Opinion

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Undecided membrane proteins insert in random topologies. Up, down and sideways: it does not really matter

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It is usually assumed that to ensure proper function, membrane proteins must be inserted in a unique topology. However, a number of dimeric small multidrug transporters can function in the membrane in various topologies. Thus, the dimers can be a random mixture of NiCi (N and C termini facing the cell cytoplasm) and NoCo (N and C termini facing the outside) orientation. In addition, the dimer functions whether the two protomers are parallel (N and C termini of both protomers on the same side of the membrane) or antiparallel (N and C termini of each protomer on opposite sides of the membrane). This unique phenomenon provides strong support for a simple mechanism of transport where the directionality is determined solely by the driving force.

Much has been said about the topology of EmrE, a small (110 residues) homodimeric multidrug transporter from *Escherichia coli* that extrudes positively charged aromatic drugs in exchange for two protons, making bacteria resistant to a variety of toxic compounds.

EmrE is a dimer. Any single dimeric membrane protein may adopt three different topologies (denoted here as 'topoforms') schematically shown in Figure 1 (top). Relative to each other, the two protomers can theoretically adopt either a parallel (P; N and C termini of both protomers on the same side of the membrane) or anti-parallel orientation (AP; N and C termini of each protomer on opposite sides of the membrane). Additionally, relative to the lipid bilayer, the ensemble of parallel dimers can theoretically adopt a single topology or a dual topology where dimers are either all in a NiCi (or NoCo) orientation or a mixture of both. Although all the potential topologies can occur, what topology makes biological sense? The answer to that question is open since there are constraints such as the way the protein is inserted into the membrane during synthesis and the requirement for the dimer to be catalytically active. Thus, determining the topology of EmrE is essential to learning how it functions and how the protein has evolved. As more complex membrane proteins could have evolved from smaller ones, this also has implications for the evolution of membrane proteins in general.

One of the reasons for the controversy about the topology of EmrE is the assumption that, to ensure proper function, membrane proteins must be inserted in a unique topology. Indeed, it is obvious that proteins such as receptors must face the environment they are probing. Channels that sense the electrical field across membranes depend on the polarity of the field and cannot be inserted randomly; pumps that derive their energy from molecules such as ATP, available only on one side of the membrane, need to have the ATP utilization machinery in the right location. However, is a unique topology a necessary prerequisite for uniporters or ion-coupled transporters? Work with EmrE demonstrates that this is not an essential prerequisite for function as will become clear in this Opinion paper.

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Looking for the right unique topology

For almost a decade, our group and others have published what looks like conflicting results regarding the topology of EmrE. I briefly describe below the evidence supporting the opposing views. I must however start by clarifying from the beginning that, even though I critically evaluate the evidence that supports the antiparallel topology, I do believe that the antiparallel topology is also possible under some conditions, and my group has presented experimental evidence that supports this view. However, I also present evidence that supports the parallel topology (critically evaluated or disregarded by others) and I conclude that small multidrug resistance (SMR) proteins can function whether parallel or antiparallel, and when parallel, they can function whether facing one side of the membrane or the other. To the best of my knowledge, this is a unique phenomenon and its significance is discussed below.

Evidence supporting antiparallel topology

The possibility of an antiparallel topology of the protomers in the EmrE homodimer was first suggested by Ubarretxena-Belandia *et al*. to explain the quasi-symmetry observed in the 3D structure of EmrE acquired by cryoelectron microscopy (cryo-EM) at 7.5 Å resolution in the membrane plane [1]. A model derived from a low-resolution 3D-crystal structure supported this suggestion [2]. Even though the structure was withdrawn [3], it stimulated investigators to pursue this unique avenue. Fleishman *et al*. used the symmetry relation mentioned above, combined with sequence conservation data, to assign the transmembrane segments in EmrE to the densities seen in the cryo-EM structure. The C-α model of the transmembrane region is constructed so that the helices of one protomer have the topology opposite to the ones of the other protomer (antiparallel) [4].

