As one of the most elegant biological processes developed in bacteria, the siderophore-mediated iron uptake demands the action of specific ATP-binding cassette (ABC) importers. Although extensive studies have been done on various ABC importers, the molecular basis of these iron-chelated-siderophore importers are still not fully understood. Here, we report the structure of a ferrichrome importer FhuCDB from *Escherichia coli* at 3.4 Å resolution determined by cryo electron microscopy. The structure revealed a monomeric membrane subunit of FhuB with a substrate translocation pathway in the middle. In the pathway, there were unique arrangements of residues, especially layers of methionines. Important residues found in the structure were interrogated by mutagenesis and functional studies. Surprisingly, the importer’s ATPase activity was decreased upon FhuD binding, which deviated from the current understanding about bacterial ABC importers. In summary, to the best of our knowledge, these studies not only reveal a new structural twist in the type II ABC importer subfamily, but also provide biological insights in the transport of iron-chelated siderophores.
Siderophores are a group of small molecules synthesized and secreted by microorganisms to chelate life-essential iron ions (Fe\(^{3+}\)) from the environment\(^1\). The importance of siderophore molecules is further highlighted by the increasing reports of their biological functions other than Fe\(^{3+}\) chelating\(^6\). These functions include binding and transporting a variety of metal ions as metallophores\(^3,4\), acting as signaling molecules for gene regulation\(^5\), protecting bacteria from oxidative stress\(^5,8,9\), as well as providing antibacterial activity as sideromycins\(^9,10\). In the extracellular space, secreted siderophores form stable complexes with Fe\(^{3+}\) ions, which are recognized and imported by the outer membrane receptors. In the periplasm, a group of soluble proteins named periplasmic substrate-binding proteins (SBP) are generally responsible for binding the siderophore-Fe\(^{3+}\) molecules and delivering them to corresponding inner membrane importers. Specifically, in the inner membrane of uropathogenic Escherichia coli, there are four such importer systems known so far: YbtPQ importing yersiniabactin\(^10\), FepBDGC importing enterobactin\(^11\), FhuCBD importing citrate-based siderophores\(^12\), as well as FhuCBD importing hydroxamate-based siderophores\(^13\). Although all of siderophore importers belong to the superfamily of ATP-binding cassette (ABC) transporters, the architecture of the four systems is not the same: YbtPQ resembles the fold of type IV exporters\(^14\), while the other three seem to be bacterial type II importers.

It is well known that ABC transporters have at least four common domains: two transmembrane domains (TMD) forming a central translocation pathway and two cytosolic nucleotide-binding domains providing necessary energy for the substrate translocation via ATP hydrolysis. For type II ABC importers in gram-negative bacteria, periplasmic substrate-binding proteins (SBP) are usually required to specifically bind and deliver substrates to their TMDs. Available high-resolution structures of type II importers, including vitamin B\(_12\) importer BtuCDF from E. coli\(^15-18\), heme importers HmuUV from Y. pestis\(^19\) and BhuUVT from B. cenocepacia\(^20\), as well as molybdate importer MolBC from H. influenzae\(^21\), have provided excellent explanation for the general mechanism of substrate import. However, how are different iron-chelated siderophores imported through related ABC importers is not well understood.

In this study, we functionally characterized the ferrichrome, a hydroxamate-type siderophore molecule, importer FlhCDB complex from E. coli and determined its structure at 3.4 Å resolution by single-particle cryo-electron microscopy (cryo-EM). As far as we know, this is the first high-resolution structure of a siderophore importer in the type II ABC importer subfamily. The structure was in the inward-open conformation as the periplasmic side of the transport pathway was closed while the cytoplasmic side was open. The substrate-binding pocket in FlhU (SBP), was fully occupied by loops from FlhB (TMD) as well as several water molecules. In all known type II importer structures, the translocation pathway is formed by homodimers of their membrane subunits. While in FlhCDB, the pathway was in the middle of the FlhB monomer, representing another type of type II importer structure. Along the surface of the pathway, there were small hydrophobic residues, uncharged polar residues, as well as unique layers of Met residues. With the FlhCDB structure and related functional experiments, our study provides important mechanical insights in the import of iron-chelated siderophores.

**Results and discussion**

**ATPase activity and transport assay of the Flh importer in proteoliposomes.** To characterize the function of Flh importer, we overexpressed the full FlhCDB complex as well as the FlhCB subcomplex (only the membrane and cytosolic components), purified them in detergent lauryl maltose neopentyl glycol (LMNG) (Supplementary Figs. 1 and 2). To perform functional experiments, we incorporated FlhCDB into liposomes made of E. coli polar extract lipids. The orientation of the reconstituted importers is determined by limited proteolysis of the proteoliposomes using thrombin, followed by western blot using anti-His probe (Supplementary Fig. 3a). The idea was that FlhC from the inside-out importers would be digested and lose its N-terminal His-tag, while FlhC from the right-side-out importers would not be digested. The results (Supplementary Fig. 3b) showed that ~64% FlhC remained intact after the treatment. Considering that the efficiency of the thrombin digestion was ~90%, thus in the proteoliposomes, ~60% of the reconstituted FlhCDB were right-side-out, and the rest ~40% of them were inside-out.

