Functional Roles of Highly Conserved Amino Acid Sequence Motifs A and C in Solute Transporters of the Major Facilitator Superfamily

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Abstract The biological membrane covers all living cells and provides an effective barrier against the passage of biologically important water-soluble solutes. This natural passage barrier is essentially overcome with the use of integral membrane proteins known as solute transporters. These transport systems translocate solutes across the membrane such as in the case of bacterial drug and multidrug resistance efflux pumps. One of the largest groups of transporters is referred to as the major facilitator superfamily. This group contains secondary active transporters such as symporters and antiporters and passive transporters such as uniporters. The transporters within the major facilitator superfamily share conserved structures and primary amino acid sequences. In particular, several highly conserved amino acid sequence motifs have been discovered and studied extensively, providing substantial evidence for their critical functional roles in the transport of solutes across the membrane.

1 Importance of Solute Transport in Living Organisms

All known living cells are surrounded by a biological membrane that provides an effective barrier against the passage of aqueous-based solutes and ions. Living cells, however, must be able to acquire helpful substances while also extruding harmful ones. Biological membranes solve this barrier problem by using integral membrane proteins that selectively catalyze the acquisition and efflux of helpful

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and harmful water-soluble molecules, respectively. Therefore, integral membrane solute transporters are important for all life on Earth (Broome-Smith 1999).

When solute transporters are defective, medical disease may occur such as those seen in glucose–galactose malabsorption (Wright et al. 2002), Fanconi–Bickel syndrome (Santer et al. 2002), and De Vivo disease (De Vivo et al. 1991), which are genetic diseases involving impaired transport of glucose across the membranes of cells and develop from inheritable mutations which occur in the genes that encode monosaccharide sugar transporters, thus impairing the uptake of monosaccharides into cells.

Bacteria use solute transporters to efflux multiple antimicrobial agents, often causing loss of chemotherapeutic efficacy during treatment of infectious diseases (Chopra 1992; Kumar and Varela 2013; Li et al. 2015). Solute transporters that multidrug-resistant bacteria use to efflux antimicrobial agents can be grouped into several protein families, such as the ABC (ATP-binding cassette) transporters (Higgins 1992), the resistance-nodulation-cell division (RND) superfamily (Tseng et al. 1999), the small multidrug resistance (SMR) superfamily (Chung and Saier 2001), the multidrug and toxic compound extrusion (MATE) superfamily (Kuroda and Tsuchiya 2009; Kumar et al. 2013), and the major facilitator superfamily (MFS) (Paulsen et al. 1996b; Pao et al. 1998; Saier et al. 1999; Kumar and Varela 2012; Andersen et al. 2015). This review will focus on the antimicrobial agent efflux pumps of the MFS and especially MFS pumps of known structures. Particular attention will be paid to studies which have involved amino acid residues that belong to highly conserved sequence motifs A and C of the MFS (Griffith et al. 1992; Marger and Saier 1993).

2 Acquisition of Helpful Nutrients and Efflux of Harmful Solutes

Substances are routinely transported across biological membranes of living organisms. These substances include an extremely diverse range of water-soluble solutes such as amino acids, Krebs cycle intermediates, sugars, nucleic acids, neurotransmitters, antimicrobial agents, and other small molecules (Henderson et al. 1998). Nutrient uptake via solute transport is a crucial process in which living cells acquire and accumulate molecules from the external environment in order to support metabolism, cell growth, and cell maintenance. On the other hand, living organisms must be able to efflux toxic substances from the inside of their cells into the extracellular milieu in order to maintain growth and survival. Living bacterial cells, for example, have developed integral membrane proteins to facilitate efflux of toxic molecules, a trait that confers antimicrobial resistance (Kumar and Varela 2013).

3 Types of Solute Transporter Systems

Transport systems play important roles in the cellular uptake of helpful molecules such as nutrients, ions, and small molecules and in the exit of harmful or inhibitory molecules. Cellular entry and exit of solutes can occur in two general ways: passive and active transport. Passive transport entails the movement of small molecules across the membrane and does not require biological energy to do so (Mitchell 1967; West and Mitchell 1972). Active transport systems move solutes across the membrane against their own solute concentration gradients (i.e., from low to high concentrations), using integral membrane proteins, called pumps or active transporters. This type of solute transport is referred to as active because of the energy required to conduct transport across the biological membrane (Henderson 1991; Hediger 1994).

3.1 Passive Solute Transport

In passive transport systems, solutes are translocated across the membrane from a side of the membrane with relatively high solute concentration toward the side with relatively low solute concentration, i.e., down the solute concentration gradient (Hediger 1994). The passive solute transport systems generally do not require the expenditure of biological energy. Transport systems use integral membrane carriers to catalyze solute uniport, a facilitative diffusion process that enables a single molecular species to be transported down their concentration gradients (Henderson 1991; Saier 2000).

3.1.1 Facilitated Diffusion

Facilitated diffusion refers to solute transport involving pore- or carrier-forming molecules. In this process, solute reversibly binds to a solute-specific carrier protein that resides integral to the membrane. The complex of solute and carrier oscillates between the inner- and outer-facing surfaces of the biological membrane, thus causing binding and release of the solute to the other side of the same membrane (Henderson 1991).

A special class of integral membrane proteins, called porins, form large nonspecific water-filled channels within the outer membrane to allow the acquisition of nutrients from the periplasm of Gram-negative bacteria. These channels are also associated with the efflux of the waste products (Nikaido 1994). Many so-called classical porins examined so far are OmpC, OmpF, and PhoE from *Escherichia coli* (Nikaido and Vaara 1985; Nikaido 1992). These porins exist as closely associated trimeric complexes that cannot be dissociated even with sodium dodecyl sulfate (SDS), unless heated denatured beforehand (Reid et al. 1988).

These porins show preferences on the basis of solute size and charge. In the case of charge, OmpC and OmpF prefer cations slightly more compared to anions, and PhoE prefers anions. OmpF allows translocation of relatively larger solutes compared to OmpC, showing preferences according to the size of the solute (Nikaido 2003).

3.2 Active Transporter Systems

Two main energy-requiring solute transporter systems, i.e., primary active transport (energized by hydrolysis of ATP) and secondary active transport (energized by ion gradients), are used to efflux biomolecules from bacteria (Mitchell 1966, 1972, 1991, 2011; Harold 2001). Among the dozens of primary and secondary active transporter families, two such superfamilies in particular occur in a ubiquitous manner across all taxonomic categories of living organisms. These systems include a superfamily called the ATP-binding cassette (ABC) transporters and another group called the major facilitator superfamily (MFS) of transporters (Pao et al. 1998; Saier et al. 1999; Davidson and Maloney 2007; Law et al. 2008).

