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Review

Molecular mechanisms of antimicrobial transport across the membrane by multidrug efflux pumps of the major facilitator superfamily in bacterial pathogens

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ABSTRACT

Bacterial pathogens confer virulence by resisting the inhibitory nature of antimicrobial agents during infection. Membrane transporters of the major facilitator superfamily (MFS) mediate the active efflux of structurally distinctive antimicrobial agents. Treatment failures in patients with bacterial infectious disease frequently involve multidrug efflux systems of the MFS. These multidrug transporters are energized by secondary active transport mechanisms involving conformational changes tied to drug transport catalysis to mediate efflux across the bacterial membrane. This review article briefly summarizes recent developments regarding the molecular nature of the MFS multidrug efflux in bacterial pathogens. These active transporter systems represent promising targets for biotechnological approaches to reduce the conditions that foster infections that are recalcitrant to chemotherapy.

KEYWORDS: major facilitator superfamily, antimicrobial transport, multidrug resistance, bacteria, pathogens, drug efflux pumps, solute transport, transporter proteins, multidrug efflux.

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1. Introduction

Pathogens in seafood can cause diverse infections, mostly gastrointestinal in nature, ranging in severity from mild to fatal. Every year, an estimated 600 million people fall ill due to foodborne infections, resulting in 420,000 deaths, 40% of which (about 125,000) are children, and the loss of 33 million disability-adjusted life years (DALYs) [1]. All food types are involved in outbreaks, such as meat, fish, vegetables, fruits, milk, or their products. Pathogens are either naturally present in the food, such as the diverse bacteria associated with meat, or introduced into food during various stages of growing, harvesting, processing, transportation, or preparation [2, 3]. Unhygienic food preparation facilities or personnel contribute to food contamination with pathogens, and their numbers are amplified when the foods are improperly stored and prepared. The major foodborne pathogens of public health significance, often involved in foodborne disease outbreaks, are the pathogenic *Escherichia coli*, *Salmonella enterica*, *Campylobacter jejuni*, *Listeria monocytogenes*, and *Vibrio* species [4]. Over 250 different types of infections are associated with food. Children, older populations, and the immunocompromised are at greater risk from foodborne infections. In most cases,

foodborne bacterial infections cause mild to severe gastroenteritis and other associated symptoms, which may be self-limiting or require hospitalization, which is a primary reason for underreporting.

A report from the United States suggests that 25,606 infection cases, 5,893 hospitalizations, and 120 deaths from foodborne infections occurred in 2018 [5]. Among bacterial pathogens, *Campylobacter* spp. cause the most number of infections (19.5 per 100,000 population), followed by *Salmonella* (18.3), Shiga toxin-producing *Escherichia coli* (STEC) (5.9), *Shigella* (4.9), *Vibrio* (1.1), *Yersinia* (0.9), and *Listeria monocytogenes* (0.3) [5]. The Centers for Disease Control (CDC) estimates that each year 48 million people get sick with foodborne infections, 128,000 are hospitalized, and 3,000 die in the United States [6]. Furthermore, in the European Union, 3,086 foodborne outbreaks by bacteria in 2020 involved 20,017 human cases, with *Salmonella* as the leading agent of illness, followed by *Campylobacter* spp., STEC, and *Listeria monocytogenes* [7].

2. Important foodborne bacterial pathogens

2.1. *Campylobacter* spp.

Campylobacter jejuni is the most common *Campylobacter* spp. responsible for human infections, causing gastroenteritis, meningitis, and bloodstream infections. Less than 0.25% of the cases develop into a serious state of infection called Guillain-Barré syndrome (GBS), a chronic neurological disorder characterized by ascending paralysis and related complications [8].

In the United States, *Campylobacter* causes 850,000 infections each year, leading to 8,500 hospitalizations and 76 deaths [9], while in the European Union, about 1% of the Western European population is infected by *Campylobacter* annually [10]. Campylobacters are microaerophilic, requiring 10-15% of CO_2 environment for optimum growth. Animals and birds are the reservoirs of *Campylobacter* spp., and the consumption of insufficiently cooked poultry meat, raw egg, or unpasteurized milk are the common causes of *Campylobacter* infections.

2.2. *Salmonella* spp.

