JOURNAL OF BACTERIOLOGY, Apr. 1992, p. 2305–2311
0021-9193/92/072305-07$02.00/0

Vol. 174, No. 7

Iron(III) Hydroxamate Transport in *Escherichia coli* K-12: FhuB-Mediated Membrane Association of the FhuC Protein and Negative Complementation of *fhuC* Mutants

GABRIELE SCHULTZ-HAUSER,[1] WOLFGANG KÖSTER,[1] HEINZ SCHWARZ,[2] AND VOLKMAR BRAUN[1]*

Mikrobiologie II, Universität Tübingen,[1] and Max-Planck Institut für Entwicklungsbiologie,[2] D-7400 Tübingen, Germany

Received 28 October 1991/Accepted 22 January 1992

Iron(III) hydroxamate transport across the cytoplasmic membrane is catalyzed by the very hydrophobic FhuB protein and the membrane-associated FhuC protein, which contains typical ATP-binding domains. Interaction between the two proteins was demonstrated by immunoelectron microscopy with anti-FhuC antibodies, which showed FhuB-mediated association of FhuC with the cytoplasmic membrane. In addition, inactive FhuC derivatives carrying single amino acid replacements in the ATP-binding domains suppressed wild-type FhuC transport activity, which arose either from displacement of active FhuC from FhuB by the mutated FhuC derivatives or from the formation of mixed inactive FhuC multimers between wild-type and mutated FhuC proteins. Inactive FhuC derivatives containing internal deletions and insertions showed no phenotypic suppression, indicating conformational alterations that rendered the FhuC derivatives unable to displace wild-type FhuC. It is concluded that the physical interaction between FhuC and FhuB implies a coordinate activity of both proteins in the transport of iron(III) hydroxamates through the cytoplasmic membrane.

Iron(III) hydroxamates are transported across the outer membrane of bacteria in an energy-coupled process that involves specific receptor proteins, the TonB protein and the ExbBD proteins (6, 18). Ferrichrome and its structural analog the antibiotic albomycin use the FhuA receptor, coprogen uses the FhuE receptor, and aerobactin uses the Iut receptor (8, 9, 16, 28). These iron hydroxamates are transported across the cytoplasmic membrane by the same transport system composed of the periplasmic FhuD protein, the very hydrophobic transmembrane FhuB protein, and the membrane-associated FhuC protein (8, 10, 11, 16, 23, 24). The properties and the locations of these proteins suggest a periplasmic-binding protein-dependent transport mechanism across the cytoplasmic membrane. In support of this notion, specific binding of ferrichrome, coprogen, and aerobactin to the FhuD protein has been demonstrated (26). Furthermore, the FhuC protein contains two domains which are typical for ATP-binding sites (10, 11). Substitution of the lysine residue in domain I with glutamine or glutamate and of the aspartate in domain II with asparagine or glutamate resulted in a complete inactivation of FhuC (5). ATP energization of the transport of histidine and maltose, taken up via periplasmic-binding protein-dependent transport, has been demonstrated (7, 13); hence it has been inferred that ATP serves as an energy source for periplasmic-binding protein-dependent transport systems in general (1, 2, 4, 24, 32), including the one for iron hydroxamates.

It is conceivable that the iron hydroxamates are transferred from FhuD to FhuB, which then translocates them across the cytoplasmic membrane. Translocation is energized by FhuC-catalyzed ATP hydrolysis, which induces a conformational change in FhuB (22). This model implies an interaction between the FhuB and FhuC proteins since ATP hydrolysis at FhuC somehow has to be transmitted to FhuB. Alternatively, binding of iron hydroxamate-loaded FhuD to FhuB triggers a transport-competent FhuB conformation, and dissociation of the unloaded FhuD, or restoration of a FhuD-binding conformation of FhuB, consumes energy. It is also possible that FhuC spans the membrane and interacts with FhuD, as has been proposed for the ATP-hydrolyzing HisP protein based on *hisP* mutations that render D-histidine transport independent of the periplasmic HisJ-binding protein (31) and suppress transport-defective *hisJ* mutations (3, 29).

In this paper we report data that suggest a physical interaction between FhuB and FhuC. We investigated the subcellular location of FhuC by electron microscopy of ultrathin-sectioned cells in which FhuC was immunogold labeled. This technique requires a protein concentration of at least 10^4 molecules per cell, which is greater than the amount of FhuC protein expressed by the chromosomal genes. The low level of expression of the *fhuCDB* genes made it necessary to overexpress these genes in order to identify the gene products by sensitive fluorography of [^{35}S]methionine-labeled cells (24). To see whether FhuB is involved in binding of FhuC to the cytoplasmic membrane, various combinations of the *fhu* genes (*fhuC*, *fhuC fhuB*, *fhuA fhuC*, and *fhuACDB*) were cloned and overexpressed for immunogold labeling with anti-FhuC serum. Further, the amount of FhuB was varied relative to that of FhuC to examine whether the amount of FhuC associated with the cytoplasmic membrane depended on the concentration of FhuB in the membrane.

