Supporting Information for

IroT/MavN is a *Legionella* transmembrane Fe(II) transporter: metal selectivity and translocation kinetics revealed by *in-vitro* real-time transport

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AUTHOR INFORMATION

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MATERIALS AND METHODS

Wild type *Lp*IroT and mutant expression and purification

A pET-52b(+) plasmid encoding for the codon optimized IroT homologue from *Legionella pneumophila subsp. pneumophila* (strain Philadelphia 1 / ATCC 33152 / DSM 7513); Gene: lpg_2815) was generated by Genscript Inc.. The construct was transformed in to *E. coli* BL21(DE3) GOLD competent cells (Agilent Technologies) for recombinant protein expression. O/N pre-cultures were inoculated at 1% (v/v) in Terrific Broth (TB) media supplemented with 1% glycerol and grown in the presence of 50 μg mL^{-1} ampicillin at 37 °C with orbital shaking (140 rpm) to an OD_{600}=2. Cells were subsequently cooled to 18 °C and protein expression was induced by addition of isopropyl thiogalactopyranoside (IPTG) to a final concentration of 0.30 mM. The protein was expressed for 19 h, 18 °C at a shaking speed of 100 rpm.

Cells were harvested by centrifugation (20 min, 4 °C, 20000 x g) (Thermo Scientific Sorvall LYNX 6000 centrifuge). The cell pellet was suspended in buffer (20 mM Tris/HCl, pH 8, 150 mM NaCl, 5 mM $MgCl_2$, 30 μg/mL deoxyribonuclease I from bovine pancreas (Sigma-Aldrich), and 2x EDTA-free protease inhibitor cocktail tablets (Thermo Scientific). Cells were lysed with an ice-chilled microfluidizer at 20000 psi by flowing the cells through a Z-shaped diamond chamber for three cycles (Microfluidics M-110P). The lysate was subsequently centrifuged) to remove cell debris (20 min, 4 °C, 20000 x g, Thermo Scientific Sorvall LYNX 6000 centrifuge). The membrane fraction containing *Lp*IroT was isolated by ultracentrifugation (1 h, 4 °C, 205,100 x g, Beckman Optima XPN80).

The membrane pellet was suspended in a resuspension buffer (20 mM Tris/HCl, pH 8, 500 mM NaCl, 1% (w/v) glycerol supplemented with EDTA-free protease inhibitor cocktail) to a final membrane concentration corresponding to 1g of cells per 1.0 mL of buffer. The membrane suspension was flash frozen in liquid N_2 and stored at -80 °C until purification.

Mutants were generated by site directed mutagenesis (Genscript Inc.) and expressed following the same protocol of *wtLp*IroT.

*wtLp*IroT and all mutants were purified by following the same IMAC chromatography methods with a Ni-NTA 5 mL HisTrap affinity column (GE Health care). For purification, 10 mL of the membrane suspension was diluted to a final volume of 50 mL with ice-cold protein extraction buffer (20 mM Tris/HCl, pH 8, 500 mM NaCl, 35 mM imidazole, 5 mM β-mercaptoethanol, and EDTA-free protease inhibitor cocktail (Thermo Scientific)). n-Tetradecylphospnocholine (Fos-Choline-14) was added to a final concentration of 1% (w/v) in buffer and vigorously stirred for 1 h at 4 °C to extract membrane proteins. Residual membrane debris was removed by ultracentrifugation (20 min, 4 °C, 205,100 xg). The supernatant was loaded onto a pre-equilibrated Ni-NTA column with wash buffer (20 mM Tris/HCl, pH 8, 500

mM NaCl, 35 mM imidazole, 1 mM dithiothreitol (DTT), 0.05% (w/v) 7-cyclohexyl-1-heptyl-β-D-maltoside (Cymal-7)) using an AKTA Pure FPLC system (GE Health care). The column was washed with 50 CV wash buffer (20 mM Tris/HCl, pH 8, 500 mM NaCl, 35 mM imidazole, 1 mM dithiothreitol (DTT), 0.05% (w/v) 7-cyclohexyl-1-heptyl-β-D-maltoside (Cymal-7) and bound protein was eluted with a linear imidazole gradient (0-100%) obtained by mixing wash and elution buffer (20 mM Tris/HCl, pH 8, 500 mM NaCl, 500 mM imidazole, 0.05% (w/v) Cymal-7, 1 mM DTT) over 8 CV. The protein containing fractions were combined and the buffer immediately exchanged to final 20 mM MOPS/NaOH, pH 7, 500 mM NaCl, 1 mM dithiothreitol (DTT), 0.05% (w/v) Cymal-7, to remove imidazole on a HiPrep 26/10 desalting column (GE Healthcare). EDTA was added to a final concentration of 2 mM and samples concentrated to ~5–10 mg/mL using a 100,000 MWCO cutoff filter (Sartorious VIVASPIN 20) by centrifugation (2100 x g, 4 °C). Low molecular weight impurities and protein aggregates were removed by size exclusion chromatography using Superdex 200 10/300 or HiLoad Superdex 200 16/600 columns (GE Health care) in 20 mM MOPS/NaOH, pH 7, 500 mM NaCl, 1 mM dithiothreitol (DTT), 0.05% (w/v) Cymal-7. After all the purification steps the protein was concentrated to ~5–10 mg/mL for proteoliposome reconstitution and purity checked by SDS-PAGE (4-15% Tris-Glycine Mini-PROTEAN gels, BioRad). Protein concentration was determined by absorption at 280 nm on a Nanodrop spectrometer (Thermo Scientific Nanodrop One) utilizing the extinction coefficient of ε_{280} = 122395 M^{-1} cm^{-1} for the wild type protein (calculated based on Tyr and Trp content in the primary sequence using the Expasy Protparam software), or the respective extinction coefficient for mutants. All purified proteins were directly reconstituted in proteoliposomes without freezing.

