

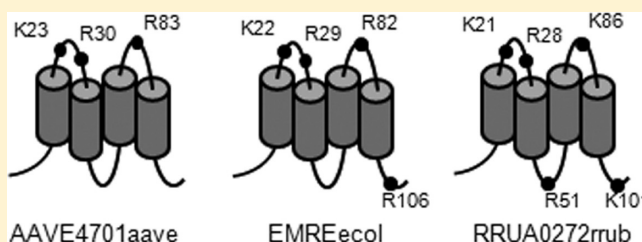
Role of Individual Positive Charges in the Membrane Orientation and Activity of Transporters of the Small Multidrug Resistance Family

Magdalena A. Kolbusz,[†] Dirk Jan Slotboom,[‡] and Juke S. Lolkema^{*,†}

[†]Molecular Microbiology and [‡]Membrane Enzymology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Groningen, The Netherlands

S Supporting Information

ABSTRACT: The effect of individual positively charged residues on the orientation in the membrane was analyzed in three dual-topology transporters of the small multidrug resistance (SMR) family: AAVE4701aave of *Acidovorax avenae*, EMREecol of *Escherichia coli*, and RRUA0272rrub of *Rhodospirillum rubrum*. It is shown that (i) individual positive charges have different impacts on the orientation, (ii) positive charges that are conserved in the three different proteins do not have the same impact on the orientation, (iii) positive charges in odd- and even-numbered loops have different impacts, (iv) for some, but not all, the impact depends on the presence of other positive charges, and (v) proteins from which all positive charges are removed in some cases are dual-topology proteins and in other cases have a single orientation. A small number of positive charges placed in the loops of the latter proteins results in the violation of the so-called positive-inside rule that has been reported previously [Kolbusz, M. A., et al. (2010) *J. Mol. Biol.* 402, 127–138]. We conclude that each positive charge shifts the distribution between the two orientations toward the state that has the positive charge in the cytoplasm but that intrinsic factors other than positive charges determine the orientation as well. The ability of the mutants of AAVE4701aave and EMREecol to confer resistance against ethidium bromide revealed an essential role in catalysis for a conserved pair of positive charges in the second loop. No significant relation between activity and the relative orientation of the monomeric subunits in the dimer could be demonstrated.



Most of the proteins located in cellular membranes have adapted a unique orientation in the membrane. More than 20 years ago, the major factor governing the orientation of the proteins in the membrane was described in the so-called positive-inside rule.^{1,2} Accordingly, loops rich in positively charged residues, lysine (K) and arginine (R), tend to be located at the cytoplasmic side of the membrane. Statistical analysis showed a surplus of positive charges in cytoplasmic over periplasmic loops.^{1,3} A marked exception is formed by a small group of membrane proteins that insert into the membrane in both orientations and, therefore, were termed dual-topology proteins. These proteins may represent an important step in the evolution of membrane proteins consisting of two homologous domains with opposite orientations in the membrane.^{4–10} In line with the positive-inside rule, dual-topology proteins do not have a bias of positive charges in cytoplasmic over periplasmic loops. The role of positive charges in membrane topology was experimentally confirmed by manipulating the orientation of dual-topology proteins by adding lysine (K) and arginine (R) residues to the loops and/or removing them from the loops.^{11–15}

Members of the small multidrug resistance (SMR) family and in particular EmrE from *Escherichia coli* (EMREecol) have been studied extensively.^{6,16–19} The transporters, which excrete toxic compounds from the cell, function either as heterodimers or as homodimers.^{20–22} The former are encoded by a pair of homologous genes that encode proteins with opposite

orientations in the membrane and, subsequently, interact to form an antiparallel dimer. The homodimers are encoded by a single gene that encodes a dual-topology protein. Thus, the protein has both orientations in the membrane, and evidence has been presented that the proteins interact to form an antiparallel homodimer; however, the matter is still under debate.^{7,23–25} A study²⁶ involving 27 members of the SMR family (including both homo- and heterodimers) showed a good correlation between the distribution of positively charged residues over internal and external loops and the orientation in the membrane that was experimentally determined by the reporter fusion technique using green fluorescent protein (GFP)^{27,28} and alkaline phosphatase (AP/PhoA)²⁹ as reporters. The positive-inside rule predicted the genes in the pairs to encode proteins with opposite orientations while the single genes encoded proteins with a weak charge bias corresponding to dual topology.²⁶ There were two exceptions where the correlation was not seen: the homodimeric proteins AAVE4701aave of *Acidovorax avenae* and EMREecol of *E. coli*. While both proteins showed a clear charge bias over odd- and even-numbered loops, they, nevertheless, inserted in both orientations into the membrane. The discrepancy appeared to

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correlate with the low total number of positive charges in the loops. While on average, the loops of the homodimeric subunits contained approximately five to eight positive charges, this number was only three or four for AAVE4701aave or EMREecol, respectively. Clearly, in these two cases, factors other than positive charge distribution must play a role in determining membrane topology.

Here, a systematic study of the positive charges in the loops of AAVE4701aave and EMREecol and the control protein RRUA0272rrub of *Rhodospirillum rubrum* is reported. The positive charges were removed one by one and in several combinations. The effect of the mutations on the distribution between the two orientations of the proteins and the ability to confer drug resistance was analyzed. The discrepancy between the positive charge bias and the orientation in the membrane of the AAVE4701aave and EMREecol proteins seems to be related to the single orientation of the proteins in the absence of positive charges in the loops.

■ EXPERIMENTAL PROCEDURES

Growth Conditions. *E. coli* strain SF100³⁰ harboring pLIC and pBAD24 vectors (see below) was grown in Luria-Bertani broth (LB) medium supplemented with 50 μ g/mL ampicillin at 37 °C while being shaken continuously. Overnight cultures were diluted 1:30 in 5 mL of fresh medium. Protein expression from vectors pLIC1 and pLIC2 results in C-terminal transcriptional fusions with alkaline phosphatase (AP) and green fluorescent protein (GFP), respectively. Expression of proteins encoded on the pLIC and pBAD24 plasmids was induced by adding 0.004% (w/v) arabinose when the optical density of the cultures measured at 660 nm reached a value of 0.60, after which growth was continued for 1.5 h for GFP and AP measurements and 1 h for the drug resistance activity assay.