Salmonella enterica, particularly of the non-typhoidal group, is commonly responsible for foodborne infections. Salmonellae are a diverse group of bacteria with over 2,500 serotypes, determined by the lipopolysaccharide (O), flagellar (H), and capsular (K) antigens. Every year, *Salmonella* causes an estimated 78 million cases of illness, 59,000 deaths, and 4 million DALYs globally [11]. The three most common serovars involved in foodborne infections are Enteritidis, Newport, and Typhimurium [5]. Chicken and eggs are the most likely sources of *Salmonella* infections, although various other food products, including milk and milk products, seafood, fruits, and vegetables, are also involved in large outbreaks of *Salmonella* food poisoning. Gastroenteritis caused by non-typhoidal *Salmonella* infections is usually self-limiting. However, invasive salmonellosis leads to more severe health complications such as septicemia, meningitis, osteomyelitis, and even death [12].

Several large outbreaks of foodborne infections have been associated with non-typhoidal *Salmonella* involving various foods. These include *Salmonella* Thompson with smoked salmon [13], *Salmonella* Agona with powdered infant formula [14], *Salmonella* Braenderup, *Salmonella* Newport, *Salmonella* Typhimurium with tomatoes [15], *Salmonella* Thompson with Rucola Lettuce [16], *Salmonella* Poona with cucumbers [17], *Salmonella* Tennessee with contaminated peanut butter [18], *Salmonella* Oranienburg with chocolates [19], multidrug-resistant *Salmonella* Typhimurium Definitive Type 104 with commercial ground beef [20], *Salmonella* Enteritidis with eggs [21], to mention a few.

2.3. *Escherichia coli*

Among five pathogroups of *E. coli* identified by their distinct mechanisms of infection, virulence gene composition, and serotypes, *viz*. Enteropathogenic (EPEC), Enterotoxigenic (ETEC), Shiga toxin-producing or Enterohaemorrhagic (STEC/EHEC), Enteroaggregative (EAEC) and Enteroinvasive (EIEC) *E. coli* [22], STEC are more commonly associated with large outbreaks

involving various food types, particularly meat and meat products [23]. The STEC/EHEC group causes bloody diarrhea, which occasionally can progress into more severe complications such as haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) [24].

Infection of STEC usually occurs through contaminated food and water. Further, the pathogenic strain can transmit from person-to-person and infected animals to humans *via* close contact [25]. Domestic and farm animals such as cattle, sheep, and goats are known to be the major reservoir of STEC [26]. Raw/undercooked/frozen ground beef products, seafood, raw milk products, different salads, vegetables such as raw leeks and potatoes, sprouts, fruits, raw prepacked cookie dough, etc. have been identified as etiological agents in most of the outbreaks [27, 28]. Of 400 serotypes of STEC, 160 serotypes have been recovered from patients with HC or HUS in which O157:H7, O26, O103, O121, O111, O145, O45, and O4 are the most virulent serotypes involved in food poisoning outbreaks [29]. Ingesting as few as 1 to 10 cells may cause illness in humans. A large foodborne outbreak by STEC O104:H4 through contaminated raw sprouts was reported from Germany in 2011, involving 3,816 cases of infection and 54 deaths [30], followed by a report of 8,313 confirmed cases from 29 EU/EEA countries in 2019 [31]. Approximately 265,000 STEC infections happen in the United States annually, of which 64% of the cases are caused by non-O157 STEC [32]. The figures might be underestimated as not all STEC infections are diagnosed and reported.

2.4. *Listeria monocytogenes*

L. monocytogenes can frequently be isolated from soil, water, and vegetation, and they can cause serious foodborne infections in susceptible populations [33]. Listeriosis is of considerable concern for the elderly, pregnant women, neonates, and immuno-compromised individuals. The disease manifests as septicemia, meningitis, encephalitis, and death; in pregnant women, listeriosis may result in miscarriages or stillbirths [34]. The recent listeriosis outbreak associated with raw milk cheese in the US in 2017 led to the infection and hospitalization of eight people, out of which two causalities were reported. Although *L. monocytogenes* is widely found in nature, more than 14 serotypes have been identified based on the immune-reactivity of two cell surface structures, the O and H antigens, of which three serotypes (1/2a, 1/2b, and 4b) cause approximately 95% of human illness [35].

