Figures and figure supplements

An ECF-type transporter scavenges heme to overcome iron-limitation in *Staphylococcus lugdunensis*

Angelika Jochim *et al*

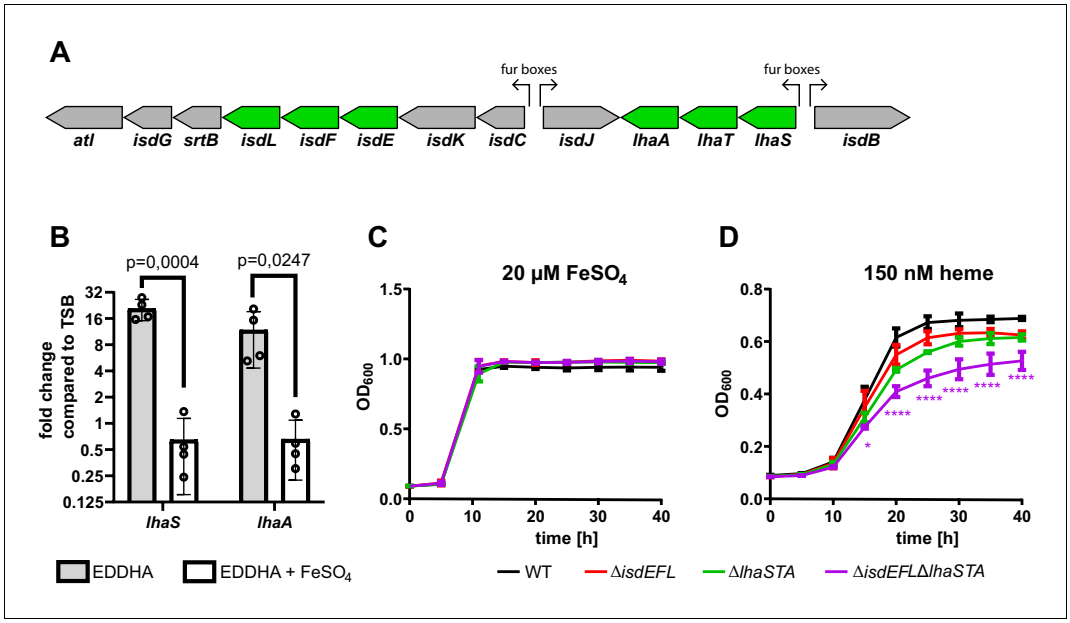

Figure 1. LhaSTA represents an iron-regulated heme transporter. (**A**) Schematic diagram of the *isd* operon of *S. lugdunensis* N920143. Coding sequences, direction of transcription and Fur-binding sites are indicated. ABC membrane-transporters are shown in green. *lhaS* - SLUG_00900; *lhaT* - SLUG_00910; *lhaA* - SLUG_00920 (**B**) Iron-regulated expression of Lha: *S. lugdunensis* was grown overnight in TSB, TSB + 200 µM EDDHA or TSB + 200 µM EDDHA + 200 µM $FeSO_4$. Gene expression was quantified by qPCR. Expression was normalized to 5srRNA and to the TSB standard condition using the ΔΔCt method. Fold differences in gene expression are shown. Data represent mean and SD of four independent experiments. Statistical evaluation was performed using students unpaired t-test (lhaS: t = 7,045, df = 6; lhaA: t = 2,979, df = 6) C/D Growth curves of *S. lugdunensis* N920143 and isogenic mutants. The wild type (WT) *S. lugdunensis* N920143 strain and the indicated isogenic null mutant strains were grown in the presence of 20 µM $FeSO_4$ (**C**) or 150 nM heme (**D**) as a sole source of iron. 500 µl of bacterial cultures were inoculated to an OD_{600} = 0,05 in 48 well plates and OD_{600} was monitored every 15 min using an Epoch1 plate reader. For reasons of clarity values taken every 5 hr are displayed. Mean and SD of three experiments are shown. Statistical analysis was performed using one-way ANOVA followed by Dunett's test for multiple comparisons. * - p<0,05, ****p<0,00001.

