Biochemical Characterization of MsbA from *Pseudomonas aeruginosa*®

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Lipopolysaccharide of *Pseudomonas aeruginosa* is a major constituent of the outer membrane, and it is composed of three distinct regions: lipid A, core oligosaccharide, and O antigen. Lipid A and core oligosaccharides (OS) are synthesized and assembled at the cytoplasmic side of the inner membrane and then translocated to the periplasmic side of the membrane where lipid A-core becomes the acceptor of the O antigens. Here we show that MsbA encoded by *pA4997* of the *P. aeruginosa* genome is a member of the ABC transporter family, but this protein has distinctive features when compared with other MsbA proteins. *msbA* is an essential gene in this organism since mutation in this gene is lethal to the bacterium. Disruption of the chromosomal *msbA* was achieved only when a functional copy of the gene was provided in trans. *msbA* from *Escherichia coli* (*msbAEc*) could not cross complement the *msbA* merodiploid cells of *P. aeruginosa*. MsbA was expressed and purified, and the kinetic of its ATPase activity is vastly different than that of MsbA Ec. The activity of MsbA could be selectively stimulated by lipid A, suggesting that of MsbA Ec, but not MsbA Ec, conferred resistance to erythromycin in *P. aeruginosa*.

Lipopolysaccharide (LPS)3 is a major constituent of the outer leaflet of the outer membrane of Gram-negative bacteria and is composed of three distinct regions, namely, lipid A, core oligosaccharide, and O antigen (1). This macromolecule plays an important role in maintaining the structural integrity of the outer membrane (2, 3). The enzymes involved in the biosynthetic pathways of lipid A and core oligosaccharide of *Escherichia coli* have been reviewed by Raetz and Whitfield (1).

Homologues of these enzymes are also found in *P. aeruginosa* and other Gram-negative bacteria. Biosynthesis of lipid A-core begins at the cytoplasmic side of the inner membrane, and all of the enzymes involved are either localized in the cytoplasm or associated with the membrane. In *E. coli*, the minimal LPS structure required for growth has been known to be two 2-keto 3-deoxy-α-mannooctulosonic acid residue of the inner core attached to lipid A (Kdo2-lipid A or Re LPS) (4). The requisite LPS structure has been redefined by Meredith et al. (5) who had shown in their recent study that an *E. coli* mutant producing only the lipid A precursor known as lipid IV A in the outer membrane was viable. In contrast, *P. aeruginosa* requires sugars of the full inner core and at least part of the outer core in addition to lipid A to be viable (3, 6). The lipid A-core moiety must be flipped to the periplasmic side of the inner membrane where it is either directly transported to the outer membrane or serves as acceptor of the O antigens. Earlier work on *E. coli* showed that the transport of this molecule is ATP-dependent and is most likely carried out by an ATP binding cassette transporter, named MsbA (7). This protein exhibited structural similarities to mammalian multidrug-resistant ABC transporters as well as several bacterial exporters (8). MsbA was therefore classified as a member of the ABC transporter superfamily. Polissi and Georgopoulos (9) showed that msbA Ec is an essential gene and the only essential bacterial ABC transporter known in *E. coli*. The MsbA protein was localized to the inner membrane and shown to possess ATP binding and hydrolysis properties. Studies by Zhou et al. (10) and Doerrler and Raetz (11) showed that MsbA can transport fully acylated lipid A. Interestingly, the ATPase activity of MsbA can be stimulated by lipid A, suggesting that this protein could be involved in transport of lipid A across the inner membrane. Homologues of MsbA have been identified in almost all Gram-negative bacteria as well as some Gram-positive bacteria. Because Gram-positive bacteria do not produce LPS, the function of the MsbA homologues in these bacteria has been implicated in multidrug resistance (12, 13). To date, information on the biochemical characteristics of MsbA proteins is limited and MsbA Ec is the only one that has been characterized biochemically. Moreover, the sequence conservation within the membrane-spanning domains (MSDs) of MsbA proteins is low (supplemental Fig. S1), likely caused by differences in their substrate recognition and the kinetics of ATPase activity.

