Function of the Membrane Fusion Protein, MexA, of the MexA, B-OprM Efflux Pump in Pseudomonas aeruginosa without an Anchoring Membrane*

(Received for publication, August 20, 1999, and in revised form, November 22, 1999)

Hiroshi Yoneyama‡, Hideaki Maseda, Hiroshi Kamiguchi, and Taiji Nakae

Department of Molecular Life Science, Tokai University School of Medicine, Isehara 259-1193, Japan

Resistence of Pseudomonas aeruginosa to multiple species of antibiotics is largely attributable to expression of the MexA, B-OprM efflux pump. The MexA protein is thought to be located at the inner membrane and has been assumed to link the xenobiotics-exporting subunit, MexB, and the outer membrane channel protein, OprM. To verify this assumption, we analyzed membrane anchoring and localization of the MexA protein. [9,10-3H]Palmitic acid incorporation experiments revealed that MexA was radiolabeled with palmitic acid, suggesting that the MexA anchors the inner membrane via the fatty acid moiety. To evaluate the role of lipid modification and inner membrane anchoring, we substituted cysteine 24 with phenylalanine or tyrosine and tested whether or not these mutant MexAs function properly. When the mutant mexAs were expressed in the strain lacking chromosomal mexA in the presence of [9,10-3H]palmitic acid, we found undetectable radiolabeling at the MexA band. These transformants restored antibiotic resistance to the level of the wild-type strain, indicating that lipid modification is not essential for MexA function. These mutant strains contained both processed and unprocessed forms of the MexA proteins. Cellular fractionation experiments revealed that an unprocessed form of MexA anchored the inner membrane probably via an uncleaved signal sequence, whereas the processed form was undetectable in the membrane fraction. To assure that the lipid-free MexA polypeptide could be unbound to the membrane, we analyzed the two-dimensional membrane topology by the gene fusion technique. A total of 78 mexA-blaM fusions covering the entire MexA polypeptide were constructed, and all fusion sites were shown to be located at the periplasm. To answer the question of whether or not membrane anchoring is essential for the MexA function, we replaced the signal sequence of the MexA protein with that of the azurin protein, which contains a cleavable signal sequence but no lipid modification site. The signal sequence of the azurin-MexA hybrid protein was properly processed and bore the mature MexA, which was fully recovered in the soluble fraction. The transformant, which expressed azurin-MexA hybrid protein restored the antibiotic resistance to a level indistinguishable from that of the wild-type strain. We concluded from these results that the MexA protein is fully functional as expressed in the periplasmic space without anchoring the inner membrane. This finding questioned the assumption that the membrane fusion proteins connect the inner and outer membranes.

Pseudomonas aeruginosa often infects immuno-compromised patients with cancer, dialysis, cystic fibrosis, and transplantation. Problems associated with P. aeruginosa infection are that this organism is naturally resistant to many noxious compounds such as β-lactam antibiotics, fluoroquinolones, chloramphenicol, and tetracycline (1). Upon exposure to antibiotics, the organism easily elevates the antibiotic resistance to a higher level than the wild type strain (2). Recent studies have suggested that both basal and elevated levels of intrinsic multiantibiotic resistance in this organism are mainly attributable to interplay between the antibiotic efflux pumps and low outer membrane permeability (3, 4). Among several efflux pumps reported in P. aeruginosa, the MexA, B-OprM pump plays a central role in antibiotic resistance because it is expressed in the wild-type strain and up-regulated upon nalB mutation (5).

A MexA, B-OprM pump, encoded by the mexA, B-oprM genes, consists of two inner membrane-associated components, MexA and MexB, and an outer membrane component, OprM (6). It has been assumed on the basis of computer-aided structural prediction that MexA belongs to a membrane fusion protein family that is thought to connect the inner and the outer membranes (7, 8). It has been suggested that each of MexB and OprM functions as an antibiotic-exporting component and an antibiotic exit outer membrane channel, respectively (3, 4, 8). Both MexA and OprM have been assumed to be lipoproteins, since they contain a consensus signal sequence for amino-terminal lipid modification, and amino-terminal amino acids seem to be blocked (6, 9). However, the membrane topology and precise localization of these proteins remain obscure, except for the two-dimensional structure of the MexB protein, which spans the cytoplasmic membrane 12 times and forms 2 large hydrophilic domains extending toward the periplasmic space (10). This protein was predicted to function as the substrate-exporting subunit across the cytoplasmic membrane.