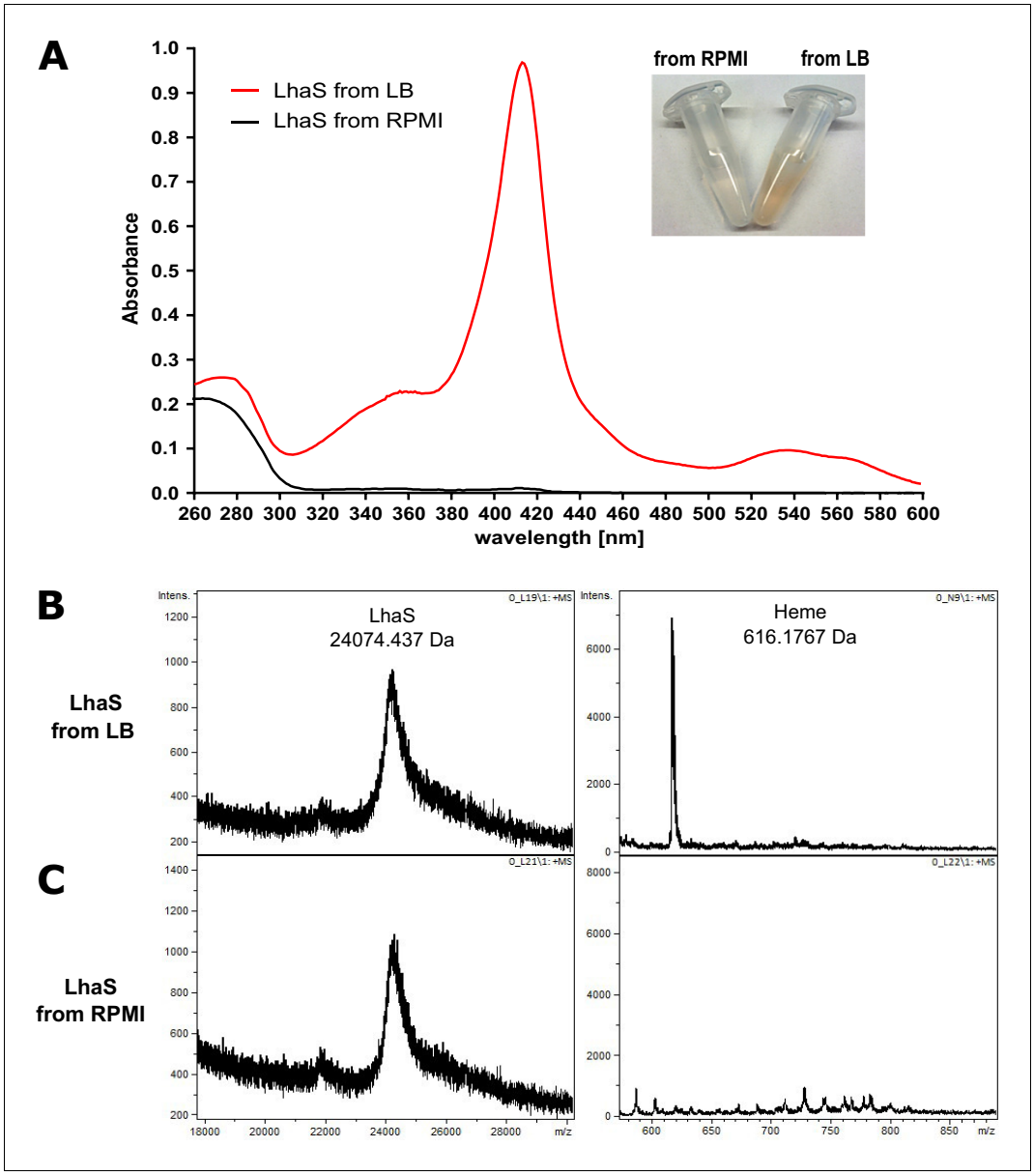

Figure 2. LhaS binds heme. (**A**) Ultraviolet-visible (UV-vis) spectrum of recombinant LhaS. C-terminal His-tagged LhaS was heterologously expressed in *E. coli* and purified from heme-containing LB medium or heme-free RPMI medium. The UV-vis spectrum of the purified LhaS was measured with a BioPhotometer. (**B**) and (**C**) MALDI-TOF mass spectra of recombinant LhaS. LhaS (**B**) was purified out of LB medium and apo-LhaS (**C**) was purified out of RPMI medium. Mass spectra were recorded with a Reflex IV in reflector mode. All spectra are a sum of 50 shots. Prior to measurements the protein samples were mixed with a 2,5-dihydroxybenzoic acid matrix dissolved in water/acetonitrile/trifluoroacetic acid (50/49.05/0.05) at a concentration of 10 mg ml^{-1} and spotted onto the MALDI polished steel sample plate.