In addition, if FhuC binds to FhuB, mutant FhuC should displace wild-type FhuC, resulting in transport inactivation. The same phenotype would be expected if FhuC forms a dimer (or multimer), and mutated FhuC combined with wild-type FhuC inactivates wild-type FhuC. To study negative complementation, the FhuC point mutants in the ATP-binding domains were used on the assumption that these amino acid replacements did not distort the overall conformation of FhuC, so that either binding to FhuB or FhuC dimer formation had been retained. In addition, FhuC mu-

* Corresponding author.

TABLE 1. *E. coli* K-12 strains and plasmids used in this study

Strain or plasmid	Genotype	Source or reference
E. coli		
AB2847	*aroB thi malT tsx*	This institute
KO281	AB2847 *fhuC zif*::Tn*10*	5
H1443	*aroB rpsL lac araD*	This institute
BL21(DE3)	pLysS, F$^-$, *hsdS gal*, phage T7 polymerase under *lacUV5* control, Cm^r, Amp^r	34
W3110	Wild type	This institute
KS474	*degP41 galE galH rpsL phoA*	33
GS29	B121(DE3) with a deletion in *fhuC*	This study
Plasmids		
pT7-5	Promoter of phage T7 gene 10, Amp^r	35
pT7-6	Promoter of phage T7 gene 10, Amp^r	35
pGP1-2	Phage T7 polymerase under *c*I857 control	35
pWK380	pT7-5 *fhuC*	24
pWK346	pT7-6 *fhuB*	24
pWK388	pT7-6 *fhuA fhuC fhuD fhuB*	24
pWK412	pT7-5 *fhuC* (deletion from Ile-180 to Asp-207)	24
pWK464	pT7-5 *fhuC* (duplication from Gly-23 to Asp-177	This study
pKB35	pT7-5 *fhuC* (Lys-50–Glu)	5
pKB36	pT7-5 *fhuC* (Lys-50–Gln)	5
pKB39	pT7-5 *fhuC* (Asp-172–Glu)	5
pKB33	pT7-5 *fhuC* (Asp-172–Asn)	5
pGS64	pT7-6 *fhuA fhuB*	This study
pGS65	pT7-6 *fhuC fhuB*	This study
pGS78	pWK380 with a 33-bp insertion in the *Eco*RV1 site of *fhuC*	This study
pGS79	pWK380 with a 33 bp insertion in the *Eco*RV2 site of *fhuC*	This study
pGS85	pACYC184 (*fhuC*)	This study

tant proteins which contained insertions and deletions leading to inactive FhuC derivatives were constructed. They were expected to display strong conformational defects and should thus be unable to compete with wild-type FhuC.

We obtained convincing evidence for an increase in the amount of membrane-associated FhuC that was dependent on the presence of FhuB. We take this as evidence that FhuC catalyzes iron hydroxamate transport through a functional interaction with FhuB. We also obtained suppression of wild-type FhuC activity by overexpression of FhuC derivatives carrying single amino acid replacements, showing either competition between mutant FhuC and wild-type FhuC at FhuB or, possibly, mixed FhuC dimer formation.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *Escherichia coli* K-12 strains and the plasmids used are listed in Table 1. The new plasmids were constructed as follows. pGS64 contains the 3,544-bp *Hin*dIII-*Sal*I fragment of pWK365, encoding *fhuA* and *fhuC*, in the *Hin*dIII-*Sal*I site of pT7-6 (24, 35). pGS65 was obtained by cloning the 2,398-bp *Pvu*II fragment of pWK405 in pT7-6. The 784-bp *Sal*I-*Pst*I fragment was excised and blunt-end ligated. The resulting plasmid, pGS60, encoded *fhuC*, was lacking *fhuD*, and contained about half of *fhuB* (*'fhuB*). The 977-bp *Sma*I-*Aat*II fragment was excised and cloned into the 5,973-bp *Sma*I-*Aat*II fragment of pWK405, yielding pGS65 (*fhuC fhuB*). pGS85 (*fhuC*) contained the 1,071-bp *Dra*I-*Sal*I fragment of pWK405 in the 3,778-bp *Eco*RV-*Sal*I fragment of pACYC184. pGS78 and pGS79 were constructed by cloning the synthetic oligonucleotide UACCCAUACGACGUUCCGGACUACGCAGGAUCC (17) into the 5′ *Eco*RV (*Eco*RV1) and the 3′ *Eco*RV (*Eco*RV2) sites of pWK380, respectively.

Growth and transport assays. Sensitivity to albomycin was examined by spotting 10 μl of albomycin solutions in water (1, 0.3, and 0.03 mg/ml and 3 and 0.3 μg/ml) onto tryptone-yeast (TY) agar plates (16) seeded with 2×10^8 cells of the strain to be tested in 3 ml of TY soft agar. Growth promotion by ferrichrome, coprogen, and dihydroxybenzoic acid was tested by placing filter paper disks containing 15 μl of the substances (1, 0.1, and 0.01 mM and 1 μM) onto nutrient broth–0.2 mM dipyridyl agar plates (23). The plates were inspected after incubation for 6 and 15 h at 37°C. The diameter of the growth zone and the cell density were recorded.

