Structures and transport dynamics of a *Campylobacter jejuni* multidrug efflux pump

Chih-Chia Su, Linxiang Yin, Nitin Kumar, Lei Dai, Abhijith Radhakrishnan, Jani Reddy Bolla, Hsiang-Ting Lei, Tsung-Han Chou, Jared A. Delmar, Kanagalaghatta R. Rajashankar, Qijing Zhang, Yeon-Kyun Shin, & Edward W. Yu

Resistance-nodulation-cell division efflux pumps are integral membrane proteins that catalyze the export of substrates across cell membranes. Within the hydrophobe-amphiphile efflux subfamily, these resistance-nodulation-cell division proteins largely form trimeric efflux pumps. The drug efflux process has been proposed to entail a synchronized motion between subunits of the trimer to advance the transport cycle, leading to the extrusion of drug molecules. Here, we use X-ray crystallography and single-molecule fluorescence resonance energy transfer imaging to elucidate the structures and functional dynamics of the *Campylobacter jejuni* CmeB multidrug efflux pump. We find that the CmeB trimer displays a very unique conformation. A direct observation of transport dynamics in individual CmeB trimers embedded in membrane vesicles indicates that each CmeB subunit undergoes conformational transitions uncoordinated and independent of each other. On the basis of our findings and analyses, we propose a model for transport mechanism where CmeB protomers function independently within the trimer.
C. jejuni is responsible for more than 400 million cases of human enterocolitis each year worldwide\(^1,2\). This infection is capable of triggering an autoimmune response and initiating the development of Guillain-Barre syndrome\(^2\). C. jejuni is frequently found in the intestinal tracts of animals and can be spread to humans through contaminated food, water, or raw milk. Fluoroquinolone and macrolide antibiotics are commonly used as treatment strategies for human campylobacteriosis\(^3\). However, Campylobacter has become resistant to these antimicrobials \(^4\)–\(^6\). Recently, the Centers for Disease Control and Prevention have listed drug-resistant Campylobacter as a serious threat in the United States.

Resistance of Campylobacter to antibiotics is mediated by multiple mechanisms\(^7,7\) and multidrug efflux is one of the major causes of failure of drug-based treatments. Multidrug efflux pumps contribute significantly to both intrinsic and acquired resistance to various antimicrobials in C. jejuni. The resistance compromises the effectiveness of clinical therapy and affects the duration of clinical treatment. It has been suggested that acquisition of fluoroquinolone resistance provides a fitness advantage on C. jejuni in the animal host\(^8\). Needless to say, new therapeutic strategies are needed to develop to strive antibiotic resistant Campylobacter.

The best characterized multidrug efflux system in C. jejuni is the Cme (Campylobacter multidrug efflux) tripartite system\(^9\)–\(^11\). The Cme locus consists of three tandemly linked genes (cmeABC) encoding protein components of the tripartite Cme efflux pump (CmeA, CmeB, and CmeC), where all three components are absolutely required for substrate expulsion. This tripartite system is composed of the CmeB efflux pump, an inner membrane resistance-nodulation-cell division (RND)\(^12\) transport protein that contains substrate-binding sites and transduces the electrochemical energy required for pumping drugs out of the cell; the CmeA periplasmic protein, a member of the membrane fusion protein family; and the CmeC outer membrane-associated protein that is integral to the outer membrane. Mutations on this tripartite system have been found to have a drastic effect on drug susceptibility.

To understand the transport mechanism of the CmeB efflux pump from C. jejuni, we here define the X-ray structures of this membrane protein, which assembles as a trimer. Using single-molecule fluorescence resonance energy transfer (sm-FRET) imaging, we demonstrate that each CmeB protomer within the trimer is able to function independently.

**Results**

**Structures of C. jejuni CmeB.** Two distinct conformations of CmeB with space groups C2 (form I) and P1 (form II) were captured in two different forms of crystals (Fig. 1, Table 1 and Supplementary Figs. 1 and 2). Overall, CmeB adopts the fold of a
Interestingly, 22 mutated residues are found to localize within this drug-binding cavity. The correspondence of the CmeB pump forms a large internal cavity (Supplementary Fig. 3b), which plays a crucial role in drug binding. In AcrB, this cavity has been shown to form an extrusion pathway. The CmeB pump is able to confer high-level bacterial resistance to multiple antibiotics, including oxacin, erythromycin, and tetracycline. Interestingly, the other CmeB molecule of the form II structure has a different conformational state in the periplasm (Fig. 2c, d). We labeled this conformer as the "extrusion" form. No channel was found in this conformer (Fig. 2c, d and Supplementary Fig. 4). This conformer was classified as the "extrusion" form. All of the extrusion protomers in both form I and form II crystals share very similar structural features, which include the conformation of transmembrane helices (Supplementary Table 3 and Supplementary Fig. 5a) and position of side chains of residues within the proton-relay network such as D409 and D410 of TM4 and K935 of TM10 (Supplementary Fig. 5b). Interestingly, the other CmeB molecule of the form II structure displays a distinct conformation, forming a new state that is different from the "extrusion" form. No channel was found in this conformer (Fig. 2c, d and Supplementary Fig. 4c). This conformation probably represents one of the intermediates that the CmeB pump must go through during the transport cycle. However, the conformation of this conformer is similar to the "resting" state of apo-CusA, a specific heavy-metal RND efflux pump that recognizes Cu(I) and Ag(I) ions. A similar conformational state in the AcrB pump has also been found based on molecular dynamics simulations. Thus, we designed this conformation as the "resting" state of the CmeB efflux pump.

