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Topological analysis of an RND family transporter, MexD of *Pseudomonas aeruginosa*

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Abstract The membrane topology of a resistance-nodulation-division (RND) family transporter, MexD of *Pseudomonas aeruginosa*, was determined. Although it had been predicted previously that most RND proteins contain 12 transmembrane helices, three independent computer programs used in the present study predicted that MexD possessed 11, 14 or 17 transmembrane segments. To investigate the topology of MexD more thoroughly, 25 MexD-PhoA (alkaline phosphatase) and 18 MexD-Bla (β-lactamase) fusion plasmids were constructed and analyzed. The resulting topological model had just 12 transmembrane helices and two periplasmic loops of about 300 residues between helices 1 and 2 and helices 7 and 8. It is therefore proposed that the N- and C-termini are located in the cytoplasm and the predicted orientation is consistent with the 'positive-inside rule'. This topological model can be applied to other RND proteins.

Key words: Multidrug efflux; MexD; Topology; Resistance-nodulation-division family; *Pseudomonas aeruginosa*

1. Introduction

The resistance-nodulation-division (RND) family proteins have been identified in many bacteria involving multidrug resistance [3,10,11,17,18,30], heavy-metal ion export [16,22,33], transport of oligosaccharides [1] and extrusion of hydrophobic solvent [14]. These proteins require the members of two further protein families in order to function: the MFP (membrane fusion protein) family proteins, which are probably localized in the periplasmic space, and the OMF (outer membrane factor) family proteins, which facilitate passage of a variety of substrates into external medium [27]. Members of the RND family are considered to be proton-motive-force-dependent transporters because no ATP-binding cassette has been identified in their primary sequences and because the reduced accumulation of substrates in the cells was restored by addition of inhibitors of the cytoplasmic membrane proton gradient [23–25]. Expression of functional RND transporters in pathogenic bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* is a significant problem because it confers intrinsic and acquired multiple drug resistance to these bacteria. However, there is little information about mechanisms for substrate recognition and transport across the cell envelope. A prerequisite for a full investigation of these mechanisms is the determination of the structure of the RND protein. To this end computer-aided analyses of hydropathy and multiple sequence alignment suggested that most of the RND family proteins form 12 membrane spanning structures and have two large periplasmic loops [10,26,28,31]. Here we report for the first time actual experimental evidence which confirms that the membrane topology of an RND family transporter, MexD from *P. aeruginosa* (an opportunistic pathogen exhibiting innate multidrug resistance), consists of 12 transmembrane segments and two large periplasmic loops.

2. Materials and methods

2.1. Bacterial strains and culture conditions

P. aeruginosa strain PAO1 was used as a source of chromosomal DNA for PCR amplification of the *mexD* gene. *E. coli* strains TG1 [34] and 8 [12] were used as hosts for plasmid construction and for alkaline phosphatase assays, respectively. Bacterial strains were cultured aerobically at 37°C in LB medium [32] supplemented with appropriate antibiotics if needed [8].

2.2. DNA techniques

Preparation of chromosome and plasmid DNA, and related in vitro manipulation, agarose gel electrophoresis, transformation, restriction endonuclease digestion, ligation and PCR were performed according to established procedures [32].

2.3. Construction of mexD-phoA and mexD-bla fusion plasmids

To construct in-frame fusion of *mexD-phoA* and *mexD-bla*, the C-terminally truncated *mexD* gene was amplified by PCR using chromosome DNA extracted from *P. aeruginosa* PAO1 as a template. PCR products containing truncated *mexD*-coding regions of various sizes were blunt-ended with T4 DNA polymerase and phosphorylated with T4 DNA kinase, followed by ligation to a unique *Sma*I site on pPAB307 [4] for *phoA* fusion. These expressed alkaline phosphatase (*phoA*) lacking signal sequences, under regulation by the *tac* promoter. The same PCR fragments were also cloned into blunt-ended *Eco*RI sites on pKMV011 for *bla* fusions. In this study, the construct was prepared by slightly modifying pHSGamp1 [13], so that β-lactamase without signal sequences was expressed dependent on the *tac* promoter and constituting a unique *Eco*RI site between the promoter and the *bla* gene. *E. coli* TG1 transformed with these plasmid constructs were screened by PCR amplification for the presence of the fusion junction using the following primers: #mexD4, 5′-ATGTCC-GAATTCTTCATCAAGC-3′, which anneals to the 5′ terminus downstream of *mexD* gene: and #phoA1, 5′-TTTATCGCTAAGA-GAATCACGC-3′ or #BLA1, 5′-AATAGTGTATGCGGC GACC-G-3′, which anneal to the middle of the *phoA* or *bla* genes, respectively, upstream of each gene. The presence of the fusion junctions of the plasmids employed was confirmed by dideoxy chain termination nucleotide sequencing using the Autocycle Sequencing kit (Pharmacia).