SEC-MALS analysis for molecular weight determination

20 μM IroT was injected into a Superdex S200 Increase 10/300 GL column (GE healthcare) connected to miniDawn TREOS and Optilab T-rEX refractive index detectors (Wyatt). The column was pre-equilibrated with buffer (20 mM MOPS/NaOH pH=7, 100 mM NaCl, 1 mM TCEP, 0.05% (w/v) Cymal-7) and the data was analyzed using the ASTRA 7.02 software (for details: Wyatt Technology ASTRA Technical Note TN1007). UV extinction coefficients were calculated using Expasy Protparam (122395 M^{-1} $cm^{-1)}$, and detergent dn/dc values were obtained from Anatrace (Cymal-7: dn/dc = 0.1328 mL/g). The standard protein dn/dc value of 0.185 mL/g was used for the analysis as recommended by the manufacturer. Analysis revealed a polydispersity index (PDI) equal to 1.008 ± 0.037%.

***Lp*IroT and mutants reconstitution in proteoliposomes**

Purified wild- type *Lp*IroT and all mutants were reconstituted at a 1:25 (w/w) protein-to-phospholipid ratio. *E. coli* polar lipid extract (Avanti Polar Lipids) and L-α-phosphatidylcholine (from chicken egg; Avanti Polar Lipids) were used in a 3:1 ratio (w/w). The *E. coli* polar lipid extract and L-α-phosphatidyl-choline mixture dissolved in chloroform was dried under a nitrogen stream under rotation, and further dried overnight in a vacuum desiccator.

The lipids were subsequently hydrated and suspended in a 1 mM TCEP solution (treated with Chelex (Bio-Rad) to remove metal contaminations. The suspended lipids were buffered to final 20 mM MOPS/NaOH, pH 7, 100 mM NaCl, 1 mM TCEP, by addition of a 10-times concentrated Chelex treated buffer solution (200 mM MOPS/NaOH, pH 7, 1 M NaCl, 1 mM TCEP). The final lipid concentration was 25 mg mL^{-1}.

Small unilamellar vesicles (SUVs) were prepared by three freeze thaw cycles in liquid nitrogen followed by 11 extrusions through polyethersulfone (PES) membranes of decreasing pore sizes (1 µm, 400 nm, and 200 nm) using a 1 mL air tight syringe system (Avanti, Polar Lipids, Inc). Unilamellar vesicles were then destabilized by adding 0.02% (w/v) Cymal-7 and tilted for 1 h at room temperature.

To reconstitute the purified protein into the liposomes, a concentrated protein solution stock (~5–10 mg/mL) was added to the detergent-destabilized liposomes on ice to a final 1:25 (w/w) protein:lipid ratio. Control liposomes were also generated by buffer addition to the detergent destabilized unilamellar lipid vesicles. The mixtures were tilted at 4 °C for 1 h.

Bio-Beads (SM-2; Bio-Rad) were activated by sequential washing with methanol, ethanol and water followed by drying by vacuum filtration.

The detergent was removed by addition of the pre-activated Bio-Beads to the proteoliposomes and control liposomes to a final slurry Bio-Bead concentration of 40 mg mL^{-1}, and tilted at 4° C. Bio-beads were exchanged after 1, 12, 14, and 16 h, and the proteoliposomes and control liposomes were pelleted by ultracentrifugation at 160,000 x g in a Sorvall mX120+ Micro-Ultracentrifuge. The proteoliposomes and control liposome pellets were collected and resuspended in 20 mM MOPS/NaOH, pH 7, 100 mM NaCl, 1 mM TCEP (treated with Chelex) to a final lipid concentration of 25 mg mL^{-1} (*Lp*IroT =1 mg mL^{-1}). Protein incorporation in the proteoliposomes was estimated by analyzing the ultracentrifugation supernatant and final suspended proteoliposomes by SDS-PAGE (4-15% Tris-Glycine Mini-PROTEAN gels, BioRad). The proteoliposomes and control liposomes were flash frozen and stored at -80° C prior to use for the transport assay.

The number of *Lp*IroT molecules present in each proteoliposome particle was estimated by calculating the particle concentration based on the determined average SUVs diameter (determined by dynamic light scattering, see below) and the total lipid concentration. Assuming spherical particles, the total lipid head group area (S_{TOT}) is the sum of the proteoliposome outer layer surface area (S_{out}) and the inner layer surface area (S_{in}). Assuming a bilayer thickness of 3 nm (h=3nm), S_{TOT} could be estimated:

$$S_{TOT} = S_{out} + S_{in} = 4\pi r^2 + 4\pi(r-h)^2$$

The total number of lipid molecules per SUV (N_{TOT}) has been calculated assuming an average polar head surface (S_L) of 0.7 nm^2/phospholipid:

$$N_{TOT} = S_{TOT}/S_L$$

The estimated MW for a single proteoliposome particle was calculated to determine the total SUV concentration in the samples, assuming the average phospholipid MW= 750 g/mol. By knowing the *Lp*IroT concentration in the preparations, the number of *Lp*IroT molecules per SUV was estimated.

Dynamic Light Scattering measurements

Fluozin-3 encapsulated proteoliposomes and control liposomes were analyzed by UV-Vis dynamic light scattering (DLS) with Zetasizer Nano ZS (Malvern Panalytical) to determine the vesicle size and the size distribution. Fluozin-3 encapsulated proteoliposomes and controls were prepared and harvested as descried. Measurements were collected on samples loaded in a disposable micro cuvette at 25.0° C using a 633 nm laser wavelength, 175° scattering angle and calculated using a medium refractive index of 1.33 and a material refractive index of 1.51. The determined control liposome and IroT proteoliposome PDIs, are 0.195 and 0.275 respectively.