Computer-aided hydropathy analysis of membrane fusion proteins suggested that they might be highly hydrophilic (7, 11). Preliminary analyses of the topology of the MexA homologues, such as CzeB of Alcaligenes eutrophus and CvaA, HlyD, and AcrA of Escherichia coli, suggested that they might be largely located at the periplasmic space (12–15). However, the precise membrane topology and localization of the MexA protein remained unclear. We analyzed whether or not the MexA protein can function without a lipid moiety and report that the
lipid-free MexA protein was soluble in the periplasmic space and fully functional in the antibiotic export.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Growth Conditions**—Bacterial strains and plasmids used are listed in Table I. Cells were grown aerobically in L-broth containing 10 g of Tryptone, 5 g of yeast extract, and 5 g of NaCl/liter (pH 7.2) at 37 °C. pKM1 is a new version of pYZ5 (16).

**Incorporation of [9,10-2H]Palmitic Acid**—Cells were grown in L-broth overnight and diluted with 1 ml of fresh L-broth to OD600 = 0.2. To this was added 1.48 × 108 Bq of [9,10-2H]palmitic acid (Amersham Pharmacia Biotech), and the tubes were incubated at 37 °C overnight. Cells were harvested by centrifugation, washed once with 1 ml of 50 mM sodium phosphate buffer containing 150 mM NaCl (pH 7.2; 50 mM PBS), suspended in 800 μl of 50 mM sodium phosphate (pH 7.2). The resulting cell lysate was incubated for 135 min after adding 23 μg of DNase I and centrifuged at 37 °C overnight. pKM1 was a mature derivative of PAO4290 (17) and was transformed into pBluescript II SK(+). A series of nested deletions of the mexA-blaM gene was obtained by exonuclease III digestion and subcloning into the above plasmid DNA.

**Manipulation of the DNA**—Preparation of the plasmid DNA, the restriction enzyme treatment, ligation, and transformation were carried out essentially as described by Sambrook et al. (18). A series of nested deletions of the mexA gene was obtained by exonuclease III (TOYOBO) treatment (19). Construction of mexA-blaM fusions was carried out as follows. A 1.7-kilobase KpnI-BamHI fragment encompassing the entire mexA gene was subcloned into pYZ4 pretreated with BamHI and NcoI to yield pMXEA2. pMXEA2 treated with KpnI and BamHI was incubated with exonuclease III for various lengths of time, then treated with SacI. A nested series of the deleted mexA gene was ligated with the blaM gene (coding for ampicillinase without a signal peptide) isolated by digesting pKM1 with SmaI and SacI to obtain mexA-blaM fusions. Transformants harboring the mexA-blaM fusions were screened on L-agar plates containing 12.5 μg/ml kanamycin. The oligonucleotide primers used to confirm the junction between the mexA and the blaM was TGCGTGCACCAAACGTA or TTGTGAGATCCGTTGACGAGAAGTTGAGTCCG for the anticyoding strand.

### Table I

| Strains or plasmids | Relevant properties | Reference |
|---------------------|---------------------|-----------|
| **Strains** | recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lacF, proAB, lacYIzM15, Tn10 (Tet') | Stratagene |
| E. coli XL1-Blue | | |
| P. aeruginosa PAO4290 | MexA’ derivative of PAO4290 | 17 |
| P. aeruginosa TNP070 | | Stratagene |
| **Plasmids** | | |
| pBluescript II SK+ | Cloning vector, ABPC' | 20 |
| pHSG397/398 | Cloning vector, CP' | 16 |
| pYZ4 | Expression vector, KM' | 16 |
| pKM1 | Mature β-lactamase (BluM) cassette plasmid, TET | 16 |
| pMMB67EHE4HE | Broad host range vector, ABPC', IncQ | 21 |
| pMEXA1 | MexA expression plasmid | 32 |
| pMEXA2 | pYZ4 derivative carrying mexA gene | This study |
| pMEXA-His-397 | pHS937 derivative carrying MexA-His6 gene | This study |
| pMEXA-His57 | pMMB67EH derivative carrying MexA-His6 gene | This study |
| pMEXA(C24F) | pMMB67EH derivative carrying C24F-MexA gene | This study |
| pMEXA(C24Y) | pMMB67EH derivative carrying C24Y-MexA gene | This study |
| pAZU398 | pHS938 derivative carrying signal sequence of azurin gene | This study |
| pAzU-MexA | pMMB67EH derivative carrying Azu-MexA gene | This study |
| pMexF-MexA | pMMB67EH derivative carrying Mex-F-MexA gene | This study |