The capacity to persist in the environment for long periods and over a wide range of challenging conditions makes *L. monocytogenes* extremely difficult to eradicate, especially in the food processing environment where many reservoirs have been found to harbor the pathogen. Floors, drains, pipes, cleaning tools (sponges or brushes), conveyor belts, packaging equipment, slicers/ dicers/blenders, etc., frequently contain high numbers of *Listeria* spp [36]. The capability of this pathogen to grow at broad ranges of temperatures (1^{o}C to 45^{o}C) and pH conditions (4.3-9.5) and its ability to survive at a relatively low water activity (as low as 0.90) and high salt concentrations (up to 10%) make it one of the hardiest human pathogens to control [37].

3. Importance of antimicrobial and multidrug resistance

The emergence of bacteria resistant to multiple, clinically relevant antibiotics has challenged the currently prevalent treatment strategies for infectious diseases, with many pathogenic bacteria becoming resistant to almost all the antibiotics available. Many known pathogens have acquired abilities to tolerate multiple antibiotics [38]. Such bacteria can also contaminate the food systems through different pathways and end up in the human food chain.

The breakthrough of the antibiotic penicillin in the 1920s heralded an era of antimicrobial chemotherapy, which seemed to conquer several infectious diseases and halted the pandemics that bothered the human race for thousands of years [39]. The 1960s and the 70s were the golden periods when many more antibiotics were discovered. However, with the increased use of antimicrobials, bacterial resistance to antibiotics started appearing in clinical settings, which was also found in animal production systems and human communities [40]. The emergence of

antibiotic-resistant bacteria in clinical settings has been rapid, and through various sources of contamination, such bacteria enter the environment and human food chain. Several human pathogenic bacteria have become extremely resistant to clinically relevant antibiotics. The most important group of such multidrug resistant (MDR) bacteria is represented by the so-called ESKAPEE pathogens, exemplified by *Enterobacter* spp., *Staphylococcus aureus, Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterococcus faecium*, and *Escherichia coli* [41, 42].

Bacterial mechanisms of antimicrobial resistance include enzymatic hydrolysis of antibiotics, alteration of antibiotic targets, reduced permeability, and antibiotic extrusion by efflux pumps [43]. The genetic factors responsible for antibiotic resistance usually dwell in mobile genetic elements such as plasmids and transposons [44]. Related or unrelated bacteria can acquire the genes through horizontal gene transfer (HGT), a mechanism responsible for rapidly disseminating resistance genotypes [45]. However, a similar phenomenon can occur in the aquatic environment when MDR bacteria are introduced from anthropogenic sources [46]. Resistance genes can be transferred to co-occurring pathogenic and non-pathogenic bacteria in the aquatic environment resulting in wider dissemination of MDR bacteria.

4. Types of solute transport systems

4.1. Passive diffusion versus active transport

The movement of macromolecules across the bacterial membrane can occur either by passive diffusion or active transport. In passive diffusion, the solute migrates across the cellular membrane from a region of higher solute concentration to a region of lower concentration till an equilibrium is reached [47]. In another type of passive diffusion called facilitated passive diffusion, the diffusion of the solute across the membrane barrier is facilitated by transmembrane protein carriers or gated channels [47]. Passive diffusion of any type does not require additional energy other than that present in the electrochemical gradient of solute across the membrane. While passive transport occurs from a region of higher solute concentration to a lower concentration, active transport requires additional energy in the form of ATP or the proton motive force (PMF) to transport solutes against the gradient resulting in the net accumulation of solute(s) [48]. The active transport types of machinery which directly hydrolyze ATP to power the solute transport process are known as primary active transporters, while those which use the electrochemical gradient of ions, such as H^+, Na^+, or K^+, across the membrane occur by a process called chemiosmotic coupling [49, 50]. The cellular metabolism generates the protein motive force (PMF) across the membrane, and the energy gradient established by the PMF drives the transport process, which is classified into uniport, symport, and antiport mechanisms [51].

In uniport, a single substrate moves in one direction across the membrane. This process enables the movement of non-diffusible solutes across the membrane, usually facilitated by a carrier protein, such as the GLUT1 glucose carrier [52]. In symport, two molecules are transported in the same direction across the membrane, one being the proton H^+ or Na^+, which energizes the process. The classic example is the lactose carbohydrate/H^+ symport system of *Escherichia coli* [51, 53]. In the antiport transport mechanisms, substrate and proton move in opposite directions, as in the case of antibiotic efflux pumps such as the QacA or NorA proteins [54].

