In vivo reconstitution of an active siderophore transport system by a binding protein derivative lacking a signal sequence

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ORIGINAL PAPER

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In vivo reconstitution of an active siderophore transport system by a binding protein derivative lacking a signal sequence

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Abstract Transport of iron(III) hydroxamates across the inner membrane of *Escherichia coli* depends on a binding protein-dependent transport system composed of the FhuB,C and D proteins. The FhuD protein, which is synthesized as a precursor and exported through the cytoplasmic membrane, represents the periplasmic binding protein of the system, accepting as substrates a number of hydroxamate siderophores and the antibiotic albomycin. A FhuD derivative, carrying an N-terminal His-tag sequence instead of its signal sequence and therefore not exported through the inner membrane, was purified from the cytoplasm. Functional activity, comparable to that of wild-type FhuD, was demonstrated for this His-tag-FhuD in vitro by protease protection experiments in the presence of different substrates, and in vivo by reconstitution of iron transport in a *fhuD* mutant strain. The experimental data demonstrate that the primary sequence of the portion corresponding to the mature FhuD contains all the information required for proper folding of the polypeptide chain into a functional solute-binding protein. Moreover, purification of modified periplasmic proteins from the cytosol may be a useful approach for recovery of many polypeptides which are normally exported across the inner membrane and can cause toxicity problems when overproduced.

Key words Solute-binding protein · Periplasmic binding protein · ABC transporter · Iron(III) hydroxamate transport · *E. coli*

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Introduction

Transport of Fe^{3+} siderophores of the hydroxamate type across the outer membrane of *Escherichia coli* requires highly specific receptor proteins such as FhuA, FhuE or Iut (Braun and Hantke 1990). Energy coupling of the transport process is dependent on the cytoplasmic membrane-bound TonB, ExbB and ExbD proteins (Hancock and Braun 1976; Eick-Helmerich and Braun 1989). For subsequent translocation across the cytoplasmic membrane a single periplasmic binding protein-dependent (PBT) system, composed of the FhuC,D,B proteins, accepts all ferric hydroxamates and the antibiotic albomycin (Braun and Hantke 1990; Köster 1991). In this system FhuC represents the membrane associated cytosolic component which energizes the system via ATP hydrolysis (Burkhardt and Braun 1987; Coulton et al. 1987; Schultz-Hauser et al. 1992a, b). FhuB was identified as the integral membrane component (Köster and Braun 1986, 1989, 1990a; Köster and Böhm 1992). FhuD, synthesized as a precursor with a typical signal sequence, was found to be processed and exported to the periplasmic space (Fecker and Braun 1983; Burkhardt and Braun 1987; Coulton et al. 1987; Köster and Braun 1989). Binding of different iron(III) hydroxamates to the mature FhuD protein has been reported (Köster and Braun 1990b).

FhuD is absolutely essential for ferric hydroxamate transport. But even under iron-limiting conditions it is produced in extremely low quantities when expressed from the chromosome. In order to examine the properties of FhuD in vitro it was necessary to overproduce and purify the protein. On the chromosome, the *fhuABCD* genes are organized in an operon (Fecker and Braun 1983); *fhuC* and *fhuD* are likely to be translationally coupled since translation stop and start codons overlap. Moreover, a weak ribosome-binding site was identified upstream of *fhuD*. This may explain why the wild-type gene (including its own translation initiation

Table 1 Bacterial strains and plasmids

Strain or plasmid	Relevant genotype	Source or reference
AB2847	*aroB malT thi tsx*	This institute
KO293	As AB2847 but *zif*::Tn10 *metE fhuD*	Köster and Braun 1990 b
KO295	As AB2847 but *zif*::Tn10 *metE fhuD*	This study
MR1	As KO295 but *tonB*	This study
BL21(DE3)	pLysS F^- *hsdS gal* phage T7 polymerase under *lac*UV5 control, Cm^r	Studier and Moffat 1986
pGP1-2	T7 polymerase under cI857 control	Tabor and Richardson 1985
pT7-6	ColE1 ori, T7 promoter, Amp^r	Tabor and Richardson 1985
pT7-7	ColE1 ori, T7 promoter, Amp^r	Tabor and Richardson 1985
pWK480	pT7-6, $fhuD^+$	Köster and Braun 1990b
pSP22	pT7-7, $'fhuD^+$	This study
pET19-b	ColE1 ori, T7 /*lac* promoter/operator, "His-tag" region, "fusion cloning site", $lacI^+$, Amp^r	Novagen, Germany
pMRE	pET19-b, modified fusion cloning site	This study
pMR21	pMRE, $'fhuD^+$	This study
pCG752	pT7-5, $tonB^+$	Fischer et al. 1989

region), cloned downstream of a strong phage T7 promoter (Tabor and Richardson 1985), rendered only a moderate FhuD overexpression in the T7 system.

In the present study, when *fhuD* was cloned into plasmid pT7-7, thereby taking advantage of vector-borne DNA structures optimal for transcription and translation initiation, expression of a FhuD derivative containing an altered signal sequence was found to be lethal for host strains KO293 (*fhuD*, pGP1-2) and KO295 (*fhuD*, pGP1-2). After overexpression in a suitable host strain (BL21 DE3 pLys; Studier and Moffat 1986) predominantly unprocessed protein was found, presumably because the Sec export system was overloaded. In order to overcome these difficulties, we investigated whether a protein that is normally exported could be isolated in an active conformation from the cytoplasm, even when synthesized as a derivative lacking its signal sequence. It was of special interest to find out whether components that are thought to be involved in a late translocation step across the inner membrane (Bieker-Brady and Silhavy 1992; Thome and Müller 1991; Matsuyama et al. 1993) and/or components from the periplasm which may act as potential chaperones (Baichwal et al. 1993; Kamitani et al. 1992; Missiakas et al. 1993; reviewed in Wülfing and Plückthun 1994) contribute to folding, substrate binding and transport activity of FhuD. Consequently, a DNA fragment encoding only the mature FhuD protein was inserted into the modified expression vector pET19-b. In the resulting FhuD derivative, the signal sequence of wild-type FhuD is replaced by a His-tag sequence. His-tag-FhuD was overproduced and subsequently purified on a Ni-chelate resin. Biological activity, comparable to that of wild- type FhuD, was shown in vitro by protease protection experiments, and in vivo by restoration of iron transport in a *fhuD* mutant strain. To our knowledge this is the first report of a transport-active periplasmic binding protein that is devoid of a functional signal peptide and can be purified from the cytoplasm.

