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Structure and Function of *Neisseria gonorrhoeae* MtrF Illuminates a Class of Antimetabolite Efflux Pumps

**Graphical Abstract**

MtrF is a sulfonamide efflux transporter

**Highlights**

- MtrF is a dimeric molecule with a unique architecture
- Each subunit of the transporter has nine transmembrane helices and two hairpins
- MtrF is capable of removing the metabolite *p*-aminobenzoic acid from bacterial cells
- *N. gonorrhoeae* MtrF is an antimetabolite efflux pump

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**In Brief**

Su et al. report the crystal structure of the *Neisseria gonorrhoeae* MtrF membrane protein, which belongs to the AbgT family of transporters. A combination of structural information and functional data indicate that MtrF is an antibiotic efflux pump mediating bacterial resistance to sulfonamide drugs.

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**SUMMARY**

*Neisseria gonorrhoeae* is an obligate human pathogen and the causative agent of the sexually transmitted disease gonorrhea. The control of this disease has been compromised by the increasing proportion of infections due to antibiotic-resistant strains, which are growing at an alarming rate. *N. gonorrhoeae* MtrF is an integral membrane protein that belongs to the AbgT family of transporters for which no structural information is available. Here, we describe the crystal structure of MtrF, revealing a dimeric molecule with architecture distinct from all other families of transporters. MtrF is a bowl-shaped dimer with a solvent-filled basin extending from the cytoplasm to halfway across the membrane bilayer. Each subunit of the transporter contains nine transmembrane helices and two hairpins, posing a plausible pathway for substrate transport. A combination of the crystal structure and biochemical functional assays suggests that MtrF is an antibiotic efflux pump mediating bacterial resistance to sulfonamide antimetabolite drugs.

**INTRODUCTION**

*Neisseria gonorrhoeae* is a gram-negative diplococcus that is found only in humans and causes the sexually transmitted disease gonorrhea. Given that it is a strictly human pathogen and can colonize both male and female genital mucosal surfaces and other sites, it has developed mechanisms to overcome antimicrobial systems of the host’s innate defense. One major mechanism that *N. gonorrhoeae* uses to resist antimicrobial agents is the expression of multidrug efflux pumps that recognize and actively export a variety of structurally unrelated toxic compounds from the bacterial cell, including antibacterial peptides, long-chain fatty acids, and several clinically important antibiotics (Lee and Shafer, 1999; Rouquette-Loughlin et al., 2003; Shafer et al., 1998, 2001; Unemo and Shafer, 2014).

The best characterized efflux system in *N. gonorrhoeae* is the MtrC-MtrD-MtrE multidrug efflux system (Delahay et al., 1997; Lucas et al., 1995; Hagman et al., 1995, 1997; Veal and Shafer, 2003; Warner et al., 2008). This system is similar to other efflux pumps of the resistance-nodulation-cell division (RND) superfamily (Tseng et al., 1999) possessed by many Gram-negative bacteria. MtrD (Bolla et al., 2014; Hagman et al., 1995, 1997; Hagman and Shafer, 1995) is the inner membrane transporter component of the tripartite RND pump. The complex is formed by interactions between MtrD, the periplasmic membrane fusion protein MtrC (Hagman et al., 1995, 1997; Veal et al., 2002; Janganan et al., 2013), and the outer membrane channel MtrE (Delahay et al., 1997; Lei et al., 2014b; Janganan et al., 2011, 2013). This powerful efflux complex mediates the export of several structurally diverse hydrophobic antimicrobial agents, such as antibiotics, nonionic detergents, antibacterial peptides, bile salts, and gonadal steroidal hormones (Delahay et al., 1997; Hagman et al., 1995, 1997; Shafer et al., 1998). In addition, the Mtr efflux system includes another inner membrane protein MtrF (Veal and Shafer, 2003; Folster and Shafer, 2005), which belongs to the AbgT family of transporters (Prakash et al., 1998, 2001; Unemo and Shafer, 2014). It has been proposed that MtrF cooperates with the MtrC-MtrD-MtrE complex to export certain antimicrobials by a yet-unknown mechanism (Veal and Shafer, 2003).