Open reading frame *pa4997* from the annotated *Pseudomonas* PA01 genome encodes a protein that shares 40% sequence identity with *E. coli* MsbA. MsbA protein (msbA/MsbA) is either directly transported to the outer membrane or serves as acceptor of the O antigens. Earlier work on *E. coli* showed that the transport of this molecule is ATP-dependent and is most likely carried out by an ATP binding cassette transporter, named MsbA (7). This protein exhibited structural similarities to mammalian multidrug-resistant ABC transporters as well as several bacterial exporters (8). MsbA was therefore classified as a member of the ABC transporter superfamily. Polissi and Georgopoulos (9) showed that msbA Ec is an essential gene and the only essential bacterial ABC transporter known in *E. coli*. The MsbA protein was localized to the inner membrane and shown to possess ATP binding and hydrolysis properties. Studies by Zhou et al. (10) and Doerrler and Raetz (11) showed that MsbA can transport fully acylated lipid A. Interestingly, the ATPase activity of MsbA can be stimulated by lipid A, suggesting that this protein could be involved in transport of lipid A across the inner membrane. Homologues of MsbA have been identified in almost all Gram-negative bacteria as well as some Gram-positive bacteria. Because Gram-positive bacteria do not produce LPS, the function of the MsbA homologues in these bacteria has been implicated in multidrug resistance (12, 13). To date, information on the biochemical characteristics of MsbA proteins is limited and MsbA Ec is the only one that has been characterized biochemically. Moreover, the sequence conservation within the membrane-spanning domains (MSDs) of MsbA proteins is low (supplemental Fig. S1), likely caused by differences in their substrate recognition and the kinetics of ATPase activity.

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MsbA of *P. aeruginosa*

nated with a suffix in subscript font) possesses conserved ABC transporter protein motifs (supplemental Fig. S1) and as such is classified as a member of this family. In this study, we describe the characterization of the putative MsbA of *P. aeruginosa*, and test the hypothesis that this protein is involved in the transport of lipid A-core, and not just lipid A, from the cytoplasmic side of the inner membrane to the periplasmic side. We observed that msbA is an essential gene and msbAE cannot be used to cross-complement an msbA mutation in *P. aeruginosa*. The differences between these two genes and their products are substantiated by the observation that the kinetic parameters of purified and reconstituted MsbA are considerably different than that of MsbAE. Finally, this is the first report to show that the phosphate substituents in the lipid A-core play a role in the transport of this molecule across the membrane in model bilayers (liposomes).

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Culture Conditions**—Bacterial strains and plasmids used in this study are described in the supplemental Table S1 (50). Bacterial strains were routinely propagated in Luria-Bertani (LB) broth (Invitrogen Canada Inc., Burlington, ON) at 37 °C. Pseudomonas Isolation Agar (PIA; DIFCO Becton Dickson, Sparks, MD) was used for the bacterial mating experiments.

**DNA Procedures**—All restriction enzymes were used according to the suppliers’ specifications. Small-scale plasmid DNA preparations were carried out using a plasmid Mini-Prep kit (Sigma-Aldrich Canada). Plasmid DNA was electroporated into *P. aeruginosa* with a Gene Pulser instrument (Bio-Rad). Genomic DNA was isolated from *P. aeruginosa* (PA01) and *E. coli* K12 (W3110) by the method described by Ausuble et al. (14).

Early attempts to amplify *pa4997* of strain PA01 using a standard PCR procedure with primers designed to sequences flanking the 5′- and 3′-end of the open reading frame were unsuccessful. Subsequently, we amplified two segments of the gene and ligated them to produce an intact gene. The intact gene product was cloned into pEX18Ap and pQE80 for protein expression experiments. Southern blot analysis was performed following the procedure described by the manufacturer (Roche Applied Science). To generate a knock-out mutation in msbA, the method described by Hoang et al. (18) was followed.

To construct a chimera of msbA, the membrane-spanning domain (MSD) of MsbA was fused to the nucleotide-binding domain (NBD) of MsbA, by using the Splice Overlap Extension (SOE) method as described previously (15). The length of each of the two domains was determined by the Simple Molecular Architecture Research Tool (SMART) domain prediction program (16, 17). Chimeric-MsbA (Chi-MsbA) was overexpressed as an N terminus histidine-tagged fusion protein using pQE80 and purified as described below for MsbA.

**Overexpression of MsbA**—N-terminal histidine-tagged MsbA was expressed in *E. coli* BL21 (DE3) cells. Several expression conditions were tested including those reported for BmrA (12) and MsbAE (11). The bacterial cultures were grown at 37 °C until the OD600 reached 0.6. The bacterial cells were induced with 1 mM isopropyl-thio-β-D-galactopyranoside (IPTG) for 16 h at 15 °C. Cells were harvested by centrifugation at 10,000 × *g* for 10 min, the cell pellet was washed with ice-cold 100 mM phosphate buffer, pH 7 and resuspended in 40 ml of the suspension buffer (50 mM HEPES pH 7.5, 5 mM 2-mercaptoethanol, and 5 mM MgCl2). Cell breakage was achieved by 3 passages through a French Press cell at 20,000 psi. Unbroken cells and cellular debris were then removed by centrifugation at 20,000 × *g* for 10 min. Inside-out membrane vesicles were sedimented by centrifugation at 125,000 × *g* for 30 min. Membrane vesicles were resuspended in 50 mM HEPES pH 7.5, 5 mM 2-mercaptoethanol and 10% glycerol at protein concentration of 10–30 mg/ml and stored at −80 °C in small aliquots.

