Membrane topology of ABC-type macrolide antibiotic exporter MacB in *Escherichia coli*

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Abstract MacB is an ABC-type membrane protein that exports only macrolide compounds containing 14- and 15-membered lactones, cooperating with a membrane fusion protein, MacA, and a multifunctional outer membrane channel, TolC. We determined the membrane topology of MacB by means of site-specific competitive chemical modification of single cysteine mutants. As a result, it was revealed that MacB is composed of four transmembrane (TM) segments with a cytoplasmic N-terminal nucleotide binding domain of about 270 amino acid residues and a periplasmic large hydrophilic polypeptide between TM segments 1 and 2 of about 200 amino acid residues.

Key words: ABC transporter; Membrane topology; Site-directed mutagenesis; Cysteine modification

1. Introduction

Although drug resistance mediated by active drug efflux is mainly due to ATP binding cassette (ABC) transporters such as *P*-glycoprotein and MRP [1] in eukaryotes, no drug-exporting ABC transporters are known in bacteria except for LmrA in *Lactococcus lactis* [2] and its homolog [3], while many ABC transporters exist. We previously reported that MacB is a macrolide antibiotic-specific drug exporter in *Escherichia coli*, which was the first ABC-type drug exporter experimentally identified in a Gram-negative bacterium [4]. MacB exports macrolide compounds containing 14- and 15-membered lactones. It requires a membrane fusion protein (MFP) [5], MacA, and a multifunctional outer membrane channel protein, TolC, for its function [4].

MacB is a half-type ABC transporter having only one nucleotide binding domain (NBD). Although in most half-type ABC transporters the NBD domain is at the C-terminus, in MacB it is at the N-terminus, similar to in the ABCG subfamily [6], while MacB shows no sequence homology to any ABCG family proteins. In addition, judging from the results of amino acid sequence analysis, MacB comprises only four transmembrane (TM) segments [4]. The number of predicted TM segments of MacB is minimum in half-type ABC transporters, most of which comprise six TM segments [7].

In this study we try to determine the precise number of TM segments of MacB on the basis of the experimental results of competitive site-directed chemical modification. This method has been proved to be the most reliable one for topology determination of a membrane protein [8–10]. This method is based on the fact that the binding of a membrane-permeable SH reagent, NEM (*N*-ethyl maleimide), to a cysteine residue located on the outside surface of the membrane is prevented by a membrane-impermeable SH reagent, AMS (4-acetoamide-4′-maleimidylstilbene-2,2′-disulfonic acid), while the binding of NEM to a cysteine residue located on the inside surface of the membrane is not affected by AMS when intact cells are incubated with NEM and AMS [9,10].

On the basis of the results of competitive site-directed chemical modification of 33 Cys mutants with NEM and AMS, we determined that the MacB polypeptide chain spans the membrane four times, and that its N-terminal NBD (about 260 amino acids) and the large polypeptide loop (about 200 amino acids) between TM1 and TM2 are actually located on the cytoplasmic and periplasmic sides of the membrane, respectively.

2. Materials and methods

2.1. Bacterial strains and plasmids

E. coli strains W3104 [11], CJ236 [12], and JM109 [13] were used for gene disruption, single-stranded DNA preparation, and plasmid construction, respectively. Plasmids pBAD24 and pBAD33 were described previously [14] and obtained from the National Institute of Genetics (Mishima, Japan). Plasmid pUC118 (Takara Shuzo Co.) was obtained from a commercial source. Plasmid pKO3 was a gift from George M. Church [15]. Plasmids pBAD24 [14] and pBAD33 [14] are arabinose-induced expression vectors that contain the P_{BAD} promoter and *araC* gene. Plasmid pBAD24 contains amp^r and the pBR322 origin of replication. Plasmid pBAD33 contains Cm^r and the pACYC184 origin of replication. Plasmid pKO3 is a gene replacement vector that contains a temperature-sensitive origin of replication, and markers for positive and negative selection for chromosomal integration and excision.