To date, approximately 13,000 putative transporters of the AbgT family have been identified. AbgT-type proteins are commonly found in Gram-negative bacteria such as *Salmonella enterica*, Gram-positive bacteria such as *Staphylococcus aureus*, as well as yeasts such as *Saccharomyces arboricola*. Surprisingly, among proteins in this diverse family, only *Escherichia coli* AbgT (Hussein et al., 1998; Carter et al., 2007) and *N. gonorrhoeae* MtrF (Folster and Shafer, 2005; Veal and Shafer, 2003) have been partially characterized. Thus far, there is no structural information available for this family of membrane proteins, obscuring the details of their function and mechanism.
The products of the *E. coli* *abg* genes have been shown to catalyze the uptake and cleavage of the folate catabolite *p*-aminobenzoyl-glutamate (Carter et al., 2007). Particularly, *E. coli* AbgT has been demonstrated to import the catabolite *p*-amino-benzoyl-glutamate for de novo folic acid synthesis (Carter et al., 2007). As *E. coli* AbgT and *N. gonorrhoeae* MtrF belong to the same family of transporters, there is a chance that the MtrF protein may also act as an importer to uptake *p*-aminobenzoyl-glutamate and related small molecules for the synthesis of the essential folate vitamin. However, since MtrF is needed for the high-level resistance of gonococci to hydrophobic antimicrobials, including erythromycin and TX-100 (Folster and Shafer, 2005), it seems more likely that their conformations are nearly identical to each other. The dimeric form of MtrF in the crystal lattice is in good agreement with the oligomerization state of this membrane protein in detergent solution in which it also assembles as a dimer (Figure S3).

**RESULTS AND DISCUSSION**

**Overall Structure of *N. gonorrhoeae* MtrF**

The *N. gonorrhoeae* MtrF transporter is composed of 522 amino acids, sharing 38% identity with *E. coli* AbgT (Figure S1). The crystal structure of this membrane protein was determined to a resolution of 3.95 Å (Table S1). Two molecules of MtrF, which assemble as a dimer, are found in the asymmetric unit (Figure S2; Figure 1). Superimposition of these two MtrF molecules gives an RMSD of 0.5 Å over 506 Cα atoms, indicating that their conformations are nearly identical to each other. The dimeric form of MtrF in the crystal lattice is in good agreement with the oligomerization state of this membrane protein in detergent solution in which it also assembles as a dimer (Figure S3).

(462–471) and TM9 (480–506)]. It should be noted that five of these TMs (TM1, TM2, TM3, TM7, and TM8) are broken into segments within the membrane. In addition, HP1 and HP2 are formed by relatively short helices, which are only long enough to span half of the membrane (Figures 1A and 1B). Interestingly, the intramembrane loops of several of these TMs and HPs are right next to each other, allowing the transporter to form an internal cavity within the membrane (Figures 1C and 1D). Noticeably, our structure indicates that the N- and C-terminal ends of MtrF are located at different sides of the inner membrane (Figures 1A and 1B). As there was no signaling peptide added when expressing the MtrF protein, the N-terminal end of this protein should be located at the cytoplasm. Thus, the C-terminal end of MtrF should be positioned within the periplasmic space. This is in good agreement with the predicted E. coli AbgT topology that the C-terminal end of this protein is located at the periplasm (Daley et al., 2005).

Each protomer of MtrF contains a relatively small periplasmic domain. This domain is made up of two long loops formed between TMs 1 and 2, and TMs 5 and 6, respectively. Below the inner leaflet of the membrane, a small cytoplasmic domain links TMs 4 and 5 together. This domain comprises a relatively long random loop and helix (α1) (Figure 1A).

Viewed in parallel to the membrane, the MtrF dimer is bowl shaped with a concave aqueous basin facing the intracellular solution (Figure 1B). The dimer is about 75 Å tall, 80 Å wide, and 50 Å thick, with the transmembrane portion of the transporter lying approximately in the middle. The rim of the basin is as large as 45 Å. The bowl-shaped structure is 25 Å in depth and deeply penetrates into the inner leaflet of the cytoplasmic membrane (Figure 1C). This basin probably allows aqueous solution to reach the midpoint of the membrane bilayer. Interestingly, a similar bowl-shaped structure has also been found in the glutamate transporter (Yernool et al., 2004). However, the bowl shape, which deeply penetrates into the membrane, is formed within the trimer of this transporter (Yernool et al., 2004).

The structure of the MtrF dimer can be roughly divided into the inner and outer cores (Figure 2). The inner core contains TMs 1, 2, 5, 6, and 7, creating a frame-like structure to house the outer core of the protein (Figure 2A). A portion of this inner core is responsible for forming a dimerization domain of the transporter. Viewed along the membrane normal, TMs 1b, 2a, 2b, 6, 7a, and 7b, as well as their counterparts from the next subunit, contribute to this distinct dimerization domain.