**Solubilization and Purification of HsΔc-MsbA from Membrane Vesicles**—Several detergents including N-laurylsarcosine (Sarcosyl), octylglucoside (OG), lauryldimethylamine-oxide (LDAO), and dodecyl β-D-maltoside (DDM) were tested for their ability to solubilize functionally active MsbA. The following procedure was used to reproducibly produce relatively large quantities of MsbA (5 mg per 500 ml of culture) that exhibited very high level of ATPase activity. Briefly, frozen membrane vesicles were quickly thawed at 37 °C and diluted to a final concentration of 5 mg/ml with the solubilization buffer (100 mM potassium phosphate pH 8.0, 200 mM NaCl, 5 mM 2-mercaptoethanol, 15% glycerol, and 1% (w/v) DDM), and the sample was stirred for 1 h at 4 °C. Insoluble materials were removed by centrifugation at 125,000 × *g* for 30 min. The supernatant containing the solubilized membranes were adjusted to a final concentration of 10 mg/ml imidazole. Prior to purification, detergent-solubilized protein was gently stirred for 1 h at 4 °C in 1 ml of TALON resin (ClonTech). After that MsbA was eluted with 300 mM imidazole. Imidazole was removed from the purified protein using a PD-10 desalting column (GE Healthcare BioSciences Inc., Baie d’Urfe, QC), and the buffer was exchanged to protein storage buffer (50 mM HEPES pH 7.5, 200 mM NaCl, 10 mM MgCl2, 15% glycerol, and 0.05% DDM) and stored at −80 °C.

**Reconstitution of Purified MsbA**—Commercially available *E. coli* polar lipids (Avanti Polar Lipids Inc, Alabaster, AL) were dissolved in chloroform:methanol (1:4, v/v) and dried under nitrogen gas and vacuum for 30 min. The resulting lipid film was resuspended in a HEPES buffer containing 50 mM HEPES pH 7.5 containing 20 mM NaCl and 5 mM MgCl2 (HEPES buffer) at a concentration of 20 mg/ml. The lipid suspension was then passed through a 100-nm filter several times using an extruder device (Avestin, Ottawa, ON) to produce unilamellar liposomes. The liposomes were then diluted to 4 mg/ml and saturated with DDM to a final concentration of 2 mM. At this concentration of detergent, there was no noticeable change in the OD540 of the sample, indicating the saturation point has been reached before onset of solubilization (19). Purified MsbA was then added to a lipid to protein ratio of 50:1 (w/w) and incubated at room temperature for 30 min with gentle stirring. BioBeads SM-2 (Bio-Rad) were then added to the sample at 100 mg/ml followed by incubation for 2 h at room temperature. The above step was repeated once more followed by a third addition of BioBeads and the incubation period was continued for 16 h at 4 °C. Proteoliposomes were harvested by centrifugation at
molecule was calculated as 1:1. photometric detection based on an optimum extinction coeffi-
cient would release a free Kdo from the inner core oligosaccharide
previously (25). Briefly, the activity of the detergent-solubilized MsbA, 2
was used as described previously (25). Briefly, and reconstituted protein samples were quantified by the Bradford assay (Bio-Rad).

Colorimetric ATPase Assay—The method of Chifflet et al. (20) was employed for the ATPase assay. To determine the activity of the detergent-solubilized MsbA, 2 μg of the protein was added to a 100-μl reaction mixture in a buffer containing 50 mM HEPES pH 7.5, 200 mM NaCl, 10 mM MgCl₂, 5 mM 2-mercaptoethanol, and 0.05% DDM. For assays with the liposome-reconstituted protein, the above buffer was replaced with one without the 0.05% DDM. The amounts of ATP used in the entire experiments are as follows: 0–20 mM for establishing standards curves, 0.2–6 mM for assaying intrinsic ATPase activity of detergent-solubilized and liposome-reconstituted MsbA, and 4 mM for assessing the stimulatory effect of different forms of lipid A- core and for evaluating the effect of MsbA on antibiotic susceptibility. The reactions were incubated at 37 °C for 1 h and stopped by the addition of 100 μl of 12% (w/v) SDS, 3% (w/v) ascorbic acid and 1% (w/v) ammonium molybdate tetrahydrate. After 5 min incubation at room temperature, 100 μl of a solution containing 2% (v/v) acetic acid, 2% (w/v) sodium arsenite, and 2% sodium citrate was added to stop the reaction. Reactions were incubated for an additional 20 min and measured for absorbance at OD750.