2.4. Immunodetection of MexD-PhoA fusions

To confirm whether strain 8 of *E. coli*, which lacks PhoA [12], produces fused MexD-PhoA protein in-frame after transformation with mexD-phoA fusion plasmids, cell envelopes were prepared after induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C for 2 h as described previously [6]. The envelope preparation was solubilized with SDS-PAGE sample buffer [6] and subjected first to electrophoresis and then to Western immunoblotting for detection of

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the PhoA component. For this purpose, rabbit anti-*E. coli* alkaline phosphatase polyclonal antibody was used (Chemicon International). Membrane-bound antibody was detected as described elsewhere [7].

2.5. PhoA assay and MIC measurement

The activity of PhoA expressed by *E. coli* strain 8 cells transformed with *mexD-phoA* fusion plasmid was determined by the method of Brickman and Beckwith [2]. Minimum inhibitory concentrations (MICs) of ampicillin for *E. coli* TG1 transformed with *mexD-bla* fusion plasmid were measured as described previously [6]. In both cases, expression of fusion genes was induced by supplementation of the culture media with 1 mM IPTG.

3. Results and discussion

3.1. Prediction of transmembrane helices of MexD

To predict the structure of the transmembrane region of MexD, the amino acid sequence of MexD was analyzed using computer programs for the prediction of hydrophobicity and the presence of transmembrane helices. The proposed structure of RND proteins, for example *E. coli* AcrB, *Neisseria gonorrhoeae* MtrD and *P. aeruginosa* MexB, consists of 12 transmembrane segments with two large periplasmic loops [10,25,27]. The MexD amino acid sequence revealed a similar hydropathy profile to MexB [27] (Fig. 1), suggesting that it has a 12 membrane-spanning structure. However, in spite of the consistency of these findings, the computer programs used for predicting transmembrane segments in this study provided inconsistent models (Fig. 1). Therefore, to determine the membrane topology of MexD experimentally, one sense primer at the start of the MexD-coding sequence and 26 antisense primers at various positions as indicated in Fig. 1 were designed and synthesized. These primers enabled the analysis of all probable transmembrane segments predicted in this study

3.2. Construction of mexD-phoA and mexD-bla fusion plasmids

Twenty-six PCR-amplified fragments encoding C-terminally truncated MexD were cloned into plasmids carrying a gene for PhoA lacking a signal sequence in order to construct translational fusions. To test successful construction of these MexD-PhoA fusions, cell envelopes prepared from transformants harboring candidate plasmids were subjected to SDS-PAGE for detection of fusion proteins by Western immunoblotting. Using an antibody specific for PhoA, probable fusion proteins with molecular masses greater than native PhoA protein were detected (Fig. 2). However, one clone which was expected to produce a fusion with the whole length of MexD failed to demonstrate PhoA polypeptide and was therefore excluded from the following analysis. Some of the fusions showed proteolytic degradation products; this instability of fusion proteins has been observed in other studies [21,35]. In addition, protein bands with higher molecular masses than expected were observed; this might result from a low