These proteoliposomes were used to measure the ATPase activity of the importers. For the FlhCDB subcomplex alone, the initial rate of ATP hydrolysis of the inside-out importers in the first 4 min of reaction was measured and appeared to be linear (Supplementary Fig. 4). We then plotted the initial rates as a function of the ATP concentration (Fig. 1a). Here, the ATPase null-mutant (E173A, in the Walker-B domain of FlhC) was used as a negative control as it had no detectable activity. In addition, vanadate was able to inhibit more than 90% of the ATPase activity. While for the wild-type FlhC, the data were fit using an expanded version of the Michaelis–Menten equation with Hill coefficient, which yielded the following kinetic constants: V\(_{\text{max}}\) of ~745 ± 31 nmol/mg/min, K\(_{\text{m}}\) of ~0.69 ± 0.08 mM, and Hill coefficient of ~1.97 ± 0.08. When comparing to known Type II ABC importers (Supplementary Table 1), we found that FlhCDB had a relatively high K\(_{\text{m}}\) value, suggesting a lower binding affinity to ATP. Furthermore, the sigmoidal shape of the curve with a Hill coefficient of ~1.97 indicated a strong cooperativity between the two ATP binding sites in FlhC. To test if the importer’s ATPase activity was affected by FlhD, we performed the same experiment using FlhCDB proteoliposomes with enclosed ferrichrome-loaded FlhD and FlhD only. Surprisingly, the results (Fig. 1b) showed that the ATPase activity of the FlhCDB + FlhD was decreased as its kinetic constants were V\(_{\text{max}}\) of ~127 ± 8 nmol/mg/min, K\(_{\text{m}}\) of ~0.74 ± 0.05 mM, and Hill coefficient of ~1.45 ± 0.04. While with the ferrichrome-loaded FlhD, the importer’s ATPase activity was also decreased as the kinetic constants were V\(_{\text{max}}\) of ~155 ± 19 nmol/mg/min, K\(_{\text{m}}\) of ~0.77 ± 0.06 mM, and Hill coefficient of ~1.73 ± 0.06. The consensus notion in the field is that docking of the SBP to the TMD stimulates the ATPase activity of all known ABC importers\(^22\). However, in the case of Flh importer, upon FlhD binding, its ATPase activity was apparently decreased as the V\(_{\text{max}}\) dropped more than five times. In the meantime, the cooperativity of the ATP binding was weakened, although the ATP binding affinity seemed to be at the same level as indicated by similar K\(_{\text{m}}\) values. These results suggest that the molecular mechanism of FlhCDB might deviate from the standard model of type II ABC importers derived from BtuCDF.

For the transport assay, an ATP-regenerating system with ATP and MgCl\(_2\) was included inside the reconstituted proteoliposomes by following the established protocol\(^23\). The transport activity of the right-side-out FlhCDB was quantified by liquid scintillation counting as \(^{55}\)Fe-ferrichrome was used as the substrate. The results (Fig. 1c) showed that the \(^{55}\)Fe-ferrichrome uptake followed the Michaelis–Menten equation and reached saturation in ~10 min. The initial rate of the transport was ~7.5 nmol/mg/min. Ideally, the coupling efficiency of the ATPase activity and transportation rate of ABC transporters is 2, meaning two ATP are hydrolyzed when one substrate is translocated. In the case of FlhCDB, if assuming that FlhCDB is the main species in vivo, the calculated coupling efficiency is ~20, which is higher than the...
theoretical value of two but reflects the universal characterization of inefficient translocation among most ABC transporters24.

Overall architecture of FhuCDB in the inward-open conformation. To understand the molecular basis of this importer, we determined the structure of FhuCDB in LMNG by single-particle cryo-EM at 3.4 Å resolution (Supplementary Table 2, Supplementary Figs. 5–7). The overall size of the complex was ~130 × 100 × 80 Å³, with one FhuD on the periplasmic side, one FhuB in the membrane, and two FhuC on the cytosolic side (Fig. 2a). The overall fold of the complex indicated that the importer belonged to the type II ABC importer subfamily. However, a unique feature in FhuCDB was the single membrane subunit of FhuB, comparing to two subunits of homodimers in other known subfamily members. FhuB had 20 transmembrane helices (TM) folding into two domains: N-half with the first ten TMs and C-half with the last ten TMs (Fig. 2b). In between the two halves, there was a central translocation pathway. Based on a tunnel calculation using the MoleOnline server25, the FhuB central pathway was open to the cytosolic side and closed to the periplasmic side, indicating an inward-open conformation (Fig. 2a). Right at the cytosolic opening of the central pathway,
there was a loop of ~24 residues (residues 324–347) connecting TM10 from N-half and TM11 from C-half that are 45 Å away from each other. Unfortunately, the cryo-EM density of this loop was mostly missing, suggesting that the loop was fairly flexible. Although the loop was unlikely to block the channel opening in this inward-open conformation, it is unclear at this point whether its local state would change in the occluded or outward-open conformations.

