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Analysis of the Topology of *Vibrio cholerae* NorM and Identification of Amino Acid Residues Involved in Norfloxacin Resistance▽†

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NorM, a putative efflux pump of *Vibrio cholerae*, is a member of the multidrug and toxic compound extrusion family of transporters. We demonstrate that NorM confers resistance to norfloxacin, ciprofloxacin, and ethidium bromide. Inactivation of *norM* rendered *V. cholerae* hypersensitive towards these fluoroquinolones. Multiple sequence alignment of members of its family identified several regions of high sequence conservation. The topology of NorM was determined using β-lactamase and chloramphenicol acetyltransferase fusions. The amino acid residues G^{184}, K^{185}, G^{187}, P^{189}, E^{190}, G^{192}, and G^{195} in the periplasmic loops and L^{381}, R^{382}, G^{383}, Y^{384}, K^{385}, and D^{386} in the cytoplasmic loops, as well as all the acidic and cysteine residues of NorM, were mutated. Mutants G184V, G184W, K185I, P189S, E190K, and E190A lost the norfloxacin resistance-imparting phenotype characteristic of NorM. Mutants E124V, D155V, G187V, G187R, C196S, Y384H, Y384S, and Y384F exhibited partial resistance to norfloxacin. Mutants with replacements of G^{184} or G^{187} by A, K^{185} by R, and E^{190} by D retained the norfloxacin resistance phenotype of NorM. Analysis of the accumulation of norfloxacin in intact cells of *Escherichia coli* expressing NorM or its mutants in the presence or absence of carbonyl cyanide *m*-chlorophenylhydrazone supported the results obtained through susceptibility testing and argued in favor of NorM-mediated efflux as the determining factor in norfloxacin susceptibility in the genetically manipulated strains. Taken together, these results suggested that E^{124}, D^{155}, G^{184}, K^{185}, G^{187}, P^{189}, E^{190}, C^{196}, and Y^{384} are likely involved in NorM-dependent norfloxacin efflux. Except for D^{155}, C^{196}, and Y^{384}, all of these residues are located in periplasmic loops.

Multidrug efflux pumps extrude a variety of structurally unrelated drugs from cells (3, 23, 30), and pumps such as AcrAB have been associated with the intrinsic reduced susceptibility of organisms such as *Proteus mirabilis* to certain drugs (26, 27, 37). A family of multidrug efflux proteins has been identified which utilize the electrochemical potential of Na^+ transport across membranes as the driving force (8, 21, 24, 25, 34) and which show sequence similarity. These transporters constitute the *m*ultidrug *a*nd *t*oxic compound *e*xtrusion (MATE) family, which contains more than 30 proteins present in all three kingdoms (7), including NorM proteins from *Vibrio parahaemolyticus* (21), *Neisseria gonorrhoeae*, *Neisseria meningitidis* (27), and *Brucella melitensis* (4); YdhE, a NorM homologue in *Escherichia coli* (21); VmrA from *V. parahaemolyticus* (8); VcmB, VcmD, VcmH, VcmN, VcmA, and VcrM of non-O1 *Vibrio cholerae* (2, 24); PmpM of *Pseudomonas aeruginosa* (11); and BexA of *Bacteroides thetaiotaomicron* (20).

Recently, quinolone resistance was reported for clinical isolates of *V. cholerae* from Calcutta, India (10). In our laboratory, we have demonstrated that an efflux pump-dependent mechanism imparts fluoroquinolone (FQ) resistance to clinical isolates of *V. cholerae* (1), making it important to understand the mechanism of action of FQ-specific pumps.

Chromosome I of *V. cholerae* encodes a putative counterpart of NorM (12) that has a high level of sequence similarity (Fig. 1) to the NorM protein of *V. parahaemolyticus*. In this report, we demonstrate that disruption of the *norM* gene of *V. cholerae* confers sensitivity towards fluoroquinolones, making it likely that NorM is one of the FQ resistance-determining efflux pumps of this organism. Otsuka et al. (29) have only recently demonstrated that three conserved acidic residues in the predicted transmembrane region of NorM of *V. parahaemolyticus* are involved in the Na^+-dependent drug transport process. However, no detailed information is available on the topology of this transporter or the role of conserved residues in the periplasmic and cytoplasmic loops of NorM in FQ efflux. With this in view, we have carried out a detailed mutational and topological analysis of *V. cholerae* NorM, with the particular aim of identifying residues crucial for imparting FQ resistance. We focused on residues conserved in the MATE family of transporters, particularly acidic residues, and determined the effects of mutating these residues on the norfloxacin (NOR) sensitivity of the resulting transformants. Our results demonstrate the importance of E^{124}, G^{184}, K^{185}, G^{187}, P^{189}, and E^{190}, which are located in periplasmic loops, of D^{155} and Y^{384}, which are located in cytoplasmic loops, and of C^{196}, which is located in a transmembrane segment (TMS), in the norfloxacin resistance-imparting property of NorM.

MATERIALS AND METHODS

Bacteria and growth. The clinical isolate *V. cholerae* AM54, (1), *V. cholerae* N16961, and *Escherichia coli* TG1Δ*acrAB* (a gift from K. Nishino, Osaka University, Osaka, Japan) (28) were used in this study. Cells were grown in Luria broth (LB) at 37°C. Cell growth was monitored by measuring the optical density at 600 nm.