**Construction of Polyhistidine-tagged mexA Gene**—We incorporated six histidines at the carboxyl-terminal glycine residue by polymerase chain reaction using forward and reverse primers, TCGAATTCCTC-GCGGTTTCCG and GAATCCCCCTTCGACTGTGGTTGCGTGCGTGCG-ATGCGCCCTTGTGCTGCGTTTGC, respectively. Polymerase chain reaction was performed with AmpliTaq Gold (Applied Biosystems, Inc.) according to the manufacturer’s instructions. Amplified products were treated with T4-DNA polymerase (New England Biolabs) and EcoRI and cloned into pBluescript II SK+ pretreated with SmaI, EcoRI, and shrimp alkaline phosphatase (Roche Molecular Biochemicals). The nucleotide sequence was confirmed subsequently for all the polymerase chain reaction products. Next, an HindIII-EcoRI fragment (about 1,100 base pairs) covering the amino-terminal half of mexA was subcloned into the pBluescript II SK+ (Stratagene) pretreated with HindIII, EcoRI, and shrimp alkaline phosphatase. A HindIII-BamHI fragment (about 1,570 base pairs) covering the entire mexA gene plus six histidines was subcloned into pHS937 (20) pretreated with HindIII and BamHI (pMEXA-His-397), then into pMMB67EHE (21) pretreated with HindIII and BamHI to yield pMEXA-His.

**Site-directed Mutagenesis of Cysteine Residue**—We replaced cysteine 24 with phenylalanine or tyrosine by site-directed mutagenesis using forward primers GTTGGCAGATTTGGCCCTTTCCGGTTCG and GTTGGCAGATTTGGCCCTTTCCGGTACGG for phenylalanine and tyrosine, respectively, and reverse primer GACTGCAGGTAGGCGGGTCGCGATTTCGGCCCTTTCCGGGTCG for phenylalanine and tyrosine, respectively. Amplified products were treated with T4-DNA polymerase followed by PolI digestion and subcloning into pBluescript II SK+ digested with EcoRV and PolI. Next, the NruI-PolI fragment from the mutant mexA was subcloned into the NruI and PolI sites of mexA, contained upstream of the mexA gene through one of the two PolI sites in the mexA gene. A PolI-SmaI (about 900 base pairs) fragment containing the second PolI site was subcloned into the above plasmids pretreated with PolI and EcoRV. Next, the SacI-HindIII fragment of these plasmids containing the entire mexA gene with the respective mutation was subcloned into pMMB67EHE (21) pretreated with SacI and HindIII to yield pMEXA(C24F) and pMEXA(C24Y), respectively. To construct histidine-tagged mexA mutation with a mutation at residue 24, a SacI-BamI fragment (about 1,100 base pairs) from each mutant plasmid was subcloned into pMEXA-His-397 pretreated with SphI and SacI. A SacI-BamHI fragment of each mutated version of mexA was then subcloned into pMMB67EHE pretreated with SacI and BamHI.

**Site-specific Insertion of the blaM Gene**—To insert the blaM gene next to Lux-43 of mexA, the amino-terminal region of the mexA gene was amplified by AmpliTaq Gold (PE Applied Biosystems) using forward, TCGAATATGGGCACTGGC, and backward, TACTTTGCAAGTACTCG, primers. The amplified products were digested with KpnI and NcoI, then ligated to pYZ4 pretreated with KpnI and NcoI. The recombinant plasmid DNA was treated with KpnI, then with T4 DNA polymerase to trim the 3’-protruding nucleotides. This was followed by SacI digestion. Next, the blaM gene, isolated from...
Localization and Topology of the Membrane Fusion Protein MexA

pKM1 by digestion with SmaI and SacI, was ligated directionally to the above site.