Fluozin-3 encapsulation in proteoliposome lumen

To encapsulate in the proteoliposome lumen the fluorescence chelator Fluozin-3 (Invitrogen), proteoliposomes were diluted to a final lipid concentration of 12.5 mg mL^{-1} in transport buffer (20 mM MOPS/NaOH, pH 7, 100 mM NaCl, 1 mM TCEP). Fluozin-3 was added to the proteoliposome suspension to a final concentration of 10 µM and encapsulated by three subsequent freeze-thaw cycles in liquid N_2. Fluozin-3 containing SUVs were prepared by sequential 11 extrusions through 1 µm, 400 nm, and 200 nm membrane filters using an air tight syringe system.

Free Fluozin-3 was removed via proteoliposome ultracentrifugation at 160000 x g in a Sorvall mX120+ Micro-Ultracentrifuge. The proteoliposome pellets were washed by an additional suspension and ultracentrifugation round. The proteoliposome pellets were suspended in transport assay buffer (20 mM

MOPS/NaOH, pH 7, 100 mM NaCl, 1 mM TCEP) to determine *in- vitro* transition metal transport. Control liposomes were prepared in the same manner with protein-free liposomes.

In vitro Fe^{2+} transport assay

MilliQ water and buffer solutions (20 mM MOPS/NaOH, pH 7.0, 100 mM NaCl, 1mM TCEP) were made oxygen-free on a Schlenk-line by at least three vacuum/nitrogen cycles. All Fe^{2+} stock solutions were freshly prepared prior to each experiment in an anaerobic glove box purged with constant N_2 flow. To prepare the Fe^{2+} stock solution series, a 100 mM Fe^{2+} solution was prepared by dissolving iron (II) sulfate heptahydrate (ACROS ORGANICS) in miliQ water and 10x Fe^{2+} stocks for transport assays were prepared by serial dilution in transport buffer. All stock solutions were stored in sealed tubes prior to use in the proteoliposome assays.

The Fe(II) transport assays were conducted at 25 °C in a sub-micro quartz cell (Starna Cells) in a spectrofluorometer (Horiba scientific Fluoromax-4), using excitation and emission wavelengths of 480 and 515 nm, respectively. 108 µL of the Fluozin-3 encapsulated *Lp*Irot proteoliposomes were placed in the quartz cuvette and the baseline florescence was measured for 50 s prior to Fe^{2+} addition. Fe(II) transport was initiated by addition of a freshly prepared Fe^{2+} solution (12 µL) and the fluorescence change monitored for 600 s with 0.5 s interval time. Measurements on control protein-free liposomes were conducted with the same procedure.

Fe^{2+} transport was determined as $\Delta F/F_0$, where ΔF is the difference between the fluorescence measured at time *t* (F_t) and the fluorescence recorded immediately before Fe^{2+} addition (F_0).

Fe^{2+}-coupled proton counter-transport monitored with pyranine

Fe^{2+}-dependent H^+ counter-transport was determined on *wtLp*IroT proteoliposomes and control liposomes by encapsulating the fluorescent pH-dependent indicator pyranine (Alfa Aesar) in the lumen. Prior to each experiment *wtLp*IroT proteoliposomes and control liposomes were ultracentrifuged at 160,000 x g for 45 min and resuspended in transport assay buffer (10 mM MOPS/NaOH, pH 7.0, 100 mM NaCl, 1mM TCEP) to a final lipid concentration of 12.5 mg mL^{-1}. Pyranine was added to a final concentration of 1 mM and encapsulated by three freeze-thaw cycles in liquid N_2. Pyranine-encapsulated SUVs were prepared by extrusion as described above. After 2 washing steps, proteoliposomes and control liposomes were resuspended in transport assay buffer (10 mM MOPS/NaOH, 100 mM NaCl, 1mM TCEP) to a final lipid concentration of 25 mg mL^{-1}. Oxygen-free Fe^{2+} stock solutions were freshly prepared in anaerobic atmosphere prior to each experiment. Kinetic measurements were carried out on a stopped-flow spectro-

fluorometer at 25° C (SX-20, Applied Photophysics), by mixing equal volumes (70µL) of *wtLp*IroT proteoliposomes (or control liposomes) with 40µM Fe^{2+} stock solutions, and the fluorescence kinetic traces recorded using λ_{exc}= 450 nm in the presence of an emission filter with λ_{cut}-off > 515 nm. Fluorescence spectra was recorded for 601s.

Metal selectivity transport assays

To determine *Lp*IroT selectivity, the transport assay was conducted utilizing the same procedure as described for Fe(II) with different metal stocks. Buffers were made oxygen-free by at least three vacuum/nitrogen cycles on a Schlenk-line and Ni^{2+}, Co^{2+}, Fe^{2+} and Mn^{2+} stock solutions were freshly prepared in an anaerobic glove box purged with constant N_2 flow. Nickel(II) chloride hexahydrate (Alfa Aesar), cobalt(II) chloride hexahydrate (Alfa Aesar), iron(II) sulfate heptahydrate (ACROS ORGANICS), and manganese chloride tetrahydrate were utilized, respectively. Zn^{2+} and Cd^{2+} stocks were prepared in a similar manner using $ZnCl_2$ and $CdCl_2$ under ambient conditions (air atmosphere, 20 °C).

To test Fe^{2+} selectivity over Fe^{3+}, transport assays were conducted with Fluozin-3 excluding the reducing agent TCEP from the assay buffer. Buffer exchange on *Lp*IroT proteoliposomes and control liposomes was performed by collecting the SUVs by ultracentrifugation (160,000 x g, 45 min, 4 °C) followed by resuspension in TCEP-free buffer (20 mM MOPS/NaOH pH= 7.0, 100 mM NaCl). Ferric ammonium citrate (MP Biomedicals,LCC) stock solutions were prepared and transport assays perfomed at Fe^{3+} = 5µM. For comparison, Fe^{2+} transport was tested under the same conditions in the absence of reducing agents.

