Tat Pathway-Mediated Translocation of the Sec Pathway Substrate Protein MexA, an Inner Membrane Component of the MexAB-OprM Xenobiotic Extrusion Pump in *Pseudomonas aeruginosa*[▽]

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***Pseudomonas aeruginosa* is equipped with the Sec and Tat protein secretion systems, which translocate the xenobiotic transporter MexAB-OprM and the pathogenic factor phospholipase C (PlcH), respectively. When the signal sequence of MexA was replaced with that of PlcH, the hybrid protein was successfully expressed and recovered from the periplasmic fraction, suggesting that the hybrid protein had been translocated across the inner membrane. MexA-deficient cells harboring the plasmid carrying the *plcH-mexA* fusion gene showed antibiotic resistance comparable to that of the wild-type cells. This result suggested that MexA secreted via the Tat machinery was properly assembled and functioned as a subunit of the MexAB-OprM efflux pump. A mutation was introduced into the chromosomal *tatC* gene encoding an inner membrane component of the Tat protein secretion machinery in *mexA*-deficient cells, and they were transformed with the plasmid carrying the *plcH-mexA* fusion gene. The transformants showed antibiotic susceptibility comparable to that of *mexA*-deficient cells, indicating that the hybrid protein was not transported to the periplasm. Whole-cell lysate of the *mexA-tatC* double mutant harboring the *plcH-mexA* plasmid produced mainly unprocessed PlcH-MexA. The periplasmic fraction showed no detectable anti-MexA antibody-reactive material. On the basis of these results, we concluded that MexA could be translocated across the inner membrane through the Tat pathway and assembled with its cognate partners, MexB and OprM, and that this complex machinery was fully functional. This hybrid protein translocation system has the potential to be a powerful screening tool for antimicrobial agents targeting the Tat system, which is not present in mammalian cells.**

Infectious diseases caused by bacteria seemed to be well controlled at one time by the use of effective therapeutic agents, but now these are becoming less useful due to resistance. Problems have arisen through the reckless use of antimicrobial agents, causing the emergence of drug-resistant bacteria. Special attention has been paid to the spread of multidrug-resistant bacteria, including *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*, methicillin-resistant *Staphylococcus aureus* (MRSA), and vancomycin-resistant MRSA (28). To combat these multidrug-resistant pathogens, an intensive ongoing search for new compounds active against them has been carried out, yet the discovery of new lead compounds seems to be more and more difficult (12). An alternative approach that differs from the traditional means may be possible (14, 32). If the cell machinery essential for the expression of bacterial virulence but not the life-sustaining processes was the target of *in vitro* screening systems, it might be possible to discover a compound(s) that attenuates bacterial virulence. Importantly, it is unlikely that such a compound(s) would cause the emergence of resistant cells (2).

A potential antibiotic target appears to be protein secretion machinery that is present in bacterial cells but not in mamma-

lian cells (17). One such candidate may be the twin-arginine translocation (Tat) system that was originally discovered in plant cells (21); more recently, the presence of a similar Tat system has been confirmed in bacterial cells (24). The Tat system is an electrochemical-gradient-driven protein secretion system that transports the proteins across the cytoplasmic membrane (17, 21, 24). Proteins to be translocated by the Tat system have a consensus signal sequence with an SRRXFLK motif, where the presence of consecutive arginine residues is essential and X could be replaced with any polar amino acid (17, 18). Lines of evidence have been accumulating that show the Tat system plays an important role in the secretion of virulence factors in pathogenic bacteria (5, 16, 19). Therefore, a Tat system inhibitor could act as a potent pathogenicity attenuator.

Besides the Tat system, most bacterial cells are equipped with the general protein secretion (Sec) system, which translocates a wide variety of proteins across the cytoplasmic membrane (20). The Sec system secretes proteins having a consensus signal peptide that is similar to, but distinct from, that of the Tat system. The Sec signal sequence lacks an N-terminal consecutive-arginine sequence and has a relatively hydrophobic central region and a relatively short signal sequence compared with that of Tat (17, 18). Thus, whether proteins are secreted via the Sec system or the Tat system largely depends on the characteristic features of their signal sequences.