3.2.1 Primary Active Solute Transporters

In primary active transport, the free energy required for solute transport against the electrochemical gradient is provided by the very protein performing the transport. They do so by the hydrolysis of adenosine triphosphate (ATP) (Tarling et al. 2013). Often referred to ABC transporters (Higgins 1992), these primary active transporters represent a large group of integral membrane proteins that couple the transport of a substrate like amino acids, ions, sugars, lipids, and drugs across the membrane (Chang 2003) to the hydrolysis of the phosphate bond between the γ- and the β-phosphate of ATP (ter Beek et al. 2014). It includes both importers and exporters (Locher 2009), bringing nutrients and other molecules into cells or exporting toxins, drugs, and lipids across membranes (Rees et al. 2009). To attain export, ABC transporters use four types of subunits called domains, two transmembrane domains (TMDs) plus two nucleotide binding domains (NBDs). TMDs provide specificity and form the binding sites for ligand, and NBDs undertake ATP hydrolysis to accomplish the translocation across the membrane of its bound solute. However, import requires an additional periplasmic binding domain (PBP) (Linton 2007; Procko et al. 2009). A conformational change in the TMDs occurs once substrate binds, followed by transmission to the NBDs to initiate ATP hydrolysis (Higgins 2001). ABC transporters adopt at least two conformations, i.e., the cis-side or the trans-side. The binding site for the solute is exposed when the transporter is in either one of these two conformations. Alternation between the two conformations allows substrate translocation to occur across the membrane (ter Beek et al. 2014).

3.2.2 Secondary Active Transporters

Secondary active solute transport systems have significant roles in the uptake and efflux of biologically important molecules. Metabolic and bioenergetic systems of organisms convert the energy stored in nutrients during catabolism into an electrochemical energy of protons or sodium ions, generating proton-motive or sodium-motive forces (Mitchell 1967, 1991). These energies are then used to drive biological work such as the translocation of solutes across the membrane against their concentration gradients to accumulate solute on one side of the membrane (Poolman and Konings 1993; Krämer 1994; Wilson and Ding 2001). In the chemiosmosis mode of biological energy generation during respiration and fermentation, light, chemical, or redox energies are converted to electrochemical energies, which in turn are used to drive other biological work. This bioenergetic process takes place by coupling biochemical reactions to the transport of solutes, ions, and other small molecules across the cell and plasma membranes. In bacteria, protons, and sodium are the coupling ions that are used during energy transduction (Krämer 1994).

4 The Major Facilitator Superfamily

The MFS has become an extremely well-studied and important compilation of solute transporters across all taxa of living organisms (Maloney 1994; Paulsen et al. 1996b; Saier et al. 1999; Pao et al. 1998; Law et al. 2008). The substrates or solutes of these MFS transporters are extremely diverse and include structurally distinct small molecules like sugars, amino acids, intermediary metabolites, nucleic acids, antimicrobial agents, and ions. To date, the MFS encompasses thousands of members conveniently stored and organized in a well-maintained database called the Transporter Classification Database (TCD) www.tcdb.org (Saier et al. 2014), which currently includes well over 15,000 proteins of the MFS (Saier et al. 2014).

4.1 Discovery of the MFS

As integral membrane solute transporters were refractory to isolation and purification by traditional biochemical approaches, making their study difficult, molecular biological approaches became available and, thus, quite useful in the cloning of the genes that encoded solute transporters (Teather et al. 1978). Gene cloning, in turn, allowed almost the immediate determination of the nucleotide sequences encoding solute transporters (Büchel et al. 1980). Soon after the cloning and DNA sequence determinations of additional genes that encoded solute transporters became available, a remarkable discovery was made by Henderson and colleagues in which

comparison of the sequences between several sugar transporters from prokaryotic and eukaryotic organisms demonstrated that these seemingly distinct proteins were in fact homologous (Maiden et al. 1987), indicating a shared or common evolutionary origin. As many more transporter gene sequences were determined and compared, investigators began to compile these transporters in families and superfamilies, referred to initially as the transporter superfamily (TSF) (Henderson 1993), the uniporter–symporter–antiporter (USA) family (Goswitz and Brooker 1995), and the generally accepted term major facilitator superfamily (MFS) (Marger and Saier 1993).

4.2 General Features of the MFS

These transporter members of the MFS include *(a)* uniporters, which catalyze facilitated diffusion of solute across the membrane down their solute concentration gradients; *(b)* symporters, which catalyze ion-driven secondary active transport of solutes in the same directions across the biological membrane; and *(c)* antiporters, which catalyze ion-driven secondary active solute transport across the membrane in opposite directions (Mitchell 1991). These transporters have on average between approximately 400 and 600 amino acids along their polypeptide chains (Pao et al. 1998; Law et al. 2008).

The MFS transporters catalyze the translocation of water-soluble solutes across the membrane using the energy stored in chemiosmotic ion gradients (Marger and Saier 1993). The ions, for instance, are either protons (i.e., H^+) or sodium (i.e., Na^+), and their gradients across the membrane are formed by the respiratory chain during catabolism of nutrients (Mitchell 1991; Harold 2001). The substrate will accumulate extracellularly in an energy-dependent fashion. Thus, these substrate/H^+ antiport (efflux) systems allow all cells, including bacteria, to survive and grow while in the presence of potentially inhibitory molecules. Therefore, these biomolecule efflux systems allow bacteria to tolerate unusually high concentrations of potentially lethal molecules, such as antimicrobial agents, heavy metals, industrial waste molecules, etc. An interesting and unique property of several MFS efflux systems is that they have the ability to transport multiple structurally different substrates (Levy 1992, 2002; Lewis 1994; Piddock 2006). Also known as uniporter–symporter–antiporter superfamily (Goswitz and Brooker 1995), members include both passive and secondary active transport systems.

4.3 Key Secondary Active Transporters of the MFS

The energy of ion gradients drives solute transport across the membrane during secondary active solute transport. Many of the solute transporters that are members of the MFS use these particular types of ion gradient energies for the cellular uptake

and efflux of solutes (Poolman and Konings 1993; Krämer 1994; Kumar and Varela 2013). The term symport is used to describe the co-transport movement of solute and ion in the same direction across the cell or plasma membrane; that is, ion translocation down its gradient drives solute transport up its gradient. On the other hand, the term antiport is used to describe the co-transport of solute and its driving ion in the opposite directions across the same types of biological membranes; again, the ion moves down its concentration gradient to mediate solute transport against its own gradient. In both of these symport and antiport systems, the transported solute accumulates on one side of the membrane (Saier 2000).