Fluozin-3 titration with M^{2+} ions.

M^{2+} (M^{2+}=Mn(II), Fe(II), Co(II), Ni(II), Zn(II) or Cd(II)) stock solutions were freshly prepared prior to each titration experiment as described for metal selectivity transport assays. Fluozin-3 solutions were prepared in Chelex-treated transport assay buffer. Metal titrations were conducted in a 1 mL sub-micro quartz fluorometer cell (Starna Cells) using a Fluoromax-4 spectrofluorometer (Horiba scientific). 10 µM Fluozin-3 was placed in the quartz cell and the initial florescence measured for 50s (λ_{exc}= 480 nm ; λ_{em}= 515 nm). After 50s, the M^{2+} solutions were added and resulting fluorescence intensity was measured. Similarly, titration experiments were performed in the presence of 12.5 mg/mL liposomes in the assay buffer to test the effect of lipids on the dye turn-on response to different M^{2+} ions.

Emission spectra for Fluozin-3 (10 µM) in the presence of equimolar M^{2+} concentrations (10 µM) were collected between 490-650nm ($\lambda_{exc.}$= 480nm) on samples prepared as above.

Similar experiments were also conducted with Ferric ammonium citrate solutions to test the response of Fluozin-3 to Fe^{3+}.

His_{10}-tag removal

Purified *wtLp*IroT and *Δ*C-term IroT were rebuffered in 20 mM MOPS/NaOH, pH = 7.0, 100 mM NaCl, 1 mM TCEP, 0.05% (w/v) Cymal-7). Protein concentration was adjusted to 0.5 mg mL^{-1}. Bovine Thrombin (EMD Millipore Crop.) was added (4 Units/mg IroT) and cleavage carried out at 25° C for 8h, with tilting. Imidazole was then added to the mixture to a final concentration of 35 mM. The mixture was subsequently incubated for 1 h with 2 mL of Ni-NTA Resin (Biosciences) equilibrated in the same buffer. Uncleaved protein bound to the resin was removed by centrifugation and 2 mM EDTA added to the supernatant. Impurities and protein aggregates were removed by size-exclusion chromatography using a HiLoad Superdex 200 16/600 column and eluted with chelex-treated buffer (20 mM MOPS/NaOH, pH = 7.0, 100 mM NaCl, 1 mM TCEP, 0.05% (w/v) Cymal-7).

Determination of overall apparent dissociation constants by ligand competition.

The overall apparent IroT-Fe^{2+} dissociation constants (K_D) were determined by ligand competition using MagFura-2 (MF-2) using previously developed methods[1-3]. Experiments were carried out in a 1.0 cm quartz cuvette using chelex-treated oxygen-free buffer (25 mM MOPS/NaOH, pH = 7.5, 150 mM NaCl, 1 mM TCEP, 0.05% (w/v) Cymal-7). All the mixing steps were conducted in an anaerobic glove-box purged with N_2. Tag-free *wtLp*IroT and MF-2 were mixed to final 20 μM and 10 μM, respectively, in a quartz cuvette. The actual final MF-2 concentration was determined using ε_{366} = 29900 M^{-1} cm^{-1} [1,2]. UV-Vis spectra were recorded on a Cary 300 UV-Vis Spectrophotometer (Agilent). Fe^{2+} competition titrations were performed in an anaerobic glovebox, by sequential Fe^{2+} addition in 2 μM increments and the resulting absorption spectra recorded. Similar determination were carried out with *Δ*C-term IroT.

Overall apparent K_D determination was performed as described previously[2,3]. Briefly, the variables Q and P are calculated using equation 1 and 2.

$$Q = \frac{\varepsilon - \varepsilon_{IL}}{\varepsilon_I - \varepsilon} \quad \textbf{(1)} \qquad P = L_t - {}^{1}/_{QK_I} - {}^{I_t}/_{Q+1} \quad \textbf{(2)}$$

Free indicator to metal bound indicator ratio is given by Q. ε, ε_I and ε_{IL} are the extinctions coefficients of observed, free and MF-2-Fe^{2+} bound states. P is the concentration of IroT-Fe^{2+} complex. I_t is total MF-2 concentration and K_I is the association constant of MF-2-Fe^{2+} complex (K_D= 1.5 μM)[1]. Assuming a 1:1 protein-metal complex formation, variables are related as in equation 3.

$$\frac{S_t}{P} = \frac{K_I}{K_{prot}} Q + 1 \quad (3)$$

Dissociation constant ($K_{D,Fe(II)}$) for the IroT-Fe^{2+} complex can be estimated by plotting S_t/P vs Q. S_t is the total protein concentration. K_{prot} is the Fe(II) association constant for IroT [2,3].

1. Patel, S.J.*, et al.* Fine-tuning of Substrate Affinity Leads to Alternative Roles of Mycobacterium tuberculosis Fe^{2+}-ATPases. *J. Biol. Chem.,* **291**, 11529-11539 (2016).
2. Walkup, G.K. & Imperiali, B. Fluorescent Chemosensors for Divalent Zinc Based on Zinc Finger Domains. Enhanced Oxidative Stability, Metal Binding Affinity, and Structural and Functional Characterization. *J. Am. Chem. Soc.* **119**, 3443-3450 (1997).
3. Connors, K.A. *Binding Constants: The Measurement of Molecular Complex Stability*, (Wiley-Interscience: New York, 1987).