To search for antimicrobial agents that can knock out the Tat system, it is necessary to develop a high-sensitivity reporter

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant properties[a]	Reference or source
Strains		
E. coli JM109	*recA1 endA1 gyrA96 thi hsdR17*(r$_K^-$ r$_K^+$) *e14*$^-$ (*mcrA*$^-$) *supE44 relA1* Δ(*lac-proAB*)/F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	Laboratory strain
E. coli S17-1	RP4-2-Tc::Mu-Km::Tn7 *pro res*$^-$ *mod*$^+$	26
P. aeruginosa PA04290	*leu-10 argF10 aph-9004* FP$^-$	Matsumoto collection
P. aeruginosa TNP070	Δ*mexA* derivative of PAO4290	35
P. aeruginosa TNP080	Δ*tatC* derivative of TNP070	This study
Plasmids		
pBluescript II SK(+)	Cloning vector; Ap^r	Stratagene
pUC18	Cloning vector; Ap^r	Takara
pTH18ks1	pSC101 derivative; *repA$_{ts1}$* Km^r	9
pK18mob	pK18 derivative carrying RP4 *mob* region; Km^r	25
pMMB67HE	Broad-host-range vector; Ap^r IncQ	8
pMEXA1	pMMB67HE derivative carrying wild-type *mexA* gene; Ap^r	34
pBlueMexA	pBluescript II SK(+) derivative carrying *mexA* gene; Ap^r	This study
pTH18-PlcMexA	pTH18ks1 derivative carrying chimeric *mexA* gene with *plcH* signal sequence; Km^r	This study
pPlcMexA	pMMB67HE derivative carrying chimeric *mexA* gene with *plcH* signal sequence; Ap^r	This study
pK18ΔTat	pK18mob derivative carrying internal *tatC* gene and *mob* gene; Km^r	This study
pTH18-TatPlcMexA	pTH18ks1 derivative carrying entire *tatC* gene and the chimeric *mexA* gene; Km^r	This study
pTatPlcMexA	pMMB67HE derivative carrying entire *tatC* gene and the chimeric *mexA* gene; Ap^r	This study

[a] Ap, ampicillin; Km, kanamycin.

assay system. Our strategy was as follows. If the signal sequence of the protein to be secreted via the Sec system was replaced with that of the protein to be translocated by the Tat system, the Sec system-dependent protein could be translocated via the Tat system. If the candidate protein was a component involved in antibiotic resistance, the hybrid proteins secreted via the Tat system would render the cells resistant to antibiotics. If a compound that blocked or inactivated the Tat system was present, the reporter protein would not be translocated across the inner membrane, and therefore, the cell would become antibiotic susceptible. We employed the MexA subunit of the MexAB-OprM antibiotic efflux pump of *P. aeruginosa* as a reporter of the Sec system and phospholipase C (PlcH) as a donor of the Tat system signal sequence.

The MexA protein is a component of the MexAB-OprM efflux pump and functions as a molecular clamp in the pump assembly (1, 35). Though the MexAB-OprM pump confers multiantibiotic resistance on cells, the absence of MexA totally inactivates the pump function, thus rendering the cells hypersusceptible to antibiotics (35). MexA anchors the inner membrane via fatty acids attached to the N-terminal cysteine residue (33). When the N-terminal signal sequence plus an adjacent cysteine residue of MexA was replaced with the signal sequence of periplasmic azurin, this hybrid protein was properly translocated, processed, and folded in the periplasmic space as the soluble form (33). In addition, the cells producing the hybrid protein but lacking the chromosomal *mexA* gene restored antibiotic resistance, indicating that membrane anchoring is not essential for the function of MexA (33).