Construction of Soluble and Membrane-bound MexA—To construct a mexA gene in which the signal sequence was replaced with that of the azurin gene, we annealed the sense and antisense oligonucleotides, AGCTTGCCTAGGAGCTGCTCCATGTCAGTAAACCTGCTGGG-GATCCTCGCTGTCCTCGTCTAGTGCGCCGTGCTGGCTGGCGAG and TCGACTGCGACCAGCCAGGCGGCGACTGAGGAGGGACGCGG-GAGTGATACCGAGTTATGAGCTTACGGAGCGGCTCTAGGCA, respectively, followed by HindIII and SalI digestion to subclone into pHSG398 (20) pretreated with HindIII and SalI (pAZU398). The DNA fragment encoding mature MexA was amplified by LA Taq (Takara, Japan) using the forward and reverse primers, CCGGTGAGGCCAAAGGCGGACGGTAGAA-GAACCAGAT and CAAGTCGACTTCCGGGTATTCGCTGATGG, respectively, and subcloned into the above plasmid premade with BamHI and SalI. A BamHI-HindIII fragment containing the whole recombinant gene of this plasmid was subcloned into pMMB67HE pretreated with BamHI and HindIII to yield pAzu-MexA.

To construct a mexA gene containing the first transmembrane segment of the MexF gene, we first amplified the DNA fragment encoding mature MexA by LA Taq (Takara) with forward and reverse primers, TCCCTGAAAGGGAGGAGGCCGCG and GGTTAGCCTGTGATGCGGC, and subcloned it into pHSG398. Next, the first transmembrane segment of the MexF gene was amplified by LA Taq using forward and reverse primers, CCGGTGAGGCCAAAGGCGGACGGTAGAA-GAACCAGAT and CAAGTCGACTTCCGGGTATTCGCTGATGG, respectively, and the amplified fragments treated with BamHI and SalI were subcloned into the above plasmid premade with BamHI and SalI. Next, the HindIII-KpnI fragment containing the entire fusion gene was subcloned into pMMB67HE pretreated with HindIII and KpnI to yield pMexF-MexA.

Single-cell Minimum Inhibitory Concentration (MIC) of Antibiotics—E. coli XL1-Blue harboring the fusion plasmid was grown in Mueller-Hinton broth, diluted with 200 ml of the same fresh medium containing 12.5 µg/ml kanamycin, and rotated at 37 °C for 1 h. Next, 1 ml isopropyl-

1-thi-β-D-galactopyranoside was added, and the cells were grown for an additional 4 h at 37 °C. Cells were harvested by centrifugation, washed once with 20 ml of sodium phosphate buffer (pH 7.2) containing 150 mM NaCl (20 mM PBS), and resuspended in 20 ml of 20 mM PBS containing 0.5 mM p-toluenesulfonyl fluoride. The suspension was subjected to sonic oscillation in an ice bath for 15 min with 24-s exposure and 36-s intermittent cooling/min (Cell Disrupter 200, Branson). Unbroken cells and large fragments were removed by centrifugation at 10,000 × g for 15 min at 23 °C, and the crude membrane fraction was obtained by centrifugation at 130,000 × g for 1 h at 15 °C. The membrane fraction was washed once with 20 mM PBS and suspended in 2.2 ml of PBS. To the membrane fraction (1.84 ml), 0.16 ml of 10% sodium sarcosinate was added, and the mixture was incubated at 30 °C for 30 min, then centrifuged at 130,000 × g for 1 h at 15 °C. The supernatant was used as the inner membrane fraction. The inner and outer membrane fractions of P. aeruginosa were also obtained by the method described earlier (22).

One-dimensional SDS-PAGE was carried out as described by Laemmli (23). MexA-BluA fusion products were visualized with the anti-ampicillinase antibody (5 Prime → 3 Prime, Inc., Boulder, CO) using alkaline phosphatase-conjugated secondary antibody (16). Protein concentration was quantified by the method of Lowry (24).