Transport of [$^{55}Fe^{3+}$]ferrichrome was measured as described previously (25).

Preparation of FhuC antiserum. *E. coli* BL21 transformed with pWK380 (*fhuC*$^+$) was grown in the presence of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h. Membranes were prepared (15), and the components were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (27) with ultra-pure chemicals (Bio-Rad, Munich, Germany). After electrophoresis the gel was washed for 3 min with distilled water. The protein bands were then visualized by incubation in 0.3 M $ZnCl_2$ for 5 min, after which the gel was washed three times with water (14). The gel segment containing FhuC was excised and destained by three 5-min incubations in 0.25 M EDTA–0.25 M Tris-hydrochloride (pH 9). Then the gel segment was sliced up and FhuC was eluted with 0.4 ml of 0.125 M Tris-hydrochloride–0.05% SDS–20% glycerol–10% mercaptoethanol (pH 7.0) for 24 h. The gel slices were removed by centrifugation, and the solution was dialyzed for 2 days against water and then concentrated with Aquacide IA (Calbiochem, San Diego, Calif.). The protein concentration was determined (30), and the sample was examined by SDS-PAGE. Rabbits were

injected subcutaneously three times at 4-week intervals, each time with 0.3 mg of protein in complete Freund's adjuvant. Serum was obtained by centrifugation after the blood had been allowed to clot overnight at 4°C. For removal of nonspecific antibodies, 1.5 ml of serum was incubated four times for 1 h at room temperature with the membrane fraction of the *fhuC* deletion mutant GS29. The incubation mixture was centrifuged for 1 h at 100,000 × *g* after each intermediate incubation step and for 1.5 h after the last step. The membrane fraction used was prepared from a 500-ml overnight culture of strain GS29. Cells were pelleted by centrifugation, suspended in 30 ml of 10 mM Tris-hydrochloride (pH 7.5), supplemented with 0.6 mg of DNase I, and then disrupted by ultrasound. Residual cells and large fragments were pelleted at 650 × *g* for 15 min. The membrane fraction was sedimented at 200,000 × *g* for 90 min and suspended in 1.5 ml of 1.5 mM KH_2PO_4–8.1 mM Na_2HPO_4–137 mM NaCl–2.7 mM KCl (pH 7.4) (PBS) containing 1% bovine serum albumin.

Immunoelectron microscopy. Synthesis of phage T7 RNA polymerase of *E. coli* BL21(DE3, pLysS), carrying various *fhu* plasmids, was induced at an optical density of 0.6 (578 nm) with 0.5 mM IPTG. Culture samples (10 ml) were taken after 0, 30, 75, and 180 min and centrifuged, and the sedimented cells were suspended in 1 ml (0- and 30-min probes) and 1.5 ml (75- and 180-min probes) of 4% formaldehyde–0.015% glutaraldehyde dissolved in PBS. The cells were incubated for 5 min at room temperature and for 30 min at 0°C, after which they were spun down, washed twice with 3 ml of PBS, and finally suspended in 1 ml of PBS. The fixed cells were embedded in 2% agarose and dehydrated stepwise with 30 to 100% ethanol at 0 to −35°C and then subjected to UV polymerization in Lowicryl HM20 at −32°C. Ultrathin sections were transferred to vapor-blasted copper nets and treated with preadsorbed anti-FhuC antiserum. The bound immunoglobulins were detected with 15-nm *Staphylococcus aureus* gold-protein A particles.

RESULTS

Subcellular localization of the FhuC protein. (i) Immunoelectron microscopy. To determine the subcellular location of the FhuC protein we used immunocytochemistry with anti-FhuC antibodies to which gold particles were adsorbed. In addition, we examined whether FhuC binds to or inserts into the cytoplasmic membrane alone or with the help of the FhuB protein. For this purpose, we used various combinations of the *fhuACDB* genes cloned downstream of the phage T7 gene 10 on plasmids pT7-5 and pT7-6. Transcription was started by IPTG induction of the T7 RNA polymerase in *E. coli* BL21(DE3). After 0, 30, 75, and 180 min cells were withdrawn, fixed with aldehyde, ultrathin sectioned, and labeled with anti-FhuC antibodies which had been preadsorbed on whole membranes of a *fhuC* deletion mutant to remove nonspecific antibodies. The antiserum reacted exclusively with the FhuC protein, as revealed by Western immunoblots with cells which overexpressed FhuC and FhuC derivatives with altered electrophoretic mobility.

