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Functional Cloning and Characterization of the Multidrug Efflux Pumps NorM from *Neisseria gonorrhoeae* and YdhE from *Escherichia coli*▽†

Feng Long,[1] Corinne Rouquette-Loughlin,[2] William M. Shafer,[2,3] and Edward W. Yu[1,4]*

Molecular, Cellular, and Developmental Biology Interdepartmental Graduate Program, Iowa State University, Ames, Iowa 50011[1]; Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, Georgia 30322[2]; Laboratories of Microbial Pathogenesis, VA Medical Center, Decatur, Georgia 30033[3]; and Department of Physics and Astronomy, Iowa State University, Ames, Iowa 50011[4]

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Active efflux of antimicrobial agents is one of the most important adapted strategies that bacteria use to defend against antimicrobial factors that are present in their environment. The NorM protein of *Neisseria gonorrhoeae* and the YdhE protein of *Escherichia coli* have been proposed to be multidrug efflux pumps that belong to the multidrug and toxic compound extrusion (MATE) family. In order to determine their antimicrobial export capabilities, we cloned, expressed, and purified these two efflux proteins and characterized their functions both in vivo and in vitro. *E. coli* strains expressing *norM* or *ydhE* showed elevated (twofold or greater) resistance to several antimicrobial agents, including fluoroquinolones, ethidium bromide, rhodamine 6G, acriflavine, crystal violet, berberine, doxorubicin, novobiocin, enoxacin, and tetraphenylphosphonium chloride. When they were expressed in *E. coli*, both transporters reduced the levels of ethidium bromide and norfloxacin accumulation through a mechanism requiring the proton motive force, and direct measurements of efflux confirmed that NorM behaves as an Na^+-dependent transporter. The capacities of NorM and YdhE to recognize structurally divergent compounds were confirmed by steady-state fluorescence polarization assays, and the results revealed that these transporters bind to antimicrobials with dissociation constants in the micromolar region.

Neisseria gonorrhoeae is a gram-negative diplococcus which is found only in humans and causes the sexually transmitted disease gonorrhea. Since it is a strictly human pathogen and can colonize male and female genital mucosal surfaces and other sites, it has developed mechanisms to overcome host antimicrobial systems that are important in the innate host defense. One important mechanism that *N. gonorrhoeae* uses to subvert antimicrobial agents is the expression of multidrug efflux pumps that recognize and actively export a wide variety of toxic (often structurally unrelated) compounds, including antibacterial peptides, long-chain fatty acids, and several clinically useful antibiotics, from the bacterial cell (17, 32, 35, 36).

Recently, four efflux pumps have been identified in *N. gonorrhoeae*. One such pump is the MtrD inner membrane protein (21) that exists as a component of a tripartite resistance nodulation cell division (RND) efflux system (41). MtrD interacts with a periplasmic membrane fusion protein, MtrC, and an outer membrane channel, MtrE, to mediate the export of hydrophobic antimicrobial agents, including antibiotics, nonionic detergents, certain antibacterial peptides, bile salts, and gonadal steroidal hormones (7, 9, 10, 36). The FarB efflux pump (17), which belongs to the major facilitator (MF) family (8, 22, 31), recognizes antibacterial long-chain fatty acids and exports them out of the cell in conjunction with the FarA membrane fusion protein and the MtrE outer membrane protein channel (17). The MacB transporter was recently described (33), and it belongs to the ATP binding cassette transporter family (13). It is poorly expressed in wild-type gonococci due to a natural mutation in its promoter, but it can recognize and export certain macrolide antibiotics. Finally, *N. gonorrhoeae* contains the NorM efflux pump (32), which is a member of the multidrug and toxic compound extrusion (MATE) family of efflux transporters (5, 25, 32). The *N. gonorrhoeae* NorM transporter is homologous to NorM of *Vibrio parahaemolyticus* (25), YdhE of *Escherichia coli* (2, 25, 42), VmrA of *V. parahaemolyticus* (6), and VcrM of *Vibrio cholerae* (1, 27). As a multidrug efflux pump, the gonococcal NorM appears to recognize a number of cationic toxic compounds, such as ethidium bromide, acriflavine, 2-*N*-methylellipticinium, and ciprofloxacin (32). The capacity of NorM to export ciprofloxacin was suggested to be of clinical relevance in the development of fluoroquinolone resistance in *N. gonorrhoeae*, especially when isolates have other mutations that raise the MIC to a level near that seen in treatment failures.

Members of the MATE family of transporters characteristically possess 12 putative transmembrane domains and have been reported in all three kingdoms of life (*Eukaryae*, *Archaeae*, and *Eubacteriae*) (5). Phylogenetic tree analysis suggests that this family possesses three distinct clusters: (i) the bacterial multidrug efflux pumps, including *N. gonorrhoeae* NorM and *E. coli* YdhE; (ii) the eukaryotic efflux proteins found in fungi and plants, such as Erc1; and (iii) a branch

* Corresponding author. Mailing address: Department of Physics and Astronomy, A115 Physics, Iowa State University, Ames, IA 50011. Phone: (515) 294-4955. Fax: (515) 294-6027. E-mail: ewyu@iastate.edu.

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containing *E. coli* DinF (5). Among these three clusters, the putative NorM branch exhibits overall identity and similarity of 26 and 47%, respectively.

In order to directly test the capacity of the *N. gonorrhoeae* NorM pump to recognize putative substrates, including ciprofloxacin, which was, until recently, widely used in the United States to treat gonococcal infections (15), we expressed the gonococcal *norM* gene in *E. coli* and investigated its function by using drug susceptibility and transport assays. We also studied the homologous protein YdhE in *E. coli* and compared its function with that of *N. gonorrhoeae* NorM. Using a sensitive fluorescence polarization assay (12, 18), we characterized the interactions of these two transporters with various drugs. The results revealed that NorM and YdhE bind to a variety of structurally dissimilar agents in the micromolar range.

MATERIALS AND METHODS

Cloning of *norM* and *ydhE*. The *norM* open reading frame from the genomic DNA of *N. gonorrhoeae* strain FA19 was amplified by PCR with primers 5′-GAG GAATAATAAATGCTGCTCGACCTCGACCGC-3′ and 5′-GTTTAAACTCAA TGGTGATGGTGATGATGGACGGCCTTGTGTGATTTGACC-3′ to generate a product that would encode a NorM recombinant protein with a six-His tag at the C terminus (32). The corresponding PCR product was extracted from the agarose gel and cloned into pBAD-TOPO, as described by the manufacturer (Invitrogen). To remove the V5 epitope and polyhistidine region of the vector, the plasmid was digested with PmeI and then self-ligated to form the expression vector pBADΩ*norM*. The recombinant plasmid was transformed into DH5α cells, and transformants were selected on LB agar plates containing 100 μg/ml ampicillin. The presence of the correct *norM* sequence in the plasmid construct was verified by DNA sequencing.