2.2. Materials

N-[Ethyl-1-^{14}C] maleimide (1.5 GBq/mmol) was purchased from Perkin-Elmer Life Sciences. AMS was purchased from Molecular Probes. All other materials were of reagent grade and obtained from commercial sources.

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Abbreviations: ABC, ATP binding cassette; NEM, N-ethyl maleimide; AMS, 4-acetoamide-4′-maleimidylstilbene-2,2′-disulfonic acid; NBD, nucleotide binding domain; TM, transmembrane

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2.3. Gene disruption

Deletions of the chromosomal *macAB* and *acrAB* genes were created by in-frame crossover polymerase chain reaction (PCR) [15], and then the deletions were placed in the *E. coli* chromosome by means of plasmid pKO3. The resulting strain was named *E. coli* W3104ΔacrABΔmacAB. Gene deletions were confirmed by the lengths of the PCR fragments of the chromosomal *acrAB* and *macAB* genes.

2.4. Mutagenesis

First of all, five new silent restriction sites (*Sac*I, *Kpn*I, *Bgl*II, *Xba*I and *Spe*I) and a 3′-terminal poly-histidine tag were introduced into the *macB* gene, and the resulting cassetted *macB* gene was cloned into the *Nco*I–*Sal*I sites of pBAD24. The resulting plasmid, pB2BKN (Fig. 1A), was used for MacB expression. In order to construct Cys-introduced mutants, the *macB* gene was cloned into pUC118 and the resulting pUC8macBKUNKEL was used for site-directed mutagenesis by the method of Kunkel [12] as a template. The mutation site was replaced in pB2BKN by means of restriction enzyme–cassette exchange. Plasmid pB3A (Fig. 1B) carrying the *macA* gene was constructed by cloning of the *macA* gene into pBAD33. *E. coli* W3104ΔacrABΔmacAB was co-transformed by pB3A and pB2BKN or one of its mutational derivatives.

2.5. Drug resistance assays

The resistance of cells harboring the indicated plasmids with *macA* and *macB* constructs under the control of the arabinose promoter was measured by the agar dilution method [16] with the indicated concentrations of arabinose.

2.6. Prevention of NEM binding with AMS

The experiment was performed as previously reported [10]. Without any exceptions, W3104ΔacrABΔmacAB harboring mutant plasmids was grown in minimal medium supplemented with 0.1% casamino acids and 0.4% glycerol. In the middle of the logarithmic phase, *macB* gene expression was induced with 0.2% (w/v) arabinose for 2 h, followed by solubilization with 2% Triton X-100 in place of 1% Triton X-100 and 0.1% sodium dodecyl sulfate (SDS).

3. Results

3.1. Construction and expression of Cys mutants of MacB in acrAB and macAB double-knockout E. coli cells

We first removed the *acrAB* gene from the *E. coli* W3104

Table 1
Erythromycin resistance, MacB expression and NEM binding of *E. coli* cells harboring multicopy plasmids encoding Cys mutant *macB* gene and plasmid pB3A encoding wild-type *macA* gene

