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Bacterial ABC transporters of iron containing compounds.

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Abstract

Iron acquisition is an essential aspect of cell physiology for most bacteria. Although much is known about how bacteria initially recognize the various iron sources they can encounter, whether siderophore, heme, host iron/heme binding proteins, much less is known about how the iron containing compounds (Fe^{2+} , Fe^{3+} , Fe^{3+} -siderophore complex or heme) are transported across the cytoplasmic membrane. This last transport step is powered by specific ABC (ATP-Binding-Cassette) transporters, made up of a substrate binding protein (SBP) that delivers its cargo to the TMD (TransMembrane Domain) of the ABC transporter triggering the entry of the substrate inside the cytoplasm upon catalytic activity of the ABC module. This review focuses on structural aspects of the functioning of such ABC transporters with the most part devoted to the substrate binding proteins.

Keywords

Iron, siderophore, heme, substrate-binding protein.

Introduction

This review deals with the structure and function of ATP-binding cassette (ABC) transporters specifically involved in heme/iron transport across membranes. Whether monoderm or diderm, bacteria devote a substantial part of their genomes to the acquisition of iron containing compounds. Iron is needed as an essential component of many key enzymes and also takes part under the heme form to several constituents of respiratory chains. Each *Escherichia coli* cell contains ca. 200000-800000 iron atoms [1], which it must acquire from its environment during a cell division time. Iron being also toxic under its Fe^{2+} form (due to the Fenton reaction), its homeostasis is strictly controlled at several levels [2, 3]. At near-neutral and basic pH, and in the presence of oxygen, Fe is essentially found under the form of highly insoluble ferri-hydroxy compounds, and although iron is an important component of terrestrial media, its soluble concentration is extremely low, well below the needs of the bacteria. Under acidic and/or reducing conditions iron is mostly found under the readily soluble form Fe^{2+} , that is acquired by many bacteria. When developing in mammalian hosts, bacteria face the so-called nutritional immunity imposed by the host, sequestering Fe in very high affinity components [4], that has to be overcome by the bacterium to ensure a successful colonization. Under iron starvation, most bacteria synthesize organic compounds with very high affinity for Fe^{3+} , named siderophores and able to displace Fe^{3+} from its initial sources [5]. In the case of Gram-negative bacteria, the Fe^{3+} -siderophore complex is internalized in the periplasmic space by specialized outer membrane (OM) transporters. They are called TonB-Dependent Transporters (TBDT), and the TonB complex that sits in the cytoplasmic membrane conveys the energy of the proton-motive force (pmf) to the TBDT to allow entry of the substrate bound to the extracellular site of the receptor into the periplasm [6, 7]. Most bacterial species also express receptors for so-called xenosiderophores, synthesized by other microorganisms. Among the most potent siderophores is enterobactin (the *E. coli* siderophore, also named enterochelin) with an affinity constant for Fe^{3+} over 10^{50} M^{-1} [8]. Transport of the Fe^{3+} containing compound across the cytoplasmic membrane is ensured in most cases by specific ABC transporters consisting of at least one soluble substrate binding protein (SBP, also named periplasmic binding protein for Gram-negative bacteria, or PBP [9]) and a membrane bound ABC complex, which in turn in most cases contains two integral transmembrane domains (TMD) and two peripheral ABC modules [10]. In the case of Gram-

positive bacteria, the Fe^{3+} -siderophore complex is recognized by substrate binding proteins that are in most cases retained in the cytoplasmic membrane by a lipid anchor. Although heme is also ultimately bound to a SBP in the case of Gram-positive bacteria, heme/heme source recognition may involve several previous steps occurring at the cell surface by proteins either secreted in the milieu and/or anchored at different levels of the cell wall [4, 11, 12]. The siderophore/heme loaded SBP is recognized by the TMD subunit of the ABC complex, that internalizes the substrate during its cycle, powered by ATP binding and hydrolysis, by the ABC subunit. The TMD, as well as the ABC might be encoded by either one or two genes. These ABC transporters belong to the larger class of ABC importers that have been subdivided at least into two subfamilies [13, 14]. The type I ABC importer is exemplified by the *Escherichia coli* Maltose transporter (MalEFGK₂, MalF and MalG being the TMD, MalK the ABC and MalE the SBP) whereas the type II is exemplified by the BtuC₂D₂F from *E. coli* that transports the B12 vitamin (BtuC being the TMD, BtuD the ABC, and BtuF the SBP). Most often the TMD's of type II ABC importers have 10 TM helices, whereas their number is smaller for type I ABC importers. Differences are also found in the mode of action of the two families, where in the « resting state » of the transporter, the cavity at the interface of the TMD's is open toward to the cytoplasm in type I importers and towards the periplasm in type II importers [13-15]. This review will specifically deal with some recent results concerning the structure and function of Fe/siderophores/heme transport via ABC transporters. Fig. 1 shows the general architecture of those systems across the Gram-negative envelope.

I. Substrate binding proteins: structural aspects

This part will mostly deal with the promiscuity displayed by binding proteins, that can bind several substrates, as opposed to the rather strict specificity of the siderophore outer membrane receptors that often bind only one substrate with quite high affinity. *Escherichia coli* K12 synthesizes one siderophore (enterobactin or enterochelin, of the catecholate type), whose OM receptor is FepA. It synthesizes several other outer membrane receptors, that recognize various siderophores CirA (Fe-DHBS) (DHBS, dihydroxybenzoylserine, a breakdown product of enterobactin), YoeA (the coding region is interrupted by an IS2), YncD (the substrate is unknown), YbiL or Fiu (DHBS3Fe), FhuA (ferrichrome), FecA (Fe-dicitrate), FhuE (Fe-rhodotorulic acid/ coprogen). Substrate binding proteins are bilobate proteins that

bind their substrate at the boundary between the two lobes before transferring it to the ABC TMD domain of the ABC transporter. An initial classification of substrate-binding proteins recognized three classes, according to the number of links between the two domains. Class I SBP, exemplified by the MBP (Maltose Binding Protein) has three links between the two lobes. Class II SBP exemplified by Histidine-Binding Protein has two links between the two lobes, and class III SBP, exemplified by BtuF, the vitamin B12 binding protein has only one link between the two lobes. A more recent classification based on structural alignments of SBP's enlarged the number of classes/clusters up to 6, the initial class II being subdivided in four structurally distinct clusters [16]. Up to now, no class I SBP has been identified as involved in iron/iron complex transport in bacteria, and all of them belong either to class III (structural class A from [16]) or class II (structural classes C and D from [16]).

Most structural data related to SBP's came from crystallographic studies of the free and substrate-bound forms, whereby they are most likely trapped in low-energy states that do not reflect their dynamic properties.

The binding protein has to fulfill several functions, first of efficiently binding the substrate when it arrives the periplasm, then to bind to the ABC transporter in order to release its cargo, and finally to cycle back to its initial free state in the periplasmic space. There is the common belief that binding proteins are in stoichiometric excess as compared to the ABC transporters [17].

As compared to class I or class II SBP's, the main characteristics of the class III SBP's are the much more modest, or even absent, large movements of one domain with respect to the other upon substrate binding. However, it is also well possible that the free forms of the binding proteins undergo larger relative movements of the two domains than the bound forms, this being a basis for the discrimination by the TMD domains.

Siderophores have been divided in at least four classes according to the metal chelating groups, catecholates, carboxylates, hydroxamates, or mixed type. Despite this rather simple classification, there is a huge structural diversity of siderophores even in a single class, arising from the modifications added on the siderophore backbone during its synthesis [18, 19]. As a consequence the binding proteins have adapted to this huge diversity, and the following chapter will underline this diversity. The situation is somewhat different for heme binding proteins, as the iron atom is bound by the four pyrroles of the heme molecule, leaving two potential valences available for binding.

IA Class III (structural class A) siderophore/heme binding proteins (supplementary tables S1 and S2)

Up to now all siderophore binding proteins identified belong to the class III, with only one alpha-helix connecting the two domains, that are each made of a distorted beta-sheet surrounded by alpha-helices. The binding site for the siderophore lies at the intersection of the two domains. The Table SI presents a list of PDB entries corresponding to siderophore binding proteins, as complete as possible at the present time. The class III was initially defined by the structures of both PsaA and TroA, two divalent metal binding proteins of *Streptococcus pneumoniae* and *Treponema pallidum* respectively [20, 21]. PsaA, which has been identified as an essential virulence factor in an animal model is likely a Mn^{2+} binding protein, whereas TroA is a Zn^{2+} binding protein.

IA1 Catecholate binding proteins

- *Escherichia coli* enterobactin binding protein (PDB codes 2M6K, 2M6L, 3TLK).

Enterobactin (Ent) is the main siderophore synthesized by *E. coli*. It belongs to the catecholate family (it is essentially a cyclic siderophore made up of three molecules of dihydroxybenzoylserine, DHBS), and the complex Fe^{3+} -Ent is transported across the OM by the TBDT FepA, and then bound to the FepB binding protein, before its transport across the IM by the FepCDG complex. Surprisingly structural studies on FepB either in its free form or complexed to enterobactin, have been scant and only appeared recently one by X-ray crystallography [22] the other one by NMR in solution [23]. In the X-ray study FepB was crystallized in the presence of enterobactin, and an unusual form was observed, with three FepB-FeEnt molecules in the asymmetric unit with their binding sites facing each other, together with another FeEnt molecule shared between the three FepB molecules. Each subunit has ca. 1100 Å² buried upon interaction. It is not completely clear whether the supplementary FeEnt molecule is necessary for complex formation. This crystal (PDB 3TLK) form was obtained with a 3-fold excess of FeEnt as compared to FepB (30 vs 10 μM). Although there is no doubt that this form might exist, its biological meaning is questionable, as there was no study as a function of concentration of the appearance of this FepB₃FeEnt₄ complex. However a very crude estimation of the number of FepB molecules (under full induction, a few thousand)

given the periplasmic space volume, is actually within the 10 μ M range (assuming a periplasmic volume of 0,1 fL) used in this study. The use of a FepDG mutant would clarify this point.

A similar observation has been made with a linear siderophore shared by two binding protein molecules is the case of CeuE of *Campylobacter jejuni* in complex with FeMECAM (1,3,5-N,N',N''-tris-(2,3-dihydroxybenzoyl)-triaminomethylbenzene, a tri-catecholate synthetic siderophore) [24]. However in this last case, there is no extensive contact between the two polypeptide chains of the asymmetric unit, that are linked together via the linear FeMECAM, each MECAM molecule respectively contributing 4 and 2 valences for holding the iron atom (see below).