RESULTS

Deletion of the Lipid Moiety by Replacing Cysteine 24 with Another Amino Acid—Since the amino-terminal region of MexA contains the lipid modification consensus sequence SLSG (9), we carried out a [3H]palmitic acid incorporation experiment (Fig. 1). Fluorography showed that the extracts of TNP071 exhibited a radiolabeled protein band corresponding to the MexA, 42 kDa (Fig. 1, lane 2). In contrast, radiolabeling
The mature form of MexA is indicated at the right side.

**Function of Soluble and Membrane-bound MexA**—Since the above result showed that the lipid moiety is not essential for the pump function and the processed and unprocessed forms of C24F-MexA and C24Y-MexA were not acylated, the results indicated that both C24F-MexA and C24Y-MexA were not acylated. TNP070 transformed with either one of these modified mexA genes fully restored the susceptibility to azthreonam and chloramphenicol to the level of their parent strain PAO4290 (Table II), indicating that lipid modification is not essential for the MexA function.

**Antibiotic susceptibility** of MexA variants was determined, and the results are shown in Table II. The TNP070 strain expressing C24F-MexA or C24Y-MexA fully restored the antibiotic susceptibility to the level of the wild-type strain. However, it is not certain whether the soluble form of MexA or the membrane-bound form is functional as the pump subunit, because these transformants expressed both forms of MexA. To ascertain this point, we tested to see if the antibiotic susceptibility of TNP070 expressed either one of Azu-MexA or MexF-MexA and found that the susceptibility to the bands corresponding to these recombinant MexAs was undetectable (data not shown). Thus, we were successful in constructing the lipid-free MexA variants localized either only in the periplasm or inner membrane.

**Construction and Expression of the Reporter Gene, mexA-blabM Fusion**—The results described above suggested that processed forms of the cysteine 24-modified MexA and Azu-MexA were fractionated as described under "Experimental Procedures." Inner and outer membrane (2 μg of protein) were subjected to SDS-PAGE (12% gel) and visualized with anti-MexA antibody. Lane 1, whole cell lysate of MexF-MexA; lane 2, whole cell lysate of wild-type MexA; lane 3, inner membrane fraction (5 μg of protein); lane 4, inner membrane fraction (5 μg of protein); lane 5, EDTA-lysozyme-treated supernatant (5 μg of protein); and lane 6, delipidated MexA. Whole cell lysate of MexF-MexA; lane 2, whole cell lysate of wild-type MexA; and lane 3, inner membrane fraction (5 μg of protein). The results described above suggested that the lipid modification is not essential for the function of MexA, at least for the pump activity. As expected, the radiolabeling in both MexAs were completely undetectable, although the wild-type MexA expressed from the plasmid was heavily radiolabeled (Fig. 2c). The signals were visualized by fluorochrome analyzer FLA-2000 (Fuji Film Co., Japan). Lane 1, TNP070 harboring pMexA(C24Y) and pMexA(C24F); lane 2, TNP070 harboring pMexA(C24Y); lane 3, TNP070 harboring pMexA1; lane 4, TNP070. The lower panel shows the same gel stained with Coomassie Brilliant Blue. The mature form of MexA is indicated at the right side.
Localization and Topology of the Membrane Fusion Protein MexA