Materials and methods

Bacterial strains

The bacterial strains used in this study are derivatives of *E. coli K12*, except for the *E. coli* B derivative BL21, and are listed in Table 1. KO293 and KO295 are *fhuD* strains isolated as two independent clones that had spontaneously become resistant to albomycin. MR1 *fhuD*, *tonB* was isolated as a KO295 derivative which was resistant to phage Φ 80 and colicin B. A *fhuA* mutation was excluded since MR1 was still sensitive to phage T5. The *tonB* function could be complemented by plasmid pCG752 containing the wild-type *tonB* gene.

Media and growth conditions

Bacteria were routinely grown at 37° C in TY and NB media as described previously (Fecker and Braun 1983; Köster and Braun 1986, 1989). Albomycin sensitivity was determined on TY plates according to Fecker and Braun (1993).

Recombinant DNA techniques

DNA isolation, digestion with restriction endonucleases, ligation, transformation and agarose gel electrophoresis were as outlined by Maniatis et al. (1982).

Plasmids

Construction of pSP22

A 1.4 kb *Hpa*I-*Cla*I fragment containing the 5′ truncated *fhuD* gene from pWK480 (Köster and Braun 1990b) was inserted into the

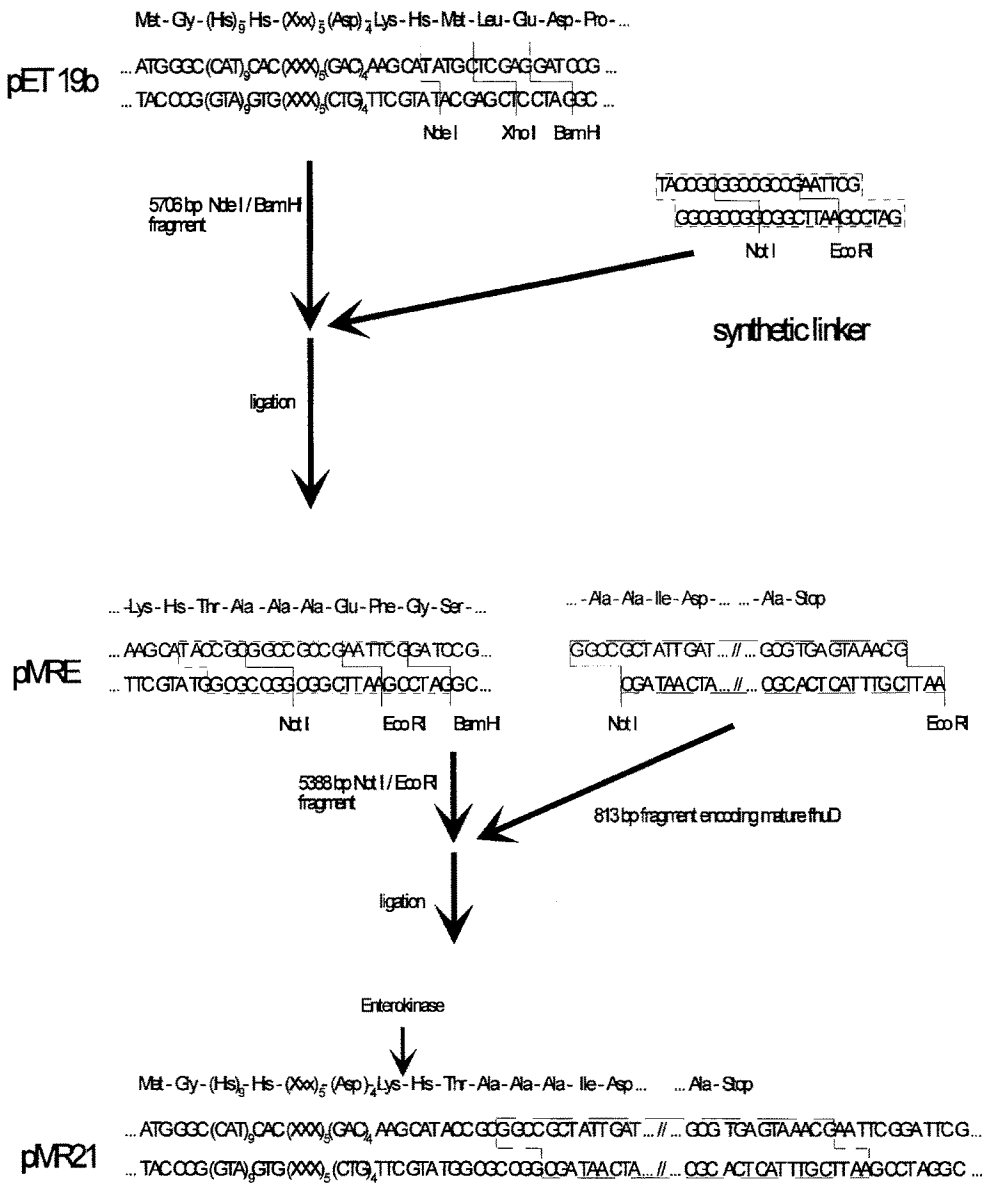

Fig. 1 Construction of pMR21 encoding His-tag-FhuD (details of experimental procedures are given in the Materials and methods)

*Sma*I/*Cla*I sites in the polylinker region of expression vector pT7-7 (Tabor and Richardson 1985) downstream of the phage T7 phi 10 promoter. Moreover, the vector provided an ideal Shine-Dalgarno Box located an optimal distance from the translational ATG start codon. The resulting plasmid, pSP22, coded for a FhuD polypeptide with an altered signal sequence: (MSGLPLISRRRLLTAMAL-SPLLWQMNTAHA, ... changed to MARIRARPTAMAL-SPLLWQMNTAHA, ... the cleavage site is indicated by the comma. Synthesis of this FhuD construct was strictly dependent on the presence of T7 RNA polymerase.

Construction of pMR21

Expression vector pET19-b (Novagen, Germany) offers the possibility to fuse any gene in-frame to a DNA region which codes for a histidine-rich N-terminal amino acid sequence. Ten histidine residues within that His-tag sequence allow for easy purification of the fusion protein (see below). The His-tag sequence $[MG[H]_{10}SSGHI[D]_4K]$ can be removed from the purified protein by enterokinase cleavage (after the lysine residue at position 21; Fig. 1).