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Vp  ---------MHRYKEEASSLIKLATPVLIASVAQTGMGFVDTVMAGGVTQTDMAAVSVAS  51
Vc  -----MENSVHRYKKEASNLIKLATPVLIASVAQTGMGFVDTIMAGGVSAIDMAAVSIAA  55
Ec  ---------MQKYISEARLLLALAIPVILAQIAQTAMGFVDTVMAGGYSATDMAAVAIGT  51
Nm  MLLDLNRFSFSVFLKEVRLLTALALPMLLAQVAQVGIGFVDTVMAGGAGKEDLAAVALGS  60
                .  : .*.  *  ** *:::*.:**..:*****:****    *:***::.:

Vp  SIWLPSILFGIGLLMALVPVVAQLNGSARREKIPFEIQQGVVLALLISIPIIGVLLQTQF  111
Vc  SIWLPSILFGVGLLMALVPVVAQLNGAGRQHKIPFEVHQGLILALLVSVPIIAVLFQTQF  115
Ec  SIWLPAILFGHGLLLALTPVIAQLNGSGRRERIAHQVRQGFWLAGFVSVLIMLVLWNAGY  111
Nm  SAFATVYITFMGIMAALNPMIAQLYGAGKTDEVGETGRQGIWFGLFLGVFGMVLMWAAIT  120
    * : .  :   *:: ** *::*** *:.: ..:    :**. :. ::.:  : ::  :

Vp  ILQLMD-VEAVMADKTVGYIHAVIFAVPAFLLFQTLRSFTDGMSLTKPAMVIGFIGLLLN  170
Vc  IIRFMD-VEEAMATKTVGYMHAVIFAVPAYLLFQALRSFTDGMSLTKPAMVIGFIGLLLN  174
Ec  IIRSMQNIDPALADKAVGYLRALLWGAPGYLFFQVARNQCEGLAKTKPGMVMGFIGLLVN  171
Nm  PFRNWLTLSDYVEGTMAQYMLFTSLAMPAAMVHRALHAYASSLNRPRLIMLVSFAAFVLN  180
     ::    :.  :  . . *:     . *. :..:. :   ..:  .:  *::.* .:::*

Vp  IPLNWIFVYGKFGAPELGGVGCGVATTIVYWVMFALLLAYVMTSSRLKSINVFGEYHKPQ  230
Vc  IPLNWIFVYGKFGAPELGGVGCGVATAIVYWIMLLLLLFYIVTSKRLAHVKVFETFHKPQ  234
Ec  IPVNYIFIYGHFGMPELGGVGCGVATAAVYWVMFLAMVSYIKRARSMRDIRNEKGTAKPD  231
Nm  VPLNYIFVYGKFGMPALGGAGCGLATMAVFWFSALALWIYIAKENFFRPFGLTAKFGKPD  240
    :*:*:**:**:** * ***.***:**  *:*.    :  *:     :  .       **:

Vp  WKAQVRLFKLGFPVAAALFFEVTLFAVVALLVSPLGPIIVAAHQVAINFSSLVFMLPMSV  290
Vc  PKELIRLFRLGFPVAAALFFEVTLFAVVALLVAPLGSTVVAAHQVALNFSSLVFMFPMSI  294
Ec  PAVMKRLIQLGLPIALALFFEVTLFAVVALLVSPLGIVDVAGHQIALNFSSLMFVLPMSL  291
Nm  WAVFKQIWKIGAPIGLSYFLEASAFSFIVFLIAPFGEDYVAAQQVGISLSGILYMIPQSV  300
         :: ::* *:. : *:*.: *:.:.:*::*:*   **.:*:.:.:*.:::::* *:

Vp  GAAVSIRVGHRLGEENVDGARVASRVGIMVGLALATITAIITVLSRELIAELYTNNPEVI  350
Vc  GAAVSIRVGHKLGEQDTKGAAIAANVGLMTGLATACITALLTVLFREQIALLYTENQVVV  354
Ec  AAAVTIRVGYRLGQGSTLDAQTAARTGLMVGVCMATLTAIFTVSLREQIALLYNDNPEVV  351
Nm  GSAGTVRIGFSLGRREFSRARYISGVSLVSGWMLAVITVLSLVLFRSPLVSMYNNDPAVL  360
    .:* ::*:*. **. .   *   : ..:: *   * :*.:  *  *. :. :*.::  *:

Vp  TLAMQLLLFAAVYQCTDAVQVIAAGALRGYKDMRAIFNRTFIAYWILGLPTGYILGRTDW  410
Vc  ALAMQLLLFAAIYQCMDAVQVVAAGSLRGYKDMTAIFHRTFISYWVLGLPTGYILGMTNW  414
Ec  TLAAHLMLLAAVYQISDSIQVIGSGILRGYKDTRSIFYITFTAYWVLGLPSGYILALTDL  411
Nm  SIAATVLLFAGLFQPADFTQCIASYALRGYKVTKVPMFIHAAAFWGCGLLPGYLLAYR--  418
    ::*  ::*:*.::*  *  * :.:  *****     :     ::*  ** .**:*.