Expression of *fhuC* on pWK380 resulted in an even distribution of the immunogold label throughout the cell. Only areas containing DNA remained unlabeled. No preferential labeling of the cytoplasmic membrane was seen. Overexpression, in particular when all *fhuABCD* genes were on plasmids, yielded small, optically denser areas in which the label accumulated, suggesting a preferential location of FhuC in these regions (Fig. 1, compare panel A, showing cells expressing chromosomally encoded FhuC, with panel F, showing plasmid-encoded overexpressed FhuACDB). A similar FhuC distribution was obtained with BL21 transformants carrying plasmid pGS64 (*fhuA*$^+$ *fhuC*$^+$) (Fig. 1C). In this case strongly overexpressed FhuA outer membrane receptor, encoded on a runaway plasmid, has previously been found to accumulate at certain sites on the inner side of the cytoplasmic membrane (20). In the system used in this study, FhuA deposition was observed but not to the same extent as previously found, probably because less protein was synthesized. Under these conditions, no redistribution of cytoplasmic FhuC to the membrane occurred. In contrast, coexpression of *fhuC* with *fhuB* resulted in a preferential association of the label with the cytoplasmic membrane (Fig. 1D). In Fig. 1E the label precisely follows the invagination of the cytoplasmic membrane (arrow). A similar picture emerged in cells transformed with plasmid pWK388 (*fhuA fhuC fhuD fhuB*). The label was arranged mostly along the cytoplasmic membrane and in protein aggregates (Fig. 1F).

In an additional experiment we examined whether the preferential association of the FhuC protein with the cytoplasmic membrane depended on the amount of FhuB synthesized. Strain BL21 was doubly transformed with plasmid pWK346, carrying *fhuB* downstream of the strong T7 gene 10 promoter, and with plasmid pGS85, containing *fhuC* downstream of the *tet* promoter of the medium-copy plasmid pACYC184. Transcription of *fhuB* was initiated by IPTG induction of the T7 RNA polymerase. Samples were withdrawn after 0, 30, 75, and 180 min, and immunogold-labeled ultrathin sections were examined under the electron microscope. The originally even distribution of the label changed substantially after *fhuB* expression. FhuC appeared almost exclusively on the cytoplasmic membrane (Fig. 1, compare panels G and H). The amount of FhuC was somewhat reduced after induction of FhuB synthesis. This can be explained by the strong expression of FhuB, which presumably uses more of the capacity of the cells for protein synthesis.

Negative complementation. *E. coli* H1443 (*aroB*) was unable to synthesize the siderophores enterochelin and aerobactin and therefore depended on the addition of siderophores to grow on nutrient broth (NB) in which the available iron was reduced by 2,2′-dipyridyl (NBD medium). When the cells were seeded on an NBD agar plate, a growth zone developed around filter paper disks containing ferrichrome, coprogen, or dihydroxybenzoic acid. Dihydroxybenzoic acid is the missing precursor of the *aroB* mutant for synthesis of enterochelin (a catecholate compound) and was added as a control to test the exclusive dependence of iron hydroxamate transport on FhuC. Sensitivity to albomycin, which is taken up by the same transport system as ferrichrome (16), was also used to examine uptake via FhuCDB. Transformation of H1443 with plasmids pKB36 (Lys-50–Gln [i.e., Lys at position 50 is replaced by Gln]) and pKB39 (Asp-172–Glu) had no effect on albomycin sensitivity and growth stimulation by ferrichrome and coprogen. pKB36 and pKB39 contain the mutated *fhuC* genes on plasmid pT7-5 downstream of the gene 10 promoter of phage T7 (5). The mutated *fhuC* genes were probably expressed, since an identically constructed plasmid containing wild-type *fhuC* complemented *fhuC* mutants in the absence of T7 RNA polymerase (24). However, no FhuC protein was seen on autoradiographs of gels, indicating a very low expression (data not shown). Plasmid pGP1-2, carrying the temperature-inducible T7 RNA polymerase gene, was therefore transferred into H1443 containing pKB36 and pKB39. The resulting cells, grown at