Strain	Plasmid 1	Plasmid 2	MIC (μg/ml)/arabinose (%)		MacB expression	NEM binding
			0.2	2		
W3104	No	No	200	200	slight	N.D.[a]
W3104ΔacrAB	No	No	6.25	6.25	slight	N.D.
W3104ΔacrABΔmacAB	No	No	3.13	3.13	mp	N.D.
	pB2ABNH	No	25	50	yes	no
	pB2BKN	No	3.13	3.13	yes	no
	pB3A	No	3.13	3.13	no	N.D.
	pB2 C56A	pB3A	12.5	50	yes	no
	pB2 S13C	pB3A	3.13	3.13	no	N.D.
	pB2 Y14C	pB3A	6.25	25	yes	yes
	pB2S28C	pB3A	12.5	50	yes	yes
	pB2 S62C	pB3A	12.5	50	yes	yes
	pB2 L98C	pB3A	12.5	50	yes	yes
	pB2 L128C	pB3	12.5	50	yes	yes
	pB2 Q151C	pB3A	12.5	50	yes	no
	pB2 G173C	pB3A	6.25	12.5	yes	yes
	pB2 I198C	pB3A	12.5	50	yes	no
	pB2 A207C	pB3A	3.13	3.13	no	N.D.
	pB2 A227C	pB3A	12.5	50	yes	yes
	pB2 S252C	pB3A	12.5	25	yes	yes
	pB2 A261C	pB3A	3.13	3.13	no	N.D.
	pB2 A267C	pB3A	12.5	50	yes	yes
	pB2 S306C	pB3A	6.25	12.5	yes	yes
	pB2 Y327C	pB3A	12.5	50	yes	yes
	pB2 A347C	pB3A	12.5	50	yes	yes
	pB2 T383C	pB3A	25	50	yes	yes
	pB2 L412C	pB3A	25	50	yes	no
	pB2 A417C	pB3A	25	50	yes	yes
	pB2 S442C	pB3A	3.13	3.13	yes	yes
	pB2 F444C	pB3A	3.13	3.13	yes	yes
	pB2 S446C	pB3A	3.13	3.13	no	N.D.
	pB2 S467C	pB3A	12.5	50	yes	yes
	pB2 S494C	pB3A	12.5	50	yes	yes
	pB2 T517C	pB3A	12.5	25	yes	yes
	pB2 T520C	pB3A	12.5	25	yes	no
	pB2 T523C	pB3A	25	50	yes	no
	pB2 T552C	pB3A	12.5	50	yes	yes
	pB2 V561C	pB3A	12.5	50	yes	no
	pB2 S566C	pB3A	12.5	50	yes	yes
	pB2 L602C	pB3A	12.5	50	yes	yes
	pB2 G604C	pB3A	25	100	yes	yes
	pB2 A633C	pB3A	12.5	25	yes	no
	pB2 A637C	pB3A	12.5	50	yes	no
	pB2 D643C	pB3A	12.5	50	yes	yes
	pB2 A646C	pB3A	12.5	50	yes	yes

The resistance levels were depicted as minimum inhibitory concentration (μg/ml) determined by the agar dilution method.
[a]N.D., not done.

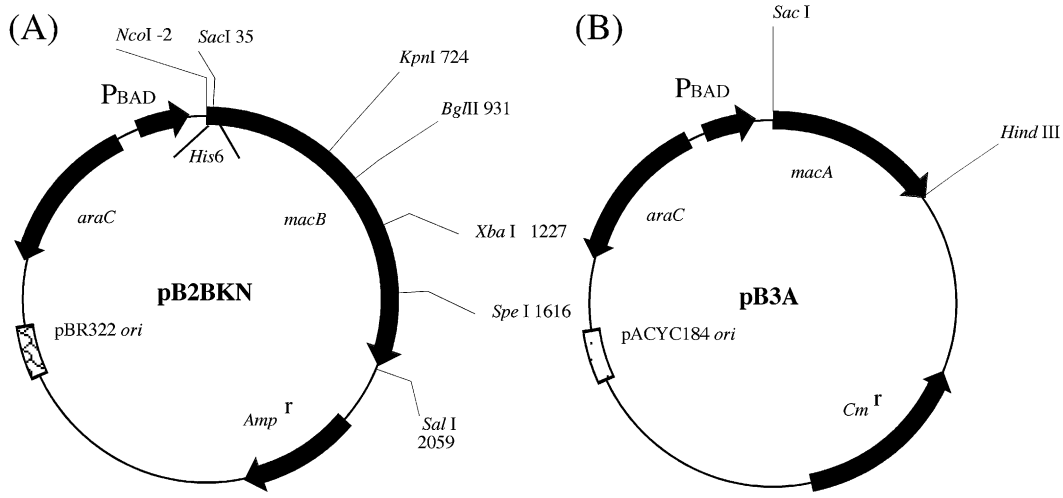

Fig. 1. Plasmid maps of pB2BKN and pB3A. Numbers given with the restriction enzyme sites are positions from the *macB* start codon. Abbreviation: ori, origin of replication.