The PlcH protein, an important virulence factor of *P. aeruginosa*, has the consensus signal sequence RRRTFLK and appears to be secreted into the extracellular medium via the Tat system (27). Accordingly, we designed the experiment to replace the signal sequence of MexA with that of PlcH and assessed whether MexA could be translocated via the Tat machinery.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used are listed in Table 1. Cells were grown aerobically in L broth containing 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter (pH 7.2) at 37°C. For the pyoverdine assay, cells were grown in medium containing 5 g per liter of Casamino Acids, 5 mM K$_2$HPO$_4$, and 1 mM MgSO$_4$ with shaking at 37°C overnight (16). The MICs of aztreonam (Sigma) and chloramphenicol (Wako Pure Chemical, Japan) were determined by the agar dilution method using Mueller-Hinton agar (35).

Replacement of the signal peptide of *mexA* with that of *plcH*. The modified overlap extension method (10) was used to construct recombinant *mexA* having the signal sequence of the *plcH* gene (27). The sequence including the *plcH* signal sequence was amplified by ExTaq polymerase (Takara, Japan) using forward (PlcH-F) and reverse (PlcH-R) primers (Table 2) from the *P. aeruginosa* PAO1 chromosomal DNA as a template. The gene encoding mature MexA was amplified by ExTaq using forward (MexA-F) and reverse (MexA-R) primers from a derivative of pBluescript II SK(+) (Stratagene) carrying the MexA gene as a template. The PCR products were fused by a second DNA amplification using primers PlcH-F and MexA-R, followed by cloning onto pTH18ks1 (9) digested with BamHI and KpnI to yield pTH18-PlcMexA. We next amplified the fused gene using the PlcH-F and MexA-R primers and pTH18-PlcMexA as a template, and then the fragment was cloned onto a shuttle vector, pMMB67HE (8), pretreated with BamHI and KpnI to yield pPlcMexA. The nucleotide sequence of the fused gene was determined by the dideoxy chain termination method (23). *P. aeruginosa* TNP070 and TNP080 were transformed with the plasmid pPlcMexA by electroporation with a GenePulser II (Bio-Rad) at 2.5 kV, 200 Ω, and 25 μF.

Construction of a *tatC*-deficient mutant. To inactivate the *tatC* gene in the *mexA*-deficient strain, TNP070, we first amplified an internal region of *tatC* (446 bp) with ExTaq using forward (TatC-F1) and reverse (TatC-R1) primers and the PAO1 chromosomal DNA as a template. The DNA fragment produced was cloned into the BamHI site of pK18mob (25) to generate pK18ΔTat. *Escherichia coli* S17-1 was transformed with pK18ΔTat (36), and the chromosomal *tatC* of *P. aeruginosa* TNP070 was disrupted by integration of the plasmid by conjugation with selection on an agar plate containing 12.5 μg/ml of kanamycin (Wako Pure Chemical, Japan) and 50 μg/ml of tetracycline (Wako Pure Chemical, Japan), yielding TNP080. Mutation in the *tatC* gene was confirmed by Southern blot analysis as described previously (36).

Construction of the *tatC* expression vector. The *tatC* gene was amplified with PrimeStar (Takara) using forward (TatC-F2) and reverse (TatC-R2) primers, with the PAO1 chromosomal DNA as a template. The amplified fragment was cloned into the BamHI site of pUC18, and the presence of the cloned *tatC* was verified by nucleotide sequencing. The BamHI fragment excised from the result-

TABLE 2. Primers

Primer	Sequence[a]	Tag
PlcH-F	5′-GA<u>GGATCC</u>TCGCTGTATTCCCTGCC-3′	BamHI
PlcH-R	5′-CGGCGCCTCGCTTTTTCCCTCGACGGCCAGGGCGCG-3′	
MexA-F	5′-CGCGCCCTGGCCGTCGAGGGAAAAAGCGAGGCGCCG-3′	
MexA-R	5′-AG<u>GGTACC</u>CTTGATCAGCCCTTGC-3′	KpnI
TatC-F1	5′-GT<u>GGATCC</u>ACCTGACCGAACTGCGTACG-3′	BamHI
TatC-R1	5′-AC<u>GGATCC</u>GTCAGGACGAAGTCCTAGGTAG-3′	BamHI
TatC-F2	5′-GT<u>GGATCC</u>ACCTGACCGAACTGCGTACG-3′	BamHI
TatC-R2	5′-AC<u>GGATCC</u>GTCAGGACGAAGTCCAGGTAG-3′	BamHI