Substrate-binding site in FhuD occupied by FhuB loops and water molecules. FhuD is a Cluster A type periplasmic SBP for the FhuCDB import system. It has two lobes (not identical to each other) connected by a hinge α-helix (backbone), which ensures a rigid overall fold and allows only small movement upon substrate binding (Fig. 3a). In our structure, although there was no substrate bound, FhuD could be superimposed well to all previously determined substrate-loaded FhuD structures with a root mean square deviation (r.m.s.d) of 2.3–2.5 Å (Fig. 3a & Supplementary Fig. 8). Using the gallichrome-loaded FhuD (PDB:1EFD) as a comparison, the C-lobe of our FhuD moved marginally with most substrate-interacting residues (hydrophobic I183, L189, and W273) remaining in position except W217, which was flipped 180°. In contrast, the N-lobe moved substantially away from the center, leaving only one residue (W68) unchanged while most other substrate-interacting residues (R84, S85, Y106) pulled away. For instance, the OH group of the Y106 side chain moved as far as ~9 Å. The movement of these residues effectively deformed the original substrate-binding pocket in FhuD and allowed it to be occupied by two loops from FhuB: L5 in between TM5 and H5a as well as L15 in between TM15 and H15a (Fig. 3a). Such loop was originally described in the crystal structure of maltose transporter MalFGK29, as the P3 loop from MalG clashed into the maltose-binding site in MBP. After that, similar loops were found in BtuCDF and BhuUVT complex structures. Thus, it appears to be a common theme for ABC importers to use these loops to scoop out substrates from their SBP (called “scoop loop”) and subsequently deliver them to their TMD. In FhuCDB, three residues in L5 (F167, D170, Q171) were directly interacting with FhuD residues (N64, W68) via hydrogen bonding. L15 loop protruded into the FhuD pocket without apparent interactions. However, an interesting observation here is that there were three structured water molecules. Specially, two of them were trapped in the interface by the hydrophobic bubble created by L15 and the surrounding FhuD loops. Although such water molecules had been observed in multiple MBP-MalFGK crystal structures, they had never been described in type II importers such as BtuCDF. It is possible that the water molecules are critical for the association of FhuD and FhuB.

