[Frontiers in Bioscience 10, 462--477, January 1, 2005]

CYTOPLASMIC MEMBRANE IRON PERMEASE SYSTEMS IN THE BACTERIAL CELL ENVELOPE

Wolfgang Koster

Environmental Microbiology and Molecular Ecotoxicology (MIX), Swiss Federal Inst. for Environmental Science and Technology (EAWAG), Uberlandstrasse 133, CH-8600 Dubendorf, Switzerland, VIDO – Vaccine & Infectious Diseases Organization, University of Saskatchewan, 120 Veterinary Rd., Saskatoon, Saskatchewan S7N 5E3, Canada (present address)

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1. ABSTRACT

Iron is a vital nutrient for the vast majority of organisms. Gram-positive bacteria, Gram-negative bacteria and archaea established different strategies to utilize iron from various sources. This article concentrates on the cytoplasmic membrane-associated uptake systems involved in the acquisition of iron. Genetic, biochemical and structural data as well as computational analyses were taken into consideration. Besides divalent metal transporters of the Nramp type and ferrous iron transport proteins of the Feo type, four distinct families of ABC transporters related to iron uptake are known. Their components mediate the transfer of iron, siderophores, heme and vitamin B_{12} into the cytosol of prokaryotes.

2. INTRODUCTION

Iron is not always easily bio-available, despite the fact that it is the fourth most abundant metal on earth. In the environment it is mainly found as a component of insoluble hydroxides; in biological systems it is complexed by high-affinity iron binding proteins (e.g. transferrins, lactoferrins, ferritins) or found as a component of heme and hemoproteins. Iron is an essential nutrient for most living bacteria. As a component of key molecules such as cytochromes, ribonucleotide reductase, and other metabolically linked compounds, iron plays an important role. Only few organisms are known which do not depend on iron. In certain bacteria such as lactobacilli iron can be functionally replaced by manganese (1). Also, strains of *Borrelia* (e.g. *B. burgdorferi*) are claimed to have no need for iron. Despite the fact that an outer membrane protein (28 kDa) from *B. burgdorferi* B31 had been detected that bound holo-Transferrin but not apo-Transferrin (2), and although the genes encoding an iron-containing superoxide dismutase (*sodA*) (3) and a putative ferric uptake regulator (*fur*) had been identified in the bacterial genome, it was calculated from growth experiments that the intracellular iron concentration was significantly below a physiologically relevant concentration (4). Thus the Lyme disease bacterium seem to use the concept of iron independence to bypass a major host defence strategy: generating iron limiting conditions for pathogens.

Due to the redox potential of Fe^{2+}/Fe^{3+} (1300 mV to 2500 mV, depending on the protein environment and the ligand), iron is predestinated to participate in many electron-transfer reactions. Microorganisms established various sequestering strategies for this essential nutrient. Notably, a sufficient iron supply contributes significantly to the virulence of a number of bacterial pathogens (5, 6, 7, 8, 9).

Under anaerobic conditions, ferric iron is soluble enough to be transported without the assistance of any chelating agents. Similarly, at pH 3 ferric iron can support growth of acid-tolerant bacteria without major difficulties.

In contrast, iron is mostly found in insoluble compounds at higher pH values. As an answer to the extreme low concentration of free iron, many bacterial species and certain fungi produce a great variety of low-molecular-weight iron ligands, termed siderophores, suitable to bind Fe^{3+} with very high affinity. Four major structural types that are involved in complexing ferric iron are described: catecholates, hydroxamates, α-hydroxycarboxylates, and siderophores with thiazoline/thiazolidine rings (10, 11, 12, 13). For a long time the mechanism of siderophore secretion remained poorly understood. In 1998 putative export components were identified in mycobacteria (14). Recently, the *Escherichia coli entS* gene, located within a cluster of enterochelin (= enterobactin) synthesis genes, was found to encode a membrane protein with similarity to the 12-transmembrane segment major facilitator superfamily of export pumps. EntS represents an important component of the enterochelin secretion machinery (15). A predicted gene encoding a putative 45 KDa protein with significant similarity to EntS was identified in an *Acinetobacter baumannii* chromosomal region harbouring genes related to siderophore biosynthesis and transport (16). Similarly, the vibrioferrin synthesis operon of *Vibrio parahaemolyticus* contains the *pvsC* gene that encodes a putative membrane associated siderophore exporter of the EntS type (17). In general, the chelators are released in their ligand-free forms and subsequently transported into the cell as ferric-siderophore-complexes. Many bacteria are capable of using a variety of (structurally) different siderophores including those produced by other bacterial species, certain fungi, and plants.

Sometimes the iron acquisition by siderophores seems to be inappropriate and often the availability of free iron is strongly limited in vertebrates. Thus, a number of pathogenic microbes can use heme-bound iron from hemoglobin, hemopexin, and haptoglobin (6, 9, 18). Some bacteria (e.g. *Serratia marcescens, Pseudomonas aeruginosa*) secrete a heme-binding protein, the hemophore HasA, in its apo-form which is then taken up as heme bound form by a set of transport components (18). The *S.* marcescens hemophore binds heme as a monomer with a stoichiometry of 1 and an affinity lower than 10-9 M. The crystal structure oft the holoprotein has been solved and found to consist of a single module with two residues interacting with heme (19). In addition, transferrins and lactoferrins serve as major iron sources for certain bacterial species. The assimilation of these compounds also involves specific uptake systems in the cell envelope. The mode of iron release from transferrins and lactoferrins and its further transport is not yet well understood at the molecular level (20, 8).

3. TRANSPORT TO THE CYTO-PLASMIC MEMBRANE

Before the nutrient iron (either in its ionic ferric or ferrous form or as part of a complex) becomes available for the uptake systems located in the cytoplasmic membrane (CM), it has to overcome specific obstacles: the different groups of bacteria are surrounded by characteristic envelopes functioning to various extents as permeability barriers.

3.1. Transport across the outer membrane of Gram-negative bacteria

The outer membrane (OM) typical of Gram-negative bacteria constitutes a permeability barrier for a large variety of molecules. Highly specific receptors in the OM are essential for the internalisation of siderophores, heme and vitamin B_{12}. They are also indispensable for the binding of hemoglobin, haptoglobin, hemopexin, hemophores, lactoferrins and transferrins. The term TonB-dependent receptors is used to emphasize their interaction with the Ton complex composed of the proteins TonB, ExbB, and ExbD anchored in the CM. The Ton complex components couple the receptor-mediated transport through the OM to the electrochemical potential across the inner membrane. (for review see 11, 12,22, 23, 24, 8, 20, 25 and references therein). A more detailed insight into energy coupled uptake mechanisms was possible after investigating the crystal structures of selected TonB-dependent OM transporters. They form a barrel anchored in the membrane. A channel-like structure is (partialy) closed by a globular portion of the receptor referred to as 'cork', 'plug', or 'hatch' domain. The proteins FhuA, FepA, FecA and BtuB from *E. coli* are strikingly similar with respect to their overall organisation in the lipid bilayer (26, 27, 28, 29, 30, 31). In *E. coli* the translocation through the (OM) is the rate limiting step for internalisation of the siderophores (32). Remarkably, the TonB-dependent receptors display higher substrate specificity than the proteins of the ABC systems mediating further uptake of siderophores into the cytoplasm. Nonetheless, a given chelator can be transported via several OM receptors (e.g. studies with *Salmonella enterica* serovar Typhimurium revealed that 2,3-dihydroxybenzoylserine can be taken up by any of the catecholate receptors FepA, IroN and Cir) (33) In general, it seems to be the rule that there exist more different TonB dependent OM receptors in a bacterial cell than corresponding permeases. It has to be mentioned that some iron uptake is TonB independent in Gram-negative bacteria. In experiments carried out under iron-deficient conditions *tonB* mutants show some growth and reduced but detectable iron uptake. The murein sacculus in the periplasmic space is made up by a single layer of peptidoglycan and can be largely neglected as potent barrier with respect to the transported molecules described in this article.

