Supplementary Information

Protein expression and purification

The *shuUV* genes were subcloned into pET22b via the *MscI* and *XhoI* sites. A His_6-tag was encoded at the C-terminus of ShuV for subsequent purification. The ShuUV proteins were co-expressed in *E. coli* BL21 (DE3) cells in Terrific Broth (TB)-carbenicillin (100 μg/ml) at 25°C for 36h. The cells were harvested by centrifugation at 10,000 rpm for 10 minutes. Following lysis in 100 ml Bugbuster® HT (Novagen) containing 4% (w/v) Triton X-100, 20% (w/v) glycerol, 1 mM phenlymethylsulfonyl fluoride, 10 mM benzamidine, and one mini-EDTA free protease inhibitor cocktail tablet (Roche Diagnostic GmbH) the lysate was centrifuged at 20,000 rpm for 1hour. The pellet was resuspended in 50 mM Tris-HCl (pH 7.3) containing 4% (w/v) Triton X-100, 20% (w/v) glycerol, and 10 mM benzamidine and centrifuged again. Both soluble fractions were pooled and applied to a Ni-NTA agarose column (1 x 10 cm) equilibrated in 50mM Tris-HCl (pH 7.3) containing 150 mM NaCl, 0.1% (w/v) Triton X-100 and 20% (w/v) glycerol. The column was washed with 100 ml of the same buffer containing 10 mM imidazole, and the complex was eluted in 50 mM Tris-HCl (pH 7.3) containing 250 mM imidazole, 0.1% Triton X-100, and 20% (w/v) glycerol.

Fractions containing ShuUV as determined by SDS-PAGE were pooled, concentrated and applied to a S-300 Sephadex size exclusion column (1 x 15 cm) equilibrated in 20 mM Tris-HCl (pH 7.3) containing 0.1% (w/v) Triton X-100 and 20% (w/v) glycerol. The ShuUV proteins were concentrated and stored at -80°C till further use. The purification yielded on average 4-5mg ShuUV per liter of cells. The ShuT and ShuS proteins were purified as previously described (*1, 2*).

Preparation of the ShuUV Proteoliposomes.

Liposomes were prepared from acetone/ether precipitated *E. coli* lipids and L-α-phosphatidylcholine (Avanti Polar Lipids) from egg yolk in a ratio of 3:1 (w/w). The lipids were

hydrated at 45°C at 20 mg/ml in 50 mM Tris-HCl (pH 7.0) containing 0.14% (w/v) Triton X-100. The lipids were allowed to hydrate for 2-3h and then sonicated three times on ice until a clear solution was observed. The liposomes were filtered through a 400 nm polycarbonate membrane and diluted to 4 mg/ml. The ShuUV proteins were added at a ratio of 1:100 (w/w) of protein/lipids. The protein-lipid mixture was incubated at room temperature for 30 minutes with gentle shaking. The detergent was removed by the addition of 40 mg/ml wet weight of BioBeads SM2 (BioRad, Hercules, CA), with incubation at room temperature for 15 minutes with gentle shaking. Similar aliquots were added four more times, with incubation times of 15 minutes, 30 minutes, overnight, and 1h at 4°C. The solution was diluted 5 fold and filtered over glass silk to remove the BioBeads SM2. The proteoliposomes were collected by centrifugation at 150,000 x *g* for 20 min at 4°C. The collected proteoliposomes were resuspended at 0.4 mg/ml protein and 10 mg/ml lipid in 50 mM Tris-HCl (pH 7.5) and stored at -80°C (*3-5*). Based on SDS-PAGE it is estimated that 50% of the ShuUV is incorporated into the proteoliposomes giving an overall ratio of 0.01:1.0 mg protein: phospholipid.

In order to incorporate ShuT into the lumen of the vesicles, ShuUV proteoliposomes and purified apo- or holo-ShuT were mixed and flash frozen in liquid nitrogen, and then thawed at room temperature. The proteoliposome preparations were freeze-thawed three additional times, centrifuged at 150, 000 x *g*, and then finally passed through a 400 nm polycarbonate filter.