Vp  IVEP-MGAQGFWLGFIIGLTAAALMLGVRLRWMHRQEPDVQLNFSLQ  456
Vc  LTEQPLGAKGFWLGFIIGLSAAALMLGQRLYWLQKQSDDVQLHLAAK  461
Ec  VVEP-MGPAGFWIGFIIGLTSAAIMMMLRMRFLQRLPSAIILQRAAR  457
Nm  ---FDMGIYGFWTALIASLTIAAIALVWCLELCSR--EMVRSHKAV-  459
         :*  *** .:* .*: **: :   :    :    :  : :
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FIG. 1. Multiple alignment of the amino acid sequence of NorM with those of representative homologs in *Vibrio parahaemolyticus* (Vp), *Vibrio cholerae* (Vc), *Neisseria meningitidis* (Nm), and *Escherichia coli* (Ec), using CLUSTAL W. *, identical residues; :, >60% homologous residues. The conserved regions $G^{184}KFGXP^{189}$ and $L^{381}RGYKD^{386}$ are overlined.

Chemicals. Ampicillin, NOR, streptomycin, erythromycin, doxorubicin, novobiocin, and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were purchased from Sigma Chemical Co. Kanamycin, tetracycline, and chloramphenicol were purchased from Roche Applied Sciences, Germany. Ciprofloxacin (CIP) was a gift from Ranbaxy Laboratories, India. Ethidium bromide (EtBr) was purchased from Stratagene.

Molecular biological procedures. Standard procedures for cloning, analysis of DNA, PCR, and transformation were used (32). Enzymes used to manipulate DNA were obtained from Roche Applied Sciences. All constructs made by PCR were sequenced to verify their integrity. Kanamycin was used at a concentration of 50 μg/ml, and ampicillin was used at a concentration of 100 μg/ml.

The *norM* gene was amplified from genomic DNA of *V. cholerae* AM54, using the primer pair 5′-AT**GCTAGC**TTGGAGAACTCTGTGCATCGTT-3′ (sense) and 5′-AT**GAATTC**TTATGCTGCAAGGTGTAACTGTACG-3′ (antisense), containing asymmetric NheI and EcoRI sites (in bold), and cloned between the NheI and EcoRI sites of the vector pET28a (Novagen) to generate pVC101. The NcoI and EcoRI fragment excised from pVC101 was then cloned between the NcoI and EcoRI sites of the vector pBADHisC (Novagen) to generate pVC102.

Mutants of NorM were generated by overlap extension PCR. The initial rounds of PCR were carried out using the primer pairs a-b and c-d (see Table S1 in the supplemental material), with pVC102 as the template. The products of each PCR were purified and used as templates for the second round of PCR, using primers a and d. The final products were cloned between the NcoI and EcoRI sites of pVC102 to generate *norM* mutants in pBADHisC.

Western analysis of expressed protein. *E. coli* cells transformed with pVC102 or its mutants were grown to mid-log phase, and crude cell extracts were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by electrophoretic transfer to a polyvinylidene difluoride membrane and Western blotting with an anti-His antibody.

Construction and analysis of β-lactamase fusions with truncated NorM derivatives. pJBS633 (carrying the mature TEM β-lactamase-encoding *blaM* gene) (5) was used for constructing in-frame fusions of TEM β-lactamase with the C-terminal ends of truncated NorM derivatives. These derivatives were generated by PCR using the forward primer 5′-TT**GGATCC**AATTGGAGAACTCT GTGCATCGT-3′, containing a BamHI site (in bold), and reverse primers carrying portions of the *norM* gene (see Table S2 in the supplemental material). The

PCR products were cloned between the BamHI and PvuII sites of pJBS633. *E. coli* JM105 was transformed with the ligation mixture, and transformants growing on LB agar plates containing 50 μg/ml kanamycin were chosen for further analysis. Transformants containing in-frame NorM–β-lactamase fusions were detected by the ability to grow when patched with toothpicks onto agar containing 200 μg/ml ampicillin (6). MICs of ampicillin for individual cells of *E. coli* JM105 containing NorM-Xaa–TEM β-lactamase fusions (where Xaa represents the amino acid residue of NorM at the fusion junction) were determined by spotting 4-μl samples of $1:10^5$ diluted overnight cultures (approximately 40 cells) on LB agar plates containing a range of doubling concentrations of ampicillin.

Construction and analysis of targeted CAT fusions. The *cat* gene was PCR amplified from pACYC184, using the forward primer 5′-AG**GGTACC**AAAAAAATCACTGGATATA-3′ (KpnI site is shown in bold) and the reverse primer 5′-ATA**AAGCTT**CGCCCCGCCCTGCCACTC-3′ (HindIII site is shown in bold), and inserted between the KpnI and HindIII sites of pBADMycHisA, generating pBAD-CAT. Chloramphenicol acetyltransferase (CAT) constructions were created by targeted PCR fusion with derivatives of NorM, inserting KpnI sites after the amino acids of interest by using the forward primer 5′-AT**ACTGCAG**ATTTGGAGAACTCTGAG-3′, containing a PstI site (in bold), and reverse primers (see Table S2 in the supplemental material) carrying portions of the *norM* gene. The constructs were transformed into *E. coli* TG1 containing 100 μg/ml ampicillin and tested for resistance to chloramphenicol as follows. Overnight cultures in LB medium were diluted 10-fold with fresh medium and allowed to grow for 3 h. Induction was then carried out by the addition of 0.05% L-arabinose for 1 h, and 4-μl samples of a 10^4 dilution of the cultures were spotted onto plates containing different concentrations of chloramphenicol in combination with 0.2% L-arabinose. Growth was observed after 16 h.

