Membrane Topology of the DrrB Protein of the Doxorubicin Transporter of Streptomyces peucetius*

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Daunorubicin and doxorubicin, two commonly used anticancer agents, are produced by the soil bacterium Streptomyces peucetius. Self-resistance to these antibiotics in S. peucetius is conferred by the drrAB locus that codes for two proteins, DrrA and DrrB. DrrA is an ATP-binding protein. It belongs to the ABC family of transporters and shares sequence and functional similarities with P-glycoprotein of cancer cells. DrrB is an integral membrane protein that might function as a transporter for the efflux of daunorubicin and doxorubicin. Together, DrrA and DrrB are believed to form an ATP-driven pump for the efflux of these drugs. The drrAB locus has been cloned, and the two proteins have been expressed in a functional form in Escherichia coli. A topological analysis of the DrrB protein was performed using gene fusion methodology. Random and site-directed fusions of the drrB gene to lacZ, phoA, or gfp reporter genes were created. Based on the fusion data, a topological model of the DrrB protein is proposed in which the protein has eight membrane-spanning domains with both the N terminus and the C terminus in the cytoplasm.

Daunorubicin and doxorubicin, two antibiotics produced by the soil bacterium Streptomyces peucetius, are used in chemotherapy for treatment of various cancers. Self-resistance in S. peucetius to its own antibiotics results from the action of two proteins, DrrA and DrrB. The DrrA protein belongs to the ATP-binding cassette (ABC) family of proteins (1), whereas DrrB bears homology to the membrane components of some bacterial ABC transporters (2). Together, DrrA and DrrB are proposed to form an ATP-dependent efflux pump for the export of these antibiotics (3–5). Based on the nucleotide sequence analysis, it has been proposed that DrrB belongs to a novel subfamily of ABC transporters (2). This subfamily has been termed ABC-2, and it includes four other members, namely NodJ, KpsM, BexB, and CtrC. The membrane components of members of this subfamily (ABC-2) show significant sequence homology to each other but not to other ABC transporters (2). Within this subfamily, DrrB and NodJ are more closely related to each other than to the other three members. Although DrrAB is a drug export system, the other four transporters form the polysaccharide export systems in Gram-negative bacteria (6–10). DrrA, the ABC component, shows homology to the ATP binding components of these four systems, however, DrrA also shows homology to other members of the ABC family, including the P-glycoproteins (Pgps) from mammalian tumor cells (2, 3). Therefore, it has been proposed that DrrAB and the other four systems, mentioned above, comprise a novel subfamily of permeases that utilize an ABC-type ATPase to provide energy for export.

It is well known that the prolonged use of chemotherapeutic drugs, including daunorubicin and doxorubicin, results in multidrug resistance in cancer cells due to the overexpression of Pgp in their cell membranes (11). Pgp, like DrrA, is a member of the ABC family of transporters (1) and is known to bring about efflux of the anticancer drugs in an ATP-dependent manner. It is a large protein, which consists of two similar halves, each half containing one ABC domain and one membrane domain made of six alpha helices. Because DrrAB and Pgp show sequence homology and carry out a similar function, DrrAB in the producer organism and Pgp in the target cells, the bacterial DrrAB system may serve as a good model system to understand the mechanism and evolution of multidrug resistance in cancer cells. Furthermore, elucidation of the structure and function of DrrAB is expected to help in understanding the function of members of the ABC-2 subfamily and elucidate their evolutionary relationships to other ABC transporters.

The drrAB locus has previously been subcloned in an Escherichia coli expression vector, and the proteins have been identified (4). The expression of the two genes was also shown to confer doxorubicin resistance in a sensitive strain of E. coli (4). DrrA, a peripheral membrane protein, contains one consensus ATP-binding domain. It binds ATP in a doxorubicin-dependent manner. DrrB, an integral membrane protein, is believed to form a channel for the export of daunorubicin and doxorubicin (4). Previous studies have shown that the complex of DrrA and DrrB may have a stoichiometry of two subunits of DrrA and two of DrrB (5). Thus the complex of DrrA/DrrB should be structurally similar to a molecule of Pgp as well as to most other ABC transporters that are known to consist of four domains, two ATP-binding and two integral membrane domains, assembled from homo- or heterodimers of each type of domain (2, 12).

Even though the catalytic and membrane domains in DrrAB are present on separate subunits, there is evidence for strong interaction between them so that their stability and function are dependent on each other (5). It has previously been shown that DrrB protein is not maintained in the cells in the absence of DrrA (5). Moreover, the ATP-binding ability of DrrA is dependent on the presence of DrrB in the membrane (5). Because the two domains, present on separate molecules, are tightly coupled, this property of the DrrAB system offers an advantage in employing this system to understand interaction between the catalytic and the membrane domains, which will also help...
in elucidating the interaction of domains within Pgp and shed light on its mechanism.