There is no crystal structure of the apo form of *E. coli* FepB. The NMR study (which, by the way was quite a tour de force, given the size of the protein) has provided many interesting insights in particular into the dynamic of the protein. The alpha-helices are well defined, whereas this is not so true for the beta-sheets (perdeuteration effect). The comparison of the chemical shift perturbations between GaEnt-FepB and FepB allows to see three zones (145-150, 225-245 and 290-310) where GaEnt binding affects the structure of the protein (see Fig. S1); GaEnt was used instead of FeEnt, as FeEnt perturbs too much the NMR spectra ; GaEnt binds to FepB with a similar affinity compared to FeEnt. There is no large effect on overall structure of FepB upon binding of GaEnt, however several residues in the 225-242 loop region, as well as 77-81 and 293-303 appear more rigid upon ligand binding. HoloFepB also appears slightly more compact than apoFepB. Contrarily to class I and II SBP's where domain contacts vary greatly between the unbound and bound forms of the SBP, this is not the case for the class III SBP : contacts between the N- and the C-domains already exist in the apo form, and they are slightly rearranged upon ligand binding. Also the ligand is much more accessible in the class III SBP's than in the class I or II, where it is deeply buried at the domain interface.

- *Bacillus subtilis* FeuA [25, 26] (PDB codes 2WHY, 2WI8, 2XUZ, 2XV1)

In this case, the protein is held on the cytoplasmic membrane via a lipid anchor, as it is the case for the Gram-positive BP's [27]. Upon binding of the natural substrate, bacillibactin, there is a slight closing and rotation movement of the two domains and the substrate adopts a single conformation constrained by the binding site (Fig. 2, I); other substrates (enterobactin and Fe-MECAM are bound by mostly the same residues, with a few exceptions. Bacillibactin

binds with a high affinity to FeuA (K_d ca 27 nM), as well as enterobactin (K_d 40 nM), Fe-MECAM (K_d 1030 nM). Vibriobactin, the tris catecholate siderophore from *Vibrio cholerae* does not bind specifically. In the same study, the two surface exposed residues E90 and E220 were proposed as interacting with the membrane components FeuBC. A double mutant FeuAE90A-E220A, although still able to bind bacillibactin, showed impaired iron acquisition. Both Fe-bacillibactin and Fe-enterobactin promote efficient growth of *B. subtilis*. This is not the case with Fe-MECAM ; this might be due to the difficulty in hydrolysing the MECAM backbone. The binding of MECAM is not the same as for the *C. jejuni* CeuE binding protein (see below).

- *Campylobacter jejuni* CeuE (PDB codes 2CHU, 3ZKW, 5A1J, 5A5D, 5A5V, 5AD1, 5ADV, 5ADW, 5LWH, 5LWQ, 5MBQ, 5MBT, 5MBU, 5OAH, 5OD5, 5TCY)

Sixteen structures of CeuE have appeared, making it particularly interesting [24, 28-30]. There is a very slight closure movement upon substrate binding (3ZKW, apo vs 5ADV two DBS molecules, Fig. S2). This is a particularly interesting case, as most strains of *C. jejuni* do not appear to synthesize their own siderophores and therefore rely on xenosiderophores. Fe-enterobactin is a source of iron for *C. jejuni*. It appears that the trilactone ring of enterobactin can only be hydrolyzed in the periplasm of *C. jejuni*, as there is only one esterase, Cee residing in this compartment [31]. This points to the fact that the substrate transported across the inner membrane might be different from the one transported across the outer membrane (see *Pseudomonas* case below).

The first structure of a hexadentate siderophore bound to CeuE was that of Fe-MECAM. In this particular case, a dimer of Fe-MECAM binds two molecules of CeuE. In this case the Fe^{3+} coordinations are completely fulfilled by the siderophore catecholate groups, two coming from one MECAM molecule, the others from the other molecule. Such an arrangement is actually incompatible with further transport. It happens that Fe-MECAM is poor iron source for *C. jejuni*. Whether or not this is due to this arrangement is not known. No details were given in the purification protocol. Several other crystals were obtained in the presence of tetradentate synthetic siderophores as well as with dihydroxybenzoylserine (DHBS). In all those forms, besides the Arg and Lys residues that contribute to bonds with the catecholate, two residues, His227 and Tyr288 make direct contacts with the Fe^{3+} to fulfill its coordination requirements (Fig. S2). A very interesting observation was made, as it was shown that the K_d of CeuE for Fe-ENT was much higher than for Fe-bisDHBS (400 vs 10 nM). The 10

nM K_d is in the same range as that observed for FepB-Fe-ENT, for example. The double mutant His227Leu-Tyr288Phe has a K_d of ca 500 nM, indicating that the presence of the two residues H227 and Y288 are crucial for the high affinity towards the Fe-bisDHBS.

- *Escherichia coli* FitE [32] (PDB codes 3BE5, 3BE6)

This represents an interesting case : FitE was identified as a putative ferrichrome binding protein from *E. coli* O157 :H7 [33], it behaves as a dimer in solution, which is already unusual for a binding protein. The natural substrate is unknown, but given its very hydrophilic nature and its lining with mostly positive residues, a catecholate derivative is more likely to represent the natural substrate. Its sequence is actually closer to *E. coli* K12 FepB. However, enterobactin (and Fe-citrate) are not substrates of the fit system. Two crystal forms were obtained and for each, the asymmetric unit contained two dimers and the four monomers do not share the same conformation, mimicking the open and closed conformations (Fig. 2, II). This indicates that, in the absence of substrate, the binding protein can undergo quite substantial conformational changes, and that the structure is not as rigid as previously thought.

- *Vibrio cholerae* ViuP [34] (PDB codes 3R5S, 3R5T)

This represents also an interesting case. Vibriobactin is a catecholate siderophore having, besides the three catechols, also two oxazolines, and questions have arisen whether the oxazoline nitrogen could coordinate the iron. The structure of the Fe-vibriobactin-ViuP complex shows that one of the oxazoline nitrogen coordinates the iron atom, leading to the rotation and the exposure of one of the catecholate hydroxyl groups towards the solvent (Fig. S3). The structure of the binding site is quite polar with many residues establishing bonds with the siderophore backbone (Table S2). The structure of the vibriobactin also, with one free catechol hydroxyl group, is also in line with the fact that siderocalin-2 (a component of the innate immunity system of the host, able to bind enterobactin) [35] is not able to bind it with high affinity, most likely due to steric hindrance (K_d of 1.4 μM for Fe-Vib2-, compared to 2.6 nM for Fe-Ent3-). This might contribute to the escape of the innate immunity provided by siderocalin-2. In this case, there is also quite a sizeable movement of one lobe with respect to the other upon vibriobactin binding. ViuP shows low (20%) level of primary sequence conservation, as compared to the other Fe-catecholate binding proteins.

271

272 - *Vibrio cholerae* VctP[36] (3TEF)

273 VctP is a SBP from *V. cholerae* that binds both vibriobactin and enterobactin. Based on
 274 structural alignments and docking studies of both siderophores, the residues potentially
 275 involved in siderophore binding have been identified. The surface potential shows a binding
 276 site surrounded by positive residues (Fig. S4).

277

278

279 IA2 Hydroxamate binding proteins

280 - *Bacillus subtilis* Bsu3320 (FhuD)[37] ferrichrome (PDB code 3G9Q)

281 *B. subtilis* is able to use several hydroxamate siderophores (shizokinen, ferrioxamine B
 282 and ferrichrome) and mutants were isolated, that allowed to identify the genes involved. It
 283 was shown that the cloned gene could complement an *E. coli* mutant defective in FhuD, and
 284 that the protein was a lipoprotein. The binding site is not surrounded by positives charges, as
 285 is the case for the catecholate siderophores (Fig. S5).

286

287 - *Escherichia coli* hydroxamate binding protein FhuD [38, 39] (PDB codes 1EFD, 1ESZ, 1K2V,
 288 1K7S).

289 There is no structure for the apoprotein, so the movements upon siderophore binding
 290 are not known. The siderophore binding site is globally more hydrophobic than for
 291 catecholates and is lined with several Trp/Phe residues. This was the first structure of a
 292 siderophore binding protein. Contrarily to Fe-enterobactin which is negatively charged, Fe-
 293 ferrichrome is not charged, and it has been hypothesized that the negative charges around
 294 the binding site would repel catechol siderophores (Fig. S6). In the case of albomycin (a mixed
 295 ferrichrome-like/uracil analog antibiotic that enters the periplasm via the FhuA receptor [40]),
 296 the antibiotics moiety was not seen in the electron density. As can be seen from the Fig. S6,
 297 although the Fe atom occupies almost exactly the same place (not exactly the same as Ga in
 298 gallichrome), the backbone of the siderophore is not always in the same position, and it is
 299 clear that more space is still available in the binding site.

300

301 - *Staphylococcus aureus* FhuD2[41, 42] (PDB codes 4B8Y, 4FIL, 4FKM, 4FNA)

Four structures have appeared, two in the apo form (one with methylated lysines residues, which shows a slightly more closed form than the non methylated one (green)), one with Fe-Ferrioxamine B (magenta), the other one with Fe-Ferrichrome (cyan) (Fig. S7), the two of them more closed than the apo forms, and the one with Fe-Ferrichrome being the most closed form.

The K_d of FhuD2 for ferrichrome (derived from SPR experiment) is ca. 10 nM, and this interaction is strictly dependent upon the presence of iron. FhuD2 is a potential vaccine candidate. As compared to the *E. coli* FhuD protein, the main residues that establish H-bonds with the siderophore are found on different lobes of the protein, on the N-terminal lobe in *E. coli* (see above), on the C-terminal lobe in *S. aureus*; in the *B. cereus* case (3TNY) residues from both lobes establish bonds with the siderophore backbone (in this case shizokinen). FhuD2 can bind ferrichrome, as well as ferrioxamine B; as can be seen on the Fig. S7, the siderophore backbone does not occupy the same position being closer to the C-terminal lobe in the ferrioxamine case. Two residues E97 and E231 have been identified as potentially important for the interaction with the ABC transporter; FhuD2 with mutations in either E97 and/or E231, still allow efficient ferrichrome transport, but not of ferrioxamine B [43].

- *Streptococcus pneumoniae* PiaA[44-46] (PDB codes 4H59, 4HMO, 4HMP, 4HMQ, 5JJ5)

PiaA recognizes ferrichrome with a higher affinity than *E. coli* FhuD (K_d of 5 vs 100 nM, respectively) [45], and together with PiaB and PiaC imports ferrichrome inside the cell [44]. In the crystal structures, the conformational changes upon substrate binding appear quite modest (Fig. S8). The binding pocket is rather hydrophobic, with few polar residues. The Trp63 residue contributes to the binding pocket, without establishing a H-bond with the siderophore. Its mutation for Ala leads to a 6-fold increase in K_d . As in FhuD2 compared to *E. coli* FhuD, the residues stabilizing the backbone of the siderophore are in the C-terminal lobe of PiaA. Somewhat conflicting results regarding the K_d 's of PiaA (and its mutants) have been obtained depending upon the technique used for the measure, with much higher K_d 's obtained by fluorescence quenching, as opposed to ITC (Isothermal Titration Calorimetry). Two other hydrophobic residues (Trp158 and Phe255) have been mutated to Ala and also affect the binding of Ferrichrome, the Trp158 mutation having a marked effect on the thermal stability of the protein, whether in the absence or presence of the substrate [46].

