J Infect Chemother 21 (2015) 357–362

Contents lists available at ScienceDirect

Journal of Infection and Chemotherapy

journal homepage: http://www.elsevier.com/locate/jic

Original article

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*

ARTICLE INFO

Article history:
Received 24 September 2014
Received in revised form
3 December 2014
Accepted 2 January 2015
Available online 9 January 2015

Keywords:
MDR pump
MFS
Salmonella Typhi
Efflux pump
Multidrug resistance

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).

http://dx.doi.org/10.1016/j.jiac.2015.01.002
1341-321X/

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).