[a] Restriction site tags are underlined.

ing plasmid was cloned into the BamHI site of pTH18-PlcMexA, yielding pTH18-TatPlcMexA, which carried the intact *tatC* gene in front of the *plcH-mexA* fusion. After digestion of pHT18-TatPlcMexA with HindIII and KpnI, the fragment encompassing *tatC* and the *mexA* gene fusion was purified using the QIAEXII gel extraction kit (Qiagen). Next, the isolated fragment was cloned into pMMB67HE pretreated with HindIII and KpnI to generate pTatPlcMexA. TNP080 lacking chromosomal *mexA* and *tatC* was transformed with pTatPlc-MexA.

Fractionation of the periplasmic proteins. Crude periplasmic materials were obtained by cold shock treatment (15). Briefly, overnight-grown cells in 20 ml of L broth containing 12.5 μg/ml of kanamycin and 50 μg/ml of sulbenicillin (Takeda, Japan), if needed, were diluted with 200 ml of prewarmed (37°C) L broth containing 2 mM isopropyl-1-thio-β-D-galactopyranoside and incubated at 37°C until late log phase. The cells were harvested by centrifugation ($9,000 \times g$; 10 min; 23°C) and suspended in 50 mM Tris-HCl, 0.2 M MgCl$_2$ (pH 7.3) at the wet-cell-weight/buffer ratio of 1 to 5. The cell suspension was alternatively immersed in a 37°C water bath for 10 min and an ice water bath for 15 min for two cycles. After centrifugation at $17,500 \times g$ for 15 min at 10°C, the supernatant was centrifuged at $108,000 \times g$ for 60 min at 10°C (Beckman rotor TLA-120.1). The supernatant was saved as the periplasmic protein fraction.

Other methods. Visualization of the chromosomal *tatC* region was carried out using the DIG labeling and detection system (Roche) according to the manufacturer's instructions. Pyoverdine was detected by measuring the absorption spectra of the culture supernatants from 380 to 500 nm (30). For Western blotting, cellular extracts were subjected to electrophoresis and blotted to a polyvinylidene fluoride membrane (Millipore), and the protein band was probed with the anti-MexA antibody using alkaline phosphatase-conjugated secondary antibody (35). SDS-PAGE was carried out as described by Laemmli (11). The protein concentration was quantified by the method of Lowry et al. (13).

RESULTS AND DISCUSSION

The function of MexA encoded by the *plcH-mexA* fusion gene. The hybrid protein encoded by the fused gene of the azurin signal sequence and the mature *mexA* sequence had been found to be properly secreted into the periplasmic space and fully functional (33). We assumed, therefore, that the signal sequence of MexA might be replaced with that of phospholipase C (PlcH) and that the hybrid PlcH-MexA might be transported via the Tat secretion machinery. TNP070 cells lacking the chromosomal *mexA* gene were transformed with a plasmid carrying the fused gene of the *plcH* signal peptide and the *mexA* sequence encoding the mature form of MexA. The MICs of aztreonam and chloramphenicol in the transformant TNP070(pPlcMexA) were 2 and 32 μg/ml, respectively (Table 3). These values were 8 and 4 times, respectively, higher than the MICs of aztreonam and chloramphenicol in cells lacking MexA (TNP070) and were fully comparable to the MICs in cells producing a full amount of MexA, TNP070(pMEXA1). These results suggested that the hybrid MexA had been expressed and translocated across the inner membrane via the Tat protein secretion machinery.