334 - *Staphylococcus pseudointermedius* FhuD [47] (PDB code 5FLY)

335 This binding protein was crystallized in the presence of PEG400, that sits in the binding
336 site of the protein and makes many contacts with residues of the binding sites, which appears
337 to be somewhat hydrophobic/negatively charged (Fig. S9). The protein is quite stable in its
338 apo form and is even more stabilized by its substrate.

339

340 - *Vibrio cholerae* FhuD (PDB codes 5GGX, 5GGY)

341 The structure of *V. cholerae* FhuD has been solved in the apo and Fe-desferal
342 (ferrioxamine) bound forms (Fig. 2, III). There is virtually no relative movement of the two
343 lobes upon ligand binding. The binding site contains two residues making polar bonds with the
344 siderophore and several hydrophobic residues.

345

346 IA3 Mixed type, polycarboxylate, siderophore binding proteins

347 - *Acinetobacter baumannii*: BauB (PDB code 6MFL)

348 *A. baumannii* is among the « ESKAPE » WHO (*Enterococcus faecium*, *Staphylococcus*
349 *aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*,
350 *Enterobacter* spp. World Health Organization) list, and as such has received deep attention, in
351 particular for its many iron acquisition systems. Three classes of siderophore have been
352 identified in clinical isolates of *A. baumannii*: acinetobactin, fimsbactin, both mixed type
353 siderophores and baumannoferrin, hydroxamate. Acinetobactin [48], synthesized by the vast
354 majority of clinical isolates is the most studied one. Acinetobactin is synthesized (on NRPS) as
355 a preacinetobactin that spontaneously isomerizes to acinetobactin. Both acinetobactin and
356 preacinetobactin bind iron (under the form of Acb2Fe or PreAcb2Fe) and can serve as iron
357 source for the bacterium, via the TBDT BauA. Each siderophore contributes 3 iron ligands (two
358 O from the catechol, one N from the imidazole ring). The periplasmic binding protein is BauB.

359 The binding site of the siderophore is relatively neutral, with positive charges on the
360 C-terminal half (Fig. S10); only one residue (Y301) makes a direct H-bond with the siderophore
361 moiety. Whereas both PreAcb and Acb have similar K_d 's for BauB (380 vs 300 nM), BauB
362 strongly prefers FeAcb2 over FePreAcb2 (160 vs 750 nM). BauB can also bind Fimsbactin (a
363 mixed bis catechol-hydroxamate siderophore), both in its free and Fe bound forms with similar

affinities (360 vs 240 nM). It has also been shown in vivo that Fim inhibits growth promotion of FeAcb2, whereas the converse (Acb inhibition of growth promotion by FeFim) is much less true. Whereas the ABC transporter associated with BauB is likely to be BauCDE, much less is known about the Fim pathway; it is hypothesized that FbsP, a putative periplasmic reductase, encoded in the fim locus might release the iron from the FeFim (to be then taken up by a Feo type transporter). Whether all these observations are relevant in a clinical setting is not known, but they point to a potential role of the BauB protein in the regulation of iron acquisition. By analogy with *Pseudomonas*, it is proposed that Fimsbactin is equivalent to pyoverdine, that never enters the cytoplasm, whereas Acinetobactin is equivalent to pyochelin, being transported to the cytoplasm [49]. This is also a case where the substrate transported across the OM is not the same as across the IM as the one transported across the OM by BauA is a preAcb-AcbFe complex, not having the same configurations of the iron binding sites.

- *Bacillus subtilis* YclQ (PDB code 3GFV) [50]

One of the siderophores transported by YclQ is petrobactin, a mixed bis-catechol hydroxyacid siderophore synthesized by *B. anthracis* for example. The transporter is YclNOPQ. The binding protein presents a binding site surrounded by positive charges (Fig. S11).

- *Thermobifida fusca*, fuscachelin binding protein FscJ [51] (PDB codes 5DH0, 5DH1, 5DH2)

Fuscachelin is a mixed catechol/hydroxamate type of siderophore. Only the apo structure has been solved, on different crystal forms at different pH's, and, as for previous examples there is some conformational variability, with a slight closing of the two domains on each other. The K_d of fuscachelin for FscJ is in the μM range. The binding site is also quite hydrophobic and mostly surrounded by a rather negatively charged surface (Fig. S12).

- *Staphylococcus aureus* HtsA [52, 53] (PDB codes 3EIW, 3EIX, 3LHS, 3LI2)

S. aureus synthesizes two siderophores, named staphyloferrin A and B, which are of the polycarboxylate type. They are respectively synthesized by the *sfa* and *sfn* loci, encoding NIS synthases (NRPS Independent Siderophore synthases). A double *sfa sfn* mutant is no longer able to grow in serum [52]. Contiguous to the *sfa* locus is the *htsABC* locus that encodes the HtsA substrate-binding protein, and HtsB and HtsC two membrane subunits of an ABC

transporter. No ABC subunit is encoded in this locus. The ABC subunit, common to several siderophore ABC transporters is FhuC, encoded in the *fhuCBG* operon. HtsA was initially supposed to be the binding protein for heme, as a *htsA* mutant is slightly affected in growth on heme as an iron source. It was later conclusively shown that HtsA is in fact the staphyloferrin A binding protein and not a heme-binding protein. The structure of HtsA, without or with Fe-staphyloferrin A has been determined (Fig. S13). Two forms have been obtained in the presence of the siderophore, one closely resembling the apo form, the other one slightly more closed on the substrate. The movements between the two forms are minor, and the highly polar nature of the binding site and the absence of a potential heme iron binding residue are not in favour of heme binding ability. HtsA has a very high affinity for Staphyloferrin A (subnanomolar Kd) and does not bind Staphyloferrin B, although it is a hydroxy-carboxylate type siderophore.

- *Staphylococcus aureus* SirA (PDB codes 3MWF, 3MWG)[54]

Like HtsA for staphyloferrin A, SirA is the specific lipoprotein for staphyloferrin B. Like for HtsA, there is little difference between the free and the siderophore loaded forms (Fig. S14). SirA binds Fe-staphyloferrin B with a high affinity (subnanomolar range Kd), and high specificity. This is opposite for example to the FhuD case of *E. coli* that binds several hydroxamate type siderophores. Although the overall structure of SirA is highly similar to HtsA (top hit in DALI server), the residues involved in the siderophore binding and the position of the siderophore in its binding pocket are not the same for both proteins, explaining their high specificity.

IA4 Heme binding proteins[55-59] (supplementary tables S3 and S4)

- *Pseudomonas aeruginosa* and *Shigella dysenteriae* PhuT/ShuT (PDB codes 2R79, 2RG7, 2RTA)

The first structures of two periplasmic heme-binding proteins were solved in 2007, in the group of T. Poulos, namely PhuT from *Pseudomonas aeruginosa* and ShuT from *Shigella dysenteriae* (Fig. S15) [55]. They show the typical class III SBP fold, with one heme molecule bound inbetween the two lobes of the protein. The heme molecule has opposite orientations in the two structures, with the propionates respectively pointing inward and outward in the PhuT and ShuT structures. Otherwise, a single Tyr residue coordinates the iron atom leading

to a pentacoordinated heme iron. In the case of the ShuT protein, which also crystallized in the apo form, there is a small closing movement upon ligand binding. In both cases, the binding site is lined with a few apolar/hydrophobic residues, and the heme is quite accessible from the medium.

- *Yersinia pestis* HmuT (PDB codes 3MD9, 3NU1)

HmuT was the first heme-SBP to be crystallized with two heme molecules in the binding pocket [56]. The two molecules are in an anti-parallel arrangement, with the propionates of one heme facing outward, whereas those from the other facing inwards (Fig. S16). One heme iron is coordinated via a Tyr70 residue, whereas the other one is coordinated by a His167 residue. ITC titrations ruled out this resulting from a potential crystallization artifact. Two successive binding sites were putatively identified with K_d 's of respectively 0.3 and 30 nM, the second K_d , being also quite low. This was also confirmed by UV-visible absorption spectroscopy, since the second heme does not absorb (Soret band) at the same wavelength as the first (375 vs 404 nm). Whether this is physiologically relevant will be discussed in the transport section.

- *Corynebacterium glutamicum* HmuT [57] (PDB codes 5AZ3, 5B4Z, 5B50, 5B51)

The WT HmuT protein from *C. glutamicum* was crystallized in the presence of heme as well as three mutants, two of the axial ligands (His141 and Tyr240), and one of the Arg242 establishing an H-bond with Tyr240, to stabilize its tyrosinate form, able to bind the iron atom. The WT protein presents the classical class III SBP fold, with heme at the junction of the two lobes. The binding site is lined with non polar residues, and His141 and Tyr240 constitute the heme iron axial ligands. The heme adopts two preferential orientations (rotation around the two heme iron axial ligands) positioning the propionates in two distinct positions, besides also rotation along the alpha-gamma meso axis. In the WT protein, the heme cannot be reduced by dithionite. There are only marginal conformation changes upon the mutations, and the heme is held in the same position (Fig. S17). However, there is only one orientation of the heme in the His140Ala and Arg242Ala mutants. There were unfortunately no measure of the affinities of the various HmuT mutants for heme, precluding further in vivo characterization.

- *Burkholderia cenocepacia* BhuT[58] (PDB codes 5GIZ, 5Y89, 5Y8A) *Roseiflexus* sp. RhuT (PDB codes 5GJ3, 5Y8B)

BhuT represents a very interesting case, where, as for *Y. pestis* HmuT, two heme molecules can bind to a BhuT monomer and where an in-depth characterization of the binding was made. The first heme binds to BhuT with a K_d of 18 nM, mostly driven by entropic gain, whereas the second heme binds with a lower K_d (360 nM), and driven by enthalpic gain. The two species can also be distinguished by their UV-visible (as well as resonance Raman) spectral properties. The crystal structures show that heme 1 iron is bound to Tyr87 residue (in interaction with Arg89 to stabilize the tyrosinate), and that upon binding of the second heme, the first one reorients along the Fe-Tyr87 axis so that its propionates are now pointing outwards. The propionates of the second heme point towards the binding cleft. In this form, the second heme iron is not coordinated to a protein residue, the second heme molecule is parallel to the first one. The propionates of the second molecule are in interaction with several residues of the C-lobe. The structure also shows that both apo and two-heme bound forms are in a more open conformation than the one-heme bound form (Fig. 3). This is evident when one measures the distance between the $C\alpha$ of Glu96 and Glu231 that point towards the TMD extracellular loops. Each protein crystallized with two monomers per asymmetric unit and distances vary from 45.7-47.4 Å (apo form) to 40.2-40.7 Å (holo1) and 46.8-46.5 Å (holo2). This will be discussed further.