4.2. Active efflux of antimicrobials

Bacteria show resistance to antibacterial agents and other toxic compounds by a mechanism known as active efflux, where the integral membrane transporters, known as drug efflux pumps, prevent the accumulation of drugs inside the bacterial cells [43]. Efflux pumps are membrane proteins that have the function of detoxifying cells by expelling harmful molecules. The efflux pump genes and proteins are present in antibiotic-susceptible and antibiotic-resistant bacteria [55, 56]. The extrusion of antimicrobial compounds, such as biocides and antibiotics, is considered to be an accidental function of such efflux systems. These efflux pump-specific genes can be found either in the chromosome or on transmissible genetic elements such as plasmids.

Multidrug efflux pumps are mainly located on the chromosomes, except, for instance, QacA/B and Smr, which only have been described in plasmids [57, 58].

Efflux pumps consist of hydrophobic proteins capable of exporting specific drug classes or structurally diverse compounds and toxins utilizing ATP or ion gradients as a source of energy [59]. Efflux proteins are categorized by their energy requirements and structural and subcellular organizational differences. Bacterial efflux systems can be either specific, extruding only one antibiotic or class of antibiotics or ejecting several types of antimicrobial compounds, designated multidrug resistance (MDR) efflux pumps [58, 60].

The third category of drug pumps, the phosphotransferase system (PTS), catalyzes the drug's transport with concomitant phosphorylation, usually for cellular entry of the drug substrate. Efflux systems are widely distributed in Gram-positive and -negative bacteria [61]. Efflux pumps are either single-component transporters or multiple-component systems containing an inner membrane transporter, an outer membrane channel, and a periplasmic adaptor protein [62, 63].

4.3. Superfamilies of antimicrobial transporters

The discovery of the lactose/H^+ mechanism spearheaded the discovery of numerous secondary active transport proteins that were distantly related structurally and functionally, suggesting a common ancestor for these proteins [64]. Proteins with similar functions and amino acid sequences were considered phylogenetically similar, and a database comprising various families of transporter proteins called the transporter classification database (TCDB) was established [65]. The TCDB consists of 20,653 proteins classified into 1,752 transporter families, 26% of which are members of 82 recognized superfamilies [66]. The database also hosts 1,567 tabulated 3D structures [66]. The secondary active transport proteins in the TCDB encompass four families or superfamilies, namely, i) the major facilitator superfamily (MFS), ii) the resistance-nodulation-cell division transporter superfamily (RND), iii) the small multidrug resistant transporter superfamily (SMR), and iv) the multiple antimicrobial extrusion protein superfamily (MATE) [67]. The MFS, RND, and SMR efflux pumps are H^+/drug antiporters, while the MATE group of efflux proteins are Na^+/drug antiporters [54].

4.4. Major facilitator superfamily

4.4.1. Discovery and history

The relatedness between distinctive solute transport systems in a wide array of organisms ranging between prokaryotes and eukaryotes was a crucial insight in the discovery of the major facilitator superfamily (MFS) by Henderson and colleagues [68, 69]. The advent of gene cloning methods and sequencing of the genetic determinants permitted protein structure prediction and comparative sequence analyses [70]. Similarities in amino acid sequences, energy modes, predicted secondary structures within the membrane, and specific conserved sequence motifs permitted the grouping into families of homologous and related transporters and the incorporation of these families into the MFS [68, 71]. These MFS proteins transport diverse substrates, including sugars, ions, amino acids, nucleic acids, fatty acids, metabolic intermediates, and structurally different antimicrobial agents [72]. Furthermore, as the proteins of the MFS harbor similarities in sequence motifs and structures, a common solute and ion transport mechanism across the membrane is predicted [70]. Early studies focused on establishing the functional importance of the classes of highly conserved sequence motifs in symporters and antimicrobial efflux pumps [73, 74]. Early investigators examined the substrate-ion cotransport and antiport mechanisms as modes for the energization of transport based on physiological studies [69, 75, 76].