Similarly, the open reading frame of *ydhE* from *E. coli* K-12 genomic DNA was amplified by PCR and TA cloned into pBAD-TOPO with primers 5′-GAGGA ATAATAAATGCAGAAGTATATCAGTG-3′ and 5′-GTTTAAACTCAATG GTGATGGTGATGATG GCGGGATGCTCGTTGCAGAATG-3′ to generate a PCR product that would encode a recombinant protein containing a six-His tag at the C terminus. The recombinant plasmid (pBADΩ*ydhE*) was transformed into DH5α cells as described above, and the presence of the correct insert in a representative plasmid transformant was verified by DNA sequencing.

Drug susceptibility assays. The MICs to various antimicrobial agents of *E. coli* strains (inoculum, 10^4 CFU/spot) harboring pBADΩ*norM*, pBADΩ*ydhE*, or the pBAD vector were determined by the twofold dilution method with LB agar containing the desired antimicrobial agent (28). Bacterial growth was recorded after 18 to 24 h of incubation at 37°C. Each assay was repeated at least four times to ensure the reproducibility of the results.

Drug accumulation assays. Assays of norfloxacin and ethidium bromide accumulation were performed as described previously (20, 24, 25). In brief, *E. coli* AG100AX cells carrying pBADΩ*norM*, pBADΩ*ydhE*, or pBAD were grown in LB broth with 0.01% L-arabinose at 37°C to an optical density at 600 nm (OD_{600}) of 1.0, harvested, washed three times with buffer containing 0.1 M Tris-HCl (pH 7.0), and suspended in the same buffer to an OD_{600} of 1.0. Norfloxacin was then added to a final concentration of 100 μM. Samples of 1 ml were taken at intervals, centrifuged at 10,000 rpm for 30 s at 4°C, and washed once with the same buffer. After 15 min, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added to a final concentration of 100 μM to disrupt the proton gradient across the membrane. Then, 15 min later, CCCP was removed by centrifugation at 10,000 rpm for 30 s and the cells were resuspended in buffer containing 0.1 M Tris-HCl (pH 7.0) and 0.4% glucose. Each pellet was resuspended in 1 ml of 100 mM glycine-HCl (pH 3.0), shaken vigorously for 1 h at room temperature to release the fluorescent content, and then centrifuged at 15,000 rpm for 10 min at room temperature. The fluorescence of the supernatants was measured at an excitation λ (λ_{ex}) and an emission λ (λ_{em}) of 277 and 448 nm, respectively, by using a Perkin-Elmer LS55 spectrofluorometer equipped with a Hamamatsu R928 photomultiplier. The amount of maximum fluorescence was normalized to 100%.

For ethidium bromide accumulation, the cells were prepared in a manner similar to that described above. To initiate the assay, ethidium bromide was added to the cell suspension at a final concentration of 20 μg/ml. Samples of 1 ml were taken at different time points. After 15 min of incubation, CCCP was added to a final concentration of 100 μM. Then, 15 min later, CCCP was removed by centrifugation at 10,000 rpm for 30 s and the cells were resuspended in buffer containing 0.1 M Tris-HCl (pH 7.0) and 0.4% glucose. The fluorescence of the samples was measured at a λ_{ex} and a λ_{em} of 500 and 580 nm, respectively (3). The amount of maximum fluorescence was normalized to 100%.

Accumulation of ethidium bromide in the presence of Na^+. *E. coli* AG100AX cells containing pBADΩ*norM* or pBAD were grown in LB broth with 0.01% L-arabinose at 37°C to an OD_{600} of 1.0, harvested by centrifugation, washed three times with buffer containing 0.1 M Tris-HCl (pH 7.0), and suspended in the same buffer to an OD_{600} of 1.0. Ethidium bromide was added to the cell suspension at a final concentration of 20 μg/ml, followed by the addition of 100 mM NaCl or KCl. At each time point, a 1-ml sample was removed, centrifuged at 10,000 rpm for 30 s at 4°C, and washed once with the same buffer. The fluorescence signal was measured at a λ_{ex} and a λ_{em} of 500 and 580 nm, respectively.

Efflux of norfloxacin in the presence of Na^+. For the determination of norfloxacin efflux, cells were grown in LB broth with 0.01% L-arabinose at 37°C to an OD_{600} of 1.0, harvested, and washed twice with buffer containing 0.1 M Tris-HCl (pH 7.0). To load the cells with norfloxacin, the bacteria were incubated in the same buffer supplemented with 100 μM norfloxacin and 20 μM CCCP at 37°C for 30 min, as described previously (30). The cells were then pelleted, washed twice, and resuspended in the same buffer to an OD_{600} of 2.0. Samples of 1 ml were taken at intervals, centrifuged at 10,000 rpm for 30 s at 4°C, and washed once with the same buffer. After 10 min, NaCl or KCl was added to a final concentration of 100 mM. Fluorescence measurement of norfloxacin was performed by a procedure similar to that described above.

Purification of transporters. The *N. gonorrhoeae* NorM protein containing a six-His tag at the C terminus was overproduced in *E. coli* TOP10 cells (Invitrogen) possessing pBADΩ*norM*. The cells were grown in 12 liters of LB medium with 100 μg/ml ampicillin at 37°C. When the OD_{600} reached 0.5, the culture was treated with 0.2% L-arabinose to induce *norM* expression and cells were harvested within 3 h. The bacteria collected were resuspended, as described previously (43), in low-salt buffer containing 100 mM sodium phosphate (pH 7.2), 10% glycerol, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (PMSF) and were then disrupted with a French pressure cell. The membrane fraction was collected and washed twice with a high-salt buffer containing 20 mM sodium phosphate (pH 7.2), 2 M KCl, 10% glycerol, 1 mM EDTA, and 1 mM PMSF and once with 20 mM HEPES–NaOH buffer (pH 7.5) containing 1 mM PMSF. The membrane protein was then solubilized in 1% (wt/vol) *n*-dodecyl-β-D-maltoside (DDM). Insoluble material was removed by ultracentrifugation at 370,000 × *g*. The extracted protein was loaded into an Ni^{2+}-affinity column; washed with a buffer containing 20 mM HEPES–NaOH (pH 7.5), 50 mM imidazole, and 0.02% DDM; and eluted with a buffer consisting of 20 mM HEPES–NaOH (pH 7.5), 400 mM imidazole, and 0.02% DDM. The eluted protein was then concentrated to 5 mg/ml and loaded into G-200 sizing columns preincubated with 20 mM HEPES–NaOH (pH 7.5) and 0.02% DDM for further purification. The YdhE protein that contains a six-His tag at the C terminus was overproduced in *E. coli* TOP10/pBADΩ*ydhE* and purified as described above for NorM. The purities (>90%) of the NorM and YdhE proteins were judged by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis with Coomassie brilliant blue staining.