The multiple cloning site of pET-19b was modified by inserting a synthetic linker into the *Nde*I/*Bam*HI restriction sites (Fig. 1). The resulting construct, pMRE, contained an additional *Not*I and an additional *Eco*RI site. The *fhuD* sequence of pWK480 (Köster and Braun 1990b) coding for the mature protein was amplified by the polymerase chain reaction (PCR) method using the primers FDH: 5′-GCCCAC**GCGGCCGC**TATTGAT-3′ and FD2: 5′-GAAAAG-CG**GAATTC**GTTTACCT-3′. FDH creates a unique *Not*I site at the position which corresponds to the cleavage site of the leader peptidase within FhuD (Fig. 1). FD2 inserts a unique *Eco*RI site downstream of *fhuD*. The amplified DNA fragment was then inserted into the *Not*I + *Eco*RI-digested pMRE, resulting in pMR21 (Fig. 1). pMR21 allowed the expression of His-tag-FhuD from the phage T7 Φ10 promoter located upstream of the *lac* operator. Transcription was induced with isopropylthiogalactoside (IPTG) to prevent LacI (the gene for which was also present on pMR21) from binding to the operator region. With the ribosome binding site at an

optimal distance, translation starts at the first codon of the His-tag sequence.

Overproduction of wild-type FhuD and derivatives

Protein expression was performed in the host strain BL21 DE3 pLys as described (Studier and Moffat 1986). For expression, cells were grown in TY medium and induction of the T7 system was carried out at an $OD_{578\ nm}$ of 0.8–1.0 with 1 mM IPTG. After further incubation for 2 h at 37° C cells were collected by centrifugation, washed once with 0.9% NaCl and stored at −20° C.

Purification of His-tag-FhuD

Crude cell extracts were obtained by disrupting cells in a French pressure cell using binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM TRIS-HCl, pH 7.9). All buffers used during the purification procedure contained 0.1 mM *p*-amino-benzamidine and 0.2 mM phenylmethylsulphonyl fluoride (PMSF). The disrupted cells were sedimented and the supernatant containing soluble proteins was loaded onto a Ni-chelate column (bed volume 1.5–2 ml; Diagen, Germany), equilibrated with binding buffer. After two wash steps of ten bed volumes binding buffer and ten volumes wash buffer (binding buffer, supplemented with 60 mM imidazole), bound protein was eluted with five bed volumes of elution buffer (binding buffer, supplemented with 200 mM imidazole). For purification to near homogeneity, purified protein was again loaded onto a fresh Ni-chelate resin and purified a second time. Purified protein was stored in a solution containing 15% glycine at −20° C.

Protease protection experiments

Twenty micrograms of purified His-tag-FhuD were digested with 0.2 µg proteinase K for different times at 56° C in the presence or absence of 30 µM aerobactin, ferrichrome, schizokinen, and coprogen, respectively. As a control, the protein was incubated without proteinase K and substrates for 30 min at 56° C to demonstrate its stability. After incubation, proteins were precipitated with 10% trichloroacetic acid (final concentration) and separated on a sodium dodecyl sulphate (SDS)-polyacrylamide gel according to the method of Lugtenberg et al. (1975), (see also Köster and Braun 1986, 1989).

Protein sequencing

Proteins isolated from SDS-polyacrylamide gels were sequenced by Edman degradation according to the method described by Matsudaira (1987).

Restoration of iron transport

For introduction of purified His-tag-FhuD into the periplasm of strain KO295 *fhuD*, a method for partial, reversible permeabilization of the outer membrane was applied (Brass 1986). Cells of the logarithmic growth phase (1×10^9 cells), grown under slightly iron-limiting conditions in nutrient broth were collected by centrifugation, resuspended and washed at room temperature in 1 ml 100 mM TRIS-HCl buffer, pH 7.5, and then in 1 ml 100 mM potassium phosphate buffer, pH 7.5. The supernatant was completely removed, and the cells were resuspended in 50 µl of ice-cold 100 mM TRIS-HCl buffer, pH 7.5, containing 300 mM $CaCl_2$ and 0.2 mg purified His-tag-FhuD. The reconstitution mixture was shaken at 0° C for 30 min, washed once with 1 ml 0.9% NaCl, resuspended in 1 ml M9 (Maniatis et al. 1982) and kept at room temperature until uptake assays, with 10 mM [^{55}Fe] ferrichrome, were performed as described (Köster and Braun 1990a). As controls, iron transport assays were performed with cells permeabilized in the presence of bovine serum albumin (BSA) or in the absence of any protein. The amount of radioactivity in the cells was determined by liquid scintillation counting.

Results

High-level overexpression of pro-FhuD results in inefficient processing and cell death

Under physiological conditions, a large number of proteins is present in very low amounts, even under inducing (or derepressing) conditions. In order to obtain enough material for an efficient purification and characterization, the genes encoding such proteins are usually cloned into high copy-number plasmid vectors downstream of a strong promoter. Such an approach with *fhuD* resulted in plasmid pSP22, a derivative of expression vector pT7-7, encoding a FhuD derivative with an N-terminally altered signal sequence (for details see the Materials and methods). Iron(III) hydroxamate transport and albomycin sensitivity were restored in strains KO293 *fhuD* and KO295 *fhuD* carrying pSP22, when the heat-inducible T7 RNA polymerase gene on plasmid pGP1-2 was provided. Unfortunately, FhuD production killed the hosts, even when only small amounts of the T7 polymerase were present at the non-inducing temperature of 27° C. After transformation of KO293 *fhuD* and KO295 *fhuD* with pGP1-2 and pSP22, transformants were able to form colonies overnight on agar plates but survived for only a single albomycin sensitivity test. Therefore, a different host strain, BL21 DE3 pLys (Studier and Moffat 1986), was used. Overproduction of the FhuD derivative from pSP22 was much more efficient than that of wild-type FhuD from pWK480, but processing was very inefficient (data not shown). Whether the accumulation of pro-FhuD (from pSP22) was only the result of the strong overproduction and/or due to the altered N-terminus of the signal sequence was not determined.