chromosome as described in Section 2, since constitutively expressed AcrAB masks the macrolide resistance phenotype of MacB [17,18]. Then, the *macAB* gene was removed from *E. coli* W3104ΔacrAB. The resulting double-knockout strain, W3104ΔacrABΔmacAB, was used as the host for the expression of Cys mutant MacB. The minimum inhibitory concentrations of erythromycin for *E. coli* cells are listed in Table 1. *E. coli* W3104ΔacrAB was 32-fold more sensitive to erythromycin than wild-type W3104. W3104ΔacrABΔmacAB was two-fold more sensitive to erythromycin than W3104ΔacrAB. The contribution of the chromosomal *macAB* gene to erythromycin resistance appears to be less than that of the *acrAB* gene, probably because the expression of MacAB from the chromosome under normal conditions is less than that of AcrAB.

MacB has three intrinsic Cys residues, that is, Cys56, Cys580 and Cys620. Among them, Cys56 is located in the first putative loop region, and Cys580 and Cys620 in the putative TM region. First of all, we replaced Cys56 with Ala. The resulting C56A mutant showed no reactivity with NEM (Fig. 2), indicating that Cys580 and Cys620 are embedded in the hydrophobic region of the membrane. Thus, we used the C56A mutant to introduce a new Cys residue by site-directed mutagenesis. We constructed 37 Cys mutants in which a single Cys residue was introduced at 30–40 amino acid intervals. Since MacB requires MFP MacA for its function, *E. coli*