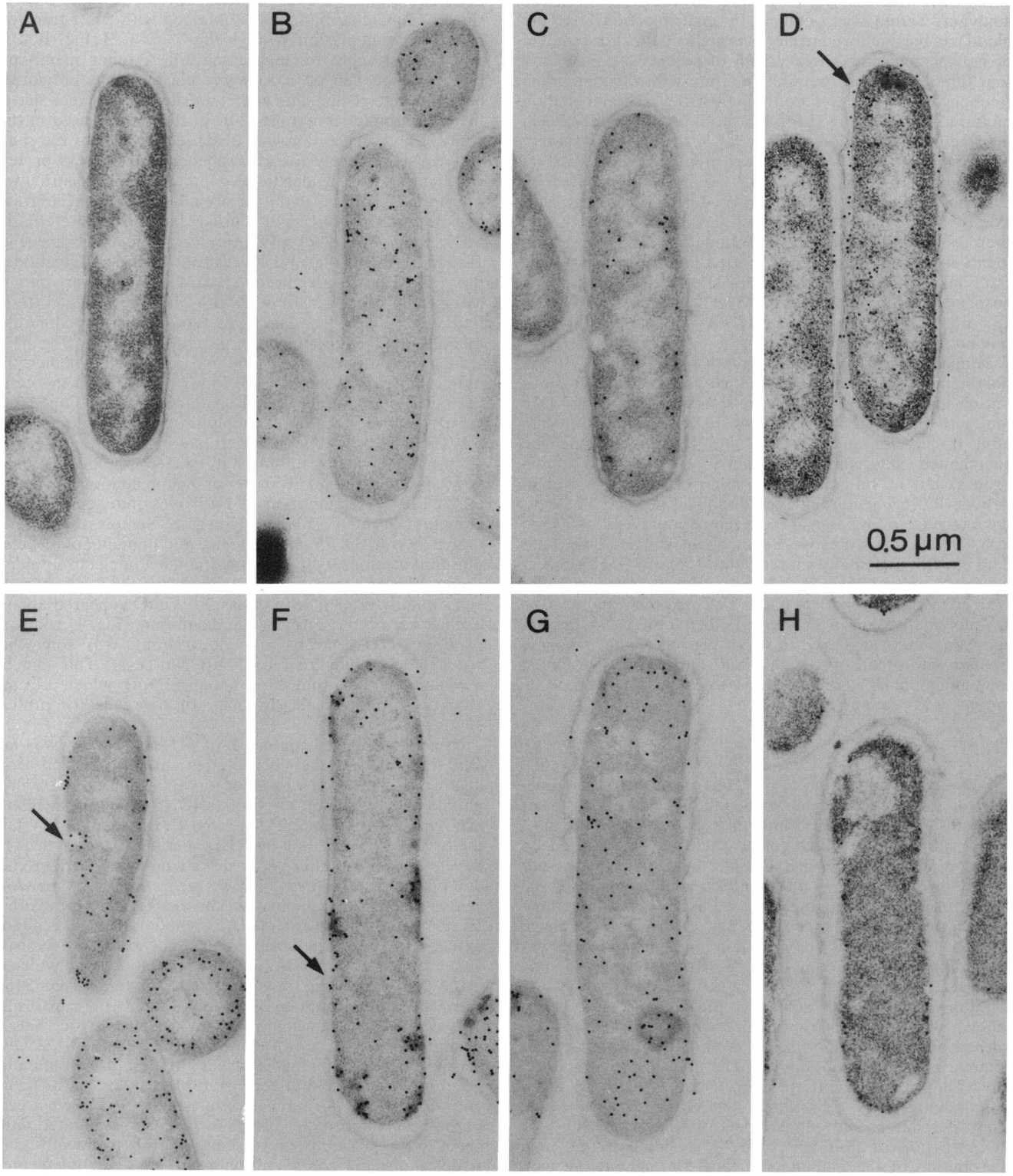

FIG. 1. Immunoelectron microscopy of ultrathin-sectioned cells of *E. coli* BL21(DE3) transformed with plasmids pT7-5 (A), pWK380 (*fhuC*) (B), pGS64 (*fhuA fhuC*) (C), pGS65 (*fhuC fhuB*) (D and E), pWK388 (*fhuA fhuC fhuD fhuB*) (F), and pGS85 (*fhuC*) and pWK346 (*fhuB*) after 0 min (G) and 180 min (H) of *fhuB* transcription initiation by IPTG. The arrows point to the preferential location of FhuC at the cytoplasmic membrane.

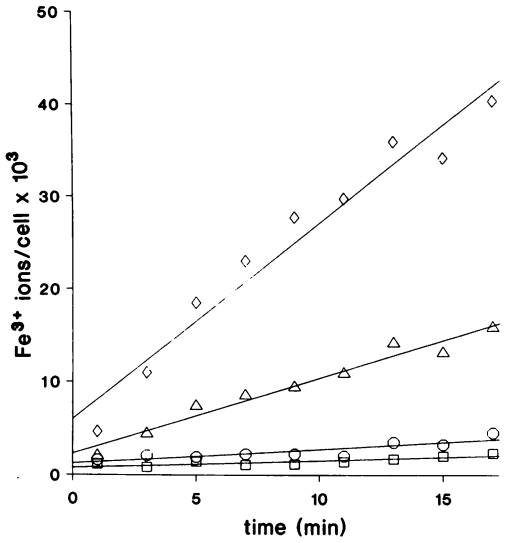

FIG. 2. Suppression of [$^{55}Fe^{3+}$]ferrichrome transport into cells of *E. coli* H1443 (*fhuC*$^+$) (◇) and H1443(pGP1-2, pWK380) (*fhuC*$^+$) (△) by plasmids carrying *fhuC* point mutations, H1443(pGP1-2, pKB39) (*fhuC* Asp-172–Glu) (○), and H1443(pGP1-2, pKB36) (*fhuC* Lys-50–Gln) (□).