We analyzed the whole-cell lysate of the transformant harboring pPlcMexA by the Western blotting method, probing with anti-MexA antibody, and found that there were two anti-MexA antibody-reactive bands. (Fig. 1, lane 3). The lower band corresponded to the mature form of MexA, and therefore, the upper band was most likely the unprocessed form of MexA. If this was the case, it could be expected that at least the mature form of MexA would be localized in the periplasmic aqueous phase. To confirm this prediction, the periplasmic materials were released from the cells into the aqueous phase by the cold osmotic shock method, and then the extracts were subjected to Western blotting and probed with the anti-MexA antibody. The electrophoretogram showed only one major band reactive with the anti-MexA antibody and corresponding to the location of the mature form of MexA (Fig. 1, lane 8). This result suggests that hybrid MexA was translocated across the inner membrane, most likely through the Tat secretion machinery. As a control experiment, the wild-type cell, PAO4290, which produces fatty acid-modified MexA and hence contains MexA, which anchors the inner membrane, was subjected to the osmotic shock experiment. The result showed that MexA was undetectable in the periplasmic materials, confirming the previous results (33).

Translocation of the hybrid MexA through the Tat pathway. The results described above could not distinguish whether the hybrid protein was translocated via the Tat pathway or the Sec pathway. If the hybrid protein was secreted via the Tat pathway, introduction of a mutation into the subunit protein of the Tat machinery should abolish the secretion. Therefore, we disrupted the *tatC* gene by single-crossover mutagenesis in the *mexA*-deficient mutant (TNP080). Disruption was confirmed by Southern blot analysis (data not shown). The function of the

TABLE 3. MICs of aztreonam and chloramphenicol for transformants expressing chimeric MexA

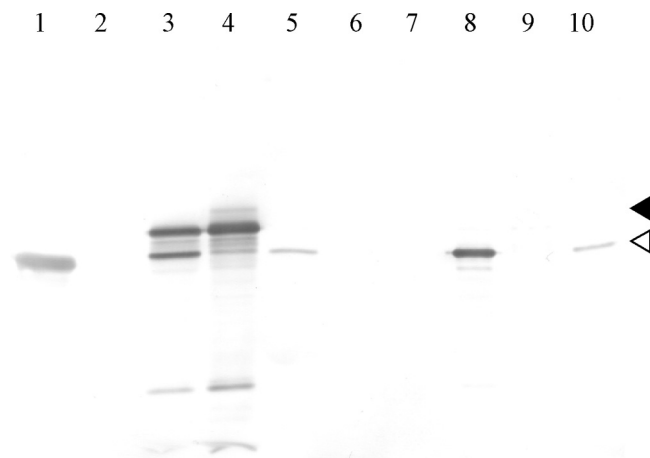| Strain	Plasmid	MIC (μg/ml)	
		Aztreonam	Chloramphenicol
PAO4290	−[a]	4.0	64
TNP070	−	0.25	8.0
TNP070	pMEXA1	2.0	32
TNP070	pPlcMexA	2.0	32
TNP080	−	0.13	4.0
TNP080	pMexA1	2.0	32
TNP080	pPlcMexA	0.25	4.0
TNP080	pTatPlcMexA	2.0	32

[a] −, no plasmid.

FIG. 1. Western blotting of periplasmic materials extracted from cells expressing hybrid MexA proteins. Whole-cell lysate (4 μg protein) (lanes 1 to 5) and periplasmic materials (1 μg protein) (lanes 6 to 10) were subjected to SDS-PAGE (12% gel), blotted onto a polyvinylidene fluoride membrane, and visualized with anti-MexA antibody. Lanes 1 and 6, PAO4290; lanes 2 and 7, TNP070; lanes 3 and 8, TNP070(pPlcMexA); lanes 4 and 9, TNP080(pPlcMexA); lanes 5 and 10, TNP080(pTatPlcMexA). The white and black arrowheads indicate mature and unprocessed MexA.

