# Electronic Supplementary Information

# Chiral separation of D/L-arginine with whole cells through an engineered FhuA nanochannel

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## Chemicals and Materials

All chemicals were of analytical grade or higher quality and were purchased from Sigma-Aldrich (Steinheim, Germany) and Applichem (Darmstadt, Germany); all enzymes were purchased from New England Biolabs (Frankfurt, Germany), Fermentas (St.Leon-Rot, Germany), or Sigma–Aldrich (Darmstadt, Germany) unless stated otherwise.

Thermal cycler (Mastercyler gradient; Eppendorf, Hamburg, Germany) and thin-wall polymerase chain reaction (PCR) tubes (Multi-ultra tubes; 0.2 mL; Carl Roth, Germany) were used in all PCRs. The amount of DNA in cloning experiments was quantified by using a Nano-Drop photometer (NanoDrop Technologies, Germany).

## Computationally guided design of chiral FhuA

We have added a workflow (Fig. S1) and a detailed description for the computationally guided designing of chiral FhuA in the revised manuscript. The computational guided FhuA enginnering was performed in three main steps:

**(i) In silico identification of loops hindering transport of amino acids and stabilization of stabilization of cork domain in interior of channel**

Intitially, the water diffusion channel was computationally identified using the crystal structure of FhuA WT (PDB ID: 1BY3) followed by the shortening of two loops via deleting residues 35-40 in loop1 (blue color) and residues 135-145 in loop2 (green color) hindering the diffusion of water molecules (Fig. S2). After the deletion of two loops all residues at the inner interface located between barrel and cork-domain were investigated by full computational saturation mutagenesis utilizing FoldX for stabilization of the cork domain withing FhuA barrel. Three best substitutions (Q62D, R81W, and N117L) with highest stabilization energy (ΔΔG<- 1kcal/mol) and largest distance from the generated loop cavity were selected. Finally, steered molecular dynamics simulation was performed with FhuA WT and FhuAΔL variant (with loops deletion and stabilizing substitutions) by placing D-/L-arginine at FhuA channel entrance. A constant force along the diffusion path through FhuA passing the selectivity filter regions (indicated with two circles in dark blue and light blue) near loop 1 and loop 2 (Fig. S5) was applied during steered MD simulation using AMBER99 force field at 298K. The simulation results indicated no diffusion of arginine enantiomers through FhuA WT whereas diffusion of arginine was observed through FhuAΔL which is controlled by a longer residence time in the two selectivity filter regions near the engineered loop 1 and 2 (Fig. S5).

**(ii) Identification of arginine contact residues (within 5 Å) in filter regions to design and generate OmniChange libraries**

Based on the selectivity filter regions identified in FhuAΔL variant, six OmniChange libraries with up to five saturation positions were selected. The geometric design criteria were introduced by identification of contact residues (sidechain distance < 5 Å) of D-/L-arginine placed within the respective selectivity filter region. The generated OmniChange libraries were screened and FhuAF4 variant showing an improvement in chiral separation of arginine recemate mixture was identified and characterized.

**(iii) SMD simulations to understand separation mechanism at molecular level**

After the identification of FhuAF4 variant, steered molecular dynamics simulations were carried out to understand the separation mechanism at molecular level for resolution of D- and L-arginine. FhuAΔL variant was used as a control to see the differences in diffusion of arginine enantiomers compared to FhuAF4 variant.

## Experimental procedure

**Selection of filter regions.** The starting coordinates of FhuA was downloaded from protein data bank (PDB ID: 1BY3[1]). Water channel was visually inspected using YASARA Structure Version17.8.9[2] and two loops (residues 35-40 and 135-145) hindering the flow of water within the cork domain were identified. The selected residues were deleted and the fragments of the cork domain were manually rejoined. Afterwards, the structure was initially minimized using first steepest descent without electrostatics to remove steric clashes and subsequently relaxed by steepest descent minimization and simulated annealing from 298 K (time step 2 fs, atom velocities scaled down by 0.9 every $10^{th}$ step) until convergence was reached, i.e. the energy improved by less than 0.05 kJ/mol per atom during 200 steps.

**Channel generation in FhuA WT.** The identified loops predicted to open up a small channel in FhuA WT (loop 1: 35-40 and loop 2: 135-145) were shortened by PCR using two set of primers. Primer pairs (loop 1 del_Fwd: 5´-CGCGACAGTCTGCTACGCCGATTCAAAAAG-3´ and loop 1 del_Rev: 5´-CTGTGGCACTTTTTGAATCGGCGTAGCAGA-3´) were used to shorten loop 1 and primer pairs (loop 2 del_Fwd: 5´-CGCTGAAATTATGCGTGGCGGCGGCCTGTT-3´ and loop 2 del_Rev: 5´-GCTGACCATATTCAACAGGCCGCCGCCACG-3´) were used to shorten loop 2. Resulting PCR products were digested (20 U *Dpn*I: 16 h, 37°C), purified using PCR cleanup kit (Macherey-Nagel) and transformed into *E. coli* $B^E$ BL21 (DE3) Omp8 competent cells. Plasmid isolation was performed using a plasmid purification kit (Macherey-Nagel NucleoSpin Extract-II DNA purification kit, Dueren, Germany). Deletion of loops was subsequently verified by sequencing.

**Stabilization of the cork domain.** A structural model of the FhuA after shortening of loops was constructed in YASARA Structure version17.8.9[2] using the YASARA-FoldX plugin and employing the FoldX method.[3] The starting coordinates for the FoldX *in silico* mutagenesis experiment were taken from the X-ray structure of the FhuA protein (PDB ID: 1BY3[1]). A FoldX mutation run including rotamer search, exploring alternative conformations (3 independent runs) were performed during the FoldX energy minimization employing a probability-based rotamer library. Stabilization energy calculations (defined as the difference in free energy (ΔΔG) given by $\Delta G_{variant}$-$\Delta G_{wt}$) were computed with FoldX version 3.0 Beta[3] using default settings. Primer pairs listed in Table S1 were used for substitution of amino acids (Q62D, R81W, and N117L) identified with FoldX to generate variant FhuAΔL. Resulting PCR products were treated as described in earlier section.