Purification of a signal sequence-free FhuD protein from the cytoplasm

In spite of the problems of overproduction (see above), we still wished to isolate a functional, mature binding protein and not its precursor. Therefore we, first addressed the question whether a normally periplasmic protein purified from the cytoplasm would be able to adopt a substrate binding-competent formation. The assumption that components involved in late stages of *sec*-dependent translocation (Bieker-Brady and Silhavy 1992; Matsuyama et al. 1993; Thome and Müller 1991) as well as specific or general chaperones

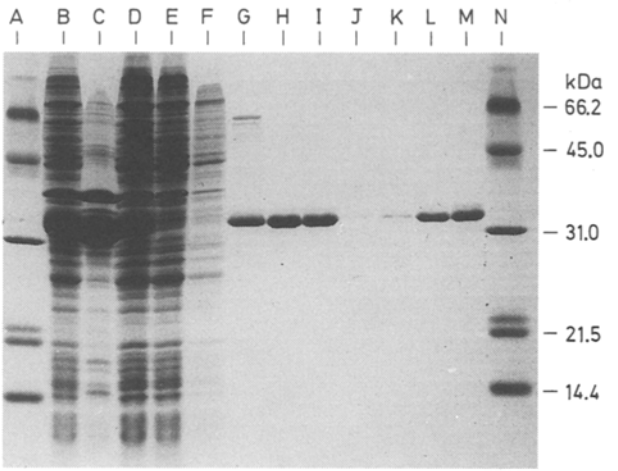

Fig. 2 Purification of His-tag-FhuD. Fractions are displayed on a Coomassie blue-stained sodium dodecyl sulphate (SDS)-polyacrylamide gel. Lanes A, and N, molecular weight standard; lane B, whole-cell protein; lane C, insoluble part of cell lysate; lane D, crude cell extract loaded onto the column; lane E, unbound proteins; lane F, wash step with binding buffer; lane G, wash step with wash buffer; lane H, eluted protein; lane I, protein loaded onto a fresh column after change of buffer; lane J, K, wash steps; lane L, second elution; lane M, protein after desalting

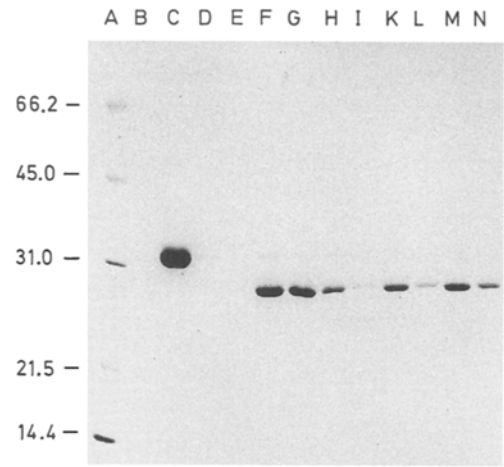

Fig. 3 In vitro protease protection of purified His-tag-FhuD. Treated samples were fractionated on a Coomassie Blue-stained SDS-polyacrylamide gel. Lane A, Molecular weight standard; lane B, 0.2 μg proteinase K; lane C, untreated protein; lanes D, E, 20 min, 40 min plus proteinase K without ferric siderophores; lane F, G, 20 min, 40 min plus proteinase K plus aerobactin; lanes H, I, 20 min, 40 min plus proteinase K plus ferrichrome; lanes K,L, 20 min, 40 min plus proteinase K plus coprogene; lanes M,N, 20 min, 40 min plus proteinase K plus schizokinen

in the periplasm (Baichwal et al. 1993; Bardwell et al. 1993; Kamitani et al. 1992; Missiakas et al. 1993) contribute to formation of the functional conformation of a variety of exported proteins (Wülfing and Plückthun 1994), made this question a topic of interest in its own right.

For this purpose, a fusion protein was constructed in which a cleavable His-tag sequence was linked to the mature form of FhuD (see the Materials and methods; Fig. 1). His-tag-FhuD, when expressed in strains KO293 and KO295, was found in whole cell preparations and in the cytoplasmic fraction, but was not detected in the periplasmic fraction (supernatant of spheroplasts) and was not able functionally to complement the transport function — to allow growth with ferrichrome and coprogen as sole iron source – in these chromosomal *fhuD* mutant strains (data not shown). Therefore it was concluded that the FhuD derivative remained trapped in the cytoplasm. For the purification, His-tag-FhuD was produced in strain BL21 DE3 pLys. Lanes B to M of Fig. 2 show the results of a typical purification procedure (for experimental procedures see the Materials and methods) for His-tag-FhuD. Approximately 50% of the protein produced was isolated from the soluble moiety of the disrupted cells. A notable amount of protein was lost, probably due to the formation of inclusion bodies which could be collected by low-speed centrifugation. Attempts to purify the protein under denaturing conditions (6 M guanidine-HCl) to increase the yield of isolated protein were not successful, since only biologically inactive protein was obtained. As confirmed on SDS-polyacrylamide gels, the purified protein had an apparent molecular weight of 32 kDa, in good agreement with the size predicted from the DNA sequence. On a Coomassie Blue stained SDS-polyacrylamide gel (Fig. 2, lanes L,M) the protein appears as a single homogenous band; moreover no impurities were detected even by silver staining (data not shown). From a 500 ml culture about 6 mg of purified protein was routinely obtained.

Substrate binding to His-tag-FhuD in vitro

Purified His-tag FhuD (Fig. 3, lane C), like the wild-type FhuD polypeptide (Köster and Braun 1990b), was totally degraded by proteinase K in the absence of ferric siderophores (Fig. 3, lanes D, E). Proteinase K alone (Fig. 3, lane B), applied in the same amounts as used for the protease protection assay, was not detected by Coomassie Blue staining. Digestion of purified His-tag-FhuD with proteinase K (for 20 min and 40 min) in the presence of ferric hydroxamates resulted in a stable protein band with an apparent molecular weight of 28.5 kDa (Fig. 3, lanes F – N). The best protection was achieved with aerobactin (Fig. 3, lanes F, G), while the other ferric hydroxamates tested, although allowing a significant amount of protein to remain undigested, had less stabilizing effect. With respect to FhuD protection, the order of effectiveness was schizokinen (Fig. 3, lanes M, N), followed by coprogen (lanes K, L) and ferrichrome (lanes H, I). In summary, these data were consistent with the results obtained for wild-type FhuD, thus indicating that the interaction with

appropriate substrates induces a conformational change or stabilizes a protease-resistant structure.

Protein representing the "protected band" was isolated from the gel and subsequently sequenced by Edman degradation. It is noteworthy that, the amino acid sequence determined from the truncated protein (Ala-Ile-Asp-Pro-Asn-Arg-Ile-Val...) was identical to the predicted N-terminus of mature FhuD (Ala-Ala-Ile-Asp-Pro-Asn-Arg-Ile-Val...) except for one missing alanine residue. Obviously, for the experiments described above, removal of the His-tag sequence by enterokinase prior to the assay was not necessary to obtain a substrate binding-competent FhuD.