The lactose permease, LacY, a secondary active transporter from *E. coli*, has been studied in the laboratories of Brooker (Brooker 1990), Kaback (Guan and Kaback 2006), and Wilson (Varela and Wilson 1996) and is considered to be a useful model system for investigation of newer transport systems of the major facilitator superfamily, such as novel multidrug efflux pumps (Floyd et al. 2013). LacY was originally described as an important component of the well-known *lac* operon and is encoded by *lacY*, a regulated structural gene contained within operon itself (Müller-Hill 1996; Varela and Wilson 1996). Using protons, the LacY symporter transports lactose and other related sugars across the inner membrane, and it uses the energy of the electrochemical gradient of protons to couple this movement of sugar and proton symport. This causes sugar to accumulate against a concentration (Mitchell 1967, 1991; Varela and Wilson 1996).

EmrD is a proton-dependent multidrug efflux pump of *E. coli* that belongs to MFS family (Sulavik et al. 2001). EmrD transports detergents, such as benzalkonium chloride and sodium dodecyl sulfate (Nishino and Yamaguchi 2001). Not only does it confer resistance to detergents, the EmrD efflux pump influences the formation of biofilm (Matsumura et al. 2011). The X-ray crystal structure of EmrD exhibits hydrophobic interiors which is a means for transporting various substrates in the drug efflux mechanism. An additional area consisting of two long helical regions that are located on cytoplasmic side can provide additional substrate specificity and transport (Yin et al. 2006).

TetA(B) is the most extensively studied efflux pump of the MFS family, members of which transport sugar, intermediate metabolites, and drugs (Buivydas and Daugelavièius 2006). The gene has been encoded on transposon Tn10 and represents a metal–tetracycline/H^+ antiporter (Tamura et al. 2003). The efflux of tetracycline from bacteria is driven by a proton gradient as the driving force (Kaneko et al. 1985). The presence of TetA(B) in *Bacillus cereus* represents the transfer of the antibiotic resistance genes from other bacteria (Rather et al. 2012). This efflux pump actively expels tetracycline by a membrane-associated protein, resulting in the reduction in the accumulation of tetracycline (Levy 1992; Nelson and Levy 2011).

The bacterial pathogen *S. aureus* harbors many antimicrobial agent efflux pumps that are members of the MFS of transporters, and several are well studied (Hooper 2000; Brown and Skurray 2001; Costa et al. 2013; Andersen et al. 2015). One of the most intensively studied is QacA (Brown and Skurray 2001; Saidijam et al. 2006), a plasmid-encoded multidrug pump that confers resistance to multiple antiseptics, diamidines, and dyes (Tennent et al. 1989). The deduced sequence shows

514 residues, and QacA is the first MFS discovered to have 14 TMS instead of 12 as has previously been observed in other superfamily members. The 14-transmembrane domain topology was supported by fusion studies of QacA with enzymatic reporters (Paulsen et al. 1996a). Presently, many MFS efflux pumps have the 14 TMS motif (Saidijam et al. 2006). QacA transports ethidium bromide using the proton gradient as the driving force (Littlejohn et al. 1992).

Another MFS efflux pump for multiple structurally distinct antimicrobial agents is NorA of *S. aureus* (Ubukata et al. 1989; Yoshida et al. 1990). NorA has 388 amino acid residues and 12 predicted transmembrane segments (Yoshida et al. 1990). Originally discovered in a clinical isolate (Ubukata et al. 1989), NorA was thought to be a single-drug efflux pump for the antimicrobial agent norfloxacin. NorA is now well known to be a multidrug transporter (Neyfakh et al. 1993) which is closely related to Bmr from *Bacillus subtilis* (Neyfakh 1992). Physiological studies show that NorA transports structurally different antimicrobial agents like the fluoroquinolones (e.g., ciprofloxacin and norfloxacin), dyes (e.g., rhodamine and ethidium), and quaternary ammonium compounds (e.g., benzalkonium chloride and tetraphenylphosphonium) (Yoshida et al. 1990; Kaatz et al. 1993; Neyfakh et al. 1993; Kaatz and Seo 1995). Recent primary studies of NorA have emphasized on efflux pump inhibitors of NorA (Holler et al. 2012a, b; Kalia et al. 2012; Roy et al. 2013; Shiu et al. 2013; Thai et al. 2015) and regulation of NorA expression (Fournier et al. 2000, 2001; Truong-Bolduc et al. 2003, 2005; Kosmidis et al. 2010; Deng et al. 2012), both topics of which are beyond the scope of this review but have been reviewed elsewhere (Zhang and Ma 2010; Costa et al. 2013).

The protein MdeA from *S. aureus* is predicted to have 479 amino acids, 14 transmembrane domains (Huang et al. 2004; Yamada et al. 2006), and transport Hoechst 33342 and ethidium bromide (Yamada et al. 2006). Predictions also indicate that MdeA confers resistance to tetraphenylphosphonium chloride, norfloxacin, rhodamine 6G, doxorubicin, and daunorubicin (Yamada et al. 2006; Huang et al. 2004). The MdeA efflux pumps of *S. aureus* N315 (Yamada et al. 2006) and *S. aureus* Buttle (Huang et al. 2004) are 99 % identical, differing at five key residues and likely explaining why MdeA from *S. aureus* Buttle confers resistance to benzalkonium chloride while MdeA from *S. aureus* N315 does not. Additionally, it was shown that piperine inhibits MdeA transport activity and potentiates the effects of the antimicrobial agent mupirocin (Mirza et al. 2011).

A more recently discovered multidrug efflux pump, LmrS, encoded on the chromosome and cloned from a clinical isolate of a methicillin-resistant *S. aureus* (MRSA) strain, actively transports ethidium bromide and confers resistance to structurally dissimilar substrates, such as linezolid, lincomycin, tetraphenylphosphonium chloride, chloramphenicol, erythromycin, florfenicol, fusidic acid, gatifloxacin, kanamycin, oxytetracycline, streptomycin, and trimethoprim (Floyd et al. 2010). The LmrS multidrug efflux pump is predicted to harbor 14 transmembrane domains, which is identical to that predicted for QacA (Paulsen et al. 1996a; Floyd et al. 2010). Furthermore, LmrS shares homology with LmrB of

B. subtilis (Kumano et al. 1997), VceB from *V. cholerae* (Colmer et al. 1998), and EmrB from *E. coli* (Lomovskaya and Lewis 1992).