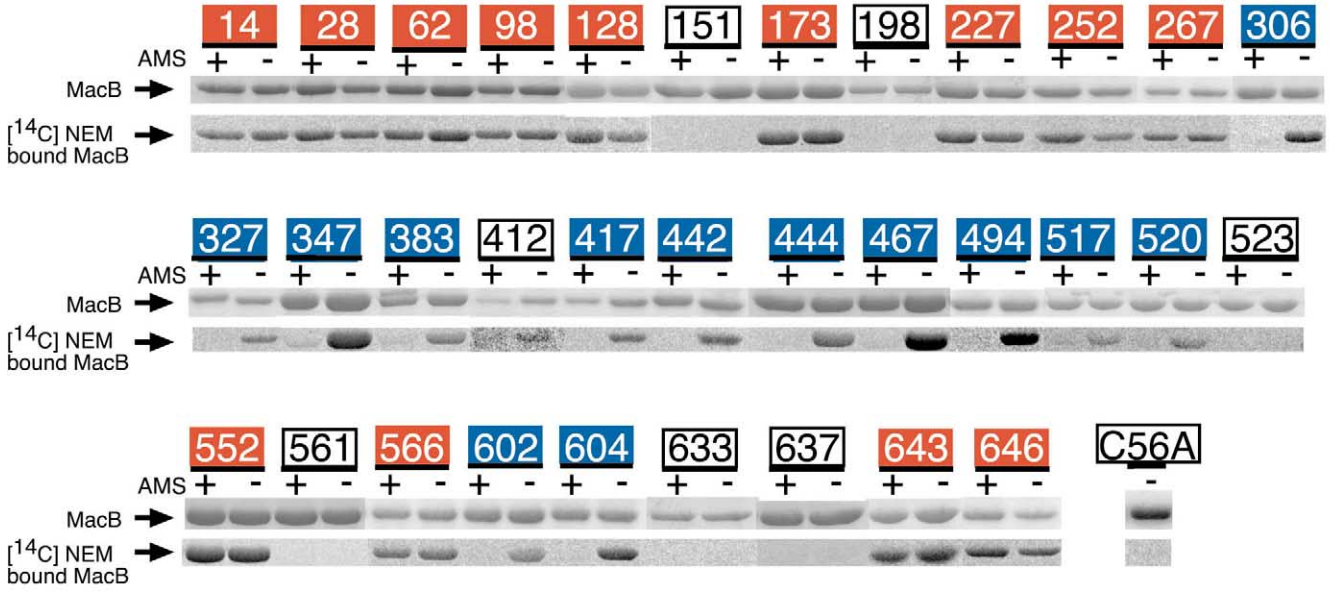

Fig. 2. The expression of single cysteine mutants of MacB and the effect of AMS on [^{14}C]NEM binding to these mutants. *E. coli* cells carrying MacB proteins were incubated in the presence (+) or absence (−) of 5 mM AMS, and then incubated with 0.5 mM NEM containing 62.5 μM [^{14}C]NEM. Cells were disrupted by sonication. After membrane fractions had been collected by ultracentrifugation, the MacB proteins were solubilized with 2% Triton X-100 (v/v) and then purified on Ni-chelating Sepharose (Amersham Pharmacia Biotech.). The purified MacB proteins were subjected to SDS–PAGE. The protein bands (upper panel) and radioactive bands (lower panel) were visualized by Coomassie brilliant blue staining and autoradiography with a BAS-1000 Bioimaging analyzer, respectively.