Tat system in the mutant was assessed by measuring the pyoverdine in the medium, the production of which is Tat dependent (31). Though the culture supernatant of the wild-type cells, PAO4290, and the MexA-deficient cells, TNP070, showed the typical absorption spectra of pyoverdine (Fig. 2), the cells carrying the mutation in the *tatC* gene secreted an undetectable level of pyoverdine, suggesting that the Tat secretion machinery was not functional. This result was consistent with what had been seen before (31). We also noted that

the growth rate of the TNP080 cells was slowed down compared with that of the parent strain, TNP070 (data not shown), consistent with the characteristic feature of the *tatC*-deficient mutant of *P. aruginosa* (16). When the *tatC*-deficient TNP080 cells were transformed with the plasmid carrying unimpaired *tatC*, pTatPlcMexA, the pyoverdine-secreting capability of the cells was restored (Fig. 2).

We then investigated whether inactivation of the Tat machinery interfered with the function of the MexAB-OprM efflux pump by determining the MICs of the antibiotics. The TNP080 cells with double mutations in *mexA* and *tatC* and harboring pPlcMexA showed aztreonam and chloramphenicol MICs of 0.25 μg/ml and 4.0 μg/ml, respectively, and these values were comparable with those in MexA-deficient TNP070 cells (Table 3). This result suggested that the hybrid MexA protein was not transported to the periplasmic space, implying that the hybrid protein was not the substrate of the Sec system. It is likely that the unprocessed form of the hybrid MexA remained in the cytoplasmic space or stacked at the inner membrane. In fact, a significant amount of unprocessed MexA was recovered from the whole-cell lysate of TNP080 harboring pPlcMexA (Fig. 1, lane 4), whereas nearly no detectable processed form of MexA was recovered from the periplasmic fraction (Fig. 1, lane 9). These results clearly indicated that the hybrid MexA was translocated across the inner membrane exclusively through the Tat pathway. In contrast, TNP080 harboring pMEXA1, and thus expressing wild-type MexA, restored resistance to aztreonam (2.0 μg/ml) and chloramphenicol (32 μg/ml) to levels similar to those of TNP070 harboring pMEXA1, confirming that MexA was translocated through the Sec system.

Restoration of hybrid MexA translocation by TatC expressed from the plasmid-borne *tatC* gene. We next designed an experiment to recover the antibiotic resistance in TNP080
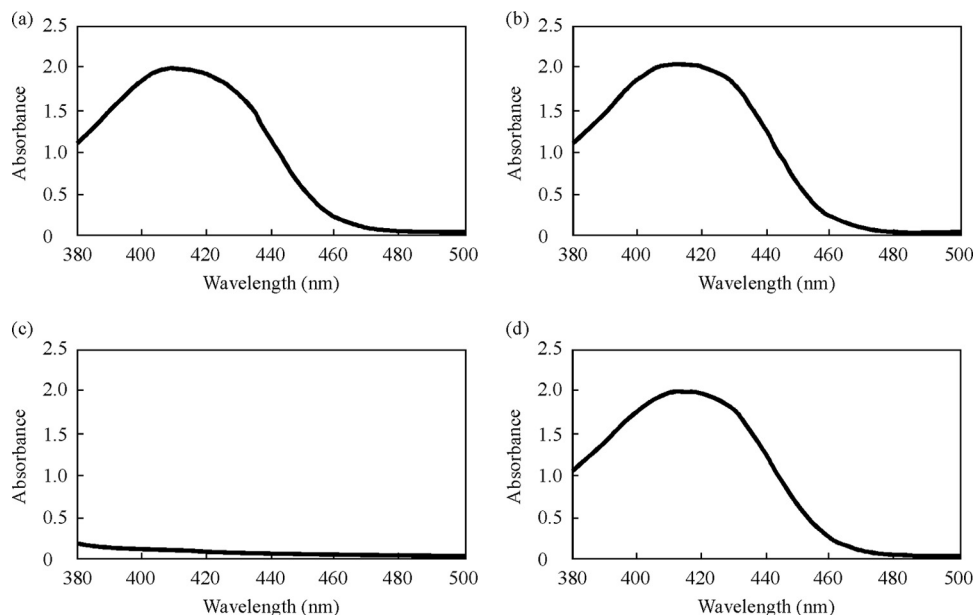