Our structures of CmeB do not show the typical conformation of an asymmetric trimer with two periplasmic clefts open and one cleft closed. In both the "access" and "binding" states of AcrB, the periplasmic cleft created by subdomains PC1 and PC2 are open. However, this cleft is closed in the "extrusion" protomer. Thus, the asymmetric trimer of AcrB features a conformational state of two periplasmic clefts open and one cleft closed. During drug binding and extrusion, it was proposed that the three periplasmic clefts within the trimer have to open and close accordingly in order to advance the transport cycle.

Both crystal structures of CmeB depict that this pump also forms an asymmetric trimer. In the form I structure, the three periplasmic clefts of the CmeB trimer are closed (Fig. 1b). Although the conformations of the three monomers are different from each other, they are more similar to the "extrusion" form of AcrB (Supplementary Table 1). A channel for extrusion is found in each protomer of our form I structure (Fig. 2a, b). We therefore assigned the conformational state of these three protomers as the "extrusion" form. Surprisingly, in the form II structure, the periplasmic cleft of one of the protomers is open (Fig. 1c), albeit similar to the conformation of the "binding" state of AcrB (Supplementary Table 2). An elongated channel is found in the periplasmic domain of this protomer. It was observed that this channel leads through the opening of the periplasmic cleft, exposed to solvent in the periplasm (Fig. 2c, d). We labeled this conformer as the "binding" form of CmeB. However, the periplasmic clefts of the other two protomers are closed in configuration. One of these two CmeB molecules is more related to the structure of the "extrusion" conformers of the form I structure. Like the three protomers of the form I structure, the periplasmic domain of this conformer also creates an extrusion channel (Fig. 2c, d and Supplementary Fig. 4). Thus, this conformer was classified as the "extrusion" form. All of the extrusion protomers in both form I and form II crystals share very similar structural features, which include the conformation of transmembrane helices (Supplementary Table 3 and Supplementary Fig. 5a) and position of side chains of residues within the proton-relay network such as D409 and D410 of TM4 and K935 of TM10 (Supplementary Fig. 5b). Interestingly, the other CmeB molecule of the form II structure displays a distinct conformation, forming a new state that is different from the "extrusion" form. No channel was found in this conformer (Fig. 2c, d and Supplementary Fig. 4c). This conformation probably represents one of the intermediates that the CmeB pump must go through during the transport cycle. However, the conformation of this conformer is similar to the "resting" state of apo-CusA, a specific heavy-metal RND efflux pump that recognizes Cu(I) and Ag(I) ions. A similar conformational state in the AcrB pump has also been found based on molecular dynamics simulations. We thus designated this conformation as the "resting" state of the CmeB efflux pump.

Our structures of CmeB do not show the typical conformation of an asymmetric trimer with two periplasmic clefts open and one cleft closed (Supplementary Fig. 6). We then went through the existing crystal structures of these RND proteins, including AcrB, MexB, MtrD, and CusA, available in the protein data bank. We found that the trimer can have three periplasmic clefts open or closed at a time, in addition to the asymmetric conformation of the AcrB trimer (Supplementary Fig. 7). On the basis of this structural information, we postulated that individual protomers of these trimeric RND pumps could bind and export substrates independently instead of operating in a synchronized fashion. Thus, each protomer may autonomously...
go through a sequence of conformational transitions, which lead to the extrusion of substrates through a particular protomer. The structures of individual protomers of AcrB and CmeB captured by crystallography may simply reflect the conformation of various transient states that these protomers may go through within the transport cycle. To this point, the conformations of the three protomers within the trimeric pump can be identical with three periplasmic clefts open or closed as shown in the case of the symmetric structures of AcrB22 and CusA19. However, the structures of individual protomers of the trimeric pump can also be distinct from each other as indicated in the cases of the asymmetric AcrB structures 14, 17, 18, where the three protomers are in different transient states with two open and one closed periplasmic clefts within the trimer. For the asymmetric CmeB trimer, the conformations of the three protomers display in such a way that either only one out of the three periplasmic clefts is open or all of these clefts are closed.