A basic requirement for understanding interaction between DrrA and DrrB is that we first understand how DrrB is oriented in the membrane, which is the topic of the present study. Gene fusions have been successfully used to determine topology of membrane proteins in E. coli (13–15). In this study, gene fusions of drrB to the promoterless lacZ or phoA reporter genes were created. Because the phoA gene product alkaline phosphatase (AP) is active only in the periplasm and the lacZ gene product β-galactosidase is active only in the cytoplasm, active fusions can be localized to the periplasm or cytoplasm of these cells. A third type of fusion with gfp (green fluorescent protein) was also constructed, especially at regions of anomaly where results with lacZ or phoA were either unclear or contradictory. Based on the prediction tools, such as Kyte-Doolittle and Top-pred, DrrB is predicted to contain at least six hydrophobic regions. The information obtained from the complementary gene fusions and the computer-generated hydropathy plots was used in the present work to determine the approximate locations of periplasmic, transmembrane, and cytoplasmic regions, and a model of DrrB in the membrane was developed. We propose that DrrB consists of eight transmembrane domains with both the N and the C termini of the protein in the cytoplasm.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids**—Strains, plasmids, antisera, and phages used in this study are listed in Table I.

| Strains | Genotype or description | Reference or source |
|---------|-------------------------|---------------------|
| CC118   | F′ araD139 Δ(ara-leu76897) lacX74 Δ(nalR)AD2 galK16 thi rpsE rpoB argB1(Am) recA1 | 13 |
| TG 1    | K12 Δ(lec-pro), supE, thi, hsdS2 F′traD36, proAB lacY, lacZD15 | 16 |

**Plasmids**
- pDx101: drrAB-gfp
- pMLB1069: pBR322 Δ(tet) lacZY
- pMLB1070: pBR322 Δ(temphoA)
- pGFPuv: gfp in pUC19 derivative
- pGFpUVAB: drrAB in pGFP–
- pDxGFP: drrAB-gfp in pSU2718
- pDXP series: Random phoA fusions in pDX101
- pDXP66: Random phoA fusion in pDX101 at the 66th amino acid in DrrB
- pDXL series: Random lacZ fusions in pDX101
- pMPL series: phoA fusions in pMLB1070
- pMll series: lacZ fusions in pMLB1069
- pDxG series: GFP fusions at different positions in DrrB in pDxGFP

**Phages**
- λp2oA
- λTnAlacZ

**Antisera**
- Anti-βGal: Antibody to β-galactosidase
- Anti-PhoA: Antibody to alkaline phosphatase
- Anti-GFP: Antibody to green fluorescent protein

**Construction of the PhoA Reporter Plasmid**—A phoA reporter vector was constructed by substituting the lacZ gene in pMLB1069 with the phoA gene. pMLB1069 (Table I) was digested with HindIII and CellIII to remove the 3.25-kb lacZ fragment. PhoA gene was cloned into the remaining 3.25-kb pBR322 fragment as described below. PhoA gene was PCR-amplified using pDxP66 (Table I) as the template. The upstream and downstream primers were HindIII and CelI site, respectively, had the following sequence: upstream primer, 5′-CCCAAGCTTCCTCTCTTATTTATAACAAC-3′; downstream primer, 5′-CCCGTCACTGATTTCATACGTGACAGCGCCGC-3′. The 1.4-kb amplified PCR product was digested with HindIII and CellIII and ligated to the 3.25-kb pBR322 fragment from above to generate 4.7-kb plasmid containing the promoterless phoA gene.

**Construction of the Gfp Reporter Plasmid**—The drrAB genes were PCR-amplified using pDx101 as a template and cloned in front of the gfp gene in pGFpUV (pUC19-based plasmid, Table I) using the XbaI and KpnI restriction sites. This generated a clone called pGFPuvAB. The upstream and downstream primers for amplification of drrA and drrB contained the XbaI (5′-CTAGTCTAGACATATGAACACGCAGCCGC-3′) and the KpnI (5′-GGGGGTACCAAGTGGGCGTTCTTGTTGC-3′) sites, respectively. The drrAB-gfp fragment was then amplified using pGFpUVAB as a template and employing the same upstream primer as used above and a downstream primer containing a HindIII site as follows: 5′-CCCCAACGCTC-TCTCTCTTATATATAACAAC-3′. The 1.2-kb amplified PCR fragment was digested with HindIII and KpnI and ligated to the 3.25-kb pBR322 fragment from above to generate 4.7-kb plasmid containing the promoterless phoA gene.