Preparation of LPS—The standard hot aqueous-phenol method of Westphal and Jann (21) was used to prepare LPS from two well-defined LPS-mutants of P. aeruginosa PAO1, namely, a rmlC mutant which produces LPS that is truncated in the core region, designated as RT-LPS; and a wapR mutant that produces rough LPS with a complete core-oligosaccharide (R-LPS) (22). Both forms of LPS are devoid of O antigens. Dephosphorylation of the R-LPS was prepared by treating 100 mg of lyophilized LPS sample with concentrated hydrofluoric acid (HF, 48%) at 4 °C for 48 h. HF was then removed by evaporation under a stream of nitrogen until the sample was dried (about 20 h). Lyophilized LPS was then resuspended in water and dialyzed against 16 liters of deionized water for at least 48 h. Finally, the sample was lyophilized to recover the LPS and it was resuspended in 50% dimethylsulfoxide (Me₂SO). LPS prepared in this manner was stored at 4 °C until use. LPS was quantified by 3-deoxy-D-26941

MsbA of P. aeruginosa

Msba Is Essential to the Viability of P. aeruginosa—To determine the functional role of msbA, constructions of a knock-out mutant of msbA was attempted using insertional mutation and allelic replacement as described previously by Hoang et al. (18). A large number of gentamicin-resistant colonies were obtained indicating that the Gm<sup>+</sup> insertion was effective. However, upon streaking the gentamicin-resistance colonies on PIA containing 5% sucrose as counter selection, none of the colonies tested were able to grow on the specific medium, suggesting the presence of sacB from the gene-replacement vector and that the loss of functional msbA would result in a lethal phenotype. The genotype of these colonies was determined by colony PCR. As expected, the PCR results showed that the colonies were merodiploids containing a wild-type copy of msbA as well as a disrupted copy of this gene in their chromosome (data not shown). These merodiploid colonies were also resistant to carbenicillin, further indicating the incorporation of the entire vector (pEX18Ap) into the chromosome. The inability of these colonies to grow on sucrose even after extensive passages was the first evidence to suggest that msbA is an essential gene in P. aeruginosa. msbA was cloned into a low-copy-number plasmid pRK404 (26) and the resulting construct was transformed into a sucrose-sensitive merodiploid. Transformants were then subjected to growth in PIA containing sucrose and gentamycin to select for excision of the plasmid carrying sacB and the wild-type copy of msbA, leaving the mutated copy on the chromosome. Southern hybridization analysis showed that the sucrose-resistant colonies have lost the functional chromosomal copy of msbA and retained the disrupted copy (Fig. 1). However, the loss of the wild-type msbA could only occur when another functional copy is present in trans suggesting that msbA is an essential gene. Thus, in P. aeruginosa true mutants are not viable. Complementation of merodiploid isolates followed by Southern blot further confirmed the essential nature of msbA in this organism.

In Trans Supplementation of the Merodiploid Colonies with msbA<sub>Ec</sub>—To test whether msbA from E. coli K12 (W3110) could substitute for msbA, msbA<sub>Ec</sub> was PCR-amplified from genomic DNA and cloned into pRK404. This construct was then transformed into P. aeruginosa msbA<sub>Ec</sub> merodiploid cells. Most of the transformants were not able to grow on medium containing 5% sucrose. A few colonies that grew were streaked on LB agar supplemented with carbenicillin and LB agar supplemented with gentamicin. All the colonies tested were resistant to both carbenicillin and gentamicin. Upon repeating the experiment the colonies were still resistant to carbenicillin.
MsbA of P. aeruginosa

This suggested that the presence of msbA<sub>Ec</sub> in trans was unable to substitute for msbA and a functional copy of msbA is still required for viability. These results indicated that there are sufficient differences between msbA in these two bacterial species. To ensure that MsbA<sub>Ec</sub> is active in P. aeruginosa, ATPase activity of MsbA<sub>Ec</sub>-enriched membrane vesicles was measured, and the activity was observed to compare closely with those of MsbA-enriched membrane vesicles (supplemental Fig. S2).