Transport dynamics of C. jejuni CmeB. To elucidate if a CmeB protomer can export drugs individually within the trimer, we decided to directly observe the transport dynamics and conformational changes of the periplasmic domain movements using total internal reflection sm-FRET imaging. The CmeB protein has three cysteines (C453, C496, and C544) located at the transmembrane. We replaced these three cysteines by serines to create the cysteineless CmeB membrane protein. This cysteineless CmeB pump is fully functional as suggested by in vivo susceptibility assay (Supplementary Notes, Supplementary Fig. 8 and Supplementary Table 4). On the basis of the crystal structures of CmeB, we then introduced a single cysteine mutation on the cysteineless protein at a position of high-solvent accessibility as well as low-sequence conservation. The resulting three cysteine residues within each CmeB trimer were derivatized with a mixture of maleimide-activated Alexa Fluor 546 (AF546) and Alexa Fluor 647 (AF647), which served as a molecular ruler for measuring the distance between two inter-subunit cysteines. Our goal was to measure the relative change in distance instead of absolute distance as the finite size, orientation, and length of fluorophores bring difficulty of measuring a precise distance through FRET 28, 29. Thus, we mainly focused on the conformational movement of the CmeB transporter and made point mutation to observe the functional dynamics during drug export. As CmeB is a proton-motive-force (PMF)-dependent transporter, we reconstituted the purified and derivatized CmeB protein into liposomes, where we could generate the proton gradient required for substrate translocation. These proteoliposomes were immobilized on streptavidin-decorated surfaces for FRET signal recording (Fig. 3a).

We chose to mutate residue K843 of the cysteineless CmeB protein to a cysteine in order to anchor the dyes. This residue is located right outside subdomain PC2, facing the periplasm, and at a position where the inter-subunit distances are quite different...
between the conformations as suggested by the crystal structures (Fig. 3b, c). Both in vitro proton transport and in vivo susceptibility assays indicated that the K843C mutant is fully functional (Supplementary Notes, Supplementary Fig. 9 and Supplementary Table 4). We selected the CmeB trimers that only contained one donor (AF546) and one acceptor (AF647) dyes for FRET measurements. If CmeB functions by means of the proposed rotating mechanism 14, 17, 18, sequentially transitioning through three different states, then the distance between the two inter-subunit K843 residues should sequentially vary in a manner of ~74, ~79, and ~69 Å, respectively. This should lead to the observation of three distinct signals, which correspond to the intermediate-, low-, and high-FRET states, in the FRET trajectory and transition density plot (Fig. 3d, e). However, if CmeB employs a different mechanism to recognize and export substrates, then the characteristic of this transition density plot should be different.

We adjusted the extra- and intra-vesicular pHs of the CmeB K843C proteoliposomes to 6.5 and 7.5, respectively. We then performed sm-FRET experiments both in the absence and presence of 1 or 10 μM taurodeoxycholate (Tdc), which is the CmeB substrate. In the absence and presence of Tdc, the FRET state values are very similar. We also find that the frequency of transitions is more or less the same with and without the substrate Tdc. The majority of the populations of apo-CmeB are largely in favor of the low FRET state. However, the addition of substrates seems to shift the state occupancies more favorable to the higher FRET states (Fig. 4). At least four distinct states can be observed, indicating that the trimeric pump is transitioning between various states. These four states are labeled as low (L), intermediate-1 (I₁), intermediate-2 (I₂), and high (H) FRET states, which correspond to ~0.20, ~0.35, ~0.45, and ~0.60 FRET efficiencies. Interestingly, our sm-FRET data do not seem to agree with the proposed rotating mechanism. On the basis of the traces and symmetrical nature of the density plots, it is more likely that the three protomers function independently of each other.

As a negative control, we selected to mutate residue K781 of the cysteineless CmeB pump to a cysteine. This residue is located at the top of the funnel region (subdomain DC). According to our crystal structures, it was predicted that this region should not have any significant motions. The inter-subunit distance between the two K781 residues is ~52 Å. This mutant behaves the same as the wild-type pump as suggested by the in vivo susceptibility assay (Supplementary Table 4). FRET efficiency distributions histogram yielded a single narrow peak centered at 0.85
proton translocation for energy coupling. A single point mutation most probably establishes the proton-relay network and relays...

In the transmembrane region of CmeB, the conserved charged...

At the population level, a dominant mutant is found to cluster in the low FRET state regardless of the presence of ligand (Fig. 5). At the population level, a dominant mutant is found to cluster in the low FRET state regardless of the presence of ligand (Fig. 5). At the population level, a dominant mutant is found to cluster in the low FRET state regardless of the presence of ligand (Fig. 5). At the population level, a dominant mutant is found to cluster in the low FRET state regardless of the presence of ligand (Fig. 5). At the population level, a dominant mutant is found to cluster in the low FRET state regardless of the presence of ligand (Fig. 5). At the population level, a dominant mutant is found to cluster in the low FRET state regardless of the presence of ligand (Fig. 5). At the population level, a dominant mutant is found to cluster in the low FRET state regardless of the presence of ligand (Fig. 5). At the population level, a dominant mutant is found to cluster in the low FRET state regardless of the presence of ligand (Fig. 5).