DNA Manipulations. Point mutants were obtained by site-directed mutagenesis or were artificially synthesized (Invitrogen). Wild-type genes were cloned using the ligation-independent cloning technique into pLIC1 and pLIC2 plasmids as described previously.²⁶ Plasmids pLIC1 and pLIC2 contain transcriptional fusions with genes encoding an N-terminal His₆ tag and C-terminal alkaline phosphatase (AP) and green fluorescent protein (GFP), respectively.³¹ To obtain the mutants by site-directed mutagenesis, whole pLIC1 and pLIC2 plasmids carrying genes encoding the wild-type proteins were used as a template for the polymerase chain reaction (PCR). The desired mutations were placed on the forward primer. The PCR products with blunt ends were first phosphorylated using T4 polynucleotide kinase (Fermentas) and circularized by T4 DNA ligase (Fermentas). The synthesized genes were cloned into pLIC1 and pLIC2 plasmids using NcoI and KpnI restriction sites located at the 5' and 3' ends, respectively. The genes were recloned into pBAD24 (Invitrogen), resulting in the genes without the reporters, by amplification with *pfu* polymerase (Fermentas) using primers, in which NcoI (forward primer) and XbaI (reverse primer) restriction sites were introduced. The PCR product and plasmid pBAD24 were restricted with NcoI and XbaI and ligated using T4 DNA ligase (Fermentas). All the constructs were checked by sequencing (Macrogen).

GFP Fluorescence of Whole Cells. The GFP fluorescence of cells containing GFP fusion proteins was measured as described previously.²⁶ Briefly, cells from 2 mL of culture grown as described above were harvested by centrifugation, washed once, and resuspended in a buffer containing 50 mM

Tris-HCl (pH 8.0), 200 mM NaCl, and 15 mM EDTA to an OD₆₆₀ of 0.2. The cell suspension was supplemented with *N*-dodecyl β -D-maltoside (DDM) to a final concentration of 0.5% (w/v). Fluorescence was measured using an Aminco-Bowman Series 2 spectrometer. In the experiment, an excitation wavelength of 468 nm and an emission wavelength of 507 nm were used. For each sample, the background fluorescence of cells expressing plasmid carrying no insert and no gene encoding reporter protein was subtracted. Every sample was measured at least three times. Data are presented as means with the standard deviation.

Alkaline Phosphatase Activity of Whole Cells. The alkaline phosphatase activity of cells containing AP fusion proteins was measured as described previously.²⁶ The culture was grown as described above. Cells from 2 mL of culture were harvested by centrifugation, washed once, and resuspended in 1 mL of 1 M Tris-HCl (pH 8.0), and the OD₆₆₀ was measured. An aliquot of 500 μ L of the suspension was incubated at 37 °C for 5 min, and *p*-nitrophenyl phosphate (Sigma Aldrich) was added to a final concentration of 1.4 mg/mL. The reaction was stopped by addition of 100 μ L of 1 M K₂HPO₄ when a yellow color appeared. Alkaline phosphatase activity was expressed in Miller units.³² For every mutant, at least three measurements were taken. For each sample, the background activity of cells harboring a plasmid carrying no insert and no gene encoding reporter protein was subtracted. AP activities were normalized to GFP activities as described previously.²⁶ Data are presented as means with the standard deviation.

Resistance Assay. Cells were grown and harvested as described above and diluted to an OD₆₆₀ of 0.6. Serial 10-fold dilutions were prepared in fresh medium lacking ampicillin. Five microliters of the 10⁻² to 10⁻⁶ dilutions was spotted on LB plates supplemented with the indicated amounts of ethidium bromide (Bio-Rad). Ampicillin was omitted from the plates. The plates were incubated overnight at 37 °C. Within one batch of plates, the results were highly reproducible. Between batches, small differences were observed. Growth on the plates was quantified using a Fuji LAS-4000 imager and ImageJ. Data are presented as means with the standard deviation.

Data Analysis. For each mutant, both AP activity and GFP fluorescence were measured, and after AP activity (nAP) had been normalized to GFP fluorescence as described previously,^{26,31} the values were plotted against each other. In the plot, each mutant is represented by a vector, the tangent of which measures the orientation of the protein in the membrane, and the length is a measure of the expression level of the protein. Tangent of 1, 0, and infinity indicate dual topology, a SMR protein that is N_{in}C_{in}, and a SMR protein that is N_{out}C_{out}, respectively. A change in orientation relative to that of the wild-type protein is expressed as the ratio of the tangents of the vectors representing the protein and the wild type (relative orientation).

■ RESULTS

Positive Charge Distribution in Three SMR Proteins. Proteins AAVE4701aave (GI:120613332) from *A. avenae* strain AAC00-1, EMREecol (GI:16128526) from *E. coli* strain K-12, and RRUA0272rrub (GI:83591612) from *R. rubrum* strain ATCC 11170 are members of the SMR family that are encoded by a single gene. The sequence of EMREecol is 55 and 32% identical with those of AAVE4701aave and RRUA0272rrub, respectively, and the latter two share 33% identical residues (Figure 1). The proteins contain three, four, and five positively