ATP-Hydrolysis.

The ATPase activity of ShuUV was stimulated by addition of ATP to a 500 µl proteoliposome preparation. The reaction was started upon the addition of 2 mM ATP and 10 mM $MgCl_2$ and aliquots (50 µl) were taken at various time points and mixed with 50 µl of 12% SDS. The amount of inorganic phosphate generated was calculated from a NaH_2PO_2 calibration curve generated by the modified molybdate method (*6*). All measurements of ATP hydrolysis were done in triplicate and averaged.

Estimation of heme content by the pyridine hemochrome method.

The proteolipososmes were pelleted by centrifugation at 150,000 x g for 10 min. The supernatant was removed and the pellet washed with 2 ml 50 mM Tris-HCl (pH 7.0). The supernatants were combined and the heme content measured by pyridine hemochrome (*7*). Heme concentration was determined in the presence of pyridine and dithionite by measuring the absorbances at 418.5, 526, and 555 nm and the respective extinction coefficients of 170, 17.5, and 34.4 mM^{-1} cm^{-1}. Within each experiment the concentration was determined at each wavelength and averaged. Each experimental condition was performed in triplicate and the heme concentrations averaged. Holo-ShuT incorporated into the lumen was calculated by subtracting the total holo-ShuT added from that remaining in the supernatant following the freeze thawing process.

Homology models of ShuUV and ShuS

The structure of ShuUV as presented in Fig S1 was prepared using the SSM Superpose function as implemented in Coot as part of the CCP4 suite (*8*) using the structural coordinate file for BtuCD (PDB accession number 1L7V) as the template (*4*). The ShuS structure was similarly constructed from the HemS coordinate file (PDB accession number 2JOR) (*9*). The cytoplasmic heme binding protein HemS from the heme uptake system of *Yersinia entercolitica* is 64 % homologous to ShuS (*9*). In addition the crystal structure of the *E.coli* homolog ChuS confirmed the structural as well as sequence homology within this family of proteins (*10*). The holo-ShuT structure was obtained from PDB file 2R7A (*2*). The schematic model of the transport system shown in Figure S1 shows the vectorial orientation of the transport system and does not necessarily represent the true orientation by which ShuS interacts with the ShuUV transporter. It is likely that ShuS interacts with the transporter at the ShuUV interface. Further work to elucidate sites of interaction of the ShuUV with ShuS is currently underway.

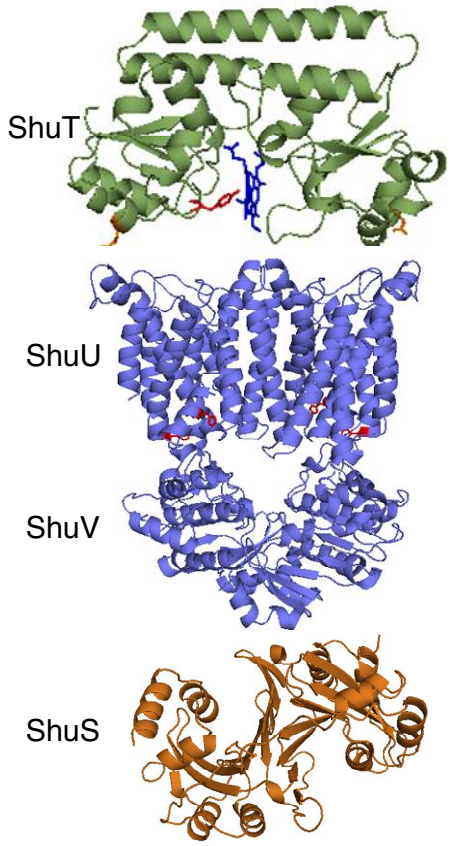

Fig S1. Schematic model of the cytoplasmic ABC-transport system. The holo-ShuT is shown in green with the heme in blue and the conserved Glu-74 and Glu-207 in red. Homology models of ShuUV and ShuS are shown in blue and gold, respectively. The ShuU conserved His-252 and His-262 are shown in red. Homology models of ShuUV and ShuS were created with the CCP4 suite.

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