3.2. Transport across the cell wall of Gram-positive bacteria

Gram-positive bacteria, which are devoid of an outer membrane possess a thick peptidoglycan network instead, consisting of up to 40 layers making up to 90% of the cell wall.

Many aspects of receptor mediated iron transport across the OM of Gram-negative bacteria are fairly well understood, but comparatively little information is available on suitable systems for iron uptake in the envelope of Gram-positve bacteria. Siderophores can diffuse through the multi-layered murein sacculus without major problems, but many questions remain open as to how pathogenic bacteria acquire iron from host iron sources. A number of surface exposed proteins of Gram-positives are

characterised by a signature sequence located near their carboxy-terminus. Proteins containing such a cell wall-anchoring motif ("LPXTG" or "NPQTN") will be attached to the murein. The sorting step involves specific enzymes, sortases, acting as transpeptidases (34, 35). A group of the cell wall-anchored proteins may act as receptors for iron containing compounds such as heme, hemoglobin, transferrin and lactoferrin (36, 37, 38, 39, 40, 41, 42).

3.3. Transport across the envelope of mycobacteria

The cell walls of mycobacteria are featured by their remarkably high impermeability, which is the result of an outer lipid barrier based on a monolayer of characteristic mycolic acids and a capsule-like coat of polysaccharide and protein (43, 44). More lipophilic molecules can diffuse through the lipid layer, whereas hydrophilic nutrients are believed to cross the cell wall through special porins (45). *Mycobacterium tuberculosis* possesses at least two porins in addition to the low activity channel protein OmpATb. OmpATb is essential for adaptation of *M. tuberculosis* to low pH and survival in macrophages and mice and its channel activity might be of importance for the defence of *M. tuberculosis* against acidification within the phagosome of macrophages (46). MspA, the main porin of *Mycobacterium smegmatis,* differs obviously from the known tetrameric channels of Gram-negative bacteria in that it constitutes a tetrameric complex with a single central pore of 10 nm length and a cone-like structure (47). Some strains of mycobacteria are known to acquire iron from siderophores. To our knowledge it is still unknown as to how these molecules traverse the outermost part of the envelope, since predicted genes resembling receptor proteins of the TonB-dependent type have not been found in the mycobacterial genomes sequenced so far.

4. TRANSPORT THROUGH THE CYTOPLASMIC MEMBRANE

Six possible pathways involved in (or related to) the uptake of iron into the cytosol of bacteria will be described in the following sections. The different groups of transport systems can be classified by their characteristic structural and functional features.

Transporters of the Nramp type are found throughout all kingdoms of life. The bacterial members of this group cluster in a prokaryotic group that is clearly distinguishable from the eukaryotic cluster. The eukaryotic Nramp proteins are often found to have an N-terminal extended region which might act as a regulatory domain.

Ferrous irons transport systems belonging to the Feo type have been reported exclusively from bacterial species. They form a rather uniform class with only little variability.

Four uptake routes represent systems of the binding protein-dependent type (a subfamily of ABC transporters or traffic ATPases (48, 49)). The typical composition of a prokaryotic ABC importer is: (i) one or several extracellular binding proteins (BP), (ii) one or two different (homodimer, heterodimer, or pseudo-heterodimer) polytopic integral membrane proteins (IMP), and (iii) one or two different ATP-hydrolases (ATPase) that face the cytoplasm and supply the system with energy. It is generally accepted that all ABC systems, in particular those being involved in the uptake of various solutes, originate from a common ancestor, which is reflected by their almost identical design. Nonetheless, it is possible to clearly distinguish the members of the four iron transport families, the siderophore/heme type, the ferric iron (Fbp/Sfu) type, the metal (Fe, Zn, Mn) type, and the fused-IMP/ATPase type, with respect to the primary structure of the different components. The ligand binding proteins and the integral membrane proteins display significant similarity only within their families. In particular, BPs and IMPs of the siderophore family seem to be totally unrelated to those of any other known ABC transport systems. In contrast, the ferric iron type proteins display a low but significant homology to the equivalent components that are involved in the utilisation of e.g., sulfate, spermidine and putrescine. Although the ATPases of different families show a higher degree of conservation, they still cluster in distinctive groups. All major ABC importer families presented in this overview can be divided in subfamilies. The alignments of the BPs, the ATPases, and the IMPs give similar results, thus indicating a series of subsequent duplication events of complete systems - and less often of single components - during evolution. The formation of sub-families is not species specific, and components of a given cluster can be found in Gram-positives, Gram-negatives and archaeae. The most obvious difference concerns the binding proteins of ABC transporters. In Gram-negatives, the binding proteins are predominantly synthesized with a cleavable signal sequence and subsequently released into the periplasm. Only a few cases are known where the binding proteins are anchored to the cytoplasmic membrane via a lipoprotein modification (50, 51, 52, 53, 54). In Gram-positives the latter case seems to be the rule and the binding proteins are tethered to the membrane.

Systems involved in (or related to) the acquisition and assimilation of iron are shown in Figure 1 that provides a schematic view on a typical Gram-negative bacterium. The OM receptors as well as the proteins of the Ton complex are absent in Gram-positive bacteria and have not been identified so far in mycobacteria and members of the mycoplasma group (Figure 2).

4.1. Divalent metal transporters of the Nramp type

The Nramp (natural resistance associated macrophage proteins) transporters are integral membrane proteins found in many prokaryotic organisms including archaea and bacteria, but originally identified in eukaryotic organisms (mammals, higher plants, insects and yeast). The first identified gene of this family, Nramp1, was characterised in mice as a protein involved in host resistance to certain pathogens (55, 56, 57). Nramp2 was recognized as the major transferrin-independent iron uptake system of the intestine in mammals mediating influx of transition metal divalent cations, including Fe^{2+}, Mn^{2+}, and probably, Cd^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+} (58). Like