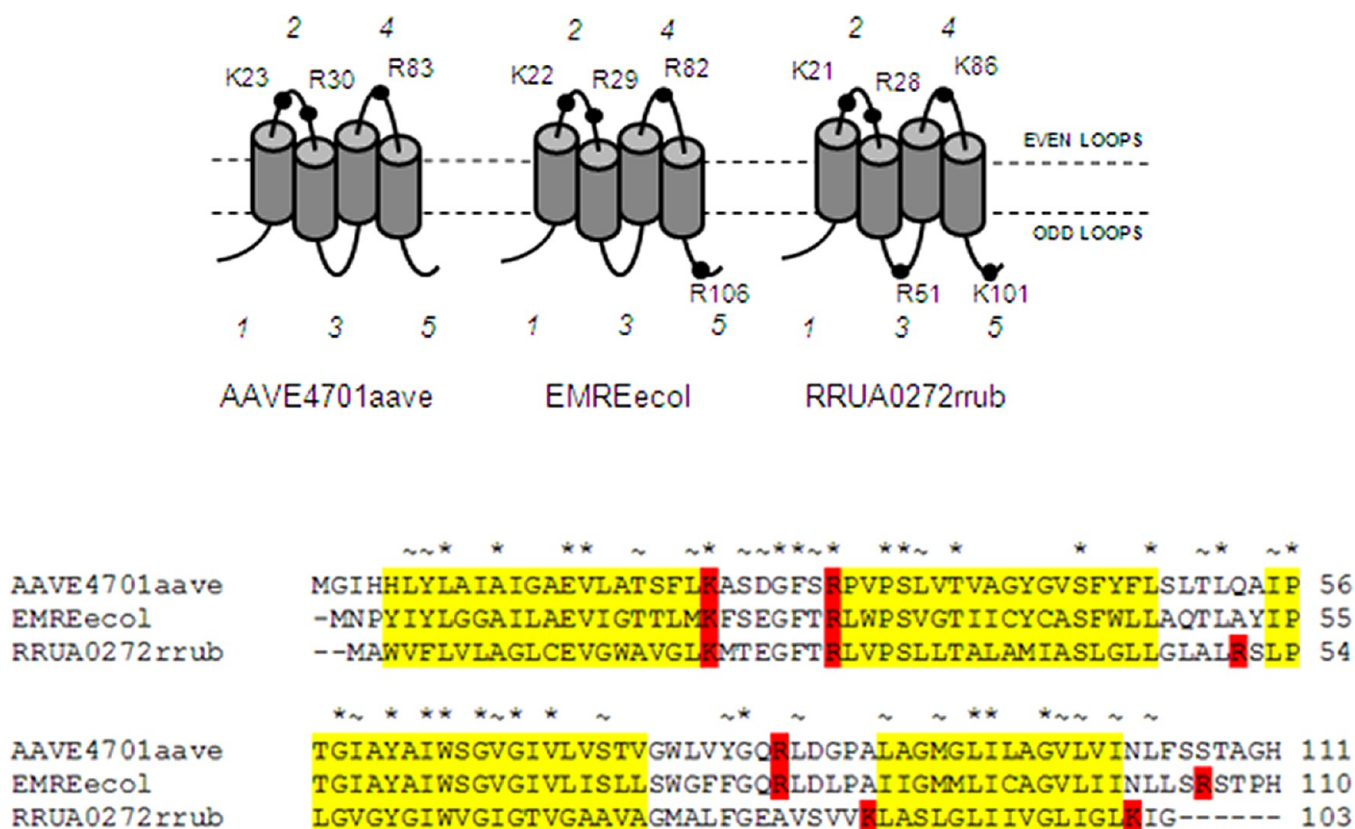


Figure 1. Membrane topology models (top) showing the distribution of positively charged residues in the AAVE4701aave, EMREecol, and RRUA0272rrub proteins. Loop regions are numbered 1–5. For the sake of simplicity, both the N- and C-termini were considered loops as well. Positively charged residues (R and K) are depicted as black dots. Transmembrane segments are depicted as gray cylinders. Multiple-sequence alignment (bottom) of the AAVE4701aave, EMREecol, and RRUA0272rrub proteins. Transmembrane segments are highlighted in yellow and positively charged residues in red. Similar and identical residues are denoted by ~ and *, respectively.

charged residues, respectively, located in hydrophilic loops. In membrane topology models, three of the positive charges are located at one side of the membrane in the even-numbered loops (Figure 1). The lysine and arginine residues in the first even-numbered loop are highly conserved throughout the family and always separated by six other residues. The positive charge in loop 4 is conserved between AAVE4701aave and EMREecol, but at a different position in RRUA0272rrub. Though not conserved, a positive charge is found in this loop throughout the family. At the other side of the membrane, no positive charges are found in AAVE4701aave, a single positive charge is found in the C-terminal loop of EMREecol, and two positive charges are found in RRUA0272rrub (one in the second odd-numbered loop and another in the C-terminal loop). The charge distribution over odd- and even-numbered loops results in an imbalance of +3, +2, and +1 positive charges for AAVE4701aave, EMREecol, and RRUA0272rrub, respectively. In spite of the imbalance, experimental evidence indicated that the proteins insert into the membrane in both orientations, i.e., are dual-topology proteins.²⁶ The role of the individual positive charges in membrane topology was studied by creating mutants in which the positive charges were replaced with neutral residues, alone and in different combinations. The orientation of the mutants in the membrane was determined by fusing the reporters alkaline phosphatase (AP) and green fluorescent protein (GFP) at the C-termini. The alkaline phosphatase activity of the fusion protein reports an extracellular location of the C-terminus ($N_{out}C_{out}$), whereas

the GFP fluorescence reports an intracellular location of the C-terminus ($N_{in}C_{in}$).

Positive Charges in the Odd-Numbered Loops. The arginine residue at position 106 (R106) in the C-terminus of EMREecol of *E. coli* is the only positive charge in the odd-numbered loops of the protein (Figure 1). Fusions of the wild-type protein to AP and GFP resulted in significant activity of both reporters indicative of dual topology (Figure 2A, left). The mutant in which R106 was replaced with alanine (R106A) resulted in slightly lower AP and GFP activities, but there was no significant change in the orientation in the membrane measured as the ratio of AP to GFP activity (see Experimental Procedures; Figure 2, middle panel). The expression level of the R106A mutant was slightly lower than that observed for wild-type EMREecol (Figure 2A, right panel).

RRUA0272rrub has two positively charged residues in the odd-numbered loops, R51 and K101 in loops 3 and 5, respectively (Figure 1). The residues were mutated into alanine one at the time as well as together, yielding mutants R51A, K101A, and R51A/K101A. The AP and GFP activities of the wild-type and mutant proteins (Figure 2B, left panel) showed that the mutations did not result in significant changes in the orientation of the proteins in the membrane (Figure 2B, middle panel). Similarly, the levels of expression of the three mutants were comparable to the level of expression of wild-type RRUA0272rrub (Figure 2B, right panel).

