fluorescence intensity of acriflavine increased upon glucose addition, showing that this dye is also actively exported by STY4874 (Fig. 2B). These results demonstrate that both ethidium bromide and acriflavine are true substrates of STY4874.

To verify that substrate transport by STY4874 depends on a proton gradient as energy source, the ionophore CCCP (Carbonyl cyanide m-chlorophenyl hydrazone) was used to dissipate the transmembrane proton gradient. Addition of CCCP increased the fluorescence of ethidium bromide and decreased the fluorescence of acriflavine, indicating that STY4874 requires a proton gradient to export the dyes from the cells (Fig. 2). These findings are in agreement with previous studies that showed that MFS-type MDR transporters are secondary active transport proteins that function as proton/antimicrobial antiporters [37].

4. Discussion

The current study was aimed to establish whether some of the uncharacterized MFS proteins in *S.* Typhi function as MDR transporters. Based on sequence analysis, topology prediction, homology and motif searches, 16 putative MFS protein were selected (Fig. 1A). These targets were cloned into pTTQ18-based vectors with a C-terminal oligohistidine tag and expressed in the drug-hypersensitive *E. coli* strain KAM42 (Fig. 1B). Extensive substrate profiling experiments identified three transporters that conferred resistance against some of the tested antimicrobials (Table 1). One of them, STY0901, unambiguously transports benzalkonium chloride, a cationic substrate. As this was the only one of the 25 antimicrobials we tested that was transported by STY0901, further tests

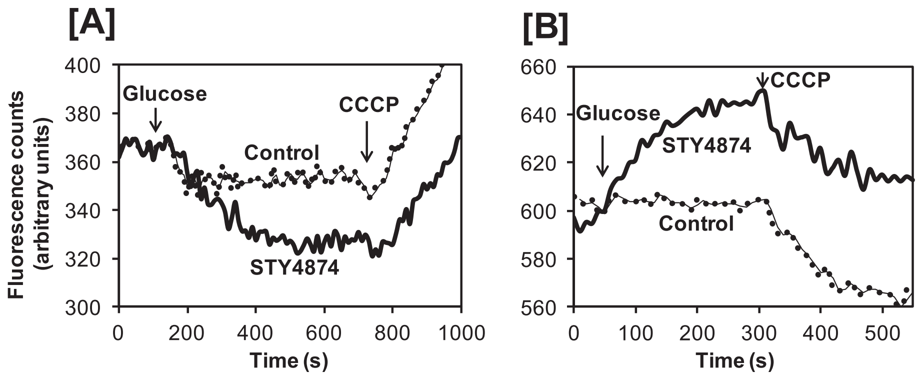

Fig. 2. Analysis of STY4874-mediated export of ethidium bromide and acriflavine. (A) Efflux of ethidium bromide from *E. coli* KAM42 cells expressing STY4874 (thick line) and from control cells (thin line with circles). Fluorescence was monitored at 595 nm. After 100 s, cells loaded with ethidium bromide were energized by addition of 0.5% glucose (first arrow), resulting in a decrease of fluorescence of cells expressing STY4874. Dissipation of the transmembrane proton gradient by addition of 40 μM CCCP (second arrow) resulted in the inhibition of active transport by STY4874 and a concomitant increase in fluorescence. (B) Efflux of acriflavine from *E. coli* KAM42 cells expressing STY4874 (thick line) and from control cells (thin line with circles). Fluorescence was monitored at 535 nm. After 50 s, cells loaded with acriflavine were energized by addition of 0.5% glucose (first arrow), resulting in an increase of fluorescence of cells expressing STY4874. Dissipation of the transmembrane proton gradient by addition of 40 μM CCCP (second arrow) resulted in the inhibition of active transport by STY4874 and a concomitant decrease in fluorescence.

will be needed to confirm the assignment in TransportDB [38] that this protein is indeed a poly-substrate specific pump. STY0901 shares homology with several uncharacterized transporters, and our study now suggests that these may also transport benzalkonium. Another putative efflux transporter, STY2458, a homolog of the *E. coli* bicyclomycin transport protein (Bcr) [39], conferred resistance to the tetracycline antibiotics doxycycline and tetracycline, and the aminoglycoside antibiotic kanamycin. In contrast to *E. coli* Bcr, however, which also confers a two-fold resistance to acriflavine [27], STY2458 had no effect on acriflavine sensitivity under the conditions tested in this study.