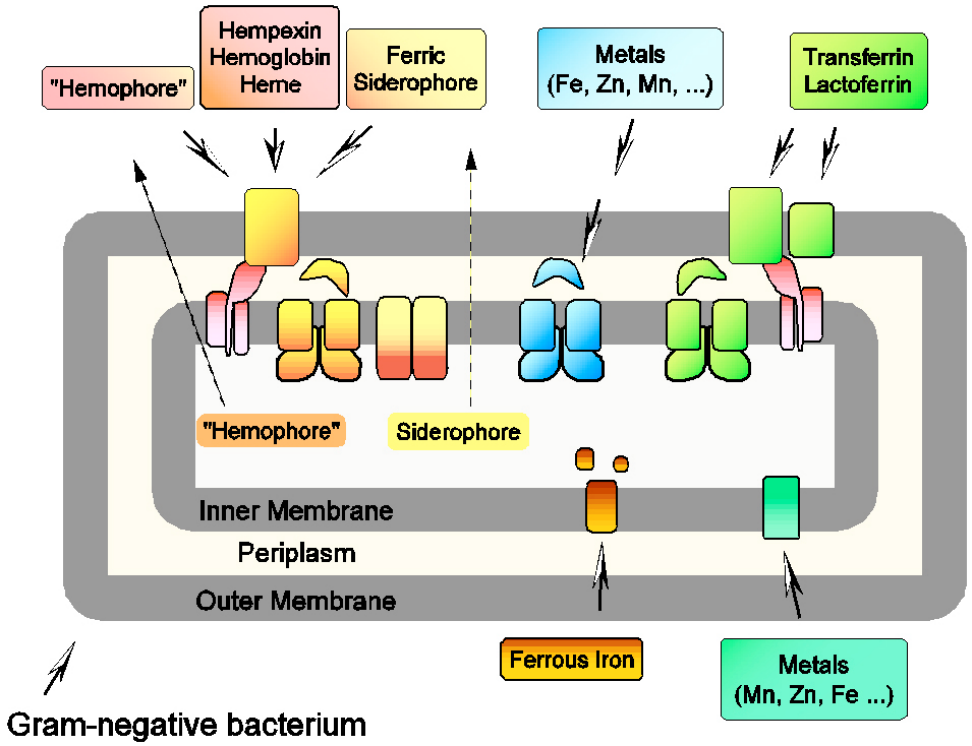

Figure 1. Iron acquisition systems in Gram-negative bacteria. The summary of systems involved in (or related to) the uptake and assimilation of iron represents a schematic view on a typical Gram-negative bacterium. The OM receptors as well as the proteins of the Ton complex (TonB, ExbB, ExbD) are not present in Gram-positive bacteria and were not found in mycobacteria and members of the mycoplasma group. For details see text.

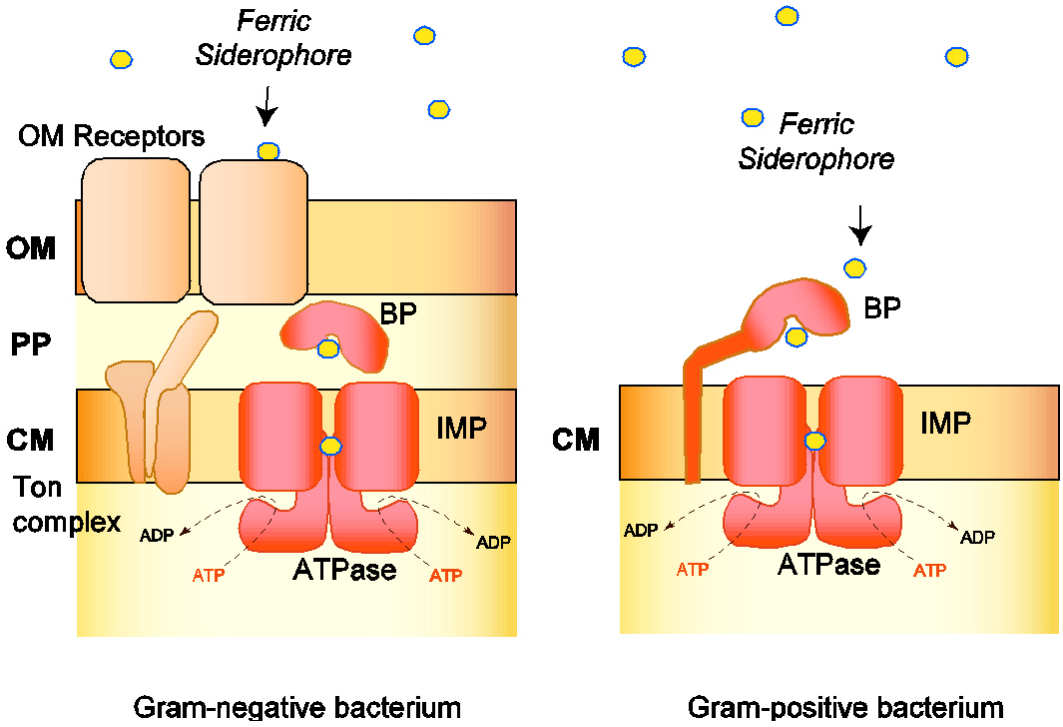

Figure 2. Schematic view on the uptake of ferric siderophores by Gram-positive and Gram negative bacteria. Please note that the murein (peptidoglycan) network associated with the cytoplasmic membrane is not shown. For details see text.

mammalian Nramp transporters yeast Smf proteins exhibit a broad specificity for both essential and nonessential toxic metals: Smfp1, Smfp2, and Smfp3 are rather selective for Mn^{2+} but have been recently linked to the uptake of other heavy metals including copper, cobalt and cadmium (59).

The bacterial Nramp protein MntH from *E.coli* is a proton-dependent divalent cation co-transporter with a preference for Mn^{2+} (Km approximately 0.1 μM)(60, 61). In silico predictions and experimental data based on reporter-fusion proteins suggest a two-dimensional topology model with the N-terminus facing the cytoplasm followed by 11 transmembrane domains (62) The *mntH* genes in *Salmonella enterica* serovar Typhimurium and in *E. coli* are regulated at the transcriptional level by both substrate cation and H_2O_2. In the presence of Mn^{2+}, MntH expression is blocked mainly by the manganese transport repressor, MntR, which interacts with an inverted-repeat motif on the DNA located between the -10 region and the ribosome binding site. In the presence of ferrous iron, the Fur repressor prevents expression of *mntH*, acting through a Fur-binding motif overlapping the -35 region. (63, 64). The OxyR protein, which binds to a consensus motif just upstream of the putative promoter activates *mntH* expression in the presence of hydrogen peroxide (64). Genetical and biochemical experiments demonstrate that the MntH transporter in the Gram-positive bacterium *Bacillus subtilis* is selectively repressed by Mn(II). This regulation requires the MntR protein acting under high Mn(II) conditions as repressor of *mntH* transcription (65). The role in iron acquisition of the bacterial MntH proteins studied so far seems to be of extremely low importance under physiological conditions, in contrast to some eukaryotic Nramps that are clearly involved in iron transport.