Fluorescence polarization assays. Fluorescence polarization assays (12, 18) were used to determine the drug binding affinities of NorM and YdhE. The experiments were done with a ligand binding solution containing 20 mM HEPES–NaOH (pH 7.5), 0.02% DDM, and 1 μM ligand (rhodamine 6G, ethidium bromide, proflavine, ciprofloxacin, or norfloxacin). The protein solution, which consisted of NorM or YdhE in 20 mM HEPES–NaOH (pH 7.5), 0.02% DDM, and 1 μM ligand, was titrated into the ligand binding solution until the polarization was unchanged. As this is a steady-state approach, the fluorescence polarization measurement was taken after a 5-min incubation for each corresponding concentration of the protein and drug to ensure that the binding had reached equilibrium. It should be noted that the detergent concentration was kept constant at all times to eliminate the change in polarization generated by the drug-DDM micelle interaction. All measurements were performed at 25°C with a Perkin-Elmer LS55 spectrofluorometer equipped with a Hamamatsu R928 photomultiplier. The excitation wavelengths were 527, 483, 447, 330, and 277 nm for rhodamine 6G, ethidium, proflavine, ciprofloxacin, and norfloxacin, respectively. Fluorescence polarization signals (as the change in polarization) were measured at emission wavelengths of 550, 620, 508, 415, and 448 nm for these ligands, respectively. Each titration point recorded was an average of 15 measurements. Data were analyzed by using the equation $P = \{(P_{bound} - P_{free})[protein]/(K_D + [protein])\} + P_{free}$, where P is the polarization measured at a given total protein concentration, P_{free} is the initial polarization of free ligand, P_{bound} is the maximum polarization of specifically bound ligand, [protein] is the protein concentration, and K_D is the dissociation constant. The titration experiments were repeated three times to obtain the average K_D value. Curve

TABLE 1. Drug susceptibility

Drug	MIC (μg/ml)		
	AG100AX/ pBADΩ*norM*	AG100AX/ pBADΩ*ydhE*	AG100AX/ pBAD
Ethidium bromide	15.63	15.63	7.81
Ciprofloxacin	0.006	0.006	0.003
Rhodamine 6G	12.5	12.5	3.125
Chloramphenicol	0.625	0.625	0.625
TPP	25	25	12.5
Norfloxacin	0.032	0.016	0.008
Ofloxacin	0.013	0.013	0.013
Lomefloxacin	0.032	0.016	0.016
Tetracycline	1.25	1.25	1.25
Enoxacin	0.063	0.063	0.031
Doxorubicin	6.25	6.25	1.56
Benzalkonium	0.625	0.312	0.156
Novobiocin	3.125	3.125	1.56
Nalidixic acid	0.625	0.625	0.625
Crystal violet	2.5	2.5	0.625
CCCP	6.25	6.25	6.25
Rifampin	6.25	6.25	6.25
Streptomycin	>50,000	>50,000	>50,000
Methyl viologen	156.3	156.3	156.3
Vancomycin	250	250	250
Minocycline	0.625	0.625	0.625
Berberine	250	250	31.25
Acriflavine	7.82	7.82	3.91
Proflavine	12.5	12.5	6.25
Gentamicin	2.5	2.5	2.5

fitting was accomplished with the ORIGIN program (version 7.5; OriginLab Corporation, Northampton, MA).

RESULTS AND DISCUSSION

Drug specificity of NorM and YdhE in vivo. To determine if the *N. gonorrhoeae* NorM multidrug transporter is functionally expressed in *E. coli* and to compare its activity to that of a second member (YdhE) of the MATE family of efflux pumps, we transformed *E. coli* AG100AX, an AcrAB-deficient strain (29), with the empty pBAD vector, pBADΩ*norM*, or pBADΩ*ydhE*. We then tested the susceptibilities of the representative transformants bearing these plasmids to a panel of 24 structurally divergent antimicrobials (Table 1). These antimicrobials were selected on the basis of whether or not they are putative substrates of MATE family exporters in the NorM subclass. It is important to stress that in many instances the expression of drug efflux pumps, including members of the MATE superfamily (5), can result in only modest changes (twofold) in bacterial susceptibility to certain antimicrobials, while more significant changes in susceptibility to other agents can be observed in the same system. Indeed, in our drug testing experiments, we detected a range of changes in the susceptibilities of *E. coli* cells producing either NorM or YdhE.

Among the 24 antimicrobials tested, AG100AX cells expressing the *N. gonorrhoeae* NorM were less sensitive (two- to eightfold) than AG100AX cells containing pBAD to a subset of antibiotics (norfloxacin, doxorubicin, novobiocin, lomefloxacin, enoxacin, and ciprofloxacin), dyes (rhodamine 6G, ethidium bromide, acriflavine, and crystal violet), and quaternary ammonium compounds (berberine, benzalkonium, and tetraphenylphosphonium chloride [TPP]). The YdhE-producing cells displayed a similar drug susceptibility profile (with the exception of that to lomefloxacin) as that of the NorM-producing *E. coli* cells, suggesting that these two MATE transporters are functionally similar in vivo.

Like YdhE of *E. coli*, our drug susceptibility experiments showed that NorM functions as a multidrug efflux pump when it is expressed in *E. coli*. The MICs to 14 of the 24 antimicrobials (Table 1) were observed to increase by two- to eightfold in NorM-producing AG100AX bacteria, which lack the AcrB efflux pump. Similar differences (data not presented) in antimicrobial susceptibility were obtained in $AcrB^+$ *E. coli* TOP10 cells that were used to obtain recombinant NorM. In that the MICs of norfloxacin, ciprofloxacin, enoxacin, and lomefloxacin were raised in cells expressing NorM or YdhE, our results suggest that, like other MATE transporters, such as BexA of *Bacteroides thetaiotaomicron* (23), VcmA of *Vibrio cholerae* (16), NorM of *Vibrio cholerae* (37) and AbeM of *Acinetobacter baumannii* (40), fluoroquinolones might be a common substrate for MATE transporters in the NorM cluster.

NorM and YdhE reduce drug accumulation in bacteria. The observed (Table 1) capacity of NorM and YdhE to reduce the susceptibility of *E. coli* to norfloxacin, ciprofloxacin, and ethidium bromide is consistent with the drug recognition profiles of other members of the MATE efflux transporter family. To confirm the drug susceptibility testing results, we measured the levels of accumulation of norfloxacin and ethidium bromide in AG100AX cells that carried pBADΩ*norM*, pBADΩ*ydhE*, or pBAD. The results showed lower levels of norfloxacin (Fig. 1a) or ethidium bromide (Fig. 1b) accumulation in AG100AX cells producing NorM or YdhE compared to those in control cells harboring the empty pBAD vector.