Drug susceptibility testing. The MICs of drugs were determined in Mueller-Hinton agar (Hi-Media, India) containing different drugs at various concentrations. *E. coli* TG1Δ*acrAB* cells transformed with the wild-type or mutant *norM*-harboring constructs were grown at 37°C in LB supplemented with ampicillin (100 μg/ml) to an optical density at 600 nm of 1. A series of 10-fold dilutions were spotted on plates containing ampicillin (40 μg/ml) and different concentrations of test drugs (38), and growth was observed after 16 h.

Assay of norfloxacin accumulation in cells. The assay of norfloxacin accumulation in cells was carried out as described earlier (1). Briefly, *E. coli* TG1Δ*acrAB* cells were grown in LB supplemented with 100 μg/ml ampicillin to an A_{600} of 1. The cells were harvested, washed with 50 mM sodium phosphate buffer (pH 7.2), and suspended in the same buffer to an A_{600} of 20. Cells were energized with 0.2% glucose for 20 min at 30°C. Norfloxacin was added to a concentration of 10 μg/ml. Where necessary, after 10 min, CCCP was added to the assay mixture at 100 μM. Samples (100 μl each) were taken at intervals, centrifuged at 6,000 rpm on a table-top centrifuge for 30 s, and washed with the same buffer. The pellet was suspended in 1 ml of 100 mM glycine-HCl buffer (pH 3.0). The suspension was shaken vigorously for 1 h at 37°C and then centrifuged at 13,000 rpm in a microcentrifuge for 5 min at room temperature. The fluorescence of the supernatant was measured with excitation at 277 nm and emission at 448 nm (13, 22) in a Hitachi spectrofluorimeter (model S4500). For each experiment, an aliquot of cells was filtered separately, before the addition of norfloxacin, using Whatman GF/C filters. The filters were dried and weighed to calculate the dry weight of the cells.

Assay of ethidium bromide accumulation in cells. Cells were grown as described above and suspended to an A_{600} of 0.5. The cell suspension (2.5 ml) was mixed with 5 μM ethidium bromide and placed in a cuvette. Fluorescence was measured at excitation and emission wavelengths of 500 and 580 nm, respectively (19).

Inactivation of *V. cholerae norM*. The *norM* deletion construct was generated by PCR, using genomic DNA of *V. cholerae* N16961 as the template. The primer pair sense 1 (5′-AT**GAATTC**GAGCTCAACATGACAGTTGATGAG-3′) and antisense 1 (5′-AT**GGTACC**GATATTGAGCAATAACCCAA-3′), containing asymmetric EcoRI and KpnI sites (in bold), was used to amplify an 842-bp fragment (fragment 1) encoding N-terminal amino acids 1 to 175 along with a 300-bp sequence upstream of *norM*. Similarly, the primer pair sense 2 (5′-AT**GGTACC**CTTGGCTTGCCCACCGGTTA-3′) and antisense 2 (5′-AT**GGATCC**CCAGTAAGCAGCAAAAGTGC-3′), containing asymmetric KpnI and BamHI sites (in bold), was used to amplify a 433-bp fragment (fragment 2) encoding amino acids 401 to 461 along with a 250-bp region downstream of *norM*. PCR-amplified fragment 1 was cloned between the EcoRI and KpnI sites of pUC19 to generate pAS101. Fragment 2 was then digested with KpnI and BamHI and cloned between the same sites of pAS101 to generate pAS102. The deleted *norM* gene from pAS102 was digested with BamHI and SacI and cloned into the suicide vector pWM91 (17; kindly provided by J. J. Mekalanos) between the same sites to generate pAS103. pAS103 was introduced into recipient *V. cholerae* N16961 cells by electroporation (15). Colonies were selected on LB plates supplemented with streptomycin and ampicillin. The streptomycin- and ampicillin-resistant colonies were patched on LB agar overnight to allow homologous recombination between flanking regions of pAS103. Cells were then selected for the ability to form colonies on 5% sucrose to select for excision of the integrated plasmid (9). Genomic DNAs isolated from the wild type and the knockout construct of *V. cholerae* were used for PCR amplification, using primers sense 1 and antisense 2, to identify the clones harboring the deletion in *norM*. PCR products were checked by sequencing.

TABLE 1. Susceptibility to and accumulation of fluoroquinolones in *V. cholerae* and *E. coli* strains

Strain	MIC (μg/ml)[a]		Accumulation of NOR (μg mg^{-1} cell dry wt)[b]	
	NOR	CIP	Before addition of CCCP	After addition of CCCP
V. cholerae strains				
Wild type (N16961)	0.0156	0.0039	0.031 ± 0.0012	0.394 ± 0.022
NorM-KO	0.001	0.0004	0.331 ± 0.0019	0.415 ± 0.0025
E. coli strains[c]				
TG1Δ*acrAB* harboring pBADHisC (control)	0.016	0.004	0.367 ± 0.009	0.428 ± 0.012
norM wt/pBADHisC*	1	0.125	0.191 ± 0.012	0.436 ± 0.011
norM G187R/pBADHisC*	0.063	0.032	0.298 ± 0.009	0.432 ± 0.009
norM E190K/pBADHisC*	0.016	0.004	0.386 ± 0.011	0.425 ± 0.012
norM G192V/pBADHisC*	0.5	0.125	0.188 ± 0.008	0.433 ± 0.008

[a] All experiments were carried out thrice with the same results.
[b] Data are means ± standard deviations for three independent determinations.
[c] *, *norM* wt/pBADHisC refers to the wild-type *norM* gene cloned into pBADHisC, *norM* G187R/pBAD HisC refers to the mutant *norM* gene encoding the G187R mutation cloned into pBADHisC, and so on.