27 or 37°C on TY agar plates, were albomycin resistant and showed only very weak growth stimulation zones around iron hydroxamate-loaded filter paper disks on NBD agar plates. The growth rate and the number of viable cells (CFU) were not reduced, showing that the partially induced polymerase did not harm the cells. In contrast, overnight incubation at 42°C reduced the number of viable cells to 1% of the input number. Strain H1443 transformed with pGP1-2 and either pKB35 (Lys-50–Glu) or pKB33 (Asp-172–Asn) were, upon induction of the T7 polymerase, also resistant to albomycin (the other properties were not determined), demonstrating that overexpression of all four FhuC derivatives with single amino acid replacements in the supposed ATP-binding sites inhibited chromosomally encoded wild-type FhuC.

The very sensitive growth assays were complemented by determination of [$^{55}Fe^{3+}$]ferrichrome transport rates. Transport of iron was already reduced when strain H1443(pGP1-2) was transformed with pWK380 (*fhuC*$^+$) (Fig. 2). We usually observe a decrease in transport rates when one of the transport components is synthesized in greater amounts than the others, with the exception of the FhuA receptor, whose overexpression increases transport rates (19, 25). Transformants carrying pKB36 and pKB39 were virtually devoid of transport activity, showing that the mutated FhuC derivatives interfered with the transport activity of wild-type FhuC.

Inactive FhuC derivatives carrying a deletion (pWK412, from Ile-180 to Asp-207) and a duplication (pWK464, from Gly-23 to Asp-177) did not affect chromosomally encoded wild-type FhuC activity. In addition, a synthetic DNA fragment encoding the undecapeptide Tyr Pro Tyr Asp Val Pro Asp Tyr Ala Gly Ser was inserted in frame into the two *Eco*RV sites of *fhuC*. The resulting plasmids, pGS78 and pGS79, were introduced into strain KO281 (*fhuC*). The transformants remained albomycin resistant and growth deficient on ferrichrome as the sole iron source, showing that the plasmid-encoded *fhuC* derivatives were inactive and unable to complement the chromosomal *fhuC* mutation. The pGS78 and pGS79 transformants of the *E. coli fhuC*$^+$ strains W3110, AB2847, H1443, and KS474 retained their albomycin sensitivity and their ability to grow on ferrichrome, demonstrating a lack of negative complementation. Apparently, the larger alterations in the *fhuC* gene resulted in FhuC derivatives which were unable to compete with active FhuC protein. All transformants behaved similarly, excluding recombination between plasmid and chromosomal *fhuC*. Fluorograms of [^{35}S]methionine-labeled cells of *E. coli* BL21 showed the mutated FhuC proteins with slower and faster electrophoretic mobilities, depending on whether the FhuC derivatives contained insertions or deletions, respectively (data not shown). Therefore, the lack of positive and negative complementation did not result from the lack of mutated FhuC proteins.

DISCUSSION

To understand iron(III) hydroxamate transport across the cytoplasmic membrane, it is important to know whether the transport components act sequentially as individual polypeptides or whether they interact with each other and catalyze transport in a cooperative manner. The presence of physical interactions between the proteins suggests that they interact functionally as well. Immunoelectron microscopy revealed a preferential association of FhuC with the cytoplasmic membrane in the presence of FhuB. The amount of FhuC protein synthesized from chromosomal *fhuC* was too small for detection by immunogold labeling (Fig. 1A). Overexpression resulted in a random distribution of FhuC in the cytoplasm, and some FhuC was associated with the cytoplasmic membrane (Fig. 1B). Although FhuB was encoded chromosomally, it was present in amounts too small to affect FhuC distribution. No difference in FhuC localization was observed after these experiments had been repeated with a *fhuB* deletion mutant. The same picture emerged when FhuC was overexpressed together with FhuA (Fig. 1C). Although strongly overexpressed FhuA forms inner-membrane-associated inclusion bodies (20), the smaller amount of FhuA synthesized in the experiments described in this paper did not form visible inclusion bodies, but FhuA may still be membrane bound. In this context it is of interest that FhuA did not direct FhuC to the membrane. Direction of FhuC to the membrane was seen only after simultaneous overexpression of FhuB (Fig. 1D and E). The requirement of a certain amount of FhuB to bind FhuC was demonstrated by the time-dependent overexpression of FhuB while the amount of FhuC remained constant (Fig. 1G and H). After induction of the T7 polymerase, *fhuB* cloned downstream of a T7 promoter was expressed and led to the association of FhuC, synthesized constitutively (*fhuC* on pACYC184), with the cytoplasmic membrane. This result suggests a stoichiometric relationship between FhuC and FhuB. If FhuC itself binds to or inserts into the cytoplasmic membrane, no dependence on the amount of FhuB would be expected. We take these data as evidence that FhuB mediates the association of FhuC with the cytoplasmic membrane. This does not exclude the possibility that a portion of FhuC is able to bind to the membrane without the help of FhuB. However, the amount of membrane-bound FhuC present in the cells is strongly increased when similar amounts of FhuB are synthesized. The Kyte-Doolittle hydropathy plot of the FhuC sequence reveals nine rather short hydrophobic segments, none of which are longer than 14 residues (10), so that an α-helical transmembrane arrangement is unlikely, but the segments

could well be involved in membrane insertion. The clear evidence for FhuB-mediated binding of FhuC to the membrane demonstrates a physical interaction between the two proteins, making it very likely that they also interact functionally in the transport of iron(III) hydroxamates across the cytoplasmic membrane.