### Table III

| Isolate No. | Fusion site | MIC µg/ml | Isolate No. | Fusion site | MIC µg/ml | Isolate No. | Fusion site | MIC µg/ml |
|-------------|-------------|-----------|-------------|-------------|-----------|-------------|-------------|-----------|
| 1           | Gly-383     | 200       | 27          | Ser-248     | 400       | 53          | Asn-154     | 800       |
| 2           | Thr-379     | 800       | 28          | Phe-247     | 400       | 54          | Ile-153     | 800       |
| 3           | Asn-365     | 800       | 29          | Lys-245     | 400       | 55          | Lys-145     | 800       |
| 4           | Gln-350     | 400       | 30          | Glu-242     | 800       | 56          | Glu-143     | 800       |
| 5           | Ile-345     | 200       | 31          | Gln-238     | 800       | 57          | Leu-142     | 800       |
| 6           | Ile-344     | 400       | 32          | Asp-235     | 800       | 58          | Ala-140     | 800       |
| 7           | Lys-343     | 400       | 33          | Lys-232     | 800       | 59          | Asp-136     | 800       |
| 8           | Asp-342     | 400       | 34          | Leu-231     | 800       | 60          | Ala-135     | 800       |
| 9           | Lys-331     | 200       | 35          | Lys-228*    | 800       | 61          | Glu-132     | 800       |
| 10          | Asp-330     | 400       | 36          | Gly-223     | 800       | 62          | Ala-129     | 800       |
| 11          | Gln-315     | 800       | 37          | Gln-218     | 400       | 63          | Leu-122     | 800       |
| 12          | Asn-312     | 200       | 38          | Ala-215     | 400       | 64          | Gln-118     | 800       |
| 13          | Ala-308     | 800       | 39          | Pro-203     | 800       | 65          | Asn-109     | 800       |
| 14          | Glu-305     | 800       | 40          | Tyr-197     | 800       | 66          | Asp-102     | 800       |
| 15          | Lys-302     | 400       | 41          | Pro-195     | 800       | 67          | Thr-98      | 800       |
| 16          | Asp-301     | 800       | 42          | Leu-193     | 800       | 68          | Pro-96      | 800       |
| 17          | Gly-297     | 800       | 43          | Ala-188     | 800       | 69          | Ile-94      | 800       |
| 18          | Gln-283     | 400       | 44          | Ala-184     | 800       | 70          | Gln-89      | 800       |
| 19          | Gln-281     | 200       | 45          | Asn-181     | 800       | 71          | Gly-88      | 800       |
| 20          | Phe-277*    | 100       | 46          | Thr-180     | 800       | 72          | Lys-80      | 800       |
| 21          | Met-276*    | 100       | 47          | Val-179     | 800       | 73          | Leu-78      | 800       |
| 22          | Asn-269     | 100       | 48          | Glu-175     | 800       | 74          | Val-70      | 800       |
| 23          | Pro-268     | 50        | 49          | Thr-174     | 800       | 75          | Ala-60      | >800       |
| 24          | Pro-266     | 50        | 50          | Ala-172     | 200       | 76          | Leu-43      | >800       |
| 25          | Gly-255     | 400       | 51          | Ser-171     | 400       | 77          | Ser-27      | >800       |
| 26          | Ser-251     | 100       | 52          | Thr-158     | 800       | 78          | Gly-23      | 400       |

* These clones lacked CCC codon accidentally, resulting in the loss of the first amino acid residue, proline, of the mature BlaM. The MIC of ampicillin for E. coli XL1-Blue harboring pYZ4 was 3.13 µg/ml.

MexA could be soluble in the periplasmic space and functioned properly in the intact cell. Hydropathy analysis by the TOP-PRED II program (25) predicted that the MexA protein bears two hydrophobic segments at a site proximal to the amino-terminal end. One is likely to be the topology-determining signal sequence. Another is a stretch of segment immediately downstream of the signal peptide. To verify whether or not MexA contains membrane-anchoring segments, we constructed mexA-blaM fusions as described under “Experimental Procedures.” We obtained 77 independent fusion genes with correct framing (Table III). The gaps between one fusion site to the nearest neighboring sites were consistently less than 21 amino acid residues, excepting that there were 33 amino acid residues between Ser-27 and Ala-60. To cover this region we constructed an additional fusion at Leu-43. Accordingly, we obtained a total of 78 clones covering the residue Gly-23 to the carboxyl-terminal end.

To ascertain proper expression of the hybrid proteins in the E. coli host, we ran SDS-PAGE of the inner membrane fraction prepared from the cells harboring the plasmids, and the protein was visualized by the immunoblotting method using an antibody raised against ampicillinase (Fig. 4). The result shown in Fig. 4 depicted that the hybrid proteins from G383-BlaM through G23-BlaM were lined up in decreasing molecular mass as the fusion sites became more distal from the carboxyl-terminal end. The size of all hybrid proteins was consistent with the size predicted from the length of the truncated MexA plus the size of the reporter protein. The size of the G23 hybrid protein was slightly larger than the S27 hybrid protein, suggesting that the sequence LGSC acts as a cleavage and lipid modification signal sequence in the E. coli host (Fig. 4, lanes 12 and 13). In fact, the [3H]palmitic acid radiolabeling was positively demonstrated in isolate 77 but not in isolate 78 (data not shown). Faint protein bands with a low molecular mass below that of the expected hybrid proteins were most likely the degradative products of the hybrid proteins often seen in blaM fusion in other proteins (16, 26). A faint protein band seen in Fig. 4, lane 13 with the mass about 30 kDa might be an unidentified protein derived from the E. coli host.