**Construction of the Site-directed Gene Fusions**—Different lengths of the drrB gene were inserted into the plasmids pMLB1069 or pMLB1070 to create fusions between drrB and lacZ or phoA, respectively. Plasmid pDx101 was used as a template to PCR-amplify drrA along with different portions of drrB. The amplified DNA fragment was digested with XcoII and HindIII. The resulting drrB-gfp fragment was inserted in place of the drrB gene in pDx101 (pSU2718 clone, Table I). The resulting plasmid, pDxGFP, contains wild type drrA and an in-frame fusion of gfp at the 3′-end of the drrB gene.

**Construction of the Site-directed Gene Fusions**—Different lengths of the drrB gene were inserted into the plasmids pMLB1069 or pMLB1070 to create fusions between drrB and lacZ or phoA, respectively. Plasmid pDx101 was used as a template to PCR-amplify drrA along with different portions of drrB. Each amplification employed the same upstream primer but a different downstream primer. Both primers contained a sequence for HindIII restriction site. The upstream primer containing the sequence 5′-GGGGGTACCAAGTGGGCGTTCTTGTTGC-3′, corresponded to a region upstream of the plac region of the plasmid pSU2718 so that the amplified fragment contains the lac promoter and the operator region. The downstream primers are shown in Table II. The purified PCR products were digested with HindIII and inserted into the HindIII site of the reporter vector. A total of twelve site-directed lacZ fusions and seven phoA fusions were made. The subsequent fusion proteins are referred to as “L” or “P” (Table II). The
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number next to the notation indicates the last residue in DrrB to which the fusion with β-galactosidase or AP was made.
gfp fusions at different locations in the drrB gene were made by PCR-amplifying different lengths of the drrB gene from pDxGFP using the same upstream primer but a different downstream primer for each amplification. The upstream primer, containing the sequence 5′-TGACATGACGCTCCCG-3′, corresponds to a region bearing an AatII site at the 5′-end of the drrB gene. The downstream primers corresponded to different regions in drrB and each incorporated a KpnI site (Table II). The amplified products were digested with AatII and KpnI. Fragments containing different lengths of the drrB gene were inserted into the same sites in pDxGFP to create in-frame fusions of gfp to different lengths of the drrB gene. This procedure replaced the wild type drrB gene in pDxGFP with a smaller version of drrB in each case. A total of six different GFP fusions (to residues 84, 117, 232, 236, 245, and 283) in DrrB were created. The subsequent fusion proteins are referred to as “G.”

Measurement of β-Galactosidase or AP Activity—The activity of the fusion proteins was determined by the protocols published earlier (17, 18). Briefly, CC118 cells containing the fusion plasmid were grown to mid-log phase and induced with 0.01 mM IPTG for 3 h. The cells were harvested and lysed by a single passage through a French pressure cell at 20,000 p.s.i. The lysate was then centrifuged at 10,000 × g for 15 min to remove unbroken cells. 100 μg of lysate was suspended in buffer A and the volume was made up to 3 ml. Fluorometric measurements of triplicate samples were recorded using the Photon Technology International Fluorometer at an excitation wavelength of 485 nm. The emission peaks were recorded at 508 nm. Readings were taken at varying slit widths ranging from 1.0 to 2.5 mm.

Western Blot Analysis of the Fusion Proteins—Cell lysates containing fusion proteins were prepared as described above for the measurement of activity. Indicated amounts of the protein were analyzed on 8.5% β-galactosidase fusions), 10% (AP fusions), or 12% (GFP fusions) polyacrylamide-SDS gels. Western blot analysis was then carried out using either the anti-AP (5′-3′ Inc.), anti-β-galactosidase (5′-3′ Inc.), or anti-GFP (Abscam, Inc.) antiserum. The intensity of the bands was determined by densitometric analysis using GS-710 Calibrated Imaging Densitometer (Bio-Rad). Anti-AP and anti-β-galactosidase antibodies were used at a dilution of 1:1,500, whereas anti-GFP antibody was used at a dilution of 1:5,000. Detection was done with a chemiluminescence kit from Bio-Rad.

RESULTS

Random Gene Fusions

To determine the topology of the DrrB protein in the membrane, random gene fusions of drrB with the reporter gene lacZ or phoA were generated. ΔTnlacZ or ΔTnphoA were used to create transposon-mediated random gene fusions in the clone pDx101 (Table I), which contains both drrA and drrB genes in cis. The two genes in pDx101 are also translationally coupled, exactly as they occur in the wild type situation. Translational coupling of DrrA and DrrB has previously been shown to enhance translation of DrrB about 2- to 3-fold (5). Furthermore, DrrA has been shown to be required for the stability of DrrB (5). Thus, in the present study, we used plasmid pDx101 to provide optimum conditions for expression of DrrB fusion proteins.