Table 1
PhoA activities and MICs of ampicillin expressed from fusions

Fusion junction[a] (residue no.)	PhoA activity[b] (units μg^{-1} of protein)	MIC of ampicillin ($\mu g\ ml^{-1}$)
46	84.33 ± 7.28	ND[c]
85	52.01 ± 7.22	25
137	63.65 ± 16.23	50
191	42.33 ± 12.59	12.5
326	47.18 ± 10.15	50
361	7.91 ± 3.53	1.56
366	14.67 ± 2.73	ND
392	55.86 ± 15.53	50
424	5.99 ± 3.30	1.56
437	7.99 ± 2.03	ND
472	58.06 ± 6.50	ND
506	5.79 ± 1.78	ND
520	5.76 ± 0.84	1.56
530	12.58 ± 7.49	ND
579	50.95 ± 25.67	50
614	49.91 ± 2.63	ND
653	56.65 ± 17.59	50
707	37.32 ± 5.46	50
768	50.41 ± 14.08	12.5
840	65.32 ± 12.64	12.5
891	16.01 ± 2.26	1.56
920	53.26 ± 12.66	50
957	6.44 ± 2.82	1.56
968	7.42 ± 3.96	ND
998	60.73 ± 14.25	25
1043	ND	1.56

[a]Fusion junctions are shown by the residue number of the MexD amino acid sequence.
[b]PhoA activities are indicated as means ± S.D. from at least three independent experiments.
[c]ND, not determined.

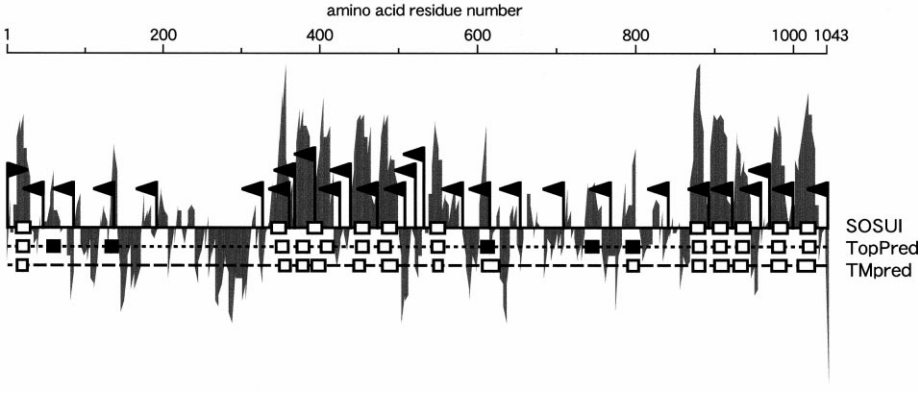

Fig. 1. Hydropathy profile, prediction of transmembrane helices of MexD and positions corresponding to PCR primers synthesized. The Protein Analysis program included in the MacVector software (Oxford Molecular Group) employs the Kyte and Doolittle algorithm [15] and was used to assess the hydropathy (vertical axis) of an 11-amino acid interval as a function of the position in the protein (N- to C-terminus; horizontal axis). Corresponding positions of transmembrane helices proposed by SOSUI (http://www. tuat.ac.jp/–mitaku/adv_SOSUI/), TopPred II [5], and TMpred (http://ulrec3.unil.ch/software/TMPRED_form.html) programs are indicated by boxes. Shaded boxes show five putative transmembrane segments predicted by TopPred II. Arrowheads indicate positions and directions corresponding to synthesized PCR primers.