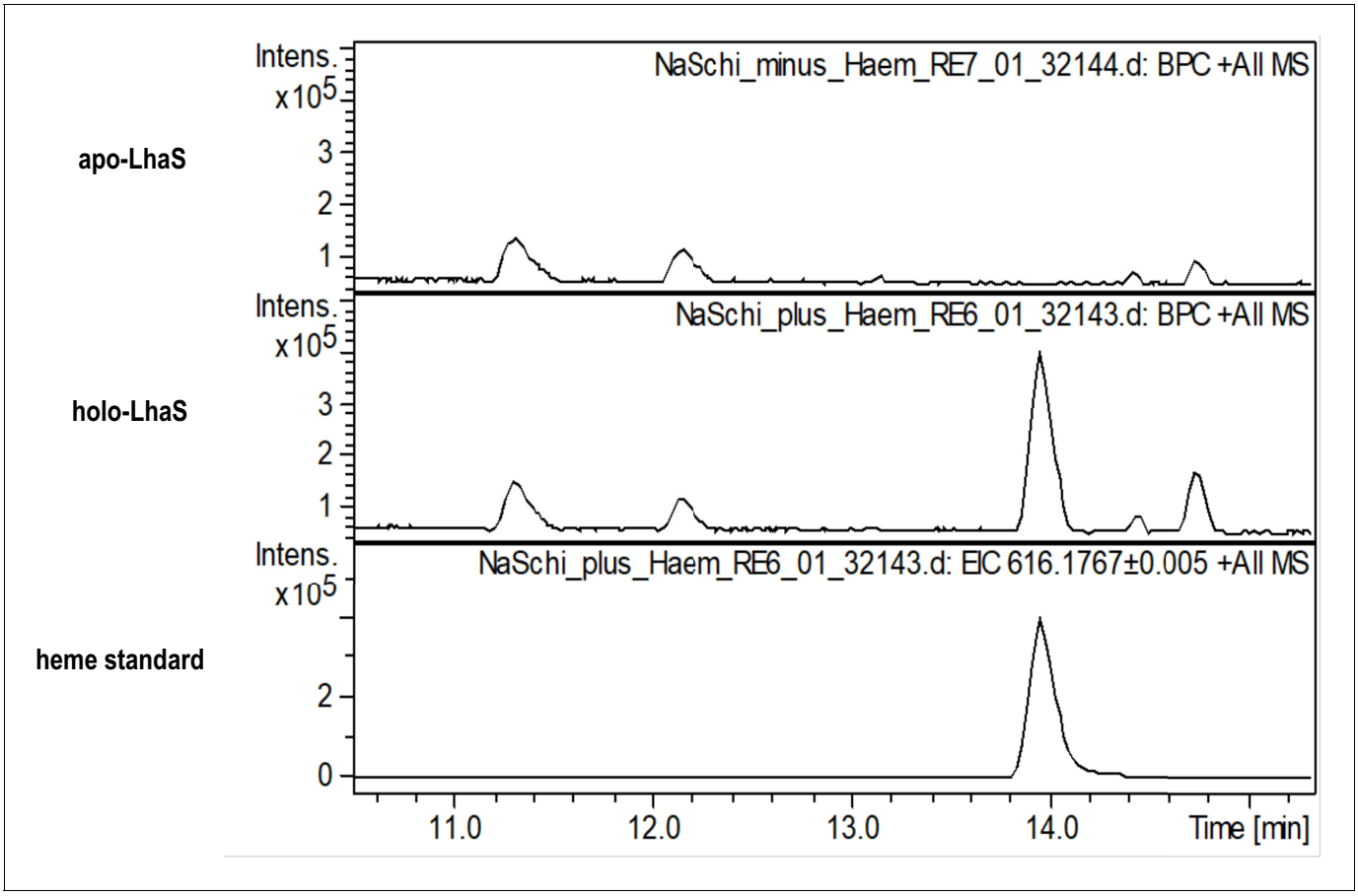

Figure 2—figure supplement 1. High resolution mass spectra of apo- and holo-LhaS. Spectra of apo-LhaS isolated from *E. coli* grown in RPMI medium (upper panel), holo-LhaS isolated from LB-medium (middle panel) and a heme standard (lower panel) were recorded on a HPLC-UV-HR mass spectrometer. The samples were diluted with MilliQ-H_2O and applied to a Dionex Ultimate 3000 HPLC system that is coupled to the MaXis 4G ESI-QTOF mass spectrometer.

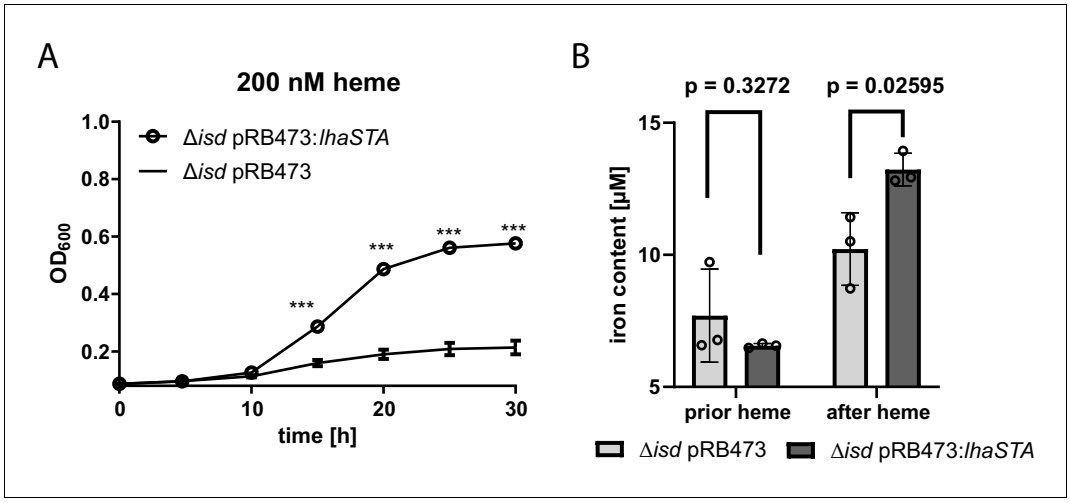

Figure 3. LhaSTA represents a functionally autonomous iron acquisition system. (**A**) LhaSTA-dependent proliferation. *S. lugdunensis* N920143 deletion mutant strains lacking the entire *isd* operon and expressing LhaSTA (Δ*isd* pRB473:*lhaSTA*) or not (Δ*isd* pRB473) from the plasmid pRB473 were grown in the presence of 200 nM heme as a sole source of iron. 500 µl of cultures were inoculated to an OD_{600} = 0,05 in 48 well plates and OD_{600} was monitored every 15 min using an Epoch1 plate reader. For reasons of clarity values taken every 5 hr are displayed. Mean and SD of three experiments are shown. Statistical analysis was performed using students unpaired t-test. ***p<0,0001 (**B**) Intracellular accumulation of iron. Strains were grown in iron limited medium to OD_{600} = 0,6 and 5 µM heme were added for 3 hr. Cell fractionation of 1 ml OD_{600} = 50 was performed and the iron content of the cytosolic fraction was determined using the ferrozine assay. Data represent the mean and SD of three independent experiments. Statistical analysis was performed using students unpaired t-test (t = 5,12729, df = 4).