Restoration of ferrichrome transport in KO295 *fhuD*

Based on the results of the in vitro studies, we addressed the question whether purified FhuD was functional if added to the periplasm from outside the cell, instead of taking the usual export route from the cytoplasm across the inner membrane. Moreover, we wanted to know whether the His-tag sequence in vivo interfered with substrate binding and/or the interaction with other Fhu transport components. In vivo activity of purified His-tag-FhuD was studied by measuring ferrichrome uptake into strain KO295 carrying a chromosomal *fhuD* mutation. By reversible and partial permeabilization of the outer membrane, purified binding protein was introduced into the periplasm of the mutant cells and iron ferrichrome transport was assayed using [^{55}Fe] (Fig. 4).

Significant transport of iron, as [^{55}Fe] ferrichrome, was found in KO295 with His-tag-FhuD trapped in the periplasmic space (Fig. 4). This transport was comparable to that of KO295 expressing plasmid-encoded wild-type *fhuD* (data not shown). A chromosomal *fhuD*$^+$ strain could not serve as an appropriate control since the *fhuD*295 mutation displayed a strong polar effect and therefore resulted in drastically reduced expression of the *fhuB* and *fhuC* genes, which are located in the same operon (W. Köster, unpublished results). Unfortunately, the reconstitution experiment with wild-type FhuD protein, a control that would be helpful to evaluate the quality of the reconstitution, could not be carried out, since there is no methodology available for efficient purification of the extremely low amount of wild-type mature FhuD in the periplasm. As further controls, KO295 treated with bovine serum albumin (data not shown) or buffer instead of the purified protein (Fig. 4), allowed no detectable uptake of [^{55}Fe].

In order to examine whether the accumulation of iron in KO295 (+ His-tag-FhuD) was the result of an active transport process or due to an unspecific effect, such as binding to the cell surface or, leakage into the periplasm, the same experiment was carried out with a strain carrying an additional *tonB* mutation. No

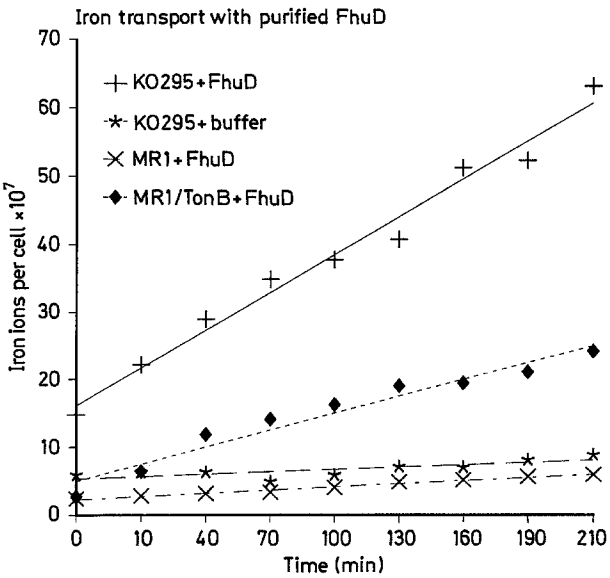

Fig. 4: In vivo restoration of [^{55}Fe] ferrichrome transport in KO295 *fhuD* with purified His-tag-FhuD (for details see text).

His-tag-FhuD mediated [^{55}Fe] ferrichrome transport was observed with MR1 (*fhuD*, *tonB*) indicating that the outer membrane integrity of the reconstituted cells was not impaired during the transport assay. Additional evidence that iron uptake in strain KO295 (+ His-tag-FhuD) indeed depended on functional TonB, ExbB, D and FhuA proteins, was provided by data obtained with MR1 pCG752. In strain MR1 pCG752 with His-tag-FhuD trapped in the periplasm the chromosomal *tonB* mutation was partially complemented by the plasmid-encoded TonB. The finding that transport function was not fully restored relative to KO295 (+ His-tag-FhuD; Fig. 4) was not surprising, since previous results had shown that TonB, expressed from a multicopy plasmid, has a negative impact on uptake of ferrisiderophores and vitamin B_{12} (Mann et al. 1986).

Discussion

The "osmotic shock-sensitive" (Berger and Heppel 1974; Furlong 1987) or "periplasmic binding protein-dependent" (Ames 1986; Furlong 1986; Higgins et al. 1990) transport systems are also termed ABC transporters (Higgins 1992) or traffic ATPases (Ames et al. 1990). Among the components the periplasmic solute-binding proteins are by far the best characterized polypeptides (for an overview see Ames and Joshi 1990; Quiocho 1990; Nikaido and Saier 1992; Tam and Saier 1993). Substrate binding is generally known to induce a substantial conformational change (Newcomer et al. 1981a, b; Olah et al. 1993) or to stabilize a so-called closed formation (Oh et al. 1993). Despite little homology in their primary structure, very similar three-dimensional structures were reported for all binding

proteins that have been studied in detail (Adams and Oxender 1989; Quiocho 1990; Mowbray et al. 1990; Sharff et al. 1993; Spurlino et al. 1991; Vyas et al. 1991; Zou et al. 1993). According to the "venus fly trap" model (Ames 1986), two major lobes are connected by a flexible hinge region (Gilliland and Quiocho 1981). Solute-binding proteins of gram-positive and gram-negative bacteria are known to interact with specific integral cytoplasmic membrane proteins (Gilson et al. 1988; Treptow and Shuman 1988; Saurin et al. 1989; Dean et al. 1989; Prossnitz et al. 1989; Mimmack et al. 1989; Kerppola et al. 1991; Doige and Ames 1993; Higgins et al. 1990; Hor and Shuman 1993), triggering directly (Baichwal et al. 1993) or indirectly (Davidson et al. 1992) the ATP-driven translocation (Bishop et al. 1989; Davidson and Nikaido 1990, 1991) of substrates across the inner membrane.

In the *fhu* system, FhuD has been identified as the solute-binding protein in the periplasm (Köster and Braun 1989). Binding of various iron(III) hydroxamates to FhuD has been demonstrated (Köster and Braun 1990b). Resistance to proteinase K upon substrate binding indicated a conformational change, thus suggesting that the "two-lobe model" may in principle be valid for FhuD. Data obtained from FhuD characterization may be of general importance, at least for the other members of the siderophore-binding protein family which are closely related in structure and function: FecB and FepB from *E. coli*, FatB from *Vibrio anguillarum*, HemT from *Yersinia enterocolitica*, and FhuD from *Bacillus subtilis* (Staudenmaier et al. 1989; Elkins and Earhart 1989; Friedrich et al. 1986; Actis et al. 1988; Köster 1991; Köster et al. 1991; Stojiljkovic and Hantke 1994; Schneider and Hantke 1993).