The third putative *S.* Typhi MFS-type MDR protein, STY4874, has 39% amino acids sequence identity with the well-characterized *E. coli* transporter MdfA [37,40–43], and 87% with MdtM, another *E. coli* homolog [44]. STY4874 shows the clearest characteristics of an MDR transporter, conferring resistance to ten of the tested antimicrobials: ciprofloxacin, norfloxacin, levofloxacin, kanamycin, streptomycin, gentamycin, nalidixic acid, chloramphenicol, ethidium bromide, and acriflavine. We also found that STY4874 does not confer resistance to benzalkonium chloride (Table 1), which is consistent with an earlier study by Nishino and Yamaguchi on MdtM (or YjiO), the *E. coli* homolog of STY4874, that also did not show resistance against benzalkonium chloride [27]. In another study, Holdsworth and Law recently reported, however, that MdtM from *E. coli* does confer resistance to various quaternary ammonium compounds (QACs), including benzalkonium chloride [45]. This difference might reflect different experimental conditions. Nishino and Yamaguchi and our studies were performed on LB agar plates using the related *tac* and *trc* promoters, whereas Holdsworth and Law conducted their assays in liquid media using the arabinose promoter.

Like with the putative MFS-type MDR transporters in *E. coli* [27], many of the putative *S.* Typhi MFS proteins we selected did not confer resistance to any of the 25 tested antimicrobials (Table 1). Possibly some of these proteins transport other antimicrobials that we did not test for, but only a subgroup of MFS proteins function as MDR transporters while most MFS proteins are involved in the transport of a wide variety of other kinds of substrates. However, numerous MFS proteins have been annotated as MDR transporters merely on the basis of sequence homology. For instance, the membrane transport database annotated STY1491 as a methyl viologen resistance protein [38], but our study now shows that this protein does not transport this compound. This finding emphasizes the need to experimentally verify the substrate specificity of putative MFS-type MDR transporters.

Of the *S.* Typhi MFS proteins tested in this study, STY4874 was the most likely MDR transporter, as it conferred resistance to ten of the 25 tested antimicrobials. To verify its function as an MDR transporter, we confirmed by real-time measurements that cells expressing STY4874 transported ethidium bromide and acriflavine out of the cells (Fig. 2). This transport was proton-dependent, as addition of the uncoupler CCCP abolished efflux of the compounds (Fig. 2). Proton-dependent transport of ethidium bromide was also seen for the *E. coli* homolog MdtM [44], but efflux of acriflavine has not been reported before for STY4874 or any of its homologs.

In conclusion, we have identified that STY4874 is a proton-dependent efflux pump that transports several of the tested fluoroquinolone antibiotics, which were drugs of choice, alone or in combination with other antimicrobials, to treat *S.* Typhi infections [5,6,8,9,46]. Our finding suggests that STY4874 may be an important drug target. This notion can now be tested by studying the antimicrobial susceptibility of an *S.* Typhi strain, in which the STY4874 gene has been deleted or inactivated.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jiac.2015.01.002.

References

[1] Tran TC, Campbell JI, Galindo C, Tran TH, Nguyen TC, Acosta CJ, et al. Antimicrobial drug resistance of *Salmonella* Typhi in Asia. Int J Infect Dis 2008;12: E120.

[2] Afzal A, Sarwar Y, Ali A, Haque A. Current status of fluoroquinolone and cephalosporin resistance in *Salmonella enterica* serovar Typhi isolates from Faisalabad, Pakistan. Pak J Med Sci 2012;28:602–7.

[3] Ochiai RL, Acosta CJ, novaro-Holliday M, Baiqing D, Bhattacharya SK, Agtini MD, et al. A study of typhoid fever in five Asian countries: disease burden and implications for controls. Bull World Health Organ 2008;86: 260–8.

[4] Madukosiri CH, Edike T, Ghandi EO. Comparative studies of susceptibility of *Salmonella* Typhi to antibiotics and some plant extracts. Niger J Biochem Mol Biol 2009;24:16–21.

[5] Chau TT, Campbell JI, Galindo CM, Hoang NVM, Diep TS, Nga TTT, et al. Antimicrobial drug resistance of *Salmonella enterica* serovar Typhi in Asia and molecular mechanism of reduced susceptibility to the fluoroquinolones. Antimicrob Agents Chemother 2007;51:4315–23.