Four inactive FhuC derivatives, which carried single amino acid replacements in the two domains displaying sequence homology with ATP-binding sites, suppressed wild-type FhuC activity. Overexpression of the mutated derivatives was required to displace wild-type FhuC from its site of action. The target site could be at FhuB, or it could be at FhuC if the active form of the latter is a dimer or a multimer. A direct biochemical study of whether active FhuC consists of a single polypeptide or several polypeptides is prevented by the very low chromosomal expression of the protein. Plasmid-mediated overexpression leads to the formation of aggregates (inclusion bodies), as revealed by the immunoelectron-microscopic study. The four FhuC derivatives containing either a deletion of 27 amino acids or insertions of 11 and 154 amino acids showed no phenotypic suppression. They were probably grossly altered in their conformation such that the interprotein-binding region was distorted, rendering the proteins unable to compete with wild-type FhuC. Lack of FhuC displacement by the insertion and deletion derivatives was expected and is considered a control to demonstrate the specificity of the phenotypic suppression by the FhuC derivatives carrying single amino acid substitutions. At present, it cannot be determined whether negative complementation results from interaction of FhuC with FhuB or with another FhuC, or with both.

The immunocytochemical results presented in this paper shed new light on previous interpretations of FhuC localization data. In our earlier study, the FhuC protein was found in the total membrane fraction of transformants carrying the *fhuACDB* genes on plasmid pBR322 (16). FhuC was removed by washing with 2% Triton X-100 in the presence of 10 mM $MgCl_2$, from which a FhuC location in the cytoplasmic membrane was deduced (16). However, a substantial amount of overexpressed FhuC in the cytoplasm, most probably deposited as inclusion bodies, was shown by our latest immunocytochemical results. It is not known how much of this cytoplasmic FhuC fraction is solubilized during membrane preparation or whether remnants are removed by treatment with Triton X-100. In this study, the immunoelectron microscopy data have clearly localized FhuC in the cytoplasmic membrane.

Very recently, physical interaction of the cytoplasmic membrane components was shown for two other periplasmic-binding protein-dependent transport systems. The MalF, MalG, and MalK proteins of the *E. coli* maltose transport system were solubilized as a 1:1:2 complex. All three proteins were precipitated with an anti-MalF antibody, and specific interactions between MalF and MalG and between MalF and MalK were revealed by chemical cross-linking (12). Coprecipitation was also shown for the three transport components HisQ, HisM, and HisP of the *Salmonella typhimurium* histidine transport system with antibodies against any one of the proteins. Chemical cross-linking revealed a 1:1:2 stoichiometry of the three proteins. Although HisP (equivalent to MalK and FhuC) was membrane associated in the absence of HisQ and HisM, it was much more tightly bound in their presence (21). It is not known whether the ATP-binding proteins form dimers. Two FhuC proteins may bind individually to one FhuB protein, which is twice the size of the corresponding nonpolar components of most of the other periplasmic-binding protein-dependent transport systems, or two FhuC polypeptides may form a dimer and interact as such with FhuB. Isolation of a FhuC-FhuB complex by cross-linking is hampered by the pronounced degradation of FhuC by cellular proteases upon cell disruption.

ACKNOWLEDGMENTS

We thank R. Tsolis for comments on the manuscript. We are grateful to C. Herrmann and S. Paul for excellent technical assistance.

This work was supported by the Deutsche Forschungsgemeinschaft (grant SFB 323) and the Fonds der Chemischen Industrie.