**Steered Molecular Dynamics (SMD) simulation.** FhuAWT based loop deletion variant (FhuAΔL.) was generated to create a larger cavity and steered molecular dynamics simulation was performed with both variants (FhuA WT and FhuAΔL). In a simulation box, the D-/L-arginine was placed at the tunnel entrance and a constant force along the diffusion path through FhuA passing the

selectivity filter regions (indicated with two circles in dark blue and light blue) near loop 1 and loop 2 was applied during steered MD simulation using AMBER99 at 298K. The simulation results indicated no diffusion of arginine enantiomers through FhuA WT whereas diffusion of arginine was observed through FhuAΔL which is controlled by a longer residence time in the two selectivity filter regions near the engineered loop 1 and 2 (Fig. S5).

**Expression and extraction of FhuA WT and FhuAΔL.** *E. coli* $B^E$ BL21 (DE3) Omp8 cells were used as a host for expression of FhuA WT and variant FhuAΔL. Precultures were grown overnight in LB media supplemented with 0.1 mM ampicillin (30°C, 250 rpm, 20 ml/ 250 ml shaking flask). Main-culture media TY with supplement of 0.1 mM ampicillin was inoculated with the preculture to obtain an initial $OD_{600}$ of 0.1 and incubated (37°C, 250 rpm, 200 ml/1 l shaking flask) until $OD_{600}$ of 0.6 was reached. Expression was induced by addition of isopropyl-β-D-1- thiogalactopyranoside (IPTG, final concentration of 1 mM). Samples were taken for SDS-PAGE analysis before induction (BI) and after induction (AI) to confirm expression. Extraction and refolding of both FhuA variants was carried out as previously described.[4]

**Calcein release assay**

*Polymersome preparation with entrapped calcein*. ABA tri-block copolymer poly(2-methyloxazoline)–poly(dimethylsiloxane)–poly(2-methyloxazoline) (PMOXA–PDMS–PMOXA; Mn X 103: 1.2-4.8-1.2) was used to generate polymersomes. 10 mg polymer was dissolved in 1 mL of 50 mM calcein solution dissolved in Tris buffer (10 mM Tris, 100 mM KCl, pH 7.4) by gently stirring at room temperature (16 h) to form self-assembled nanocompartments. After incubation, generated polymersomes were subjected to freeze thaw cycles by freezing in liquid nitrogen for 3 min and heating at 60°C for 3 min. Unilamellar vesicles were obtained by extrusion (4-6 times) through 0.2 µm membrane (Millipore) using 2 ml syringe. Polymersomes with encapsulated calcein (50 mM) were collected by performing size exclusion chromatography through a sepharose 6B column.

*Insertion of FhuA variants*. Extracted and refolded FhuA samples were diluted in FhuA refolding buffer (10 mM sodium phosphate buffer, 50 mM 2-methyl-2,4-pentandiol (MPD), 1 mM EDTA, pH 7.4) to get final concentration of 3.91 µM. 50 µL of prepared polymersomes was supplemented with 150 µL Tris buffer (10 mM Tris, 100 mM KCl, pH 7.4) in black microtiterplate and measurement were taken with Tecan infinite M200 Pro (Tecan Group AG, Zürich, Switzerland). The release of calcein was determined by applying the following settings: number of cycles: first 15 cycles and then 45 cycles, shaking: 3 sec, number of flashes: 10, integration time: 20 µs, manual gain: 49, excitation: 495 nm, emission: 525 nm. After the first 15 cycles without addition FhuA samples, 3.91 µM of FhuA WT and FhuAΔL were separately added stepwise in 10 µL batches (after every 45 cycles) to the polymersome suspension and the fluorescence increase was recorded at 520 nm. Higher release of calcein in case of FhuA

WT compared to FhuAΔL supports the stabilization effect of inserted substitutions (Q62D, R81W, and N117L) by reducing fluctuations of cork domain

**Structure guided OmniChange library generation.** Six libraries with up to five targeted positions were selected on the basis of the two selectivity filter regions of FhuAΔL variant. The geometric design criteria were introduced by identification of contact residues (sidechain distance to aa < 5 Å) of arginine placed within the respective selectivity filter region.

Multiple site saturation mutagenesis libraries were generated with previously described OmniChange[5] protocol using FhuAΔL as a template. Primers containing NNK saturation codon are listed in Table S2. The template plasmid DNA was digested by adding 1 µl (20 U) of *Dpn*I to each PCR sample. After incubation at 37°C for 4 h, PCR products were purified using the Macherey-Nagel NucleoSpin Extract-II DNA purification kit.

Purified vector fragment and insert fragments were diluted to 0.02 pmol/µl and 0.11 pmol/µl respectively using Milli-Q water. The PCR products were cleaved by iodine at alkaline conditions. All cleaved fragments were assembled by stepwise hybridization at room temperature for 5 min and transformed into 100 µl chemical competent *E. coli* DH5 α cells. Verification of the correct assembly was done by colony PCR and saturation at target sites were confirmed by sequencing of plasmid isolated from few clones.

**Cloning and expression of FhuA with amino acid utilizing enzymes.**

*Cloning and expression for screening system.* For generation of screening system, a D- and L-arginine specific reporter enzyme (arginine deiminase, ADI) was coexpressed with FhuA (ADI-FhuA system). Vector pET-42b(+) containing ADI M21[6] variant was transformed into *E. coli* $B^E$ BL21 (DE3) Omp8 competent cells. Subsequently chemical competent cells were prepared from the cells already containing ADI gene (*E. coli* $B^E$ BL21 (DE3) Omp8_pET42b_ADI M31 competent cells). OmniChange libraries of FhuA were transformed into these competent cells and were used for screening. Coexpression was confirmed by transforming FhuA gene into *E. coli* $B^E$ BL21 (DE3) Omp8_pET42b_ADI M31 competent cells followed by cultivation in ZYM-5052 autoinduction media[7] (37°C., 250 rpm, 200 ml/1 l shaking flask).

*Cloning and expression for characterization system.* In combination with ADI-FhuA system another coexpression system was generated with D-arginine specific enzyme (pig kidney D-amino acid oxidase, DAO) for characterization of identified FhuA variants from screening. Synthetic gene of DAO mutant 222D224G[8] (GeneArt Gene Synthesis, ThermoFisher Scientific, Regensburg, Germany) was cloned into pET-42b(+) vector using *Nde*I and *Hind*III restriction enzymes. The vector pET-42b(+)_DAO was transformed into *E. coli* $B^E$ BL21 (DE3) Omp8 competent cells. Afterwards, chemical competent cells were prepared from the cells already containing DAO gene (*E. coli* $B^E$ BL21 (DE3) Omp8_pET42b_DAO competent cells). Coexpression was confirmed by transforming FhuA gene into *E. coli* $B^E$ BL21 (DE3) Omp8_pET42b_DAO competent cells (DAO-FhuA system) followed by cultivation in ZYM-5052 autoinduction media[7] (37°C., 250 rpm, 200 ml/1 l shaking flask).