It follows that mutation of the positive charges located in the odd-numbered loops to neutral residues had little effect on the

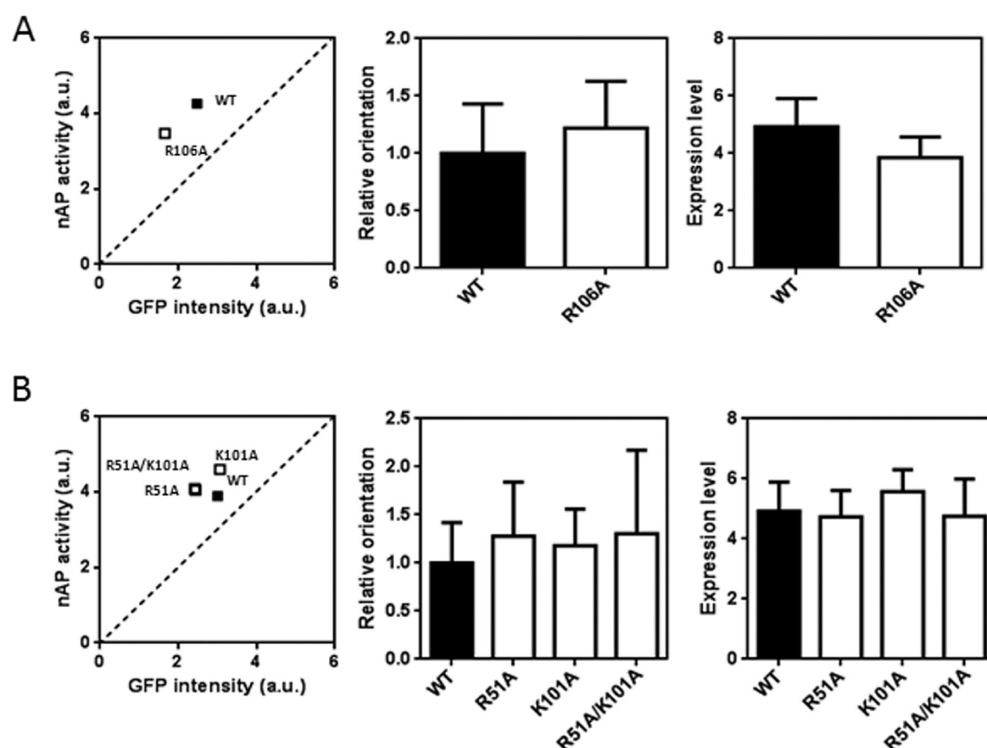


Figure 2. Effect of the positively charged residues located in the odd-numbered loops of EMREecol (A) and RRUA0272rrub (B) on the orientation of the proteins in the membrane (left and middle) and level of expression (right). Data for wild-type (WT) and mutant proteins are indicated with black and white bars, respectively. The relative orientation and expression level are defined in Experimental Procedures.

orientation of EMREecol and RRUA0272rrub in the membrane or on their expression levels. The mutants inserted in both orientations like the wild-type proteins (Figure 2). Moreover, three proteins, wild-type AAVE4701aave, the R106A mutant of EMREecol, and the R51A/K101A double mutant of RRUA0272rrub, share the same positive charge imbalance of +3 but, nevertheless, are dual-topology proteins.

Positive Charges in the Even-Numbered Loops. Using the same strategy described above, the role of the positive charges in the even-numbered loops was evaluated. The positive charges were replaced with neutral residues either alone or in combination, resulting in a total of seven mutants per protein: three single mutants, three double mutants, and one triple mutant (Figure 3 and Table S1 of the Supporting Information). Mutants of AAVE4701aave, in which residues K23 and R30 in loop 2 were substituted with uncharged amino acids (one by one or together), showed by and large the same orientation in the membrane that was observed for the wild-type protein. The largest shift to a more $C_{out}N_{out}$ orientation by a factor of 1.3 was observed for the K23M/R30G double mutant (Figure 3A, left panel). It follows that the two charges in the first even-numbered loop do not significantly affect the orientation in the membrane. In contrast, mutation of residue R83 in loop 4 resulted in a decrease in the relative orientation by a factor of 4, indicating a change in the orientation from dual topology for the wild-type protein to an orientation with the C-terminus in the cytoplasm ($N_{in}C_{in}$) for the mutant. All combinations of mutations containing the R83A mutation (K23M/R83A, R30G/R83A, and K23M/R30G/R83A) showed this shift to the $N_{in}C_{in}$ orientation, indicating that the mutation of R83 to alanine was responsible for the effect in a manner independent of the other mutations. The mutants exhibited levels of expression similar to that observed for the wild-type

protein (Figure 3A, right panel) except for the K23M/R30G/R83A triple mutant, which had an almost 2-fold lower level of expression.

The corresponding mutants involving the two positive charges in loop 2 of EMREecol behaved differently. Mutations K22M and R29G shifted the orientation of the protein significantly to the $N_{in}C_{in}$ orientation, both to the same extent (Figure 2B, left panel). Moreover, a strong cooperative effect was observed rendering the K22M/R29G double mutant essentially completely in the $N_{in}C_{in}$ orientation. Mutation R82S in loop 4 shifted the orientation by a factor of ~ 4 , as observed for the corresponding mutation in the AAVE4701aave protein. In all combinations of mutations, i.e., K22M/R29G, K22M/R82S, R29G/R82S, and K22M/R29G/R82S, the shift toward the $N_{in}C_{in}$ orientation was stronger than that observed for the single mutants. The expression levels of the mutants were similar to that observed for the wild type, except for the K22M/R29G/R82S triple mutant, for which the level of expression was reduced by half, as observed for the corresponding AAVE4701aave mutant (Figure 3B, right panel).