Members of the MATE family use energy from the proton motive force (PMF) during export; and loss of the PMF inactivates pump activity, resulting in the enhanced accumulation of substrates, which translates to increased susceptibility to the agents normally recognized by the transporter. Accordingly, we also measured the levels of norfloxacin and ethidium bromide accumulation after the addition of CCCP, a decoupler of the membrane proton gradient. After the addition of CCCP into the assay solution, the levels of accumulation of norfloxacin (Fig. 1a) and ethidium bromide (Fig. 1b) increased drastically in the NorM- and YdhE-expressing cells, with the levels of accumulation being nearly the same in the three different strains within 5 min. It should be noted that the effect of CCCP is reversible simply by removing the CCCP and adding glucose to reenergize the AG100AX cells. The experiments were performed at least three times. Statistical comparisons were assessed by Student's *t* test, and *P* values of <0.05 were considered to indicate statistically significant differences (Fig. 1a and b).

NorM expressed in *E. coli* behaves as an Na^+ ion-dependent transporter. It has been suggested but not proven that NorM of *N. gonorrhoeae* is an Na^+-drug antiporter (32). Thus, we investigated the levels of accumulation of ethidium bromide in isogenic strains AG100AX/pBADΩ*norM* and AG100AX/pBAD in the presence of Na^+ or K^+. In AG100AX/pBADΩ*norM* cells, a lower level of ethidium bromide accumulation was observed in the presence of 100 mM NaCl than in the presence of 100 mM KCl (Fig. 2a). In contrast, we did not observe a difference in the levels of ethidium bromide accumulation in AG100AX/pBAD cells, regardless of the presence of NaCl or KCl (Fig. 2b). Furthermore, in the presence of NaCl, the level of accumulation of

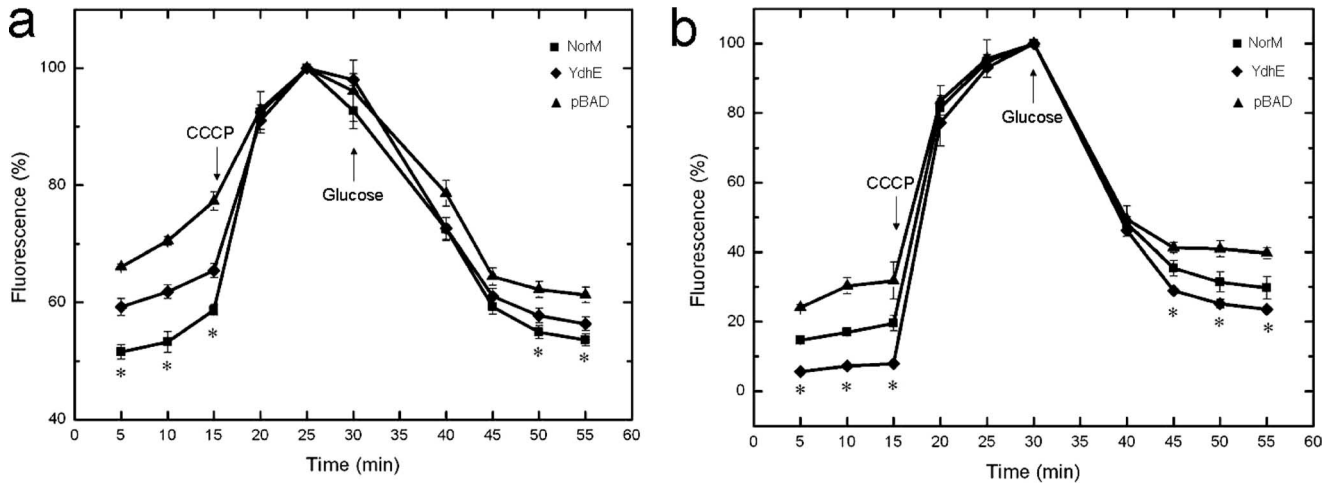

FIG. 1. NorM and YdhE reduce the levels of accumulation of norfloxacin and ethidium bromide in *E. coli* cells. (a) Accumulation of norfloxacin in cells transformed by NorM (AG100AX/pBADΩ*norM*), YdhE (AG100AX/pBADΩ*ydhE*), or an empty vector (AG100AX/pBAD). CCCP was added to the suspensions (first arrow) at a final concentration of 100 μM. After 15 min, glucose was added (second arrow) at a final concentration of 0.4%. (b) Accumulation of ethidium bromide in the same strains. CCCP was added to the suspensions (first arrow) at a final concentration of 100 μM. After 15 min, glucose was added (second arrow) at a final concentration of 0.4%. ∗, the values for AG100AX/pBADΩ*norM* and AG100AX/pBADΩ*ydhE* cells were significantly different from those for the control (AG100AX/pBAD) ($P < 0.05$).

ethidium bromide in the NorM-producing strain was lower than that in AG100AX/pBAD.

We next measured the efflux of norfloxacin that had accumulated in strain AG100AX/pBADΩ*norM* or AG100AX/pBAD in the presence of Na^+ or K^+. Cells were first loaded with norfloxacin; thereafter, 100 mM either NaCl or KCl was added to test the effect on drug efflux. As shown in Fig. 3a, the addition of 100 mM NaCl to AG100AX/pBADΩ*norM* cells resulted in a greater efflux of the drug from the cells compared to the levels of efflux observed in the presence of KCl or without added salt. This effect was NorM dependent because the addition of either NaCl (100 mM) or KCl (100 mM) to AG100AX/pBAD cells did not affect norfloxacin efflux (Fig. 3b). Taken together with the ethidium bromide accumulation results, our observations support the earlier proposal (32) that the gonococcal NorM protein is an Na^+-dependent transporter.