**Cultivation and expression in 96-well plates.** Colonies obtained from OmniChange libraries were cultivated (16 h, 37°C, 900 rpm, 70 % humidity; Multitron Pro, Infors AG, Bottmingen, Switzerland) in 96-well microtiter plates (F-bottomed, polystyrene plates; Greiner bio-one GmbH, Frickenhausen, Germany) containing 150 µl of LB media (0.1 mM ampicillin and 0.05 mM kanamycin). Glycerol stocks were prepared by adding 50 µl of 50 % glycerol solution to each well. Expression (14 h, 30°C, 900 rpm, 70 % humidity; Multitron II, Infors GmbH, Einsbach, Germany) of ADI-FhuA system in 96-deep well microtiter plate (round bottom, polypropylene plates, VWR, Langenfeld, Germany) was performed by inoculating 5 µl of glycerol stock to 500 µl ZYM-5052 autoinduction media (0.1 mM ampicillin and 0.05 mM kanamycin). After cultivation, these clones were used for screening.

**Screening of OmniChange libraries.** Cell cultures obtained after 14 h of incubation were pelleted by centrifugation (3200 g, 20 min, 4°C; Eppendorf centrifuge 5810 R, Eppendorf AG, Hamburg, Germany). Pelleted cells were washed once with PBS and again resuspended in 400 µl of PBS. Content of plate were divided into 4 flat bottom microtiter plates and $OD_{600}$ was measured for each plate. Substrate (5 mM L-arginine or 5 mM D-arginine) was added separately to two plates and PBS was added to respective control plates. Plate with L-arginine (and control) was incubated for 20 min and plate with D-arginine (and control) was incubated for 3 h respectively. The arginine deiminase used converts D- and L-arginine, whereby L-arginine is reacted faster by a factor of 10; the overall lower absorption values during the reaction of D-arginine were largely compensated by prolonged incubation times.

Quantification of arginine enantiomer transported through FhuA was done by modified citrulline detection assay.[9] After incubation entire content was transferred to a 96-well PCR plate (VWR international bvba, Leuven, Germany) and 60 µl of ferric acid solution and 20 µl of DAM:TSC (1:1) was added. The reaction mixture was further incubated 30 min at 70°C in homemade incubation system for PCR microtiter plate. Colour development was stopped by 5 min incubation on ice followed by centrifugation (3200 g, 20 min, 4°C; Eppendorf centrifuge 5810 R, Eppendorf AG). Supernatant was transferred to F-bottom microtiter plate and absorbance was measured at 530 nm (Tecan Sunrise, Tecan Group AG, Zürich, Switzerland) (Fig. S8). A standard deviation in the 96-well MTP format of 13.6 % and 9.3 % was achieved after optimization for L-arginine and D-arginine, respectively.

**Chiral HPLC.** Resolution of arginine enantiomers was done by using a chiral column (Astec CHIROBIOTIC T, 25 cm x 4.6 mm I.D., 5 µm particles) in analytical HPLC (Nexera X2, Shimadzu Deutschland GmbH, Duisburg, Germany). Both L-arginine and D-arginine were dissolved in PBS (5 mM each). Mobile phase was prepared by mixing PBS, methanol and formic acid (30:70:0.02, pH 3.9). Unless otherwise indicated, the mobile phase flow rate was 1 ml/min and the UV detection wavelength was 202 nm (Fig. S11).

**Characterization of identified variants.** Parental FhuA variant (FhuAΔL) and identified variant from screening (FhuAF4) were expressed separately in ADI-FhuA system and DAO-FhuA system as explained above. Coexpressing cells were $OD_{600}$ normalized to 4 and centrifuged (3200 g, 20 min, 4°C; Eppendorf centrifuge 5810 R, Eppendorf AG). Pellets were once washed by resuspending in PBS followed by centrifugation. Obtained pellets were finally resuspended in PBS and mixed together in 1:4.5 ratio (ADI-FhuA:DAO-FhuA cells) according to catalytic properties of ADI and DAO. Mixed cells were incubated (room temperature, rotatory shaking) with racemic mixture of arginine (5 mM of each enantiomer). 200 µl samples were taken at regular interval and centrifuged. Supernatant obtained were analyzed with chiral HPLC column and calculations of enantiomeric excess and conversion was performed according to reference.[10]

**Quantification of FhuAΔL and FhuAF4.** The amount of outer membrane expressed FhuA was determined by utilizing StrepTactin-Chrome546-conjugate which binds specifically to StrepTactinII sequence (WSHPQFEK).[11] StrepTactinII recognition sequence was introduced in previously identified outer loop 5 (between P405 and V406)[12] by overlap extension PCR with PTO-overlaps and subsequent PLICing. The primer sequences are summarized in Table S3. The generated genetic constructs were transformed in *E. coli* $B^E$ BL21 (DE3) Omp8 competent cells for expression. Quantification of FhuA WT_Strep, FhuAΔL_Strep and FhuAF4_Strep was done according to reference.[12]

**Steered Molecular Dynamics (SMD) Simulations.** A SMD simulation protocol was developed for diffusion of D- and L- arginine through the channel of FhuAΔL and FhuAF4 variants. These variants were generated *in silico* within YASARA Structure version17.8.9[2] based on the crystal structure of FhuA WT (PDB ID: 1BY3[1]). The D- and L- arginine was placed above the channel entrance of FhuAΔL and FhuAF4 variants, respectively. For the pulling of arginine from the top of the FhuA channel, D487 located at the bottom of the FhuA channel was defined as an end point. The size of the simulation box was 100.39 x 76.00 x 61.16 Å with alpha = 90.00, beta = 90.00 and gamma = 90.00. AMBER03 force field[13] was applied at a temperature of 298 K. During SMD simulations, distances of the arginine and the end point (D487) were analyzed. A pulling force of 5000 pm/ps$^2$ on the transported arginine was applied per time step as implemented in YASARA steered MD macro. The arginine was passed through the channel and the selectivity filter regions, which was monitored by time recorded with md_runsteered.mcr script within YASARA.