Subunit interactions within FhuCDB. The rearranged FhuD substrate-binding pocket was not the only communication between FhuD and FhuB in the structure. In fact, FhuD docked onto FhuB through a complicated network of interactions. On one hand, the N-lobe of FhuD docked onto the C-half of FhuB via the following specific interactions FhuD-E90/FhuB-R390, FhuD-N88/FhuB-Q636, FhuD-N88/FhuB-Y517, FhuD-E86/FhuB-S515, as well as a hydrogen-bonding network involving a third water molecule (Supplementary Fig. 9a). This water not only connected FhuB-T510 and the backbone of FhuD-T85, but also stabilized the side chain of FhuB-Q507 which in turn interacted with the backbone of FhuB-G107. On the other hand, the C-lobe of FhuD docked onto the N-half of FhuB involving the following FhuD residues H187, S223, D225, R226, and FhuB residues S57, T182, T184, E304 (Supplementary Fig. 9b). Most FhuB residues interacting directly with FhuD were highly conserved.
Similar to other ABC transporters, the coupling helices from FhuB-N-half (residues 209-226) matched those from FhuC. The association between FhuB and FhuC was symmetrical: the FhuC subunits were almost identical (Supplementary Fig. 13a). FhuB halves were superimposed reasonably well, while the two FhuD lobes were completely misaligned, the two sides of inward-open BhuUVT (PDB:5B58), the translocation pathway in FhuB was vividly narrower as TM15 moves ~4.5 Å towards the center while other adjacent TMs stayed at similar positions as in BhuU (Supplementary Fig. 15). The pathway in FhuB was thus unsymmetrically formed by three main TMs: TM3, TM5, and TM15, without TM13 as in BhuUVT (Fig. 4). On the periplasmic side, the pathway was closed by residues from the following regions: TM5 ~ H5a and TM15 ~ H15a. Specifically, these four residues: V165, F176 on the N-half and their counterparts Q498, L509 on the C-half, defined the narrowest position in the pathway, which was then directly closed on top by H169 and M505 (Fig. 4a). Below the periplasmic gate, residues along the path could be grouped into three types: hydrophobic residues with mostly small side chains (A96, G150, L151, L155, G158, A159, L494, I512), polar residues (T91, T92, T97, Q100, S154, N161, Q162, S421, E423, S487, T488, T491, S513) capable of forming hydrogen bonds, as well as Met residues (M175, M495, M492, M484) (Fig. 4c). How do the specific arrangements of residues contribute to the translocation of substrate such as ferrichrome? First, it has been demonstrated that ferrichrome favorably interacts with aromatic rings and thus there are adequate aromatic residues found in the ferrichrome-binding site in FhuD and its outer membrane receptor FhuA. While in FhuB, no aromatic residues are found pointing directly towards the central pathway, suggesting that the binding between FhuB and ferrichrome will be rather weak and thus favors rapid translocation. Second, to minimize the energy barrier of translocation, oligosaccharide transporters, such as the maltoporin channel and Wzm-Wzt transporter, employ a combination of aromatic hydrophobic interactions with a continuous pattern of hydrogen bond donors and acceptors along their translocation pathways. While in the case of FhuCDB, ferrichrome is not inherently hydrophobic, especially its hexapeptide ring. Thus, its translocation should be facilitated by the continuous layers of hydrogen bond donors and acceptors along the way, which are the abundant polar residues especially Thr and Ser. Third, a similar arrangement of Met residues is also observed in another siderophore ABC importer YbtPQ (Supplementary Fig. 16). To confirm the functional importance of the Met residues along the pathway, we mutated all four of them (M175, M484, M492, M495) to Ala and performed 55Fe-ferrichrome transport assay using proteoliposomes. The results (Supplementary Fig. 17) show that the Met mutant FhuCB reconstituted into the liposomes as efficiently as wild-type FhuCB. Although the ATPase activity remains at the similar level, the transport efficiency of the Met to Ala mutant drops ~80%, suggesting potential interactions between the substrate and Met residues. We then mutated all four
Met to Leu and found that the transport efficiency drops ~50% comparing to the wild-type FhuB, confirming that the hydrophobic nature of the Met side chains is important for the substrate translocation. However, as the transport efficiency of Met to Ala mutant drops more, these Met residues must provide more than just hydrophobic interactions to the substrate. It is known that the sulfur atom in the Met side chain is generally not active but can occasionally react with some electrophilic centers, even forming hydrogen bonds. Thus, a slight possibility is that in the FhuCDB complex, the Met side chains may transiently attract the chelated Fe\(^{3+}\) in ferrichrome using its sulfur atom, which could also contribute to the substrate translocation.

Previous studies on BtuCDF complex revealed two cytoplasmic gates: gate I of the TM5 that was closed in the structure of asymmetrically closed BtuCDF (PDB: 2QI9)\(^{16}\), and gate II of the L2 loop between TM2 and TM3 that was closed in the structure of outward-open BtuCD (PDB: 4R9U)\(^{18}\). Here, we compared those two structures with our FhuCDB (Supplementary Fig. 18). The comparison showed roughly the same position for the cytosolic half of these TMs: FhuB-TM15 and BtuC-TM5'. However, the positions of TM3, TM5, and L2 in FhuB were drastically different. Specifically, FhuB-TM3 was much closer to the center of the molecule than BtuC-TM3 in both Btu structures, and thus actively participated in the formation of the translocation pathway. As cytoplasmic gate I, the cytoplasmic half of FhuB-TM5 was tilted away from the center, making it different from the closed TM5 in the 2QI9 structure (Supplementary Fig. 18a) but similar to the open TM5 in the 4R9U structure (Supplementary Fig. 18b). Thus, the cytoplasmic gate I was open in our structure. In the meantime, the cytoplasmic gate II in FhuB...
was also open, because the distance between the L2 and L12 loops were much larger than the closed gate II in the outward-open BtuCD (Supplementary Fig. 18b). The closed gate II in BtuCD was formed by two conserved residues N83 and L85, equivalent to residues N85, N417, and L87, M419 in FhuB, respectively. While in our structure, the narrowest position, which is still open, in the cytoplasmic side of the pathway was defined by residues T92, M484, L151, and E89 (Fig. 4c).

**Conclusions**

In this study, we determined the structure of the ferrichrome ABC importer FhuCDB from *E. coli* in the inward-open conformation at 3.4 Å resolution. The structure revealed critical residues regarding subunit interactions, periplasmic gating, as well as transport pathway. The importance of these residues was confirmed by mutagenesis and functional assays. Specifically, along the transport pathway, a continuous layer of Met residues as transport pathway. The importance of these residues was confirmed by mutagenesis and functional assays. Specifically, along the transport pathway, a continuous layer of Met residues was proposed to form a gate. This gate is likely to interact with layers of Met and small polar residues in the periplasmic region. The structure also revealed that the substrate is translocated down the pathway via transient interactions with layers of Met and small polar residues.