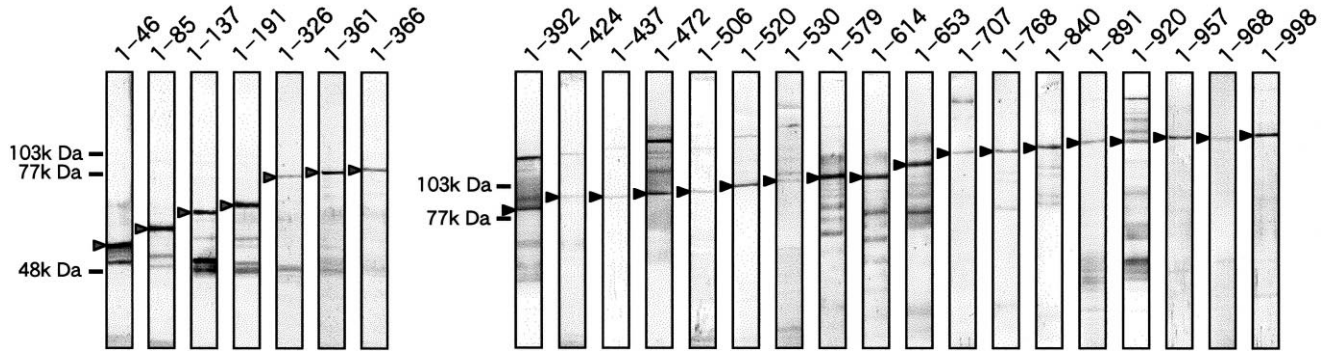

Fig. 2. Western immunoblotting of MexD-PhoA fusions. After induction with 1 mM IPTG, cell envelopes of *E. coli* 8 transformants prepared as described previously [6] were subjected to SDS-PAGE and analyzed by immunodetection using anti-PhoA antibody. Lanes contained 20 μg of total envelopes of strains harboring the *mexD-phoA* fusion plasmids carrying the indicated MexD amino acid residues. Calculated molecular masses of fusions are: 1–46 (52.3 kDa), 1–85 (56.3 kDa), 1–137 (61.9 kDa), 1–191 (67.9 kDa), 1–326 (82.4 kDa), 1–361 (86.5 kDa), 1–366 (87.1 kDa), 1–392 (89.9 kDa), 1–424 (93.4 kDa), 1–437 (94.7 kDa), 1–472 (98.3 kDa), 1–506 (101.9 kDa), 1–520 (103.6 kDa), 1–530 (104.8 kDa), 1–579 (110.2 kDa), 1–614 (114.0 kDa), 1–653 (118.2 kDa), 1–707 (123.8 kDa), 1–768 (130.4 kDa), 1–840 (138.2 kDa), 1–891 (143.7 kDa), 1–920 (146.6 kDa), 1–957 (150.8 kDa), 1–968 (152.0 kDa), and 1–998 (155.0 kDa). The left panel shows fractionations done in 10% polyacrylamide gels and the right panel 8% gels. The positions of molecular mass markers are indicated in kDa. Arrowheads indicate the positions of each fusion protein.

solubility of these fusion proteins, because the samples were subjected to SDS-PAGE without heating in sample buffer [9]. Although different amounts of fusion proteins were detected (as shown in Fig. 2), there was no correlation between the level of PhoA activity and the amount of MexD-PhoA fusion protein (data not shown). On the other hand, 18 of 26 PCR fragments used above were also cloned into plasmids carrying a gene for β-lactamase lacking a signal sequence. Thus, 25 MexD-PhoA fusions were successfully constructed and an additional 18 *mexD-bla* clones were prepared to analyze the membrane topology of MexD.

3.3. PhoA activity and ampicillin susceptibility of fusions in intact cells

The alkaline phosphatase activity of the intact *E. coli* cells producing MexD-PhoA fusions was measured. These assays revealed various levels of activity which could be classified roughly into two groups of relatively high ($\geq 37.32 \pm 5.46$ units, fusion at residue 707) and low ($\leq 16.01 \pm 2.26$, at residue 891) values (Table 1) consisting of 15 and 10 clones, respectively. Moreover, these two groups were clustered into six and five consecutive regions. Since alkaline phosphatase functions in the periplasmic space, but not in the cytoplasm [19], the fusion sites of MexD-PhoA belonging to the high-activity group were presumably located in the periplasm, and in contrast, those of the low-activity group were expected to be distributed in the cytoplasm. Taken together, the results from analyses of the PhoA-fusion constructs suggest that MexD has six periplasmic and five cytoplasmic regions; in other words, it must contain at least 10 membrane-spanning moieties.

To confirm this hypothesis, 18 clones harboring *mexD-bla* fusion plasmids were analyzed. On the basis of the MICs of ampicillin against *mexD-bla*-carrying strains, these clones were divided into two groups: (1) sensitive (MIC: 1.56 μg ml^{-1}) and (2) resistant (MIC: over 12.5 μg ml^{-1}), consisting of 6 and 12 fusions, respectively (Table 1). Because β-lactamase also has hydrolytic activity when translocated into the periplasm, it is suggested that the resistant strains produce MexD-Bla fusion proteins in which the β-lactamase moiety is located in the periplasmic space. Although it was not directly determined whether all 18 clones successfully produce MexD-Bla fusion proteins, at least 12 clones revealing ampicillin resistance were very likely to have produced the fusion proteins. This result was completely consistent with that from PhoA fusion experiments and strongly supported the prediction that MexD has at least 10 membrane-spanning segments.