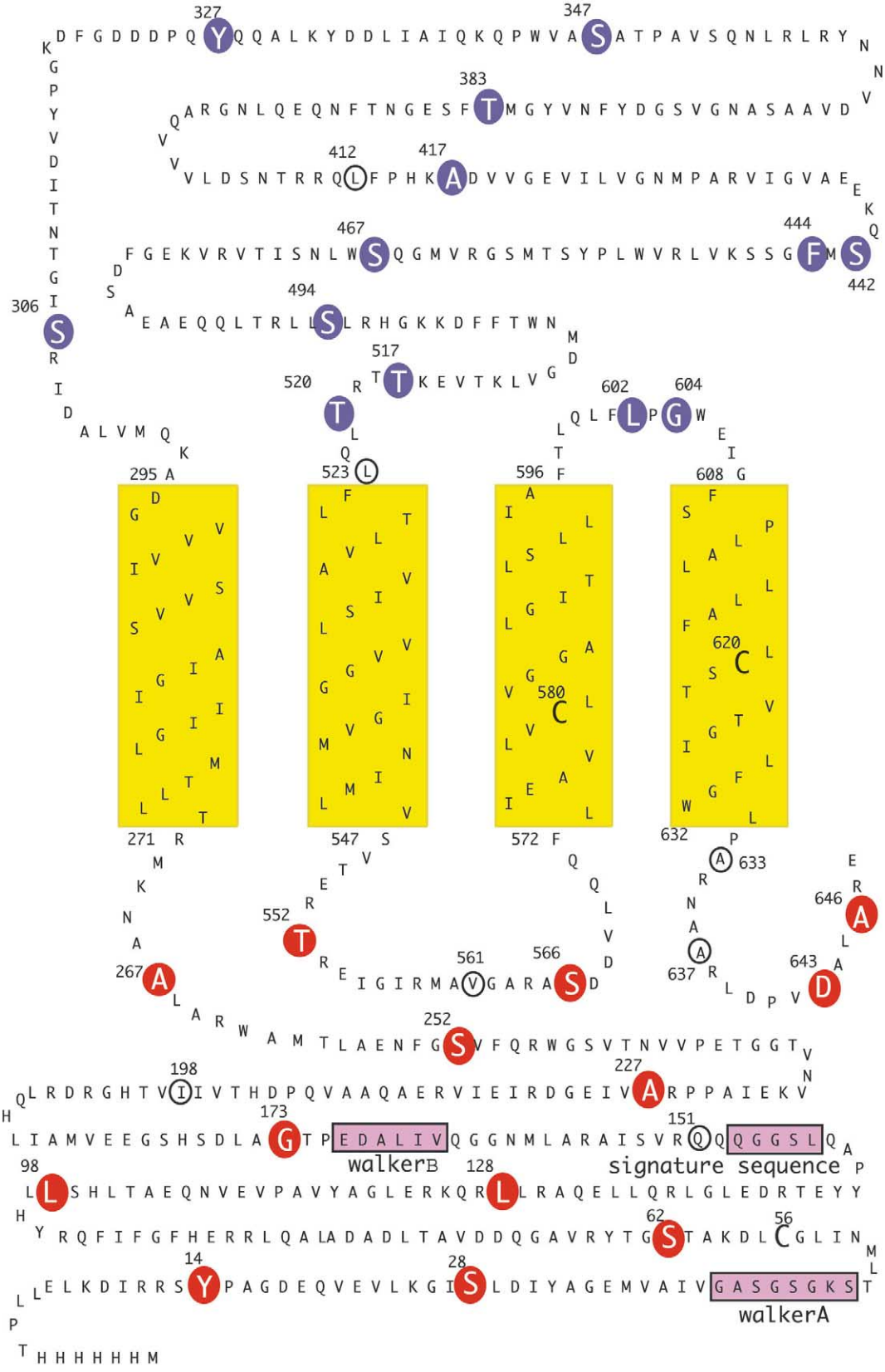

Fig. 3. Membrane topology of MacB determined in this study and the locations of the introduced cysteines. Cys56, which was replaced by Ala, and the other two intrinsic cysteines are depicted as *bold letters*. *Blue ovals*, NEM labeling was completely or largely blocked by AMS; *red ovals*, labeling was not affected by pretreatment with AMS; *open circles*, [^{14}C]NEM was not reacted regardless of pretreatment or not with AMS; *pink squares*, nucleotide binding motifs. *Yellow squares* indicate the most probable membrane-spanning domain determined with SOSUI [27].

W3104ΔacrABΔmacAB was co-transformed with a plasmid carrying a Cys mutant *macB* gene and pB3A that carries a *macA* gene. Both the *macB* and *macA* genes are under the control of the arabinose promoter. Among the 37 Cys mutants, only six (S13C, A207C, A261C, S442C, F444C and S446C) had lost the ability to confer erythromycin resistance to W3104ΔacrABΔmacAB (Table 1).

Four (S13C, A207C, A261C and S446C) of them showed no MacB expression at all, while the other two (S442C and F444C) expressed MacB proteins (Table 1 and Fig. 2). Ser442 and Phe444 might be essential for the MacB function. The 33 MacB-expressing mutants including S442C and F444C were used for topology determination.

3.2. Prevention of [^{14}C]NEM binding to the Cys mutants of MacB by AMS in intact cells

E. coli W3104ΔacrABΔmacAB cells expressing mutant MacB proteins were pre-incubated in the absence or presence of 5 mM AMS, and then treated with 0.5 mM NEM containing 62.5 μM [^{14}C]NEM, as described in Section 2. Seven mutants (Q151C, I198C, L412C, L523C, V561C, A633C and A637C) were not labeled at all by NEM regardless of the presence or absence of AMS (Table 1 and Fig. 2), indicating that these residues might be embedded in the hydrophobic region of the membrane or the interior of the protein. The NEM binding to the 13 mutants (Y14C, S28C, S62C, L98C, L128C, G173C, A227C, S252C, A267C, T552C, S566C, D643C and A646C) was not affected by pretreatment with AMS at all, indicating that these residues are located on the cytoplasmic surface of the membrane. The binding of NEM to the other 13 mutants (S306C, Y327C, S347C, T383C, A417C, S442C, F444C, S467C, S494C, T517C, T520C, L602C and G604C) was significantly inhibited by pretreatment with AMS, indicating that these residues are located on the outside surface of the membrane.