Noticeably, the outer core of the MtrF protomer, comprising TMs 3, 4, 8, and 9 as well as HPs 1 and 2, folds into a cylindrical structural feature (Figure 2A). Based on the crystal structure, the outer core cylinder forms a substrate-binding site and transport pathway. Based on the crystal structure, the outer core cylinder contributes to form a tunnel spanning approximately from the middle of the inner membrane up to the periplasmic space (Figures 2B and S4). This tunnel is surrounded with HPs 1 and 2, as well as TMs 3 and 8. Interestingly, this tunnel is connected to the cytoplasmic space through an opening located at the basin of the bowl-shaped structure. The loop regions of HP1, HP2, TM3, and TM8 form this opening. Several conserved residues, including D193, S417, W420, P438, R446, D449, and P457, are found to line the wall of the channel (Figure 2B). These residues may play an important role for the function.

**MtrF Is Capable of Exporting p-Aminobenzoic Acid from the Cell**

As E. coli AbgT has been shown to enable uptake of the folate catabolite p-aminobenzoyl-glutamate (Carter et al., 2007), we investigated whether E. coli cells expressing N. gonorrhoeae MtrF can grow in liquid minimal medium (containing 90.4 mM Na2HPO4, 22.0 mM KH2PO4, 8.5 mM NaCl, 0.1 mM CaCl2,
The data showed in (A) and (B) are the cumulative average of three successive type MtrF (p < 0.009).

Mutants of the MtrF transporter. Cells possessing the mutant transporter mtrF pressing double-knockout cells transformed with pET15b/abgT/D/pabA cells, transformed with either pET15b/abgT/D/mtrF or pET15b, could not grow in this liquid medium supplemented with p-aminobenzoyl-glutamate up to 1 mM. However, these cells were capable of growing in liquid minimal medium when supplemented with 30 nM PABA. Within the E. coli abg operon, there are two additional genes, abgA and abgB, located upstream of abgT. The products of these two genes are the AbgA and AbgB aminoaoyl aminohydrolases, which cleave the catabolite p-aminobenzoyl-glutamate imported by the AbgT transporter to form PABA for folic acid synthesis (Carter et al., 2007). In contrast, the mtr operon of N. gonorrhoeae does not possess abgA- or abgB-related genes (www.genome.ou.edu). Thus, our data suggest that MtrF and AbgT may function differently even though these two membrane proteins belong to the same family.

Given that PABA is an important precursor for folic acid synthesis, we thought that N. gonorrhoeae MtrF might enable uptake of PABA. It should be noted that PABA is capable of diffusing into bacterial cells through the membrane, participating as an intermediate in de novo synthesis of the essential vitamin folic acid. Nonetheless, we compared the radioactive PABA content in cells transformed with either pET15b/abgT/D/mtrF or the empty vector pET15b. Surprisingly, E. coli cells producing N. gonorrhoeae MtrF showed a significant decrease in the level of [3H]-PABA, in contrast to cells transformed with the empty pET15b vector (Figure 3A). Instead of an importer, the data strongly suggested to us that MtrF may act as an exporter, capable of expelling the intracellular PABA metabolite from the cell.

In order to determine whether the conserved MtrF residues, D193, S417, W420, P438, D449, and P457, lining the inner wall of the tunnel formed by each protomer are important for the function of the transporter, we mutated each residue to alanine, individually (Table S2). We expressed these mutant transporters in BL21(DE3)/JabgT/D/pabA. Western analysis suggested that the expression levels of these mutant transporters are comparable with that of wild-type MtrF (Figure S5). In addition, western analysis on the crude cell lysates indicates that the level of expression of the wild-type MtrF transporter was ~200 copies per cell (Figure S6). In comparison with the expression level of the AcrB pump, which is ~500 copies per cell (Tikhonova and Zgurskaya, 2004), the expression level of our MtrF proteins should be low enough for physiological measurement. We then measured the accumulation of [3H]-PABA in cells harboring the mutant transporters, D193A, S417A, W420A, P438A, D449A, and P457A. The results showed a significant increase in the levels of [3H]-PABA accumulation in cells possessing the mutant transporter D193A, W420A, P438A, D449A, or P457A, when compared with cells expressing wild-type N. gonorrhoeae MtrF (Figure 3B). However, cells expressing S417A and R426A only indicated a modest change in the [3H]-PABA concentration when compared with cells carrying wild-type MtrF (Figure 3B).