## Figures

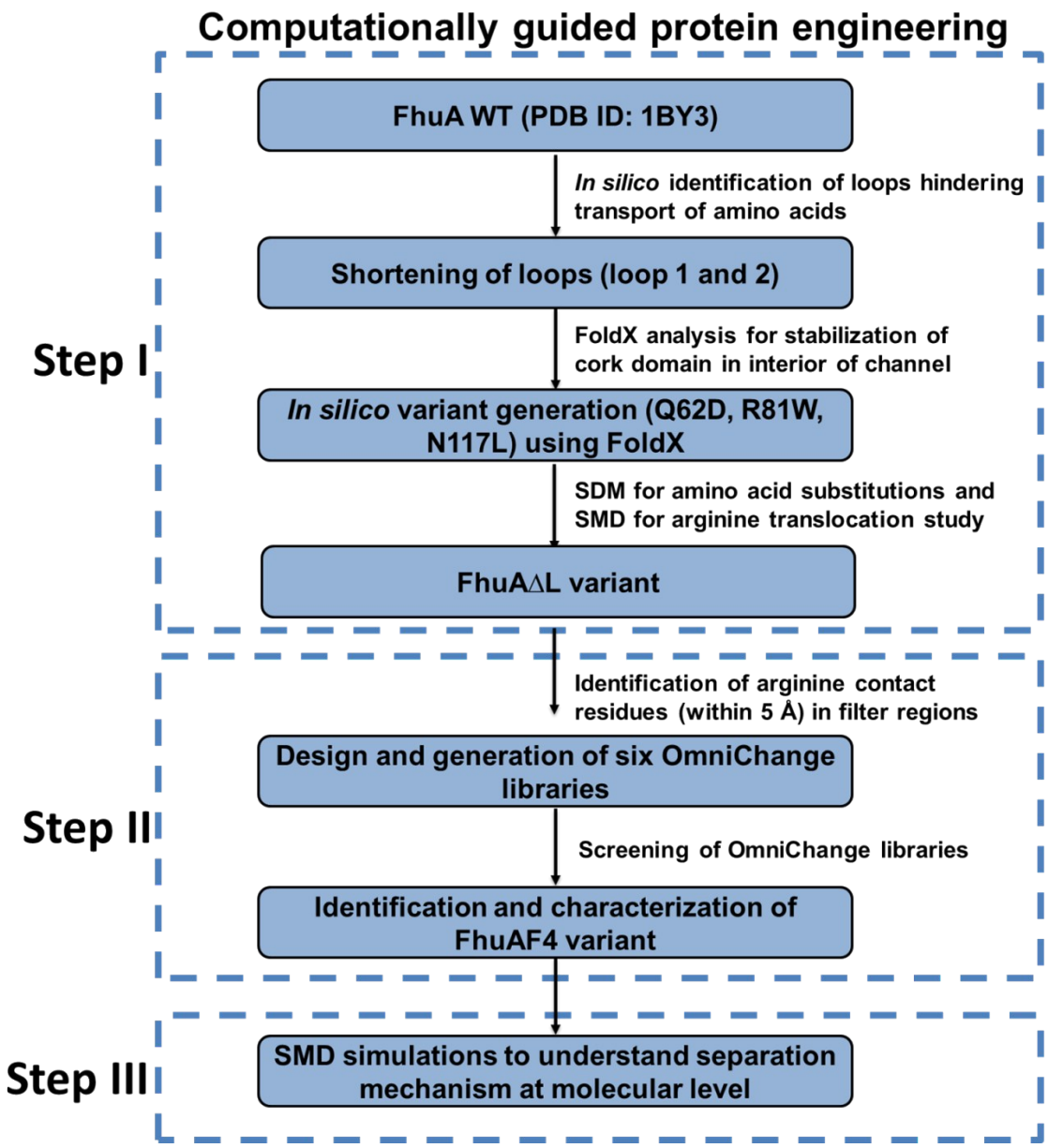
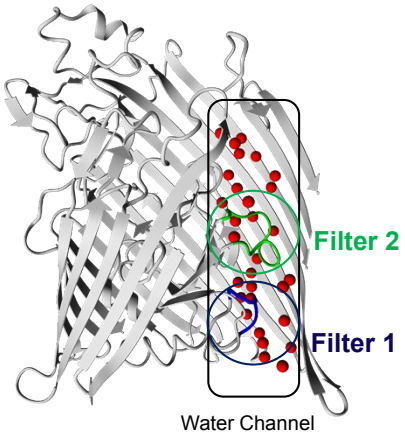



**Fig. S1** An overview of computationally guided engineering procedure for designing of chiral FhuA. The process was divided in three steps. Step I: *In silico* identification of loops hindering transport of amino acids and stabilization of stabilization of cork domain in interior of channel, Step II: Identification of arginine contact residues (within 5 Å) in filter regions to design and generate OmniChange libraries and Step III: SMD simulations to understand separation mechanism at molecular level

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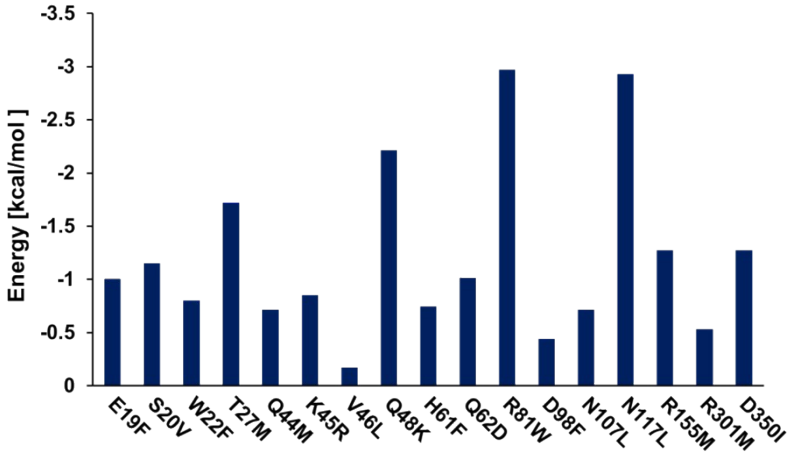
230 **Fig. S2** Crystal structure of FhuA WT (PDB ID: 1BY3[1]) in a cartoon representation. Two flexible loops (loop 1: blue (34-40) and loop
231 2: green (134-146)) in water channel (waters in red VdW-ball representation). These two loops were shortened to generate filter
232 1 and filter 2 regions (inside blue and green circles).