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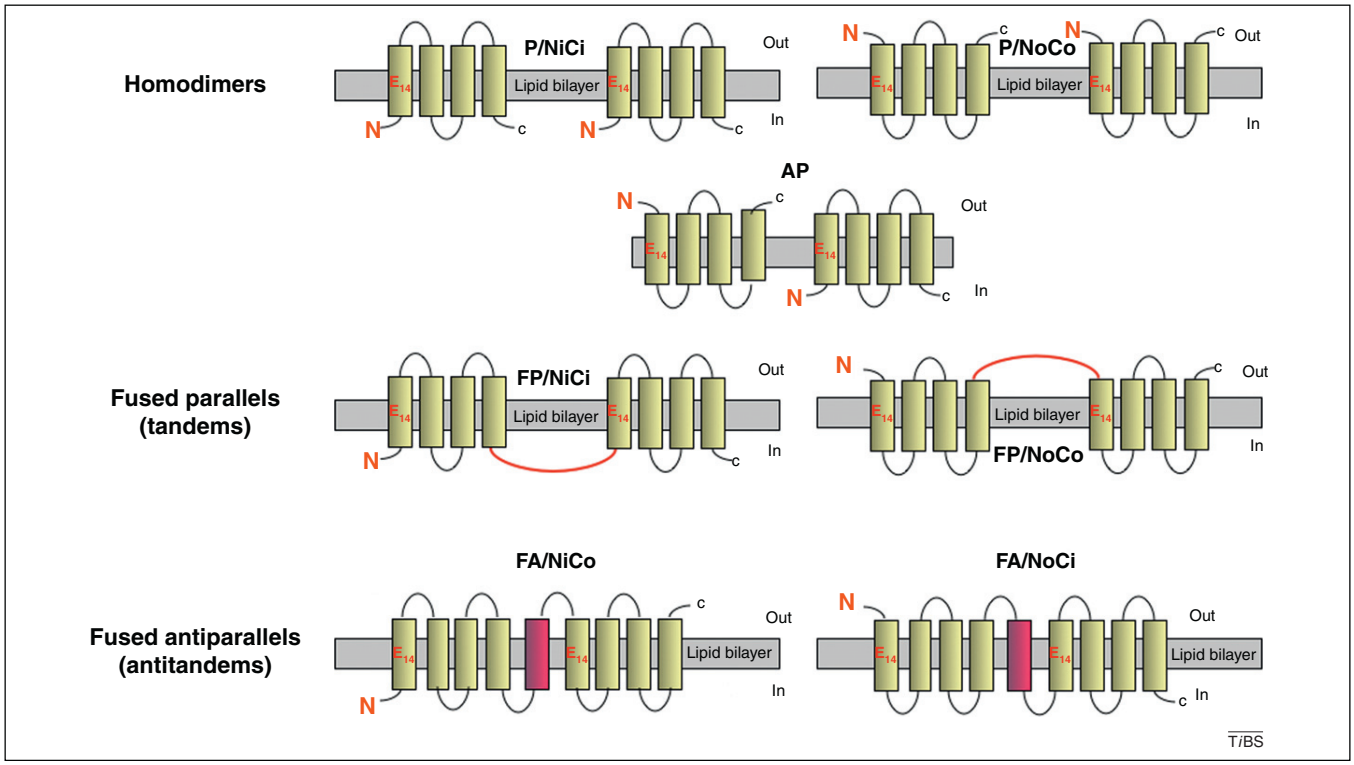

Figure 1. Native and artificially generated topoforms of EmrE. Topoforms are dimers in which the protomers have parallel (P), antiparallel (AP), fused parallel (FP) or fused antiparallel (FA) orientation relative to each other. Each individual protomer can have of several possible orientations, based on whether the N and C termini face the outside of the cell (No and Co, respectively) or the cytoplasm (Ni and Ci). Four of them (P/NiCi, P/NoCo, FP/NiCi and FA/NiCo) have been experimentally demonstrated, and a fifth one (AP) has been suggested from models based on low-resolution 2D and 3D crystals and from FRET and crosslinking experiments in bicelles. The Glu residues located in the central binding domain are shown in red (E_{14}).

A recalculated model based on the 3D-crystals is remarkably similar to the model suggested from the EM data [5]. The similarity is notable because the resolution of the structures used to build the models is low in both cases. Moreover, the crystals used to derive the C-α model X-ray structure were obtained with protein solubilized with detergents that inhibit activity by dissociating monomers, as shown by us and in the crystallization paper [5,6]. Protomers arrange in the crystal in a conformation that minimizes the energy needed for crystal formation and do not necessarily reflect the topology in the membrane.