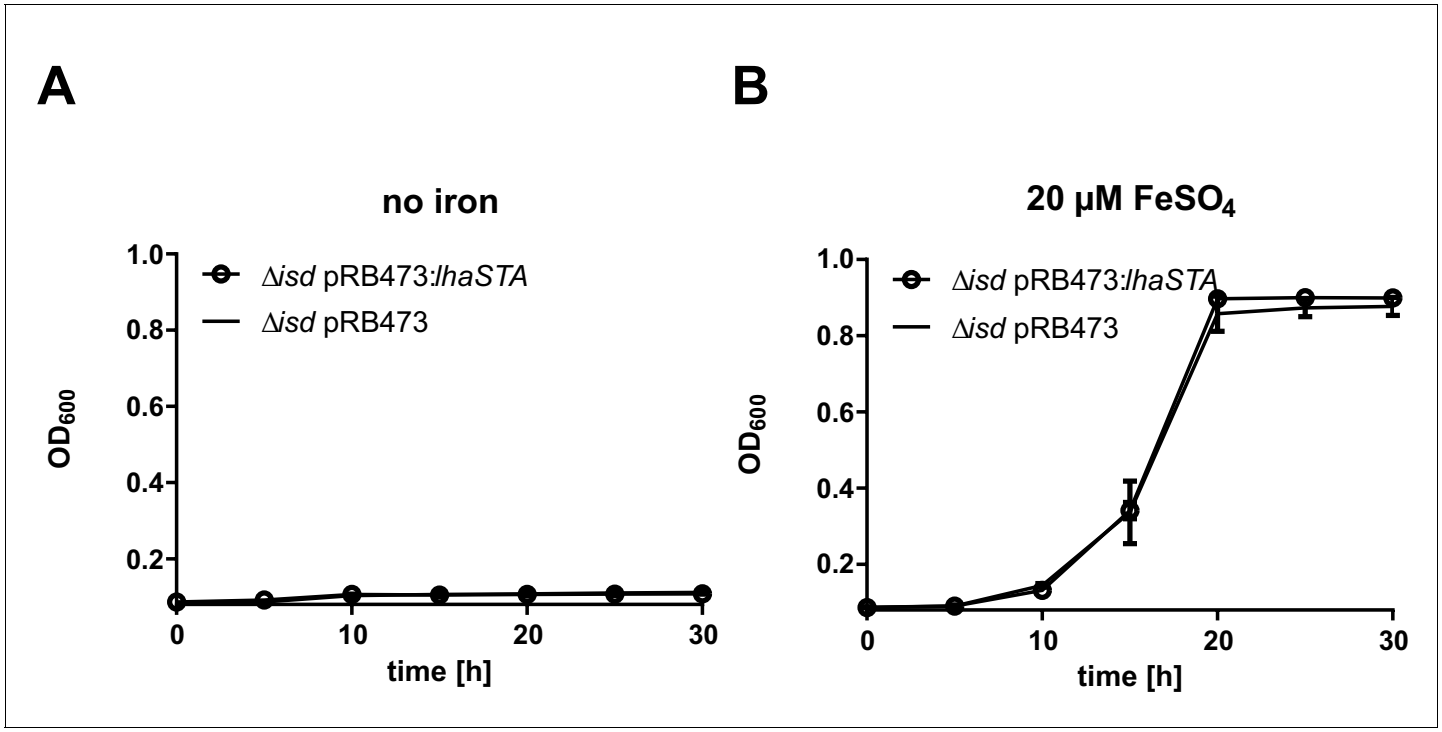

Figure 3—figure supplement 1. LhaSTA dependent growth. (**A/B**) Proliferation of *S. lugdunensis* N920143 Δ*isd* pRB473 and Δ*isd* pRB473:*lhaSTA* strains. The indicated strains were grown in the absence of nutritional iron (**A**) or in the presence of 20 µM $FeSO_4$ (**B**). 500 µl of cultures were inoculated to an OD_{600} = 0,05 in 48 well plates and OD_{600} was monitored every 15 min using a Epoch1 plate reader. For reasons of clarity values taken every 5 hr are displayed. Mean and SD of three experiments are shown. Statistical analysis was performed using students unpaired t-test.