Figure S1

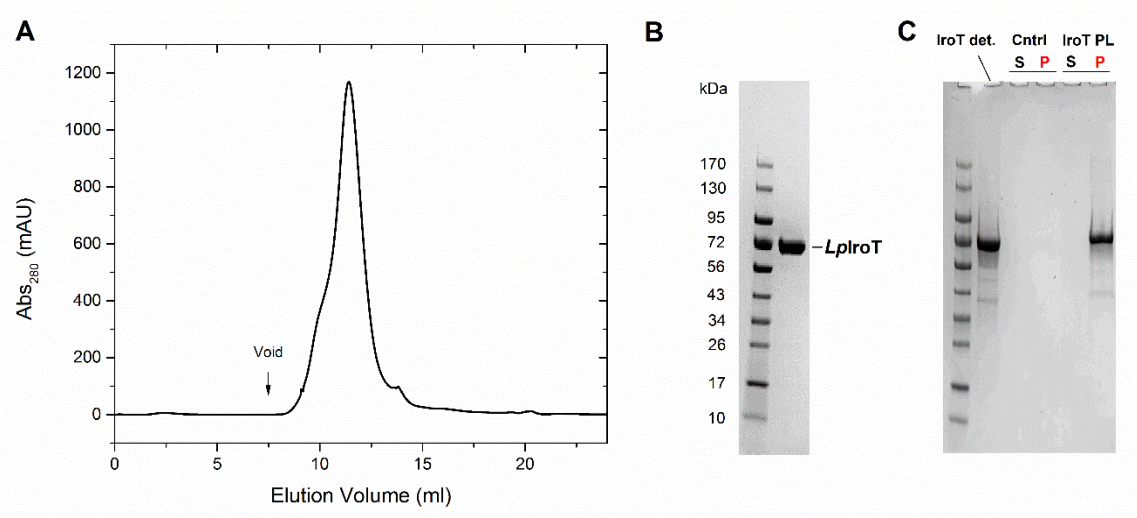


Figure S1: **(A)** Size exclusion chromatography elution profile for *wtLp*IroT purified in in Cymal-7 micelles. (**B**) SDS-PAGE analysis of *Lp*IroT upon SEC. (**C**) SDS-PAGE analysis of *wtLp*IroT incorporation in pelleted proteoliposomes (P) compared to non-incorporated protein (S, supernatant) *vs. wtLp*IroT-Cymal-7 micelles, and corresponding analysis of control liposomes.

Figure S2

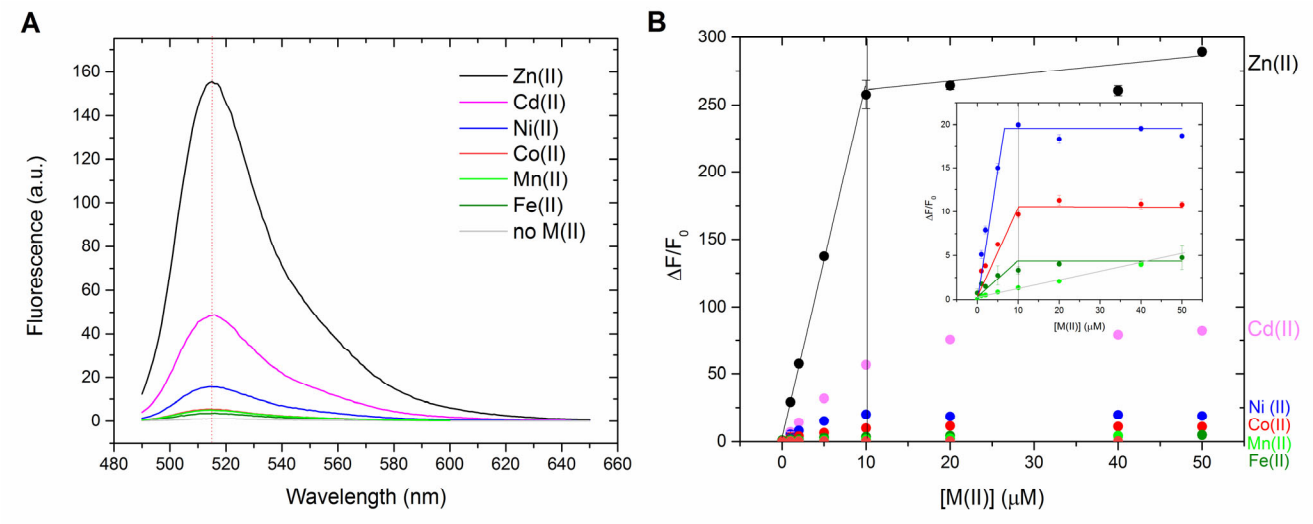


Figure S2 (**A**) Fluozin-3 (10 μM) fluorescence emission turn-on response in the presence of equimolar concentration of Mn(II), Fe(II), Co(II), Ni(II), Zn(II) and Cd(II) (λ_{exc}= 480nm) in absence of liposomes. (**B**) Fluozin-3 (10 μM) fluorescence turn-on response upon metal titration (λ_{exc}= 480nm; λ_{em}= 515nm, line color corresponding to Figure 2A) in absence of liposomes, reported as $(F-F_0)/F_0$ (where F_0 is the Fluozin-3 fluorescence in absence of metals). The response to Mn(II), Fe(II), Co(II) and Ni(II) is enlarged in the *Inset.*