4.4.2. Importance of the MFS

In the modern era, the MFS constitutes thousands of characterized passive and secondary active transporters from all known living taxa [77, 78]. The transporters of the MFS have served useful roles in the investigations of structure-function relationships, especially those with antimicrobial substrates [79, 80]. More recently, much effort

has been devoted to studying the promiscuous nature of the substrate specificity profiles in multidrug efflux pumps [80, 81]. Similarly, gene expression programs for the genetic determinants encoding the MFS multidrug efflux pumps have been the focus of intense study [82]. In addition, the MFS transporters are targets for antimicrobial resistance modulation to restore the clinical efficacy of chemotherapeutic agents that are compromised by multidrug transporters during infection [80, 83-86]. Towards this avenue, various efflux pump inhibitors have been developed against MFS antimicrobial transporters but require further development in the clinical setting [87].

4.4.3. MFS efflux pumps and biofilms

Bacteria in biofilms are attached to a solid surface, either biological or non-biological surfaces. Biofilms are covered by thick and slimy matrices made up of bacterial exopolysaccharides. A biofilm may contain a single species of bacteria, or more than one species may aggregate to form a biofilm [88]. Biofilm formation constitutes a protective mode of growth that permits microorganisms to survive in hostile environments. Biofilm is responsible for persistent chronic infections due to its inherent resistance to antimicrobial agents [89]. Biofilms can cause infections in humans that are persistent and difficult to treat, such as *S. epidermidis* and *S. aureus* infections of central venous catheters, contact lens- and intraocular lens-associated eye infections, *Pseudomonas aeruginosa* airway infections in cystic fibrosis patients, dental plaque and otitis media. Biofilm production occurs in many loci, such as wounds, water environments, and food processing surfaces [90]. As such, microorganisms in biofilms are protected from the entry of various types of antimicrobial agents. In the biofilm formation mechanism, the growth of bacteria conferring less susceptibility towards drugs could be due to a protective mutation in plasmids, mobile genetic elements, or horizontal gene transfer among bacteria [91]. For example, enzymes produced by bacterial cells in biofilms inactivate antibiotic action by degrading or breaking chemical bonds and increasing the expression of an efflux pump with a broad range of structurally different substrates. Other mechanisms, like poor diffusion of antibiotics through the biofilm polysaccharide matrix and phenotypic changes in biofilm-forming bacteria, are reportedly responsible for the antimicrobial resistance in biofilm structures [43, 83].

4.4.4. Structural biochemistry of MFS transporters

Before the advent of crystal structure elucidation of the MFS transporters, much was predicted regarding their biochemical and structural properties [70]. For instance, highly conserved amino acid residues in transporters of the MFS were demonstrated by site-directed mutagenesis to be functionally required for solute transport and antimicrobial resistance [63]. Furthermore, the predicted secondary topology of the MFS transporters consisted of 12 to 14 transmembrane segments with the N- and C-termini facing the intracellular section of the cell [70]. Along these lines, the so-called “MFS fold” structure was predicted [87] and later confirmed by protein structure elucidation studies [80]. The canonical MFS fold is characterized by a two-bundled domain structure connected by an intracellular loop segment [92]. Four so-called inverted repeated topology units are embedded within the overall MFS fold structure in several 12-helix MFS transporters [31]; see Figure 1.

In general, the protein structures of the MFS transporters are known to harbor large centrally-located cavities by residues from each of the two bundle structures, forming an interdomain interface [93-95]. These cavity structures in the known transporter structures are widely considered to form substrate recognition moieties [96]. Crystal structures of *E. coli* MdfA with the drug-binding cavity in its inward conformation and bound to one of its substrates [97], and in an outward open conformation bound to a blocking Fab antibody have been deduced [98]. These alternating structural variants represent intermediates in the overall substrate translocation mechanism that occurs during multidrug efflux [54].

The first MFS multidrug efflux pump for which crystal structural information became available was the EmrD protein, reported in 2006 [94], followed by the structure elucidations of YajR in