During the same work the authors also explored another heme-SBP from *Roseiflexus* sp. which is also able to bind two hemes per monomer. ITC graphs yielded complex patterns that were difficult to fit using a simple two-site model, but contrarily to the BhuT case, the first binding step was clearly driven by an enthalpic gain, indicating very diverse modes of binding. As compared to the apo form, the holo2 form was more open ; the two hemes are in the same orientation with the propionates pointing towards the solvent. Both heme irons are liganded by Tyr residues that are stabilized in the tyrosinate form by Arg residues and a Gln residue for heme 1. The propionates of the first heme are also in interaction with an Arg residue.

The HutB protein from *V. cholerae* was also crystallized in the apo form [60] (PDB code 5KHL) and shows a quite wide cleft putatively able to harbour two heme molecules. It was actually shown by UV-visible heme titration that Vc HutB could fix successively two heme

molecules and potential heme iron ligands were identified by comparing the structure of *Y. pestis* HmuT with that of *V. cholerae* (Fig. S18). The residues Tyr65 and His164 are the putative heme iron ligands. A mutant Tyr65Ala showed altered UV-visible absorption spectrum as compared to the WT. Molecular dynamics studies also showed that HutB could explore a large range of twisting motions of one lobe versus the other, with correspondingly very high b-factors.

IA5 Unidentified substrate

- *Mycobacterium tuberculosis* Mtb RV0265c (also called FecB2, structure 4PM4)[61, 62]

The substrate is unknown ; it is possible it to be heme, as Rv0265c has been shown to bind heme, although with rather low affinity (detected by SPR on immobilized his-tagged protein), and to be involved in heme acquisition. In this respect there is a conspicuous Tyr residue that could potentially bind the heme iron atom (Tyr39). Rv0265c is most likely a lipoprotein anchored in the inner leaflet of the mycomembrane (Fig. S19).

- *Saccharomonospora viridis* (PDB code 5CR9)

The substrate is unknown and there is one Mn atom in the binding site.

- *Yersinia pestis* YiuA[63] (PDB codes 6B2X, 6B2Y)

This putative SBP has been crystallized in its apo form, in two crystal forms that show some variations, one being slightly more « open » than the other. The substrate has not been identified, although from the surface potential it likely appears to be a catecholate, or a polycarboxylate, given its highly positive charge near the binding site (Fig. S20).

IB Class II iron/heme binding proteins

IB1 Ferric ion binding proteins [64-66]

These proteins (named FbpA) were amongst the first SBP's identified, in *Haemophilus influenzae* or *Neisseria gonorrhoeae* (see for [66] a review); the initial substrate recognized by the OM TBDT is transferrin or lactoferrin[67]. They belong to class II SBP's. The iron binding site resembles that of transferrin [64]; however there is at one point a change in the co-ion, from carbonate to phosphate or sulphate. As for the siderophore SBP's there is some

movement of the two domains relative to each other, in the iron bound forms (3ODB/3OD7, or mutants vs WT, Fig. S21). Likewise the apo form is in a more open conformation. Several mutants of the iron binding site have been made without affecting too much in some cases, the affinity of the mutant for iron. For example Tyr195Ala and Tyr196Ala mutants, have barely one log difference of Fe³⁺ binding, as measured by citrate competition (logK_a from 19,62 to 19,2) [65]. Those mutants are inactive in *H. influenzae*, yet they are active in a reconstituted system in *E. coli*. The reason why they are inactive in *H. influenzae* is far from being clear.

There are also two other types of ferric ion binding proteins, that can be distinguished on the basis of the Fe³⁺ coordination in the binding site. A first type is exemplified by *Mannheimia hemolytica* FbpA, that displays two Fe³⁺ binding modes, one without a synergistic anion, in an open conformation of the two lobes, the other with a synergistic anion, in the closed conformation, different from the one found in the case of *H. influenzae* : the synergistic anion is carbonate, providing two coordinations to the Fe³⁺ and three Tyr residues (structures 1Q35, 1SI1 and 1SI0 in the PDB). A second variation is found in the YfuA protein from *Y. enterocolitica* or SfuA from *Serratia marcescens* (structures 1XVY and 1XVX in the PDB) in which there is no longer a synergistic anion (replaced by an Asp residue), but all the other coordinating residues are the same as in *H. influenzae* FbpA (two Tyr oxygen, one Glu oxygen, one water molecule oxygen, and one His nitrogen) [68, 69].

IB2 Heme-binding proteins

- *Escherichia coli*

In *E. coli* K12 that has no heme outer membrane TBDT, extracellular heme can still reach the cytoplasm and be used as an iron source, provided the expression of a functional OM heme TBDT. Two SBP's were shown to bind heme respectively DppA and MppA (the binding proteins for dipeptides and tripeptides derived from murein respectively)[70] and donate heme to the Dpp ABC transporter, showing the promiscuity of these SBP's. Heme is, roughly speaking, the condensation product of 8 molecules of delta-amino-levulinic acid, an

amino-acid that is also transported by the Dpp ABC transporter in *E. coli* [71]. This might explain the promiscuity of peptides binding protein for this compound.

-Haemophilus influenzae

Haemophilus influenzae (Hi), is a strict heme auxotroph with a reduced genome. The RdKW20 strain (an avirulent d serotype strain, devoid of capsule) was the first completely sequenced bacterial genome and has been used in many studies. It expresses many different heme/hemoprotein receptors, and there are several reports of periplasmic heme-binding proteins[59, 72]. Whereas several Pasteurellaceae species do contain class III heme SBP's, this is not the case for *H. influenzae*. Instead, four SBP's with other functions have been reported as potential heme SBP's ; they are all belonging to class II SBP's. HbpA (heme binding protein A, also named HI0853 in RdKW20) was the first periplasmic heme binding protein reported in this organism[73, 74]. It was identified in the Hib strain DL42 (a virulent b serotype strain) as a lipoprotein, most likely anchored in the outer leaflet of the cytoplasmic membrane. Its main function might be to transport glutathione[59]. The gene encoding HI0853 was not identified to be induced under heme/iron starvation[75]. Three other SBP's were also identified as heme binding proteins : HI0213 (also described as OppA, and subject in some strains to heme/iron starvation regulation, also a lipoprotein, most likely anchored to the outer leaflet of the cytoplasmic membrane)[76], HI1124 (identified as OppA) and finally SapA[77] (HI1638) that also binds cationic peptides. In this last case, the authors have assigned an essential role to SapA in heme transport, based on the observation, that, upon prolonged heme-iron starvation, a $\Delta sapA$ mutant is unable to grow on heme, whereas the WT and the complemented strains are able to grow. Yet it appears that this mutant has been isolated on sBHI, under aerobic conditions, where heme is strictly necessary. It is well possible that, given the apparent redundancy of the heme binding proteins in Hi, this effect might not be direct. Furthermore, as in the *E. coli* case, where peptides compete with heme for binding to DppA or MppA, there is also a competition between heme and cationic peptides for binding to SapA. The binding affinity of heme for these various proteins has been estimated between 50 and 500 μ M. HbpA has also been shown to have a role in heme acquisition in Hi[78]. On the contrary, SapF (HI1642) appears essential for growth on sBHI, and might be part of the conduit through which porphyrins enter the cells.

Although many structures of periplasmic di-, tri-, oligo-peptide binding proteins have appeared, there is not yet a structure of such a protein cocrystallized in the presence of heme. Many peptides bind to OppA, and the binding occurs through the backbone of the peptide. However, model building has shown that it is possible to accommodate a heme molecule in these binding sites[72]. There is no structure of apoOppA^{Hi}. However, *E. coli* OppA is 50,4% identical to *H. influenzae* OppA and has been crystallized in both apo and peptide bound form. This allows to see the large movement of one part of the molecule with respect to the other (Fig. S22). Further studies also showed that both halves of the molecule have specific role regarding either heme or peptide binding, the first domain (residues 21-290+ 510-541) having preference for heme over peptides (K_d's of 0.6 vs 12 μ M respectively) whereas the second domain (residues 291-509) having a preference for peptides over heme (K_d's of 0.15 vs 2.5 μ M respectively).

II Substrate binding proteins: in vivo aspects

A special mention should be made for cases where the substrate transported across the inner membrane is not the same as the one transported across the outer membrane. This is relevant for several bacteria, *C. jejuni*, *P. aeruginosa* and *E. coli* [31, 79, 80]. In those three cases either Fe-enterobactin or Fe-salmochelin are taken up across the OM, and degraded by periplasmic esterases (Cee for *C. jejuni*, PfeE for *P. aeruginosa*, and IroE for *E. coli*), before import of a Fe containing compound in the cytoplasm. In the *Pseudomonas* case, the import system once enterochelin has been degraded by the periplasmic esterase is not identified ; in the *E. coli* case, it is very likely to be the Fep ABC transporter, and eventually the IroC cytoplasmic membrane protein ; whereas there is no apparent enterobactin esterase in *Pseudomonas* and *Campylobacter*, this is not the case in *E. coli*, where Fes is the cytoplasmic esterase, that degrades enterobactin. In *Campylobacter* that does not synthesize any siderophore and yet can use Fe-enterobactin as an iron source, the import system across the inner membrane relies on the CeuE SBP (see above), which has better affinity for linear siderophores than enterobactin. This would provide a competitive advantage to *Campylobacter* over other bacterial species.

Pyoverdine (PVDI) is one of the two siderophores synthesized by *P. aeruginosa* (the other being pyochelin), a mixed siderophore coupled to a fluorescent moiety. Fe-PVDI is transported across the OM by FpvA, a TBDT receptor. The *fpv* locus contains an operon of four genes *fpvCDEF*, FpvE being an ABC-ATPase, FpvD a TMD, and FpvC and FpvF being two SBP's with different specificities [81, 82]. FpvC belongs to the PsaA/TroA family of SBP's (metal binding) whereas FpvF belongs to the siderophore family of SBP's. In solution, FpvC is a monomer that has no affinity for PVDI and low affinity for Fe-PVDI. On the other hand, FpvF is able to form dimers, that are the only form able to bind either PVDI or Fe-PVDI. Furthermore FpvC-FpvF₂ and FpvC₂-FpvF₂ oligomers have been observed both *in vitro* and *in vivo*, that are able to bind both PVDI and Fe-PVDI [81]. A deletion of the *fpvCDEF* operon leads to accumulation of Fe-PVDI in the periplasm, indicating that this ABC transporter is required for Fe-PVDI derived iron import in the cytoplasm. It was also shown that PVDI-Fe dissociates in the periplasm, most likely by a reduction step, allowing the recycling of PVDI in the extracellular medium [83]. It is therefore possible that FpvC would bind Fe²⁺.