4.2. Ferrous iron transport systems of the Feo type

Many bacteria possess a transport system specific for ferrous iron, *feo*, which under anaerobic conditions may largely contribute to the iron supply of the cell. In *E. coli* the iron(II) transport genes *feoA* and *feoB* were identified (66). The *feoAB* upstream region harboured a binding site for the regulatory protein Fur, which acts with ferrous iron as a corepressor in iron transport systems of *E. coli*. A Fnr binding site was also identified in the promoter region. The 70 kDa FeoB protein was assigned to the cytoplasmic membrane and its sequence contained regions of similarity to ATPases. That was taken as evidence that ferrous iron uptake might be ATP driven (66). The presence of high- and low-affinity uptake systems was concluded from biphasic kinetics of Fe^{2+} transport in a wild type strain of *Helicobacter pylori*. The high-affinity system (apparent Ks = 0.54 μM) is absent in a mutant lacking the *feoB* gene. Transport via FeoB is highly specific for Fe^{2+} and was inhibited by FCCP, DCCD and vanadate, which again indicated an active process energized by ATP (67). Ferrozine inhibition of Fe^{2+} and Fe^{3+} uptake implied the concerted involvement of both a Fe^{3+} reductase and FeoB in the uptake of iron supplied as Fe^{3+}. It is concluded that FeoB-mediated Fe^{2+} represents a major pathway for *H. pylori* iron sequestration (67). Further investigations revealed that FeoB is a polytopic membrane protein (containing 12 predicted transmembrane helices) with a covalently linked G protein domain containing a guanine-nucleotide-specific nucleotide binding site. The Feo family proteins, missing from eukaryotes, cluster within the class of TRAFAC (for translation factor-related) GTPases (68). The characteristic G4-motif, "NXXD", was identified within FeoB and GTP hydrolysis was demonstrated (69).

Evidence that the FeoB transporter is important for both extracellular growth and intracellular infectivity was obtained from growth experiments with the human pathogen *Legionella pneumophila* on artificial media, as well as from replication studies within iron-depleted amoebae (*Hartmannella vermiformis)* and human U937 cell macrophages (70). In addition, recent work with *Shigella flexneri* suggests that the *feo* system plays an important role in intracellular iron acquisition: a strain carrying mutations in *feoB* and genes of two other systems associated with iron availability (*sitA*) and siderophore synthesis (*iucD*) did not form plaques on Henle cell monolayers (71). Predicted genes with significant similarity to *feoB* have been found in the genomes of many bacterial species.

4.3. ABC transport systems of the siderophore/heme/vitamin B_{12} type

In bacteria and archaea ABC transport systems involved in the acquisition of siderophores, heme, and vitamin B_{12} are well conserved. Only very few species lack representatives of this family of transporters, among them bacteria with no need for iron (e.g. lactobacilli), or species that couple, as intracellular parasites, their metabolism to the metabolism of their hosts (e.g., mycoplasma). Several different ABC transport systems can exist in parallel in a given bacterial species allowing them the use of structurally different chelators. Most systems were exclusively identified by sequence data analysis, some were characterised with biochemical methods and their substrate specificity was determined. The *fhuBCD* system and the *btuCDF* system of *E. coli* can serve as model systems of the siderophore family. In the following sub-sections, the focus will be on the components mediating ferric hydroxamate uptake (*fhu*) and vitamin B_{12} uptake (*btu*).

4.3.1. Organisation on the genetic level and ligand specificity

The *E. coli fhuABCD* genes are arranged in an operon regulated by the intracellular concentration of free iron (72). Under iron-rich conditions their expression is repressed by the action of the Fur protein, which binds to a sequence known as "fur box" upstream of the first gene, *fhuA*. FhuA is the outer membrane receptor for ferrichrome and albomycin (an antibiotic of fungal origin with structural similarity to ferrichrome). The downstream located *fhuC*, *fhuD*, and *fhuB* genes are necessary for further transport across the inner membrane. *fhuC-fhuD* and *fhuD-fhuB* are translationally coupled with overlapping stop/start codons (73), as found other genes that are part of transport operons. (74, 75, 76) This may guarantee the proper stoichiometry of the transport components. The genes encoding the ferrichrome uptake system of *Bacillus subtilis* are transcribed from divergent overlapping promoters: *fhuD* in one and *fhuBGC* in the other direction (77,78).

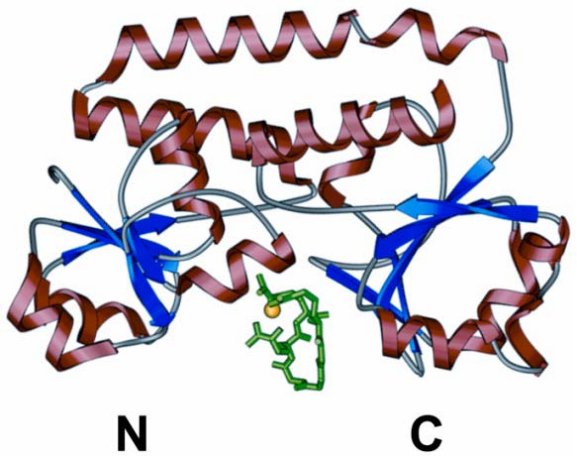

Figure 3. Ferric hydroxamate binding protein FhuD. Structure of *E.coli* FhuD with bound gallichrome at 1.9Å solution (90). The polypeptide chain traverses only once between the N-terminal and C-terminal domains. Please note that the α-helix connecting the two lobes (N and C) spans the entire length of the protein.

The organisation of siderophore family related genes in operons seems to be the rule in most Gram-negative bacteria, particularly in enterobacteriaceae (with only a few exceptions). There is no preference for any gene order: genes encoding the integral membrane components have been identified in first, middle or end positions of an operon. The same holds true for the corresponding genes coding for binding proteins or ATP-hydrolases. Siderophore/heme related ABC transporter genes are also found in clusters containing genes engaged in iron regulation and siderophore biosynthesis. However, the components of a certain transport system are not always organised in a single operon or clustered in a specific region. In Gram-positive bacteria, genes belonging to the same transport system are sometimes found at various positions in the genome. Genes contributing to iron acquisition can also be located on mobile genetic elements (e.g. pathogenic islands) inserted into the chromosome or on plasmid DNA. Examples have been reported for a variety of bacteria including pathogenic strains of *E. coli* (79, 80, 81), *Shigella flexneri* (82), *Klebsiella pneumoniae*, *Citrobacter diversus*, *Salmonella enterica, Yersinia enterocolitica, Y. pestis* (83), and *Streptococcus pneumoniae* (84). Interestingly, the plasmid-encoded anguibactin uptake system (*fat*) of the fish pathogen *Vibrio anguillarum* is lacking an ATPase, which most likely is encoded on the chromosome (85, 86)..

Translocation of siderophores through the cytoplasmic membrane is less specific than their transport across the outer membrane. In *E.coli* OM receptors FhuA, FhuE, Iut are needed to transport a number of different ferric hydroxamates. In contrast, the FhuBCD proteins accept a variety of hydroxamate type ligands such as albomycin, ferrichrome, coprogen, aerobactin, shizokinen, rhodotorulic acid, and ferrioxamine B. Likewise, three different outer membrane proteins (among them FepA the receptor for enterobactin produced by most *E. coli* strains) recognise siderophores of the catechol type (enterobactin and structurally related compounds) while only one ABC system is needed for the passage into the cytosol. (11). The substrate specificity has not been elucidated for the great majority of systems. Nevertheless, it can be speculated that many siderophore ABC permeases might be able to transport several different but structurally related substrates.