Figure S3

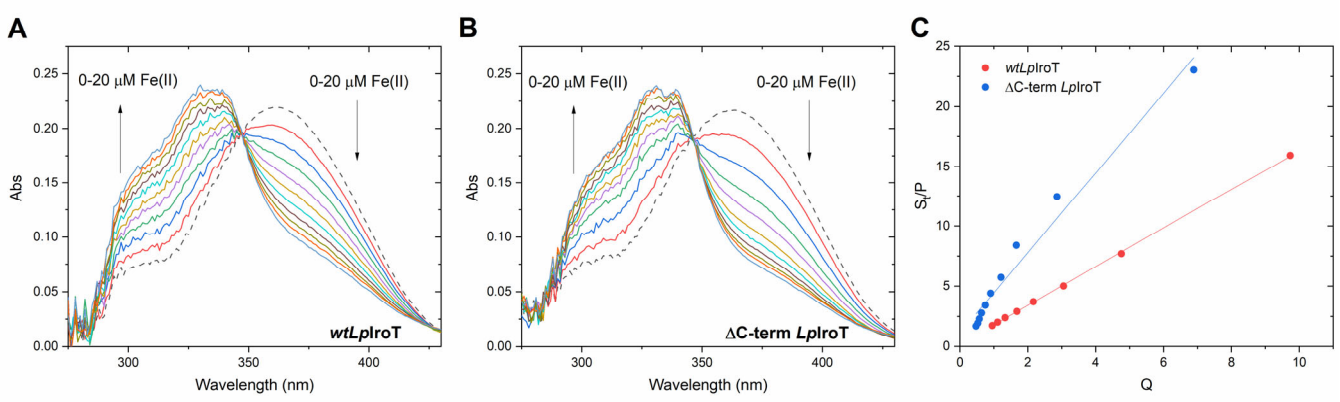


Figure S3 Competition of MagFura-2 (MF-2) for Fe (II) with IroT. (A) Representative absorption spectra obtained for the titration of 10 μM MF-2 and 20 μM *wtLp*IroT with increasing concentrations of Fe(II) (0−20 μM; 2 μM increments) in 25 mM MOPS-NaOH, pH = 7.5, 150 mM NaCl, 1 mM TCEP, 0.05% (w/v) Cymal-7. (B) Representative absorption spectra obtained for the titration of 10 μM MF-2 and 20 μM ΔC-term *Lp*IroT. (C) Representative plots obtained for fitting of S_t/P *vs.* Q values calculated as described in Materials and Methods.

Figure S4

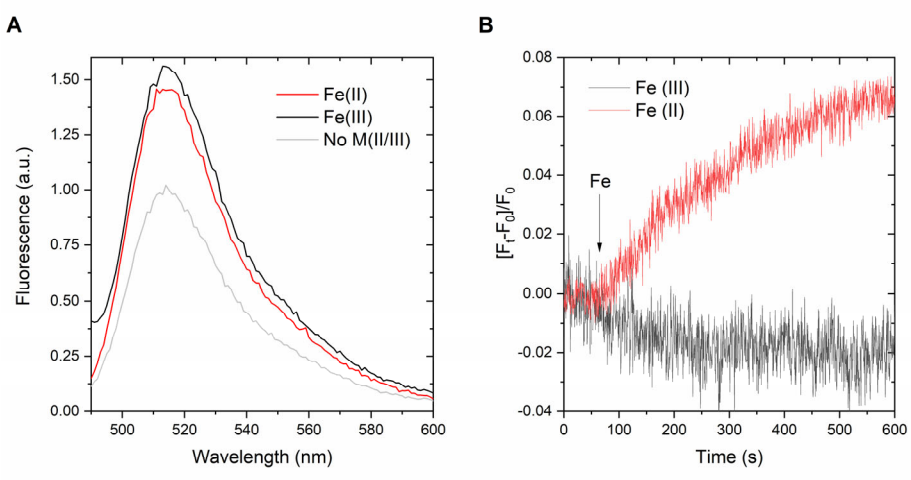


Figure S4 *wtLp*IroT substrate selectivity towards Fe(II) over Fe(III) (A) Fluozin-3 (10 µM) fluorescence emission turn-on response for equimolar concentrations of Fluozin-3 and Fe(II) or Fe (III) (5µM; λ_{exc}= 480nm) in the presence of 200 nm liposomes (PC/polar lipids 1:3 w/w, 12.5 mg/ml). Representative fluorescence real-time transport traces in *Lp*IroT proteoliposomes with encapsulated Fluozin-3 for Fe(II) and Fe(III) in the absence of TCEP (20 mM MOPS/NaOH, pH= 7.0, 100 mM NaCl).