In contrast to a number of solute-binding proteins involved in transport of certain sugars, the iron(III) hydroxamate binding protein is present in the cell in extremely low amounts. Even under iron-limiting conditions (with *fhuD* present as a single copy on the chromosome or expressed from multicopy plasmids) the FhuD polypeptide is not detectable on SDS-polyacrylamide gels when expressed exclusively from the *fur*-regulated promoter upstream of the *fhu* operon. In order to develop a system which allows an extensive in vitro characterization of FhuD it was necessary to overproduce and purify this protein.

The data presented in this paper show the importance of the translation initiation region for FhuD synthesis. Using its own 5′ upstream sequence, including a weak ribosome-binding site, *fhuD* was only moderately translated in the T7 system. Replacing that upstream region by an ideal Shine-Dalgarno Box led to an enormous increase of protein production. The FhuD construct carrying an altered signal sequence was functional in *fhuD* strains KO293 and KO295. Unfortunately, the protein was harmful to the cells and was found to be processed very inefficiently. At the moment we cannot decide if the abnormal amount of protein interfered with the secretory pathway of the cells, or the altered signal sequence itself delayed processing and/or export.

In order to overcome the problems mentioned above, we developed a strategy which enabled us to purify a functional iron(III) hydroxamate-binding protein devoid of its signal sequence. Moreover, replacement of the signal sequence by a (cleavable) region which allows rapid and effective purification may be a practical approach to isolation of a number of proteins that are normally exported across the inner membrane, if their overproduction causes problems. Lacking a hydrophobic leader peptide, soluble proteins will probably not be directed to the inner membrane and thus cannot interfere with essential membrane associated processes, and the tendency to form inclusion bodies may be reduced. At the same time, our strategy offered an excellent opportunity to test whether any components of the Sec apparatus (e.g. SecD, SKP; Bieker-Brady and Silhavy 1992; Matsuyama et al. 1993; Thome and Müller 1991) and/or so-called general or specific chaperones in the periplasm (Baichwal et al. 1993; Bardwell et al. 1991, 1993; Kamitani et al. 1992; Missiakas et al. 1993; for a review see Wülfing and Plückthun 1994) participate in the accurate folding of FhuD.

The data obtained in vitro from protease protection assays clearly demonstrated that the interaction with different ferric hydroxamates changes the structure of FhuD, to various extents, towards a protease-resistant conformation. At the moment we cannot decide if "better protection" automatically indicates "better binding" of the substrate or suggests a slightly different, "more resistant" conformation of FhuD. There is also no obvious correlation between the degree of protease protection and the ability to grow on the different hydroxamates as sole iron source. This becomes less surprising however, when one considers that siderophore uptake is a multifactorial process involving a number of components other than FhuD to interact with these substrates. An important finding is that FhuD isolated from the cytoplasm exhibited the same efficiency and specificity of substrate binding in vitro as mature wild-type FhuD from the periplasmic space. This implies that the FhuD polypeptide chain is able to spontaneously adopt a siderophore binding-competent conformation. For this process no components seem to be necessary which are either involved in the secretion pathway across the inner membrane or in protein folding in the periplasm. The N-terminal His-tag sequence does not detectably interfere with folding and function. Following substrate binding a protease- resistant conformation of His-tag-FhuD (identified as a 28.5 KDa polypeptide by SDS-polyacrylamide gel electrophoresis) was stabilized. This protein fragment was almost identical in size to mature wild-type FhuD. We favour the idea that the His-tag region does not fold

into the FhuD interior, but forms a kind of extra domain that it is accessible to proteinase K or other unspecific proteases.

Restoration of ferrichrome transport by purified His-tag-FhuD indicates that the polypeptide chain can fold into a binding-competent conformation, which allows a functional interaction with the integral membrane component FhuB. As was found in the case of the wild type, the high specificity of [^{55}Fe] ferrichrome uptake was demonstrated by its dependence not only on FhuD (imported into the periplasm) but also on functional *tonB*, indicating normal receptor-mediated translocation of substrate across an intact outer membrane. To our knowledge this is the first report of purification of a transport-active periplasmic binding protein, synthesized without a functional signal peptide, from the cytoplasm. Of course, correct folding in the cytoplasm does not completely exclude the possibility that the folding process occurring in vivo might still be an event which is facilitated by a specific periplasmic protein. It is however noteworthy that the N-terminal His-tag sequence did not disturb substrate binding or interaction with other Fhu transport proteins. Attempts are currently in progress to generate mutants in FhuD which are impaired in iron(III) hydroxamate binding and/or in the interaction with integral membrane protein FhuB. Now a practical methodology is available for further in vitro and in vivo characterization of such mutants.

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References

Actis LA, Tolmasky ME, Farell DH, Crosa JH (1988) Genetic and molecular characterization of essential components of the *Vibrio anguillarum* plasmid-mediated iron-transport system. J Biol Chem 263:2853–2860

Adams MD, Oxender DL (1989) Bacterial periplasmic binding protein tertiary structures. J Biol Chem 264:15739–15742

Ames GF-L (1986) Bacterial periplasmic transport systems: structure, mechanism and evolution. Annu Rev Biochem 55:397–425

Ames GF-L, Joshi AK (1990) Energy coupling in bacterial periplasmic permeases. J Bacteriol 172:4133–4137

Ames GF-L, Mimura CS, Shyamala V (1990) Bacterial permeases belong to a family of transport proteins operating from *Escherichia coli* to human: traffic ATPases. FEMS Microbiol Rev 75:429–446

Baichwal V, Liu D, Ames GF-L (1993) The ATP-binding component of a prokaryotic traffic ATPase is exposed to the periplasmic (external) surface. Proc Natl Acad Sci USA 90:620–624

Bardwell JCA, McGovern K, Beckwith J (1991) Identification of a protein required for disulfide bond formation *in vivo*. Cell 67:581–589

Bardwell JCA, Lee J-O, Jander G, Martin N, Beckwith J (1993) A pathway for disulfide bond formation *in vivo*. Proc Natl Acad Sci USA 90:1038–1042

Berger EA, Heppel LA (1974) Different mechanisms of energy coupling for shock sensitive and shock resistant amino acid permeases of *Escherichia coli*. J Biol Chem 249:7747–7755