[6] Wain J, Kidgell C. The emergence of multidrug resistance to antimicrobial agents for the treatment of typhoid fever. Trans R Soc Trop Med Hyg 2004;98: 423–30.

[7] Butler T. Treatment of typhoid fever in the 21st century: promises and shortcomings. Clin Microbiol Infect 2011;17:959–63.

[8] Afzal A, Sarwar Y, Ali A, Maqbool A, Salman M, Habeeb MA, et al. Molecular evaluation of drug resistance in clinical isolates of *Salmonella enterica* serovar Typhi from Pakistan. J Infect Dev Ctries 2013;7:929–40.

[9] Qamar FN, Azmatullah A, Kazi AM, Khan E, Zaidi AKM. A three-year review of antimicrobial resistance of *Salmonella enterica* serovars Typhi and Paratyphi A in Pakistan. J Infect Dev Ctries 2014;8:981–6.

[10] Nikaido H. Multidrug resistance in bacteria. Ann Rev Biochem 2009;78: 119–46.

[11] Piddock LJV. Multidrug-resistance efflux pumps – not just for resistance. Nat Rev Microbiol 2006;4:629–36.

[12] Paulsen IT. Multidrug efflux pumps and resistance: regulation and evolution. Curr Opin Microbiol 2003;6:446–51.

[13] Nishino K, Nikaido E, Yamaguchi A. Regulation and physiological function of multidrug efflux pumps in *Escherichia coli* and *Salmonella*. Biochim Biophys Acta 2009;1794:834–43.

[14] Law CJ, Maloney PC, Wang DN. Ins and outs of major facilitator superfamily antiporters. Ann Rev Microbiol 2008;62:289–305.

[15] Smith KP, Kumar S, Varela MF. Identification, cloning, and functional characterization of EmrD-3, a putative multidrug efflux pump of the major facilitator superfamily from *Vibrio cholerae* O395. Arch Microbiol 2009;191: 903–11.

[16] Mazurkiewicz P, Poelarends GJ, Driessen AJM, Konings WN. Facilitated drug influx by an energy-uncoupled secondary multidrug transporter. J Biol Chem 2004;279:103–8.

[17] Brown MH, Skurray RA. Staphylococcal multidrug efflux protein QacA. J Mol Microbiol Biotechnol 2001;3:163–70.

[18] Yu JL, Grinius L, Hooper DC. NorA functions as a multidrug efflux protein in both cytoplasmic membrane vesicles and reconstituted proteoliposomes. J Bacteriol 2002;184:1370–7.

[19] Floyd JL, Smith KP, Kumar SH, Floyd JT, Varela MF. LmrS is a multidrug efflux pump of the major facilitator superfamily from *Staphylococcus aureus*. Antimicrob Agents Chemother 2010;54:5406–12.

[20] Ogawa W, Koterasawa M, Kuroda T, Tsuchiya T. KmrA multidrug efflux pump from *Klebsiella pneumoniae*. Biol Pharm Bull 2006;29:550–3.

[21] Nishino K, Latifi T, Groisman EA. Virulence and drug resistance roles of multidrug efflux systems of *Salmonella enterica* serovar Typhimurium. Mol Microbiol 2006;59:126–41.

[22] Shahcheraghi F, Minato Y, Chen J, Mizushima T, Ogawa W, Kuroda T, et al. Molecular cloning and characterization of a multidrug efflux pump, SmfY, from *Serratia marcescens*. Biol Pharm Bull 2007;30:798–800.

[23] Chen J, Morita Y, Huda MN, Kuroda T, Mizushima T, Tsuchiya T. VmrA, a member of a novel class of Na^+-coupled multidrug efflux pumps from *Vibrio parahaemolyticus*. J Bacteriol 2002;184:572–6.

[24] Yin Y, He X, Szewczyk P, Nguyen T, Chang G. Structure of the multidrug transporter EmrD from *Escherichia coli*. Science 2006;312:741–4.

[25] Parkhill J, Dougan G, James KD, Thomson NR, Pickard D, Wain J, et al. Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. Nature 2001;413:848–52.

[26] Rahman MM, Matsuo T, Ogawa W, Koterasawa M, Kuroda T, Tsuchiya T. Molecular cloning and characterization of all RND-type efflux transporters in *Vibrio cholerae* non-O1. Microbiol Immunol 2007;51:1061–70.