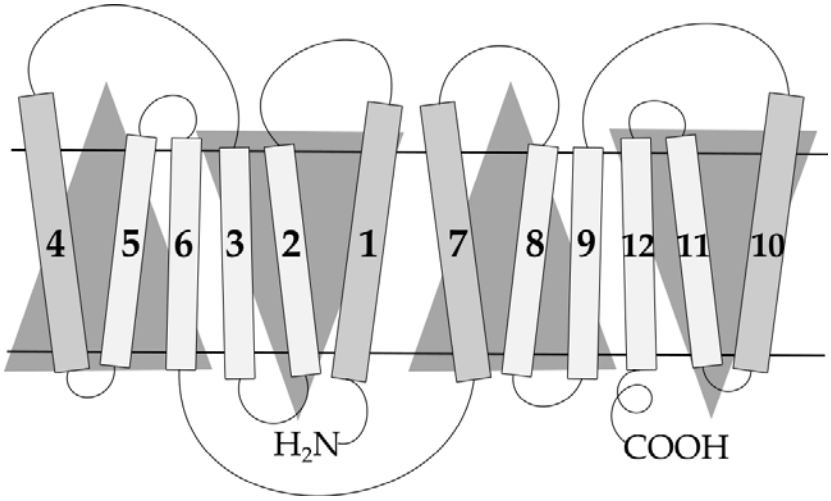

Figure 1. Inverted repeat topology of the MFS.

2013 [95] and MdfA in 2015 [99], all of which are MFS transporters from *E. coli*. As predicted by multiple sequence comparative and bioinformatics-based analyses [70, 100], the crystal structures harbor 12 α-helices that cross the cytoplasmic membrane in a zig-zag conformation where both of the termini are located in an inward-facing conformation [94, 95, 99]; see Figure 2.

The predicted two-bundle structural motif [70, 75] has also been confirmed and is a commonly found property of the MFS solute transporters [78] (Figure 3). Each bundle consists of six transmembrane domains, where one is N-terminally located and the other at the C-terminus [94]. At the interface between the two bundles, also called domains, lies a mechanistic assemblage characterized by connections between specific residues, hinges, and conformational movements that catalyze substrate and ion translocation across the membrane [78]. While the precise molecular mechanism for the transport of structurally diverse antimicrobial substrates is unclear, a new hint emerged with a recent report on MdfA structures in which the structurally conserved central cavity harbors a common substrate-binding site pocket that can accommodate antimicrobial agents of various sizes and structures [101]. The diverse nature of the antimicrobial substrate profiles is permitted by interactions between transporter and substrates that are hydrophobic, polar, and van der Waals in nature while minimizing the role of H-bonds [101, 102]. The functional roles of charged amino acids lie in mediating ion-driven coupling of the antimicrobial transport across the membrane [102]. The substrate-ion translocation catalysis system inherent in the multidrug efflux pumps of the MFS represents strategic targets for designing novel modulators to circumvent antimicrobial resistance and potentially restore the clinical efficacy of agents compromised by multidrug drug efflux [41, 85, 103].

4.4.5. Conserved amino acid motifs and transporter function

In addition to shared similarities in structures and modes of driving energy, families of transporters belonging to the MFS share conserved amino acid sequence motifs [74]. In particular, two of these signature motifs have emerged as functionally important for transporter activities [70]. Considering their ubiquitous nature amongst the MFS transporters, the structures they form, and the physiological roles they confer, these signature sequences remain relevant and the focus of study well into the foreseeable future.

While variations of the consensus sequence are extant, motif A was originally reported as “G X X X D R/K X G R R/K” [69, 70, 80]. Elements of this central motif are found in the cytoplasmic loop between transmembrane α-helices of virtually all protein members of the MFS [70, 74].

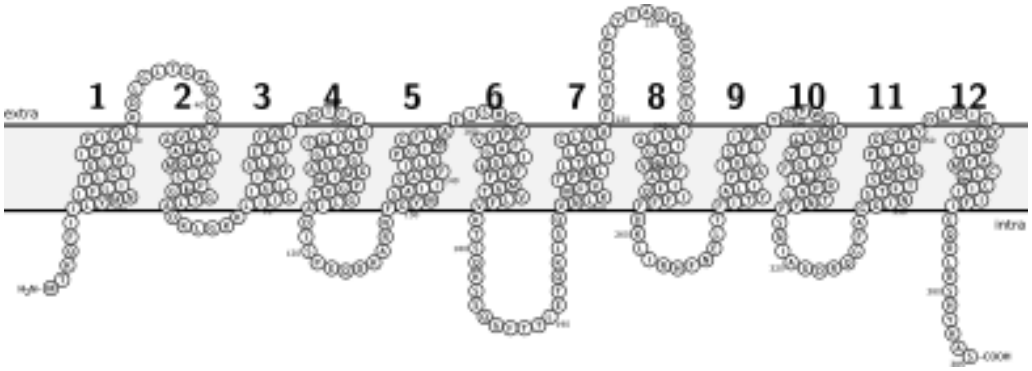

Figure 2. Secondary structure of 12-helix MFS transporter.