Optimization of Overexpression, Solubilization, and Purification of MsbA—MsbA was overexpressed as a fusion protein with an N-terminal 6-histidine tag. Doerrler and Raetz (11) have reported that overexpression of MsbA<sub>Ec</sub> was accomplished by inducing the expression strain of bacteria with 1 mM IPTG for 3 h at 30 °C, whereas Steinfels et al. (12) was able to overexpress BmrA, a homologue in the Gram-positive bacterium Bacillus subtilis, using a condition of 0.4 mM IPTG at 25 °C for 4 h. Expression of MsbA could be detected using either of these published conditions, but the protein exhibited very low enzymatic activity (data not shown). Alternatively, overexpression of MsbA was achieved by inducing the cultures with 1 mM IPTG for 16 h at 15 °C. The yield of MsbA in the membranes prepared from E. coli BL21 (DE3) cells expressing the protein appeared to be lower as compared with the yield when the other two induction protocols were used. However, MsbA purified from cells after 15 °C induction showed ATPase activity/mg protein to be about 10-fold higher than when the protein was induced using the conditions reported for MsbA<sub>Ec</sub> and BmrA, respectively (11, 12). Membrane vesicles prepared this way were frozen at −80 °C in small aliquots. The ATPase activity of MsbA was stable upon storage under these conditions for up to three months (data not shown).

To select a suitable detergent for solubilization of MsbA, a number of detergents were examined including OG at 2% (w/v), LDAO at 2% (w/v), DDM at 1% (w/v) and Sarcosyl at 1% (w/v). Samples were then incubated with gentle stirring for 1 h. No difference in the amount of solubilized proteins was observed when the membranes were incubated with detergents for longer than 1 h. Sarcosyl was most effective for solubilizing MsbA from the membranes resulting in the highest yield of solubilized protein compared with the other three detergents (Fig. 2A, lanes 3). The extent in which MsbA could be solubilized by the remaining three detergents appeared to be similar (Fig. 2A, lanes 1, 2, and 4).

Although Sarcosyl was highly effective for solubilizing MsbA from the membrane preparations, it produced a rather low yield of purified protein. In contrast, significantly higher amounts of purified proteins, i.e. ~3 mg of purified MsbA from 500 ml of culture, were obtained when DDM or OG were used as detergents (Fig. 2B, lanes 1 and 4). Interestingly, the yield of purified MsbA was the lowest, at approximately five times less when LDAO was used to solubilize the membranes (Fig. 2B, lane 2). When different preparations of purified MsbA were assayed for intrinsic ATPase activity, LDAO-solubilized MsbA showed the lowest activity (3.51 nmol/mg/min), followed by Sarcosyl (10.31 nmol/mg/min), and OG-solubilized protein (25.73 nmol/mg/min) in ascending order. DDM-solubilized MsbA exhibited the highest ATPase activity (46.37 nmol/mg/min) (Fig. 2C). It is worth noting that during the purification step for all the detergent-solubilized membranes, DDM was used at 0.1% (w/v) final concentration, which was further reduced to 0.05% (w/v) during the buffer exchange. According to our observations, overexpression of MsbA at 15 °C with 1 mM IPTG for 16 h and solubilization of the resulting membrane vesicles with 1% (w/v) DDM resulted in the highest amount of functionally active pro-
tein. This condition was used for all subsequent analyses of this protein.

**Intrinsic ATPase Activity of Purified MsbA**—Using ATP concentrations up to about 5 mM, the kinetics of ATP hydrolysis activity of detergent-solubilized MsbA follows the Michaelis-Menten equation, and $V_{\text{max}}$ of 61.4 nmol/mg/min and $K_m$ of 573 $\mu$M were observed (Fig. 3A). These parameters are consistent with those reported for MsbA$_{Ec}$ in the reconstituted system and in the presence of lipid A (11).

**Intrinsic ATPase Activity of Reconstituted MsbA**—Reconstituted MsbA showed an increase in activity that correlated with increasing ATP concentrations (Fig. 3B). At 8 mM ATP, the $V_{\text{max}}$ of MsbA was recorded at 144.5 nmol/mg/min, and $K_m$ was at 4500 $\mu$M. Interestingly, the high ATP concentration of 8 mM had no inhibitory effect on ATPase activity of the reconstituted protein. This is in contrast to the case with the detergent-solubilized form of MsbA (data not shown). The kinetic parameters of reconstituted MsbA have been summarized in Table 1.