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234 **Fig. S3** Calculated stabilization energy (ΔΔG) of the substitutions using FoldX method[3]. Three amino acid substitutions (Q62D,
235 R81W, N117L) were identified to enhance the interactions between the cork domain and the β-barrel domain. These substitutions
236 were chosen based on the highest stabilization energy and largest distance from generated filter regions.

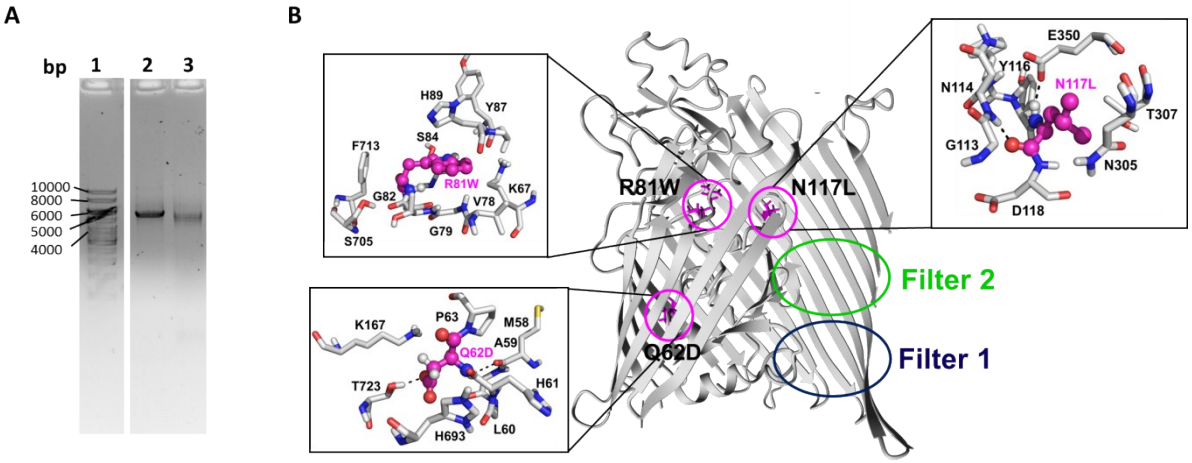



**Fig. S4** (a) Agarose gel image showing amplification of whole plasmid (pPR-IBA1) containing gene of FhuA WT and FhuAΔL (after deletion of two loops and SDM for stabilization of cork domain). Lanes: 1, ladder; 2, FhuA WT; 3, FhuAΔL. (b) Side view of FhuAΔL. FhuAΔL showing selectivity filter regions 1and 2 (blue and green circles) and the stabilizing substitutions (magenta sticks) identified by FoldX analysis. Inset showing location and interactions of selected amino acid substitutions (Q62D, R81W and N117L) within FhuA.

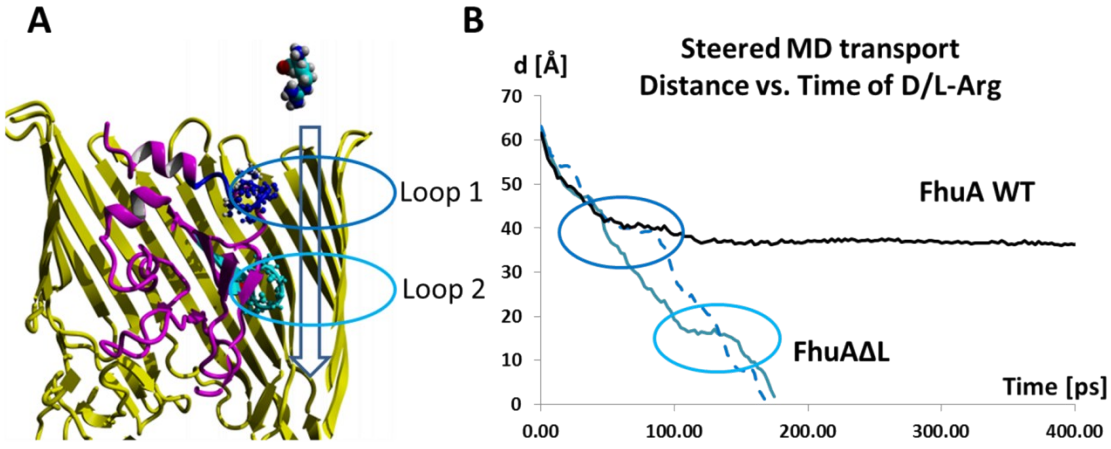



**Fig. S5** (a) The path for the steered MD simulation is indicated by an arrow, encircling the two selectivity filter regions generated by deletion of loop 1 and 2. **(b)** The diffusion time for D-/ L-arginine along the simulation path at constant force is shown for FhuA WT (black); FhuAΔL (blue); L-arginine (solid line); D-arginine (dotted line).

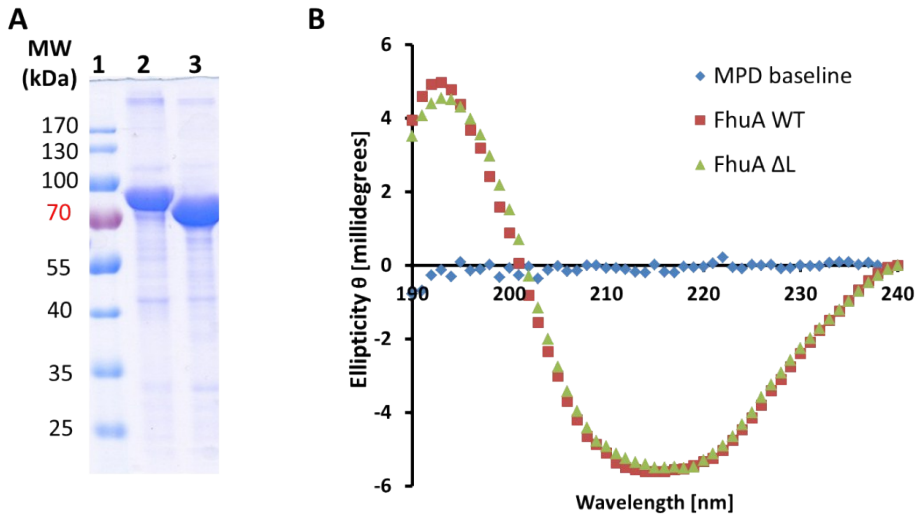



**Fig. S6** (a) SDS-PAGE showing overexpression of FhuA WT and FhuAΔL. Lanes: 1, ladder; 2, FhuA WT; 3, FhuAΔL. (b) CD spectra of FhuA WT and FhuAΔL variant refolded in MPD buffer. Both spectra show characteristic of β-barrel protein with minima at 215 nm and maxima at 195 nm.