RESULTS

Drug susceptibility testing in *V. cholerae*. In order to evaluate the role of *norM* in *V. cholerae*, the *norM* gene was inactivated. PCR amplification using primers for the flanking regions of the *norM* gene gave products of 1,842 and 1,179 bp for the wild type and the mutant, respectively (data not shown), which were sequenced to confirm the inactivation of *norM*. The knockout strain (designated NorM-KO) was >10-fold more sensitive to FQs (Table 1) than the wild type. In addition, the MIC of ethidium bromide decreased from 512 μg/ml for the wild type to 128 μg/ml for NorM-KO.

Drug susceptibility testing in *E. coli*. In order to evaluate the contribution of NorM to drug resistance, *norM* was expressed in *E. coli* TG1Δ*acrAB*, which is hypersensitive to many drugs due to a deficiency in the major multidrug efflux pump AcrAB (28). The drug susceptibilities of *E. coli* TG1Δ*acrAB* cells harboring the vector alone (control) or pVC102 (the vector carrying the *norM* gene) are shown in Table 2. *E. coli* TG1Δ*acrAB*/pVC102 was resistant to norfloxacin (60-fold), ciprofloxacin (30-fold), and ethidium bromide (16-fold) but not to sparfloxacin, a hydrophobic FQ. We also observed reproducibly low levels (two- to fourfold) of resistance to streptomycin, erythromycin, kanamycin, and doxorubicin (Table 2). Considering that the NorM protein of *V. cholerae* conferred high resistance to FQs, we designated NorM as an FQ efflux pump. These studies were repeated in the presence of CCCP (25 μM), an energy uncoupler that has been shown to inhibit the action of other efflux pumps (26). CCCP reduced the MICs of norfloxacin, ciprofloxacin, and ethidium bromide for *E. coli* TG1Δ*acrAB*/

TABLE 2. Susceptibility of *norM*-harboring *E. coli* TG1Δ*acrAB* to different compounds

Compound[a]	MIC (μg/ml)[b]	
	TG1Δ*acrAB*	TG1Δ*acrAB*/pVC102
NOR	0.016	1
CIP	0.004	0.125
EtBr	2	32
CCCP	32	32
NOR plus CCCP	0.016	0.016
CIP plus CCCP	0.004	0.004
EtBr plus CCCP	1	1
SPX	0.007	0.007
KAN	1	4
TET	0.25	0.25
CHL	1	1
Streptomycin	2	4
Erythromycin	0.5	1
Doxorubicin	8	16
Novobiocin	2	2
Rhodamine 6G	16	16
Acriflavin	2	2

[a] NOR, norfloxacin; CIP, ciprofloxacin; EtBr, ethidium bromide; SPX, sparfloxacin; KAN, kanamycin; TET, tetracycline; CHL, chloramphenicol.
[b] All experiments were carried out thrice with the same results.

pVC102, suggesting that NorM is a proton motive force-dependent pump.

Determination of the topology of NorM by analyses of NorM–β-lactamase and NorM-CAT fusions. A model of the secondary structure of NorM was developed by using the HMMTOP algorithm (35, 36) (available freely at http://www.enzim.hu/hmmtop) (Fig. 2). In order to validate the predicted topology, targeted fusions of NorM were generated with the N-terminal end of the TEM β-lactamase or CAT reporter. *E. coli* JM105 had an MIC of 4 μg/ml for ampicillin. The NorM-Xaa–TEM β-lactamase transformants, in which Xaa (the NorM amino acid at the fusion junction) was G^{41}, D^{47}, S^{52}, R^{118}, E^{124}, K^{129}, G^{184}, P^{189}, G^{192}, G^{195}, P^{268}, V^{279}, R^{340}, E^{349}, Q^{351}, T^{412}, or K^{423}, had MICs of 200 μg/ml or more for ampicillin, consistent with the view that these fusion sites were each in the periplasm, since β-lactamase fusion proteins can provide *E. coli* with ampicillin resistance only if the β-lactamase moiety is translocated to the periplasm (5). The cytoplasmic location of amino acids was studied by analyzing the NorM-Xaa–CAT fusion transformants, in which Xaa (the NorM amino acid at the fusion junction with CAT) was K^{17}, Q^{78}, E^{91}, G^{95}, Q^{148}, D^{155}, A^{163}, S^{218}, E^{228}, P^{247}, I^{300}, N^{319}, Q^{374}, V^{375}, Y^{384}, R^{393}, or Q^{442}. *E. coli* TG1 harboring these fusions showed chloramphenicol resistance, consistent with the view that these fusion sites were each in the cytoplasm, since CAT fusion proteins can provide *E. coli* with chloramphenicol resistance only if CAT is present in the cytosol. Negative validation of the predicted topology was carried out by analyzing β-lactamase constructs generated by fusion to transmembrane (C^{196} and C^{197}) or cytoplasmic (D^{155} and E^{228}) amino acids as well as by analyzing CAT constructs fused to transmembrane (C^{196} and C^{197}) or periplasmic (D^{47} and E^{124}) amino acids. Fusions to D^{155} or E^{228} were ampicillin sensitive. Fusions to D^{47} or E^{124} were chloramphenicol sensitive. Fusions to C^{196} or C^{197} were both ampicillin and chloramphenicol sensitive. We concluded that the experimentally determined topology corroborated the predicted topology depicted in Fig. 2.