Mutations K21A and R28G in loop 2 of RRUA0272rrub both shifted the orientation toward $N_{in}C_{in}$ as was observed for EMREecol, but the extent of the two mutations was different. While the K21A mutation shifted the orientation by a factor of 1.6, the R28G mutations caused a much stronger shift by a factor of 8.3. Similarly, as was observed for EMREecol, the combination of the two mutations rendered the protein completely in the $N_{in}C_{in}$ orientation (Figure 3C, left panel). The influence of the third charge, K86, in RRUA0272rrub on the orientation again was different from that observed for the other two proteins. In contrast to the EMREecol and AAVE4701aave proteins, the K86S mutation resulted in a moderate shift to the opposite $N_{out}C_{out}$ orientation by a factor

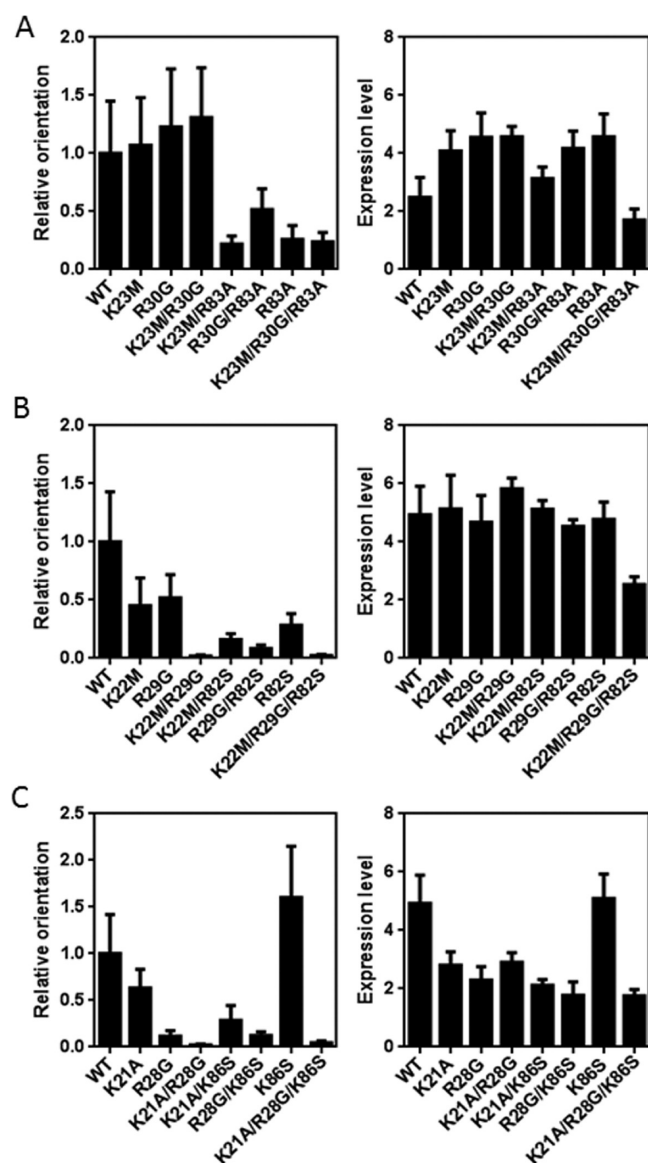


Figure 3. Effect of the positively charged residues located in the even-numbered loops of AAVE4701aave (A), EMREecol (B), and RRUA0272rrub (C) on the orientation of the proteins in the membrane (left) and the level of expression (right).

of 1.6. The orientation of multiple mutants, including K86S and residues K21A and/or R28G, i.e., K21A/K86S, K21A/R28G/K86S, and R28G/K86S, appeared to be determined by the two residues in the first even-numbered loop. The mutations in RRUA0272rrub resulted in a lowering of the expression levels by a factor of approximately 2, except for the single K86S mutation that left the expression level unaltered (Figure 3C, right panel).

The results demonstrate that different positively charged residues in loops on the same side of the membrane affect the orientation of a protein in the membrane differently and that corresponding positively charged residues in different proteins of the same family affect the orientation of the proteins differently as well.

Interaction between Positive Charges in Odd- and Even-Numbered Loops. The mutations in the even-numbered loops of EMREecol and AAVE4701aave showed different effects on the orientation of the two proteins in the

membrane. The difference in the positive charge distribution between the two proteins is the additional R106 residue located in the C-terminus of EMREecol (Figure 1). The role of R106 was evaluated by constructing the same set of seven mutants in the R106A background, which results in the same positive charge distribution as in the corresponding mutants of the AAVE4701aave protein. Note that the R106A mutation by itself did not significantly affect the orientation in the membrane (Figure 2A). The different single and multiple mutations in the even-numbered loops all showed a reduced effect on the orientation in the R106A background relative to the wild-type background (Figure 4). The compensating effect of the R106A

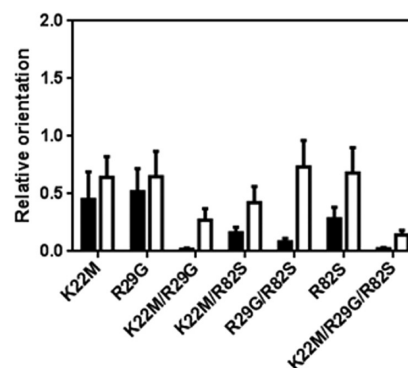


Figure 4. Effect of the positively charged residues located in the even-numbered loops of EMREecol in the wild-type background (black bars) and the R106A mutant background (white bars) on the orientation of the proteins in the membrane.

mutation increased with the extent of the effect of the mutation(s) in the even-numbered loops. Removing R106 from the K22M and R29G mutant did not result in a significantly different distribution of the proteins over the two orientations. In contrast, removing the same positive charge from the mutants that essentially insert into the $N_{in}C_{in}$ orientation (double mutants K22M/R29G and R29G/R82S and the triple mutant K22M/R29G/R82S) shifted the orientation more to the dual-topology type. Comparison of all seven mutants in the R106A background of EMREecol with the corresponding AAVE4701aave mutants showed clearly that the two proteins still behaved differently.

The results indicate that the role of particular positive charges in determining the orientation of the proteins in the membrane may be obscured by other positive charges. Whereas removing residue R106 in EMREecol does not affect significantly the orientation in the wild-type protein, it does so in combination with other mutations, for instance, the R29G/R82S double mutant.