In order to directly test whether efflux of an antimicrobial by NorM is Na^+ dependent, we then studied the extrusion of the norfloxacin that had accumulated in AG100AX/pBADΩ*norM* in the presence of CCCP. Cells were first loaded with norfloxacin. Thereafter, different concentrations of NaCl (50 to 200 mM) were added to test their effects on drug efflux. All these tests were done in the presence of 20 μM CCCP. If NorM requires proton ion as a coupler, then the level of norfloxacin accumulated in the cell should not be reduced under these conditions. However, if the coupling ion is Na^+, we should be able to observe norfloxacin efflux in an [Na^+]-dependent man-

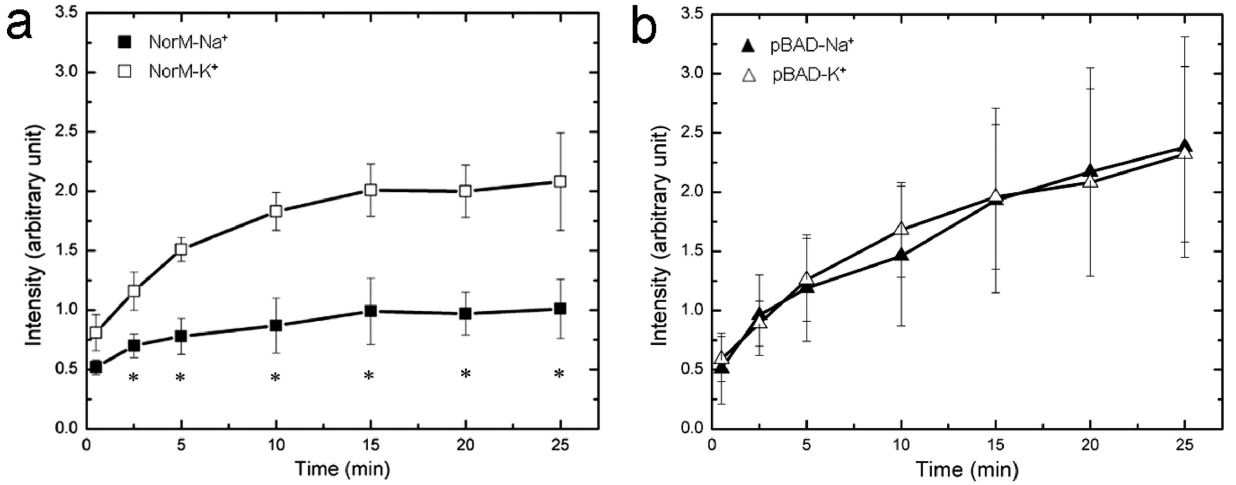

FIG. 2. Sodium ion reduces the level of accumulation of ethidium bromide in cells transformed by NorM. (a) Accumulation of ethidium bromide in AG100AX/pBADΩ*norM* cells after the addition of 100 mM NaCl or KCl; (b) accumulation of ethidium bromide in AG100AX/pBAD cells (cells carrying the empty vector) after the addition of 100 mM NaCl or KCl. ∗, the values of the ethidium bromide fluorescence intensity in the presence of 100 mM NaCl were significantly different from those in the presence of 100 mM KCl ($P < 0.01$).

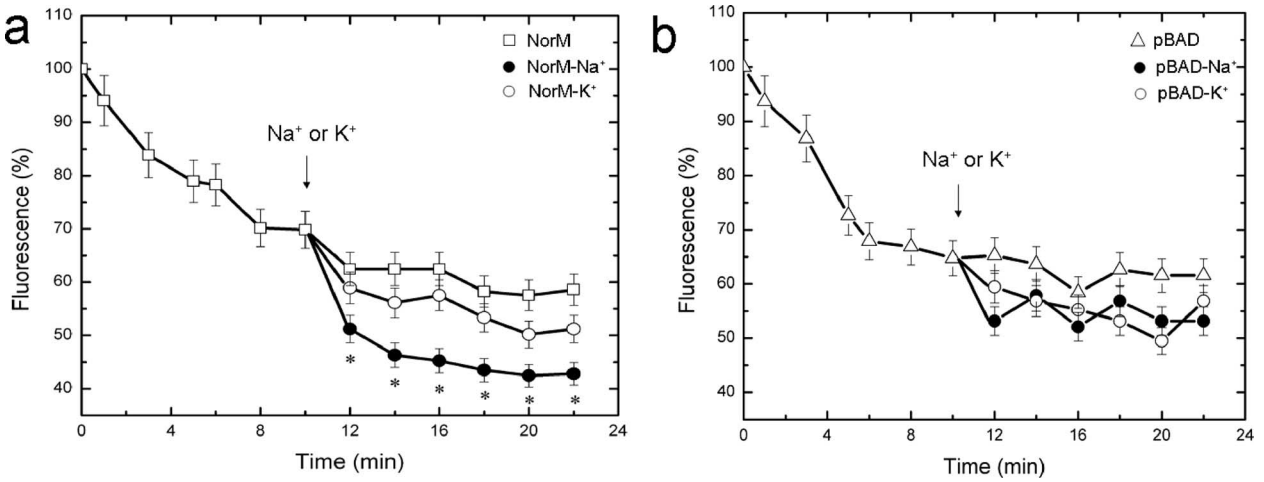

FIG. 3. Sodium ion enhances norfloxacin efflux via NorM. (a) Norfloxacin efflux from AG100AX/pBADΩ*norM* cells carrying the *norM* gene from *N. gonorrhoeae*; (b) norfloxacin efflux from AG100AX/pBAD cells carrying the empty vector. NaCl or KCl was added to the suspensions (arrow) at a final concentration of 100 mM. ∗, the values of norfloxacin fluorescence in the presence of 100 mM NaCl were significantly different from those in the presence of 100 mM KCl ($P < 0.05$).

ner. Figure 4 depicts the effect of Na^+ on the extrusion of norfloxacin. The experiment suggests that efflux activity was stimulated by the Na^+ ion. The addition of NaCl resulted in a significant change in norfloxacin efflux. A previous study with *Pseudomonas aeruginosa* PmpM, an H^+-dependent transporter in the MATE family, indicated that the Na^+ ion has no effect on drug transport (11). Thus, it is highly likely that Na^+ is the coupling cation for NorM.

Our drug accumulation and efflux assays confirm the results from an earlier report which suggested that NorM of *N. gonorrhoeae* is an Na^+-drug antiporter (32). First, Na^+ was found to enhance the ability of *E. coli* cells producing NorM to maintain a lower level of accumulation of ethidium bromide compared to the levels of accumulation by cells not producing NorM, even in the presence of Na^+ (Fig. 2). Hence, drug export mediated by NorM of *N. gonorrhoeae* is driven by an Na^+ gradient. This export mechanism is also influenced by the integrity of the PMF of the cytoplasmic membrane, since CCCP, a proton conductor, could significantly enhance the level of accumulation of ethidium bromide in NorM-producing bacteria. These accumulation assays, however, provide indirect evidence for efflux. Accordingly, direct support for efflux was obtained in experiments that measured the level of norfloxacin export by NorM-producing *E. coli* cells. The observed Na^+-dependent capacity of NorM to mediate the export of norfloxacin (Fig. 3 and 4) provides direct evidence for this model.

