Supplementary information, Data S1

Supplementary methods

Single-molecule FRET imaging experiments

First, we constructed a Cys-free variant of MdfA (denoted as WT*) by mutating all four Cys residues in WT to either Ser or Ala following a previous report [1]. Second, we screened a number of Cys-pair variants, from which the V167C/N374C/WT* variant (referred to as WT**) was selected to monitor the conformational change on the periplasmic side. Next, we confirmed the Cm-resistance activity of both WT* and WT** variants (Fig. 5a). Further, protein samples (20 μM) purified in buffer C (20 mM HEPES (pH 7.2), 100 mM NaCl, and 0.2% DM) were labeled in buffer C containing 200 μM Cy3 and 200 μM Cy5 maleimide (GE Healthcare) for 1 h at 4°C. Free Cy3 and Cy5 were removed by reloading the sample on the Ni^{+} resin. In addition, we carried out steady-state anisotropy measurements of Cy5-labelled MdfA (10 nM) using a HITACHI F-7000 spectrofluorometer with excitation and emission wavelengths of 646 nm and 662 nm respectively in a buffer (20 mM Tris-MES (pH 8.0), 100 mM NaCl, and 0.2% DM) with or without Cm. The result confirmed that dyes attached to MdfA variants had low anisotropy (Table S3).

Single molecule FRET imaging experiments were carried out as previously described [2]. Imaging chambers passivated with a mixture of PEG and biotin-PEG were incubated with 100 μg/ml streptavidin. His_6-tagged dye labeled samples were immobilized to the streptavidin treated chamber surface by Biotin-NTA-Ni^{2+}. All experiments were performed in buffer D (20 mM Tris-MES (pH 8.0) 150 mM NaCl, and 0.05% DDM, with or without 100 μM Cm) with an oxygen scavenging system (0.1% glucose, 5 mM β-mercaptoethanol, 1 unit/ml glucose oxidase, 1 unit/ml catalase, and 1 mM cyclo-octatetraene).

Fluorescence experiments were performed by using an objective based total internal reflection fluorescent (TIRF) microscope. Cy3 fluorophore was excited with 532 nm laser (Coherent Inc., Sapphire SF). Photon emitted from Cy3 and Cy5 were collected using 1.49 NA 100× objective (Olympus UAPON 100× OTIRF), and Optosplit II (Cairn Research Limited) were used to separate spatially Cy3 and Cy5 frequencies onto a cooled EMCCD (Andor iXon Ultra). Fluorescence data were acquired using the software Metamorph (Universal Imaging Corporation). Images were taken at 50 ms/frame.

Data analysis was performed using custom software written in MatLab (MathWorks). Cy3 and Cy5 channel were mapped using TetraSpeck fluorescent microsphere beads (Invitrogen, 0.1 µm). At least more than 10 beads were selected to get the transformation matrix used in mapping in MatLab. Photobleaching events in each traces were detected as a significant drop (≥ 3 times standard deviation of background noise) in the median filtered (window size = 9 frames) total fluorescence intensity ($I_{total} = I_{cy3} + I_{cy5}$) without returning to the previous average level. Signal-to-background noise ratios (SNR) are calculated as total intensity relative to the standard deviation of background noise: $I_{total}/[stdev(I_{cy3})+stdev(I_{cy5})]$. Traces were selected to meet the following criteria: a single catastrophic photobleaching event, at least 8:1 signal-to-background noise ratio, a donor-to-acceptor Pearson's correlation coefficient < 0. Spectral bleed-through of Cy3 intensity on the acceptor channel was corrected by subtracting 7.5% of donor signal from the acceptor. FRET traces were calculated as: $FRET = I_{Cy5}/(I_{Cy3} + I_{Cy5})$, where I_{Cy3} and I_{Cy5} are the instantaneous Cy3 and Cy5 fluorescence intensities, respectively. Contribution of the photophysical zero-FRET state in FRET histograms was removed by fitting the data to a two-state model ($E_1 = 0.1 \pm 0.1$ and $E_2 = 0.4 \pm 0.1$) with the segmental k-means algorithm [3].

Thermofluor stability assay

Since we observed a deoxycholate molecule in the crystal structure, a thermofluor analysis [4] was performed to verify the effects of deoxycholate on

thermal stability of MdfA. Well-known substrates of MdfA, *e.g.* TPP^+ and chloramphenicol, were tested. Analyses were performed at 0.3 mg/ml protein concentration and in 25 μl of buffers of varied pH (6.0–9.0), 100 mM NaCl, 0.2% (v/v) DM, and 100 μM sodium deoxycholate or other substrates. Thiol-specific fluorochrome N-[4-7-diethylamino-4-methyl-3-coumarinyl) phenyl] maleimide (CPM; Invitrogen, US) [5] was used as the fluorescence probe for detecting thermal denaturation, and fluorescence was measured with a 387-nm excitation and a 463-nm emission wavelength. All experiments were carried out using a qPCR instrument, Rotor-Gene 6600 (Corbett Research, Australia) equipped with a blue fluorescence channel. The result is shown in Fig. S5c.