On the basis of the transition density plot (Fig. 5b), the frequency of transitions of the K843C-D409A mutant was reduced by at least seven times compared with that of the K843C transporter in the absence of Tdc. Only a small fraction of transitions between 0.2 and 0.35 FRET efficiency states was observed. However, they are almost undetectable in the FRET histograms. This low FRET state is also seen in the K843C mutant. The FRET data indicate that the distance between the donor and acceptor dyes at this low FRET state is ~85 Å, corresponding to the 0.2 FRET efficiency. This distance is significantly longer than the distances observed in our crystal structures. However, this distance is similar to that depicted in the crystal structure of the “resting” state of apo-CusA₁₉, 2₀. In this state, the three periplasmic clefts of the CusA heavy-metal efflux pump are closed within the trimer. The data suggest that the CmeB protomers may prefer the “resting” conformation, in the absence of ΔpH, and the process of transitioning from the “resting” to “binding” states may be energy dependent.

Hidden Markov modeling was then used to quantify the transition rates of these various FRET states. In the absence of Tdc, the transition density plot of the K843C mutant indicates that the predominant FRET transitions are L → I₁ and I₁ → L, which correspond to the reversible transitions between the “resting-resting” and “resting-binding” subunits. A histogram derived from a population of dwell times was fitted with a single exponential decay, resulting in $k_{L\rightarrow I_1} = 1.64 \text{ s}^{-1}$ (0.61 s) and $k_{I_1\rightarrow L} = 3.22 \text{ s}^{-1}$ (0.31 s) transition rates for the processes “resting-resting” → “resting-binding” and “resting-binding” → “resting-resting”, respectively (Table 2 and Supplementary Fig. 11). The data suggest that the kinetics of CmeB are quite simple and can be described with a single rate constant.

In the presence of 1 μM Tdc, there is a significant decrease in the reverse transition $I_1 \rightarrow L$. The rate for this reverse transition is $k_{I_1\rightarrow L} = 1.82 \text{ s}^{-1}$ (0.55 s), which is almost two times slower than the same process without the substrate. In addition, there is a substantial increase in the forward transition $I_1 \rightarrow I_2$, suggesting that the K843C mutant may prefer to advance the transport cycle by shifting the “resting-binding” to “resting-extrusion” or “binding-binding” states. Apparently, this process is reversible as indicated by the observation of the reverse transition $I_2 \rightarrow I_1$. (Supplementary Fig. 10), indicating that this funnel region lacks a major conformational change throughout the efflux process. The data also demonstrated the robust performance of our FRET system.

Drug export by RND transporters depends upon the PMF. In the transmembrane region of CmeB, the conserved charged residues D409, D410, and K935 form a salt-bridge triad, which most probably establishes the proton-relay network and relays proton translocation for energy coupling. A single point mutation on these corresponding residues in MexB, AcrB, and CusA has been found to impair the function of these pumps. To disrupt this proton-relay network in the CmeB pump, we replaced D409 by an alanine of the K843C pump to form a K843C-D409A double-point mutant. In vitro proton transport assay has confirmed that proton transport activity in the D409A mutant was totally abolished (Supplementary Fig. 9).

This double-point mutant was then purified, derivatized with AF546 and AF647, and reconstituted into liposomes for FRET signal recording.
Fig. 5 Dynamics in the apo and substrate bound of the proton-relay mutant of CmeB. 

a. FRET efficiency population histograms of CmeB (in the absence of Tdc (n = 40 traces), top panel; in the presence of 1 μM Tdc (n = 37 traces), middle panel; in the presence of 10 μM Tdc (n = 47 traces), bottom panel).

b. Transition density plots for the CmeB efflux pump (in the absence of Tdc, top panel; in the presence of 1 μM Tdc, middle panel; in the presence of 10 μM Tdc, bottom panel).
The forward and reverse transition rates were calculated to be $k_{1 \rightarrow 2} = 1.61 \text{ s}^{-1}$ (0.62 s) and $k_{2 \rightarrow 1} = 3.45 \text{ s}^{-1}$ (0.29 s), respectively (Table 2 and Supplementary Fig. 12).

As the Tdc concentration increased to 10 µM, the transition between $I_2$ and $H$ was observed, suggesting that the transporter continues to move forward the transport cycle by switching from the “resting-extrusion” to the “binding-extrusion” forms. This process is also reversible and the transition rates for these forward and reverse processes are $k_{2 \rightarrow 1} = 1.59 \text{ s}^{-1}$ (0.63 s) and $k_{1 \rightarrow 2} = 2.86 \text{ s}^{-1}$ (0.35 s) (Table 2 and Supplementary Fig. 13).

For the K843C-D409A mutant pump, the density plots are much simpler and their characteristics are more or less the same regardless of the presence of Tdc. Both in the absence and presence of substrate, the only observed transitions are the reversible conformational change between the “resting-resting” and “resting-binding” forms, as indicated by the $L \rightarrow I_1$ and $I_1 \rightarrow L$ transitions. However, the rates of forward transitions are much slower than those for the K843C mutant. Specifically, in the absence of Tdc, the rates for the forward and reverse transitions in the K843C-D409A pump are $k_{1 \rightarrow 1} = 0.54 \text{ s}^{-1}$ (1.85 s) and $k_{1 \rightarrow 1} = 2.86 \text{ s}^{-1}$ (0.35 s) (Table 2 and Supplementary Fig. 14), suggesting that the transition process “resting-resting” $\rightarrow$ “resting-binding” may be energy dependent and needs to couple with ΔpH. All of these observed transition rates are listed in Table 2.