Fig. 3 summarizes the results as to the putative MacB topology. Viewed from the N-terminus, the NEM binding to the eight NEM-reactive Cys mutants (Y14C, S62C, L98C, L128C, G173C, A227C, S252C and A267C) was not affected by AMS and thus these residues are inside. In order to span the membrane once as an α-helical form, a polypeptide chain of about 20 amino acid residues in length is required. The intervals of the introduced Cys residues were planned to be less than 40 amino acid residues in order to exclude the possibility of spanning twice between them. Although the length of the continuous spans between Leu128 and Gly173, and Gly173 and Ala227 was longer than 40 amino acid residues, it is not likely that any residue between the N-terminus to Ala267 is located on the opposite surface of the membrane, since the former span contains the Walker motif and ABC signature sequence, and the latter one contains the essential switching histidine residue (His195 or His202) in NBD. Therefore, the N-terminus containing NBD is concluded to be inside. Between Ala267 and Ser306, the localization changed from inside to outside. There are only 39 residues between them, indicating the presence of one TM α-helix. Between Ser306 and Thr520, all NEM-reactive mutations were localized outside. For the reason stated above, there may be no TM segments between them, and the polypeptide chain between Ser306 and Thr520 must be a large periplasmic loop. Three more TM segments exist between Thr520 and Thr552, Ser566 and Leu602, and Gly604 and Asp643. In conclusion, MacB contains four TM α-helices, one cytoplasmic NBD domain and one large periplasmic loop.

4. Discussion

In this study, we showed that MacB has four TM segments with one N-terminal NBD domain and a periplasmic large loop. With respect to the number of NBD domains, MacB is a half-type ABC transporter like LmrA, which functions as a dimer [19]. However, the number of TM segments is less than that of normal half-type ABC transporters, which in general comprise six TM segments [7]. To the best of our knowledge, the LolC and LolE proteins are amongst the few examples of ABC proteins that have four putative TM segments [20]. However, they are not transporters but proteins mediating lipoprotein release from the cytoplasmic membrane surface in *E. coli* [20].

Drug transporters having the smallest number of TM segments hitherto reported are small multidrug resistance (SMR) family transporters such as EmrE [21,22] in bacteria. They have been predicted to have four TM segments [22]. Thus, MacB has the smallest number of TM segments among solute transporters, similar to SMR family transporters [23]. SMR-type transporters are estimated to each function as a dimer of dimers. Thus, the functional unit of SMR family transporters comprises 16 TM segments.

An important feature of MacB is the cooperation with TolC for drug efflux. Recently, we reported the crystal structure of multidrug efflux transporter AcrB [24] that exports a wide variety of toxic compounds, which is driven by proton motive force, in cooperation with TolC and MFP [10]. According to its crystal structure, AcrB is a trimer. Each monomer has two large periplasmic loops between TM1 and TM2, and TM7 and TM8. Thus, the large periplasmic domain of the AcrB trimer comprises six hydrophilic polypeptide loops, which are presumed to dock with the TolC trimer. If MacB acts as a hexamer, it also has six periplasmic loops and three NBD pairs. This assumption is reasonable for TolC docking and ATP hydrolysis.

MefA and MefE are well-characterized antiporter-type macrolide efflux transporters energized by proton motive force [25,26]. Interestingly, MacB shows a similar substrate specificity to Mef family transporters. Mef family exporters can transport 14- and 15-membered macrolides but not 16-membered ones, similar to MacB. Although the driving force for MacB is different from that for Mef transporters, their substrate recognition site(s) might be similar, while the amino acid sequences of these transporters exhibit almost no similarity. In any case, comparison of the substrate binding sites of MacB, AcrB and Mef family transporters should be a very interesting subject for future study.

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