4.4 Structures of MFS Transporters

Generally, these MFS transporters contain 12 (Fig. 1) or 14 transmembrane-spanning domains (TMS), with an occasional duplication of two 12 TMS to constitute 24 TMS transporters (Moir and Wood 2001; Hirai et al. 2003; Saidijam et al. 2006). Thus far, high-resolution crystal structures have been elucidated for more than a dozen of these MFS transporters. These known MFS protein crystal structures include the multiple drug efflux pump, EmrD, from *E. coli* (Yin et al. 2006); the fucose transporter, FucP, from *E. coli* (Dang et al. 2010); the glucose–H^+ symporter, $GlcP_{Sc}$, from *Staphylococcus epidermidis* (Iancu et al. 2013); the glycerol-3-phosphate transport protein, GlpT, from *E. coli* (Huang et al. 2003); the glucose transporter, GLUT1, from *Homo sapiens* (Sun et al. 2012); the lactose–proton symporter, LacY, from *E. coli* (Abramson et al. 2003); the nitrate/nitrite exchange transporter, NarK, from *E. coli* (Zheng et al. 2013); the nitrate/nitrite antiport protein, NarU, from *E. coli* (Yan et al. 2013); the oligopeptide–H^+ symport protein, $PepT_{So}$, from *Shewanella oneidensis* (Newstead et al. 2011); the phosphate transport protein, PipT, from *Piriformospora indica* (Pedersen et al. 2013); the xylose transporter, XylE, from *E. coli* (Sun et al. 2012); the multidrug transporter, YajR, from *E. coli* (Jiang et al. 2013); the peptide transport protein, YbgH, from *E. coli* (Zhao et al. 2014); the multiple drug efflux pump, MdfA, from *E. coli* (Heng et al. 2015); and, more recently, the mammalian fructose transporter, GLUT5, from *Rattus norvegicus* and *Bos taurus* (Nomura et al. 2015).

Thus far, these high-resolution protein structures support the general notion that the MFS transporters harbor two structurally symmetrical and functionally asymmetrical bundles or domains (Pao et al. 1998; Saier et al. 1999) composed of the first half (N-terminus) 6 TMDs and second half (C-terminus) 6 TMDs, at least for the 12-TMD solute transporters, which is not surprising given the early observation that the two halves of the modern MFS transporter likely arose from an internal sequence duplication and subsequent tandem repeat of a common ancestor with

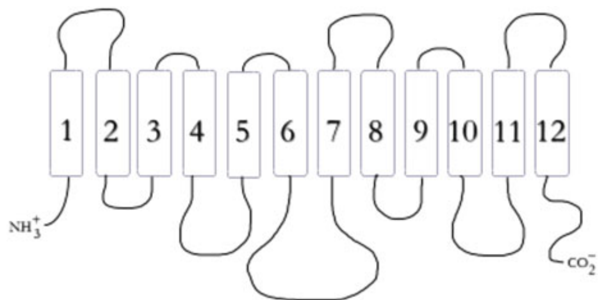

Fig. 1 Two-dimensional topology model of an MFS transporter

6 TMDs (Griffith et al. 1992). Another feature apparently common to the known crystal structures of the MFS transporters is the presence of a large central aqueous cavity formed by the two halves, supporting previous genetic analyses of the tetracycline efflux pump, TetA(C), where the N- and C-termini bundles or domains interact functionally (McNicholas et al. 1992, 1995), plus low-resolution structural data for the oxalate transporter, OxyT (Heymann et al. 2001, 2003), and Mitchell's notion of a proton gradient as an energy source for driving solute transport across the membrane (Mitchell 1977, 1991). Considering how these structural features related to the mechanism by which solute is translocated across the membrane, the so-called alternating access mechanism has been invoked to explain this important biological process in which the substrate binding site alternately faces one or the other sides of the membrane (Jencks 1980; West 1980, 1997; Tanford 1982). In principle, the substrate binding site of the MFS transporter faces one side of the biological membrane and then upon binding of the substrate orients itself via a conformational change such that the substrate binding site faces the other side to facilitate transport (Henderson 1991; Law et al. 2008), and these MFS transporters, in general, use their flexible gating structures to form inward- or outward-facing states that are occluded in order to prevent unwanted leakage and dissipation of the ion gradients (Stelzl et al. 2014). As shown in Fig. 2, intrinsic in the conserved structure is the so-called MFS fold consisting of inverted triple helices that are repeated four times to form four 3-helix inverted-topology repeats that make up the MFS fold in MFS transporters (Radestock and Forrest 2011).

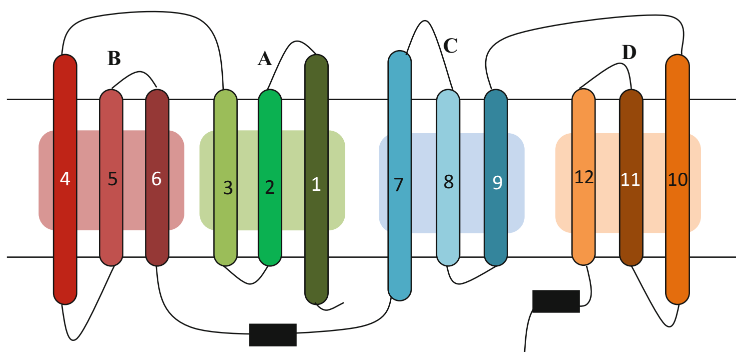

Fig. 2 The MFS fold. A transporter is shown residing in a membrane (*horizontal lines*) with the transmembrane α-helices (numbered *vertical rods*). The *shaded rectangles* A, B, C, and D depict of the four inverted triple helix structural motifs, each known as the MFS fold. Adapted from Radestock and Forrest (2011), Yaffe et al. (2013)

5 Evolutionarily Conserved Sequence Motifs Involving Amino Acid Sequences in Transporters of the MFS

Early studies that discovered the high degree of relatedness between members of the MFS also definitively demonstrated their shared evolutionary conservation of certain amino acid sequences (Fig. 3) (Henderson 1990a, b; Rouch et al. 1990; Griffith et al. 1992; Henderson et al. 1993). These investigators further discovered that members of the MFS shared similar hydrophobicity profiles and similar predicted secondary structures (i.e., 12 or 14 TMDs), suggesting that these family members share conserved three-dimensional structures and, thus, a common ancestral origin. Taken together, these findings suggested that the MFS transporters share a common solute transport mechanism, independent of the transporters' substrate specificities and modes of energy (Henderson and Maiden 1990; Rouch et al. 1990; Griffith et al. 1992; Marger and Saier 1993; Pao et al. 1998; Saier et al. 1998, 1999).