### Discussion

We have defined the crystal structures of CmeB and directly observed the transport dynamics of this membrane protein reconstituted in proteoliposomes at the single-molecule level. These data lead us to propose a simple model for the CmeB transport mechanism (Fig. 6), in which the proton pumps export substrates independently of each other. In the absence of ΔpH, the CmeB protoners may prefer the “resting” conformation, which is evidenced through our FRET data that the inter-subunit distance of the K843C-D409A double mutant measured between the two K843 residues is relatively long in comparison with that of the K843C mutant. It was found that there are relatively very little motions going on in this double mutant, suggesting that a transition from the “resting” to “binding” states may need to couple with ΔpH. In the presence of ΔpH, it appears that there are a few more observed transitions, suggesting that the pump can easily continue to advance the transport cycle by coupling with the proton-relay network. Our data indicate that the CmeB protoners can independently progress to “binding” and then “extrusion” conformations. The populations of these two states can be greatly enhanced by the addition of the Tdc ligand. Our data allow us to uncover the mechanism of drug export, where the three CmeB subunits undergo conformational changes independently of each other.

### Methods

#### Expression and purification of CmeB

The CmeB multidrug efflux pump, which contains a 6xHis tag at the N terminus was overexpressed in *E. coli* BL21(DE3) ΔacrB/Δpetl5bΔcmeB cells. These cells harbor a deletion in the chromosomal *acrB* gene and possess the PET15bΔcmeB vector. Cells were grown in 12 litres of LB broth supplemented with 100 µg ml$^{-1}$ ampicillin at 37 °C. When the OD600 reached 0.5, cells were treated with 1 mM IPTG to induce CmeB expression and harvested within 3 h. The collected cells were resuspended in buffer containing 100 mM sodium phosphate (pH 7.2), 10% glycerol, 1 mM EDTA, and 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM phenylmethylsulfonyl fluoride (PMSF). The suspension was then disrupted using 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 PMSF, and once with 20 mM HEPES-NaOH buffer (pH 7.3) containing 1 mM PMSF. The CmeB membrane protein was then dissolved in 1% (w/v) 6-cyclohexyl-1-hexyl-β-D-maltoside (Cymal-6). Insoluble material was taken away by ultracentrifugation at 100,000 x g.

#### Crystallization of CmeB

The 6xHis CmeB crystals were grown at room temperature using sitting-drop vapor diffusion with the following procedures. A 2 µl protein solution containing 20 mg ml$^{-1}$ CmeB protein in 20 mM Na-HEPES (pH 7.5) and 0.05% (w/v) Cymal-6 was mixed with a 2 µl of reservoir solution containing 4% PEG 8000, 0.1 M Na-MES (pH 6.5) and 0.1 M MgSO$_4$. The resultant mixture was equilibrated against 500 µl of the reservoir solution. Crystals of CmeB grew to a full size in the drops within a month. Typically, the dimensions of the crystals were 0.2 mm x 0.2 mm x 0.2 mm. Cryoprotection was achieved by raising the glycerol concentration stepwise to 25% with a 5% increment in each step.

### Table 2 Dwell times for CmeB transitions

| CmeB      | FRET transition dwell time (s) | L → I$_1$ | I$_1$ → L | I$_1$ → I$_2$ | I$_2$ → I$_1$ | I$_2$ → H | H → I$_2$ |
|-----------|--------------------------------|----------|----------|-------------|-------------|-----------|-----------|
| K843C     |                                | 0.61 ± 0.06 | 0.31 ± 0.05 | 0.54 ± 0.04 | 0.26 ± 0.07 |           |           |
|           |                                | 1.05 ± 0.04 | 0.55 ± 0.05 | 0.62 ± 0.03 | 0.29 ± 0.03 |           |           |
|           |                                | 0.47 ± 0.03 | 0.65 ± 0.02 | 0.50 ± 0.03 | 0.49 ± 0.07 | 0.63 ± 0.07 | 0.35 ± 0.03 |
| K843C–D409A |                              | 1.85 ± 0.13 | 0.35 ± 0.01 |           |           |           |           |
|           |                                | 1.68 ± 0.34 | 0.31 ± 0.05 |           |           |           |           |
|           |                                | 1.47 ± 0.39 | 0.39 ± 0.12 |           |           |           |           |

Fig. 6 Proposed model of drug efflux mechanism. On the basis of our experimental data, there are at least four distinct states found in the trimeric CmeB multidrug efflux pump. These four states are labeled as low (L), intermediate-1 ($I_1$), intermediate-2 ($I_2$), and high (H) FRET states, which correspond to ~0.20, ~0.35, ~0.45, and ~0.60 FRET efficiencies. During drug export, each protomer of CmeB autonomously undergoes a sequence of conformational transitions through a particular protomer. This schematic diagram indicates that each protomer within the CmeB trimer can independently go through a sequence of conformational transitions, which lead to the extrusion of substrate (R resting, B binding, E extrusion, U unlabeled).
X-ray structural determination and refinement. X-ray diffraction data were collected at 100 K at beamline 24-ID-C located at the Advanced Photon Source with a Platinum EM detector. Data were processed and scaled using DENZO and SCALEPACK33, respectively.