1.0 mM MgSO₄, 20.0 mM NH₄Cl, and 22.2 mM glucose) supplemented with p-aminobenzoyl-glutamate. Based on several studies on folic acid transporters (Eudes et al., 2010; Klaus et al., 2005; Salcedo-Sora et al., 2011), we made an E. coli knockout strain BL21(DE3)/JabgT/D/pabA that lacks the abgT gene (Hussein et al., 1998; Carter et al., 2007) to impede uptake of the catabolite p-aminobenzoyl-glutamate and pabA gene (Kaplan and Nichols, 1983) to impair intracellular synthesis of the metabolite PABA. We then transformed these double-knockout cells with pET15b/abgT/D/mtrF, expressing N. gonorrhoeae mtrF, or the empty vector pET15b. Surprisingly, the double-knockout E. coli BL21(DE3)/JabgT/D/pabA cells, transformed with either pET15b/abgT/D/mtrF or pET15b, could not grow in this liquid medium supplemented with p-aminobenzoyl-glutamate up to 1 mM. However, these cells were capable of growing in liquid minimal medium when supplemented with 30 nM PABA. Within the E. coli abg operon, there are two additional genes, abgA and abgB, located upstream of abgT. The products of these two genes are the AbgA and AbgB aminoaoyl aminohydrolases, which cleave the catabolite p-aminobenzoyl-glutamate imported by the AbgT transporter to form PABA for folic acid synthesis (Carter et al., 2007). In contrast, the mtr operon of N. gonorrhoeae does not possess abgA- or abgB-related genes (www.genome.ou.edu). Thus, our data suggest that MtrF and AbgT may function differently even though these two membrane proteins belong to the same family.

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We next investigated how residues D193, S417, W420, P438, R446, D449, and P457 affect the intracellular folic acid concentration. When transformed with plasmids expressing the mutant transporter W420A, folic acid production was restored in these cells and the level of intracellular folic acid concentration was similar to that of the double-knockout strain transformed with the empty vector (Figure 4). In addition, cells carrying the mutants, D193A, S417A, P438A, D449A, and P457A, indicated a significant increase in the level of folic acid concentration when compared with cells expressing wild-type MtrF (Figure 4). Again, the data indicate that these residues are important for the function of the MtrF efflux pump. However, for cells expressing R446A, the intracellular folic acid content was similar to that of cells carrying the wild-type MtrF transporter.

**MtrF Is an Antibiotic Efflux Pump**

As PABA is an important metabolite for producing the essential folic acid in bacteria, we questioned why the function of MtrF appears to be decreasing the intracellular PABA concentration. We postulated that the answer may lie in the ability of MtrF to protect bacterial cells by extruding antimetabolites that are structurally similar to PABA, specifically sulfonamides. Sulfonamides are antimicrobial agents, sometimes referred to as antimetabolites or growth factor analogs, which are designed to specifically inhibit the essential metabolic pathway for folic acid synthesis in bacterial pathogens. At the chemical level, sulfonamides are structurally similar to the metabolite PABA, making them ideal competitive inhibitors. Sulfonamides were used in the late 1930s and early 1940s to treat gonorrhea, but the rapid emergence of strains resistant to this class of drug resulted in its removal once penicillin became available (Unemo and Shafer, 2014); resistant strains were later found to contain mutations in _folP_ that encodes the target for sulfonamides. We therefore suspect that MtrF may be a drug efflux pump, which is capable of recognizing and extruding sulfonamide antimetabolites. In fact, we found that the minimum inhibitory concentration (MIC) of sulfanilamide for the _N. gonorrhoeae_ strain WV16 (an mtrF knockout strain) differed from that of the _N. gonorrhoeae_ parental strain FA140 by 2-fold (MIC of 250 versus 500 μg/ml, respectively). To determine whether MtrF behaves like a sulfonamide efflux pump, we used _E. coli_ as a surrogate host. We transformed BL21(DE3)_JabgT_JpabA_ with pET15b_UmtrF_ or pET15b and tested for the susceptibility of these transformants to four different sulfonamide antimetabolites, sulfamethazine, sulfadiazine, sulfathiazole, and sulfanilamide (Table S3). It is important to stress that in many instances expression of drug efflux pumps, including members of the RND (Tseng et al., 1999; Li et al., 1995; Rosenberg et al., 2000) and multidrug and toxic compound extrusion (MATE) superfamilies (Brown et al., 1999; Long et al., 2008; Rouquette-Loughlin et al., 2003), can have only modest changes (2-fold) in bacterial susceptibility to certain antimicrobials, while more significant changes in susceptibility to other agents can be observed in the same system. We found that the BL21(DE3)_JabgT_JpabA_ cells producing MtrF were 32-fold less sensitive to sulfamethazine when compared with BL21(DE3)_JabgT_JpabA_ cells containing the empty pET15b vector. In addition, this BL21(DE3)_JabgT_JpabA/pET15b_UmtrF_ transformant was found to be eight times more resistant to sulfamethazine, sulfadiazine, sulfathiazole, and sulfanilamide (Figure 4)
the dissociation constants, using isothermal titration calorimetry (ITC). The data indicate that well as PABA for the MtrF transporter were then determined using methazine, sulfadiazine, sulfathiazole, and sulfanilamide, as sensitive to sulfadiazine and sulfathiazole in comparison with cells expressing wild-type MtrF (Table S3). These data indeed agree with the idea that these residues are essential for the function of MtrF.