6 Motif A "G X X X D R/K X G R R/K" and Functional Roles

This highly conserved amino acid residue sequence motif from the MFS was discovered by Henderson and coauthors in 1987 (Maiden et al. 1987; Henderson and Maiden 1990). Now known as Motif A, it is widely accepted that elements of this motif reside in a hydrophilic loop between helices 2 and 3 of virtually all transporters of the MFS (Griffith et al. 1992; Pao et al. 1998; Saier et al. 1999; Kumar and Varela 2012; Andersen et al. 2015; see Fig. 3a). Hence, the functional importance of this motif cannot be understated. Perhaps the earliest clues to the importance of residues in Motif A arose well before it was established that elements in this protein region were conserved. First, in a series of studies working with lactose permease, LacY, a key transporter first purified from *E. coli* by Newman and Wilson (Newman and Wilson 1980), truncated LacY protein fragments were later generated by limited proteolysis and deletion mutation analyses by the laboratory of Ehring and colleagues, who found that residues of the N-terminal region where Motif A resides must be important for lactose transport across the membrane (Stochaj et al. 1986, 1988; Stochaj and Ehring 1987). Subsequent follow-up studies were conducted in which co-expression of inactive truncated nonoverlapping LacY fragments functionally complemented each other, restoring active lactose transport, thus further demonstrating the important functional roles of N-terminal residues (Wrubel et al. 1990, 1994).

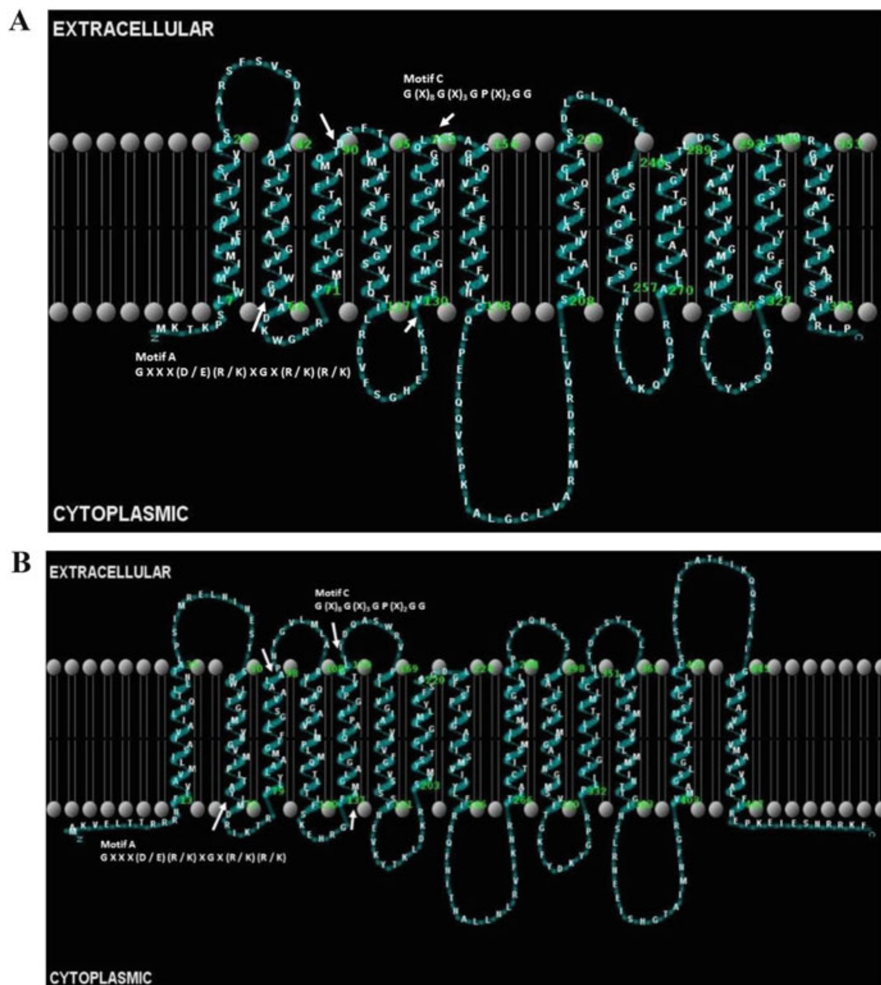

Fig. 3 Highly conserved sequence motifs A and C in 12-TMS and 14-TMS MFS transporters. Figure (**a**) indicates 12 different transmembrane helices joined together by loops. The *white arrows* point to conserved motif A [G X X X (D/E)(R/K) X G X (R/K)(R/K)] and motif C [G $(X)_8$ G $(X)_3$ G P $(X)_2$ G G] of the multidrug efflux pump EmrD-3 (Smith et al. 2009; Floyd et al. 2010) from the microorganism *Vibrio cholerae*, a pathogenic bacterium. Figure (**b**) indicates 14 different transmembrane helices joined together by intra-helical loops. The *white arrows* point to conserved motif A [G X X X (D/E)(R/K) X G X (R/K)(R/K)] and motif C [G $(X)_8$ G $(X)_3$ G P $(X)_2$ G G] in the multidrug efflux pump LmrS from the bacterial pathogen *Staphylococcus aureus*. These figures were generated using TMHMM and Tmpres2D servers

6.1 Early Studies of Motif A

Perhaps, the first site-directed mutational analysis of individual amino acid residues of Motif A in an MFS transporter was conducted by the laboratory of Yamaguchi