The lacZ gene product, β-galactosidase, is active in the cytoplasm, thus β-galactosidase fusions were used to identify cytoplasmic domains of DrrB. Complementary gene fusions with phoA result in the active gene product, AP, only if these genes are located in the periplasmic loops of a membrane protein, thus allowing us to determine periplasmic domains of DrrB. Fusions using ΔTnlacZ or ΔTnphoA were isolated by color screen on the plates. Blue colonies on medium containing X-gal (for lacZ) or 5-bromo-4-chloro-3-indolyl phosphate (for phoA) indicated active fusions and were selected for further analysis. The fusion junctions were determined by nucleotide sequence analysis and are shown in Table III.

This method generated three active β-galactosidase fusions in DrrB at acidic amino acid positions 37, 107, and 126. Quantitation of the fusion proteins was done by Western blot analysis using anti-β-galactosidase antiserum, followed by a densitometric analysis of the bands (Fig. 1) (Fig. 1 also includes additional data, which will be described later). A band corresponding to β-galactosidase itself or the fusion protein was not detected in the control CC118 cells (Fig. 1), whereas protein bands cross-reacting with the anti-β-galactosidase antiserum were seen in lanes L37, L107, and L126. Because β-galactosidase is a large protein of 116 kDa, an increase in the size of β-galactosidase, when fused with different length fragments of DrrB (total mass of about 30 kDa), could not be clearly seen on the SDS-PAGE gel shown in Fig. 1. Thus, the bands seen in lanes L37, L107, or L126 in Fig. 1 correspond either to the fusion protein or the degradation prod-

| Primer name | Primer sequence |
|-------------|-----------------|
| L15         | 5′-gcggccaggtggctatcagttctgctgcg-3′ |
| L4          | 5′-gcggccacagcggctgctggctggc-3′ |
| L84         | 5′-gcggccaggggctgctggctggc-3′ |
| L110        | 5′-gcggccaggggtgtggcctggctggc-3′ |
| L115        | 5′-gcggccaggggtgctggctggc-3′ |
| L181        | 5′-gcggccaggggtgctggctggc-3′ |
| L164        | 5′-gcggccaggggtgctggctggc-3′ |
| L220        | 5′-gcggccaggggtgctggctggc-3′ |
| L37         | 5′-gcggccaggggtgctggctggc-3′ |
| L245        | 5′-gcggccaggggtgctggctggc-3′ |
| L238        | 5′-gcggccaggggtgctggctggc-3′ |
| P197        | 5′-gcggccaggggtgctggctggc-3′ |
| P144        | 5′-gcggccaggggtgctggctggc-3′ |
| P236        | 5′-gcggccaggggtgctggctggc-3′ |
| P262        | 5′-gcggccaggggtgctggctggc-3′ |
| P264        | 5′-gcggccaggggtgctggctggc-3′ |
| P268        | 5′-gcggccaggggtgctggctggc-3′ |
| P272        | 5′-gcggccaggggtgctggctggc-3′ |
| P30        | 5′-gcggccaggggtgctggctggc-3′ |
| G4          | 5′-gggtttcctgcagctgccgctg-3′ |
| G117        | 5′-gggtttcctgcagctgccgctg-3′ |
| G232        | 5′-gggtttcctgcagctgccgctg-3′ |
| G236        | 5′-gggtttcctgcagctgccgctg-3′ |
| G245        | 5′-gggtttcctgcagctgccgctg-3′ |

Table II Sequence of downstream primers used for making site-directed fusions in drrB
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TABLE III

β-Galactosidase, alkaline phosphatase, or GFP activity of E. coli containing drrAB::lacZ, drrAB::phoA or drrAB::gfp fusion plasmids

*L* indicates a fusion with *lac Z*, *P* indicates a fusion with *PhoA*, and *G* indicates a fusion with *gfp*. The number next to the notation indicates the last residue in DrrB to which the fusion was made. β-Galactosidase activity and AP activity were determined in E. coli strain CC118. GFP activity was determined in E. coli strain TG1. CC118 and TG1 are used as controls.

