Identification of hydrophobic proteins FepD and FepG of the *Escherichia coli* ferrienterobactin permease

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In *Escherichia coli*, iron assimilation by means of the siderophore enterobactin requires two hydrophobic cytoplasmic membrane proteins, FepD and FepG, which are essential components of a binding-protein-dependent transport system. Such components are typically difficult to detect. Here we report observation of the *fepD* and *fepG* gene products in polyacrylamide gels; they appeared as diffuse bands at positions consistent with smaller sizes than those predicted by sequence analysis. Translational coupling was suggested by the lack of a detectable product from the *fepG* message in the absence of translation of the upstream *fepD* message. The orientation of FepD/FepG in the membrane was predicted based on their similarities in sequence and hydrophobicity to FhuB.

Introduction

Escherichia coli and other enteric organisms are able to thrive under conditions of low iron availability by the use of high affinity iron chelators (siderophores) either endogenously synthesized or fortuitously present. The siderophore enterobactin (Ent) is made in *E. coli* by biosynthetic enzymes encoded in the Ent cluster of genes. This cluster also contains genes for the transport of Fe(III)-Ent and for the intracellular release of the iron (Earhart, 1987). These genes are derepressed when the intracellular level of iron is low, this regulation being accomplished, at least partially, by the *fur* product which is an aporepressor (Hantke, 1981; Bagg & Neilands, 1987).

Ferri-Ent is transported into cells by means of a periplasmic permease, one of the iron/B12 subset (Shea & McIntosh, 1991) of binding protein-dependent transport systems, whose members require an outer membrane receptor and the *tonB* function (Postle, 1990). In addition to the FepA protein in the outer membrane (Neilands, 1982), the system requires FepB, the periplasmic binding protein (Pierce *et al.*, 1983; Pierce & Earhart, 1986; Elkins & Earhart, 1989), FepC, an inner-membrane-associated protein (Pierce & Earhart, 1986; Ozenberger *et al.*, 1987) with an apparent ATP-binding site (Shea & McIntosh, 1991), and two hydrophobic proteins recently sequenced and characterized, designated FepD and FepG (Chenault & Earhart, 1991; Shea & McIntosh, 1991). The *fepD* and *fepG* genes are located

just upstream of *fepC* and are transcribed with it as an operon in the direction *fepDGC*. Although the product of the distal gene *fepC* has been seen repeatedly, earlier attempts to visualize proteins encoded from the *fepDG* sequence have been unsuccessful (Pierce & Earhart, 1986; Ozenberger *et al.*, 1987; Shea & McIntosh, 1991).

It has been assumed previously that periplasmic permeases require the hydrophobic inner membrane components in very small amounts and that this explained the difficulties in identifying and studying these proteins. It now seems likely that each membrane transport complex contains one to four proteins such that the complex includes two hydrophobic integral membrane domains and two associated hydrophilic ATP-binding domains (Higgins *et al.*, 1990*a, b*). This has been directly demonstrated for the histidine permease by means of chemical cross-linking and immunoprecipitation (Kerppola *et al.*, 1991). If the same stoichiometry applies in the case of the comparable ferri-Ent transport system, this would predict that two molecules of FepC plus one each of FepD and FepG constitute the membrane transport complex. It would therefore seem likely that the difficulties in visualizing these proteins have been partially technical; for example, hydrophobic proteins are often precipitated by boiling in SDS (Beyreuther *et al.*, 1980; Ito, 1984; Koster & Braun, 1986), a commonly used procedure for preparing samples for acrylamide gels. In this paper the expression and membrane localization of FepD and FepG were studied using plasmids with a T7 RNA polymerase start site (Tabor & Richardson, 1985).

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Fig. 1. Partial map of *E. coli* ferri-Ent transport genes. Insert DNA fragments from plasmids are drawn below. Restriction site abbreviations are: A, *AccI*; C, *ClaI*; H, *HpaI*; M, *MluI*; S, *SmaI*; V, *EcoRV*.

Methods

Strains and plasmids. Protein expression experiments were done with strain K38/pGP1-2 (Tabor & Richardson, 1985) carrying plasmids with inserts shown in Fig. 1. All plasmids were derivatives of pC₄EM3Z (Promega Biotec) which carries the gene for β -lactamase in a position to be transcribed in the incorrect direction by T7 RNA polymerase. Plasmids are described more fully in Chenault & Earhart, 1991.

Iron-deficient medium. LB (Nagel de Zwaig & Luria, 1967) was made iron-deficient by adding 0.2 mM-2,2' dipyridyl from Sigma.