The form I crystals of CmeB belong to space group C2 (Table 1). The structure of CmeB was phased using molecular replacement. On the basis of the structure of the O protonator of AcbR (PDB ID: 4dx5)34, a model of CmeB generated by the FANASO program was used as the search model. The R工作中 and Rfinal were 34.9 and 42.0%, respectively. After tracing the initial model manually using Coot35, the model was refined to a resolution of 3.15 Å using PHENIX36. Iterations of refinement were done using PHENIX36 and CNS37. Model building was carried out using the O program. Excellent geometrical characteristics (Table 1).

The form II crystals took the space group P1 (Table 1). This structure was determined using molecular replacement. The structure of form I was employed as a search model. After tracing the initial model using Coot35, the model was refined to a resolution of 3.55 Å. The remaining procedures for model building and structural refinement were identical to those for the form I structure.
0.05% Tween 20 and subsequently incubated with secondary antibodies (1:1,000 dilution of goat anti-rabbit immunoglobulin G-horseradish peroxidase; Kirkegaard & Perry) at 25°C for 1 h. After washing, the blots were incubated with the four CN Membrane Peroxidase Substrate System (Kirkegaard & Perry). Prestained molecular mass markers (Bio-Rad, Hercules, CA) were co-electrophoresed and blotted to allow estimation of the sizes of the proteins.

Drug susceptibility assay. The MICs of sodium taurocholate, sodium taurodeoxycholate, and rifampin for C. jejuni 81-176 and its cmeABC mutant constructs were determined using a microtiter broth dilution method as described previously. The compounds utilized in these assays were purchased from Sigma-Aldrich (St Louis, MO).

Isothermal titration calorimetry. We used isothermal titration calorimetry (ITC) to examine the binding of taurodeoxycholate to the purified CmeB transporter. Measurements were performed on a VP-Microcalorimeter (MicroCal, Northampton, MA) at room temperature. The protein was thoroughly dialyzed against buffer containing 20 mM Na-HEPES pH 7.5 and 0.03% n-dodecyl-β-D-maltoside (DDM). Its concentration was determined using the Bradford assay and then adjusted to a final monomeric concentration of 20 μM before titration. Ligand solution consisting of 0.25 mM taurodeoxycholate in 20 mM Na-HEPES pH 7.5 and 0.03% DDM was employed as the titrant. The protein and ligand samples were degased before loading into the cell and syringe. ITC experiments were performed with the protein solution (1.5 ml) in the cell and the ligand solution as the titrant. Ten microliter injections of the ligand solution were used for a set of data collection. Injections occurred at intervals of 300 s. The duration time of each injection was 20 s. Heat transfer (μcal s−1) was recorded as a function of elapsed time (s). The mean enthalpies measured from injection of the ligand in the buffer were fit to a single-site binding isotherm according to the binding equation: ΔH = RT lnK_b. In this equation, the value of the change in free energy (ΔG) and entropy (ΔS) were obtained using the equation: ΔG = ΔH − RT lnK_b, where ΔH = ΔH_f − TAS, where T is 273 K and R is 1.9872 cal K−1 mol−1. Calorimetry trials were also carried out in the absence of the CmeB protein in the same experimental conditions. No change in heat was observed in the injections throughout the experiment.

In vitro proton translocation assay. The wild-type, K843C and D409A CmeB transporters were reconstituted into liposomes made of 75% E. coli total lipid, 25% egg-yolk phosphatidycholine (Avanti Polar Lipids, Alabaster, AL) and 2 mM 8-hydroxyquinolone-1,3,6-trisulfonic acid trisodium (Sigma-Aldrich, St Louis, MO) in a buffer containing 20 mM Na-HEPES (pH 7.5). The CmeB protein (wild-type, K843C or D409A) was mixed with unilamellar liposomes in 20 mM Na-HEPES (pH 7.5) and 0.2% DDM at a protein-to-lipid molar ratio of 1:20 (w:w). The CmeB protein (wild-type, K843C or D409A) was mixed with unilamellar liposomes in 20 mM Na-HEPES (pH 7.5) and 0.2% DDM at a protein-to-lipid molar ratio of 1:20 (w:w). The CmeB protein (wild-type, K843C or D409A) was mixed with unilamellar liposomes in 20 mM Na-HEPES (pH 7.5) and 0.2% DDM at a protein-to-lipid molar ratio of 1:20 (w:w).