| Fusion protein | Type of fusion | Fusion junction | β-Galactosidase, AP, or GFP activity | Synthesis units | Normalized activity |
|----------------|----------------|----------------|-------------------------------------|----------------|--------------------|
|                |                |                | units/mg protein | activity/synthesis units |
| **β-Gal fusions** | | | | |
| L15            | Site-directed  | tgtgacgaaagctt | 39,614 | 12.34 | 3,210.21 |
| L37            | Random         | gccacaagctt   | 37,643 | 12.60 | 2,987.54 |
| L44            | Site-directed  | cccgaagctt   | 37,643 | 12.60 | 2,987.54 |
| L84            | Site-directed  | aagtagaagctt | 22,649 | 8.47  | 2,674.03 |
| L110           | Random         | aacacggaagctt | 22,649 | 8.47  | 2,674.03 |
| L107           | Random         | aacacggaagctt | 22,649 | 8.47  | 2,674.03 |
| L110           | Site-directed  | aacacggaagctt | 22,649 | 8.47  | 2,674.03 |
| L115           | Site-directed  | aacacggaagctt | 22,626 | 9.57  | 2,364.26 |
| L126           | Random         | ttcgtcgaagctt | 31,585 | 12.93 | 2,442.77 |
| L161           | Site-directed  | gccagacggaagctt | 4,018 | 5.47  | 734.55 |
| L164           | Site-directed  | ggttcacggaagctt | 1,511 | 3.15  | 479.68 |
| L220           | Site-directed  | cccacgacggaagctt | 39,207 | 11.13 | 3,522.64 |
| L232           | Site-directed  | gacgacggaagctt | 27,797 | 9.8   | 1,836.43 |
| L245           | Site-directed  | gacgacggaagctt | 26,979 | 10.5  | 2,569.43 |
| L283           | Site-directed  | gacgacggaagctt | 17,135 | 8.6   | 1992.4 |
| CC118          |                |                | 10     | 0     | 0 |
| **AP fusions** | | | | |
| P86            | Random         | ttcgtcgaagctt | 2,412 | 11.67 | 206.68 |
| P84            | Site-directed  | ggtucacggaagctt | 2,655 | 12.6  | 210.71 |
| P91            | Random         | cccgaagctt   | 2,735 | 11.88 | 230.22 |
| P117           | Site-directed  | gtttcacggaagctt | 415  | 10.62 | 39.08 |
| P137           | Site-directed  | gatcctgacaagctt | 254  | 5.53  | 43.93 |
| P144           | Site-directed  | gatcctgacaagctt | 175  | 4.08  | 42.89 |
| P151           | Random         | gatcctgacaagctt | 250  | 5.29  | 47.26 |
| P189           | Random         | gttcagctgacaagctt | 307  | 5.21  | 58.93 |
| P197           | Site-directed  | gatcctgacaagctt | 186  | 4.84  | 40.09 |
| P263           | Site-directed  | atgcagctgacaagctt | 1,599 | 11.22 | 142.51 |
| P262           | Site-directed  | atgcagctgacaagctt | 225  | 3.08  | 73.0 |
| P270           | Random         | atgcagctgacaagctt | 282  | 7.32  | 50.35 |
| P275           | Random         | atgcagctgacaagctt | 152  | 5.87  | 84 |
| CC118          |                |                | 10     | 0     | 0 |
| **GFP fusions** | | | | |
| G84            | Site-directed  | ggtucacggaagctt | 246.2 | 19.24 | 12.8 |
| G117           | Site-directed  | gtttcacggaagctt | 401.36 | 10.43 | 38.5 |
| G232           | Site-directed  | gtttcacggaagctt | 370   | 5.72  | 64.7 |
| G236           | Site-directed  | gtttcacggaagctt | 350.2 | 5.97  | 58.6 |
| G245           | Site-directed  | gtttcacggaagctt | 120.3 | 4.08  | 29.4 |
| G283           | Site-directed  | gtttcacggaagctt | 380.3 | 4.06  | 93.6 |
| TG1            |                |                | 24     |       |       |

*Sequence from drrB is underlined.

1 β-Galactosidase, AP, or GFP activity is defined in terms of units/mg of protein.

2 The synthesis units for the fusion proteins were determined by densitometric scanning of Western blots.

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uct, β-galactosidase. Because the fusion proteins are normally subject to degradation, a sum of intensities of the full-length protein, and the degradation product is routinely used to obtain the synthesis units. This was done for quantitation of all fusion proteins in this study. Synthesis units were then used to calculate normalized activity (Table III).

High level of activity of the three β-galactosidase fusions (L37, L107, and L126), as shown in Table III, indicates that these fusions might be located in the cytoplasmic regions of DrrB. An active fusion at position 37 indicates that the N terminus of DrrB might be cytoplasmic. Because random fusions using λTnlacZ generated many more active fusions in the soluble protein DrrA (not shown) than in DrrB, site-directed fusions at desirable locations within the drrB gene were subsequently created, as described later.