Localization of the Reporter Protein and Membrane Topology of the MexA Protein—The strains expressing the MexA-BlaM hybrid protein in the periplasm are expected to be β-lactam-resistant, whereas the strains carrying the fusion in the cytoplasm will be β-lactam-susceptible. To ascertain the localization of the hybrid proteins, we determined ampicillin susceptibility of the cells harboring the mexA-blaM fusion gene. Table III shows that all the transformants carrying the mexA-blaM fusion exhibited the MICs of ampicillin 50 to more than 800 µg/ml. The MIC of ampicillin for the host cell harboring pYZ4 without BlaM fusion appeared to be 3.13 µg/ml. These
results clearly indicated that the entire MexA polypeptide is located at the periplasm.

**DISCUSSION**

All the living organisms may be exposed to the external milieu, which may contain noxious compounds such as surfactants, hydrophobic materials, heavy metals, cytotoxic agents, and drugs. Most, if not all, of them must defend against the hazards of these noxious compounds by several means of detoxification. An efficient way to avoid suffering from such agents would be to lower the intracellular concentration of the noxious compounds by active extrusion. *P. aeruginosa* appears to bear the efflux pumps, of which up-regulation renders this bacterium resistance to many antibiotics, dyes, surfactants, and organic solvents (5, 6, 27–29). Since *P. aeruginosa* is a Gram-negative bacterium, the efflux pump assembly consists of the inner membrane efflux pump, the outer membrane exit channel, and the membrane fusion protein (3, 4, 8).

The MexA subunit of the MexA,B-OprM pump has been assumed to function as the membrane fusion protein, which connects the inner membrane pump, MexB, and the outer membrane exit channel, OprM (7, 8, 11). The MexA protein contains the lipoprotein consensus sequence at the amino terminus (9), yet the biological significance of the lipid modification has remained obscure. We addressed this issue by replacing cysteine 24 with phenylalanine or tyrosine. These MexA variants had both processed and unprocessed forms of MexA (Fig. 2a), as confirmed by analysis of the amino-terminal amino acid sequence. Strains expressing only the mutant MexA restored an antibiotic susceptibility indistinguishable from that of the wild-type strain. This result is consistent with the *E. coli* membrane fusion protein, AcrA, in that modification of the cysteine residue did not grossly affect the function (30). Our result demonstrated unequivocally that lipid modification was not essential for the function of MexA but did not answer the question of whether or not membrane anchoring is essential to the function of MexA.

We addressed this issue by constructing a recombinant MexA containing the signal peptide of azurin and found that the signal peptide was properly cleaved and that the mature MexA became a soluble protein (Fig. 3). Surprisingly, MexA protein without a membrane anchor fully functioned for antibiotic export. An analogous experiment has been carried out by subcloning the signal sequence-less *acrA* gene downstream from the OmpA signal sequence (30). Since *E. coli* cells harboring this recombinant gene expressed both processed and unprocessed forms of the AcrA proteins, these materials cannot be used to test whether the soluble form of AcrA is functional or not. The periplasmic localization of the entire MexA protein was confirmed by the expression of 78 MexA-β-lactamase fusion proteins (Table III). This result is consistent with topology studies previously carried out for membrane fusion proteins.

**Acknowledgment**—We are grateful to J. K. Broome-Smith at the University of Sussex, UK, for her kind gift of the plasmids pYZ4 and pKMI and helpful discussion on the gene fusion technique.
REFERENCES