Genetic experiments were designed to support the claim for an antiparallel topology. EmrE was fused to the 'topology-reporter proteins' alkaline phosphatase or green fluorescent protein, and the results showed that the topology of the EmrE fusion proteins in the membrane was sensitive to the distribution of positive charges in the protein [7]. Manipulation of the positive charges generated a set of mutants, some with NoCo, and others with NiCi apparent topology [7,8]. Neither mutant conferred resistance to ethidium, therefore, the authors concluded that this was due to the modified topology.

Coexpression of the inactive mutants restored the ethidium resistance to the same level as seen with wild type EmrE [8]. The suggested interpretation of this finding is that coexpression results in the generation of a functional, antiparallel heterodimer. However, this assumption is based solely on the contention that the NiCi and NoCo mutants are inactive. On closer investigation, we and others have found that they are both functional as judged from the phenotype assayed following continuous growth in liquid medium [9], or in cells with the proper genetic background in which the chromosomal gene of EmrE has been inactivated and expression of the mutants induced [10].

The lower activity of the single mutants compared with the coexpressed ones could be due to impaired dimerization and not necessarily due to a different topology [10]. The coexpressed mutants display high activity because they can interact with high affinity and form a functional heterodimer where each monomer differs from the other by six amino acids [8].

A comprehensive study of the orientation in the membrane of several SMR proteins was performed using reporter genes [11]. The results suggest a fraction of the protein faces the cytoplasmic side and the other faces the periplasm. The authors conclude that the findings support an antiparallel topology but the results cannot distinguish between this and a mixed population of parallel dimers (Figure 1, top).

Amadi *et al*. performed a systematic spin labeling and electron paramagnetic resonance (EPR) analysis to study the structure and dynamics of EmrE in liposomes [12]. The study revealed at least two spin label populations arising from different packing interfaces of the EmrE dimer. One population was consistent with antiparallel arrangement of the monomers, although the EPR parameters suggested deviations from the crystal structure of substrate-bound EmrE. It is not yet clear whether the parallel dimer accounted for the rest of the population [12].

A recent study has provided support for the presence of antiparallel dimers with protein reconstituted in bicelles

[13]. The experimental evidence does not rule out the existence of parallel dimers as well. Crosslinking experiments were performed with two classes of crosslinkers: one that crosslinks only parallel dimers and another one only antiparallel ones. Despite the fact that both crosslink the dimers, it has been claimed that the parallel crosslinking reflects an artifact due to crowding and proximity between different dimers. However, even at high dilutions, the crosslinking is observed and no such control is reported for the other crosslinker. The elegant fluorescence resonance energy transfer (FRET) experiments that were carried out cannot rule out the existence of populations in which the fluorophores are very close. Energy transfer efficiency depends not only on the distance but also on the dipole orientation, and this parameter may be especially relevant in membrane proteins where the dipoles cannot always freely randomize [14].

It is possible that in bicelles, the very specific lipid environment and the manipulation of the protein pushes the equilibrium towards the antiparallel conformation [13]. This finding can provide interesting information about the determinants that drive the protein to an antiparallel conformation, and it will be interesting to see whether further experimentation demonstrates functionality of the protein under these conditions.

Evidence supporting parallel topology

To evaluate the antiparallel structural model, we tested one of its most basic predictions, the existence of antiparallel dimers, using a crosslinking approach. All our experiments were consistent only with parallel dimers.

When unique cysteines were engineered in the hydrophilic loops, or in the termini, crosslinkers that could react with residues 9–11 Å apart quantitatively crosslinked the protomers [15]. This finding is inconsistent with an antiparallel topology, which predicts the two cysteine residues to be at least 35–40 Å apart. Crosslinking could be amenable to artifacts, therefore, we also purified two proteins crosslinked in loops 1 and 3 and showed that they were fully active, supporting the contention that the crosslinking experiments reflected the protomer symmetry in functional dimers [6].