Using λTnPhoA, a total of six active random AP fusions in DrrB were isolated. These are located at amino acid positions 66, 91, 151, 189, 270, and 275 in DrrB (Table III). The fusion proteins were quantitated by Western blot analysis using anti-AP antibody, followed by densitometric analysis of the bands (Fig. 2). The synthesis units were then used to calculate the normalized activity of the fusion proteins. The data in Table III show that all the six fusions indicated above contain significant AP activity, thus suggesting that there are at least four potential periplasmic regions in DrrB. The first periplasmic region might encompass active fusions 66 and 91; second 151; third 189; and fourth 270 and 275.

Based on the data obtained from random gene fusions, some tentative conclusions about the topology of DrrB were made. These include: 1) the N-terminal end of DrrB is cytoplasmic; 2) four regions of DrrB are exposed in the periplasmic space, indicating that there may be more than six transmembrane domains; and 3) the C terminus of DrrB may be periplasmic, which may have resulted in the fourth periplasmic region identified by the active AP fusions at residues 270 and 275. These conclusions were later tested by constructing several site-directed fusions in the putative cytoplasmic or periplasmic regions, as described below.
contains a promoterless lacZ gene starting at the 11th codon with a HindIII site preceding it. Gene fragments containing drrA and different lengths of the drrB gene were amplified, and the amplified fragments were subcloned into the HindIII site to create in-frame fusions of drrB with lacZ. A total of 11 site-directed β-galactosidase fusions were generated at amino acids 15, 44, 84, 110, 115, 161, 164, 220, 232, 245, or 283 in DrrB. These residues were selected such that fusions to residues 15 and 44 would be in the N-terminal cytoplasmic tail; 84 in the first putative periplasmic loop; and 262 in the fourth periplasmic region. The fusion at residue 84 was found to be inactive (Table III). Together, these results indicate that roughly the first 50 amino acids of DrrB are cytoplasmic, while residue 84 lies in the first periplasmic loop. It is very likely that, due to its periplasmic location, L84 is degraded, thus it could not be detected by Western blot analysis, as mentioned above. All the remaining fusions showed significant β-galactosidase activity (Table III), thus confirming the presence of three cytoplasmic regions. Surprisingly, L283, a fusion at residue 283 in DrrB was also constructed.

The levels of the fusion proteins were analyzed by Western blotting using anti-β-galactosidase antibody (Fig. 1). All the β-galactosidase fusion proteins could be detected (Fig. 1), however L84 was not seen (data not shown). β-Galactosidase is known to exhibit anomalous characteristics, such as jamming of the secretion apparatus, if it is translocated through the membrane (20). Furthermore, if secreted into the periplasm, it aggregates and is degraded by the periplasmic proteases (20, 21). β-Galactosidase activities of the fusion proteins are shown in Table III. An active β-galactosidase fusion at amino acid 37 in DrrB had earlier indicated that the N terminus of DrrB is cytoplasmic (Table III). Site-directed fusions at residues 15 and 44 also showed a high level of β-galactosidase activity (Table III), whereas the fusion at residue 84 was found to be inactive (Table III). Together, these results indicate that roughly the first 50 amino acids of DrrB are cytoplasmic, while residue 84 lies in the first periplasmic loop. It is very likely that, due to its periplasmic location, L84 is degraded, thus it could not be detected by Western blot analysis, as mentioned above. All the remaining fusions showed significant β-galactosidase activity (Table III), thus confirming the presence of three cytoplasmic loops in DrrB. Surprisingly, L283, a β-galactosidase fusion to the last residue in DrrB, also showed high activity (Table III). These data suggest that the C terminus of DrrB may also be located in the cytoplasm and, in this respect, go contrary to the conclusions made earlier based on the active AP fusions at residues 270 and 275.

Alkaline Phosphatase—A phoA fusion vector, pMLB1070, was constructed as described under “Experimental Procedures.” Using this vector, seven new site-directed phoA fusions were created. Fusions were made at amino acid positions 84, 117, 137, 144, 197, 236, or 262 in DrrB. The fusions at residue 84 and 117 would be in the first periplasmic loop; 137 and 144 in the second periplasmic loop; 197 in the third periplasmic loop; and 262 in the fourth periplasmic region. The fusion proteins were quantitated by Western blot analysis (Fig. 2),
and the synthesis units were used to calculate the normalized activity (Table III). An AP fusion at amino acid 84 in DrrB showed high activity (Table III), confirming that residue 84 lies in the first periplasmic loop of DrrB. This is consistent with the low activity obtained with a β-galactosidase fusion at the same location (Table III). All the other phoA fusions also showed high AP activity (Table III), indicating that these residues lie in the periplasmic domains in DrrB.