In the *pvd* locus and just upstream of the *fpvCDEF* operon, is found another operon of four genes *fpvGHIK* [84]. It was found that deletion of *fpvG* or *fpvH* or *fpvI* but not *fpvK* had an effect on iron uptake across the inner membrane and that the strongest effect was observed with *fpvG*. *In vitro* Fe-PVDI can be dissociated upon reduction by DTT and this effect is enhanced upon addition of ferrozine, an Fe²⁺ chelator. Like ferrozine, FpvC, the metal-binding SBP encoded in the nearby operon, can enhance the dissociation of Fe-PVDI by DTT. FpvG, FpvH, FpvI and FpvK are predicted to be cytoplasmic membrane proteins with large periplasmic domains. It was also shown that *E. coli* membrane vesicles isolated from a FpvG producing strain could reduce Fe-PVDI. This strongly suggests that FpvG is the reductase that binds Fe-PVDI and dissociates it. It was however difficult to recapitulate the whole process as addition of FpvC and FpvF slowed down the process [84]. All this work highlights the potentially more complex role of siderophore SBP's in the context of iron release in the periplasm. In PAO1 genome, there are three homologs of *fpvG* all three being contiguous to genes coding for OM siderophore receptors (PA1909, PA2465, and PA4513).

Having presented the first player of the siderophore/heme ABC transporter, remains to present the membrane components and the ABC-ATPase, for which much less functional, structural and biochemical data are known. Before doing so, it is however worth mentioning

the YbtP-YbtQ case from *Y. pestis* which is involved in iron acquisition [85] by an unknown mechanism. Both YbtP and YbtQ represent fused TMD-ABC modules, and are closer to exporters than to importers, yet are required for iron entry in *Y. pestis* and for virulence in a mice model. It is not known at present whether a SBP is associated with this system. It is not known either whether those gene products are directly or indirectly involved in iron transport.

III Heme/iron siderophore ABC transporters cycle

I will first briefly present an outline of the mechanism of action for type I and type II ABC importers, exemplified by the *E. coli* maltose and *Archaeoglobus fulgidus* or *H. influenzae* molybdenum transporters (respectively MalEFGK₂ and ModAB₂C₂) for type I and the B12 vitamin transporter from *E. coli* BtuFC₂D₂ for type II. One archetype of the SBP-ABC transporter of type I is the Maltose transporter MalEFGK₂ (MalE is the class II SBP, with micromolar affinity for maltose, the two membrane proteins MalF and MalG, and MalK the ABC ATPase) on which a lot of structural, functional, genetic and biochemical data have been accumulated over the years by several groups. In the maltose system, in the resting state of the system (with very low basal ATPase activity) MalFG is open on the cytoplasmic side, and binding on the one hand of Maltose-MalE on the periplasmic side and ATP on the MalK subunits trigger conformational changes leading to opening of MalFG towards the periplasmic space and to the transfer of maltose from its MalE site to a binding site exposed on MalFG, with a mM affinity. Transfer of maltose is possible due to the relative opening of the two lobes of MalE, and to the presence of the so-called scoop loop of MalG that comes in steric clash with maltose in its binding site on MalE. Following further rearrangements that position the two MalK monomers together with two ATP molecules in a correct orientation so that the ATP hydrolysis can take place. A high energy ADP-bound state is generated with the MalFG complex open to the cytoplasm (leading to release of Maltose in the cytoplasm), and MalE still bound to MalFG. Release of ADP and MalE restores the resting state (see [15] for a review).

The situation is somewhat different in the BtuCDF B12 importer (BtuF is the class III SBP, BtuC is the membrane domain, and BtuD the ABC ATPase), where in the resting state, BtuCD is open towards the periplasm, and two « gates » have been identified on the cytoplasmic side. Somewhat conflicting results have been obtained on the association of BtuF

to BtuCD and its modulation by ATP or a non-hydrolyzable analog like AMP-PNP and the B12 vitamin substrate [86-88], whereby ATP or B12 have been found to have a destabilizing effect and ATP has also been shown as accelerating the association of B12-BtuF to BtuCD. These apparent discrepancies might result from the various conformations of the « resting BtuCD » complex and its ability to bind B12 vitamin. The BtuF-BtuCD interaction is modified by ATP [89], and as in the Maltose transport system, there is a transfer from the B12 vitamin from its binding site on BtuF into a cavity formed by BtuC (subsequently closed on the periplasmic side); ATP hydrolysis leads to opening of the cytoplasmic gates and release of the substrate in the cytoplasm[14]. There is apparently no specific binding of the B12 molecule to the BtuC cavity. Whereas there is apparently a tight coupling of ATP hydrolysis with transport in type I importers, this appears to be less the case in the Btu system where futile cycles of ATP hydrolysis occur.

Although iron acquisition is a important topic for bacterial physiology, there is a paucity of data on the functional, biochemical and structural characterization of the ABC importers of either siderophores or heme. Some metal ABC importers have been biochemically and structurally characterized, but no purification and reconstitution of iron/siderophore membrane transport system has been carried out at the present time. Several studies have however appeared on a few heme transport systems that will now be described.

-Shigella dysenteriae ShuTUV

The first one concerned the ShuTUV system from *Shigella dysenteriae* that was reconstituted in artificial membrane vesicles[90], with the SBP ShuT trapped inside the vesicle lumen, and the presence of an heme trap, ShuS in the medium. The extent of heme binding to apo ShuS was measured, as well as the ATP hydrolysis. Two types of mutants were also used, one with mutations in the two conserved glutamate residues on ShuT that serve to anchor ShuT to ShuU, and also on two histidine residues on ShuU, that were tentatively assigned a heme binding function. All mutants and the WT apo ShuT displayed a basal level of ATPase activity and no heme accumulation on ShuS, whereas the holo-ShuT led to an increase in ATPase activity, coupled to a transfer from the heme from ShuT to ShuS. Although this study

provided an interesting insight into the functioning of a type II heme importer, no further studies have appeared, to precisely measure stoichiometries, coupling parameters, and relevant kinetics.

-Yersinia pestis HmuTUV

A second study on the HmuTUV heme acquisition system from *Y. pestis* did use a slightly different way to measure heme uptake across the vesicle membrane, with the binding protein on the extracellular side of the vesicle and, trapped inside the vesicle an ATP regenerating system. Heme was measured based on its peroxidase activity, after binding to apo-HRP [91]. As in the previous case, the transport of heme was strictly dependent upon the ATP hydrolysis, and of the correct association of the SBP with the transporter. Transport rates of ca. 0.14 molecule of heme transported per min and per HmuUV transporter, were measured, comparable to those observed previously. If these rates truly represent in vivo activity they are clearly not as efficient as the maltose transporter (ca 20 maltose molecules transported by transporter by minute), which they don't need to be. The same HmuT mutant was used, unable to dock to HmuUV, and a Walker-B mutant in HmuU (E173Q) also inhibited transport. The HmuUV structure was solved by X-ray crystallography and showed the typical arrangement of type II ABC importers. There is a two fold symmetry axis, perpendicular to the membrane plane. Each HmuU subunit presents 10 TM segments, the coupling helix between TM 6 and 7 establishes contacts with the ABC subunits. At the interface of the HmuU monomers there is a cavity open to the extracellular side and delimited by helices 5, 5a and 10 of both monomers (Fig. 4). This cavity is rather apolar and large enough to accommodate a heme molecule. Neither HmuUV or HmuTUV purified in detergent displays a detectable heme affinity. Furthermore, the two histidines residues identified in the work on ShuTUV are clearly facing the cytoplasm, and given their position on the structure, their potential role in heme binding is questionable ; they could be in contact with the ABC subunits at some point of the reaction cycle. Two residues Arg176, and Gly164 completely conserved in the HmuU family, were mutated for Ala. Arg176 is exposed on the periplasmic side, at the entrance of the putative heme binding cavity and Gly 164 is lower in the cavity, at a narrowing of the cavity. A strong effect was observed with the R176A mutant, that displayed the same basal ATPase activity as the WT, but almost no stimulation by HmuT ; this correlated with a weak binding of HmuT to HmuUV. The Gly164Tyr mutant showed increased basal ATPase activity, with very

little stimulation by HmuT, and a very reduced transport activity, indicating uncoupling of the ATPase from the transport activities. Although rather unlikely, it would have been interesting to test whether this mutation might have provided a binding site for heme in the cavity.

Another study using surface plasmon resonance with immobilized HmuUV and HmuT in the running buffer showed that HmuT binds with nanomolar affinity to HmuUV, and that both ATP and an excess of heme lead to the dissociation of the HmuT-HmuUV complex [92], strengthening the similarities between the heme and B12 transporters.

Contrarily to MalFGK2 or Sav1866, that are outward facing in the presence of ATP, and inward facing in absence of nucleotide, the opposite orientation is found in the case of BtuCD or HmuUV, consistent with their classification as type II importers, where the complex is outward facing in the absence of ATP. There are at least two other characteristics of this type II ABC importers that are worth mentioning : one which is related to the number of molecules that are transported that are orders of magnitude lower in the case of either siderophore/heme or B12 vitamin as compared to a carbon source as maltose; an *E. coli* cell contains ca. a few hundred thousand iron atoms that it must acquire during a cell division time. Unfortunately, the number of transporters per cell is not known for all those systems; however the observed in vitro rate of ATPase hydrolysis is ca. 10 times higher in the case of the maltose transporter as compared to the Hmu transporter. Whether this is related to the catalytic activity of those various transporters remains to be determined. The other characteristic concerns the amplitude of the movements of the binding protein in the bound and free states in the two systems where it is much smaller in the siderophore/heme case, as compared to the maltose case. Finally, there is also a difference concerning the ligand accessibility in the SBP's of the two classes, whereby class III SBP's have their substrate at least partially exposed to the outside, which is not the case in class I or II SBP's.

-Burkholderia cenocepacia BhuUV

Another heme importer BhuUV from *Burkholderia cenocepacia* has been crystallized, in an ATP-free form, and also in the presence of the heme-SBP BhuT, in the absence of heme [58, 93]. There are several differences as compared to the HmuUV structure of *Y. pestis*. First of all, the BhuUV structure is open on the cytoplasmic side, contrarily to the HmuUV case (Fig. S23). As in the HmuUV case, there is a dimerization of BhuU along TM 5 and 10 of each BhuU

monomer. The channel that extends throughout the BhuU dimer interface is blocked on the periplasmic side by the two helices 5a, that form the periplasmic gate mentioned above. On the contrary, the channel is open on the cytoplasmic side, as it can be clearly seen on either Figure. As in the HmuUV case, the cavity is largely apolar with the exception of a conserved aspartate residue (Asp 112 in BhuU, Asp86 in HmuU), that, given its position could fulfil a specific function. Actually mutants of this residue (D112A or D112V) still display basal ATPase activity, similar to the WT protein, but are no longer able to transport heme. In the presence of the BhuT SBP, the ABC domains are slightly more close to each other.