Generation of genetic fusions (tandems) in which the C terminus of one protomer was fused to the N terminus of the second protomer has provided a paradigm to study the topology both *in vivo* and *in vitro*. The linkers were designed such that they were very short or very hydrophilic. This ensured that both termini were on the same side of the membrane and forced the dimer into a parallel topology (Figure 1, middle panel) [16]. All the tandems built as described were functional *in vivo* and after purification. To ensure that the functional unit was the dimer and not a

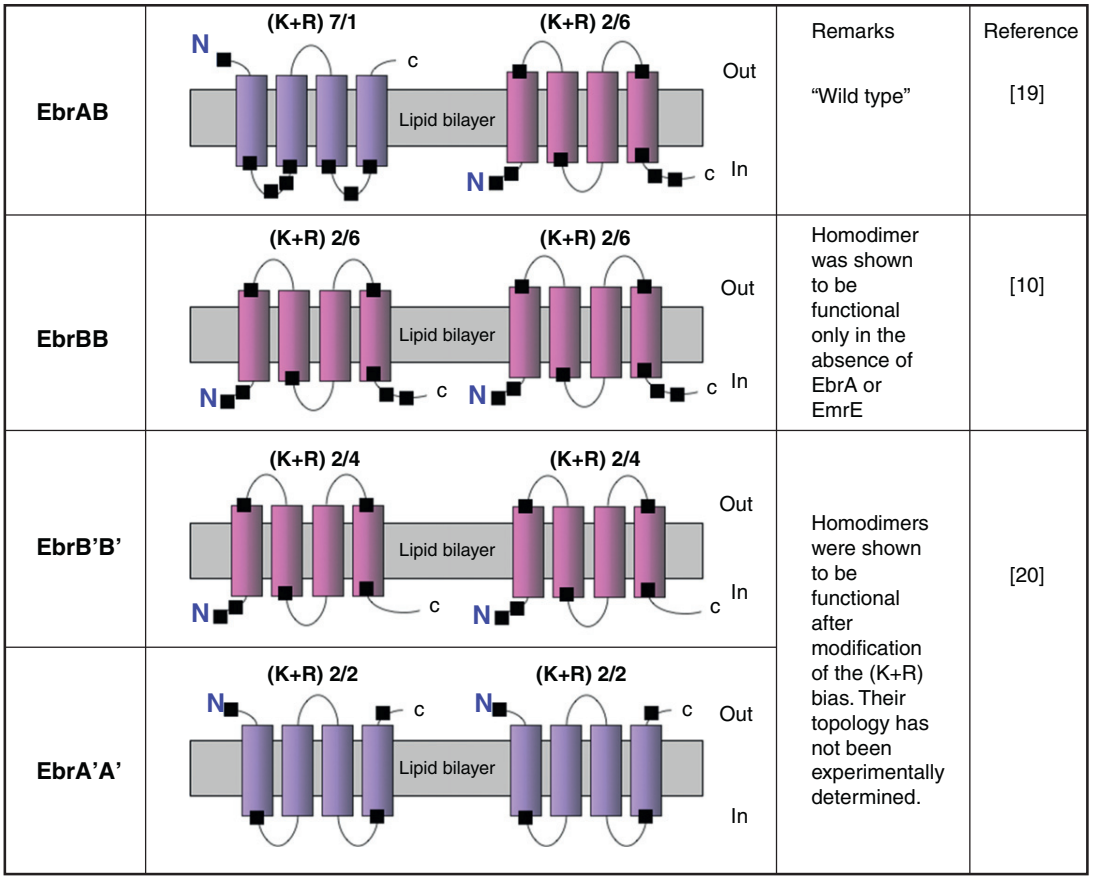

Figure 2. The many faces of a heterodimer. ***EbrAB*** is a heterodimer from *B. subtilis* [19]. EbrB can function also as a homodimer when expressed without EbrA in a Δ*emrE* host [10]. Both EbrA and EbrB can function as homodimers when their topogenic signals are properly manipulated [20]. None of the topologies has been experimentally determined.

result of interaction between dimers, we showed that, unlike the wild-type protein, the fused dimer did not interact with inactive mutants (negative dominance experiments). Furthermore, we did not detect larger oligomeric species as judged from pull-down experiments [6,16].