Most of the complementary β-galactosidase and AP fusions isolated thus far provided data consistent with each other. A few anomalous fusions were, however, isolated. These consisted of L115 and P117 (and L232, L245, and P236). Both β-galactosidase and AP fusions at these locations produced active fusions. These two regions thus needed further analysis. Moreover, high activity of a β-galactosidase fusion to the last residue 283 (Table III) indicated the cytoplasmic location for the C terminus of DrrB, which would require further verification. Therefore, at such locations where results obtained with β-galactosidase and AP either contradicted each other, or required further verification, a third type of fusion with green fluorescent protein (GFP) was created.

**GFP Fusions**

GFP has recently been used as a reporter molecule for determining the topology of membrane proteins (22). Based on the principle that the chromophore in GFP is active only in a reducing environment, GFP fusions in the cytoplasm are expected to be fluorescent, whereas those in the periplasm are not (22). To determine if GFP fusions will provide reliable results with DrrAB, a GFP fusion to residue 84 in DrrB (G84) was created. Because both β-galactosidase and the AP fusion data (Table III) support the periplasmic location of residue 84 in DrrB, a GFP fusion at this location would be expected to be non-fluorescent. G84, when tested under the fluorescence microscope, was indeed found to be non-fluorescent (Fig. 3), as expected. GFP fusions were then created at regions where contradictory results were obtained with AP and β-galactosidase fusions. There were two such regions: near residues 115 and 236 in DrrB. GFP fusions to residues 117, 232, 236, 245, or 283 were created, and fluorescence was determined both by examining under the fluorescence microscope and by UV excitation in a fluorometer. Cells containing G117, G232, or G236 were seen to be fluorescent under the microscope, whereas G245 cells were not fluorescent (Fig. 3). Cells containing G283, a GFP fusion to the last residue in DrrB, were also fluorescent under the microscope (Fig. 3). Fluorescence microscope images of cells containing G84, G117, G245, or G283 are shown in Fig. 3. Relative fluorescence of the cells containing G84, G117, G232, G236, G245, or G283 was also measured in a Photon Technology International fluorometer (excitation: 480 nm, emission: 508 nm), as described under “Experimental Procedures.” The fusion proteins were analyzed by Western blot analysis using anti-GFP antibodies (data not shown). The synthesis units were used to calculate normalized activity of the GFP fusion proteins (Table III). The data obtained with the fluorometer (Table III) are consistent with the observations made with the fluorescence microscope (Fig. 3). Cells containing G84 and G245 showed the least fluorescence activity, whereas G117, G232, G236, and G283 that resulted in fluorescence under the microscope also showed higher fluorescence by the fluorometer (Table III).

The data obtained with the GFP fusions, G117, G232, and G236 (Fig. 3 and Table III), support the data obtained with the β-galactosidase fusions, L115 and L232 (Table III). These data, thus, clear up the contradiction between β-galactosidase and AP fusions in that region (discussed earlier), indicating that these residues indeed lie in the periplasmic loops in DrrB. G245 is the only fusion that gave somewhat ambiguous results. The cells containing G245 were non-fluorescent under the microscope (Fig. 3), whereas the specific activity for G245 was found to be intermediate between G84 and other GFP fusions (Table III).

Interestingly, G283, a GFP fusion to the last residue in DrrB, showed high fluorescence activity (Fig. 3 and Table III). This supports the β-galactosidase data obtained at this location and further strengthens the evidence for the cytoplasmic nature of the C terminus of DrrB. When these data are put together with the active AP fusion data at residue 262 (and other AP fusions supporting the presence of the fourth periplasmic region), we can conclude that there are indeed four periplasmic loops in DrrB and, furthermore, that DrrB contains eight transmembrane helices with both the N and the C termini in the cytoplasm. A model based on the data generated from three different kinds of fusions is proposed in Fig. 4. All the different fusions, generated in this study, are marked on the model.