(Yamaguchi et al. 1990). The Ser-65–Asp-66 dipeptide of the motif was closely examined (Yamaguchi et al. 1990) in the Tn10 TetA(B) tetracycline efflux pump, which was discovered in the laboratory of Levy (McMurry et al. 1980). Because replacements at position Ser-65 but not at Asp-66 in the Motif A of TetA (B) showed some transport activity, it was concluded that a negative charge and the loop were both necessary for gating but not for substrate binding in the channel (Yamaguchi et al. 1990). The possibility remained, however, that the residues in the loop between helices 2 and 3 did participate in initial substrate binding, as previously postulated (Chopra 1986), as later studies involving Cys-scanning mutagenesis showed that residues in helix 3 (Asp-84) and elements of Motif A (Gly-62, Asp-66, Arg-70, and Ser-77) were also implicated in forming a tetracycline transport pathway and further interpreted as together undergoing conformational changes during transport (Yamaguchi et al. 1993a; Kimura et al. 1998b). The importance of the conserved Asp residue at this locus in TetA(B) was confirmed also in KgtP, an α-ketoglutarate permease (Seol and Shatkin 1992), and TetA(C), a plasmid-encoded tetracycline efflux pump from *E. coli* (McNicholas et al. 1992). Follow-up studies from the Yamaguchi laboratory systematically investigated the rest of the residues in Motif A of TetA(B) and found that only the Asp and Arg residues of the Motif A in the loop 2-3 were essential for tetracycline transport (Yamaguchi et al. 1992a, b), further solidifying the notion that the conserved loop structure participated in a gating function, as previously postulated (Baker and Widdas 1973), while the two Gly residues of the motif were interpreted to function in the formation of a supportive structure in order to stabilize a β-turn in the conserved loop (Yamaguchi et al. 1993b). In a study evaluating the functional roles of Arg residues of TetA(B), Arg-67, Arg-70, and Arg-71, all belong to Motif A, only replacements for Arg-70 lost both tetracycline resistance and transport (Kimura et al. 1998a). Along these lines, a defective primary mutation in TetA (B), in which Asp-66 changed to a Cys, was suppressed by a second-site mutation where Ala-40 was also changed to Asp, supporting the notion that a charged residue is an important requirement for transport (Yamaguchi et al. 1995). Similarly, a defective mutation in which Gly-62 of Motif A was changed to Leu was compensated for by a second-site mutation on the other side of the same membrane in which Leu-30 was changed to a Ser residue, and the authors interpreted this finding as the double mutation providing a "conformational hook" that blocks deleterious conformational changes at a remote location elsewhere in the protein (Kimura et al. 1997). A similar so-called remote conformational suppression effect was observed later when the primary mutation in Motif A in which Gly-62 changed to Leu in TetA(B) was suppressed by the second-site mutation where Ala-354, also on the other side of the cytoplasmic membrane, was changed to Asp (Kawabe and Yamaguchi 1999). This latter effect was interpreted as TetA(B) having a close structural proximity between helices 2 and 11 on the periplasmic side of the cytoplasmic membrane (Kawabe and Yamaguchi 1999). The seminal discovery of salt bridges in the *E. coli* lactose permease, LacY, by the Wilson laboratory, reviewed in ref Varela and Wilson (1996) and see Lee et al. (1996), prompted an evaluation of possible salt bridges in TetA(B) in which Arg-70 of the Motif A was

found to interact with Asp-120, which resides at the distal end of helix 4 (Someya et al. 2000). Similarly, using molecular simulation dynamics of the proton-coupled oligopeptide symporters $PepT_{So}$ from *Shewanella oneidensis* and $PepT_{St}$ from *Streptococcus thermophilus*, a salt bridge involving a Motif A residue, Asp-79, was predicted to form with Lys-84 which resides near helix 3 (Fowler et al. 2015). This salt bridge was further predicted to stabilize the outward-facing conformation of $PepT_{So}$, thus potentially participating in the gating topology of symporters in this closely related family (Fowler et al. 2015). In a separate study of the TetA(P) efflux pump for tetracycline from *Clostridium perfringens*, the site-directed mutations at Pro-61 and Arg-71 abolished tetracycline resistance levels (Bannam et al. 2004).

6.2 More Recent Studies of Motif A

Interestingly, a human glucose transporter, GLUT-1, expressed in red blood cells, was studied in patients with GLUT-1 deficiency syndrome, and mutations were found in elements of Motif A: Gly-91 changed to Asp and Arg-93 changed to Gln or Trp (Pascual et al. 2008). These mutations showed reduced glucose transport, and it was concluded from these findings that Gly-91 may be important for substrate docking within the recognition site and that Arg-93 may serve to help anchor GLUT-1 to the membrane (Pascual et al. 2008). Additionally, a study of autosomal dominant missense mutations showed that alteration of the Motif A residue Gly-91 to either Asp or Ala in GLUT1 from *Homo sapiens*, when expressed *Xenopus* oocytes, had severely reduced glucose transport activities (Klepper et al. 2001). In a separate study involving another eukaryotic organism, the fungus *Aspergillus nidulans*, various mutations in the high-affinity nitrate transporter, NrtA, were isolated (Kinghorn et al. 2005). Of this set of mutations, residues of Motif A were altered in which Cys-90 was changed to Phe and Gly-91 was changed to Ser, and both mutants showed reduced nitrate uptake compared to wild-type NrtA (Kinghorn et al. 2005).

The internal duplication event postulated to occur for MFS transporters (Henderson and Maiden 1990; Griffith et al. 1992), particularly the tetracycline efflux pumps (Rubin et al. 1990), prompted the evaluation of the residues of the loop between helices 8 and 9 of TetA(B) (Yamaguchi et al. 1993b). In this analysis, only Gly-273 of TetA(B) in the second loop between helices 8 and 9 was demonstrated to be essential for tetracycline transport (Yamaguchi et al. 1993b).

6.3 Studies of Motif A in Symporters

Prior to the discovery of Motif A, the roles of glycine residues along the LacY protein of *E. coli* (including glycines of Motif A) had been examined in the laboratory of Kaback (Jung et al. 1995), and it had been deemed that no such

glycines throughout the symporter were critical for the transport of lactose. The first systematic study using site-directed mutagenesis to specifically address the functional importance of Motif A residues in LacY (Brooker 1990; Varela and Wilson 1996) was conducted in the laboratory of Brooker (Jessen-Marshall et al. 1995). In their first study, most amino acid replacements for Gly-64 and Asp-68 showed dramatic losses of lactose transport activities, while replacements for Lys-69, Gly-72, Arg-73, and Lys-74 showed only moderate to no loss of lactose transport (Jessen-Marshall et al. 1995), and it was concluded that the loop 2-3 structure formed by Motif A facilitates access of lactose entry into the cell by allowing conformational changes to occur upon sugar binding to the symporter (Jessen-Marshall et al. 1995). Using the mutation in which Asp-68 was changed to Thr, second-site revertant mutants were isolated that compensated for the defect conferred by the primary mutation, and it was found that most second sites were located in proximal ends of helices 2, 7, and 11 at the periplasm–membrane juncture (Jessen-Marshall and Brooker 1996). These results were interpreted as the suppressor mutations having altered the protein topology in order to facilitate the interaction between the two bundles of the symporter and helix 2 behaving as an interface between these two symmetrical bundles (Jessen-Marshall and Brooker 1996; Pazdernik et al. 1997a), a finding later supported by extensive molecular physiological analyses (Green et al. 2000; Green and Brooker 2001). In another study, Brooker used second-site suppressor analysis with Gly-64 mutations as the first-site mutation and found second sites dispersed throughout the symporter concluding that Gly-64 allows conformational changes to occur that are necessary for lactose transport across the membrane and that this residue is at the interface between two symmetrical bundles of the LacY protein (Jessen-Marshall et al. 1997; Pazdernik et al. 1997a). As mentioned above, the primary amino acid sequences of the N-terminal halves of the MFS transporters are closely related to their corresponding C-terminal halves. Motif A in the loop between helices 2 and 3 of these transporters is thus duplicated at the cytoplasmic loop between helices 8 and 9 (Griffith et al. 1992). Thus, the functional roles of these conserved amino acids in the loop 8-9 of LacY were evaluated and determined that they, too, serve to facilitate conformational changes that are believed to occur in these transporters during solute and ion transport catalysis (Pazdernik et al. 1997b; Cain et al. 2000).