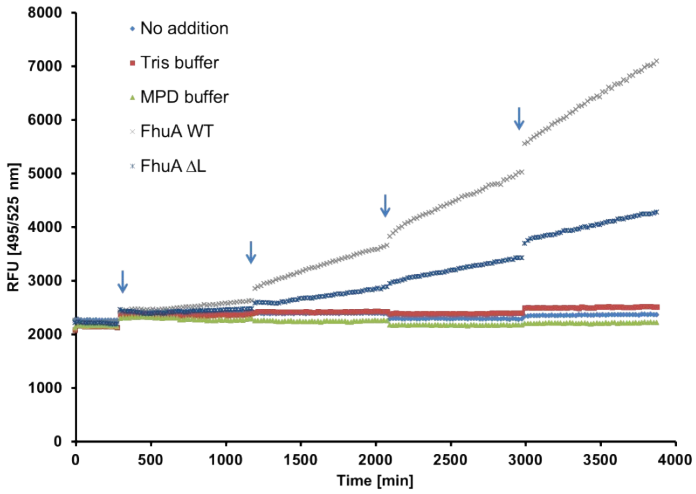
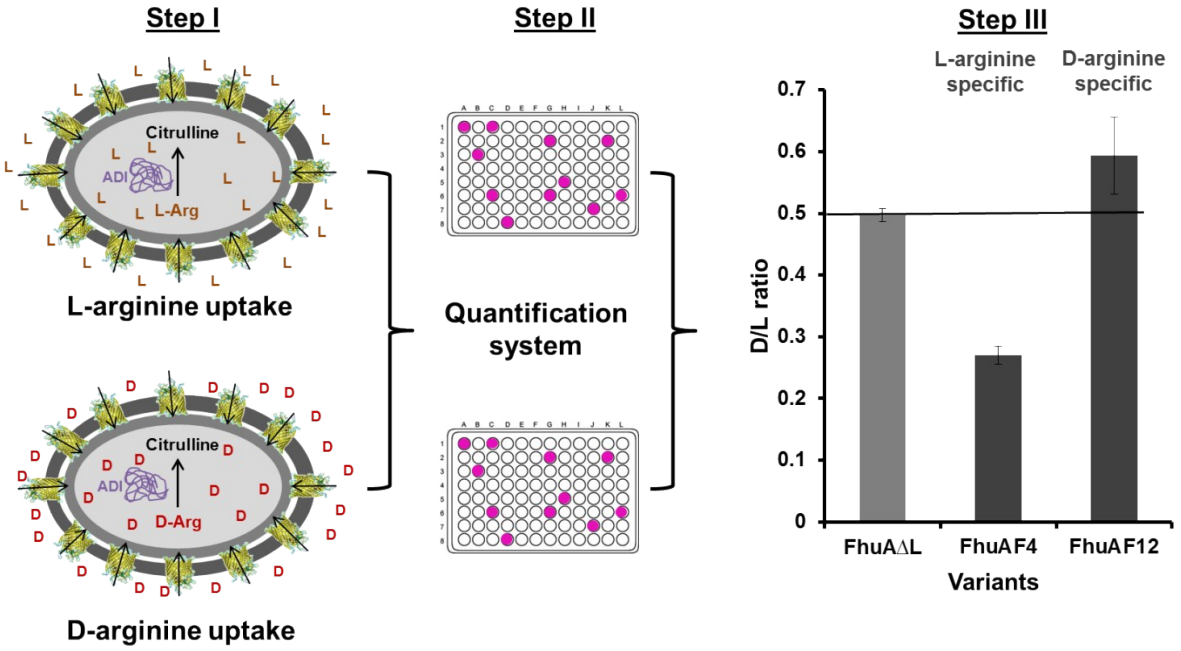
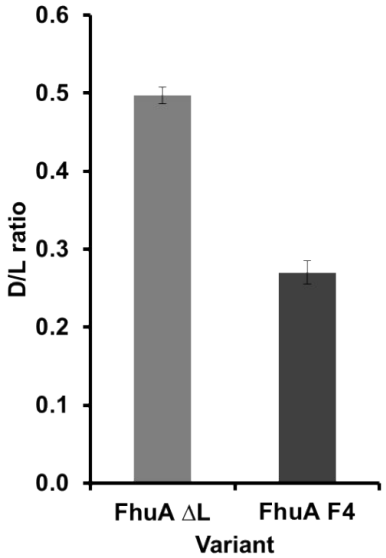



**Fig. S7** Calcein release assay. Increase in fluorescence intensity due to dilution of self-quenching concentration of calcein (50mM) as a result of diffusion through FhuA channels. Two FhuA variants (3.91 µM FhuA WT (grey) and 3.91 µM FhuAΔL (dark blue)), FhuA refolding buffer (10 mM sodium phosphate buffer, 50 mM MPD, 1 mM EDTA, pH 7.4, green) and Tris buffer (10 mM Tris, 100 mM KCl, pH 7.4; red) were added step-wise to calcein loaded polymersomes (indicated by arrows). Another control without addition of any sample (light blue) was also run.




261

262 **Fig. S8** Whole cell screening system. Microtiter plate screening technology based on cytoplasmic expression of ADI and
263 coexpression of FhuA variants in the outer membrane of *E. coli*. Step I: Overnight (~12 hrs) growth of coexpressing *E. coli* cells in
264 ZYM-5052 autoinduction media at 30°C. Cells were pelleted and resuspended in phosphate buffered saline (PBS) pH 7.4 and the
265 content was divided into two separate 96-well microtiter plates (MTPs). Each plate was incubated separately with 5 mM of D- or
266 L-arginine. ADI converts D- and L-arginine, which are essentially absorbed through the FhuA variants, to citrulline. Step II: Produced
267 citrulline was quantified by modified DAM:TSC (1:1) assay which converts citrulline to a coloured compound (absorption at
268 530 nm). Step III: The ratio of D-/L-conversion was used as benchmark to select FhuA variants with altered enantiopreference.
269 FhuAF4 showed higher transport of L-arginine and FhuAF12 showed higher transport of D-arginine (dark grey bars) when
270 compared to parent FhuAΔL.