**DISCUSSION**

This study investigates the topology of the membrane component DrrB of the doxorubicin transporter DrrAB of S. peucetius. The DrrB protein occurs in a complex with the peripheral membrane component DrrA. DrrA has been shown to bind ATP and is expected to function as the catalytic subunit in this transporter (4, 5). Even though DrrA and DrrB form separate subunits, they exhibit a tight biochemical coupling (5). This coupling results from a specific interaction between the proteins and is essential for their stability and function (5). To understand how DrrA and DrrB interact and regulate the function of each other, it is important to understand how DrrB is localized in the membrane, which was the objective of the present research. In this study, we have made use of the gene fusion methodology employing two commonly used probes, alkaline phosphatase and β-galactosidase, to determine topology of the DrrB protein expressed in E. coli. To verify the phoA and lacZ fusion data, we also made use of green fluorescent protein fusions at certain regions. Even though DrrB is originally a Streptomyces protein, our data suggest that the expression of DrrA and DrrB in E. coli results in a functional complex conferring doxorubicin resistance in this host. Moreover, DrrA expressed in E. coli binds ATP in a doxorubicin-dependent manner only if DrrB is also present, suggesting a specific interaction between DrrA and DrrB and between the proteins.
and doxorubicin (5). Thus, topology of DrrB in *E. coli* is expected to reflect the topology in *S. peucetius*. In several other instances, topology of heterologous prokaryotic and eukaryotic membrane proteins has been studied by gene fusion methodology in *E. coli* cells (23–25).

The computer-generated hydrophatic profile of DrrB shows at least six regions of 18 or more amino acid residues with a hydrophatic index greater than 1.5, suggesting the presence of six or more membrane-spanning α-helices in DrrB. Fusion data obtained in the present article suggest that DrrB traverses the membrane eight times, and it contains four periplasmic loops and three cytoplasmic loops with both the N terminus and the C terminus in the cytoplasm. A topological model for DrrB based on these results (discussed below) is presented in Fig. 4.

Three active β-galactosidase fusions in the N terminus of the protein at residues 15, 37, and 44 indicate that DrrB has a long N-terminal cytoplasmic tail. Even though these three fusions, by themselves, do not conclusively demonstrate the cytoplasmic location of the N-terminal tail, when these data are combined with the results from other fusions, such as P66, P84 or L64, and P99 (Fig. 4), it can be stated with a fair amount of certainty that the N terminus of DrrB is cytoplasmic and that the first periplasmic loop of DrrB lies between residues 66 and 91. An active β-galactosidase fusion, L283, at the end of DrrB suggests that the C-terminal of DrrB is also directed inside the cytoplasm. The orientation of the C terminus was further verified by creating a GFP fusion at residue 283. Cells containing G283 were seen to be fluorescent under the microscope, confirming the cytoplasmic location of the C-terminal end of DrrB. Furthermore, the presence of four periplasmic loops in DrrB was indicated by active AP fusions at residues 66, 84, and 91 (loop I); 137, 144, and 151 (loop II); 189 and 197 (loop III); 262, 270, and 275 (loop IV) (Fig. 4), whereas the cytoplasmic loops were shown by active β-galactosidase fusions at residues 15, 37, and 44 (N-terminal tail); 107, 110, and 115 (loop I); 161 and 164 (loop II); 220, 232, and 236 (loop III); and 283 (C-terminal tail) (Fig. 4). The current topological model of DrrB in Fig. 4 is based on the weight of the majority of the data. The results of two different types of fusions in this study were generally consistent with each other. Some anomalous fusions were isolated; however, a third type of fusion with GFP at these locations helped resolve this contradiction, as described under "Results." Some ambiguous data have also been reported in other studies (24).

Most members of the ABC superfamily are predicted to contain either six or twelve transmembrane α helices, which in several instances has been shown to be the case experimentally (26, 27). Several exceptions to this rule, however, do exist in literature. For example, Tap1, a eukaryotic ABC transporter associated with antigen presentation, has been shown to contain eight transmembrane domains so that the complex of Tap1 and Tap2 contains more than 12 α helices (23). Some other eight transmembrane domain-containing proteins include NixA, the nickel transporter in *Helicobacter pylori* (28), and HlyB, the hemolysin transporter, of *E. coli* (29). ProW, which forms the membrane component of the ProU locus, has been shown to contain seven transmembrane α helices instead of the predicted six helices (30).

To date, crystal structure information for three ABC transporters is available. These include *E. coli* MsbA (31), *E. coli* BtuCD (32), and *V. cholerae* MsbA (33). These structures have provided valuable insights into the topology of membrane components of the ABC transporters as well as suggested possible modes of their interaction with the nucleotide binding components. However, crystal structure or other topological information for members of the ABC-2 family is so far not available.

The topology of the DrrB protein reported in this study constitutes first such report. Previous studies from this laboratory have clearly shown that DrrA and DrrB form a complex and biochemically regulate the stability and function of each other (4, 5). A better understanding of the topology of DrrB will help us elucidate the domains involved in direct interaction between DrrA and DrrB. This study suggests that DrrB contains a long N-terminal cytoplasmic tail, three cytoplasmic loops, and a short cytoplasmic C-terminal tail, which may form the potential sites of interaction with DrrA. Future studies will analyze the roles of these domains.

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