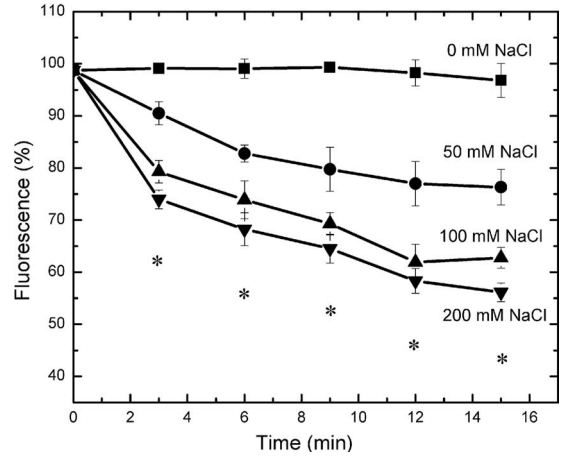

FIG. 4. Effect of Na^+ concentration on norfloxacin extrusion via NorM. Norfloxacin efflux from AG100AX/pBADΩ*norM* cells carrying the *norM* gene was measured in the presence of 0 to 200 mM NaCl. ∗, the values of norfloxacin fluorescence in the presence of 50, 100, or 200 mM NaCl were significantly different from those in the absence of NaCl ($P < 0.003$), and the values of norfloxacin fluorescence in the presence of 100 or 200 mM NaCl were significantly different from those in the presence of 50 mM NaCl ($P < 0.01$).

Binding affinities of different drugs in vitro. The results of the in vivo antimicrobial susceptibility tests (Table 1) and the accumulation/efflux studies (Fig. 1 to 4) suggested that NorM and YdhE recognize and export structurally diverse compounds. Little is known, however, about the detail of substrate binding by these MATE transporters. In order to directly test the capacities of these proteins to bind to such diverse compounds, we used a fluorescence polarization assay to quantify the binding affinities of several compounds to NorM and YdhE in vitro. This technique has been found to be sensitive and precise enough and allows us to detect for the first time the ligand binding of the *E. coli* AcrB multidrug efflux pump (an RND transporter) in a detergent environment (39). As an example of the results obtained by this protocol, Fig. 5a illustrates the binding isotherm of NorM in the presence of rhodamine 6G. As presented in Fig. 5a, a simple hyperbolic curve was observed for the binding of rhodamine 6G, and the K_D was 3.4 ± 0.2 μM. A Hill plot of the data yielded a Hill coefficient of 1.01 ± 0.03 (Fig. 5b), suggesting a simple drug binding process with a stoichiometry of one NorM monomer per rhodamine 6G ligand. When the data were presented as a Scatchard plot (data not shown), it indicated a similar K_D of 3.6 ± 0.1 μM with no sign of heterogeneity in binding sites. In addition, the titration experiments indicated that YdhE binds to

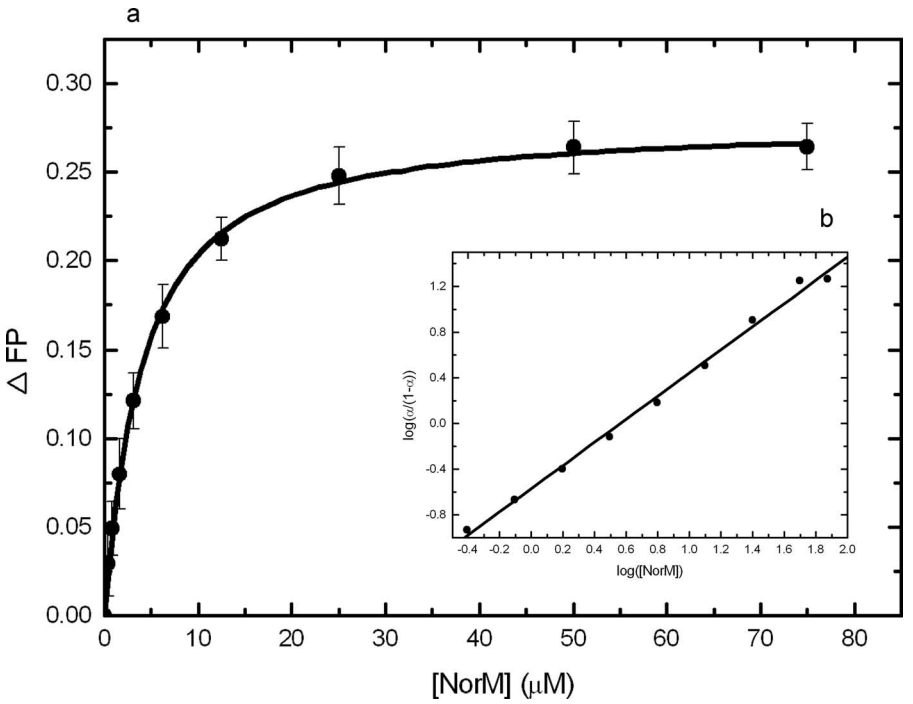

FIG. 5. Representative fluorescence polarization of NorM in 0.02% DDM with rhodamine 6G. (a) Binding isotherm of NorM with rhodamine 6G showing a K_D of 3.4 ± 0.2 μM in buffer containing 20 mM HEPES–NaOH (pH 7.5) and 0.02% DDM. (b) Hill plot of the data obtained for rhodamine 6G binding to NorM. α, the fraction of bound rhodamine 6G. The plot gives a slope of 1.01 ± 0.03, indicating a simple binding process with no cooperativity. The interception of the plot provides a K_D of 3.6 ± 0.1 μM for rhodamine 6G binding.

rhodamine 6G with a K_D of 3.0 ± 0.2 μM (Fig. 6a). This value is comparable to that of NorM, suggesting that these two transporters have similar affinities for this ligand. The Hill plots (Fig. 6b) and the Scatchard plots (data not shown) of the data indicated that the transporter employs a simple binding stoichiometry of a 1:1 monomeric YdhE-to-rhodamine 6G molar ratio.

Fluorescence polarization assays were also employed to elucidate the interaction of a variety of drugs with the NorM and YdhE transporters. The titration experiments indicated that NorM binds to ethidium bromide, proflavine, ciprofloxacin, and norfloxacin with K_Ds of 12.3 ± 1.3 μM, 33.6 ± 1.9 μM, 121.3 ± 15.7 μM, and 105.6 ± 9.8 μM, respectively (Table 2). These values are in the same range as those of YdhE, which binds to ethidium bromide, proflavine, ciprofloxacin, and norfloxacin with K_Ds of 9.8 ± 0.9 μM, 22.1 ± 0.9 μM, 90.9 ± 12.4 μM, and 98.4 ± 16.2 μM, respectively (Table 2). This binding was not affected by detergent, since similar K_Ds for the rhodamine 6G-YdhE complex were obtained in reaction mixtures that included 0.2%, 0.02%, and 0.0075% DDM.