4.3.2. Binding proteins as essential receptors

The binding proteins mediate the first steps in the passage of any siderophore across the CM. The *E.coli* FhuD protein binds and transports a variety of structurally different ferric hydroxamates. The FhuD polypeptide is synthesized as "pro-FhuD" with a cleavable signal sequence, processed by the leader peptidase, and subsequently released as mature protein into the periplasm. At the moment, FhuD is the best characterised siderophore binding protein. Binding of hydroxamate type siderophores to the mature FhuD protein has been suggested by using four different approaches: 1.) [$^{55}Fe^{3+}$]-ferrichrome accumulated in the periplasm of a FhuD overproducing strain which was devoid of the integral membrane protein FhuB and therefore blocked in the further transport of the siderophore into the cytosol (87). 2.) Radio- labeled periplasmic FhuD was protected from proteolytic digestion. Only those ferric hydroxamates resulted in proteolytic protection that supported growth of the bacterial cells under iron limiting conditions (78). 3.) A FhuD construct missing its signal sequence was purified from the cytoplasm and protected in an *in vitro* protease protection experiment indicating that ferric hydroxamates could still bind (88). 4.) Changes in the intrinsic fluorescence of purified FhuD allowed the calculation of the dissociation constants for ferric aerobactin (0.4 μM), ferrichrome (1.0 μM), ferric coprogen (0.3 μM), ferrioxamine A (79 μM), ferrioxamine B (36 μM), ferrioxamine E (42 μM), and albomycin (5.4 μM). The dissociation constants were also determined for two FhuD derivatives showing altered substrate binding: FhuD(W68L) and FhuD(A150S, P175L) (88). The results were supported by growth experiments with ferric hydroxamates and albomycin sensitivity assays. FhuD determines to a large extent the substrate specificity of transport across the cytoplasmic membrane. Interestingly, the intrinsic fluorescent measurements with the *E. coli* FepB binding protein revealed a significantly lower K_D (30 nM) for ferric enterobactin (89).

Clarke *et al.* solved the crystal structure of the FhuD protein complexed with gallichrome at 1.9 Å (90). Both hydrophilic and hydrophobic interactions are involved in the binding of the siderophore to FhuD. Previous studies showing that a mutation in tryptophane residue 68 influences the binding of hydroxamates (in particular decreased ferrichrome binding) are confirmed by the structural data. The ligand binding site appears as a shallow groove between the N- and C-terminal domains of the bilobate protein, which adopts a kidney bean-like shape (Figure 3). The polypeptide chain crosses only once between the N-terminal and C-terminal domains. The linker connecting the two lobes, a kinked α-helix, spans the entire length of the protein. The N-terminal domain consists

mainly of a twisted five-stranded parallel β-sheet and the C-terminal domain is composed of a five-stranded mixed β-sheet. Both sheets are packed between layers of α-helices. The mainly hydrophobic domain interface in FhuD suggested that binding and release of the ligands is most likely not accompanied by large scale opening and closing of the siderophore binding site. FhuD does not adopt the "classic" fold that has been published for almost all BPs that are structurally characterized to date (91). In the classical "Venus flytrap" arrangement the two domains are linked by a hinge region made up from two or three flexible β-strands at the bottom of the ligand binding cleft. For the classical arrangement it is generally accepted that ligand binding induces a substantial conformational change or stabilizes a so called closed formation of the BPs (91). In conclusion, FhuD represents a new class of BPs since it adopts a novel fold without a flexible hinge region (90). A second member of this new BP subclass is the vitamin B_{12} binding protein BtuF the crystal structure of which has been solved recently (92, 93). A significant structural similarity becomes evident when the FhuD and BtuF structures are superimposed. Vitamin B_{12} is bound in the "base-on" conformation in a deep cleft formed at the interface between the two lobes of BtuF (60).

Interestingly, although displaying no recognizable sequence similarity, the structurally characterized BPs of the iron/metal type (section 4.5.) share some topological similarities with FhuD and BtuF. The first described representatives of this group are the Mn^{2+} (and possibly Zn^{2+}) binding PsaA from *Streptococcus pneumoniae* and the Zn^{2+}, Mn^{2+} (and possibly Fe^{2+}) binding protein TroA from *Treponema pallidum* (94, 95, 96).

Sequence analysis and alignments demonstrate that FhuD forms a distinctive family, together with all BPs that transport siderophores, heme, and vitamin B_{12}. Members of this siderophore family are clearly distinguishable from any other component involved in the uptake of metals. It is most probable that all BPs of this group adopt a similar basic structure. Despite their remarkable variability in size (25 - 40 kDa) they display typical signatures (97). Interestingly, some conserved regions have been found in both halves of the proteins sharing also structural similarity. An updated alignment of 120 BPs confirmed this observation. Two examples of such distinctive signatures are given here: “ExhxxhKPDLhh” in the N-terminal lobe and “ExhxxxNPDhhh” in the C-terminal domain (with “x” being less conserved and “h” representing a hydrophobic residue). *In vivo* and *in vitro* analysis of single amino acid mutants of *E. coli* FhuD identified several residues that are important for proper functioning of the protein. The mutated residues were mapped to the protein structure to define special areas and specific amino acid residues in FhuD that are vital for correct protein function. A number of these important residues were localized in conserved regions. (98)

4.3.3. Hydrophobic integral membrane proteins as central components

The integral membrane proteins (IMPs) are characterized by their extreme overall hydrophobicity. Embedded in the CM the polytopic IMPs play a pivotal role in the design and the function of all ABC importers. They interact not only with the molecules to be transported but also with the other transport components of the system. The typical IMPs involved in siderophore uptake might form hetero dimers. The formation of homo dimers is appropriate in heme and vitamin B_{12} transport systems since only one IMP was detected in the relevant genomic regions of e.g. *E. coli, Vibrio cholerae, Pseudomonas aeruginosa, Shigella dysenteriae, Yersinia enterocolitica*, and *Y. pestis*.

FhuB and BtuC from *E. coli* are the first IMPs (related to ABC transporter mediated iron/vitamin B_{12} uptake), which have been identified and sequenced (99, 100). A special feature of FhuB is the fact that it comprises two major domains displaying significant homology to each other, thus having about double the size (70 KDa) of comparable integral membrane components from other ABC transporters and. The connection of the FhuB halves via a short linker region might be favourable for interaction. The two domains of the "pseudo-hetero dimer" are then expressed at identical levels, and the arrangement of the transport competent complex may be facilitated by their spacial proximity. Both halves (FhuB[N] and FhuB[C]) of the polypeptide are indispensable for transport; however they still function when produced as two distinct entities (101). Each half of the FhuB is able to fold autonomously into the lipid bilayer which is a prerequisite for the assembly of both halves into a transport competent formation. FhuB-like IMPs have been identified in e.g. *Actinobacter pleuropneumoniae, Azotobacter vinelandii, Rhizobium leguminosarum, Rhodobacter capsulatus, Salmonella enterica* serovar Typhimurium*, Shigella flexneri, Vibrio cholerae* and *Yersinia pestis.*.