FIG. 2. Absorption spectra of culture supernatants. Cells were grown in Casamino Acid medium overnight at 37°C under aeration by reciprocal shaking, and the culture was centrifuged at 13,700 × *g* for 10 min. The clear supernatant was scanned with a spectrophotometer (V-630 Bio; Jasco Co., Japan). (a) PAO4290. (b) TNP070. (c) TNP080. (d) TNP080(pTatPlcMexA).

cells by restoring the Tat pathway-dependent translocation of the hybrid MexA. The pTatPlcMexA plasmid carrying unimpaired *tatC* was constructed, and TNP080 was transformed with the plasmid (Table 1). The transformants harboring pTatPlcMexA excreted pyoverdine into the culture medium, as assayed by measurement of the absorption spectrum (Fig. 2), indicating that the Tat machinery was reconstituted from the plasmid expressing the TatC subunit and functioned properly. In addition, it should be noted that the lowered growth rate of the *tatC* mutant fully recovered, as the plasmid-borne TatC was expressed (data not shown). Functional restoration of the Tat machinery was assessed by determination of antibiotic susceptibility. The transformants harboring pTatPlcMexA showed the MIC of aztreonam and chloramphenicol to be 2.0 and 32 µg/ml, respectively, and these values were comparable to those in PAO4290, TNP070(pMEXA1), TNP070(pPlcMexA), and TNP080(pMexA) (Table 1), indicating again that the hybrid MexA had been translocated through the reconstituted Tat pathway, processed, and properly assembled into a functional efflux pump.

On the basis of these results, we concluded that the hybrid MexA with the Tat-directed signal sequence is exclusively translocated across the inner membrane via the Tat pathway. Thus, this system, which is a combination of the plasmid carrying the *plcH-mexA* fusion gene and the *mexA*-deficient host cell of *P. aeruginosa*, provides a high-sensitivity screening strategy for Tat pathway inhibitors. If the Tat pathway inhibitors were to be discovered, the compounds could be used as pathogenicity attenuators of *P. aeruginosa*, because the Tat machinery is involved in several aspects of virulence and pathogenic factors (16).

There are growing lines of evidence that the Tat pathway recognizes and translocates the folded proteins across the cytoplasmic membrane, in marked contrast to the Sec pathway, which translocates unfolded substrate proteins cotranslationally. Thus, the system may function as a protein-folding sensor or a folding quality control system (4). This implies that the Tat system senses the folding status of its substrate proteins, as unfolded substrate proteins are generally rejected (4, 18). It is intriguing to note that the MexA protein could be translocated in either the unfolded state via the Sec system or the folded state via the Tat system. This feature of the Tat system-mediated translocation of folded proteins has been well studied by the replacement of the Sec system-directed signal sequence of alkaline phosphatase and cytochrome *c* with the Tat system-directed signal sequence (4, 22). Another interesting reporter protein would be the green fluorescent protein (GFP) attached to the Tat-directing signal sequence (29), which is transported solely through the Tat pathway, leading to proper fluorescence of the secreted protein. GFP transported through the Sec pathway does not fluoresce (7). The translocation pathway-dependent fluorescence of the exported GFP is most likely due to the fact that GFP may be folded only in a cytoplasmic environment with reduced conditions (7). The maltose binding protein was transported by either the Sec system or the Tat system, depending upon the signal sequence attached to it (3). MexA is the first example, to our knowledge, of an RND family protein (6) that could be exported by the Tat machinery while retaining full activity. Accordingly, the hybrid MexA should prove to be

a useful tool for the study of the molecular mechanism of protein translocation of the *P. aeruginosa* Tat machinery.

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