Figure S5

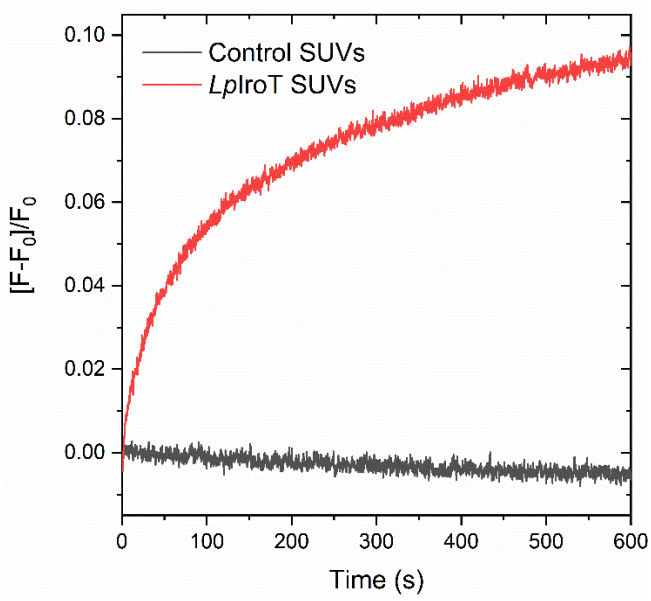
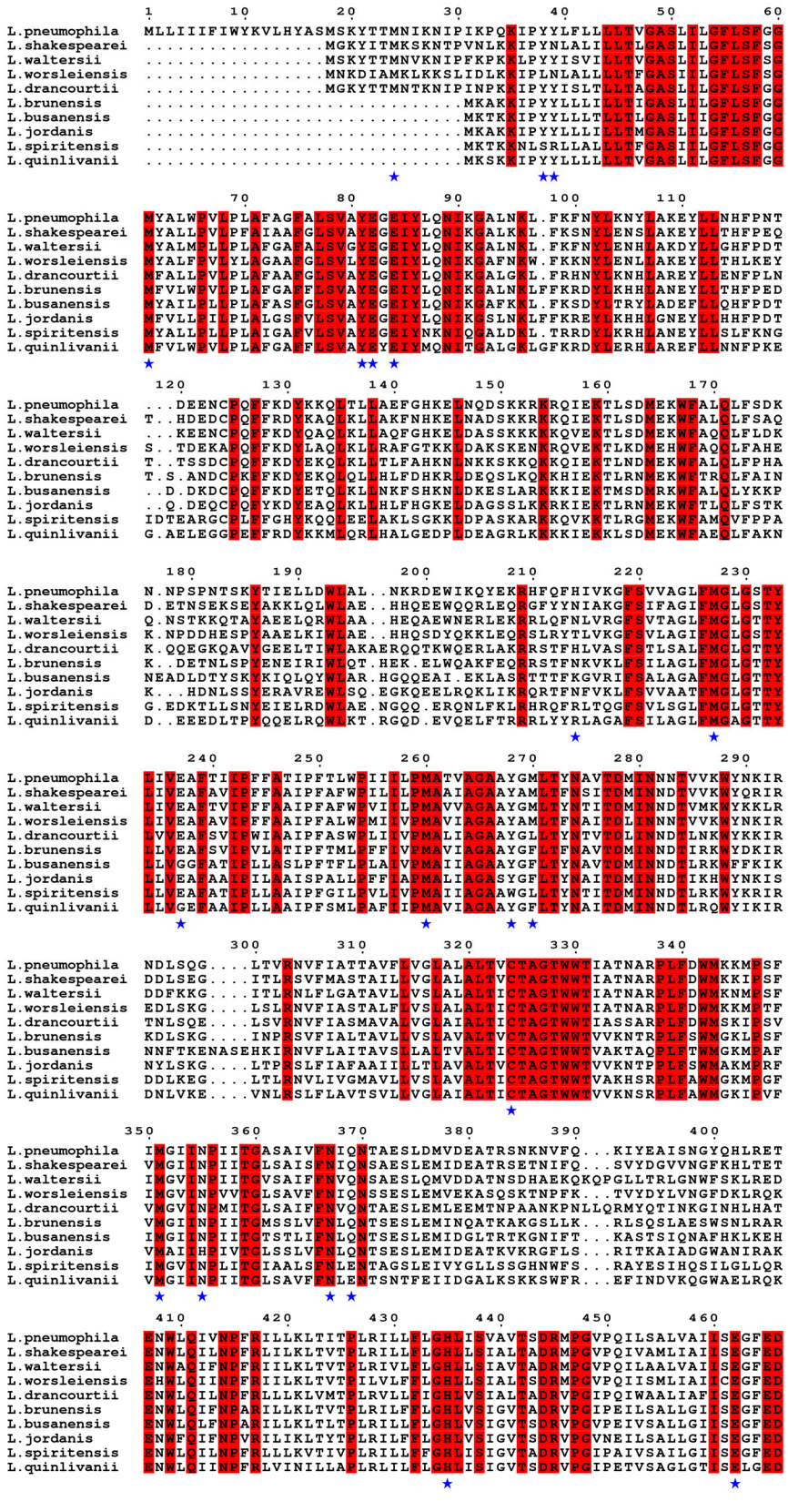


Figure S5 Fluorescence-detected (λ_{exc}= 450 nm; λ_{em} > 515 nm) proton counter-transport monitored by pyranine encapsulation in control and *Lp*IroT proteoliposomes exposed to Fe(II)= 20µM (average of two independent replicates).

Figure S6


L.pneumophila
L.shakespearei
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L.worsleiensis
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L.brunensis
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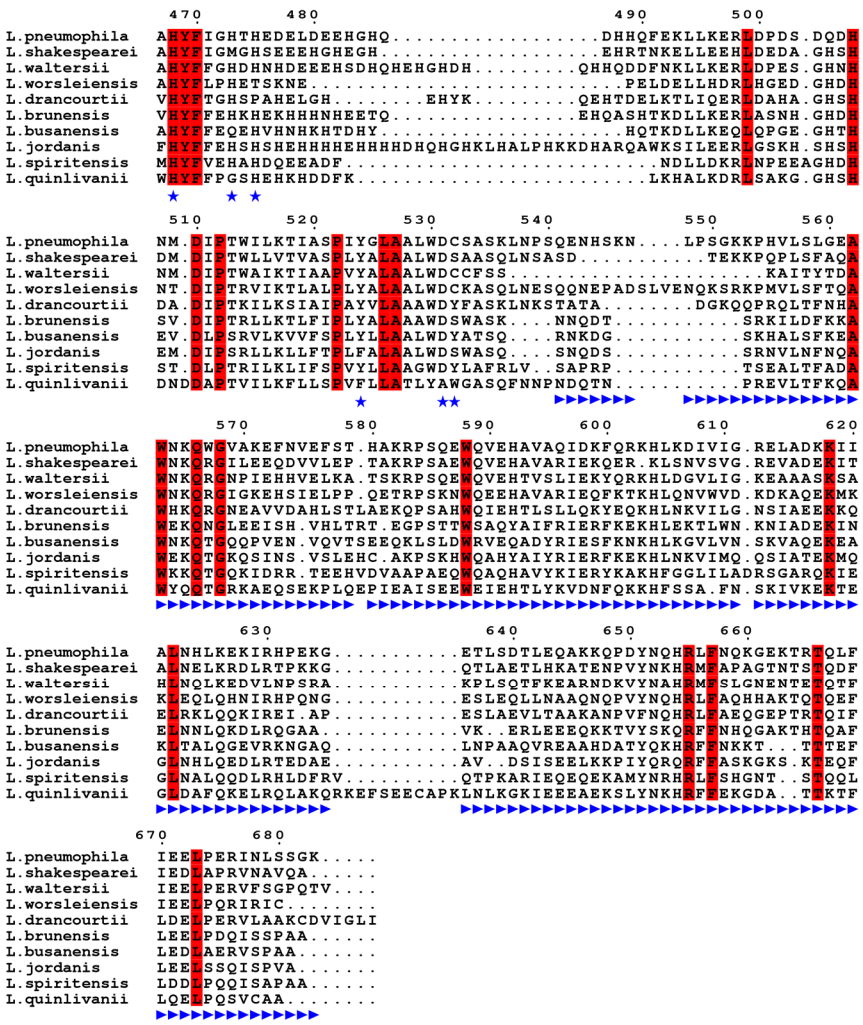