6.4 Studies of Motif A in Multidrug Efflux Pumps

In the multidrug transporter LmrP from *Lactococcus lactis* (Bolhuis et al. 1995), the functional role of Asp-68, which resides in Motif A, was explored. First, molecular physiological evidence showed that an interaction between Asp-68 and phosphatidylethanolamine, a polar head group of the biological membrane, provides a sensor mechanism for detection of a proton gradient by the cell (Hakizimana et al. 2008). This particular notion that in this position of Motif A, a conserved Asp plays a role in proton gradient sensing, is supported by an apparent lack of conservation of Asp

in this location of Motif A within MFS transporters that are not proton driven, such as in the case of the glucose facilitators (Hruz and Mueckler 2001), and the family of organic anion transporters (OATs), which are instead sodium driven (Zhou and You 2007). In another study using a biophysical analysis and molecular simulation dynamics of LmrP, it was found that during substrate transport, protonation of Asp-68 facilitated an outward-facing closed and inward-facing open conformation of the transporter, and deprotonation of Asp-68 to release protons into the cytoplasm favored a resetting back to the resting state conformation (Masureel et al. 2014); that is, Asp-68 plays a functional role in mediating conformational switching of the transporter during the multidrug efflux pump transport cycle. A study of the crystal structure of a proton-dependent oligopeptide transporter, YbgH from *E. coli*, combined with mutagenesis and comparisons with previously elucidated transporter crystal structures, found that a variant of Motif A, called Motif 1, functions as a conformational switch mechanism in order to stabilize YbgH in an outward-facing conformation (Zhao et al. 2014). An interesting development occurred with respect to Motif A and the mechanism of solute transport with the recent crystal structure determination of an *E. coli* outward-facing multidrug efflux pump, YajR, with a clearly defined loop 2-3 structure (Jiang et al. 2013). Based on this YajR crystal structure, the investigators provided structural and functional roles for individual residues of Motif A (Jiang et al. 2013). For instance, Gly-69 of YajR is believed to interact with Gly-337 and Gly-341, which are located on helix 11 of the same protein, thus forming an interface between the two domains (i.e., bundles) and allowing the formation of the outward-facing conformation of the pump (Jiang et al. 2013). Additionally, since Asp-73 was buried deep within the interface between the two bundles adjacent to helix 11 in the YajR structure, it is thus thought that this residue stabilizes both helix 11 and the bundle interface via a dipole-helix interaction; in support of this notion, the mutation Asp-73 changed to Arg decreased the melting temperature, suggesting that Asp-73 becomes solvent accessible (i.e., unburied) during the formation of an inward-facing conformation (Jiang et al. 2013). The Arg-74 residue is believed to interact with membrane phospholipid, thus possibly stabilizing the YajR protein within the membrane (Jiang et al. 2013). Gly-76 may stabilize the interaction within the N-terminal bundle, i.e., Gly-76 may confer an intra-domain stabilization (Jiang et al. 2013). Arg-77, on the other hand, is believed to form salt bridges with both Asp-73 (of Motif A) and Asp-126, the latter residue of which is located at the C-terminal end of helix 4 (Jiang et al. 2013). Incidentally, this same type of salt bridge formation is known to occur in LacY, in which Lys-319 interacts with both Asp-240 and Glu-269 to form alternating ion pairs (Lee et al. 1993). Lastly, Lys-73 of YajR is thought to interact with the C-terminal portion of helix 6 (Jiang et al. 2013). Taken together, the residues of Motif A in the YajR multidrug efflux pump are thought to stabilize the outward-facing conformation of the protein and thus participate in the conformational changes between the outward- and inward-facing stages of the transporter (Jiang et al. 2013). Strikingly, these investigators further found that elements of Motif A of loop 2-3 (called L2-3) are also present to a certain extent in three other loops of YajR, i.e., those loops between helices 5 and 6 (L5-6), 8 and 9 (L8-9), and 11 and

12 (L11-12), suggesting a widespread influence in the solute transport cycle for Motif A and Motif A-like sequences not only throughout a given MFS transporter, but in all transporters of the MFS as well (Jiang et al. 2013). Recently, the three crystal structures were elucidated for the multidrug efflux pump, MdfA, from *E. coli* in which each structure was bound to its substrate chloramphenicol or one of its analogs deoxycholate or n-dodecyl-N,N-dimethyl-amine-N-oxide (Heng et al. 2015). Since Motif A is known to stabilize the outward-facing conformation, as mentioned above for YajR (Jiang et al. 2013), the structural element conferred by this conserved motif is apparently not involved in dictating the inward-facing conformation seen in any of the three MdfA crystal structures (Heng et al. 2015).

7 Motif C "G $(X)_8$ G X X X G P X XG G" and Functional Roles

This conserved sequence motif was discovered by Rouch et al. to reside within the fifth TMD of transporters of the MFS (Rouch et al. 1990; see Fig. 3b). Initially thought to be found only with antiporters of the MFS but not in symporters or uniporters, Motif C was referred to as the "antiporter motif" (Varela et al. 1995; Varela and Griffith 1993). Recently, however, manual adjustments were performed during an extensive multiple sequence comparative analysis to surprisingly discover that sequence elements of the so-called antiporter motif are apparently found in the symporters and uniporters of the MFS as well (Yaffe et al. 2013).