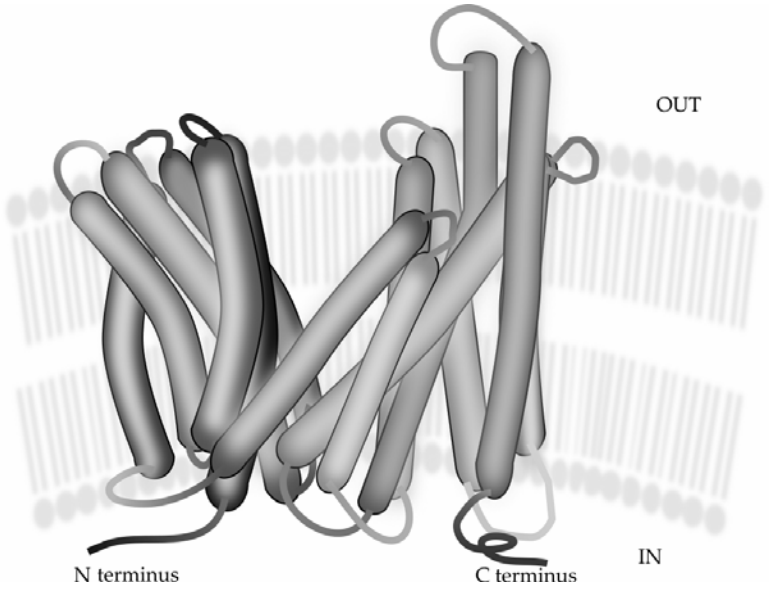

Figure 3. Three-dimensional structure of MFS efflux pump in the membrane.

The signature residues of motif A have been extensively studied in many laboratories, making the structure formed by its residues singularly critical for function in symporters, antiporters, and passive facilitative diffusers of the superfamily [74, 104].

Many early studies used site-directed mutagenesis of the non-conserved and conserved motif A residues, followed by physiological analyses of substrate and ion transport [54]. Based on these structure-function approaches, functional roles for the residues and the structures formed by these amino acids emerged [43, 54, 63, 105]. Among the various proposed functional roles in the MFS transporters for residues and structures formed by motif A, prominent are substrate binding in TetA(B) [106], the formation of the substrate translocation pathway through the TetA(B) transporter channel [107], a transporter gate structure in TetA(B) [108, 109], salt-bridge stabilizers of protein structure and mechanism of transport in YajR [95], regulation of conformation changes during transport [107, 110], interface contact mediators in YajR between the two N- and C-terminal bundles [95], an ion-gradient sensing system connecting the driving respiratory

chain to the transport activity of LmrP [111, 112], and a conformational switch permitting ion and substrate transport across the membrane [112].

A recent study has reported the role of motif A in the plant nitrate transporter MtNPF1.7 in mediating intra-bundle packing of integral membrane α-helices and structure stability [113]. While this and other studies have pointed to the various critical roles and mechanisms of motif A for achieving structure stabilization [113, 114], more recently, conserved charged residues of the motif were implicated in participating in a so-called charge-relay system involving proton and sodium for the newly discovered MdrP drug efflux pump with Na^+/H^+ antiport activity [115, 116].