Bieker-Brady K, Silhavy TJ (1992) Suppressor analysis suggests a multistep, cyclic mechanism for protein secretion in *Escherichia coli*. EMBO J 11:3165–3174

Bishop L, Agbayani RJ, Ambudkar SV, Maloney PC, Ames GF-L (1989) Reconstitution of a bacterial periplasmic permease in proteoliposomes and demonstration of ATP hydrolysis concomitant with transport. Proc Natl Acad Sci USA 86:6953–6957

Brass JM (1986) Calcium-induced permeabilization of the outer membrane: a method for reconstitution of periplasmic binding protein-dependent transport systems in *Escherichia coli* and *Salmonella typhimurium*. Methods Enzymol 125:289–302

Braun V, Hantke K (1990) Genetics of bacterial iron transport. In: Winkelmann G (ed) Handbook of microbial iron chelates. CRC Press, Boca Raton, pp 107–138

Burkhardt R, Braun V (1987) Nucleotide sequence of *fhuC* and *fhuD* genes involved in iron (III)-hydroxamate transport: domains in FhuC homologous to ATP-binding proteins. Mol Gen Genet 209:49–55

Coulton JW, Mason P, Allatt DD (1987) *fhuC* and *fhuD* genes for iron(III)-ferrichrome transport into *Escherichia coli* K-12. J Bacteriol 169:3844–3849

Davidson AL, Nikaido H (1990) Overproduction, solubilization, and reconstitution of the maltose transport system from *Escherichia coli*. J Biol Chem 265:4254–4260

Davidson AL, Nikaido H (1991) Purification and characterization of the membrane-associated components of the maltose transport system from *Escherichia coli*. J Biol Chem 266:8946–8951

Davidson AL, Shuman HA, Nikaido H (1992) Mechanism of maltose transport in *Escherichia coli*: transmembrane signaling by periplasmic binding proteins. Proc Natl Acad Sci USA 89:2360–2364

Dean DA, Davidson AL, Nikaido H (1989) Maltose transport in membrane vesicles of *Escherichia coli* is linked to ATP hydrolysis. Proc Natl Acad Sci USA 86:914–918

Doige CA, Ames GF-L (1993) ATP dependent transport systems in bacteria and humans: relevance to cystic fibrosis and multidrug resistance. Annu Rev Microbiol 47:291–319

Eick-Helmerich K, Braun V (1989) Import of biopolymers into *Escherichia coli*: nucleotide sequences of the *exbB* and *exbD* genes are homologous to those of the *tolQ* and *tolR* genes, respectively. J Bacteriol 171:5117–5126

Elkins MF, Earhart CF (1989) Nucleotide sequence and regulation of the *Escherichia coli* gene for ferrienterobactin transport protein FepB. J Bacteriol 711:5443–5451

Fecker L, Braun V (1983) Cloning and expression of the *fhu* genes involved in iron (III) hydroxamate uptake by *E.coli*. J Bacteriol 156:1301–1314

Fischer E, Günter K, Braun V (1989) Involvement of ExbB and TonB in transport across the outer membrane of *Escherichia coli*: phenotypic complementation of *exbB* mutants by overexpressed *tonB* and physical stabilization of TonB by ExbB. J Bacteriol 171:5127–5134

Friedrich MJ, DeVeaux LC, Kadner RJ (1986) Nucleotide sequence of the *btuCED* genes involved in vitamin B12 transport in *Escherichia coli* and homology with components of periplasmic binding protein-dependent transport systems. J Bacteriol. 167:928–934

Furlong CE (1986) Binding protein-dependent active transport in *Escherichia coli* and *Salmonella typhimurium*. Methods Enzymol 125:279–289

Furlong CE (1987) Osmotic-shock-sensitive transport systems. In: Neidhardt FC, Ingraham JL, Low KB, Magasanik B, Schaechter M, Umbarger HE (eds) *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington DC, pp 768–796

Gilson E, Alloing G, Schmidt T, Claverys J-P, Dudler R, Hofnung M (1988) Evidence for high affinity binding-protein dependent transport systems in Gram -positive bacteria and in *Mycoplasma*. EMBO J 7:3971–3974

Gilliland GL, Quiocho FA (1981) Structure of the L-arabinose-binding protein from *Escherichia coli* at 2.4 Å resolution. J Mol Biol 146:341–362

Hancock REW, Braun V (1976) Nature of the energy requirement for the irreversible adsorption of bacteriophage T1 and Φ 80 to *Escherichia coli*. J Bacteriol 125:409–415

Higgins CF (1992) ABC transporters: from microorganisms to man. Annu Rev Cell Biol 8:67–113

Higgins CF, Hyde SC, Mimmack MM, Gileadi U, Gill DR, Gallagher MP (1990) Binding protein-dependent transport systems. J Bioenerg Biomembr 22:571–592

Hor LI, Shuman HA (1993) Genetic analysis of periplasmic binding protein dependent transport in *Escherichia coli*: each lobe of maltose-binding protein interacts with a different subunit of the $MalFGK_2$ membrane transport complex. J Mol Biol 233:659–670

Kamitani S, Akiyama Y, Ito K (1992) Identification and characterization of an *Escherichia coli* gene required for the formation of correctly folded alkaline phosphatase, a periplasmic enzyme. EMBO J 11:57–62

Kerppola RE, Shyamala VK, Klebba P, Ames GF-L (1991) The membrane-bound proteins of periplasmic permeases form a complex – identification of the histidine permease HisQMP complex. J Biol Chem 266:9857–9865

Köster W (1991) Iron (III) hydroxamate transport across the cytoplasmic membrane of *Escherichia coli*. Biol Metals 4:23–32

Köster W, Böhm B (1992) Point mutations in 2 conserved glycine residues within the integral membrane protein FhuB affect iron (III) hydroxamate transport. Mol Gen Genet 232:399–407

Köster W, Braun V (1986) Iron hydroxamate transport of *Escherichia coli*: nucleotide sequence of the *fhuB* gene and identification of the protein. Mol Gen Genet 204:435–442

Köster W, Braun V (1989) Iron hydroxamate transport into *Escherichia coli* K12: localization of FhuD in the periplasm and of FhuB in the cytoplasmic membrane. Mol Gen Genet 217:435–442

Köster W, Braun V (1990a) Iron (III) hydroxamate transport of *Escherichia coli*: restoration of iron supply by coexpression of the N- and C-terminal halves of the cytoplasmic membrane protein FhuB cloned on separate plasmids. Mol Gen Genet 223:379–384