To confirm these drug-susceptibility testing results, we measured the accumulation of radioactive sulfonamide in BL21(DE3)::JabgT::pabA cells carrying pET15b::mtrF or pET15b. For these experiments, we compared the accumulation of $[^{3}H]$-sulfamethazine over time in these cells. As shown in Figure 5, the results indicate a much lower level of $[^{3}H]$-sulfamethazine accumulation in BL21(DE3)::JabgT::pabA cells producing MtrF, compared to controlled cells harboring the empty pET15b vector, suggesting that MtrF is capable of exporting sulfamethazine from the cell.

When transformed with plasmids expressing the mutant transporters, D193A, S417A, W420A, P438A, R446A, D449A, and P457A, the level of intracellular sulfamethazine accumulation were much higher than that of cells expressing wild-type MtrF. Each bar represents the mean of three different cultures. *Values of BL21(DE3)::JabgT::pabA/pET15b and BL21(DE3)::JabgT::pabA cells expressing the mutant transporters that are significantly higher than that of BL21(DE3)::JabgT::pabA/pET15b::mtrF expressing wild-type MtrF (p < 0.001).

MtrF Behaves as a PMF-Dependent Drug Efflux Pump

It has been suggested that members of the AbgT family use the proton-motive-force (PMF) to transport substrates across the membrane (Prakash et al., 2003). To elucidate whether N. gonorrhoeae MtrF is a PMF-dependent transporter, we measured the level of intracellular sulfamethazine accumulation in the presence of carboxyl cyanide m-chlorophenylhydrazone (CCCP), a de-coupler of the membrane proton gradient. After the addition of CCCP into the assay solution, the accumulation of $[^{3}H]$-sulfamethazine increased drastically in the MtrF-expressing cells (Figure 6A), suggesting the possibility that MtrF is PMF dependent.

We then investigated the accumulation level of radioactive sulfamethazine in strain BL21(DE3)::JabgT::pabA/pET15b::mtrF in the presence of NaCl or KCl. The concentrations of these metal ions were either 5 or 100 mM. In all cases, the levels of accumulation of $[^{3}H]$-sulfamethazine were similar to that in the MtrF-producing strain without the addition of any metal ions (Figures 5 and 6A), indicating that the function of MtrF is independent of Na$^{+}$ or K$^{+}$.

The drug accumulation data strongly suggest that MtrF is a PMF-dependent efflux pump. To test this possibility, we next measured the efflux of $[^{3}H]$-sulfamethazine that had accumulated in strain BL21(DE3)::JabgT::pabA/pET15b::mtrF over time, both in the absence and presence of Na$^{+}$. Cells were first loaded with $[^{3}H]$-sulfamethazine and CCCP was added to inhibit the pump. Cells were then re-energized by removing CCCP and

![Figure 5. Accumulation of Radioactive Sulfamethazine](image)
Conclusions

In this paper, we report the crystal structure of the *N. gonorrhoeae* MtrF transporter, which reveals a very distinct from all other families of transporters. Our experimental data strongly suggest that *N. gonorrhoeae* MtrF is a drug efflux pump, capable of removing sulfonamide antimetabolites and mediating resistance to this class of drugs in bacterial cells. It is likely that many members of the AbgT family of transporters may serve as antimetabolite efflux pumps to protect cells against these noxious agents.

**EXPERIMENTAL PROCEDURES**

Cloning, Expression, and Purification of *N. gonorrhoeae* MtrF

Briefly, the full-length MtrF membrane protein containing a 6xHis tag at the N terminus was overproduced in *E. coli* BL21(DE3)/acrB cells, which harbor a deletion in the chromosomal acrB gene, possessing pET15b<uint>mtm</uint>F. Cells were grown in 12 l of Luria broth (LB) medium with 100 μg/ml ampicillin at 25°C. When the OD<sub>600</sub> reached 0.5, the culture was treated with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to induce mtrF expression, and cells were harvested within 15 hr. The collected bacteria were resuspended in buffer containing 100 mM sodium phosphate (pH 7.2), 10% glycerol, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM PMSE and then disrupted with a French pressure cell. The membrane fraction was collected and washed twice with buffer containing 20 mM sodium phosphate (pH 7.2), 2 M KCl, 10% glycerol, 1 mM EDTA, and 1 mM PMSE and once with 20 mM HEPES-NaOH buffer (pH 7.5) containing 1 mM PMSE as described previously (Long et al., 2010). The membrane protein was then solubilized in 2% (w/v) n-dodecyl-β-D-maltoside (DDM). Insoluble material was removed by ultracentrifugation at 100,000 × g. The extracted protein was purified with a Ni<sup>2+</sup>-affinity column. The purified protein was dialyzed and concentrated to 20 mg/ml in a buffer containing 20 mM Na-HEPES (pH 7.5) and 0.05% DDM. The 6xHis tag at the N terminus was then cleaved by adding 5 U of thrombin (GE Healthcare) per mg of purified MtrF at room temperature for 20 hr. The protein was subsequently passed through a Ni<sup>2+</sup>-affinity column to remove the free 6xHis tag.