**ATPase Activity of MsbA Is Selectively Stimulated by Different Forms of Lipid A-Core**—MsbA that has been incorporated into liposomes were assayed for their ATPase activity in the presence of R-LPS and RT-LPS. Reaction conditions were similar to the assay for detergent-solubilized MsbA, except that no detergents or glycerol were included in the reaction buffer. Reaction mixtures were incubated for 1 h at a constant ATP concentration of 4 mM containing 10 mM MgCl$_2$. Both types of LPS were able to stimulate ATPase activity (Fig. 4, A and B), while empty liposomes used as negative controls showed no effect. The amount of Me$_2$SO used in the reactions never exceeded 10% of the total reaction volume because high concentration of Me$_2$SO appeared to interfere with the enzyme activity. RT-LPS stimulated ATPase activity in MsbA in a dose-dependent manner and linear relationship was observed up to 30 $\mu$M of RT-LPS (Fig. 4A). At this substrate concentration, the enzyme activity was increased about 2-fold compared with the intrinsic activity, i.e. without the influence of RT-LPS. A different pattern was observed when R-LPS were used as the substrate. ATPase activity increased about 4-fold at the lowest substrate concentration (4 $\mu$M) and then stayed at this level as the concentration of R-LPS was gradually increased to 30 $\mu$M (Fig. 4B). The effect of dephosphorylated R-LPS (HF-R-LPS) on ATPase activity of reconstituted MsbA was also investigated. This form of LPS was found to be a less potent stimulator of ATPase activity as compared with R-LPS (Fig. 4C). Only a very slight increase in ATPase activity was observed even when high concentration of dephosphorylated R-LPS (50 $\mu$M) was used. To test whether heterologous LPS was able to simulate the ATPase activity of MsbA, we used LPS from Salmonella typhi-

![FIGURE 3. Comparison of intrinsic ATPase activity of purified and reconstituted MsbA. Panel A represents the intrinsic ATPase activity of MsbA in detergent solution at low AT concentrations. The data fitted well to the Michaelis-Menten equation with a least squared value of 0.945. Panel B represents the kinetic data of reconstituted MsbA over a range of low ATP concentrations. The data were fitted to the Michaelis-Menten equation with a least square value of 0.950. No indication of cooperativity was observed when the data subjected to linear transformation according to the Hill equation. Error bars indicate S.E. (n = 3).](image)

**TABLE 1**

| Enzyme        | $V_{\text{max}}$ (nmol/mg/min) | $K_m$ (M) | $R^2$ | $V_{\text{max}}$ (nmol/mg/min) | $K_m$ (M) | $n^2$ | $R^2$ |
|---------------|--------------------------------|-----------|-------|--------------------------------|-----------|-------|-------|
| MsbA$_{Pa}$Deter$^c$ | 61.40                          | 573.50    | 0.945 | MsbA$_{Pa}$Recons$^d$           | 144.50    | 4500  | 0.950 |
| MsbA$_{Pa}$Recons$^d$ | 198.80                         | 3600      | 0.990 | Chi-MsbADeter$^e$              | 40.00     | 494.60| 0.930 |
| Chi-MsbARecom$^e$      | 40.00                          | 494.60    | 0.930 |                                 |           |       |       |

$^a$ Refers to the fit of the data to the corresponding equations.

$^b$ Refers to the Hill coefficient.

$^c$ Refers to the detergent solubilized form of protein.

$^d$ Refers to the reconstitution form of protein.

$^e$ Refers to the chimeric protein.
murium (St-LPS) that was purchased from Sigma and found that St-LPS had minimal effect on the activity of reconstituted MsbA<sub>Pa</sub>. In fact, the negligible stimulatory effect by St-LPS was similar to that observed in hydrofluoric acid-treated LPS (HF-R-LPS) from <i>P. aeruginosa</i> (supplemental Fig. S3).

**MsbA<sub>Ec</sub> but Not MsbA Increases the Resistance of <i>P. aeruginosa</i> to Erythromycin**—A recent study showed that expression of exogenous MsbA<sub>Ec</sub> in Gram-positive bacteria conferred resistance to erythromycin (27). We assessed the role of MsbA and MsbA<sub>Ec</sub> in antibiotic susceptibility of <i>P. aeruginosa</i> PAO1. Exogenous MsbA did not appear to have an effect on the susceptibility of PAO1 to the antibiotics tested. Interestingly, expression of MsbA<sub>Ec</sub> in <i>P. aeruginosa</i> decreased the susceptibility of the bacteria to erythromycin by 4-fold. Neither MsbA nor MsbA<sub>Ec</sub> had an impact on the susceptibility of <i>P. aeruginosa</i> to ciprofloxacin, tobramycin, or ofloxacin; which are commonly used in treatment of <i>P. aeruginosa</i> infections (Fig. 5A). However, both MsbA<sub>Ec</sub> and MsbA increased the susceptibility of <i>P. aeruginosa</i> to chloramphenicol (>5-fold). To investigate further, we measured the ATPase activity of reconstituted MsbA in the presence of varying concentrations of chloramphenicol (Fig. 5B). At high concentrations, this antibiotic decreased the ATPase activity of MsbA reaching half-inhibitory concentration at about 0.2 mM. Our results suggested that exogenous MsbA could confer resistance against erythromycin in Gram-negative bacteria.