**Methods**

**Plasmids.** For the FhuCDB construct, the whole operon of FhuCDB was cloned from *Escherichia coli* (*E. coli*) genome into a pET15b (Novagen) vector with a His-tag and a thrombin digestion site on the N-termini of FhuC. For the wild-type FhuC construct, FhuC in the middle of the operon was deleted directly from the FhuCDB-pET15b construct. All mutants of FhuC used in this manuscript were modified on this FhuC-pET15b construct by direct mutagenesis. For the FhuD construct, the gene was amplified and cloned into a pET15b vector.

**Protein expression and purification.** FhuCDB and FhuCB constructs were overexpressed in *E. coli* strain of BL21 (DE3) C43 (Sigma-Aldrich) at 18 °C overnight with 0.2 mM Isopropyl β-D-thiogalactopyranoside (IPTG, UBPBio). The culture was harvested and resuspended in Buffer A (20 mM HEPES at pH 7.5 and 150 mM NaCl) with 1 mM phenylmethylsulfonyl fluoride (PMSF). The cells were lysed by passing through the M110P microfluidizer (Microfluidics) two times at 15,000 psi. Cell debris was removed by centrifugation at 15,000 g for 30 min. Then, the membrane fractions were collected by ultracentrifugation at 150,000 g for 2 hr, resuspended in Buffer A, and stored at −20 °C. To solubilize the complexes in the membrane, 1% lauryl maltose neopentyl glycol (LMNG) was added, and the mixture was incubated at 4 °C for 2 hr before ultracentrifugation at 150,000 g for 1 h. Proteins in the supernatant were purified by the affinity chromatography with TALON (Clontech) resin, followed by gel filtration chromatography on a Superose 6 column (GE Healthcare Life Sciences) in Buffer A with 0.01% LMNG. The eluted peak fractions were combined and concentrated to −5 mg/ml for further experiments, respectively. FhuD was expressed and purified by following a published protocol.

**Fig. 5 Schematic representation of the transport cycle of FhuCDB.** Without the substrate, FhuCDB cycles through inward-open (states 1, 5) and outward-open (state 2) upon ATP binding, ATP hydrolysis, and ADP release. With the substrate, FhuD in FhuCDB (state 2) is replaced by the substrate-loaded FhuD (state 3). The substrate is then released to FhuB and transferred down the translocation pathway (state 4). After ATP hydrolysis, FhuB opens the cytosolic gate and releases the substrate into the cytosol (state 5).
Reconstitution of FlucB and FlucDB into proteoliposomes. The experiment was done by following a previously published protocol42. Briefly, 10 mg E. coli polar end lipids (Avanti Polar Lipids) were dissolved in chloroform, dried by nitrogen gas, and immediately resuspended in Buffer A to a final concentration of 10 mg/mL. The large unilamellar liposome vesicles were made by extruding the suspension through a 400 nm polycarbonate membrane filter using a mini extruder (Avanti Polar Lipids). To reconstitute proteoliposomes, the liposomes were destabilized by 0.015% Triton X-100 and then incubated with purified proteins (FlucDB or FlucB or FlucCB) at a ratio of 100:1 (wt/wt) for 20 min at room temperature. A total of 100 mg Bio-Beads SM-2 (Bio-Rad Laboratories) was then added to the mixture to absorb all detergents overnight at 4 °C. The reconstituted proteoliposomes were harvested by ultracentrifugation at 180,000 x g for 20 min for further experiments.

Thrombin digestion of the proteoliposomes. To estimate the ratio of inside-out and right-side-out transporters in the proteoliposomes, thrombin digestion was performed. The FlucDB and FlucB proteoliposomes were incubated with human α-thrombin (Enzyme Research Laboratories) at a molar ratio of 100:1 at room temperature for 2 h, and then subjected to western blot analysis using HisProbe-HRP conjugate (Thermo Fisher Scientific). The density of the protein bands was quantified using GelAnalyzer 19.1 (www.gelanalyzer.com).

ATPase activity assay. The ATPase activity was determined using an ATPase/GT-Pase Activity Assay Kit (Sigma-Aldrich) featuring a detectable fluorescent product from malachite green reacting with released phosphate group. Three proteoliposome samples were used: FlucB, FlucB with 10 μM ferrichrome, and FlucB with 1 μM FhuD enclosed. [35S]- labeled FlucB with 1 μM FlucB enclosed. 0.0205 mg/mL of these proteoliposomes were incubated with ATP-MgCl2 at various concentrations from 0 to 2 mM at 37 °C. Several time points between 0 and 4 min were taken. The amount of phosphate produced by inside-out transporters was detected by absorbance at 620 nm. The data were calculated in Excel. For Fig. 1, the data were fitted to the extended Michaelis–Menten equation with Hill coefficient in Excel (Microsoft).