Protein expression and labelling. Proteins were labelled by a previously described modification (Chenault & Earhart, 1991) of the method of Tabor & Richardson (1985). Cells were maintained in an iron-deficient medium to prevent Fur-binding which might interfere with transcription even by the T7 RNA polymerase. Protein samples were not boiled but were solubilized at 37 °C for 5 min in sample buffer (Koster & Braun, 1986, 1989). Polyacrylamide gel electrophoresis was carried out as described previously (Pierce & Earhart, 1986). Molecular mass standards were purchased from BRL and were as follows: lysozyme, 14.3 kDa; β -lactoglobulin, 18.4 kDa; carbonic anhydrase, 29 kDa; ovalbumin, 43 kDa; bovine serum albumin, 68 kDa; phosphorylase b, 97.4 kDa; myosin (H-chain), 200 kDa.

Results and Discussion

Identification of proteins FepD and FepG

Expression of proteins from plasmids (Fig. 1) carrying various regions of the Ent transport gene sequence is shown in Fig. 2. In the presence of rifampicin (lanes 3, 6, 9, 12, 14 and 15) there was minimal incorporation of label into chromosomally-encoded proteins; β -lactamase is not present as its gene was transcribed in the incorrect direction. The dark band running at about 30 kDa in lanes 9 and 15 has been previously identified in minicell preparations as FepC (Pierce & Earhart, 1986; Ozenberger *et al.*, 1987; Shea & McIntosh, 1991). Since pSC1100 carries only two genes, *fepD* and *fepG*, the two diffuse bands (24 kDa and 26 kDa) in lane 12, as well as the same bands in lane 9, were tentatively identified as

the products of these. Expression of the *fepD* gene alone from pSC1500 (Fig. 3, lane 6), identified the 26 kDa band as FepG and the 24 kDa band as FepD. In the gel shown, the FepD protein for unexplained reasons ran as a doublet. Both FepD and FepG appear to be smaller than the sizes predicted by sequence analysis: 33805 Da for FepD (Chenault & Earhart, 1991; Shea & McIntosh, 1991) and 34997 Da for FepG (Shea & McIntosh, 1991). When loaded onto a 15% (w/v) polyacrylamide gel the apparent sizes of the proteins were 28 kDa and 30 kDa, respectively (not shown). When samples were treated at 95 °C for 5 min, radioactivity was concentrated in the wells of the gel; FepD and FepG bands were absent (not shown).

It is typical of very hydrophobic proteins that they are precipitated by boiling in SDS and that they are found after gel electrophoresis in diffuse bands at positions not consistent with their actual size (Beyreuther *et al.*, 1980; Ito, 1984; Dassa & Hofnung, 1985; Koster & Braun, 1986). The 70 kDa FhuB protein (Koster & Braun, 1989), which has significant sequence homologies with FepD and FepG (Chenault & Earhart, 1991; Shea & McIntosh, 1991), was found on gels at size positions ranging from 41000 to 76000 Da as acrylamide concentration and cross-linking were increased (Koster & Braun, 1986). Other similar proteins, FecC and FecD, which transport ferric dicitrate, were also seen in 15% polyacrylamide gels at anomalous positions (32 kDa rather than 35.4 kDa for FecC; 29.5 kDa rather than 34.1 kDa for FecD; Staudenmaier *et al.*, 1989).

Possible translational coupling of FepD and FepG

Expression of plasmids carrying the complete *fepG* sequence but lacking the start site and 5' end of the *fepD* gene failed to produce detectable amounts of FepG (Fig. 2, lanes 14 and 15). This does not appear to be a failure of transcription, as the product of *fepC*, distal to *fepG* on the same operon (Chenault & Earhart, 1991; Shea & McIntosh, 1991) was clearly seen in lane 15 (pSC1200, FepG+FepC⁺). The *fepG* start codon is overlapped by the *fepD* stop signal (ATGA). It would not, therefore, be surprising if synthesis of FepG is closely coupled to that of FepD, causing the production of the downstream gene product to depend upon translation of the proximal *fepD* message. Predicted secondary structure (Fig. 4) could make this sequence and the preceding ribosome-binding site inaccessible unless opened up by the ribosome(s) translating the promoter-proximal *fepD* message. Obligatory translational coupling often seems to be mediated by the presence of secondary structure in the vicinity of the Shine-Dalgarno sequence and start codon of the distal gene (Hall *et al.*, 1982; Gold, 1988).