Another highly conserved amino acid sequence motif of the MFS is the antiporter motif [73], also called motif C [71]. The original consensus sequence was denoted as "G $(X)_8$ G $(X)_3$ G $P(X)_2$ G G" [117]. The antiporter motif is located in the fifth helix of MFS efflux pumps, members of which belong to three drug proton antiporter families, denoted as DHA1, DHA2, and DHA3 [118], but is seemingly lacking in symporters and uniporters of the MFS [73, 78, 117, 119]. However, in one study where a multiple sequence analysis of MFS transporters was performed using a high-throughput alignment algorithm, residues of the antiporter motif appear in symporters like the lactose permease LacY [120] and other transporters like the glycerol-3-phosphate transporter GlpT [121], in addition to drug-proton antiporters, like VMAT1 and VMAT2 (vesicular transporters of monoamines) [122]. The first experimental evidence of the functional importance of residues in the antiporter motif was provided by the laboratory of Griffith, who systematically mutagenized Gly-147 of TetA(C) tetracycline efflux pump and found only serine and alanine were somewhat acceptable replacements [73]. The same study reported that the fifth α-helix was kinked in TetA(C) [73], a structural prediction confirmed in multidrug efflux pumps by a variety of laboratories [54, 74, 80, 104]. The functional roles of the antiporter motif have been the focus of intensive study, and throughout the years, specific functions have been assigned to structural elements formed by the motif's consensus sequence [43, 105]. For example, the Gly-Pro (GP) dipeptide of motif C conferred the predicted kink, tight helical packing, and leak prevention in helix five of TetA(L) [73, 123, 124]. Motif C was implicated in dictating the direction of solute and ion transport across the membrane through MFS antiporters [73, 125, 126]. Based on its presence in MFS transporters [122], we postulate that motif C residues regulate direction transport for substrates of symporters and uniporters besides antiporters. Furthermore, residues of the antiporter motif have been shown to mediate conformation change in TetA(K) during transport [127], form a barrier to leakage from non-specific solute-ion coupling in TetA(L) [123, 124, 128], stabilize the transporter structure of TetA(B) [129, 130], bind antimicrobial agents in QacA [131], participate in forming the central solute binding cavity of CaMdr1p [132], mediate contact between the two VMAT2 bundles [122], regulate conformation switching of the vesicular acetylcholine transporter (VAChT) during transport [133], and form a hinge structure to serve in the overall transport mechanism in VAChT [133].

More recently, the highly conserved glycine of the GP dipeptide was demonstrated to interact with a conserved and critical arginine residue of helix four and specifically mediate the efflux of metal-drug complexes in Tet(38) [134]. Interestingly, motif C residues do not appear to play a role in the host cell internalization of bacterial pathogens [84].

Due to the substantially conserved nature of the signature residues of motif C in thousands of MFS transporters, we anticipate that these known functional roles are attributable to most, if not all, of the MFS members [79]. Therefore, these molecular physiological mechanisms and their structures make suitable targets for modulation, whether to restore antimicrobial therapy efficacy against infection or generate transporters with desirable substrates [60, 83, 85].

In addition to motifs A and C, two novel conserved sequences, motif-1 and motif-2, were discovered in QacA [135]. These newer motifs were demonstrated to mediate substrate binding and protein stability [135]. Amino acid sequence

conservation continues to be of major focus in investigations of solute transport across the membrane.

5. Concluding remarks

Transporters of the MFS are ubiquitous across all known living taxa. Those MFS transport systems that confer efflux of multiple structurally distinctive antimicrobial substrates are known to reduce the clinical efficacy of infectious disease treatment. Thus, the MFS multidrug efflux pumps are widely recognized as bacterial virulence factors. As such, these antimicrobial transport systems represent critical areas of study and make good targets for regulation to reduce the conditions that foster potentially untreatable bacterial infections. Therefore, transporters of the MFS continue to serve as useful molecular physiological model systems for studying solute and ion translocation across the membrane.

Areas of future study include gaining an enhanced understanding of the relationship between energy coupling and multidrug transport. These molecular systems that drive the efflux of structurally different antimicrobial agents may serve as good targets for modulation. Along these lines, the molecular mechanisms that permit the promiscuous transport of multiple antimicrobials while preventing the leakage of unwanted ions and substrates continue to be a required field of study. A related topic is the determination of substrate profiles for each of the multidrug transporters, as each member of the MFS has a unique substrate profile [136]. It is anticipated that analysis of multidrug transport systems can generate novel transporters with desirable substrates or allow modulation of known multidrug efflux pumps such that they no longer confer clinical resistance levels. Another modulatory strategy involves regulating gene expression systems for genetic determinants of multidrug resistance [137].

Future investigation is needed on generating antimicrobial agents with novel modes of action that can circumvent extant MFS transporters in pathogens. We predict that, at least for the interim, new antimicrobials are not good substrates for extant MFS multidrug efflux pumps, especially if appropriately used for treating clinical infections [41]. However, due to the evolutionary adaptation of bacterial pathogens exposed to antimicrobial agents, even novel drugs can serve as suitable substrates for MFS multidrug efflux pumps [138]. Novel avenues for chemotherapy of infectious diseases are highly preferred. Towards this goal, developing new drug combinations for infectious disease therapy seems promising. Lastly, applying genomics and metagenomics data to identify new MFS targets represents another promising avenue for dealing with persisters and antimicrobial-resistant pathogens [41].

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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