Effects of specific mutations in amino acids located in periplasmic loops on the function of NorM. The motif G^{184}KFGAP^{189} is located in the periplasmic loop between TMS 5 and 6 (Fig. 2) and is conserved among several members of the MATE family (Fig. 1). Deletion of this conserved region rendered the mutant NorM (ΔG^{184}-P^{189}) protein highly susceptible to norfloxacin (Table 3). In order to further identify important residues in this region, we replaced G^{184} with valine or tryptophan. These replacements caused a complete loss of activity of NorM, as assessed by determining the MIC of norfloxacin as well as that of ciprofloxacin for *E. coli* TG1Δ*acrAB* cells harboring the respective plasmids. However, replacement of G^{184} with alanine led to the retention of a significant amount of wild-type activity ["activity," as referred to in the following paragraphs, means the ability of the *norM*-harboring construct(s) to impart norfloxacin resistance, as assessed by the MIC]. Valine differs from glycine in being more space-filling and exhibiting a lower propensity for forming a β-turn structure. Consistent with this, the substitution G184A did not cause a large change in activity. By the same token, replacement of G^{187} with arginine or valine significantly reduced the wild-type activity, whereas replacement with alanine did not alter the activity. The positively charged lysine at position 185 was essential for NorM activity. Replacement of K^{185} with isoleucine, but not with arginine, resulted in a complete loss of NorM activity (Table 3). A positive charge therefore appeared necessary at position 185. The P189S mutant completely abolished NorM activity. Replacement of G^{192} and G^{195}, two other conserved residues in the predicted periplasmic loop between TMS 5 and 6, did not alter NorM activity (Table 3).

Effects of replacement of conserved residues in cytoplasmic loops. The region L^{381}RGYKD^{386} in the cytoplasmic loop between TMS 10 and TMS 11 (Fig. 2) is conserved (Fig. 1). Replacement of L^{381} with proline, R^{382} with glycine, G^{383} with valine, K^{385} with isoleucine, and D^{386} with valine did not alter the activity of NorM (Table 3). However, replacement of Y^{384} with phenylalanine, histidine, or serine showed reductions in

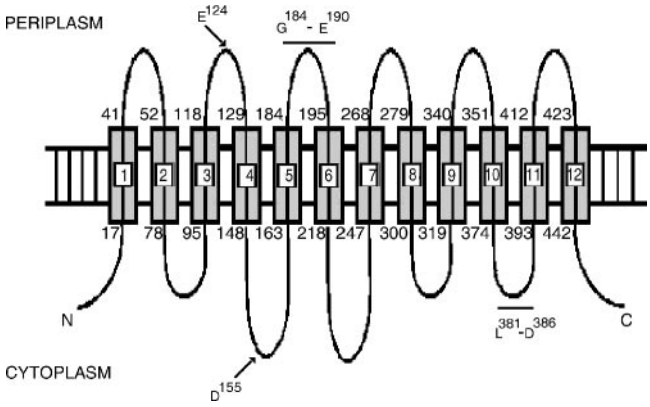

FIG. 2. Schematic representation of the predicted secondary structure of NorM. The 12 transmembrane helices are boxed. The topology was designed based on the algorithm HMMTOP (31, 32) and was confirmed by analysis of β-lactamase and chloramphenicol acetyltransferase fusions with the *norM* gene.

TABLE 3. Norfloxacin resistance of *E. coli* TG1Δ*acrAB* expressing mutant NorM proteins[a]

NorM mutant	NOR MIC (μg/ml)
Vector alone	0.016
Wild type	1
E2V	0.5
E11V	1
D36V	1
D47V	0.5
E91V	0.5
D121V	0.5
E123V	0.5
E124V	0.063
D155V	0.063
Δ[G^{184}KFGAP^{189}]	0.016
G184V	0.016
G184W	0.016
G184A	0.5
K185I	0.0156
K185R	1
G187V	0.25
G187R	0.063
G187A	1
P189S	0.016
E190K	0.016
E190A	0.016
E190V	0.016
E190D	1
G192V	0.5
G195V	1
C196S	0.125
E228V	0.5
E237V	0.5
E255V	0.5
G270V	1.0
E308V	0.5
D310V	0.5
C330S	1
E341V	0.5
E349V	0.5
C369S	1
D371V	0.5
L381P	1
R382G	1
G383V	1
Y384F	0.125
Y384H	0.063
Y384S	0.25
K385I	0.5
D386V	0.5
M387I	1
E417V	0.5
D452V	1
D453V	0.5

[a] All experiments were carried out thrice with the same results.

NorM activity corresponding to MICs of 0.125, 0.063, and 0.25 μg/ml, respectively (Table 3), indicating that Y^{384} plays an important role in NorM activity. Partial retention of activity in the Y384→S and Y384→F mutants suggested that the -OH group (common to tyrosine and serine) as well as the phenyl ring (common to tyrosine and phenylalanine) likely has a bearing on NorM activity. Replacement of Y^{384} with histidine altered NorM activity, suggesting that a positively charged functional group is not favored at this position. Mutagenesis of M^{387}, which is not a conserved residue, caused no significant change in NorM activity.