References
1. Ruiz-Palacios, G. M. The health burden of campylobacter infection and the impact of antimicrobial resistance: playing chicken. Clin. Infect. Dis. 44, 701–703 (2007).
2. van Doorn, P. A., Ruts, L. & Jacobs, B. C. Clinical features, pathogenesis, and treatment of Guillain–Barre syndrome. Lancet Neurol. 7, 939–950 (2008).
3. Blaser, M. J. & Engberg, J. in Campylobacter, 3rd edn, 99–121 (American Society of Microbiology, 2008).
4. Gibreel, A. & Taylor, D. E. Macrolide resistance in campylobacter jejuni and campylobacter coli. J. Antimicrob. Chemother. 58, 243–255 (2006).
5. Luangtongkum, T. et al. Antibiotic resistance in campylobacter: emergence, diffusion, and persistence. Future Microbiol. 4, 189–200 (2009).
6. Engberg, J., Aarestrud, F. M., Taylor, D. E., Gerner-Smidt, P. & Nachamkin, I. Quinolone and macrolide resistance in campylobacter jejuni and C. coli: resistance mechanisms and trends in human isolates. Emerg. Infect. Dis. 7, 24–34 (2001).
7. Taylor, D. E. & Courvalin, P. Mechanisms of antibiotic resistance in Campylobacter species. Antimicrob. Agents Chemother. 32, 1107 (1988).
8. Luo, N. et al. Enhanced in vivo fitness of fluoroquinolone-resistant Campylobacter jejuni in the absence of antibiotic selection pressure. Proc. Natl Acad. Sci. USA 102, 541–546 (2005).
9. Lin, J., Michel, L. O. & Zhang, Q. CmeABC functions as a multidrug efflux system in campylobacter jejuni. Antimicrob. Agents Chemother. 46, 2124–2131 (2002).
10. Lin, J., Sahin, O., Michel, L. O. & Zhang, Q. Critical role of multidrug efflux pump CmeABC in bile resistance and in vivo colonization of campylobacter jejuni. Infect. Immun. 71, 4250–4259 (2003).
11. Pumbwe, L. & Piddock, L. J. Identification and molecular characterisation of CmeA, a Campylobacter jejuni multidrug efflux pump. FEMS Microbiol. Lett. 206, 185–189 (2002).
12. Tseng, T. T. et al. The RND permease superfamily: an ancient, ubiquitous and diverse family that includes human disease and development proteins. J. Mol. Microbiol. Biotechnol. 1, 107–125 (1999).
13. Yan, M., Sahin, O., Lin, J. & Zhang, Q. Role of the CmeABC efflux pump in the emergence of fluoroquinolone resistance in Campylobacter jejuni. J. Antimicrob. Chemother. 58, 1154–1159 (2006).
14. Murakami, S., Nakashima, R., Yamashita, E., Matsumoto, T. & Yamaguchi, A. Crystal structures of a multidrug transporter reveal a functionally rotating mechanism. Nature 443, 173–179 (2006).
15. Nakashima, R., Sakurai, K., Yamasaki, S., Nishino, K. & Yamaguchi, A. Structures of the multidrug exporter AcrB under selection pressure. J. Antimicrob. Chemother. 58, 1154–1159 (2006).
16. Tseng, T. T. & Nachamkin, I. Structure of CmeB, a Campylobacter jejuni multidrug efflux pump. Structure 9, 565–569 (2001).
17. Yas, H. et al. Emergence of a potent multidrug efflux pump variant that enhances campylobacter resistance to multiple antibiotics. MBio 7, e01543–16 (2016).
18. Seeger, M. A. et al. Structural asymmetry of AcrB trimers suggests a peristaltic pumping mechanism. Science 313, 1295–1298 (2006).
19. Sennhauser, G., Amstutz, P., Briand, C., Storchenegger, O. & Gutter, M. G. Drug export pathway of multidrug exporter AcrB revealed by DARPin inhibitors. PLoS Biol. 5, e7 (2007).
20. Long, F. et al. Crystal structures of the CusA efflux pump suggest methionine-dependent metal translocation is facilitated by a multidrug efflux complex of AcrB under selection pressure. J. Antimicrob. Chemother. 58, 1154–1159 (2006).
21. Su, C. C. et al. Charged amino acids (R83, E567, D617, E625, R669, and K678) are essential for CmeB, a Campylobacter jejuni multidrug efflux pump. PLoS ONE 4, e7 (2009).
22. Fischer, N. & Kandt, C. Porter domain opening and closing motions in the multi-drug efflux transporter AcrB. Biochim. Biophys. Acta 1828, 632–641 (2013).
23. Murakami, S., Nakashima, R., Yamashita, E. & Yamaguchi, A. Crystal structures of bacterial multidrug efflux transporter AcrB. Nature 419, 587–593 (2002).
24. Yu, E. W., McDermott, G., Zgunska, H. I., Nkaiido, H. & Koshland, D. E. Jr. Structural basis of multiple drug-binding capacity of the AcrB multidrug efflux pump. Science 300, 976–980 (2003).
25. Nakashima, R. et al. Structural basis for the inhibition of bacterial multidrug exporters. Nature 500, 102–106 (2013).
26. Sennhauser, G., Bukowska, M. A., Briand, C. & Gutter, M. G. Crystal structure of the multidrug exporter MexB from pseudomonas aeruginosa. J. Mol. Biol. 389, 134–145 (2009).
27. Roy, R., Hofung, S. & Ha, T. A practical guide to single-molecule FRET. Nat. Methods 5, 507–516 (2008).
28. Guan, L. & Nakae, T. Identification of essential charged residues in transmembrane segments of the multidrug transporter MexB of pseudomonas aeruginosa. J. Bacteriol. 183, 1734–1739 (2001).
29. Takatsuka, Y. & Nkaiido, H. Threonine-978 in the transmembrane segment of the multidrug efflux pump AcrB of Escherichia coli is crucial for drug transport.
as a probable component of the proton relay network. J. Bacteriol. 188, 7234–7244 (2006).
32. McKinney, S. A., Joo, C. & Ha, T. Analysis of single-molecule FRET trajectories using hidden Markov modeling. Biophys. J. 91, 1941–1951 (2006).
33. Otwinowski, Z. & Minor, W. Processing of X-ray diffraction data collected in oscillation mode. Methods Enzymol. 276, 307–326 (1997).
34. Eicher, T. et al. Coupling of remote alternating-access transport mechanisms for protons and substrates in the multidrug efflux pump AcrB. Elife 3, e03145 (2014).
35. Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. Acta Crystallogr. D Biol. Crystallogr. 60, 2126–2132 (2004).
36. Adams, P. D. et al. PHENIX: building new software for automated crystallographic structure determination. Acta Crystallogr. D Biol. Crystallogr. 58, 1948–1954 (2002).
37. Brünger, A. T. et al. Crystallography & NMR system: a new software suite for macromolecular structure determination. Acta Crystallogr. D Biol. Crystallogr. 54, 905–921 (1998).
38. Erkens, G. B., Hanelt, I., Goudsmits, J. M., Slotboom, D. J. & van Oijen, A. M. Unsyncronised subunit motion in single trimeric sodium-coupled aspartate transporters. Nature 502, 119–123 (2013).
39. Dave, R., Terry, D. S., Munro, J. B. & Blanchard, S. C. Mitigating unwanted photophysical processes for improved single-molecule fluorescence imaging. Biophys. J. 96, 2371–2381 (2009).
40. Swoboda, M. et al. Enzymic oxygen scavenging for photostability without pH drop in single-molecule experiments. ACS Nano 6, 6364–6369 (2012).
41. Kapanidis, A. N. et al. Alternating-laser excitation of single molecules. Acc. Chem. Res. 38, 523–533 (2005).
42. Park, J. et al. Single-molecule analysis reveals the kinetics and physiological relevance of MutL–ssDNA binding. PloS ONE 5, e15496 (2010).
43. Verchere, A., Dezi, M., Adrien, V., Broutin, I. & Picard, M. In vitro transport activity of the fully assembled MexAB-OprM efflux pump from pseudomonas aeruginosa. Nat. Commun. 6, 6890 (2015).
44. Zhang, Y., Werling, U. & Edelmann, W. SLiCE: a novel bacterial cell extract-based DNA cloning method. Nucleic Acids Res. 40, e55 (2012).
45. Muraoka, W. T. & Zhang, Q. Phenotypic and genotypic evidence for L-fucose utilization by campylobacter jejuni. J. Bacteriol. 193, 1065–1075 (2011).