Transport assay. Proteoliposomes with either FlucB or FlucB Met mutants were used, and the transport rate of the right-side-out importers was measured as follows. Briefly, to enclose the ATP-regeneration system, the proteoliposomes were subjected to three cycles of freeze and thaw with 10 mM ATP, 2 mM MgCl2, 0.1 mg/mL pyruvate kinase, and 10 mM phosphoenolpyruvate followed by five times of passing through a 400 nm polycarbonate membrane filter. The proteoliposomes were harvested by ultracentrifugation at 180,000 x g for 20 min and then resuspended into Buffer A to a final protein concentration of 0.002 mg/mL. The uptake experiments were carried out at 37 °C with 1.6 μM purified FlucD and 16 μM substrate [35S]-ferrichrome. The reaction was stopped at different time points by adding 200 μL of ice-cold buffer A, followed by filtration using pre-wetted cellulose nitrate filters. The filters were washed with 1 mL of Buffer A, dried for 1 h, and then dissolved in 3 mL of Filter Count scintillation liquid (Perkin Elmer). The 35S radioactivity within the proteoliposomes was quantified by using a Tri-carb 2910 TR Scintillation counter (Perkin Elmer).

Microscale thermophoresis. To measure the binding affinity between FlucD and FlucB, MST experiments were performed with a Monolith NT.115pico (NanoTemper). A standard protocol was followed43. Briefly, the His-tag on purified FlucD was digested by trypsin and used to reconstitute proteoliposomes with FlucB. For each reaction, 40 nM FlucB was mixed with a serial dilution of 1.6 μM FlucD with substrate ferrichrome or 30 μM FlucD without substrate. Micro thermophoresis was performed using 20% LED power and medium MST power. Kd values were all calculated using the NanoTemper software with default settings.

Cryo-EM structure determination and model building. Three microlices of purified FlucDB in LMMg at ~1.2 mg/mL were applied to a plasma-cleaned C-flat carbon film containing 0.5% uranyl acetate (1.2/100 mesh, Electron Microscopy Servicing). The grids were prepared using a Vitrobot Mark IV (Thermo Fisher Scientific) with the environmental chamber set at 100% humidity and 4 °C. The grid was blotted for 3.5 s and then flash frozen in liquid ethane cooled by liquid nitrogen. Cryo-EM data were collected in the Pacific Northwest Cryo-EM Center (PNCC) on a Titan Krios (Thermo Fisher Scientific) operated at 300 keV and equipped with a K3 direct detector (Gatan) together with a Bioquantum energy filter (slit width of 20 eV used). A total of 9142 movies were recorded with a pixel size of 0.399 Å under super-resolution mode, a defocus range of −170 to −250 μm, and a total dose of ~65 electrons/A2 over 60 frames. The total exposure time was ~21.5 s with a dose rate of ~19.6 electrons/pixel/s. The data were processed using both cryoSPARC v2.145 and Relion 3.160. Specifically, in cryoSPARC, movies were processed with patch motion correction and patch CTF estimation. Approximately 2000 particles were manually picked, and ten class averages were generated by 2D classification. Five good class averages were selected as templates for auto picking. Following standard performance of particle extraction, 2D classification, ab-initio reconstruction, and heterogeneous refinement, best groups of particles were selected for a final reconstruction at 3.4 Å resolution. The reconstruction and selected particles were then imported into Relion for further 3D classification and 3D auto-refine. The final reconstruction from Relion was at the same resolution as the one from cryoSPARC. In both programs, the gold-standard FSC curves with a 0.143 cutoff were used to determine the resolution. The model building process was carried out in Coot46 using the map from Relion. X-ray structure 1ESW22 was used as the initial model to build FlucD, while other part of the FlucDB model was built manually from the scratch. Briefly, secondary structures were built with the guidance from multiple secondary structure prediction programs: iPred42 and PSIPRED43, as well as the well-known topology of the nucleotide-binding domains. Flexible loops were manually built after placing the secondary structures. The final model was refined in PHENIX46. The quality of the model was assessed by MolProbity47. Statistical details could be found in Supplementary Table 2. All superpositions of structures were calculated in the program UCSF Chimera with the alignment algorithm of Needleman-Wunsch and BLOSUM-62 matrix46.

Statistics and reproducibility. ATPase activity assays, and substrate transport experiments were independently repeated three times. All the calculations were done in Excel. For Fig. 1, the data were fitted to the extended Michaelis–Menten equation with Hill coefficient. For Supplementary Fig. 4, the data were fit to a linear function.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Cryo-EM map of FlucDB was deposited in the Electron Microscopy Data Bank under the accession code EMD-23251. Coordinates of the atomic model of FlucDB was deposited in the Protein Data Bank under the accession code 7L88. Uncropped SDP-PAGE and Western blots for Supplementary Figs. 2b, 3b and 17a are provided in Supplementary Fig. 19. Data for Fig. 1 are provided in Supplementary Data 1. All other data are available from the corresponding author upon reasonable request.