Membrane Orientation in the Absence of Positive Charges. Triple mutants K23M/R30G/R83A of AAVE4701aave and K22M/R29G/R82S in the R106A background of EMREecol do not contain any positively charged amino acid residues. The corresponding R- and K-less version of RRUA0272rrub was constructed by substituting all five arginine and lysine residues with neutral residues. The R- and K-less versions of AAVE4701aave and EMREecol inserted mostly with the $N_{in}C_{in}$ orientation (Figure 5A,B, left panel), with the relative orientations shifted by factors of 4 and 7 compared to that of the wild-type proteins, respectively (Figure 5A,B, middle panel). The result for EMREecol is in agreement with previously published data.¹⁵ The R- and K-less versions of

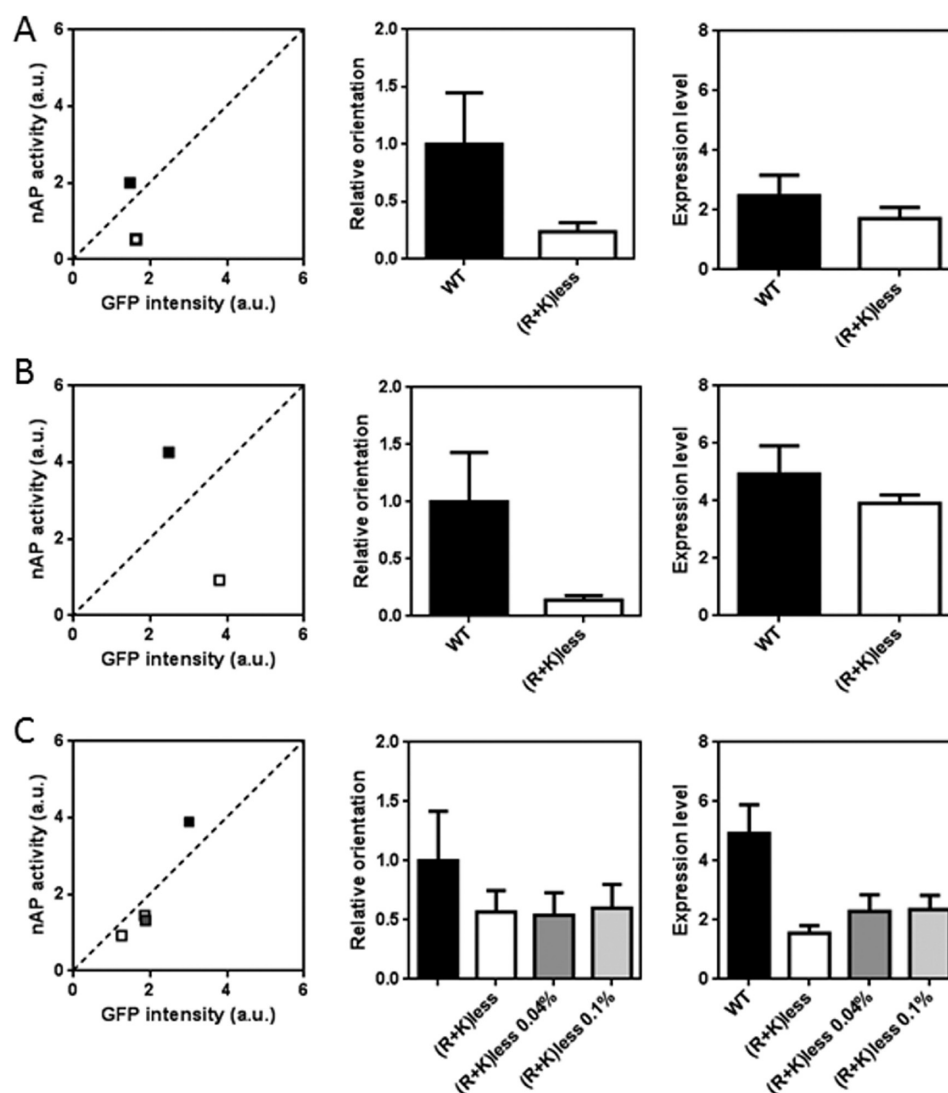


Figure 5. Orientation in the membrane (left and middle) and level of expression (right) of wild-type (black) and R- and K-less (white) versions of AAVE4701aave (A), EMREecol (B), and RRUA0272rrub (C). Panel C shows the results for three different arabinose inducer concentrations as indicated.

the two proteins were expressed at levels slightly lower than that observed for the wild-type proteins (Figure 5A,B, right panel). A similar shift in orientation was not observed for the R- and K-less mutant of RRUA0272rrub. The relative orientation of the mutant shifted by a factor of <2 (Figure 5C, middle panel), and both the mutant and the wild type inserted with dual topology (Figure 5C, left panel). Because the expression level of the R- and K-less variant of RRUA0272rrub was rather low (Figure 5C, right panel), higher concentrations of the inducer arabinose were used to enhance the expression. This resulted in an only small increase in the expression levels for which no change in orientation was observed.

It follows that for the AAVE4701aave and EMREecol proteins, factors other than positive charges in the loops pull the proteins in a single orientation in the membrane.

Activity of Mutants. The ability of the wild-type and mutant proteins to confer drug resistance was assayed by spotting serial dilutions of exponentially growing cells on plates containing the toxic compound ethidium bromide (EtBr). To prevent artifacts, the genes were recloned to a vector expressing the proteins without any tags or reporter proteins. Wild-type

AAVE4701aave and EMREecol grew well on plates containing 200 and 250 $\mu\text{g/mL}$ EtBr, respectively, while control cells containing the empty plasmid did not show growth (Figure 6). Importantly, no difference in growth was observed in the absence of EtBr. Wild-type RRUA0272rrub did not confer resistance to the EMREecol substrates ethidium bromide, methyl viologen (MV), and acriflavine, suggesting that the *R. rubrum* protein has a substrate specificity different from those of the two other proteins. Because the substrate specificity was not known, the protein was excluded from the activity analysis.

The three single mutants of AAVE4701aave, K23M, R30G, and R83A, showed resistance against EtBr that was not significantly different from that observed for the wild-type protein (Figure 6A). Remarkably, the K23M/R30G double mutant did not grow at all, suggesting that the presence of at least one positive charge in the first even-numbered loop is essential for activity. In agreement, the K23M/R30G/R83A triple mutant is inactive as well, while the two double mutants that contain either K23 or R30, i.e., K23M/R83A and R30G/R83A, showed significant activity even though the activity of the former was reduced relative to that of the wild-type protein.