Effects of replacement of cysteine residues on NorM activity. There are three cysteine residues in the deduced amino acid sequence of NorM (Fig. 1), all of which are in transmembrane segments (Fig. 2). In order to determine whether one of these cysteines is in a region of functional significance, we analyzed variants in which NorM cysteines were replaced individually by serine. In one (C196S) of the three instances, the C→S derivative displayed an eightfold higher susceptibility than the wild-type protein, while in the remaining two cases (C330S and C369S), NorM activity remained unaltered (Table 3). Multiple alignments identified the cysteine at position 196 of *V. cholerae* (Fig. 1) as a conserved residue in 17 NorM homologs (data not shown).

Effects of replacement of acidic residues on NorM activity. NorM contains 13 glutamic acid (at positions 2, 11, 91, 123, 124, 190, 228, 237, 255, 308, 341, 349, and 417) and 9 aspartic acid (at positions 36, 47, 121, 155, 310, 371, 386, 452, and 453) residues. All of the acidic residues were replaced individually with valine (Table 3). The activity of each of the mutant proteins was analyzed by drug susceptibility testing. All of the mutant proteins in which acidic residues were mutagenized were active enough to sustain a level of resistance practically identical to that of the wild type, except in the cases of E^{124}, D^{155}, and E^{190} mutants. The E124V and D155V substitutions had partial but significant effects on drug susceptibility. E^{190}, the only acidic residue in the periplasmic loop between TMS 5 and 6, led to a complete loss of activity when replaced with either lysine, alanine, or valine. The substitution E190D did not influence the norfloxacin resistance-imparting phenotype of NorM, thus highlighting the importance of an acidic residue at position 190. In order to demonstrate the expression of mutated proteins in *E. coli*, Western analysis was carried out with crude cell extracts and an anti-His antibody. The results obtained with a representative set of mutants are presented in Fig. 3. Equal levels of expression were observed for all mutants compared to the expression level of wild-type NorM, indicating that decreased expression or instability of the mutant protein was not responsible for the impaired efflux pump activity in any case.

Accumulation of norfloxacin in intact cells of *V. cholerae* and *E. coli*. The accumulation of norfloxacin in NorM-KO was higher than that in the wild-type strain (Table 1). Disruption of efflux pump activity with CCCP led to the accumulation of

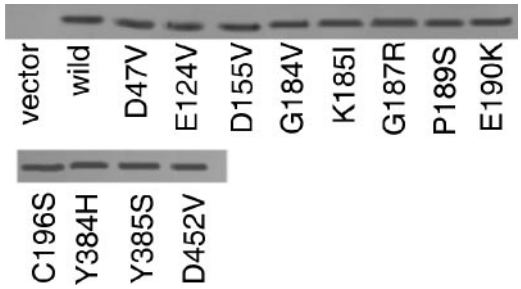

FIG. 3. Western blot analysis of recombinant NorM and its mutants. Wild-type NorM and site-directed mutants (as indicated in the figure) were expressed in *E. coli* TG1Δ*acrAB* as N-terminally His-tagged proteins. Cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by Western blotting using an anti-His monoclonal antibody.

norfloxacin in the parent strain to the level observed in NorM-KO, suggesting a role for NorM in FQ efflux. These data were in harmony with the increased sensitivity of NorM-KO to FQs compared to that of the wild-type strain (Table 1). In addition, the accumulation of EtBr was higher (1.30 ± 0.0015 μg mg^{-1} dry weight of cells) in NorM-KO than in the wild type (0.7 ± 0.0012 μg mg^{-1} dry weight of cells). After the addition of CCCP, both strains showed similar levels of accumulation of EtBr (wild type, 1.41 ± 0.0015 μg mg^{-1} dry weight of cells; NorM-KO, 1.37 ± 0.0031 μg mg^{-1} dry weight of cells). Norfloxacin accumulation was also determined for *E. coli* TG1Δ*acrAB* harboring the vector alone or a vector carrying wild-type *norM* or its mutants. The results obtained with a representative set of mutants are presented in Table 1. These mutants are as follows: (i) NorM G192V (as resistant as the wild type), (ii) NorM G187R (partially resistant), and (iii) NorM E190K (as sensitive as TG1Δ*acrAB* alone). *E. coli* TG1Δ*acrAB* cells harboring wild-type *norM* or the G192V mutant showed lower levels of norfloxacin accumulation than cells harboring the vector alone (Table 1). Accumulation was comparable in cells harboring the E190K mutant or the vector alone. The accumulation of norfloxacin in cells harboring the G187R mutant was intermediate between the levels observed in cells harboring the vector alone and those harboring the G192V mutant. We concluded that the results obtained by susceptibility testing correlated directly with the levels of norfloxacin accumulation in the cells. The addition of CCCP to the assay mixture increased the accumulation to the level in control cells (Table 1). These data argue in favor of a role of proton motive force-dependent, NorM-mediated norfloxacin efflux in norfloxacin susceptibility in the above instances.