A final purification step was performed using a G200 size exclusion column loaded with buffer solution containing 20 mM Na-HEPES (pH 7.5) and 0.05% DDM. The purity of the MtrF protein (＞95%) was judged using 10% SDS-PAGE stained with Coomassie brilliant blue. The purified protein was then concentrated to 20 mg/ml in a buffer containing 20 mM Na-HEPES (pH 7.5) and 0.05% DDM.

For 6xHis selenomethionyl-substituted (SeMet)-MtrF protein expression, a 10 ml LB broth overnight culture containing *E. coli* BL21(DE3)/acrB/pET15-b<sup>b</sup>mtmF cells was transferred into 120 ml of LB broth containing 100 μg/ml ampicillin and grown at 37°C. When OD<sub>600</sub> reached value reached 1.2, cells were harvested by centrifugation at 6,000 rev/min for 10 min and then washed two times with 20 ml of M9 minimal salts solution. The cells were re-suspended in 120 ml of M9 media and then transferred into a 12 l pre-warmed M9 solution containing 100 μg/ml ampicillin. The cell culture was incubated at 25°C with shaking. When OD<sub>600</sub> reached 0.4, 100 mg/l of lysine, phenylalanine, and threonine, 50 mg/l isoleucine, leucine, and valine, and 60 mg/l of L-selenomethionine were added. The culture was incubated with 20 ml of M9 minimal salts solution. The cells were then harvested within 15 hr after induction. The procedures for purifying SeMet-MtrF were identical to those of the native protein.

**Crystallization of MtrF**

Crystals of the MtrF transporter were obtained using vapor diffusion. The MtrF crystals were grown at room temperature with the following procedures. A 2-μl protein solution containing 20 mg/ml MtrF protein in 20 mM Na-HEPES (pH 7.5) and 0.05% (w/v) DDM was mixed with a 2 μl of reservoir solution containing 30% PEG 400, 0.1 M sodium acetate (pH 5.0), 0.1 M magnesium acetate, 3% glycerol, and 1% (w/v) octyl glucose neoepentyl glycol (OOGN). The resulting mixture was equilibrated against 500 μl of the reservoir solution. The crystallization conditions for SeMet-MtrF were the same as those for native MtrF. Crystals of both MtrF and SeMet-MtrF grew to a full size in drops within a
month. Typically, the dimensions of the crystals were 0.2 x 0.2 x 0.2 mm. Cryoprotection was achieved by raising the PEG 400 concentration to 32%. Crystals of the Ta$_6$Br$_{12}^2+$ or W$_6$(μ-O)$_6$Cl$_6$Cl$_6^-$ clusters were prepared by incubating the crystals of MtrF in solution containing 32% PEG 400, 0.1 M sodium acetate (pH 4.6), 0.05 M magnesium acetate, 5% glycerol, 1% (v/v) ONG, 0.05% (v/v) DDM, and 0.5 mM Ta$_6$Br$_{12}^2$-2Br (Jena Bioscience) or 0.5 mM (NH$_4$)$_2$W$_6$(μ-O)$_6$Cl$_6$Cl$_6^-$ for 4 h at 25°C.

Data Collection, Structural Determination, and Refinement

All diffraction data were collected at 100K at beamline 24ID-C located at the Advanced Photon Source, using a Pilatus 6M detector (Dectris). Diffraction data were integrated using DENZO and scaled using SCALEPACK (Otwinowski and Minor, 1997). Crystals of MtrF belong to space group P6$_3$ (Table S1). Based on the molecular weight of MtrF (56.3 kDa), two molecules per asymmetric unit with a solvent content of 71.9% were expected. Data from a native crystal, two heavy-atom derivatives, namely, Ta$_6$Br$_{12}^2+$ and W$_6$(μ-O)$_6$Cl$_6$Cl$_6^-$ clusters, and a selenomethionyl-substituted (SeMet) crystal were used for phase determination (Table S1). Four tantalum (Ta$_6$Br$_{12}^2+$) cluster sites were identified using SHELXD (Schneider and Sheldrick, 2002) as implemented in the HKL2MAP package (Pape and Schneider, 2004). These phases were used for density modification maps created using CCP4 were employed to ascertain loop-regions based on the microbiological procedure of Wilson and Lactobacillus casei (Faia et al., 2011; Lei et al., 2014a) (which exists as a trimer in solution with the trimeric NorM multidrug efflux pump (Lu et al., 2013; Long et al., 1995). The concentrations of folic acid in BL21(DE3)pabA expressing the mutant transporters were measured using the same procedures.