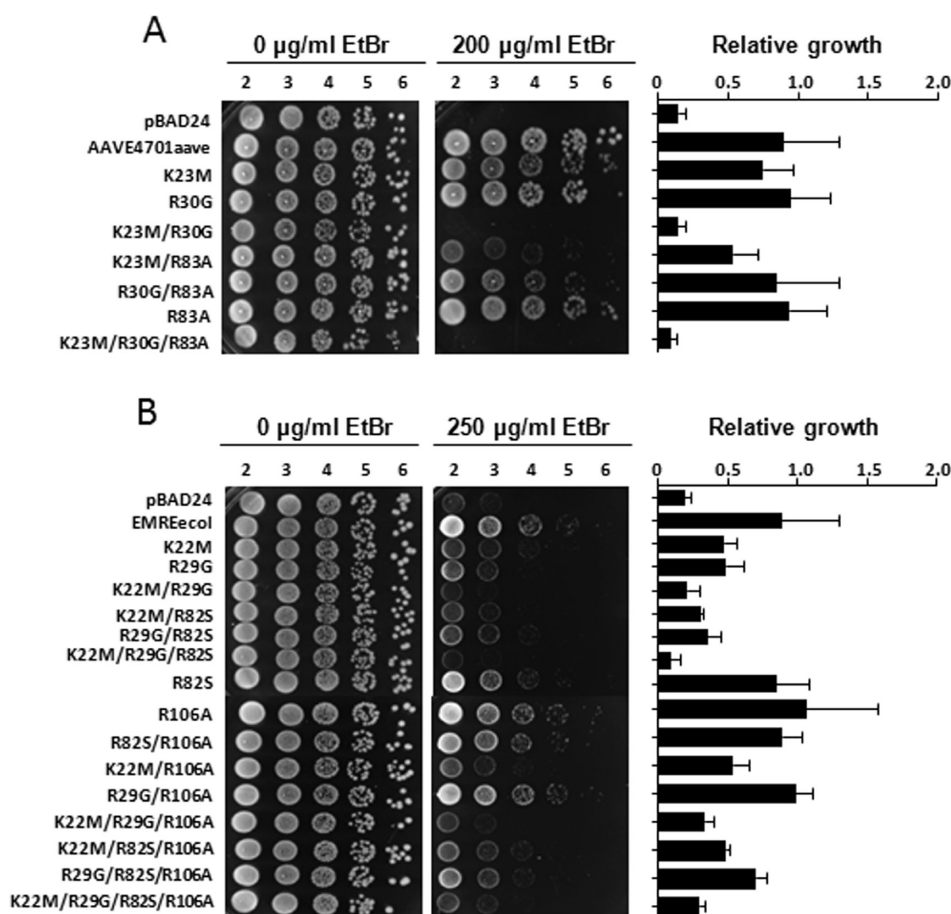


Figure 6. Ethidium bromide resistance conferred by wild-type and mutant AAVE4701aave (A) and EMREecol (B). The activity was measured by the ability of *E. coli* SF100 cells expressing the transporters from the pBAD24 plasmid to grow on the LB plates containing the indicated concentrations of ethidium bromide. A 5 µL volume of 10^{-2} to 10^{-6} dilutions of exponentially growing cultures was spotted on the LB plates and allowed to grow overnight at 37 °C. Different batches of plates were used in the experiments shown in panels A and B, which explains the slightly different resistance of the control cells. Bar plots on the right give the relative growth of the cells in the presence and absence of EtBr measured in the 10^{-2} dilutions.

A similar pattern was observed for the EMREecol mutants (Figure 6B). The single mutants, irrespective of whether the mutated charge was in the even- or odd-numbered loops, were able to confer resistance, allowing them to grow more or less as observed for the wild type. In contrast to the corresponding mutations in AAVE4701aave, the K22M and R29G mutations in the first even-numbered loop reduced the activity to some extent, but combined, EMREecol like AAVE4701 grew as the empty vector control. Cells expressing double and triple mutants, in which at least K22 or R29 was present in the first even-numbered loop, grew in the presence of ethidium bromide, while triple mutants lacking both the charges and the R- and K-less mutant K22M/R29G/R82S/R106A were unable to grow. It follows that the conserved positive charges in the first even-numbered loop of the SMR proteins play a special role in the activity of the proteins (see Discussion).

DISCUSSION

Small multidrug resistance (SMR) transporters are dimeric proteins encoded either by a single gene forming homodimers or by a pair of homologous genes forming heterodimers.^{5,21,33–37} A previous study involving 27 SMR proteins equally distributed over homodimers (singles) and heterodimers (pairs) showed a good correlation between the orientation of the proteins in the membrane and the positive

charge (R and K) bias over the hydrophilic loops at the two sides of the membrane (“positive-inside rule”).²⁶ The two proteins encoded in pairs inserted into the membrane in opposite orientations, corresponding to an average charge bias, calculated as a difference between the number of charged residues in the odd- and even-numbered loops,⁵ of +4.3 and −5.5 for the two orientations. Accordingly, the proteins are believed to form antiparallel heterodimers. The proteins encoded by single genes inserted into the membrane as dual-topology proteins, which correlated well with a small positive charge bias of −1.3 on average. The presence of both orientations in the membrane suggests that the homodimers are antiparallel as well. For two proteins encoded by single genes, AAVE4701aave of *A. avenae* and EMREecol of *E. coli*, the correlation was weak. The proteins inserted in both orientations into the membrane, even though they showed considerable positive charge biases of −3 and −2, respectively, predicting an $N_{out}C_{out}$ orientation. This study provides an explanation for this apparent discrepancy. Mutants of both proteins in which all positive charges were removed from the loops, the R- and K-less variants, inserted largely in the $N_{in}C_{in}$ orientation. The positive charge bias observed in the wild-type proteins shifts the distribution toward the $N_{out}C_{out}$ orientation, making the proteins insert in more or less equal fractions in both orientations, i.e., dual topology. In contrast, the R- and K-

less version of RRUA0272rrub was a dual-topology form, and the lack of a positive charge bias in the wild type preserves the balance between both orientations. An intriguing consequence of this explanation would be that apparently the proteins have a major advantage to insert in both orientations, which strongly supports the antiparallel organization of the two subunits in the active dimer.