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272 **Fig. S9** Change in enantiopreference of identified variant. Enantiopreference of Identified variant FhuAF4 (dark grey) compared
273 to parent variant FhuAΔL (light grey). FhuAF4 showed 1.84 times higher transport of L-arginine compared to FhuAΔL (image also
274 shown in Figure 2 Step III).

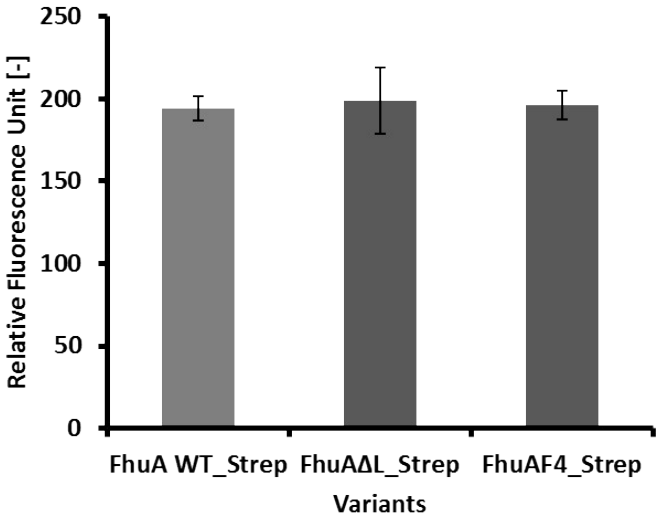


**Fig. S10** Quantification of FhuA WT_Strep, FhuAΔL_Strep and FhuAF4_Strep on *E. coli* cell surface with Chromeo546-Streptactin-conjugate. Fluorescence measurement (λex 545 nm and λem = 561 nm) in cells expressing FhuA WT_Strep (light grey) and FhuAΔL_Strep and FhuAF4_Strep (dark grey) labelled with Chromeo-Streptactin. An average of ≈23000 molecules of FhuA WT_Strep, FhuAΔL_Strep and FhuAF4_Strep were calculated on *E. coli* cell surface after 3 h of expression.

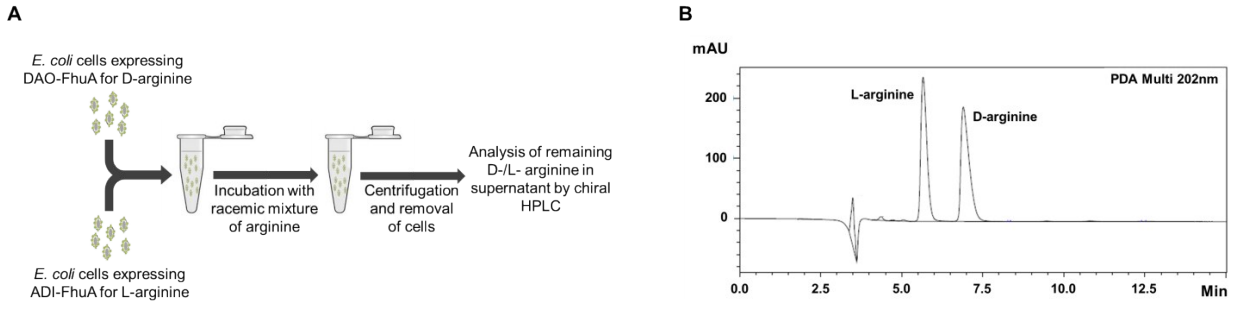


**Fig. S11** Schematic representation of work flow for characterization of parental and identified FhuA variant using chiral HPLC. (a) *E. coli* cells coexpressing DAO-FhuA and ADI-FhuA were expressed separately. Both cultures were normalized to $OD_{600}$ of 4 and resuspension in PBS buffer pH 7.4 before incubating with racemic mixture of arginine (5 mM each). Samples taken at regular intervals were analyzed with chiral HPLC. (b) Enantioresolution of arginine enantiomers (5 mM each) using chiral HPLC.

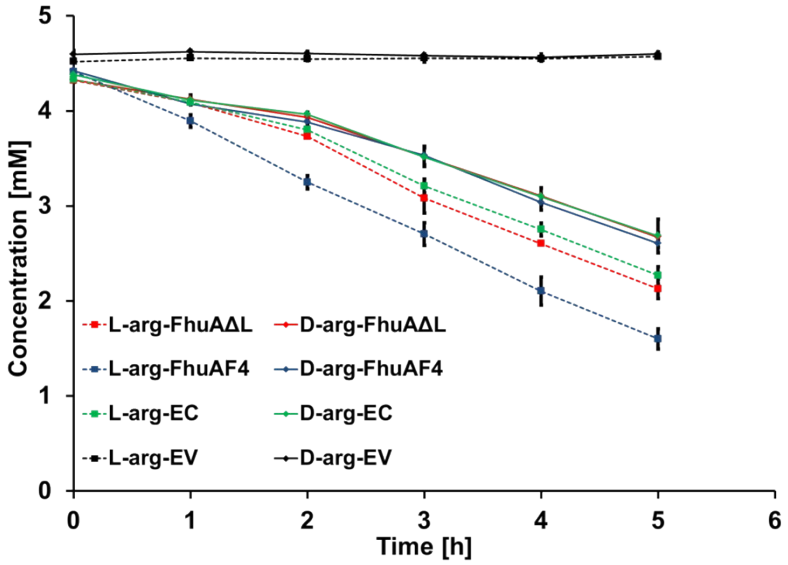


**Fig. S12** HPLC quantification of unutilized D-/L-arginine in the supernatant. Unutilized D-arginine (bold line) and L-arginine (dotted line) after incubation with coexpressing *E. coli* cells with the starting variant FhuAΔL (red lines) and identified variant FhuAF4 (blue lines). ADI/DAO enzyme mix as a control (EC) expressing only enzymes without FhuA (green lines) and empty vector (EV) control neither expressing ADI/DAO nor FhuA were also run (black lines). FhuAF4 showed higher transport of L-arginine compared to FhuAΔL after incubation with arginine racemate.