When the BhuTUV complex is formed from holo-BhuT and ATP free BhuUV, the complex is no longer coloured, and heme is not found on the complex. This is understandable, as the periplasmic gating loops from both BhuU, insert into the heme binding pocket and come into steric clash with the heme, if it were to stay on its binding site. Finally, upon BhuT fixation the heme binding pocket resembles more the open apo form than the closed form (Fig. 5). From a physiological point of view, such a situation, whereby heme would be likely ejected into the periplasmic space, upon BhuT-heme fixation on BhuUV, in the absence of ATP is highly unlikely. Two Glu residues from BhuT (E94, E231) make ion bridge with residues Arg84 from the two BhuU subunits.

In detergent solution, BhuUV has a basal ATPase activity, similar to the one observed in the HmuUV case, that is stimulated upon binding of the BhuT. Both ATP and AMP-PNP, but not ADP binding to the BhuTUV complex lead to the dissociation of BhuT from BhuUV.

The comparison of the BhuU and HmuU structures allows to see two potential states of the transporter in the inward-open and outward open states, in the absence of ATP (Fig. S24). In the absence of a defined heme binding site inside the cavity defined by the HmuU-BhuU dimer, it is extremely difficult to envision the heme transfer from its binding site on the SBP to the cavity, even though the heme binding pocket on the SBP is distorted upon interaction with the TMD subunit, lowering the affinity for heme. It is well possible that even though there might be no « true » binding site for heme inside the cavity, its rather apolar environment might drive heme inside. It is unlikely that, at one step of the transport cycle, the periplasmic loop of BhuU/HmuU joining TM's 5 and 5a might extend deep enough into the BhuT binding pocket. This could be feasible only if helix 5a might unwind and insert inside the binding pocket to act as a scoop loop.

-*Sinorhizobium meliloti* HmuUV

Finally, there is also promiscuity among heme ABC transporters: in *Sinorhizobium meliloti*, the heme transporter HmuUV is also able to work with two distinct binding proteins, HmuT for heme transport and FhuP for ferrichrome/ferrioxamine transport[94]. This is reminiscent of the Dpp transporter that can operate with two distinct binding proteins in the case of heme transport, DppA and MppA[70].

Concluding remarks

The past years have seen a wealth of structural data accumulating either on the periplasmic/extracellular siderophore/heme binding proteins, that have essentially clarified the binding specificity of those proteins, but are far from explaining the determinants for selectivity for the binding to the membrane components. There is also an urgent need to get quantitative data on the actual number of the two components of those systems (binding proteins and TMD-ABC's), in living cells under various iron stress conditions. As the reconstitution of the heme importers HmuTUV has been made, one might expect that more biochemical and biophysical data dissecting the different steps of association of the components in terms of affinity and kinetics, with regard to the nucleotide state of the ABC component should bring important data to understand these specific mechanisms. One may regret that the spectroscopic properties of heme have not been more exploited to assess its environment during the transport cycle, and the absence of a structure of a type II importer with the substrate in the transporter is a serious drawback to further characterization of this process.

Acknowledgements

I thank Elie Dassa for helpful advice and discussion during the preparation of this manuscript and the anonymous reviewer for wise comments. This work has been supported by LABEX DYNAMO (ANR-11-LABEX-0011-01).

Conflict of interest

837 None.

838

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Figure legends

Figure 1: general organization of the TonB-dependent transporters (TBDT, orange), in the outer membrane together with potential helper proteins, the TonB complex, and the siderophore/heme/iron ABC transporters in the periplasm/cytoplasmic membrane, made up of the periplasmic binding protein (SBP, bright green) and the ABC complex (TMD in two shades of magenta, ABC modules in two shades of cyan). The peptidoglycane mesh (PG) is represented as a discontinuous line in the periplasm. The various iron/heme sources are represented (Tf: transferrin, Hb: hemoglobin, Hpx: hemopexin, Hb-Hp: hemoglobin-haptoglobin, He: heme). Uptake of the iron complex (red circle) across the outer membrane (OM) is powered by the pmf, and by ATP hydrolysis across the inner membrane (IM).

Figure 2 : Structures of *B. subtilis* FeuA (I), FitE from *E. coli*O157 :H7 (II) of *V. cholerae* FhuD (III). I, top: surface potential of FeuA showing the highly positive region around bacillibactin (magenta, iron atom as an orange sphere). I, Top bottom : structure of apo (yellow, 2WHY) and Fe-bacillibactin-FeuA complex (cyan, 2WI8), showing the closing of the two lobes upon substrate binding. II, top: surface potential showing the FitE binding site surrounded by positive charges. II, bottom: Structure of two monomers of FitE, in the absence of substrate (chain C of 3BE5, closed form, red, chain B of 3BE5, open form yellow). III, top: surface potential showing the FhuD binding site with Fe-Desferal. III, bottom: superposition of apo (cyan) and Fe-Desferal-FhuD structures in ribbon representation.

Figure 3: Structures of BhuT from *B. cenocepacia* (I) and RhuT from *Roseiflexus sp.* (II). I, top : superposition of the structures of apo-BhuT (cyan), heme-BhuT (green) and bis-heme-BhuT (magenta). I, middle and bottom: surface potential of heme-BhuT and bis-heme-BhuT with heme in the binding pocket. II, top: structures of apo-RhuT (green) and bis-heme RhuT (cyan). II, bottom: surface potential of RhuT showing the two hemes in the binding site.

Figure 4 : Structure of HmuUV from *Y. pestis*. Top left : surface potential of HmuU, from the cytoplasmic side : the two negatively charged coupling helices are clearly seen. Top right : surface potential of HmuU, from the periplasmic side, with the cavity open on the periplasm. Bottom left : Ribbon structure of HmuUV, with the two transmembrane HmuU (blue and pale

blue) and the two ABC HmuV modules (tan and orange). Bottom right : same as bottom left, rotated 90° along the vertical symmetry axis, with one HmuU in thin ribbon and the other as surface with conserved residues across the family coloured labeled (magenta : Pro, yellow : Gly, green : Trp, Phe, deep green : Arg, red : Leu, Ala).

Figure 5 : Structure of BhuTUV from *B. cenocepacia*. Top left : close up on the BhuT-BhuU interface. BhuT is shown as a cyan surface, the left monomer of BhuU is shown as a deep blue surface, the right BhuU monomer as pale blue helices. Heme is represented in stick, clashing with the 5-5a helices. Top right : same as left, in cartoon representation with apo-BhuT (yellow) and heme-BhuT (magenta) superimposed to BhuT from BhuTUV (cyan). Bottom left : surface potential of BhuU dimer from BhuTUV complex, from the periplasmic side, showing the closed cavity, and the potential localisation of heme. Bottom right : surface potential of BhuT, « viewed from BhuU », with the heme placed also in its binding site.

Figure 1

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1198 Supplementary figures

1199 Figure S1 : Structures of *E. coli* FepB. Green : X-ray structure (3TLK), in complex with Fe-Ent
 1200 (magenta, iron atom as a brown sphere, only one monomer is shown). Blue : Fe-Ent complex
 1201 (2M6L), one NMR structure, yellow : apo, one NMR structure (2M6K). The orange and pale blue
 1202 regions correspond to the ones where CSP is observed upon GaEnt binding.

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1205 Figure S2 : Structures of *C. jejuni* CeuE. Left : ribbon representation of apo-CeuE (orange,
 1206 3ZKW) and holo-CeuE (Fe-DHBS2-complex, 5ADV, magenta); some of the green residues are
 1207 completing iron coordination sphere. Right : CeuE surface potential. The iron atom is
 1208 reresented as a blue-violet sphere. Bottom : structure of the Fe-MECAM-CeuE complex
 1209 (2CHU), showing the two molecules of CeuE (blue ribbon) with their binding sites facing each
 1210 other, and the two Fe-MECAM molecules (green and magenta, with the iron atoms as
 1211 orange spheres).
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Figure S3 : Structure of *V. cholerae* with and without vibriobactin (3R5S, and 3R5T respectively). Left : surface potential of ViuP complexed to vibriobactin (cyan, iron atom orange sphere). Right : comparison of the structures of apo (green) and Fe-vibriobactin ViuP (cyan).

Figure S4 : Surface potential of VctP from *V. cholerae*, in the absence of siderophore)

Figure S5 : surface potential of FhuD from *B. subtilis*

Figure S6 : surface potential of FhuD from *E.coli* with the different siderophores in the binding site (yellow : gallichrome; cyan : desferal; green : albomycin; magenta : coprogen).

Figure S7 : Structure of FhuD2 from *S. aureus*. Left : superimposition of apo-FhuD2 (green), of Fe-ferrioxamine B-FhuD2 (magenta) and Fe-ferrichrome-FhuD2 (cyan). Right : surface potential of FhuD2 with Fe-ferrichrome in the binding site.

Figure S8 : Structure of *S. pneumoniae* PiaA. Left : surface potential of PiaA with ferrioxamine E in the binding site. Right : PiaA apo (green), in complex with Fe-ferrichrome (cyan), and with Fe-ferrioxamine E (magenta).

Figure S9 : Structure of *S. pseudointermedius* FhuD : surface potential ; the binding site is occupied by a PEG400 molecule.

Figure S10 : Structure of *A. baumannii* BauB. Surface potential of the protein with Fe-Acb2 in the binding site.

Figure S11 : Structure of *B. subtilis* YclQ. Surface potential of apo YclQ

1259
1260 Figure S12 : Structure of fuscachelin binding protein FscJ from *T. fusca*. Surface potential of
1261 the protein, also showing the binding site.

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1265 Figure S13 : Structure of *S. aureus* HtsA. Top left : structures of HtsA complexed to Fe-
1266 Staphyloferrin A, in the open (green) or closed form (blue). The siderophore is represented
1267 in the binding site with the iron atom as a sphere, green or blue. Top right and bottom : two
1268 views of the surface potential, with the siderophore in the binding pocket surrounded by
1269 highly positive charges.

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1271 Figure S14: Structure of *S. aureus* SirA. Left : surface potential of the protein with Fe-
1272 Staphyloferrin B in the binding site. Right : superposition of the structures of apo-SirA (cyan)
1273 and Fe-Staphyloferrin B SirA complex (green), in ribbon representation.

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1277 Figure S15. Structures of ShuT and PhuT from *S. dysenteriae* and *P. aeruginosa*. Top left :
 1278 surface potential of ShuT with the heme in its binding site. Top right : structures of apo
 1279 (green) and holo-ShuT (cyan). Bottom left : surface potential of PhuT with heme in the
 1280 binding site. Bottom right : structures of holo-ShuT (cyan) and holo-PhuT (magenta) showing
 1281 the heme orientation in their respective pockets.

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HmuT

1284 Figure S16 : Structure of HmuT from *Y. pestis*. Left : surface potential of HmuT, with two
 1285 hemes in the binding pocket. Right : Superposition of apo-HmuT (cyan) and bis-heme-HmuT
 1286 (green).