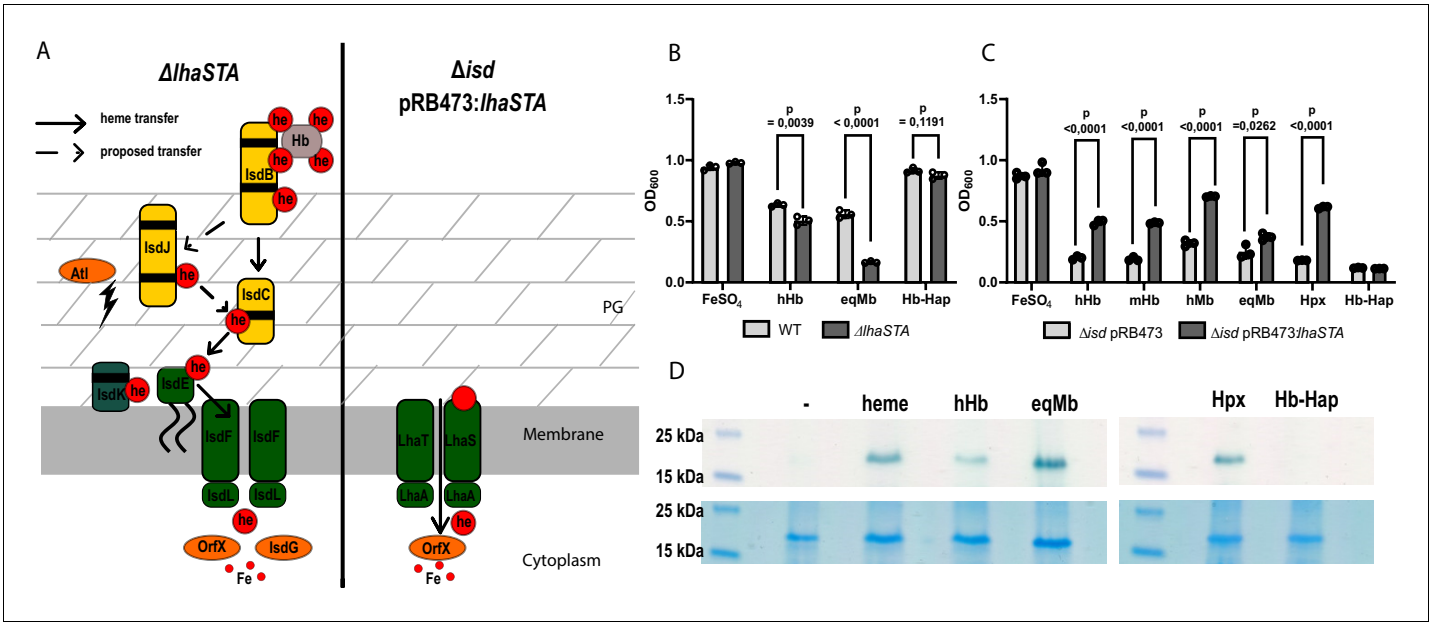

Figure 4. LhaSTA facilitates heme acquisition from a wide range of hemoprotein substrates. (**A**) Schematic diagram of known heme acquisition systems in the *S. lugdunensis* mutant strains lacking either the genes encoding LhaSTA (Δ*lhaSTA*, left) or the entire *isd* operon and expressing LhaSTA from the plasmid pRB473 (Δ*isd* pRB473:*lhaSTA*). ABC membrane transporters are shown in green. Cell wall-anchored proteins of the Isd-system are shown in yellow. Heme/hemoglobin-binding NEAT motifs within each protein are indicated as black boxes. Black arrows indicate the transfer of heme. he: heme; hb: hemoglobin; PG: peptidoglycan. (**B**) Growth of *S. lugdunensis* N920143 wild type (WT) and Δ*lhaSTA*. Strains were grown in the presence of 20 µM $FeSO_4$ or 2.5 µg/ml human hemoglobin (hHb) or 10 µg/ml equine myoglobin (eqMb) or 117 nM hemoglobin-haptoglobin complex (Hb-Hap) as a sole source of iron. 500 µl of cultures were inoculated to an OD_{600} = 0,05 in 48 well plates and OD_{600} was measured after 30 hr using an Epoch1 plate reader. Mean and SD of three experiments are shown. Statistical analysis was performed using students unpaired t-test. hHb - t = 6,0007, df = 4; eqMb – t = 20,52, df = 4; Hb-Hap – t = 1,978, df = 4. (**C**) Growth of *S. lugdunensis* N920143 Δ*isd* pRB473 and Δ*isd* pRB473:*lhaSTA*. Strains were grown in the presence of 20 µM $FeSO_4$ or 2.5 µg/ml hHb or 2.5 µg/ml murine hemoglobin (mHb) or 10 µg/ml human myoglobin (hMb) or 10 µg/ml eqMb or 200 nM human hemopexin (Hpx) or 117 nM Hb-Hap as a sole source of iron. 500 µl of cultures were inoculated to an OD_{600} = 0,05 in 48 well plates and OD_{600} was measured after 30 hr using an Epoch1 plate reader. Mean and SD of three experiments are shown. Statistical analysis was performed using students unpaired t-test hHb – t = 18,5, df = 4; mHb – t = 29,03, df = 4; hMb – t = 25,98, df = 4; eqMb – t = 3,442, df = 4; Hpx – t = 77,12 df = 4; Hb-Hap t = 2758 df = 4. (**D**) TMBZ-H_2O_2 stain of TGX gels for heme-associated peroxidase activity. Membrane vesicles were saturated with excess of hemoprotein (5.6 µM heme, 476 µg/ml hHb, 437 µg/ml eqMb, 5.6 µM Hpx, 476 µg/ml Hb-Hap) or no hemoprotein (-) for 10 min at RT. LhaS was purified, 15 µg protein was loaded on a TGX gel and stained for peroxidase activity with TMBZ-H_2O_2 (upper panel). Gels were destained and subsequently stained with BlueSafe (lower panel) to confirm the presence of the protein in all conditions.