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References
1. Mietheke, M. & Marahiel, M. A. Siderophore-based iron acquisition and pathogen control. Microbiol Mol. Biol. Rev. 71, 413–451 (2007).
2. Johnstone, T. C. & Nolan, E. M. Beyond iron: non-classical biological functions of bacterial siderophores. Dalton Trans. 44, 6320–6339 (2015).
3. Lewis, T. A. et al. Physiological and molecular genetic evaluation of the deleterion agent, pyridine-2,6-bis(monothiocarbonyl) acid (PTDC) as a secondary siderophore of Pseudomonas. Environ. Microbiol 6, 159–169 (2004).
4. Koh, E. I. et al. Metal selectivity by the virulence-associated yersiniabactin metallophore system. Metallomics 7, 1011–1022 (2015).
5. Roux, A., Payne, S. M. & Gilmore, M. S. Microbial telesensing: probing the environment for friends, foes, and food. Curr. Host Microbiol 6, 115–124 (2009).
6. Adler, C. et al. The alternative role of enterobactin as an oxidative stress protector allows Escherichia coli colony development. PLoS One 9, e84734 (2014).
7. Chaturvedi, K. S. et al. Cupric yersiniabactin is a virulence-associated superoxide dismutase mimic. ACS Chem. Biol. 9, 551–561 (2014).
8. Braun, V., Pramanik, A., Gwinner, T., Koberle, M. & Bohn, E. Sideromycins: tools and antibiotics. Biometals 22, 3–13 (2009).
9. Ghazi, I. M., Monogue, L. M., Tsui, M. & Nicolau, D. P. Humanized exposures of cedelocidor, a siderophore cephalosporin, display sustained in vivo activity against siderophore-resistant pseudomomas aeruginosa. Pharamacology 101, 278–284 (2018).
10. Fetherston, J. D., Bertolino, V. J. & Perry, R. D. YtbP and YtbQ: two ABC transporters required for iron uptake in Yersinia pestis. Mol. Microbiol. 32, 289–299 (1999).
11. Chenault, S. S. & Earhart, C. F. Organization of genes encoding membrane proteins of the Escherichia coli ferrienterobactin permease. Mol. Microbiol. 5, 1407–1413 (1991).
12. Staudenmaier, H., Van Hove, B., Yaraghi, Z. & Braun, V. Nucleotide sequences of the fecBCDE genes and locations of the proteins suggest a periplasmic-binding-protein-dependent transport mechanism for iron(III) dictate in Escherichia coli. J. Bacteriol. 171, 2626–2633 (1989).
13. Fecker, L. & Braun, V. Cloning and expression of the flu genes involved in iron(III)-hydroxamate uptake by Escherichia coli. J. Bacteriol. 156, 1301–1314 (1983).
14. Wang, Z., Hu, W. & Zheng, H. Pathogenic siderophore ABC importer YtbPQ adopts a surprising fold of exporter. Sci. Adv. 6, eaay7990 (2020).
15. Locher, K. P., Lee, A. T. & Rees, D. C. The E. coli BtuCD structure: a framework for ABC transporter architecture and mechanism. Science 296, 1091–1098 (2002).

16. Hvorup, R. N. et al. Asymmetry in the structure of the ABC transporter-binding protein complex BtuCD-BtuF. Science 317, 1387–1390 (2007).

17. Korkhov, V. M., Mireku, S. A. & Locher, K. P. Structure of AMP-PNP-bound vitamin B12 transporter BtuCD-F. Nature 490, 367–372 (2012).

18. Korkhov, V. M., Mireku, S. A., Veprintsev, D. B. & Locher, K. P. Structure of AMP-PNP-bound BtuCD and mechanism of ATP-powered vitamin B12 transport by BtuCD-F. Nat. Struct. Mol. Biol. 21, 1097–1099 (2014).

19. Woo, J. S., Zeltina, A., Goetz, B. A. & Locher, K. P. X-ray structure of the Yersinia pestis heme transporter HmuUV. Nat. Struct. Mol. Biol. 19, 1312–1315 (2012).

20. Naoe, Y. et al. Crystal structure of bacterial haem importer complex in the inward-facing conformation. Nat. Commun. 7, 13411 (2016).

21. Pinkett, H. W., Lee, A. T., Lum, P., Locher, K. P. & Rees, D. C. An inward-facing conformation of a putative metal-chelate-type ABC transporter. Science 315, 373–377 (2007).

22. Locher, K. P. Mechanistic diversity in ATP-binding cassette (ABC) transporters. Nat. Struct. Mol. Biol. 23, 487–493 (2016).

23. Geertsm, E. N., Nik Mahmood, N. A., Schuurman-Wolters, G. K. & Poolman, B. Membrane reconstitution of ABC transporters and assays of translocator function. Nat. Protoc. 3, 256–266 (2008).