1287

1288 Figure S17 : Structure of HmuT from *C. glutamicum*. Left : superposition of the structures of
 1289 the WT and three mutants of HmuT in the presence of heme. Right : surface potential of
 1290 HmuT showing heme in the binding site.

Figure S18 : comparison of apo-HutB from *V. cholerae* (green) and bis-heme HmuT from *Y. pestis* in the bis-heme conformation (magenta).

Figure S19 : Structure of Rv0265c from *M. tuberculosis*. Surface potential, showing also the putative binding site.

Figure S20 : Structure of *Y. pestis* YiuA. Surface potential with the putative binding site surrounded by positive charges.

1308 Figure S21 : Structures of Fe-FbpA of *H. influenzae* in two distinct conformations, closed
 1309 (cyan, top left) vs more open (green, bottom), together with the surface potential of the
 1310 closed form (top right).
 1311

1313 Figure S22 : Structure of *E. coli* and *H. influenzae* OppA's, in the unbound (orange, *E. coli*)
 1314 and bound (green, *H. influenzae*) forms.

Figure S23 : Structure of BhuUV from *B. cenocepacia*. Top left : surface potential of HmuU, from the cytoplasmic side : the two negatively charged coupling helices are clearly seen, as well as the cavity open on the cytoplasm. Top right : surface potential of HmuU, from the periplasmic side. Bottom left : Ribbon structure of BhuUV, with the two transmembrane BhuU (blue and pale blue) and the two ABC BhuV modules (tan and orange). Bottom right : same as bottom left, rotated 90° along the vertical symmetry axis, with one BhuU in thin ribbon and the other as surface with conserved residues across the family coloured labeled (magenta : Pro, yellow : Gly, green : Trp, Phe, deep green : Arg, red : Leu, Ala, cyan : Gln, Asn, Brown : Asp112).

Figure S24 : Superposition of BhuTU from BhuTUV (green, BhuT as a light ribbon, BhuU as a cartoon) with HmuU from HmuUV (red cartoon). The right view is rotated ca. 90° along the vertical symmetry axis.

1363 Table S1

PDB ID	Species	Protein name	Deposition date	Resolution (Å)	PubMed ID
6MFL	<i>Acinetobacter baumannii</i>	BauB	21/11/2018	1,9	30406986
6ALL	<i>Bacillus anthracis</i>	FpuA(YfmC)	16/08/2017	2,47	
3TNY	<i>Bacillus cereus</i>	YfuY	05/09/2012	1,55	
2WHY	<i>Bacillus subtilis</i>	FeuA	29/09/2009	1,7	19746494
2WI8	<i>Bacillus subtilis</i>	FeuA	22/09/2009	1,55	19746494
2XUZ	<i>Bacillus subtilis</i>	FeuA	10/08/2011	1,9	21802011
2XV1	<i>Bacillus subtilis</i>	FeuA	10/08/2011	2,15	21802011
3G9Q	<i>Bacillus subtilis</i>	Bsu3320(FhuD)	03/03/2009	2,6	
3GFV	<i>Bacillus subtilis</i>	YclQ	12/05/2009	1,75	19955416
2CHU	<i>Campylobacter jejuni</i>	CeuE	14/08/2006	2,4	16927323
3ZKW	<i>Campylobacter jejuni</i>	CeuE	03/04/2013	1,71	23512642
5A1J	<i>Campylobacter jejuni</i>	CeuE	13/05/2015	1,6	23512642
5ASD	<i>Campylobacter jejuni</i>	CeuE	29/06/2016	1,74	28383577
5ASV	<i>Campylobacter jejuni</i>	CeuE	06/07/2016	2,04	28383577
5AD1	<i>Campylobacter jejuni</i>	CeuE	28/09/2016	1,32	28383577
5ADV	<i>Campylobacter jejuni</i>	CeuE	25/05/2016	2,1	27162326
5ADW	<i>Campylobacter jejuni</i>	CeuE	25/05/2016	1,9	27162326
5LWH	<i>Campylobacter jejuni</i>	CeuE	12/04/2017	1,47	28383577
5LWQ	<i>Campylobacter jejuni</i>	CeuE	12/04/2017	1,52	28383577
5MBQ	<i>Campylobacter jejuni</i>	CeuE	12/04/2017	1,33	28383577
5MBT	<i>Campylobacter jejuni</i>	CeuE	12/04/2017	1,8	28383577
5MBU	<i>Campylobacter jejuni</i>	CeuE	12/04/2017	1,81	28383577
50AH	<i>Campylobacter jejuni</i>	CeuE	01/08/2018	1,8	
50D5	<i>Campylobacter jejuni</i>	CeuE	01/08/2018	1,9	
5TCY	<i>Campylobacter jejuni</i>	CeuE	12/04/2017	1,9	28383577
1EFD	<i>Escherichia coli</i>	FhuD	05/04/2000	1,9	10742172
1ESZ	<i>Escherichia coli</i>	FhuD	17/04/2002	2	11805094
1K2V	<i>Escherichia coli</i>	FhuD	17/04/2002	1,97	11805094
1K7S	<i>Escherichia coli</i>	FhuD	17/04/2002	2,6	11805094
2M6K	<i>Escherichia coli</i>	FepB	30/04/2014		25173704
2M6L	<i>Escherichia coli</i>	FepB	30/04/2014		25173704
3TLK	<i>Escherichia coli</i>	FepB	16/01/2013	1,85	27539322
3BE5	<i>Escherichia coli</i> O157:H7	FitE	28/10/2008	2,2	19004000
3BE6	<i>Escherichia coli</i> O157:H7	FitE	28/10/2008	1,82	19004000
4INO	<i>Helicobacter pylori</i>	CeuE	08/01/2014	1,65	24330328
4INP	<i>Helicobacter pylori</i>	CeuE	08/01/2014	2,3	24330328
4LS3	<i>Helicobacter pylori</i>	CeuE	22/01/2014	1,7	24330328
4PM4	<i>Mycobacterium tuberculosis</i>	Rv0265c	11/06/2014	2,2	
5CR9	<i>Saccharomonospora viridis</i>	?	12/08/2015	1,7	
3EIW	<i>Staphylococcus aureus</i>	HtsA	26/05/2009	1,6	19400778
3EIX	<i>Staphylococcus aureus</i>	HtsA	26/05/2009	1,35	19400778
3LHS	<i>Staphylococcus aureus</i>	HtsA	09/02/2010	1,3	20147287
3LI2	<i>Staphylococcus aureus</i>	HtsA	09/02/2010	2,2	20147287
3MWF	<i>Staphylococcus aureus</i>	SirA	01/09/2010	1,7	20810662
3MWG	<i>Staphylococcus aureus</i>	SirA	01/09/2010	2,1	20810662
4B8Y	<i>Staphylococcus aureus</i>	FhuD2	21/11/2012	1,9	23113737
4FIL	<i>Staphylococcus aureus</i>	FhuD2	12/06/2013	2,4	24606332
4FKM	<i>Staphylococcus aureus</i>	FhuD2	19/06/2013	2,2	24606332
4FNA	<i>Staphylococcus aureus</i>	FhuD2	19/06/2013	3,5	24606332
4HS9	<i>Streptococcus pneumoniae</i>	PiaA	03/10/2012	1,66	
4HMO	<i>Streptococcus pneumoniae</i>	PiaA	04/09/2013	2	23951167
4HMP	<i>Streptococcus pneumoniae</i>	PiaA	04/09/2013	2,7	23951167
4HMQ	<i>Streptococcus pneumoniae</i>	PiaA	04/09/2013	2,1	23951167
5JJ5	<i>Streptococcus pneumoniae</i>	PiaA	04/05/2016	2,3	
5FLY	<i>Staphylococcus pseudointermedius</i>	FhuD	02/12/2015	1,6	26919525
5DH0	<i>Thermobifida fusca</i>	FscJ	18/11/2015	2,44	26537767
5DH1	<i>Thermobifida fusca</i>	FscJ	18/11/2015	2,84	26537767
5DH2	<i>Thermobifida fusca</i>	FscJ	18/11/2015	2,59	26537767
3R5S	<i>Vibrio cholerae</i>	ViuP	08/02/2012	1,79	22291019
3R5T	<i>Vibrio cholerae</i>	ViuP	08/02/2012	1,45	22291019
3TEF	<i>Vibrio cholerae</i>	VctP	15/08/2012	1,7	22575663
5GGX	<i>Vibrio cholerae</i>	FhuD	22/02/2017	3,4	
5GGY	<i>Vibrio cholerae</i>	FhuD	22/02/2017	2,5	
6B2X	<i>Yersinia pestis</i>	YiuA	15/11/2017	2,2	29095164
6B2Y	<i>Yersinia pestis</i>	YiuA	15/11/2017	1,77	29095164