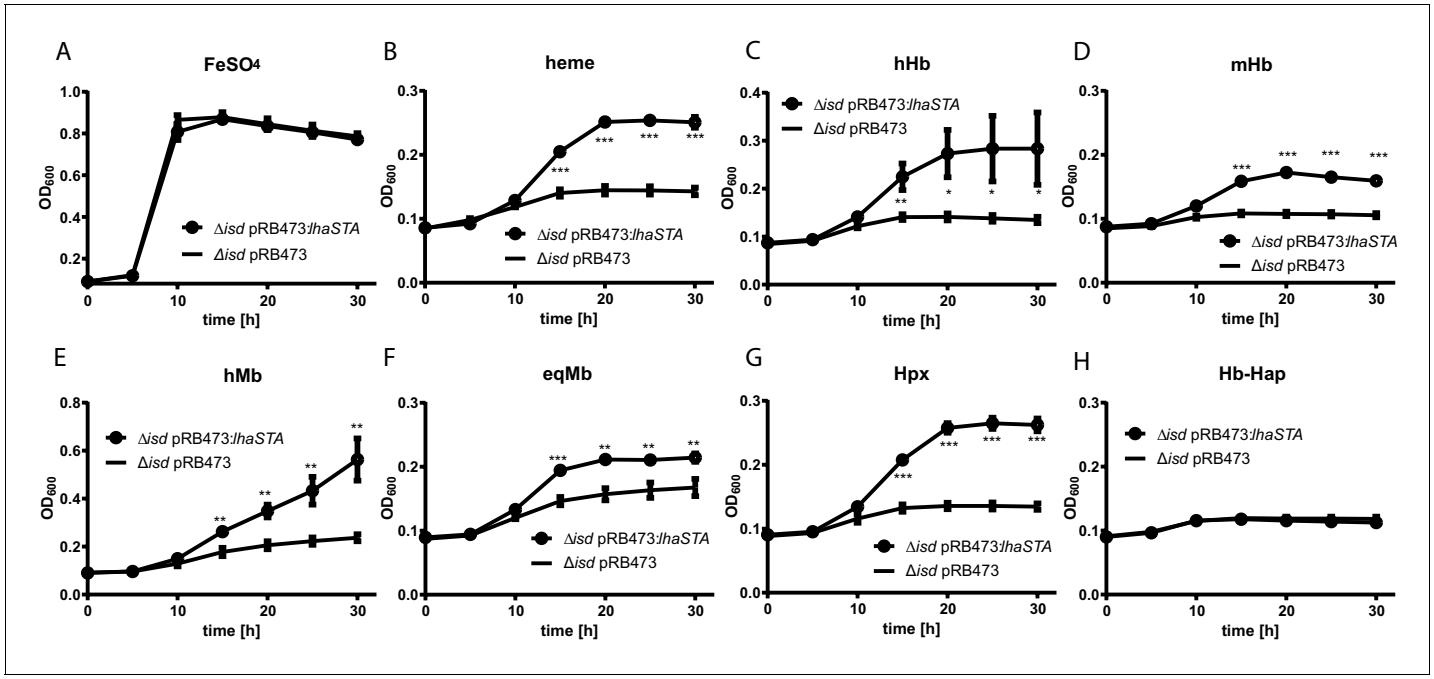

Figure 4—figure supplement 1. Growth of *S. lugdunensis* N940135 Δ*isd* pRB473 and Δ*isd* pRB473:*lhaSTA*. The indicated strains were grown in the presence of 20 µM $FeSO_4$ or 2.5 µg/ml human hemoglobin (hHb), 2.5 µg/ml or murine hemoglobin (mHb) or 10 µg/ml human myoglobin (hMb) or 10 µg/ml equine myoglobin (eqMb) or 200 nM human hemopexin (Hpx) or 117 nM Hb-Hap as a sole source of iron. 500 µl of cultures were inoculated to an OD_{600} = 0,05 in 48 well plates and OD_{600} was measured every 15 min. For reasons of clarity values taken every 5 hr are displayed. Mean and SD of three experiments are shown. Statistical analysis was performed using students unpaired t-test.

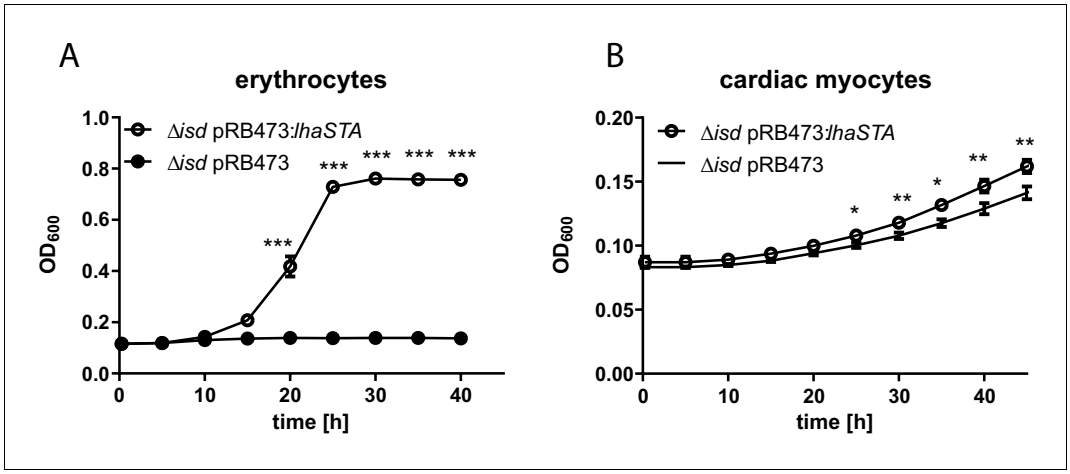

Figure 5. LhaSTA allows usage of host cells as an iron source. (**A**) Growth of *S. lugdunensis* N940135 Δ*isd* pRB473:*lhaSTA* and Δ*isd* pRB473 on human erythrocytes. Strains were grown in the presence of freshly isolated human erythrocytes (10^5 cells/ml) as a sole source of iron. 500 µl of cultures were inoculated to an OD_{600} = 0,05 in 48 well plates and OD_{600} was monitored every 15 min using an Epoch1 plate reader. For reasons of clarity values taken every 5 hr are displayed. Mean and SD of three experiments are shown. Statistical analysis was performed using students unpaired t-test. ***p<0,0001 (**B**) Growth of *S. lugdunensis* N940135 Δ*isd* pRB473 and Δ*isd* pRB473:*lhaSTA* on human cardiac myocytes. Strains were grown in the presence of 40000 primary human cardiac myocytes per well as a sole source of iron. Cardiac myocytes were detached and washed once with RPMI+200 µM EDDHA prior addition to the wells. 500 µl of cultures were inoculated to an OD_{600} = 0,05 in 48 well plates and OD_{600} was monitored every 15 min using an Epoch1 plate reader. For reasons of clarity values taken every 5 hr are displayed. Mean and SD of three experiments are shown. Statistical analysis was performed using students unpaired t-test. *p<0,05, **p<0,01.