A solution to the controversy

How can we reconcile the controversy? Our biochemical work strongly supports the existence of a parallel dimer that, in our view, is the native form of EmrE. However, we want to know if the antiparallel association also exists. We have not been able to identify any sizable fraction of the 'native' population in antiparallel configuration [6,17,18]. However, evolution has provided a partial answer to this question. Closely related SMR parallel homodimers, such as EmrE, and putatively antiparallel heterodimers, such as EbrAB from *Bacillus subtilis* [19], perform identical functions (ethidium and acriflavine efflux) even though the relative topology of the protomers is different (Figure 2, top). Moreover EbrB is capable of forming homodimers, most probably in a parallel mode because it has strong topogenic signals (Figure 2 and [10]). In addition, individual EbrA and EbrB mutants with altered (K+R) bias also function as homodimers (Figure 2 and [20]). In other words, the interaction between protomers is very promiscuous: the protomers can interact either with each other as an antiparallel heterodimer, or as a parallel homodimer in the absence of the other protomer or if the topogenic signals are modified.

If closely related heterodimers are so promiscuous and can interact parallel and antiparallel, the answer to the EmrE controversy may be simple: EmrE is also promiscuous in the interaction of the protomers and is functional in either of the proposed topologies (Figure 1, top). The topogenic determinants in the wild-type EmrE are nonexistent and therefore parallel dimers insert in random topologies: about half NiCi and half NoCo [10]. After insertion, the interaction of the protomers results in a parallel or antiparallel mode depending only on their relative affinities. The interaction in a parallel mode is the only one identified in the native protein [6,15–17], therefore, we propose that affinity for parallel association is higher, at least under the conditions tested thus far. However, the protomers can be forced to interact productively also in an antiparallel mode by fusing them head to tail with an additional membrane helix (Figure 1, bottom and [10]). This fusion results in higher local concentrations of the topologically antiparallel protomer, overcoming its lower affinity. This fused antiparallel dimer has also been shown to be functional *in vivo* and *in vitro* [10]. As with the parallel tandem, negative dominance and pull-down experiments have supported the claim that the dimer is the functional unit [10].

Topology and function

As far as we know, the diversity of functional topoforms has not yet been shown in other systems. Two important