1. Bryan, L. E. (1979) in Pseudomonas aeruginosa (Doggett, R. G., ed) pp. 219–270, Academic Press, London
2. Masuda, N., Sakagawa, E., and Ohya, S. (1995) Antimicrob. Agents Chemother. 39, 645–649
3. Nakae, T. (1995) Microbiol. Immunol. 39, 221–229
4. Nikaido, H. (1996) Science 274, 382–388
5. Moreshed, S. R. M., Lei, Y., Yoneyama, H., and Nakae, T. (1995) Biochem. Biophys. Res. Commun. 210, 356–362
6. Poole, K., Krebes, K., McNally, C., and Neshat, S. (1993) J. Bacteriol. 175, 7363–7372
7. Dinh, T., Paulsen, I. T., and Saier, M. H., Jr. (1994) J. Bacteriol. 176, 3825–3831
8. Ma, D., Cook, D. N., Hearst, J., and Nikaido, H. (1994) Trends Microbiol. 2, 489–493
9. Poole, K., Heinrichs, D. E., and Neshat, S. (1993) Mol. Microbiol. 10, 529–544
10. Guan, L., Ehrmann, M., Yoneyama, H., and Nakae, T. (1999) J. Biol. Chem. 274, 10517–10522
11. Saier, M. H., Jr., Tan, R., Reizer, A., and Reizer, J. (1994) Mol. Microbiol. 11, 841–847
12. Rensing, C., Pribyl, T., and Nies, D. H. (1997) J. Bacteriol. 179, 6871–6879
13. Schulein, R., Gentscher, I., Mollenkopf, H.-J., and Goebel, W. (1992) Mol. Gen. Genet. 234, 155–163
14. Skvirsky, R. C., Reginald, S., and Shen, X. (1995) J. Bacteriol. 177, 6153–6159
15. Ma, D., Cook, D. N., Alberti, M., Poe, N. G., Nikaido, H., and Hearst, J. E. (1993) J. Bacteriol. 175, 6299–6313
16. Zhang, Y., and Broome-Smith, J. K. (1990) Gene 96, 51–57
17. Yoneyama, H., Ocaktan, A., Tsuda, M., and Nakae, T. (1997) Biochem. Biophys. Res. Commun. 233, 611–618
18. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) in Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
19. Yoneyama, H., and Nakae, T. (1996) Microbiology (Washington D. C.) 142, 2137–2144
20. Takeshita, S., Sato, M., Toba, M., Masahashi, W., and Hashimoto-Gotoh, T. (1987) Gene 61, 63–74
21. Fürste, J. P., Pansegrau, W., Frank, W., Blöcker, R., Scholz, H., Bagdasarian, P., and Lanka, E. (1986) Gene 48, 119–131
22. Mizuno, T., and Kageyama, M. (1978) J. Biochem. (Tokyo) 84, 179–191
23. Laemmli, U. K. (1970) Nature 227, 680–685
24. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
25. von Heijne, G. (1992) J. Mol. Biol. 215, 487–494
26. Bowler, L. D., and Spratt, B. D. (1989) Mol. Microbiol. 3, 1277–1286
27. Köhler, T., Michael-Hamzehpour, M., Henz, U., Gotoh, N., Curty, L. K., and Pechere, J. (1997) Mol. Microbiol. 23, 345–354
28. Li, X., Zhang, L., and Poole, K. (1998) J. Bacteriol. 180, 2987–2991
29. Poole, K., Gotoh, N., Tsujimoto, H., Zhao, Q., Wada, A., and Yamasaki, T. (1999) Mol. Microbiol. 21, 713–724
30. Zgurskaya, H. I., and Nikaido, H. (1999) J. Mol. Biol. 285, 409–420
31. Silver, S., and Waldenhaug, M. (1992) Microbiol. Rev. 56, 195–228
32. Yoneyama, H., Ocaktan, A., Gotoh, N., Nishino, T., and Nakae, T. (1998) Biochem. Biophys. Res. Commun. 244, 898–902
Function of the Membrane Fusion Protein, MexA, of the MexA, B-OprM Efflux Pump in *Pseudomonas aeruginosa* without an Anchoring Membrane
Hiroshi Yoneyama, Hideaki Maseda, Hiroshi Kamiguchi and Taiji Nakae

*J. Biol. Chem.* 2000, 275:4628-4634.
doi: 10.1074/jbc.275.7.4628

Access the most updated version of this article at [http://www.jbc.org/content/275/7/4628](http://www.jbc.org/content/275/7/4628)

Alerts:
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/275/7/4628.full.html#ref-list-1) to choose from all of JBC’s e-mail alerts

This article cites 30 references, 10 of which can be accessed free at [http://www.jbc.org/content/275/7/4628.full.html#ref-list-1](http://www.jbc.org/content/275/7/4628.full.html#ref-list-1)