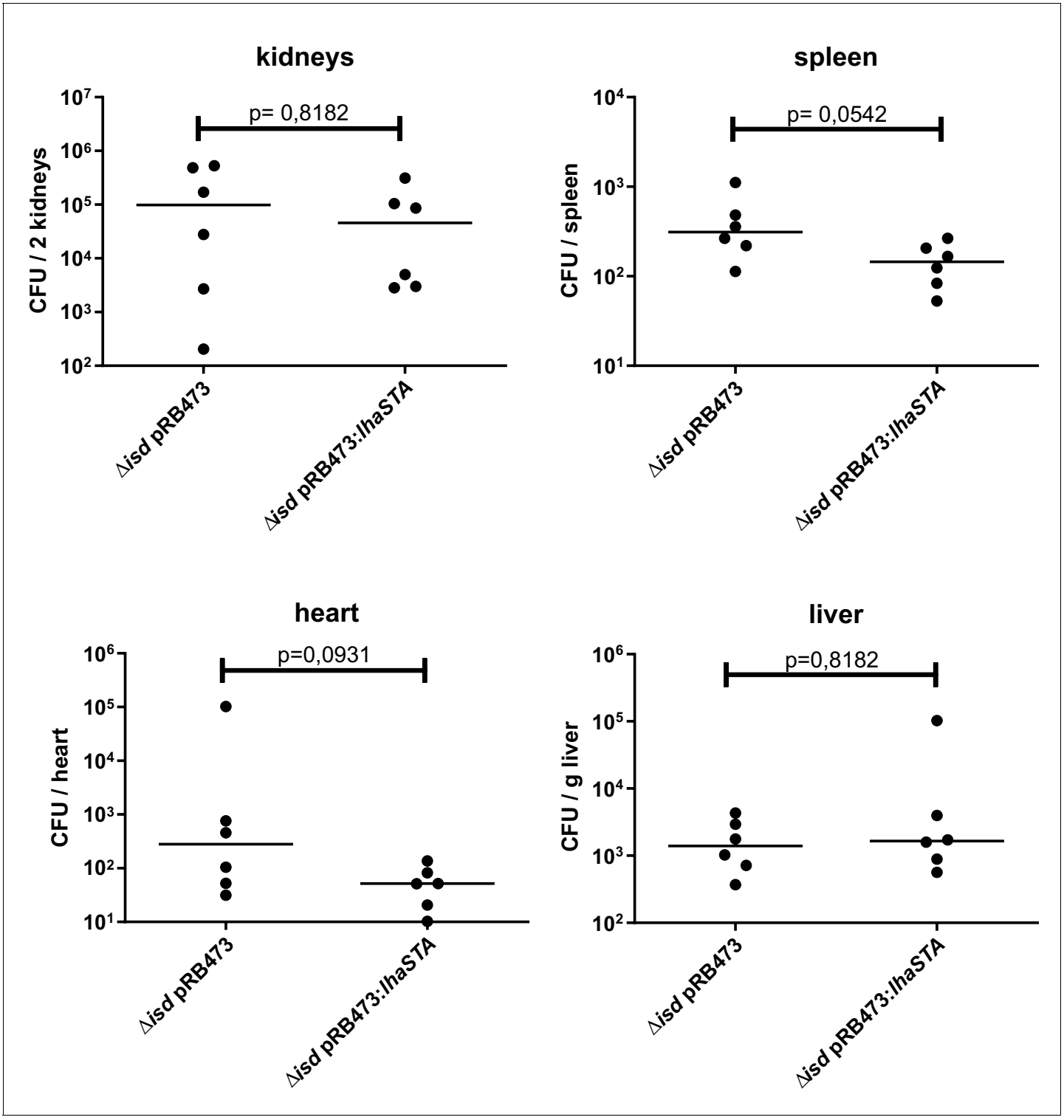

Figure 5—figure supplement 1. Mouse systemic infection model. C57BL/6 mice were infected with 3×10^7 CFU per animal. Mice were sacrificed 72 hr post infection and CFUs within the indicated organs were enumerated. Horizontal lines show the median. Statistical analysis was performed using Mann Whitney test.