**Intrinsic ATPase Activity of Purified and Reconstituted Chimeric MsbA**—At low concentrations of ATP, ATPase activity of Chi-MsbA, increased linearly similar to those shown in Fig. 6A. When the data were fitted into the Michaelis-Menten equation, the enzyme had a <i>V<sub>max</sub></i> value of 198.8 nmol/mg/min and <i>K<sub>m</sub></i> of 3600 μM. These values are much higher than those of MsbA in detergent solution but compared closely to those of the reconstituted MsbA (see Fig. 3B). The kinetic parameters of Chi-MsbA showed a Hill coefficient of 1.3, which marginally suggested cooperativity of this chimeric protein in detergent solution. The <i>V<sub>max</sub></i> at 141.20 nmol/mg/min was comparable to the <i>V<sub>max</sub></i> from the Michaelis-Menten model but the <i>K<sub>m</sub></i> value

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**FIGURE 4.** Effect of different forms of LPS on ATPase activity of reconstituted MsbA. RT-LPS (panel A) was a mild stimulator of MsbA ATPase while R-LPS (panel B) increased activity by 4-fold. SC<sub>50</sub> of R-LPS was estimated to be close to 2 μM. Dephosphorylated R-LPS (HF-R-LPS) did not have the same stimulatory effect on the ATPase activity (panel C). Error bars correspond to the S.E. (<i>n</i> = 3).

**FIGURE 5.** Role of MsbA and MsbA<sub>Ec</sub> in antibiotic susceptibility of <i>P. aeruginosa</i> PAO1. A, MsbA did not have an effect on resistance of PAO1 to the antibiotic tested. However, when exogenous MsbA<sub>Ec</sub> was introduced, the resistance of <i>P. aeruginosa</i> to erythromycin was increased by 4-fold. Both MsbA<sub>Ec</sub> and MsbA decreased the resistance of <i>P. aeruginosa</i> to chloramphenicol. The following antibiotics were used for the antibiotic susceptibility test: chloramphenicol (Chlo), erythromycin (Eryth), tobramycin (Tobr), ciprofloxacin (Cipro), ofloxacin (Oflax). B, effect of high concentrations of chloramphenicol on ATPase activity of reconstituted MsbA.
was reduced by 2-fold to 1900 μM. However, this behavior was not observed when this protein was reconstituted. No cooperativity was observed for the kinetics of MsbA in detergent solution or in the reconstituted form. Overall, there was no significant difference for the data to fit into either the Hill model or the Michaelis-Menten model. 

Reconstituted Chi-MsbA exhibited ATPase activity that followed the Michaelis-Menten curve (Fig. 6B). When data were fitted into this equation, \( V_{\max} \) at 40 nmol/mg/min and \( K_m \) at 495 μM were observed. These kinetic parameters were within a similar range as those obtained for the detergent-solubilized MsbA. In this reconstituted form no inhibition of activity of Chi-MsbA was observed, even at 8 mM ATP. The kinetic parameters of all the reactions performed in this study have been summarized in Table 1.

**DISCUSSION**

In *E. coli*, a deep rough mutant producing Kdo₂-lipid A linked to Kdo of the inner core has been isolated (28, 29). The recent study by Meredith *et al.* (5, 28, 29) showed that the requisite LPS structure in *E. coli* contains only the endotoxically inactive LPS precursor lipid IVₐ. In contrast, in *P. aeruginosa*, the core oligosaccharide region can be truncated by a few sugar residues in the outer core resulting in R-LPS phenotype, but cannot be excluded (3, 6). It is therefore critical for lipid A-core to be “flipped” to the other side of the membrane in this bacterium. From the *Pseudomonas* PAO1 genome database (the PseudoCAP project) *pa4997* is a putative msbA that its translated protein sequence shares 40% sequence identity and 64% similarity with MsbAEc, which is the best characterized MsbA so far. To assess the physiological role of msbA in *P. aeruginosa*, a well-proven gene replacement method was used to generate an msbA knock-out mutant (18). Several attempts were made to disrupt this gene with no success. The disruption of the chromosomal msbA can only be made when a functional copy of this gene was provided in trans, suggesting that msbA is an essential gene. LmrA from the Gram-positive bacterium *Lactococcus lactis* shares about 27 and 30% sequence identity to MsbAEc and MsbA, respectively. However, it shares overlapping substrate specificity with MsbAEc (30) and its mammalian homologue P-glycoprotein (31). In light of the results from these reports, we had anticipated that msbAEc could cross complement msbA. We observed the contrary as attempts to substitute msbA with msbAEc in cross-complementation experiment were not successful, suggesting that there are significant differences between them.