The mechanism by which membrane proteins render their orientation in the membrane during insertion is unknown. It is generally believed that in the mature state the two orientations are not in dynamic equilibrium. Consequently, the “decision” for either orientation is made at some point during insertion, after which the orientation of each individual protein molecule is fixed. The final distribution between the two orientations reflects the dynamic equilibrium between the two orientations just before this “point of no return” on the insertion pathway. This study shows that R- and K-less SMR proteins insert either in the $N_{in}C_{in}$ orientation (AAVE4701aave and EMREecol) or in a dual-topology orientation (RRUA0272rrub), indicating that factors other than positive charges determine the orientation equally well. Most likely, the factor involved is the primary structure as the proteins share the same fold and are inserted by the same machinery. There are no obvious differences in terms of physical properties between the proteins studied here that could account for the different behavior of AAVE4701aave and EMREecol on one hand and RRUA0272rrub on the other. Positive charges in the loop regions act on the equilibrium between the two orientations in such a way that addition to an odd-numbered loop or removal from an even-numbered loop shifts the equilibrium from $N_{out}C_{out}$ to $N_{in}C_{in}$ and vice versa. The final distribution is a continuum determined by the R and K bias over the membrane and the other “intrinsic” factors. With an increasing number of positive charges in the loops, the contribution of the intrinsic factors becomes smaller and smaller. Then, the orientation is largely determined by the positive-inside rule, which is the situation for most membrane proteins.

Two major factors appear to govern the strength by which individual positive charges of the three SMR proteins studied here affect the orientation: (i) the presence of other positive charges and (ii) the amino acid sequence context. The effect of the presence of other positive charges is most clear for the positive charges in the odd-numbered loops of EMREecol and RRUA0272rrub. Removal of R106 from wild-type EMREecol showed very little change in the orientation distribution (Figure 2), while removal from the double and triple mutants showed a significant shift toward the $N_{in}C_{in}$ orientation (Figure 4). The strength of the individual positive charge appears to be reduced in the presence of other positive charges, suggesting the resultant effect is the sum of the individual effects. Similarly, removal of K22 in the first even-numbered loop from the wild-type EMREecol changed the orientation by a factor of 2, while removal of K22 from the R29G mutant resulted in a factor of >20 change, essentially rendering the protein in the $N_{in}C_{in}$ orientation (Figure 3). Remarkably, positive charges in the even-numbered loops appear to be less sensitive to the presence of additional charges than those in the odd-numbered loops in the sense that removal from the wild-type background caused a significant shift in the distribution (R83A in AAVE4701aave, R82S in EMREecol, and R28G in RRUA0272rrub). In general, individual positive charges in the even-numbered loops appear to have a stronger effect on the

distribution than those in the odd-numbered loops, which may have mechanistic implications.

The sequence context determined strongly the strength of the individual positive charges in affecting the orientation, which is most likely a manifestation of the role of the intrinsic protein sequence in the membrane orientation mentioned above. The strength of the extremely well conserved K and R residues in the first even-numbered loop was remarkably different among the three proteins studied here. In the AAVE4701aave protein, the two residues, alone or together, did not seem to affect the orientation much. In EMREecol, both residues shift the distribution by a moderate factor of 2 while a strong effect is observed in the double mutant, and in RRUA0272rrub, the R28G mutation by itself shifted the orientation almost completely to $N_{in}C_{in}$. Similarly, the positive charge in the second even-numbered loop had little effect on the distribution between the two orientations in the case of RRUA0272rrub, while a large shift was observed in the case of AAVE4701aave and EMREecol. Apparently, it is not relevant that the position of the positive charge is in the second or fourth loop of the SMR proteins, suggesting that the “decision point” (see above) is a late event on the insertion pathway, a conclusion in agreement with previous studies of *E. coli* EMRE.¹⁵

In general, mutations in proteins may affect the activity by interfering with the catalytic mechanism or by changing the level of expression. In the case of SMR transporters, mutations may also affect the activity by changing the orientation of the protein in the membrane when the active dimer is only formed from the two opposite orientations of the monomeric subunits (antiparallel). Attempts have been made to demonstrate the latter by forcing the proteins in one orientation into the membrane,^{14,15} but the issue has still not been completely settled.^{9,25,36,37} Analysis of the correlation between activity and membrane orientation must be done with great care because the relation is weak by nature. For instance, a significant change in the distribution between the two orientations, say from 70/30 to 30/70, would result in the same concentration of antiparallel dimers. Mutant R83A of AAVE4701aave makes the protein shift from a 50/50 distribution to a 75/25 distribution (Figure 3A) while the activity is only slightly lower (Figure 6A), suggesting, at first glance, that the parallel dimers may be active as well. However, with only the antiparallel dimer active, a decrease in activity to 50% is expected, and because the level of expression is higher by a factor of ~2 (Figure 3A), the result is inconclusive. However, the most important reason why no conclusions can be reached using the present data set is that the relation between activity and the orientation of the subunits in the dimer (parallel vs antiparallel) is obscured by the essential role of the K/R pair in the first even-numbered loop in catalysis. Mutating the two residues of AAVE4701aave (K23M and R30G) alone or together does not significantly change the orientation or, therefore, the active dimer concentration regardless of the orientation in the complex. Activity measurements showed that at least one of the two residues had to be present to confer resistance. Under this condition, in which the reporter proteins do not change the orientation of the proteins, it follows that the presence of either K23 or R30, but not both, is essential for catalysis. Corresponding residues K22 and R29 of EMREecol do shift the distribution between the two orientations to some extent, but the activity measurements suggest a similar role in catalysis for this pair of residues.

■ ASSOCIATED CONTENT

■ Supporting Information

Listed values of GFP fluorescence and normalized alkaline phosphatase (nAP) activity measured for wild-type proteins and point mutants of AAVE4701aave, EMRE_{ecol}, and RRUA0272rrub and relative orientations and expression levels of all the point mutants. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

*Molecular Microbiology, Nijenborgh 7, 9747AG Groningen, The Netherlands. Telephone: 031 50 363 2155. Fax: 031 50 363 2154. E-mail: j.s.lolkema@rug.nl.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

SMR, small multidrug resistance; EtBr, ethidium bromide; MV, methyl viologen; K, lysine; R, arginine; GFP, green fluorescent protein; AP/PhoA, alkaline phosphatase.

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