7.1 Early Studies of Motif C in Efflux Pumps for Tetracycline

One of the earliest studies conducted to address the functional importance of Motif C was performed by Varela et al. in which they systematically replaced the most highly conserved residue of the motif, namely, Gly-147 of the tetracycline efflux pump, TetA(C), encoded on plasmid pBR322, with all other 19 amino acid residues (Varela et al. 1995). Interestingly, these investigators found that only Ala and Ser residues were acceptable in place of Gly-147 as tetracycline resistance was reduced to only 26 % and 19 % of the wild-type TetA(C), respectively (Varela et al. 1995). Molecular modeling analysis indicated a slight bend or kink in the fifth helix in the wild-type protein (Varela et al. 1995). Taken together, these investigators concluded that the residues of motif C dictate subtle structural differences inherent in determining substrate specificities and direction of solute transport (Varela et al. 1995). A study by Ginn et al. directly examined the structure–function relationships for all residues of Motif C of the TetA(K) tetracycline efflux pump from *S. aureus* by site-directed mutagenesis and tetracycline efflux assays (Ginn et al. 2000). These

investigators found that tetracycline efflux pump activities were moderately to severely reduced for those mutants in which only the conserved residues of the motif were altered by mutation (Ginn et al. 2000). Thus, it was demonstrated in this study that the conserved residues of Motif C confer active tetracycline efflux; furthermore, because of the relative abundance of glycine residues in the motif, it was concluded that such flexible residues mediate conformational changes necessary for the efflux pump to respond to its immediate microenvironment (Ginn et al. 2000). Cysteine-scanning mutagenesis and accessibility of such mutations to the aqueous microenvironment that were studied by the laboratory of Yamaguchi and colleagues who showed that all residues of Motif C within TMD-5 of the Tn10-derived tetracycline efflux pump, TetA(B) from *E. coli*, line a water-filled channel and are thus probably able to bind substrate to facilitate transport (Iwaki et al. 2000). Additionally, these authors concluded that residues of TMD-5 of TetA(B), along with residues of TMD-4, form a permeability barrier that serves to avoid undesirable uncoupling (Iwaki et al. 2000). The laboratory of Levy conducted a second-site suppressor study in which four second-site mutations that complemented a defective mutation at Gly-247 of TetA(B) were found in TMD-5 indicating that residues of Motif C interact with residues of TMD-8 to stabilize their close association to each other (Saraceni-Richards and Levy 2000). These authors further concluded that residues of Motif C that are forming the permeability barrier in TetA(B) mediate conformational switching that occurs during solute transport across the membrane (Saraceni-Richards and Levy 2000).

7.2 *Studies of Motif G*

As mentioned earlier, bioinformatics evidence indicated an internal tandem repeat of a primordial 6-helix ancestor to form a modern 12-helix structure (Griffith et al. 1992) implying that Motif C is duplicated as well. The duplicated Motif C, denoted Motif G, was found in TMD-11 of the 12-helix MFS transporters (Paulsen et al. 1996b). This notion was confirmed experimentally in a study by Levy and colleagues in which they characterized Mdt(A), a multiple drug efflux pump encoded on a plasmid originating from *Lactococcus lactis*, and found the two Motif C-like sequences, one residing in TMD-5 and the other in TMD-9 (Perreten et al. 2001). Remarkably, these investigators also found an ATPase domain, which is routinely found in primary active transporters (Perreten et al. 2001). In another study involving Mdt(A) from a naturally occurring drug-susceptible variant of *Lactococcus garvieae*, Motif C was found two be altered in two of the canonical residues, thus possibly explaining the observed drug susceptibilities (Walther et al. 2008).

7.3 The Glycine–Proline Dipeptide in Motif C

A molecular mechanics and modeling study showed that a glycine–proline (GP) dipeptide within Motif C specified a bend or kink within the TMD-5 of the MFS efflux pumps (Varela et al. 1995). This particular notion was evaluated by the laboratory of Krulwich in which they closely examined mutations at these two residues, Gly-155 and Pro-156, of the tetracycline efflux pump, TetA(L), from *Bacillus subtilis* and found that the replacements showed, in general, tetracycline binding and a potassium leak, but not transport of tetracycline, suggesting that the GP dipeptide from Motif C is important for tight helix packing and leak proofing of the pump and providing an explanation for observed discrepancies between transport and resistance levels (Jin and Krulwich 2002; De Jesus et al. 2005).

The sole conserved proline residue of Motif C (of the GP dipeptide) was closely studied in QacA, a 14-TMD efflux pump encoded on the chromosome of *S. aureus* in a study focusing mainly on intramembranous Pro residues (Hassan et al. 2006). Replacement of Pro-161 of Motif C with Gly, Ser or Ala residues did not abolish resistance to any QacA substrates, but did show slightly altered drug resistance levels in host cells, suggesting this Pro residue may help form the permeability barrier and allow molecular motions or interactions with substrate to occur during transport of monovalent dyes (Hassan et al. 2006).

7.4 A Conformational Switch and Motif C

An analysis of residues of Motif C in a vesicular acetylcholine transporter, VAChT, from a eukaryote, *Rattus norvegicus*, showed profound loss of acetylcholine transport across the membrane and altered kinetic behavior of transport, indicating that minor and relatively stiff kinks in TMD-5 of VAChT are formed by residues of Motif C and that the motif not only allows conformational flexibility, i.e., switching, but also confers a tight proton seal to prevent dissipation of the membrane potential (Chandrasekaran et al. 2006). Another study of VAChT using homology modeling and molecular dynamics simulations found both kinking and wobbling behavior in structures formed by residues of Motif C and a lowering of the energy barrier for structures in which residues of Motif C were mutated (Luo and Parsons 2010). The authors of this study concluded that the structure formed by Motif C is at the interface between two helical bundles, consisting of TM helices 1–6 and 7–12 of VAChT, and that Motif C forms a complex hinge region between the two helical bundles in order to provide an energy barrier during conformational changes that occur during solute transport (Luo and Parsons 2010). Motif C from another eukaryotic MFS efflux pump, CaMdr1p from *Candida albicans*, which transports antifungal agents, was studied for its functional importance (Pasrija et al. 2007), and the investigators concluded that residues of this motif possibly mediate helix packing. A recent study of VMAT2 from *R. norvegicus* discovered that Motif

C plays a significant role in forming a so-called molecular hinge structure in which helices 5 and 8 interact with helices 2 and 11 to mediate the conformational switching between the two symmetric bundles that is thought to transpire during solute transport (Yaffe et al. 2013).

In a more recent study in which the crystal protein structure was determined for the *E. coli* MdfA multidrug efflux pump, it was shown that the protein was complexed with chloramphenicol or one of two substrate analogs; and it was further demonstrated that elements of Motif C (Ala-150, Ala-153, and Pro-154) (Rouch et al. 1990; Varela et al. 1995) surrounded two critical acidic residues Glu-26 and Asp-34 that reside in helix 1 of MdfA, thus constituting part of a central aqueous substrate binding cavity, a seemingly ubiquitous property of MFS solute transporters (Heng et al. 2015).

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