ATPase activity of several ABC transporters have been investigated in a number of studies (32–36) including one on MsbAEc (11). We characterized the enzymatic (ATPase) activity of MsbA in a detergent-soluble form and a reconstituted form. The kinetic parameters of MsbA are comparable to other ABC transporters (12, 33, 36, 37). Doerrler and Raetz (11) reported very low ATPase activity for MsbA when LDAO was used to solubilize MsbA. Further increases in ATP concentration resulted in a sharp decrease in enzymatic activity. Panel B, intrinsic ATPase activity of Reconstituted Chi-MsbA. The data were fitted into the Michaelis-Menten equation with a least squared value of 0.935, \( V_{\max} \) of 40 nmol/mg/min and \( K_m \) of 0.495. Empty liposomes were run in parallel and subtracted as background ATPase activity. Error bars indicate S.E. (n = 3).
TABLE 2
Comparison of kinetic parameters of several ABC transporters

| Enzyme            | \( V_{\text{max}} \) | \( K_m \) | \( V_{\text{max}}/K_m \) | Ref.   |
|-------------------|-----------------------|-----------|---------------------------|--------|
| MsbA\(_E\) - Recons\(^a\) | 37                     | 878       | 42.14                     | (11)   |
| MsbA\(_E\) - Deter\(^b\) | 61.4                   | 573       | 107.15                    | This work |
| MsbA\(_E\) - Recons\(^c\) | 144.5                  | 4500      | 32                        | This work |
| Chi-MsbA - Deter\(^b\) | 141.2                  | 1900      | 74.31                     | This work |
| Chi-MsbA - Recons\(^b\) | 40                     | 495       | 80.81                     | This work |
| Malf500GK2-Deter\(^d\) | 203                    | 15.7      | 12929.94                  | (33)   |
| Malf500GK2-Recons\(^d\) | 1920                   | 9600      |                           |        |
| MalfK2-Recons\(^d\) | 34.3                   | 168       | 204                       | (33)   |
| CFTR\(^e\) | 51.3                   | 989.7     | 51.83                     | (37)   |
| CFTR\(^f\) | 53.8                   | 303       | 177.56                    | (37)   |

\(^a\) Refers to the detergent-solubilized form of protein.
\(^b\) Refers to the reconstitution form of protein.
\(^c\) Refers to phosphorylated form of protein.

\(V_{\text{max}}\) and \(K_m\) values that compared closely with those of detergent-solubilized MsbA (refer to Fig. 6). The \(V_{\text{max}}\) of reconstituted Chi-MsbA matched closely to that of the MsbA\(_E\), but the \(K_m\) value was lower by almost 2-fold. Besides the differences in the behavior of the Chi-MsbA and MsbA in both the solubilized state and the reconstituted forms, Chi-MsbA showed similar efficiency of intrinsic ATPase activity as its solubilized form. This is in sharp contrast to MsbA, which exhibited a 4-fold reduction in its efficiency of ATPase activity when it is reconstituted in liposomes. As mentioned above, this tight regulation of ATPase activity of MsbA could be an energy saving strategy, which may not be as tightly regulated in MsbA\(_E\). The drastic differences in activity between Chi-MsbA and MsbA suggest that the MSDs play an important role on the ATPase activity. The MSD of the MsbA might influence the NBD of the cognate protein differently than it would influence the NBD of MsbA\(_E\), which may account for the differences observed between these two proteins.

In conclusion, we showed that msbA is an essential gene in P. aeruginosa, and msbA\(_E\) cannot be used to substitute msbA in vivo in cross-complementation experiments. The kinetics of ATPase activity of MsbA is different than MsbA\(_E\), regardless of whether the soluble form or the liposome-reconstituted form of the proteins was being compared. We have also shown that the activity of MsbA can be stimulated severalfold by R-LPS (lipid A linked to complete core oligosaccharides) and to a lesser extent with RT-LPS (lipid A-truncated core). Interest...
ingly, dephosphorylated lipid A-core did not stimulate the ATPase activity of this protein suggesting an important role of the phosphate substituents in the interaction with MsbA. Chi-MsbA had enzymatic characteristics that were different than MsbA. Unlike MsbA, Chi-MsbA exhibited similar enzymatic efficiency whether it is in reconstituted or in detergent-solubilized form. Finally, our results further demonstrated that MsbAEC, and not MsbA decreased susceptibility of P. aeruginosa to erythromycin.

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*MsbA of P. aeruginosa*