[27] Nishino K, Yamaguchi A. Analysis of a complete library of putative drug transporter genes in *Escherichia coli*. J Bacteriol 2001;183:5803–12.

[28] Krogh A, Larsson B, von Heijne G, Sonnhammer EL. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol 2001;305:567–80.

[29] Putman M, Van Veen HW, Konings WN. Molecular properties of bacterial multidrug transporters. Microbiol Mol Biol Rev 2000;64:672–93.

[30] Rahman M, Ismat F, McPherson MJ, Baldwin SA. Topology-informed strategies for the overexpression and purification of membrane proteins. Mol Membr Biol 2007;24:407–18.

[31] Daley DO, Rapp M, Granseth E, Melen K, Drew D, von Heijne G. Global topology analysis of the *Escherichia coli* inner membrane proteome. Science 2005;308:1321–3.

[32] Granseth E, Daley DO, Rapp M, Melen K, von Heijne G. Experimentally constrained topology models for 51,208 bacterial inner membrane proteins. J Mol Biol 2005;352:489–94.

[33] Stark MJ. Multicopy expression vectors carrying the *lac* repressor gene for regulated high-level expression of genes in *Escherichia coli*. Gene 1987;51: 255–67.

[34] Ward A, Sanderson NM, O'Reilly J, Rutherford NG, Poolman B, Henderson PJF. The amplified expression, identification, purification, assay and properties of histidine-tagged bacterial membrane transport proteins. In: Baldwin SA, editor. Membrane transport – a practical approach. Oxford: Oxford University Press; 2000. p. 141–66.

[35] Matsuo T, Chen J, Minato Y, Ogawa W, Mizushima T, Kuroda T, et al. SmdAB, a heterodimeric ABC-type multidrug efflux pump, in *Serratia marcescens*. J Bacteriol 2008;190:648–54.

[36] Webber MA, Coldham NG. Measuring the activity of active efflux in Gram-negative bacteria. In: Gillespie SH, McHugh TD, editors. Antibiotic resistance protocols. Humana Press; 2010. p. 173–80. 642 ed.

[37] Fluman N, Bibi E. Bacterial multidrug transport through the lens of the major facilitator superfamily. Biochim Biophys Acta 2009;1794:738–47.

[38] Ren QH, Chen KX, Paulsen IT. TransportDB: a comprehensive database resource for cytoplasmic membrane transport systems and outer membrane channels. Nucleic Acids Res 2007;35:D274–9.

[39] Bentley J, Hyatt LS, Ainley K, Parish JH, Herbert RB, White GR. Cloning and sequence analysis of an *Escherichia coli* gene conferring bicyclomycin resistance. Gene 1993;127:117–20.

[40] Edgar R, Bibi E. A single membrane embedded negative charge is critical for recognizing positively charged drugs by the *Escherichia coli* multidrug resistance protein MdfA. EMBO 1999;18:822–32.

[41] Sigal N, Lewinson O, Wolf SG, Bibi E. *E. coli* multidrug transporter MdfA is a monomer. Biochemistry 2007;46:5200–8.

[42] Sigal N, Vardy E, Molshanski-Mor S, Eitan A, Pilpel Y, Schuldiner S, et al. 3D model of the *Escherichia coli* multidrug transporter MdfA reveals an essential membrane-embedded positive charge. Biochemistry 2005;44: 14870–80.

[43] Tirosh O, Sigal N, Gelman A, Sahar N, Fluman N, Siemion S, et al. Manipulating the drug/proton antiport stoichiometry of the secondary multidrug transporter MdfA. Proc Natl Acad Sci U S A 2012;109:12473–8.

[44] Holdsworth SR, Law CJ. Functional and biochemical characterisation of the *Escherichia coli* major facilitator superfamily multidrug transporter MdtM. Biochimie 2012;94:1334–46.

[45] Holdsworth SR, Law CJ. The major facilitator superfamily transporter MdtM contributes to the intrinsic resistance of *Escherichia coli* to quaternary ammonium compounds. J Antimicrob Chemother 2013;68: 831–9.

[46] Joshi YK, Joshi Maneesha. Symposium: typhoid fever; management and prevention. J Ind Acad Clin Med 2001;2:21–4.