Figure S6 Sequence alignment of 10 *Legionella* IroT/MavN homologues. Uniprot Accession Numbers: *L. pneumophila* (Q5ZRR5), *L. shakespearei* (A0A0W0YRI1), *L. waltersii* (A0A0W1A4L9), *L. worsleiensis* (A0A0W1AF00), *L. drancourtii* (G9ETW3), *L. busanensis* (A0A378JX52), *L. brunensis* (A0A0W0S175), *L. jordanis* (A0A0W0VD72), *L. spiritensis* (A0A0W0Z6K6), *L. quinlivanii* (A0A0W0Y5F3). Fully conserved residues are highlighted in red, potential Fe (II) coordinating residues mutated in this work are indicated with blue stars, and amino acids (541-683) absent in *Δ*C-term *Lp*IroT are indicated in blue triangles. Figure generated with ESPript 3.0 (http://espript.ibcp.fr/ESPript/ESPript).

Figure S7

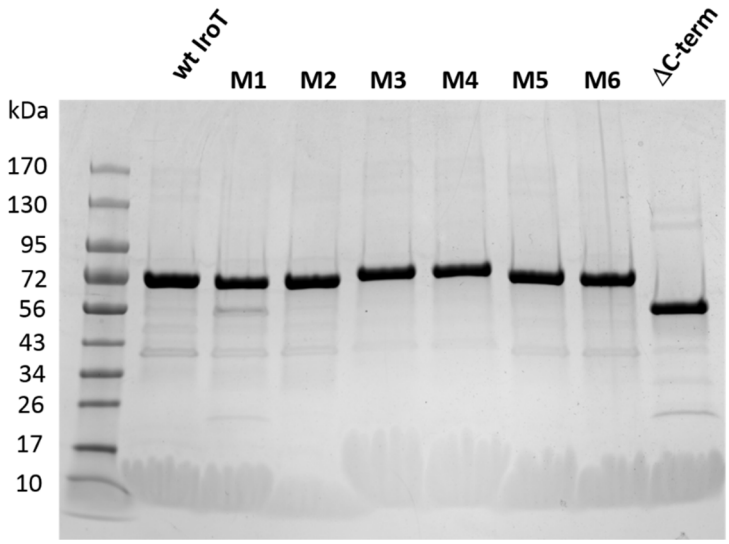

Figure S7 SDS-PAGE analysis of *wtLp*IroT and mutants (M1-6 and ΔC-term) reconstituted in proteoliposomes utilized for Fe(II) transport assays.

Figure S8

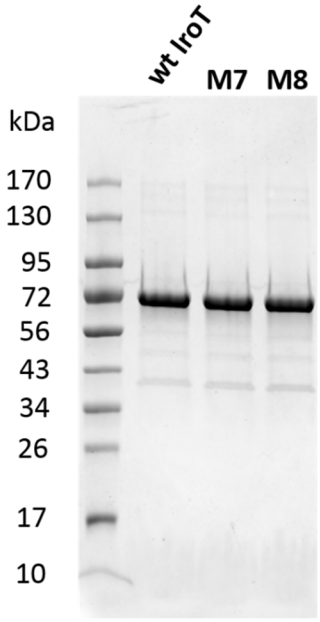

Figure S8 SDS-PAGE analysis of *wtLp*IroT and mutants M7 and M8 (E462A*Lp*IroT and E462A_H468A_H473A_H475A*Lp*IroT respectively) reconstituted in proteoliposomes utilized for Fe(II) transport assays.

Table S1

*Lp*IroT mutants and corresponding location in the *Lp*IroT topology model

Mutant Name	Mutant position	Host -side soluble do-mains	LCV-side soluble domains	Interface TM –host cytoplasm	Interface TM-LCV	TM heli-ces core
M1	E82A_E84A_H214A_D531A_C532A			**X**		
M2	E82A_E84A_H214A			**X**		
M3	M24A_Y38A_Y39A_Y81A_E82A_ E84A_H214_Y268A_M270A_N367A-Q369A_D531A_C532A			**X**		
M4	M227A_M260A_N355A_Y524A					**X**
M5	M61A_E237A_C324A_M351A_H435A				**X**	
M6	E237A_C324A_H435A				**X**	
M7	E462A		**X**			
M8	E462A_H468A_H473A_H475A		**X**			
ΔC-term	Deletion of C-terminal soluble domain ($\Delta_{541-683}$-*Lp*IroT)	**X**				

Table S2

Apparent Michaelis-Menten parameters for Fe(II) translocation in proteoliposomes obtained by fitting the maximum fluorescence intensity change ($\Delta F/F_0$) as function of Fe(II) concentrations for *wtLp*IroT and mutants

	$K_{M,\ Fe(II)}$ (μM)	$(\Delta F/F_0)_{MAX}$
***wtLp*IroT**	5.99 ± 1.70	0.25 ± 0.02
***Lp*IroT-M1**	> 25 μM	0.20 ± 0.10
***Lp*IroT-M2**	>25 μM	0.30 ± 0.10
***Lp*IroT-M3**	n.d.[a]	0.10 ± 0.02
***Lp*IroT-M4**	23.0 ± 14.8	0.22 ± 0.7
***Lp*IroT-M5**	n.d.	0.06 ± 0.01
***Lp*IroT-M6**	n.d.	0.06 ± 0.01

[a.] not determined, the mutant shows minimal transport activity.