24. Patzlaff, J. S., van der Heide, T. & Poolman, B. The ATP/substrate stoichiometry of the ATP-binding cassette (ABC) transporter OpuA. J. Biol. Chem. 278, 29546–29551 (2003).

25. Pravda, L. et al. MOLEinLite: a web-based tool for analyzing channels, tunnels and pores (2018 update). Nucleic Acids Res. 46, W368–W373 (2018).

26. Scheepers, G. H., Lycklama, A. N. J. A. & Poolman, B. An updated structural classification of substrate-binding proteins. FEBS Lett. 590, 4393–4401 (2016).

27. Clarke, T. E., Ku, S. Y., Dougan, D. R., Vogel, H. J. & Tari, L. W. The structure of the ferric siderophore binding protein FhuD complexed with gallichrome. Nat. Struct. Mol. Biol. 7, 287–291 (2000).

28. Clarke, T. E., Braun, V., Winkelmann, G., Tari, L. W. & Vogel, H. J. X-ray crystallographic structures of the E. coli periplasmic protein FhuD bound to hydroxamate-type siderophores and the antibiotic albomycin. J. Biol. Chem. 277, 13966–13972 (2002).

29. Oldham, M. L., Khare, D., Quicho, F. A., Davidson, A. L. & Chen, J. Crystal structure of a catalytic intermediate of the maltose transporter. Nature 450, 515–521 (2007).

30. Ferguson, A. D., Hofmann, E., Coulton, J. W., Diederichs, K. & Welte, W. Siderophore-mediated iron transport: crystal structure of FhuA with bound lipopolysaccharide. Science 282, 2215–2220 (1998).

31. Koster, W. & Bohn, P. Point mutations in two conserved glycine residues of the integral membrane protein FhuB affect hydroxamate transport. Acta Crystallogr A. 58, 450–456 (2002).

32. Wienen, C. J., Baaks, P., Rothbauer, U., Braun, D. & Duhr, S. Protein-binding assays in biological liquids using microscale thermophoresis. Nat. Commun. 1, 100 (2010).

33. Rohrbach, M. R., Braun, V. & Koster, W. Ferrichrome transport in E. coli K-12: altered substrate specificity of mutated periplasmic FhuD and interaction of FhuD with the integral membrane protein FhuB. J. Bacteriol. 177, 7186–7193 (1995).

34. Neilands, J. B. A crystalline organo-iron pigment from a rust fungus (Ustilago sphaerogena). J. Am. Chem. Soc. 74, 4846–4847 (1952).

35. Meyer, J. E. & Schulz, G. E. Energy profile of maltooligosaccharide permeation through maltozin as derived from the structure and from a statistical analysis of saccharide-protein interactions. Protein Sci. 6, 1084–1091 (1997).

36. Bi, Y., Mann, E., Whitfield, C. & Zimmer, J. Architecture of a channel-forming O-antigen polysaccharide ABC transporter. Nature 553, 361–365 (2018).

37. G Gregoret, L. M., Rader, S. D., Fletterick, R. J. & Cohen, F. E. Hydrogen bonds involving sulfur atoms in proteins. Proteins 9, 99–107 (1991).

38. Lewinson, O., Lee, A. T., Locher, K. P. & Rees, D. C. A distinct mechanism for the ABC transporter BtuCD-BtuF revealed by the dynamics of complex formation. Nat. Struct. Mol. Biol. 17, 332–338 (2010).

39. Punjani, A., Rubinstein, J. L., Fleet, D. J. & Brubaker, M. A. cryoSPARC: algorithms for rapid unsupervised cryo-EM structure determination. Nat. Methods 14, 290–296 (2017).

40. Scheres, S. H. A Bayesian view on cryo-EM structure determination. J. Mol. Biol. 415, 406–418 (2012).

41. Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. Features and development of Coot. Acta Crystallogr D. Biol. Crystallogr 66, 486–501 (2010).

42. Drouot, A., Cole, C. and Procter, J. & Barton, G. J. PyMOL: a protein secondary structure prediction server. Nucleic Acids Res. 43, W389–W394 (2015).

43. Yan, R., Xu, D., Yang, J., Walker, S. & Zhang, Y. A comparative assessment and analysis of 20 representative sequence alignment methods for protein structure prediction. Sci. Rep. 3, 2619 (2013).

44. Adams, P. D. et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr D. Biol. Crystallogr 66, 213–221 (2010).

45. Chen, V. B. et al. MolProbity: all-atom structure validation for macromolecular crystallography. Acta Crystallogr D. Biol. Crystallogr 66, 12–21 (2010).

46. Pettersen, E. F. et al. UCSF Chimera—a visualization system for exploratory research and analysis. J. Comput. Chem. 25, 1605–1612 (2004).

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