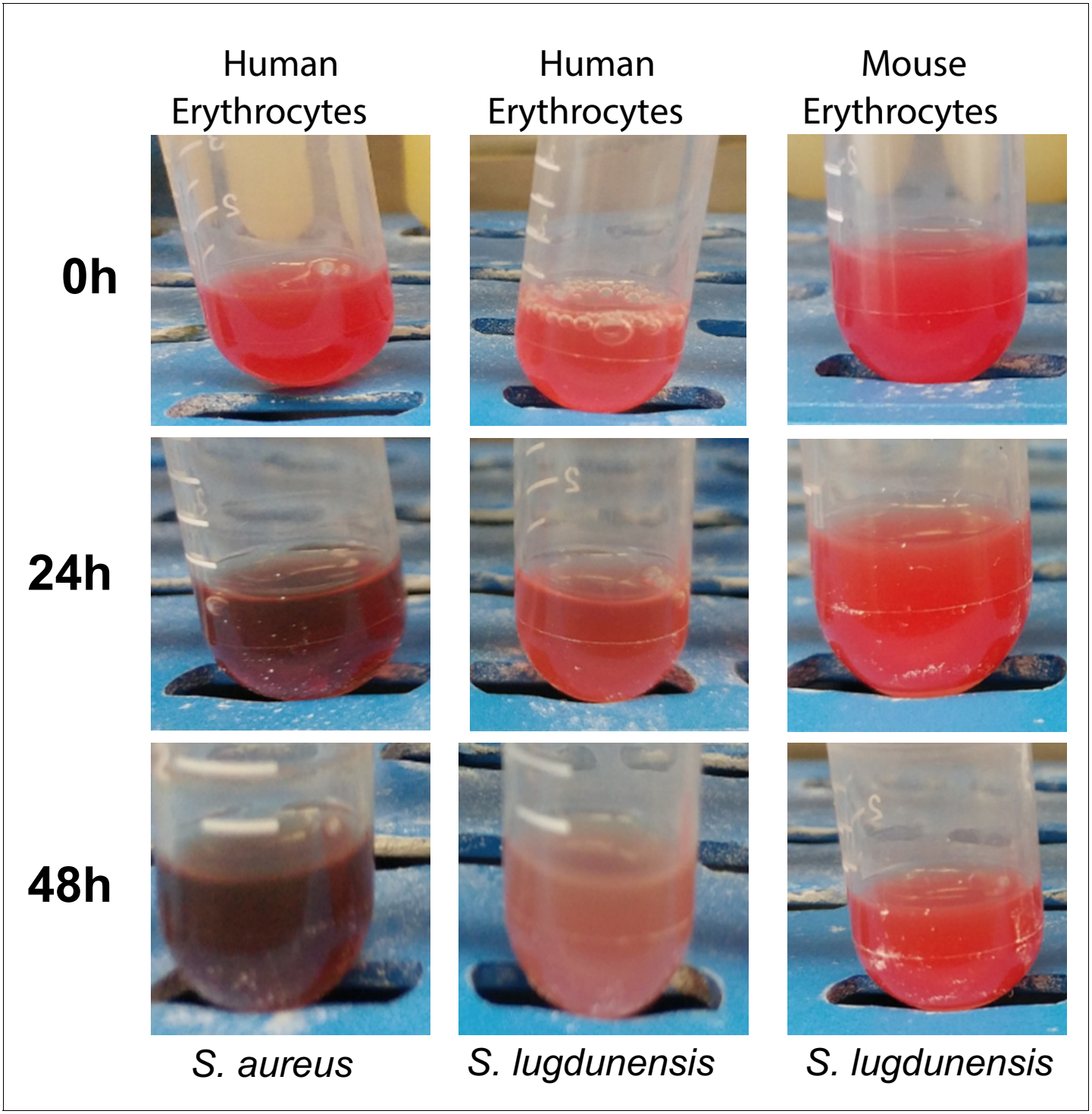

Figure 5—figure supplement 2. Hemolysis of human and mouse erythrocytes. Human (left and middle panels) or mouse erythrocytes (right panel) were incubated with the filtrated culture supernatants of *S. aureus* (left panel) or *S. lugdunensis* (middle and right panels) for 24 and 48 hr.

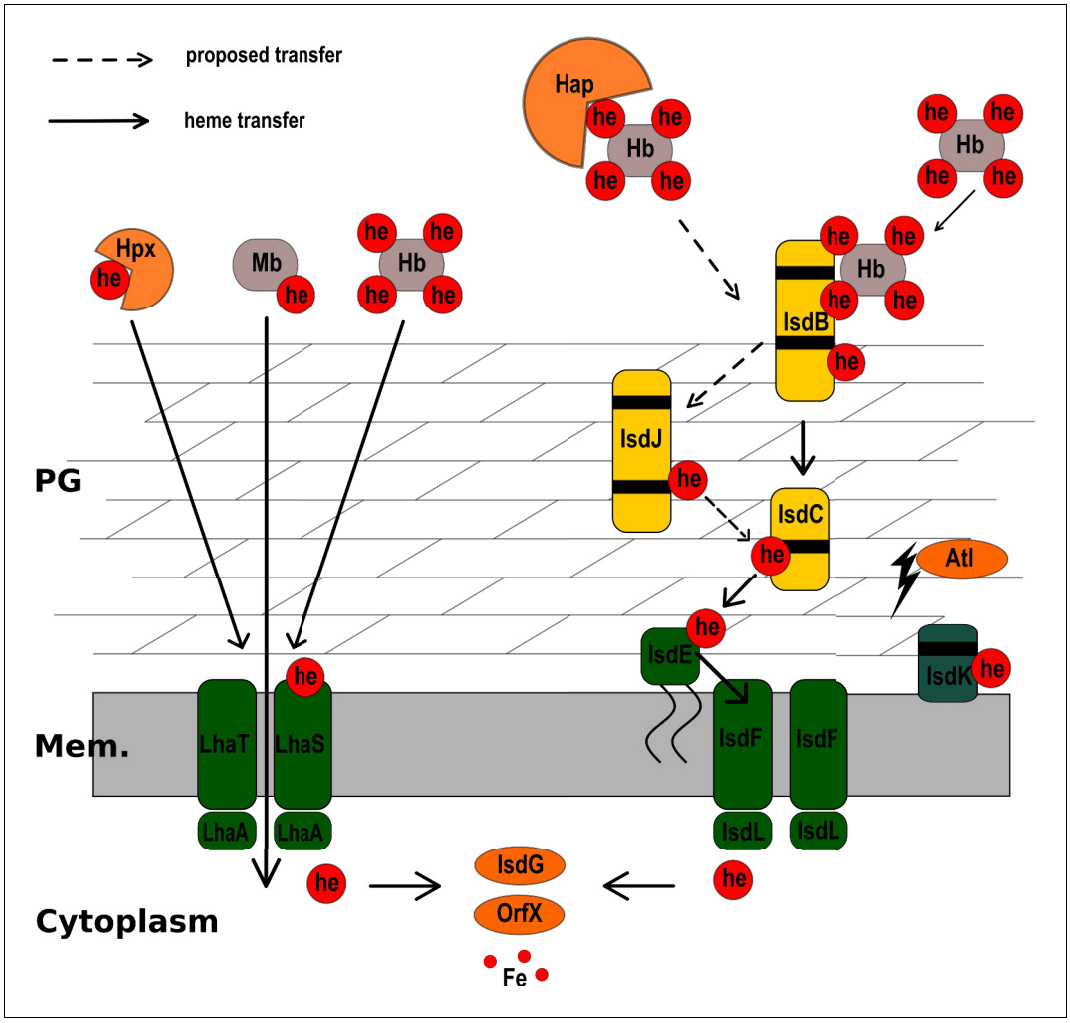

Figure 6. Model of heme acquisition in *S. lugdunensis*. ABC membrane transporters are shown in green. Cell wall-anchored proteins of the Isd-system are shown in yellow. Heme/hemoglobin-binding NEAT motifs within each protein are indicated as black boxes. Black arrows indicated the transfer of heme. he: heme; hb: hemoglobin; PG: peptidoglycan; Mem: Membrane; Hap: Haptoglobin; Hpx:Hemopexin; Atl: Autolysin.