3.4. The MexD topological model

Although the PhoA- or Bla-fusion experiments resulted in the conclusion that MexD has at least 10 transmembrane helices, distribution of the N- and C-termini could not be identified. However, all three computer programs used in this study proposed the existence of transmembrane segments between the N-terminus and the first periplasmic loop, and between the sixth periplasmic loop and the C-terminus. Therefore, it was suggested that MexD has 12 transmembrane helices with N- and C-termini located in the cytoplasm. Fig. 3 shows the MexD membrane topology model determined in this study. This model is based on the 'certain' transmembrane segments predicted by TopPred II for the following reasons. First, five 'putative' transmembrane segments predicted by TopPred II (amino acid residues 49–69, 124–144, 600–620, 732–752, and 785–805) and two transmembrane regions by TMpred (residues 603–629 and 786–805) (see Fig. 1) could not be considered membrane-spanning domains because no change in the distribution of PhoA and Bla moieties was observed between the N- and C-sides of these regions (Table 1). Second, the third transmembrane domain predicted by SOSUI (residues 382–404) could not be considered a membrane-spanning domain because PhoA fusion at residue 392 in this region exhibited apparently high activity. Third, the existence of a transmembrane domain between residues 366 and 392, and between 392 and 424 is suggested because PhoA activities at residue 366 and 424 are low, in contrast to the high activity of residue 392 (see Fig. 1 and Table 1). Taken together, this model suggest that the 1043 residues of MexD are distributed to three subcellular compartments: 130 (12.5%) residues in the cytoplasm, 666 (63.9%) residues in the periplasm and 247 (23.7%) residues in the membrane. Such a structure behaves in accordance with the positive-inside rule [36] as follows. The cytoplasmic domain consisting of