## Tables

**Table S1.** Sequences of the primers used to perform SDM for stabilization of cork domain.

| Targeted mutations | Oligoname | Sequence | % GC | $T_m$ (°C) |
|---|---|---|---|---|
| **Gln (62) → Asp** | G62D_Fwd | GAGATGGCGCTGCAT**GAC**CCGAAGTCGGTAAAAGAAGC | 55.3 | 68.3 |
| | G62D_Rev | GCTTCTTTTACCGACTTCGG**GTC**ATGCAGCGCCATCTC | 55.3 | 68.3 |
| **Arg (81) → Trp** | R81W_Fwd | GTCTCTGTTGGTACG**TGG**GGCGCATCCAACACC | 60.6 | 68.7 |
| | R81W_Rev | GGTGTTGGATGCGCC**CCA**CGTACCAACAGAGAC | 60.6 | 68.7 |
| **Asn (117) → Leu** | N117L_Fwd | GCAGGGCAACTTCTA**TCT**CGATGCGGTCATTGACC | 55.6 | 67.5 |
| | N117L_Rev | GGTCAATGACCGCATCG**AGA**TAGAAGTTGCCCTGC | 55.6 | 67.5 |

**Table S2.** Sequences of the primers used for the OmniChange libraries containing NNK degeneracy codon. Numbering of positions has been done according to FhuA WT sequence (PDB ID: 1BY3[1])

| Region | Library name | Oligoname | Sequence[a] | % GC | $T_m$ (°C) |
|---|---|---|---|---|---|
| ΔLoop 1 | Library 1 | A34 Fwd | gcgcgacagtct**NNK**ACGCCGATTCAAAAAGTG | 53.0 | 65.7 |
| | | A34 Rev | aaaggtgtcgttAAATTCGTGATCGAAGCTG | 41.9 | 60.6 |
| | | T295 and R297 Fwd | aacgacacctttt**NNK**GTG**NNK**CAGAACCTGCGCTTTG | 50.0 | 62.0 |
| | | T295 and R297 Rev | caactgggtatcAACGGAGAAGTTTTGCAG | 47.0 | 61.1 |
| | | Q362 Fwd | gatacccagttg**NNK**AGCAAGTTTGCC | 50.0 | 60.7 |
| | | Q362 Rev | agactgtcgcgcCGCAATAGTTGC | 58.3 | 63.9 |
| | Library 2 | T41 Fwd | cgacagtctgct**NNK**CCGATTCAAAAAGTG | 48.3 | 61.8 |
| | | T41 Rev | gcccgtttgtttCTGTTTATTCAGAATGCGGTAAGG | 44.4 | 63.4 |
| | | Y435 Q437 Q439 Fwd | aaacaaacgggcGTT**NNK**GTT**NNK**GAT**NNK**GCGCAGTGGGATAAAGTG | 49.0 | 69.5 |
| | | Y435 Q437 Q439 Rev | agcagactgtcgCGCCGCAATAGTTGC | 59.3 | 66.6 |
| | Library 3 | T448 G450 Fwd | aaagtgctggtc**NNK**CTA**NNK**GGTCGTTATGACTGG | 50.0 | 65.6 |
| | | T448 G450 Rev | ccaggtaaactgTTTGTCATCACGTTTATCGGTCGTCCC | 48.7 | 65.4 |
| | | R479 Fwd | cagtttacctgg**NNK**GGTGGTGTTAACTAC | 48.3 | 61.0 |
| | | R479 Rev | gaccagcactttATCCCACTGCGC | 58.3 | 62.3 |
| ΔLoop 2 | Library 4 | N103 Y105 Fwd | gcagaaggccaaAGCCAG**NNK**AAC**NNK**CTGAATGGCCTGAAG | 54.8 | 69.6 |
| | | N103 Y105 Rev | cataatttcagcGCGTTCCAGCATATACGG | 46.7 | 61.1 |
| | | G134 G146 Fwd | gctgaaattatgCGTGGC**NNKNNK**CTGTTGAATATGGTC | 46.2 | 65.1 |
| | | G134 G146 Rev | ttggccttctgcCGCAAAGCCGCGAATG | 60.7 | 68.7 |
| | Library 5 | R452 D454 Fwd | agtgggataaagTGCTGGTCACCCTAGGCGGT**NNK**TAT**NNK**TGGGCAGATCAAG | 51.9 | 70.5 |
| | | R452 D454 Rev | cgattcgctataGCTGAAGTAAGGTGTTACACC | 45.5 | 61.0 |
| | | F500 Fwd | tatagcgaatcg**NNK**GAACCTTCTTCG | 46.3 | 59.1 |
| | | F500 Rev* | ctttatcccactGCGCCTGATCCTGAACATAAAC**NNK**CGT**NNK**TTTCTGTTTATTCAG | 43.1 | 67.5 |
| | Library 6 | G134 Fwd | gaaattatgcgt**NNK**GGCGGCCTGTTG | 53.1 | 63.2 |
| | | G134 Rev | gctatagctgaaGTAAGGTGTTACACC | 44.4 | 56.9 |
| | | S499 E501 Fwd | ttcagctatagcGAA**NNK**TTT**NNK**CCTTCTTCGCAAGTTGGG | 45.2 | 66.1 |
| | | S499 E501 Rev | acgcataatttcAGCGCGTTCCAGC | 52.0 | 62.3 |

[a] small letter-phosphorothioated nucleotides, capital letter-normal nucleotides, bold letters-saturation codon. All primers sequences are in 5′ to 3′ direction. *Primer F500 Rev has two additional saturation sites (Q431 and G433)

**Table S3.** Primers for overlap extension PCR using PTOs to introduce StrepTagII into FhuA WT, FhuAΔL and FhuAF4. Small letters indicate the PTO part, underlined letters indicate the overlapping base pairs.

| Primer | Sequence | % GC | $T_m$ (°C) |
|---|---|---|---|
| **Strep_loop_fwd** | ggtctcacccgcAGTTCGAAAAATCTGGTTCTGGTGTGAATACCGATTTCGACTTCAATGCCAAAGATC | 46.4 | >75.0 |
| **Strep_loop_rev** | gcgggtgagaccAAGAACCAGAACCCGGATTGTACAGATTGAGTGG | 55.1 | >75.0 |

**Table S4.** Time required for diffusion of L- and D-arginine through FhuA ΔL and FhuA F4 variants from top of the FhuA channel towards residue D487 located at the bottom of the FhuA channel. Average values were taken from three independent SMD simulations.

| FhuA variant | Time (ps) |
|---|---|
| L-arg-FhuA ΔL | 691.50 ± 90.20 |
| D-arg-FhuA ΔL | 643.33 ± 98.94 |
| L-arg-FhuA F4 | 638.33 ± 194.23 |
| D-arg-FhuA F4 | 1186.67 ± 55.30 |

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