DISCUSSION

Recently, it was reported that NorM, a putative multidrug efflux protein of the MATE family from *V. parahaemolyticus*, confers resistance to hydrophilic FQs, such as norfloxacin and ciprofloxacin, but not to hydrophobic quinolones, such as sparfloxacin and nalidixic acid (21). Consistent with this, we report here that NorM of *V. cholerae* expressed in *E. coli* conferred high-level resistance (>10-fold) to norfloxacin, ciprofloxacin, and ethidium bromide and low-level resistance (2- to 4-fold) to kanamycin, streptomycin, erythromycin, and doxorubicin. No resistance was observed towards sparfloxacin, tetracycline, chloramphenicol, novobiocin, or CCCP (Table 2). We therefore concluded that NorM of *V. cholerae* has functional similarity to NorM of *V. parahaemolyticus* (21) and NorMI of *Brucella melitensis* (4). In the presence of CCCP (25 μM), *E. coli* TG1Δ*acrAB* harboring the wild-type *norM* gene became as sensitive to norfloxacin, ciprofloxacin, and ethidium bromide as the strain harboring the empty vector. In addition, *E. coli* TG1Δ*acrAB* harboring the wild-type *norM* gene showed less norfloxacin accumulation than the strain harboring the empty vector. The increase in accumulation upon addition of CCCP suggested that NorM is a drug/ion antiporter. The specific role of NorM in FQ resistance was further strengthened by our observations that inactivation of *V. cholerae norM* rendered the strain hypersusceptible to FQs. Efflux of norfloxacin and ethidium bromide was also impaired in the knockout strain. The increased expression of *norM* in some previously described clinical isolates (1; our unpublished observations) points in the direction of NorM having a role in FQ resistance in clinical isolates as well. Whether the inactivation of *norM* in these strains lowers the MICs for hydrophilic FQs, however, remains to be tested.

Phylogenetic analysis of prokaryotic MATE family transporters reveals two subfamilies, with one belonging to the NorM branch and the other belonging to the DinF branch (7). Phylogeny-based clustering is likely to determine the substrate specificity of the MATE family transporters (7). Consistent with this view, Begum et al. have shown that in *V. cholerae*, members of the DinF family are unlikely to be major contributors to FQ resistance (2). In the complement of MATE family transporters of *V. cholerae*, NorM would therefore be predicted to have a major role in conferring FQ resistance. The present study has validated this prediction. It is also consistent with reports from several groups (20, 21, 31) that members of the NorM family specifically confer resistance to hydrophilic but not hydrophobic FQs.

The prediction of NorM topology using the online algorithm HMMTOP 2.0 (35, 36) was validated by fusing C-terminal truncations of NorM to either the CAT or TEM β-lactamase reporter. The GXFGXP motif, which is conserved in the NorM family but not in the DinF family, was localized to a periplasmic loop between TMS 5 and 6. Deletion of $G^{184}KFGAP^{189}$ completely abolished NorM activity, as assessed by drug susceptibility testing, pointing towards a crucial role of this motif in defining the substrate specificity of NorM. Amino acids present in large periplasmic loops have also been implicated in substrate recognition of MexD, a component of the tripartite MexCD-OprJ pump of *P. aeruginosa* (14), and in the trimerization of MexB and/or its interaction with MexA (18). Substitution at G^{184} led to a loss of activity in each case, except when G^{184} was replaced by alanine, indicating that the structural requirement at this position is very strict. G^{184} probably makes a turn which does not leave enough room to tolerate bulky side chains at this position. Similarly, the loss of activity in G^{187} mutants also likely correlates with the extent of destabilization or distortion of the peptide backbone, with the substitution G187→A having no effect. The contribution of K^{185} may be due to an intraloop charge-neutralization salt bridge between K^{185} and E^{190}, because replacement of either of these residues with either a neutral or oppositely charged residue was associated with a loss of activity. Mutation of the glycines at positions 192 and 195 individually to valine did not confer any change in the norfloxacin resistance-imparting character of NorM, indicating that there is likely no turn in the NorM peptide backbone in this region. The cytoplasmic loop between TMS 10 and 11 harbors the conserved region $L^{381}RGYKD^{386}$. All of the amino acid residues in this region were targeted for mutagenesis. Y^{384} alone was found to be required for NorM activity. Among the three cysteine residues of NorM, only the replacement of C^{196} (predicted to be located within the transmembrane helix close to the periplasmic loop connecting TMS 5 and 6) with serine had a bearing on activity.

NorM conferred resistance to norfloxacin, ciprofloxacin, and the cationic substrate ethidium bromide. At physiological pH, it is expected that the piperazine rings of both ciprofloxacin and norfloxacin are positively charged (31). This led us to focus on the role of acidic residues in NorM activity. E^{124}, D^{155}, and

E^{190} were critical for the resistance phenotype of NorM. The predicted membrane topology (Fig. 2) suggested that E^{124} and E^{190} are located in the periplasmic loops connecting TMS 3 and 4 and TMS 5 and 6, respectively, whereas D^{155} is located in the cytoplasmic loop connecting TMS 4 and 5. The importance of negatively charged residues for the recognition of cationic drugs was recently demonstrated in a structural study of *Staphylococcus aureus qacR* (33). Mazurkiewicz et al. (16) also demonstrated that acidic residues in both the cytoplasmic and periplasmic loops are important for the transport of lipophilic cationic compounds such as ethidium bromide. Taken together, these results suggested that removing the positive charge on the FQ side chain could reduce its export by pumps such as NorM, thereby improving its efficacy. Our results provide information on the amino acid residues that are likely relevant for the binding of these substrates. Further detailed studies should help in defining the complete set of amino acid residues likely to be involved in the transport process.

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