To verify the validity of the fluorescence polarization assay, we studied the binding affinity of rhodamine 6G to the purified, detergent-solubilized YdhE efflux pump using equilibrium dialysis. The dialysis data suggest a K_D of 3.0 ± 0.1 μM, with a stoichiometry of a 1:1 monomeric YdhE-to-ligand molar ratio, for the binding of YdhE to rhodamine 6G (see Fig. S1 in the supplemental material). This result is in good agreement with that observed by the fluorescence polarization assay, in which the K_D of YdhE-rhodamine 6G is 3.0 μM with a 1:1 binding stoichiometry. Apparently, NorM and YdhE bind to these drugs with similar affinities. These values are similar to the K_D values for TPP binding in MdfA, an MF transporter, and EmrE, an SMR transporter, as determined by radiolabeled binding assays (19, 26), as well as the most recently determined K_Ds for AcrB, an RND transporter, with four different ligands, as determined by fluorescence polarization assays (39).

The results from the fluorescence polarization and equilibrium dialysis assays suggest that NorM and YdhE use a binding stoichiometry of a 1:1 monomeric transporter-to-ligand ratio. Thus, it is very likely that these transporters employ a simple drug binding process with no cooperativity. There is, however, a possibility that drug molecules may bind to more than one site in the proteins with similar affinities that may not be able to be distinguished by these techniques. Further experiments by different approaches may need to confirm this drug binding stoichiometry.

pH dependence of drug binding to NorM. We performed fluorescence polarization experiments to determine the binding of rhodamine 6G to NorM at different pH values. Figure 7 illustrates the decreases in the K_D values for rhodamine 6G as the pH increases from 5.8 to 8.5. Within this pH range, the K_D value decreases by about 2.6 μM. This change is quite modest compared with the effect of protons on the PMF-dependent efflux pumps AcrB (39) and EmrE (26) in *E. coli*. The binding affinities for drugs in these two transporters were strikingly dependent on the pH. In the case of AcrB, within a pH range from 5.5 to 8.4, the fluorescence polarization assay results suggest that the change in K_D for AcrB-rhodamine 6G reached 10.3 μM. As NorM shows a pH-dependent profile very differ-

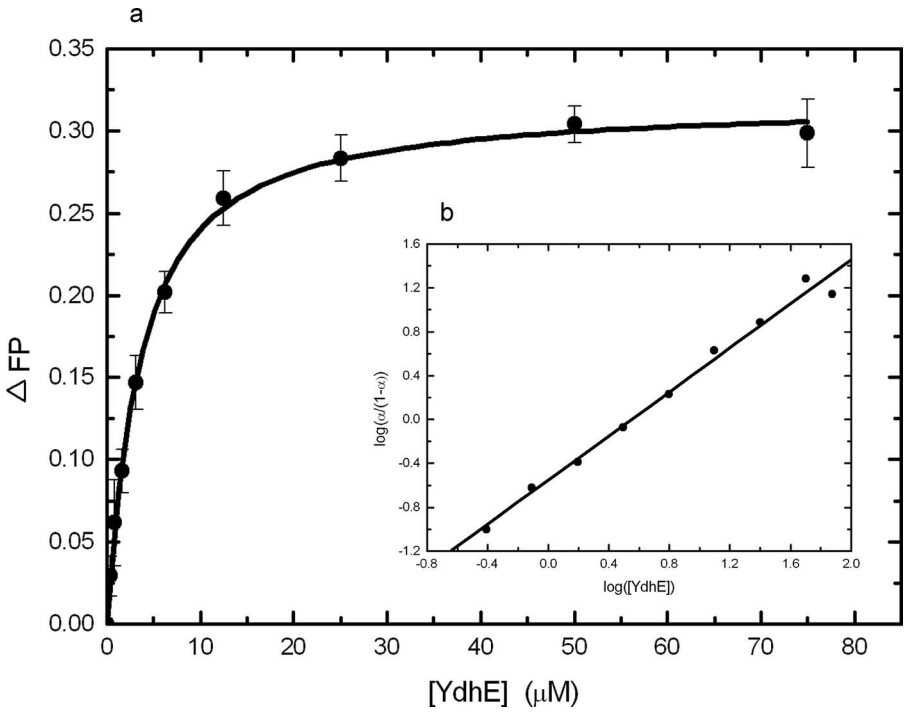

FIG. 6. Representative fluorescence polarization of YdhE in 0.02% DDM with rhodamine 6G. (a) Binding isotherm of YdhE with rhodamine 6G showing a K_D of 3.0 ± 0.2 μM in buffer containing 20 mM HEPES–NaOH (pH 7.5) and 0.02% DDM. (b) Hill plot of the data obtained for rhodamine 6G binding to YdhE. α, the fraction of bound rhodamine 6G. The plot gives a slope of 1.00 ± 0.04, indicating a simple binding process with no cooperativity. The interception of the plot provides a K_D of 3.6 ± 0.2 μM for rhodamine 6G binding.

ent from the profiles of the PMF-dependent pumps AcrB and EmrE, it is very likely that NorM does not use H^+ as an energy-coupling ion. Since NorM tends to bind to positively charged drugs, we expect that its drug-binding pocket should consist of at least one acidic residue. Indeed, the protein sequence of NorM suggests that this transporter consists of two negatively charged residues, Glu 261 and Asp 377, in the transmembrane region. These acidic residues could potentially form the multidrug binding site. Thus, the modest pH effect on NorM could be accounted for by the protonation state of the acid residue(s) in the binding pocket.

Na^+ dependence of drug binding to NorM. As the results of in vivo studies of drug accumulation and efflux suggest, NorM is a Na^+-dependent transporter, and the presence of Na^+ ions may affect the drug binding affinity in vitro. We therefore investigated the Na^+ dependence of drug binding using fluorescence polarization. As shown in Fig. 8, the K_D for rhodamine 6G is Na^+ dependent when the concentration of Na^+ is in the submicromolar range. In this region, the K_D for rhodamine 6G was a simple hyperbolic function of the Na^+ concentration. The curve showed saturability over 0.01 to 100 mM. Like the pH effect, the change in K_D for rhodamine 6G due to the presence of the Na^+ ion is quite small. Within 0 to 100 mM Na^+, the change in K_D reached only 2.5 μM (Fig. 8). We also added various concentrations of NaCl ranging from 100 to 400 mM (data not shown) and observed no significant change in the K_D.