GeL Filtration

A protein liquid chromatography Superdex 200 16/60 column (GE Healthcare) with a mobile phase containing 20 mM Na-HEPES (pH 7.5) and 0.05% (v/v) DDM was used in the gel filtration experiments. Blue dextran (Sigma-Aldrich) was used to determine the column void volume. As there are no commercially available membrane protein markers, we used the well-characterized N. gonorrhoeae NorM multidrug efflux pump (Lu et al., 2013; Long et al., 2009) (which exists as a monomer in solution with the monomeric molecular weight equals 50.6 kDa) and E. coli CusC outer membrane channel (Kušhtila et al., 2011; Lei et al., 2014a) (which exists as a trimer in solution with the trimeric molecular weight equals 147.7 kDa) as standards. The experiments were repeated for three times. Gel filtration suggested an average molecular weight of 112.1 ± 4.1 kDa for the MtrF transporter (Figure S3). This value is in good agreement with the theoretical value of 112.5 kDa for two MtrF protomers.

Construction of the Double-Knockout Strain

The double-knockout E. coli strain BL21(DE3)JabgTJpabA was produced from the BL21(DE3) strain using an RED disruption system as described by Datsenko and Wanner (2000). The JabgTJkan cassette, which was used to replace the chromosomal abgT gene, was produced by PCR and then introduced into pKD45/BL21(DE3) by electroporation. The knockout BL21(DE3)JabgTJkan strain was selected on LB plate containing 30 μg/ml kanamycin and verified by PCR. The kanamycin-resistant gene was then released to generate the BL21(DE3)JabgTJ knockout strain. The deletion of pabA from BL21(DE3)JabgTJ was done using similar procedures as described above to generate the final BL21(DE3)JabgTJpabA double-knockout strain.

Site-Directed Mutagenesis

We performed site-directed mutagenesis on residues D193, S417, W420, P438, R446, and P457 to generate single point mutants D193A, S417A, W420A, P438A, R446A, and P457A. The primers used for these mutations are listed in Table S2. All oligonucleotides were purchased from Integrated DNA Technologies in a salt-free grade.

Accumulation Assays of p-Aminobenzoic Acid

In brief, E. coli BL21(DE3)JabgTJpabA carrying pET15bSmntF or pET15b were grown in LB broth with 100 μg/ml ampicillin at 37°C. When OD$_{600}$ nm reached 0.5, the culture was treated with 0.2 mM IPTG to induce mtrF expression, and cells were harvested within 2 h. Cells were washed twice with buffer containing 50 mM potassium phosphate (pH 7.5), twice with buffer containing 100 mM Tris-HCl (pH 7.5), and then suspended in the same buffer to OD$_{600}$ nm of 1.5. [3H]-PABA (Moravek Biochemicals) was then added to a final concentration of 0.3 μM. Samples of 100 μl were taken at intervals, applied directly to prewetted glass-fiber filters, and washed twice with 5-ml aliquots of the same buffer; 0.5-μl glass-fiber filters (MFS) were used with a filter apparatus. Filters were then incubated for 30 min in scintillation fluid (ScintiVerse BD) and counted with a Packard Tri-Carb 1600TR liquid scintillation counter (PerkinElmer). For [3H]-PABA accumulations in cells expressing the MtrF transporter, the mutants, the procedures for sample preparation were the same as above.

Assays of Folic Acid

The concentration of folic acid in the double-knockout BL21(DE3)JabgTJpabA strain transformed with pET15bSmntF or pET15b was measured using Lactobacillus casei based on the microbiological procedure of Wilson and Home (1982). The susceptibilities to various drugs of E. coli were measured using the same procedures.

Drug-Susceptibility Assays

The susceptibilities to various drugs of E. coli BL21(DE3)JabgTJpabA harboring pET15bSmntF expressing the wild-type or mutant transporters, or the pET15b empty vector were tested on agar plates. Cells were grown in Luria broth (LB) medium with 100 μg/ml ampicillin at 37°C. When OD$_{600}$ nm reached 0.5, the cultures were induced with 0.2 mM IPTG and harvested in 2 h after induction. The MICs to sulfamethazine, sulfadiazine, sulfathiazole, and sulfinamide of E. coli BL21(DE3)JabgTJpabA (incoculum, 500 cells/ml) harboring these vectors were then determined using LB agar containing 50 μg/ml ampicillin, 0.1 mM IPTG, and different concentrations of these drugs.