Fig. 2. Autoradiogram of proteins synthesized in cells harbouring *fep*-bearing plasmids and resolved in a 10% (w/v) polyacrylamide gel. Positions of molecular mass standards are shown. Plasmids expressed are pSC1300, lanes 1–3; pSC1400, lanes 4–6; pSC1000, lanes 7–9; pSC1100, lanes 10–12; pSC1130, lanes 13–14; pSC1200, lanes 15–17. Conditions were as follows: lanes 1, 4, 7, 10, 13 and 17, no rifampicin, T7 polymerase not induced; lanes 2, 5, 8, 11 and 16, no rifampicin, T7 polymerase induced; lanes 3, 6, 9, 12, 14 and 15, 400 μ g rifampicin ml^{-1} , T7 polymerase induced.

Fig. 3. Autoradiogram of a 10% (w/v) polyacrylamide gel after electrophoretic separation of proteins from cells bearing *fep* genes. Plasmids expressed: lanes 1 and 2, pSC1000; lanes 3 and 4, pSC1100; lanes 5 and 6, pSC1500. Conditions: lanes 1, 3 and 5, 400 μ g rifampicin ml^{-1} , T7 polymerase not induced; lanes 2, 4 and 6, 400 μ g rifampicin ml^{-1} , T7 polymerase induced.

FepG, *FepD* and *FepC*, members of a permease family

The Ent permease proteins are members of the superfamily of 'Traffic ATPases' (Ames *et al.*, 1990) or ABC-transporters (Higgins *et al.*, 1990*a, b*) and are particularly

Fig. 4. Possible stem and loop structure involving the *fepG* translation start site. Underlined bases are probably included in the ribosome-binding site; overlapping start and stop (*fepD*) codons are within the box.

closely related to systems which transport ferric complexes. At this time there is no specific data available about interaction among the membrane-associated components FepD, FepG and FepC; however, working hypotheses concerning these interactions appear to be compatible with the growing body of information about the larger family.

Tertiary structure and membrane position

By analogy with the FhuB protein, it is very likely that the carboxy-terminus of FepD is closely associated with the amino portion of FepG. The FhuB sequence (Koster & Braun, 1986) has internal homology between the two halves (Koster & Braun, 1989; Staudenmaier *et al.*, 1989) and is highly homologous to the combined sequences of FepD (Chenault & Earhart, 1991; Shea & McIntosh, 1991) and FepG (Shea & McIntosh, 1991) with a short polar bridging sequence. Amino acid residues 1–328 of

Fig. 5. Hydropathy plots of proteins FhuB (Koster & Braun, 1986), FepD (Chenault & Earhart, 1991; Shea & McIntosh, 1991), FepG (Shea & McIntosh, 1991), FecC and FecD (Staudenmaier *et al.*, 1989) aligned to demonstrate similarities. Vertical sections represent 100 amino acid residues. Residues located above the horizontal line in each graph are hydrophobic.

FhuB are 25% homologous to residues 334–659 (Koster & Braun, 1989). If the two halves of the protein are positioned in the membrane similarly to one another, both ends of the molecule would be on the same side. Since the polar bridging sequence should not cross the membrane, FhuB sequences homologous to the FepD carboxy-terminus and the FepG amino-terminus would necessarily be positioned close together on either the cytoplasmic or periplasmic side. Hydropathy plots (Fig. 5) are very similar for FhuB and FepD/FepG as well as for FecC/FecD of the ferri-dictrate permease. It seems reasonable to assume that the FepDG and FecCD heterodimers have tertiary structure and membrane position comparable to that of FhuB. The homology between the two halves of FhuB leads to the conclusion that the ends of the molecule lie on the same side of the membrane (Koster & Braun, 1989). It then follows that the two ends of both FepD and FepG are on the same side of their respective molecules, each protein crossing the membrane an even number of times.

If the ferri-Ent transport complex has the stoichiometry of the histidine system (Kerppola *et al.*, 1991), a 1:1 ratio of FepD to FepG would be required; close translational coupling would produce this ratio as well as positioning the second translation product for immediate and correct association with the first. One further step in the histidine analogy predicts two FepC proteins to interact with the FepD-FepG heterodimer. This, of course, would imply translation starts that are independent of translation of the upstream FepG; these do seem to occur despite overlapping stop and start codons. Since submitting this manuscript we discovered the paper by

Pearce *et al.* (1992), describing the membrane topology of integral membrane proteins OppB and OppC. Each spans the membrane six times with both termini on the cytoplasmic face. These results are consistent with those which we have predicted for FepG and FepD.

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