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species	name	PDB ID		binding pocket residues
<i>Acinetobacter baumannii</i>	BauB	6MFL	Fe-acinetobactin	Y84, I104, V105, L125, N145, L203, F212, R217, R261, V264, M265, <u>Y301</u> , T302
<i>Bacillus cereus</i>	YfuY	3TNY	schizokinen	W47, M90, <u>R91</u> , L111, <u>R112</u> , <u>R162</u> , M164, <u>R169</u> , <u>Y171</u> , F197, R200, F220, <u>N261</u> , T262
<i>Bacillus subtilis</i>	FeuA	2WHY	Fe-bacillibactin	G42, <u>K84</u> , M85, <u>K105</u> , F106, P107, H125, R178, <u>R180</u> , <u>Q181</u> , Y185, Y187, V191, A214, <u>Q215</u>
	FeuA	2WI8	apo	
	FeuA	2XUZ	enterobactin	G42, <u>K84</u> , M85, <u>K105</u> , F106, P107, H125, R178, <u>R180</u> , Q181, Y187, V191, A214, <u>Q215</u>
	FeuA	2XV1	MECAM	G42, <u>K84</u> , M85, <u>K105</u> , F106, P107, H125, R178, <u>R180</u> , Y187, V191, A214, <u>Q215</u>
	Bsu3320	3G9Q	apo(ferrichrome)	
	YclQ	3GFV	apo(petrobactin)	
	CeuE		bis Fe-MECAM	<u>Q98</u> , G117, <u>R118</u> , <u>K121</u> , <u>R205</u> , <u>R249</u> , L253, <u>Y288</u>
<i>Campylobacter jejuni</i>	CeuE	2CHU	apo	
	CeuE	3ZKW	apo	
	CeuE	5A1J	Fe-4LICAM	V97, Q98, <u>R118</u> , K121, <u>R205</u> , T226, <u>H227</u> (Fe), <u>R249</u> , L253, <u>Y288</u> (Fe)
	CeuE	5A5D	Fe-SLC	V97, Q98, <u>R118</u> , K121, <u>R205</u> , T226, <u>H227</u> (Fe), <u>R249</u> , L253, <u>Y288</u> (Fe)
	CeuE	5A5V	Fe-PXJ	V97, Q98, G117, <u>R118</u> , <u>K121</u> , <u>R205</u> , T226, <u>H227</u> (Fe), <u>R249</u> , L253, <u>Y288</u> (Fe)
	CeuE	5AD1	Fe-8LC	V97, Q98, <u>R118</u> , K121, <u>R205</u> , T226, <u>H227</u> (Fe), <u>R249</u> , L253, <u>Y288</u> (Fe)
	CeuE	5ADV	Fe-DBS2	<u>Q98</u> , <u>R118</u> , <u>K121</u> , <u>R205</u> , T226, <u>H227</u> (Fe), <u>R249</u> , <u>Y288</u> (Fe)
	CeuE	5ADW	Fe-EHS	Q98, <u>R118</u> , <u>K121</u> , <u>R205</u> , T226, <u>H227</u> (Fe), <u>R249</u> , <u>Y288</u> (Fe)
	CeuE	5LWH	Apo Y288F	
	CeuE	5LWQ	apo H227L	
	CeuE	5MBQ	apo H227A	
	CeuE	5MBT	apo H227L Y288F	
	CeuE	5MBU	apo H227A Y288F	
	CeuE	5OAH	Fe-95B	V97, Q98, <u>R118</u> , <u>K121</u> , <u>R205</u> , <u>H227</u> (Fe), <u>R249</u> , L253, <u>Y288</u> (Fe)
	CeuE	5OD5	Fe- (Ir)-95B-9RT-RIR	V97, <u>Q98</u> , <u>R118</u> , <u>K121</u> , <u>R205</u> , <u>R249</u> , L253, <u>Y288</u> (Fe)
	CeuE	5TCY	H227L Fe-SLC	V97, Q98, <u>R118</u> , <u>K121</u> , <u>R205</u> , <u>R249</u> , L253, <u>Y288</u> (Fe) HOH(Fe)
<i>Escherichia coli</i>	FhuD	1EFD	Gallichrome	W43, W68, <u>R84</u> , T85, <u>Y106</u> , I183, L189, Y275, W217, W273
		1ESZ	Fe-Coprogen	E42, L44, N64, W68, <u>R84</u> , T85, <u>S103</u> , <u>Y106</u> , F124, I183, W217, W273, <u>Y275</u>
		1K2V	Fe-Desferal	W43, N64, W68, <u>R84</u> , T85, <u>Y106</u> , L189, N215, W217, W273, F274, <u>Y275</u>
		1K7S	Fe-Albomycin	E42, W68, <u>R84</u> , T85, <u>Y106</u> , I183, L189, W217, W273, Y275
	FitE	3BE5	apo	
		3BE6	apo	
<i>Mycobacterium tuberculosis</i>		4PM4	apo	
<i>Saccharomonospora viridis</i>		5CR9	apo	
<i>Staphylococcus aureus</i>	HtsA	3E1W	apo	
		3E1X	apo	
		3LHS	Fe-staphyloferrin A, open	<u>R104</u> , <u>R126</u> , F146, V200, A202, L207, <u>H209</u> , Y244, <u>R299</u> , A303, <u>R304</u> , <u>R306</u>
		3L12	Fe-staphyloferrin A, closed	<u>R86</u> , <u>R104</u> , K105, <u>R126</u> , F146, V200, A202, <u>K203</u> , <u>H209</u> , <u>Y239</u> , Y244, <u>R299</u> , A303, <u>R304</u> , <u>R306</u>
	SirA	3MWG	apo	
		3MWF	Fe-Staphyloferrin	W81, P104, V124, <u>R125</u> , <u>T144</u> , V145, F146, <u>R201</u> , <u>R206</u> , <u>Y208</u> , I238, P262, <u>N304</u> , L305
	FhuD2	4FNA	apo	
		4FKM	apo, methylated lysine residues	
		4B8Y	Fe-Ferrichrome	Y110, <u>T112</u> , Y130, F186, Y191, Y193, <u>W197</u> , <u>R199</u> , W225, <u>W279</u>
		4FIL	Fe-Ferrioxamine	P55, T56, Q74, Y110, T112, Y130, D184, F186, Y191, Y193, <u>W197</u> , <u>R199</u> , W225, W279, Y280, N281
<i>Streptococcus pneumoniae</i>	PiaA	4H59	apo	
		4HMO	apo	
		4HMP	apo	
		4HMQ	Fe-ferrichrome	W63, <u>N83</u> , Y84, <u>W158</u> , M213, W223, <u>Y225</u> , <u>R231</u> , F255
		5JJ5	Fe-ferrioxamine	W63, <u>N83</u> , Y84, Y133, <u>W158</u> , M213, W223, <u>Y225</u> , <u>R231</u> , F255, A256, Y276
<i>Staphylococcus pseudointerme</i>	FhuD	5FLY	apo	
<i>Thermobifida fusca</i>	FusA?	5DH0	apo	
		5DH1	apo	
		5DH2	apo	
<i>Vibrio cholerae</i>	ViuP	3R5S	apo	
		3R5T	Fe-vibriobactin	V59, T60, T78, T79, <u>Q80</u> , F84, I123, G124, <u>A125</u> , D126, Y146, <u>S197</u> , <u>H199</u> , <u>H239</u> , D241, F242, <u>R244</u> , F296, <u>R297</u>
	VctP	3TEF	apo	
	FhuD	5GGX	Fe-desferal	W86, <u>R102</u> , F211, W239, W293, <u>Y295</u>
		5GGY	apo	
<i>Yersinia pestis</i>	YiuA	6B2X		
		6B2Y		

1370 Table S3

PDB ID	Source	Protein name	Dep. Date	Resolution (Å)	PubMed ID
5GIZ	<i>Burkholderia cenocepacia</i>	BhuT in apo form	26/06/2016	1,5	28913898
5Y89	<i>Burkholderia cenocepacia</i>	BhuT (holo-1)	20/08/2017	2,4	28913898
5Y8A	<i>Burkholderia cenocepacia</i>	BhuT (holo-2)	20/08/2017	2	28913898
5AZ3	<i>Corynebacterium glutamicum</i>	HmuT	18/09/2015	1,42	
5B4Z	<i>Corynebacterium glutamicum</i>	HmuT H141A	20/04/2016	1,3	27240352
5B50	<i>Corynebacterium glutamicum</i>	HmuT Y240A	20/04/2016	1,65	27240352
5B51	<i>Corynebacterium glutamicum</i>	HmuT R242A	20/04/2016	1,3	27240352
3M8U	<i>Glaesserella parasuis</i>	GbpA-GSSG	19/03/2010	1,85	20628015
6DQQ	<i>Haemophilus influenzae</i>	OppA-peptide	11/06/2018	1,85	30455346
6DQR	<i>Haemophilus influenzae</i>	OppA-MGG	11/06/2018	2,08	30455346
6DQT	<i>Haemophilus influenzae</i>	OppA-LGG	11/06/2018	1,95	30455346
6DQU	<i>Haemophilus influenzae</i>	OppA-GIINTL	11/06/2018	1,65	30455346
6DTF	<i>Haemophilus influenzae</i>	OppA-KKK	16/06/2018	1,75	30455346
6DTG	<i>Haemophilus influenzae</i>	OppA-YLGANGRGC	16/06/2018	1,9	30455346
6DTH	<i>Haemophilus influenzae</i>	OppA-RPPGFSPFR	16/06/2018	1,96	30455346
2R79	<i>Pseudomonas aeruginosa</i>	PhuT	07/09/2007	2,4	17925389
5GJ3	<i>Roseiflexus sp, RS-1</i>	RhuT (holo-2)	26/06/2016	2	28913898
5Y8B	<i>Roseiflexus sp, RS-1</i>	RhuT apo	20/08/2017	2,4	28913898
2R7A	<i>Shigella dysenteriae</i>	ShuT	07/09/2007	2,05	17925389
2RG7	<i>Shigella dysenteriae</i>	ShuT	02/10/2007	2,05	17925389
5KHL	<i>Vibrio cholerae</i>	HutB	15/06/2016	2,4	
3MD9	<i>Yersinia pestis</i>	HmuT	30/03/2010	1,5	20888343
3NU1	<i>Yersinia pestis</i>	HmuT	06/07/2010	2,5	20888343

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1399 Table S4

species	name	PDB ID		binding pocket residues
<i>Pseudomonas aeruginosa</i>	PhuT	2R79	heme	T56, <u>Y71</u> (Fe), R73, Q74, I173, Y207, R228(Pr), P262, V266
<i>Shigella dysenteriae</i>	ShuT	2RG7	apo	
		2RTA	heme	G30, G31, S32, E51, T52, <u>Y67</u> (Fe), W87, R111 (Pr), A169, G170, G171, S172, S181, V182, A183, Y201,
<i>Yersinia pestis</i>	HmuT	3MD9	apo	
		3NU1	bis-heme	S54, T55, <u>Y70</u> (Fe1), M165, <u>H167</u> (Fe2), L170, M173, T179, A180, A181, R199, Y200, M255, L258
<i>Corynebacterium glutamicum</i>	HmuT	5AZ3	WT, heme	L101, Y102, V123, S124, T126, G140, <u>H141</u> (Fe), <u>Y240</u> (Fe), R242 (>Y240), V247, F249, L251, Y255, L278, M297, E299, D332, G333, L336
		5B4Z	H141A heme	L101, V123, S124, G139, G140, <u>Y240</u> (Fe), R242 (>Y240), F249, L251, Y255, M297, D332, L336
		5B50	Y240A heme	L101, Y102, V123, S124, T126, G140, <u>H141</u> (Fe), R242(Pr), L251, Y255, M297, E299, D332, G333, L401
		5B51	R242A heme	L101, Y102, V123, S124, G140, <u>Y240</u> (Fe), A242, F249, L251, Y255, M297, E299, D332, L336
<i>Burkholderia cenocepacia</i>	BhuT	5GIZ	apo	
		5Y89	WT heme	G47, T71, T72(Pr1), <u>Y87</u> (>Fe), Q88(Pr1), R89(Pr1+Y87), H133, L190, H192, T193, L198, T204, A205(Pr2), A206(Pr2), Y225(Pr2), A279, A280, L283
		5Y8A	bis-heme	T71, T72, <u>Y87</u> (>Fe1), Q88(Y87), R89(Y87), L190, H192, T195, T204, A205 (Pr He2), A206(Pr He2), H224, Y225(Pr He2), L283
<i>Roseiflexus sp</i>	RhuT	5Y8B	apo	
		5GJ3	bis-heme	S124, <u>Y140</u> (>Fe1), Q141(>Y140), R142 (>Y140), R143 (>Pr 1-2He1), <u>Y239</u> (>Fe2), R241(>Y239), F276, L295, D330, L334

1400