For the MIC studies with N. gonorrhoeae cells, we used strains FA140 and WW16 (as FA140 but mtrF::kan) described by Veal and Shafer (2003). These strains were grown on GCB agar plates. The susceptibilities to sulfanilamide were performed using the agar dilution assay described by Haggan et al. (1995).

Isothermal Titration Calorimetry

We used ITC to examine the binding of a variety of ligands to the purified MtrF transporter. Measurements were performed on a VP-Microcalorimeter (MicroCal) at 25°C. Before titration, the protein was thoroughly dialyzed against buffer containing 20 mM Tris-HCl (pH 7.5) and 0.03% DDM. The protein concentration was determined using the Bradford assay. The protein sample was then adjusted to a final monomeric concentration of 40 μM. Ligand solution consisting of 1.0 mM sulfamethazine, sulfadiazine, sulfathiazole, sulfanilamide, or PABA in 20 mM Tris-HCl (pH 7.5) and 0.03% DDM was prepared.
as the titrant. The protein and ligand samples were degassed before they were loaded into the cell and syringe. Binding experiments were carried out with the protein solution (1.5 m M) in the cell and the ligand as the injectant. Ten-microliter injections of the ligand solution were used for data collection. Injections occurred at intervals of 300 s, and the duration time of each injection was 20 s. Heat transfer (µcal/s) was measured as a function of elapsed time (s). The mean enthalpies measured from injection of the ligand in the buffer were subtracted from raw titration data before data analysis with ORIGIN software (MicroCal). Titration curves were fitted by a nonlinear least-squares method to a function for the binding of a ligand to a macromolecule. Nonlinear regression fitting to the binding isotherm provided us the equilibrium binding constant ($K_d = 1/K_0$). Calorimetry trials were also carried out in the absence of MtrF in the same experimental conditions. No change in heat was observed in the injections throughout the experiment.

Accumulation Assays of Sulfamethazine

The procedures for [3H]-sulfamethazine accumulation were the same as those for [3H]-PABA accumulation. Cells were incubated with 75 nM [3H]-sulfamethazine in the presence of NaCl or KCl (5 or 100 mM) for 15 min and then applied directly to prewetted glass-fiber filters and washed immediately twice with 5-ml aliquots of the same buffer. Filters were then incubated for 10 min in scintillation fluid (ScintiVerse BD) and counted with a Packard Tri-Carb 1600TR liquid scintillation counter (PerkinElmer). For metal ion dependent experiments, cells were incubated with 75 nM [3H]-sulfamethazine in the presence of NaCl or KCl (5 or 100 mM) for 15 min and then applied directly to prewetted glass-fiber filters and washed immediately twice with 5-ml aliquots of buffer containing 100 mM Tris-HCl (pH 7.5) and 5 or 100 mM salt (NaCl or KCl).

Efflux Assays of Sulfamethazine

E. coli BL21(DE3)JabgT, jabgA carrying pET15bcmtrF or pET15b were grown in LB broth with 100 µg/ml ampicillin at 37°C. When OD_{600, nm} reached 0.5, the culture was induced with 0.2 mM IPTG, and cells were harvested within 2 hr. Cells were washed twice with buffer containing 50 mM potassium phosphate (pH 7.5), twice with buffer containing 100 mM Tris-HCl (pH 7.5) and then suspended in the same buffer to OD_{600, nm} of 15. CCCP was added to the cell suspension at a final concentration of 100 µM. Cells were then incubated for 10 min, pelleted, washed twice, and resuspended in the same buffer to OD_{600, nm} of 10. [3H]-sulfamethazine was then added to a final concentration of 37 nM. Cells were incubated for 15 min, and a final concentration of 0.2% glucose was then added to the cell suspension. When needed, a final concentration of 5 mM NaCl was also added at the same time. At various time points, samples of 100 µl were applied directly to prewetted glass-fiber filters, and washed twice with 5-ml aliquots of buffer containing 100 mM Tris-HCl (pH 7.5). Filters were then incubated for 30 min in scintillation fluid (ScintiVerse BD) and counted with a Packard Tri-Carb 1600TR liquid scintillation counter (PerkinElmer).

ACCESSION NUMBERS

Atomic coordinates and structure factors for the structure of MtrF have been deposited at the RCSB Protein Data Bank and are available under accession number 4R11.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.cellrep.2015.03.003.

AUTHOR CONTRIBUTIONS


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