TABLE 2. K_Ds and Hill coefficients of NorM and YdhE with five different transported drugs

Drug	NorM		YdhE	
	K_D (μM)	Hill coefficient	K_D (μM)	Hill coefficient
Rhodamine 6G	3.4 ± 0.2	1.01 ± 0.03	3.0 ± 0.2	1.00 ± 0.04
Ethidium bromide	12.3 ± 1.3	0.96 ± 0.05	9.8 ± 0.9	0.97 ± 0.05
Profloxacin	33.6 ± 1.9	0.94 ± 0.03	22.1 ± 0.9	1.02 ± 0.01
Ciprofloxacin	121.3 ± 15.7	1.08 ± 0.06	90.9 ± 12.4	1.09 ± 0.06
Norfloxacin	126.3 ± 11.3	1.06 ± 0.03	98.4 ± 16.2	1.14 ± 0.05

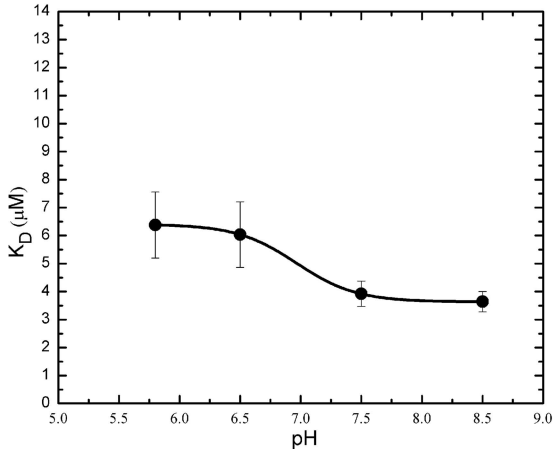

FIG. 7. Effect of pH on the K_D of rhodamine 6G binding to NorM. The resulting K_Ds were plotted against the pH.

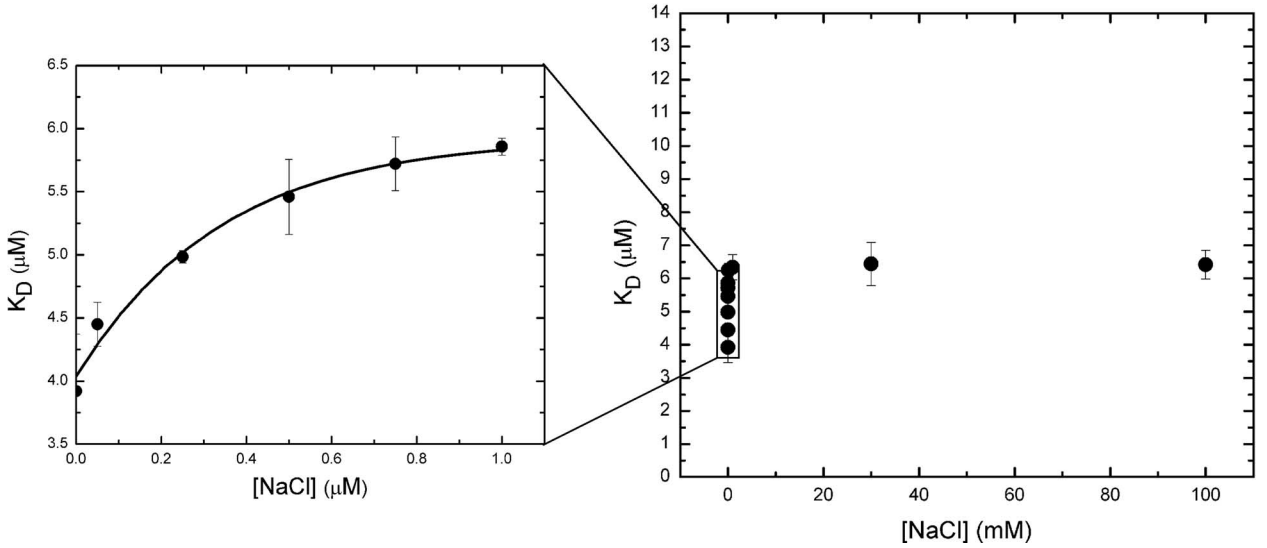

FIG. 8. Effect of Na^+ concentration on the K_D of rhodamine 6G binding to NorM. The resulting K_Ds were plotted against the NaCl concentration.

Competition binding of different drugs in YdhE. To confirm that the drug molecules bind specifically in the YdhE transporter, we performed competition experiments in which TPP was titrated into a solution containing the preformed YdhE-rhodamine 6G complex. In this case, TPP was chosen as a second ligand to knock off the bound rhodamine 6G from YdhE. The absorption spectra of TPP (from 200 to 600 nm) showed that this molecule absorbs light at wavelengths of 224.9, 268.0, and 275.9 nm. At a λ value of 527 nm, which is the λ_{ex} for rhodamine 6G, the energy is too low to excite TPP. Thus, TPP was treated as a nonfluorescent ligand in the "knock-off" experiments. The data revealed that TPP was able to bind to YdhE and replace the bound rhodamine 6G molecule from the protein, as demonstrated by the release of rhodamine 6G observed from the reduction of polarization (Fig. 9). This binding assay provides direct evidence that TPP interferes with the binding of rhodamine 6G, possibly by a direct competitive binding process for the same binding site. Alternatively, the release of rhodamine 6G by TPP may be due to an allosteric interaction between distinct binding sites of these two ligands. Regardless, the titrations demonstrate that rhodamine 6G is bound specifically in the YdhE transporter.

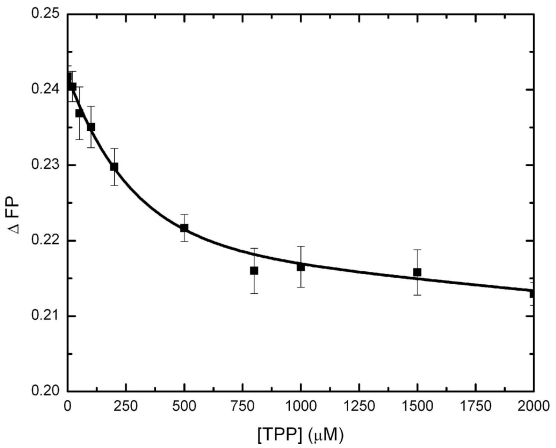

FIG. 9. YdhE binding competition experiment between rhodamine 6G and TPP. YdhE (5 μM) was preincubated with rhodamine 6G (1 μM) for 2 h before titration. The change in the fluorescence polarization signals (ΔFP) of rhodamine 6G was measured at an emission wavelength of 550 nm. TPP was nonfluorescent under the experimental conditions used. The decrease in the change in the fluorescence polarization signals showed that the bound rhodamine 6G was knocked off by TPP.

We have determined the binding affinities of five different drugs to the purified, detergent-solubilized NorM and YdhE proteins using fluorescence polarization assays. We note that fluorescence polarization has been widely used to study protein-DNA interactions (12, 14, 18) and protein-ligand interactions in transcriptional regulators (4, 34, 38). To our knowledge, this is the first attempt to use this methodology to investigate the interaction between MATE proteins and their transported substrates. This approach also allows us, for the first time, to quantify the strength of the transporter-drug interaction among the MATE family of transporters.

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