Distinctive signatures were identified in the primary structures of all siderophore family IMPs. One of these signatures contains a glycine residue in a distance of about 100 amino acids from the C-terminal end. That residue corresponds to the conserved Gly (102), which is part of the "E A A - - - G - - - - - - - - - I - L P" motif defined by Dassa and Hofnung (103). This conserved region (CR), present twice in FhuB, might play a general role in the translocation process. FhuB derivatives carrying point mutations in the highly conserved glycine residues (G226 and G559 in the N- and C-terminal half, respectively) point to the importance of these amino acids for the transport of ferric hydroxamates (104). The CRs, in particular, the conserved glycine residues are supposed to be structurally and/or functionally important for the other siderophore, heme and vitamin B_{12} uptake systems as well.

The topology of FhuB is different from the equivalent components of other ABC transporters in that each half consists of 10 membrane spanning regions. The location of N- and C-termini are facing the cytosol. The conserved region (CR) is also oriented to the cytoplasm. However, in contrast to the "classical" arrangement, this ATPase interaction loop is followed by 4 instead of 2 transmembrane spans (105). A schematic topology model is presented in Figure 4. It has been assumed that the

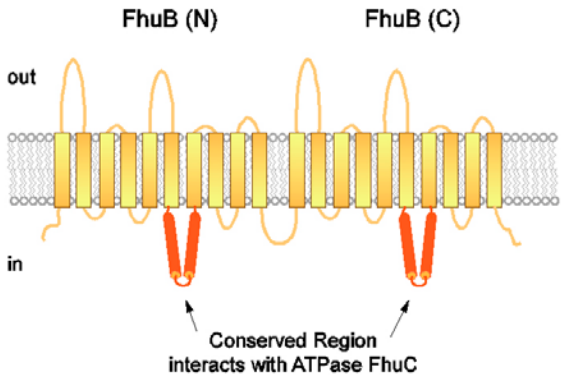

Figure 4. Topology of the FhuB protein. Transmembrane arrangement of the polytopic FhuB protein in the cytoplasmic membrane as determined by the analysis of β-lactamase proteins C-terminally fused to various portions of FhuB. The FhuB protein is composed of two halves FhuB(N) and FhuB(C) each with 10 membrane spanning regions connected by loops contacting the periplasm (in) or the cytoplasm (out). These loops were predicted to entirely or partly fold back into the overall structure. The conserved regions (CR) typical of all prokaryotic importers belonging to the ABC transporter family are shown in red. They are important for the interaction with the FhuC protein, the ATPase supplying energy for the siderophore translocation process.

hydrophilic regions may- entirely or partially - fold back into a channel like structure formed by the membrane spanning portions. It is still unclear to which extent the different "loops" are accessible from the periplasm or cytoplasm. A similar arrangement for all IMPs of the siderophore family can be concluded from sequence analysis data. This idea was further supported by the structural data of the $BtuC_2D_2$ complex (106). Each of the hydrophobic BtuC units comprises 10 transmembrane regions, which is in perfect agreement with the predicted FhuB topology model containing alltogether 20 membrane spanning segments. Notably, the CR loop is arranged as helix-turn-helix motif as it was previously predicted (102).

4.3.4. ATP-hydrolases as driving force

From the primary structure of FhuC it was concluded that this protein might function as an ATP-binding component. FhuC was one of the first ATPases (of binding protein dependent import systems) in which highly conserved residues in the 'Walker A' and 'Walker B' consensus motifs were altered by site directed mutation. A total loss of function in all the corresponding FhuC derivatives was taken as evidence that FhuC indeed acts as an ATP-hydrolase. It was assumed that energizing the transport process is accompanied by conformational changes in the components of the permease complex (107, 78). The ATP hydrolases are the units which are the most conserved among all ABC transporters. Therefore it is highly likely that the structural features and the mechanism of action are very similar in all these systems. Previous studies, in particular experiments with the histidine and the maltose uptake systems, suggested that the active ATPase subunits function as a dimer. The crystal structures of some ATPases have been solved in absence of the membrane-embedded proteins (see 108, 109, 110, 111, 112, 113). They commonly adopt a similar L-shaped structure with two arms, one containing the signature motif, and the other the Walker A and B motifs. In principle, different arrangements of the ATPase proteins in a putative dimer were possible. However, their orientation the functional permease complex remained unclear: “back to back”, “head to tail” or “head to head”. In the published structure of the $BtuC_2D_2$ complex the “head to tail” orientation is realized. Surprisingly, only a small interface was found between the two ATPase units and the Walker A motif of one monomer was facing the “LSGGQ” motif of the other. Each nucleotide-binding site contains residues from both monomers. This architecture now supports previous biochemical data obtained with the functional ABC transporters in that it provides a sound basis for the cooperativity observed in the nucleotide-binding domains (114, 115). Moreover, the participation of the highly conserved family signature motif (“LSGGQ”) in ATP binding and hydrolysis becomes more understandable.

4.3.5. Concerted interaction in a transport complex

Taking into account the situation in Gram-positive bacteria and based on studies with isolated BPs it becomes clear that siderophore binding to the BP does not necessarily require a physical interaction of BP and TonB-dependent receptor. However, it cannot be excluded that such an interaction could facilitate the release of the substrate from the OM receptor which could be favourable for transport efficiency.

The ligand-bound BP most likely delivers the ferric siderophores (or vitamin B_{12}) to the corresponding integral membrane components. A specific direct physical interaction of FhuD with FhuB was concluded from genetic and biochemical studies (e.g. protease protection and cross-linking experiments) (88, 116). From *in vitro* mapping experiments with peptides identical to parts of the FhuB sequence several regions of FhuB were proposed to interact physically with FhuD. The suggested regions comprise parts of FhuB loops oriented to the periplasm, but also loops facing the cytoplasm (including one of the CRs) as well as distinct areas from transmembrane regions (117). Further analysis will be necessary to validate these mapping results.

Like FhuD, the vitamin B_{12} binding protein BtuF is thought to direct the ligand to the hydrophobic transport units (in this case a homodimer of BtuC) in the membrane. This picture is supported by the observed formation *in vitro* of a stable complex between BtuF and BtuCD (with the stoichiometry $BtuC_2D_2F$). After the atomic structures of both the BtuF protein and the $BtuC_2D_2$ complex (the first structure of an ABC importer complex composed of integral membrane proteins and ATPases) were available at high resolution (106, 92) modeling of the individual crystal structures suggested that two surface-exposed glutamates from BtuF could interact with arginine residues on the periplasmic surface of the BtuC dimer (92). These glutamate and arginine residues had already been reported

to be conserved among BPs and IMPs related to iron and B_{12} uptake (116). Thus they may play a more general role in protein-protein interaction and the induction of conformational changes. When the ligand-bound and the apo structures of BtuF were compared it was speculated that the interaction of BtuF with the hydrophobic BtuC might induce the unwinding of a surface located alpha-helix in the C-terminal domain of BtuF. Such a conformational change could be important for triggering the release of vitamin B_{12} into the transport cavity and further passage through the $BtuC_2D_2$ complex in the CM (93).