Acknowledgements
This work was supported by NIH Grants R01AI114629 (E.W.Y.), R01GM051290 (Y.K.S.), 5U54GM087519 (Y.K.S.), and R01AI118283 (Q.Z.). This work is based upon research conducted at the Northeastern Collaborative Access Beamlines of the Advanced Photon Source, supported by an award GM103403 from the National Institutes of General Medical Sciences. Use of the Advanced Photon Source is supported by the U.S. Department of Energy, Office of Basic Energy Sciences, under Contract No. DE-AC02-06CH11357.

Author contributions
C.-C.S. and E.W.Y. designed crystallography experiments. C.-C.S., N.K., J.R.B., and H.-T. L. cloned, expressed, and purified CmeB. C.-C.S., J.R.B. and H.-T.L. crystallized form I CmeB. N.K. and A.R. crystallized form II CmeB. C.-C.S., N.K., A.R., J.R.B., H.-T.L., T.-H.C., and K.R.R. collected X-ray diffraction data. C.-C.S. performed model building and refinement of the CmeB structures. C.-C.S., L.Y., and Y.-K.S. designed single-molecule FRET experiments. C.-C.S. and L.Y. performed single-molecule FRET experiments. C.-C.S., L.D., and Q.Z. designed drug susceptibility experiments. L.D. performed drug susceptibility experiments. C.-C.S. and E.W.Y. wrote the paper. C.-C.S., J.A.D., and E.W.Y. proofread the paper. E.W.Y. supervised the research.

Additional information
Supplementary Information accompanies this paper at doi:10.1038/s41467-017-00217-z.

Competing interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s) 2017