Köster W, Braun V (1990b) Iron (III) hydroxamate transport into *Escherichia coli*. Substrate binding to the periplasmic binding protein. J Biol Chem 266:21407–21410

Köster WL, Actis LA, Waldbeser LS, Tolmasky ME, Crosa JH (1991) Molecular characterization of the iron transport system mediated by the pJM1-plasmid in *Vibrio anguillarum* 775. J Biol Chem 266:23829–23833

Lugtenberg B, Meijers J, Peters E, van der Hoeck P, van Alphen L (1975) Electrophoretic resolution of the major outer membrane protein of *Escherichia coli* K-12 into four bands. FEBS Lett 58:254–258

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Mann BJ, Holroyd CD, Bradbeer C, Kadner RJ (1986) Reduced activity of TonB-dependent functions in strains of *Escherichia coli*. FEMS Microbiol Lett 33:255–260

Matsudaira P (1987) Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J Biol Chem 262:10035–10038

Matsuyama S-I, Fujita Y, Mizushima S (1993) SecD is involved in the release of translocated secretory proteins from the cytoplasmic membrane of *Escherichia coli*. EMBO J 12:265–270

Mimmack ML, Gallagher MP, Pearce SR, Hyde SC, Booth IR, Higgins CF (1989) Energy coupling to periplasmic binding protein-dependent transport systems: stoichiometry of ATP hydrolysis during transport in vivo. Proc Natl Acad Sci USA 86:8257–8260

Missiakas D, Georgopoulos C, Raina S (1993) Identification and characterization of the *Escherichia coli* gene *dsbB*, whose product is involved in the formation of disulfide bonds in vivo. Proc Natl Acad Sci USA 90:7084–7088

Mowbray SL, Smith RD, Cole LB (1990) Structure of the periplasmic glucose/galactose receptor of *Salmonella typhimurium*. Receptor 1:41–53

Newcomer ME, Gilliland GL, Quiocho FA (1981a) L-Arabinose-binding protein-sugar complex at 2.4 Å resolution. J Biol Chem 256:13213–13217

Newcomer ME, Lewis BA, Quiocho FA (1981b) The radius of gyration of L-arabinose-binding protein decreases upon binding of ligand. J Biol Chem 256:13218–13222

Nikaido H, Saier Jr MH (1992) Transport proteins in bacteria: common themes in their design. Science 258:936–942

Oh B-H, Pandit J, Kang C-H, Nikaido K, Gokcen S, Ames GF-L, Kim S-H (1993) Three dimensional structures of the periplasmic lysine/arginine/ornithine-binding protein with and without a ligand. J Biol Chem 268:11348–11355

Olah GA, Trakhanov S, Trewhella J, Quiocho FA (1993) Leucine/isoleucine/valine-binding protein contracts upon binding of ligand. J Biol Chem 268:16241–16247

Prossnitz E, Gee A, Ames GF-L (1989) Reconstitution of the histidine periplasmic transport system in membrane vesicles. Energy coupling and interaction between the binding protein and the membrane complex. J Biol Chem 264:5006–5014

Quiocho FA (1990) Atomic structures of periplasmic binding proteins and the high affinity active transport systems in bacteria. Phil Trans R Soc Lond [B] 326:341–351

Saurin W, Martineau FP, Charbit A, Dassa E, Duplay P, Gilson E, Molla A, Ronco G, Szmelcman S, Hofnung M (1989) Periplasmic binding protein dependent transport system for maltose and maltodextrins: some recent studies. FEMS Microbiol Rev 63:53–60

Schneider R, Hantke K (1993) Iron-hydroxamate uptake systems in *Bacillus subtilis*: identification of a lipoprotein as part of a binding protein-dependent transport system. Mol Microbiol 8:111–121

Schultz-Hauser G, Köster W, Schwarz H, Braun V (1992a) Iron (III) hydroxamate transport in *Escherichia coli* K-12: FhuB-mediated membrane association of the FhuC protein and negative complementation of *fhuC* mutants. J Bacteriol 174:2305–2311

Schultz-Hauser G, Van Hove B, Braun V (1992b) 8-Azido-ATP labelling of the FecE protein of the *Escherichia coli* iron citrate transport system. FEMS Microbiol Lett 95:231–234

Sharff AJ, Rodseth LE, Quiocho FA (1993) Refined 1.8 Å structure reveals the mode of binding of β-cyclodextrin to the maltodextrin binding protein. Biochemistry 32:10553–10559

Spurlino JC, Lu G-Y, Quiocho FA (1991) The 2.3 Å resolution structure of the maltose- or maltodextrin-binding protein, a primary receptor of bacterial active transport and chemotaxis. J Biol Chem 266:5202–5219

Staudenmaier H, Van Hove B, Yaraghi Z, Braun V (1989) Nucleotide sequences of the *fecBCDE* genes and locations of the proteins suggest a periplasmic binding protein-dependent transport mechanism for Iron(III) dicitrate in *Escherichia coli*. J Bacteriol 171:2626–2633

Stojiljkovic I, Hantke K (1994) Transport of hemin across the cytoplasmic membrane through a hemin specific periplasmic binding protein-dependent transport system in *Yersinia enterocolitica*. Mol Microbiol 13:719–732

Studier FW, Moffat BA (1986) Use of bacteriophage T7 RNA polymerase to direct selective high level expression of cloned genes. J Mol Biol 189:113–130

Tabor S, Richardson V (1985) A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc Natl Acad Sci USA 82:1074–1078

Tam R, Saier Jr MH (1993) Structural, functional, and evolutionary relationships among extracellular solute-binding receptors of bacteria. Microbiol Rev 57:320–346

Thome BM, Müller M (1991) Skp is a periplasmic *Escherichia coli* protein requiring SecA and SecY for transport. Mol Microbiol 5:2815–2821

Treptow NA, Shuman HA (1988) Allele specific *malE* mutations that restore interactions between maltose-binding protein and the inner-membrane components of the maltose transport system. J Mol Biol 202:809–822

Wülfing C, Plückthun A (1994) Protein folding in the periplasm of *Escherichia coli*. Mol Microbiol 12:685–692

Vyas NK, Vyas MN, Quiocho FA (1991) Comparison of the periplasmic receptors for L-arabinose, D-glucose/D-galactose and D-ribose. Structural and functional similarity. J Biol Chem 266:5226–5237

Zou J-Y, Flocco MM, Mowbray SL (1993) The 1.7 Å refined X-ray structure of the periplasmic glucose/galactose receptor from *Salmonella typhimurium*. J Mol Biol 233:739–752