In most ABC transporters the mode of recognition and interaction of the components involved is probably very similar. However, the interaction of the integral membrane proteins at the molecular level is not well studied. The special arrangement of the two major FhuB domains allowed the isolation of point mutations at positions important for the "interdomain interaction". Under normal conditions, there seems to be a tight interaction between the two FhuB halves which cannot be disturbed by addition of an extra N- or C- terminal domain (118). However, in single site mutants FhuB(P60L) and FhuB(G426R) the mutated half, which was still linked to the rest of the protein, could be displaced by the separately synthesized wild-type half. This resulted in a transport competent complex as indicated by transport studies and growth experiments. It is suggested that the mechanism of functional complementation is a “domain displacement“ in the lipid bilayer of an existing non-functional part of the protein (118). The FhuB[N]-FhuB[C] interaction can probably serve as model for the interaction of the other siderophore family IMPs, which arrange as homo-dimers or hetero-dimers.

Interaction of IMP FhuB with ATPase FhuC was first shown by dominant negative effects on transport of FhuC derivatives with point mutations in the putative ATP-binding domains. In addition, immunoelectron microscopy with anti-FhuC antibodies showed that FhuC needs FhuB for the association with the cytoplasmic membrane (107). In a *fhu* wild-type strain moderately overexpressed FhuB derivatives (mutated in the CRs at positions G226 and G559; see above) caused a negative complementing phenotype to various extents as shown by growth tests, and transport rates. From the experimental data it was concluded that the CR is involved in the interaction with FhuC: FhuB derivatives with only one defective CR are still able to interact with FhuC, via their intact CR. As a consequence, ferric hydroxamate uptake is reduced due to the fact that there is not enough FhuC protein available to interact with the wild-type FhuB protein encoded by the chromosome. In contrast, FhuB derivatives with two defective CRs sequester FhuC to a significantly lower extent (or not at all), leading to only a slight reduction in siderophore transport (118). A more precise molecular view identifying amino acid residues as potential candidates for protein-protein interaction and suggesting possible mechanisms of ATPase-IMP interaction was possible by taking advantage of the atomic structure of the $BtuC_2D_2$ complex for vitamin B_{12} uptake. In that composition the BtuC homo dimer resembles the two halves of FhuB whereas the BtuD units are comparable to the FhuC components.

4.4. ABC transport systems of the ferric iron type

Transport systems of the ferric iron type clearly differ from the other importers. Except very few signatures, such as the CR in the IMPs or the Walker A and B motives in the ATPase components, they do not share obvious sequence similarity to the components involved in siderophore/heme/vitamin B_{12} uptake, and most of all, they function in the absence of any siderophore. The first transporter of this type characterised as iron supply system was the SfuABC system of *Serratia marcescens* (119). A comparable import system, termed FbpABC, was reported from *Neisseria gonorrhoea* and *Neisseria meningitidis* (see 6, 7, 8, 20, 120 and references therein), whereas HitABC represents the homologous uptake system from *Haemophilus influenzae* (121, 122). A similar iron- and Fur-regulated siderophore-independent system composed of the YfuABC proteins has been characterised in *Yersinia pestis,* and *Y. enterocolitica* (123, 124). More importers of this type have been detected by analysing the genomes of various bacteria e.g., *Actinobacillus pleuropneumoniae, Bacillus halodurans, Campylobacter jejuni, Ehrlichia chaffeensis, Halobacterium spec., Brachyspira hyodysenteriae, Pasteurella haemolytica, Pseudomonas aeruginosa, Vibrio cholerae.*

In certain bacterial species ferric iron is sequestered from lactoferrin or transferrin and delivered into the periplasm in a receptor-mediated Ton complex-dependent fashion. The Sfu/Fbp like systems then contribute to the virulence of pathogenic bacteria in that they mediate the further transfer of ferric iron into the cytoplasm (7, 8, 9, 125). With respect to the genetic organisation of ferric iron transport components there seems to be little variation among most bacterial species. A predicted iron regulated operon contains genes encoding - in this order - the substrate binding protein, the IMP, and the ATPase (119, 126). Transcription assays using reverse transcriptase PCR amplification confirmed that the *fbpABC* region is organized as a single expression unit in *Neisseria meningitidis* (127). The chromosomal *bit* region of *Brachyspira hyodysenteriae* constitutes an exception from the rule in that three copies of potential BP encoding genes are followed by a putative ATPase gene and two IMP genes (52).

The ferric binding protein (FbpA) is highly conserved in pathogenic *Neisseria* and represents one of the major iron regulated proteins (128, 129, 130). The first crystal structure was solved for the HitA protein which is the FbpA homologue from *Haemophilus influenzae*. (131) The structure displays all characteristics of the "classical" arrangement including a flexible hinge region. There is striking evidence that iron binding in HitA and transferrin was the result of independent convergent evolution. From structural comparison of HitA with other prokaryotic BPs and the eukaryotic transferrins it is concluded that these proteins are related by divergent evolution from an anion-binding common ancestor, not from an iron-binding

ancestor. The iron binding site of HitA incorporates an exogenous phosphate ion and a H_2O molecule as iron ligands (131, 7). Shouldice *et al.* (132) determined the first crystal structure of a new class of bacterial iron-binding proteins belonging to the transferrin structural superfamily. They report the 1.2-Å structure of the iron-free (apo) form of the FbpA homologue (PhFbpA) isolated from the Gram-negative pathogen *Pasteurella haemolytica*. According to their model for octahedral iron coordination three tyrosine residues, a glutamine and two carbonate anions are involved in metal chelation. In addition to the unique constellation of binding site residues and anions, the presence of two formate anions on opposite sides of the iron-binding pocket was an unexpected finding. These formate ions, which are thought to tether the N- and C-terminal domains of the protein and stabilize the closed structure, might also represent suitable candidates for synergistic anions in the iron-loaded state of PhFbpA. In various bacterial species more than 20 bacterial homologues were identified that probably adopt similar folds, thus playing biological roles similar to PhFbpA in their respective organisms (132).

The typical IMPs of the ferric iron type form an own distinct group showing an internal homology. Each half is significantly smaller than the homologous components of most other ABC transporters, and harbours a CR as putative interaction site with the ATPase. Breaking the "rule", the hydrophobic membrane domains of the *Brachyspira hyodysenteriae* Bit system are expressed as two separate proteins (52). The ATPases from ferric iron transport systems show the "typical" characteristics and are supposed to follow the same mechanism of energising the translocation step of substrates into the cytosplasm as described in section 3.3..

4.5. ABC transport systems of the iron/metals type

Originally, the binding proteins of some of this third group of ABC type importers related to iron uptake in bacteria have been described as adhesins in streptococcal pathogens. (133, 134, 135) Their role in pathogenesis is now understood as they indirectly influence the expression of adhesins (136, 134, 137, 138). ABC transport systems of the iron/metal type are present in many bacterial species, e.g. *Archaeoglobus fulgidus, Bacillus subtilis*, *Enterococcus faecalis*, *Erysipelothrix rhusiopathiae*, *Escherichia coli*, *Haemophilus influenzae*, *Methanobacterium thermoautotrophicum, Staphylococcus epidermidis*; *Streptococcus pneumoniae*, *S. sanguis*, *S. parasanguis*, *S. gordonii*, *S. crista*, *Synechocystis* sp., *Treponema pallidum*; *Yersinia pestis.* Many systems have a higher specificity for metals like zinc or manganese and only a small number of uptake systems seem to be primarily involved in the acquisition of iron. The genes of the iron- and Fur-regulated *sitABCD* operon of *Salmonella enterica* serovar Typhimurium are expressed under iron-deficient conditions. This observation and the fact that these genes were required for full virulence in mice led to the conclusion that the Sit system might play an important role in iron acquisition (139). Further investigations, however, revealed that the SitABCD proteins under physiological conditions function primarily as a Mn^{2+} transporter rather than as a Fe^{2+} uptake system (140). Similarly, transposon insertions and growth experiments indicate a contribution to manganese acquisition of the homologous *sitB* and *sitD* genes in *Sinorhizobium meliloti* (141).