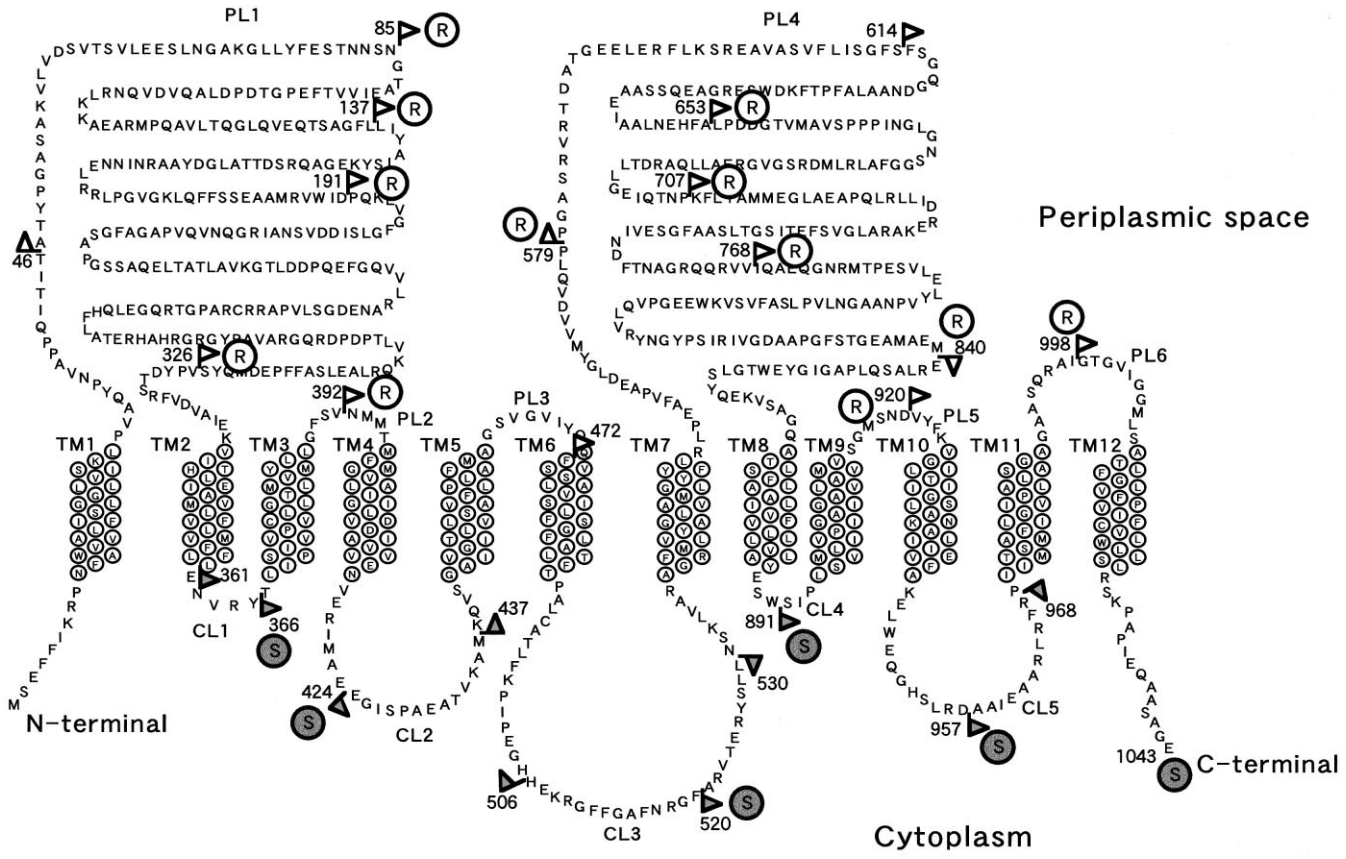

Fig. 3. Proposed model of the membrane topology of the MexD protein. Twelve transmembrane helices (TM1–TM12) are connected by six periplasmic loops (PL1–PL6) and five cytoplasmic loops (CL1–CL5). PhoA fusion junctions are labeled by white (high PhoA activity) and black (low PhoA activity) flags with amino acid residue number. MexD-Bla fusion positions are indicated by open and closed circles denoting R (MIC of ampicillin is more than 6.25 μg ml^{-1}) and S (MIC is 1.56 μg ml^{-1}), respectively. Transmembrane segments consist of amino acid residues marked with circles.

five loops (CL 1–5, Fig. 3) with N- and C-termini is constructed from 130 residues and contains 24 (18.5%) positively charged residues (R, H and K) in contrast to the six periplasmic loops (666 residues) and 12 transmembrane segments (247 residues) which have only 61 (9.2%) and four (1.6%) basic amino acids, respectively. A typical predicted structure for MexD implies the existence of two large periplasmic loops (PL1 and PL4, Fig. 3) each consisting of about 300 amino acid residues. Such a set of very large loops is likely to be a typical structure for RND family transporters but not for other proton-motive-force-dependent efflux proteins and ABC transporters [26]. MexD has a high degree of homology (38–49%) with other RND family proteins including multidrug efflux proteins, AmrB of *Burkholderia pseudomallei* [20], MexB of *P. aeruginosa* [28,29], AcrB of *E. coli* [18], MtrD of *N. gonorrhoeae* [11], CeoB of *Burkholderia cepacia* [3] and organic solvent resistance protein, SrpB of *Pseudomonas putida* [14]. The predicted sequence alignments and membrane-spanning helices indicated that these proteins have a very similar topological organization (data not shown), suggesting that the topological model proposed for *P. aeruginosa* MexD can be used to predict the topologies of the other RND family members.

The results of this study cannot distinguish between transmembrane segments and hydrophilic loops at the levels of one single amino acid residue. However, at least MexD, one of the RND family transporters, has a 12 membrane-spanning structure with two large periplasmic loops. There are no data on the function(s) of these typical domains, which may be needed for binding to other co-functional protein(s) such as MFP family members. More detailed mutational analysis taking into account the experimentally determined membrane topology of MexD in this study will enhance our understanding of the functional mechanisms utilized by members of the RND family of broad substrate-recognizing transporters.

During the preparation of this article, a paper by Guan et al. [9] appeared, in which it was demonstrated experimentally that MexB, a homologue of MexD, possesses 12 transmembrane domains. The results from this independent study accord with our own results presented here.

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