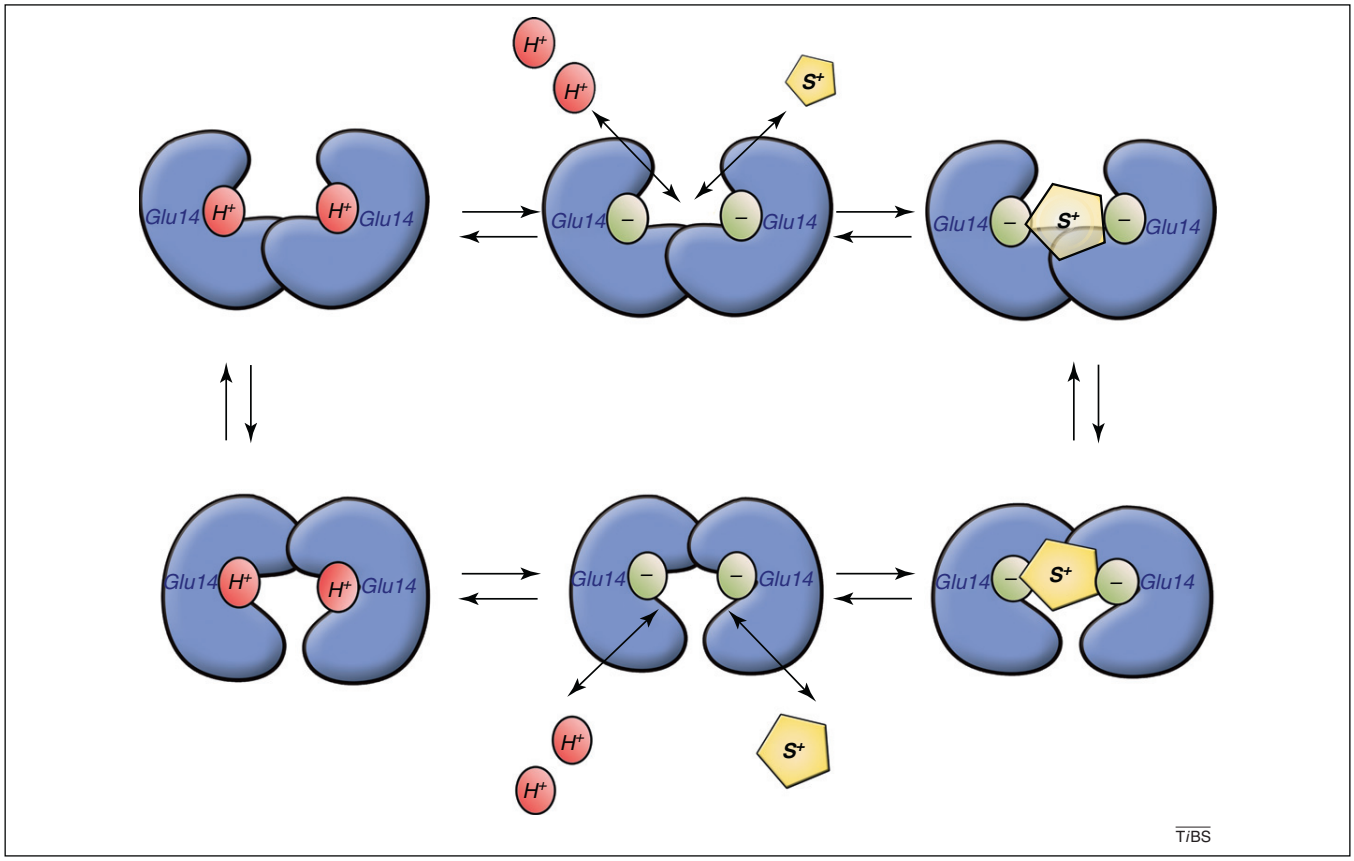

Figure 3. Transport mechanism in EmrE. ***The*** pKa of the carboxyl glutamate residue at position 14 (Glu14) is around 8.0 [27]. Substrate (S^+) binds only to the negatively charged EmrE and shifts the equilibrium so that 2 H^+ are released to the same side of the membrane. Substrate binding induces a conformational change that opens the binding chamber to the opposite side. Proton binding displaces the substrate and reorients the binding site. The substrate is represented as a yellow diamond and protons as red balls. Cartoon based on mechanism depicted in [27].

questions are posed by such behavior. First, an implicit conclusion from these findings is that the interaction between protomers is very promiscuous: homodimers can form functional heterodimers when pushed, for example, by modification of the topogenic signals [8] or by *in vitro* mixing with other SMR proteins [21]. By contrast, heterodimers can also form homodimers as described above for EbrAB [10,20]. Promiscuity of interaction between proteins is not unknown. It is a well-documented necessity in the major signaling systems in which one master protein can interact with many different partners (e.g. [22]).

Second, and to account for the observation that both parallel and antiparallel dimers are functional, we propose that it is because the mechanism of coupling ion and substrate transport is simple. This simplicity provides the robustness necessary to tolerate such a unique and unprecedented ambiguity in the interaction of the subunits and in the dimer topology relative to the membrane [17]. EmrE has a binding site that is occupied either by H^+ or by substrate, providing a basis for the coupling reaction (Figure 3). Its occupancy is mutually exclusive thanks to a carefully tuned pKa of two Glu residues located in a central location in the binding domain [17]. This essential property of the Glu residues is conserved whether the protomers interact in a parallel or antiparallel mode because both configurations provide a suitably similar environment in the hydrophobic cavity.

How are the binding determinants conserved in all the topoforms? In polyspecific proteins, binding determinants have been shown to be overlapping and even redundant so that substrates are capable of interacting with one of a number of them (e.g. [23]). We conclude that such a redundancy allows for specific binding in both, the parallel and antiparallel dimers.

The findings described here support the concept that ion-coupled proteins are by nature symmetric in their function, and the direction of the transport is determined solely by the direction of the driving force (e.g. [24]). As suggested by the alternate access model, the coupled translocation of a substrate molecule and one or more ions across the membrane involves a global conformational change that allows alternating access of substrate and H^+-binding sites to either side of the membrane. Therefore, unless dictated otherwise by regulation, only the driving force determines the directionality of transport.

The behavior observed with EmrE and other SMR proteins may represent a stage in the evolution of the topology of membrane proteins [17,25]. The evolutionary challenge of recognition and transport of a wide spectrum of substrates may have selected for SMR heterodimers that originated from gene duplication of the more ancient homodimers. After gene duplication, a small number of mutations would allow either parallel or antiparallel orientation of the protomers within the heterodimer. In this manner, one protein with only a slightly modified sequence may extend the range of the substrate specificity [25]. Inverted repeats have been identified in many large modern transporters and have been suggested to play important roles in the transport mechanism [26]. The phenomenon described here suggests a way by which these inverted repeats may have evolved.

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