Nonetheless, some ABC systems of the iron/metal type have been demonstrated to be essential for iron acquisition, e.g., Yfe of *Yersinia pestis*. The expression of the putative polycistronic *yfeABCD* operon is subject to a negative regulation by iron and manganese via Fur. The YfeABCD transporter complex has been shown to mediate the uptake of both ^{55}Fe and ^{54}Mn. Moreover, the components are required for full virulence of plague (142, 143).

Like in the ABC transport systems mentioned above, the genes encoding components of metal type ABC transporters are often organised in operons. The expression of the vast majority seems to be regulated by the degree of metals present in the environment, often depending on the metal(s) to be transported. A number of repressors (acting at the transcriptional level) with different metal binding specificity and different recognition sequences on the DNA have been identified (138.).

The crystal structures of PsaA from *Streptococcus pneumoniae* and TroA from *Treponema pallidum* have been solved at 2.0 and 1.8 Å solution, respectively (94, 95). Both proteins consist of an N- and C-lobe, each composed of β-strand bundles surrounded by α-helices. The two domains are linked together by a single helix. As also found for the structurally similar siderophore binding protein FhuD the structural topology was fundamentally different from that of other "classical" ABC-type binding proteins in that PsaA and TroA were lacking the characteristic 'hinge peptides' involved in conformational change upon solute uptake and release (94, 95). The structure of ZnuA, the periplasmic zinc binding protein from *Synechocystis* 6803, has been determined to a resolution of 1.9Å. In contrast to the previously determined structures of homologous proteins (in which the bound metal is chelated by four residues), ZnuA binds zinc via only three histidine residues and the fourth residue aspartate is replaced by a large hydrophobic cavity. In addition, ZnuA possesses a highly charged and mobile loop that protrudes from the protein in the vicinity of the metal-binding site. Similar loops seem to be typical of other zinc binding proteins and may function as a zinc chaperone to facilitate acquisition (144).

Further experimental evidence suggests that the BPs of the metal type systems do not completely share metal specificity: *Salmonella typhimurium* SitA binds primarily mangenese (140), *Yersinia pestis* YfeA iron and manganese (143). PsaA from *Streptococcus pneumoniae* is presumed to bind primarily Mn^{2+} and possibly Zn^{2+} (95), *S. pneumoniae* AdcA and *Treponema pallidum* TroA primarily Zn^{2+} and possibly Fe^{2+} (94, 96), and *Synechocystis* MntC binds Mn^{2+} (145). The variation in metal specificity amongst the metal type BPs is reflected by the variation in those residues (His, Asp, Glu) that are sequence-related to

the metal-coordinating residues thus the BPs can be grouped into several subclusters (138). The IMPs of metal type systems display characteristics typical of hydrophobic components from most ABC transporters. The same holds true for the corresponding ATPases.

4.6. ABC transport systems with fused transmembrane/ ATPase domains

A separate and unusual class of ABC transport system was first found in *Yersinia* strains. YbtP and YbtQ in *Yersinia pestis* are involved in the uptake of the siderophore yersiniabactin. They are unique as uptake components in that a membrane embedded domain and a carboxy-terminally located ATPase portion are realised in a single polypeptide chain. Before the characterization of these importers similar fused function permeases were exclusively associated with known export systems. A *Y. pestis* strain carrying a chromosomal *ybtP* mutation was impaired in iron accumulation and was avirulent in mice by a subcutaneous route of infection that mimics flea transmission of bubonic plague (146). Irp6 and Irp7 from *Yersinia enterocolitica* display significant similarity to YbtP and YbtQ. They function accordingly since it has been demonstrated that they are required for yersiniabactin utilization and mouse virulence (147). A gene displaying sequence similarity to *ybtQ* from *Y .pestis* was also found to be specifically present in the opportunistic pathogen *Pseudomonas aeruginosa,* strain PA14, but was missing in the less virulent strain PAO1. Strain PA14 mutated in the *ybtQ* homolog was significantly attenuated with respect to its virulence in both the greater wax moth, *Galleria mellonella*, and in a burned mouse model of sepsis (148). The diphtheria toxin repressor, DtxR, is a global iron-dependent regulatory protein in *Corynebacterium diphtheriae*. DtxR, acting in a similar fashion as the Fur repressor, controls gene expression by binding to 19-bp operator sequences. Most recently, CdtP and CdtQ displaying similarity to the *Y. pestis* YbtPQ transporters, were identified as members of the DtxR regulon. A mutation in *cdtP* resulted in reduced growth in iron-depleted media indicating that this system may contribute to iron uptake in *Corynebacterium diphtheriae* (149). Putative proteins with similarity to YbtP and YbtQ are present in many species (e.g. *Escherichia coli CFT073, Wolinella succinogenes, Zymomonas mobilis, Streptomyces coelicolor, Gloeobacter violaceus, Bacteroides thetaiotaomicron, Ralstonia solanacearum, Proteus mirabilis, Clostridium tetani, Staphylococcus aureus, Mycobacterium avium*) thus indicating that the fused transmembrane/ATPase import systems might be widely distributed among bacteria. At the moment it is a moot point whether the uptake of siderophores via this mechanism functions without any binding protein involved.

5. PERSPECTIVE

For the last decades many laboratories concerned with iron acquisition in bacteria concentrated their research activities on transport phenomena that were related to the OM of Gram-negatives or/and components and mechanisms involved therein. Less effort was made to study processes associated with further transfer of iron into the cytoplasm. There is no doubt, however, that the different systems mediating the passage of iron (and other metals) through the cytoplasmic membrane represent interesting research topics of general interest. The existing data are very promising and it is still challenging and important to understand more details of protein structure and folding of polypeptides, topology and membrane insertion of proteins, intra- and inter-molecular interactions, and substrate binding and translocation mechanisms at the molecular level. Moreover, the unique features of iron uptake systems still make the components involved suitable candidates to examine their potential as targets for antimicrobial agents and to investigate their role in virulence mechanisms of pathogens. Since proteins trapped in a crystal structure always represent a snap shot of a dynamic process, there is no doubt that more structural data and further studies will be needed in order to unravel the details of the actual translocation processes. Further surprises can be expected (like the novel fold of BPs).

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Key Words: ABC transporter, Binding Proteins, Iron Uptake, Siderophore, Heme, Vitamin B_{12}, Review

Send correspondence to: Dr. Wolfgang Köster, VIDO - Vaccine & Infectious Diseases Organization, University of Saskatchewan, VIDO Building, 120 Veterinary Rd., Saskatoon, Saskatchewan S7N 5E3, Canada, phone: +1-306-966-7479, fax: +1-306-966-7478, e-mail: wlk498@mail.usask.ca

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