REFERENCES

1. **Ames, G. F.-L., and A. Joshi.** 1990. Energy coupling in bacterial periplasmic permeases. J. Bacteriol. **172:**4133–4137.
2. **Ames, G. F.-L., C. Mimura, and V. Shyamala.** 1990. Bacterial periplasmic permeases belong to a family of transport proteins operating from Escherichia coli to human: traffic ATPases. FEMS Microbiol. Rev. **75:**429–446.
3. **Ames, G. F.-L., and E. N. Spudich.** 1976. Protein-protein interaction in transport: periplasmic histidine-binding protein J interacts with P protein. Proc. Natl. Acad. Sci. USA **73:**1877–1881.
4. **Angerer, A., S. Gaisser, and V. Braun.** 1990. Nucleotide sequences of the *sfuB* and *sfuC* genes of *Serratia marcescens* suggest a periplasmic-binding protein-dependent iron transport mechanism. J. Bacteriol. **172:**572–578.
5. **Becker, K., W. Köster, and V. Braun.** 1990. Iron(III)hydroxamate transport of *Escherichia coli* K 12: single amino acid replacements at potential ATP-binding sites inactivate the FhuC protein. Mol. Gen. Genet. **223:**159–162.
6. **Bell, P. E., C. D. Nau, J. T. Brown, J. Konisky, and R. J. Kadner.** 1990. Genetic suppression demonstrates interaction of TonB protein with outer membrane transport proteins in *Escherichia coli*. J. Bacteriol. **172:**3826–3829.
7. **Bishop, L., R. Agbayani, Jr., S. V. Ambudkar, P. C. Maloney, and G. F.-L. Ames.** 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.
8. **Braun, V., R. Gross, W. Köster, and L. Zimmermann.** 1983. Plasmid and chromosomal mutants in the iron(III)-aerobactin transport system of *Escherichia coli*. Use of streptonigrin for selection. Mol. Gen. Genet. **192:**131–139.
9. **Braun, V., K. Günter, and K. Hantke.** 1991. Transport of iron across the outer membrane. Biol. Metals **4:**14–22.
10. **Burkhardt, R., and V. Braun.** 1987. Nucleotide sequence of the *fhuC* and *fhuD* genes involved iron(III)hydroxamate transport: domains in FhuC homologous to ATP-binding proteins. Mol. Gen. Genet. **209:**49–55.
11. **Coulton, J. W., P. Mason, and D. D. Allatt.** 1987. *fhuC* and *fhuD* genes for iron(III)-ferrichrome transport into *Escherichia coli* K-12. J. Bacteriol. **169:**3844–3849.
12. **Davidson, A. L., and H. Nikaido.** 1991. Purification and characterization of the membrane-associated components of the maltose transport system from *Escherichia coli*. J. Biol. Chem. **266:**8946–8951.
13. **Dean, A. D., A. L. Davidson, and H. Nikaido.** 1989. Maltose transport in membrane vesicles of *Escherichia coli* is linked to ATP hydrolysis. Proc. Natl. Acad. Sci. USA **86:**9134–9138.
14. **Dzandu, J. K., J. F. Johnson, and G. E. Wise.** 1988. Sodium dodecyl sulfate-gel electrophoresis: staining of polypeptides using heavy metal salts. Anal. Biochem. **174:**157–167.
15. **Eick-Helmerich, K., K. Hantke, and V. Braun.** 1987. Cloning and expression of the exbB gene of *Escherichia coli*. Mol. Gen. Genet. **206:**246–251.
16. **Fecker, L., and V. Braun.** 1983. Cloning and expression of the *fhu* genes involved in iron(III)-hydroxamate uptake by *Escherichia coli*. J. Bacteriol. **156:**1301–1314.

17. **Field, J., J.-I. Nikawa, D. Broek, B. MacDonald, L. Rodgers, I. A. Wilson, R. A. Lerner, and M. Wigler.** 1988. Purification of a *RAS*-responsive adenylyl cyclase complex from *Saccharomyces cerevisiae* by use of an epitope addition method. Mol. Cell. Biol. **8:**2159–2165.
18. **Fischer, E., K. Günter, and V. Braun.** 1989. Involvement of ExbB and TonB in transport across the outer membrane of *Escherichia coli*: phenotypic complementation of *exb* mutants by overexpressed *tonB* and physical stabilization of TonB by ExbB. J. Bacteriol. **171:**5127–5134.
19. **Fischer, E., W. Köster, H. Hoffmann, and V. Braun.** Unpublished data.
20. **Hoffmann, H., E. Fischer, H. Schwarz, and V. Braun.** 1986. Overproduction of the proFhuA outer membrane receptor protein of *Escherichia coli* K12: isolation, properties, and immunocytochemical localization at the inner side of the cytoplasmic membrane. Arch. Microbiol. **145:**334–341.
21. **Kerppola, R. E., V. K. Shyamala, P. Klebba, and G. F.-L. Ames.** 1991. The membrane-bound proteins of periplasmic permeases form a complex. Identification of the histidine permease complex. J. Biol. Chem. **266:**9857–9865.
22. **Köster, W.** 1991. Iron(III) hydroxamate transport across the cytoplasmic membrane of *Escherichia coli*. Biol. Metals **4:**23–32.
23. **Köster, W., and V. Braun.** 1986. Iron hydroxamate transport of *Escherichia coli*: nucleotide sequence of the *fhuB* gene and identification of the protein. Mol. Gen. Genet. **204:**435–442.
24. **Köster, W., and V. Braun.** 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:**233–239.
25. **Köster, W., and V. Braun.** 1990. 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.
26. **Köster, W., and V. Braun.** 1990. Iron(III) hydroxamate transport of *Escherichia coli*. Substrate binding to the periplasmic FhuD protein. J. Biol. Chem. **265:**21407–21410.
27. **Lugtenberg, B., J. Meijers, R. Pters, P. van der Hock, L. van Alphen.** 1975. Electrophoretic resolution of the major outer membrane proteins of *Escherichia coli* K12 into four bands. FEBS Lett. **58:**254–258.
28. **Neilands, J. B.** 1981. Microbial iron compounds. Annu. Rev. Biochem. **50:**710–731.
29. **Prosnitz, P.** 1991. Determination of a region of the HisJ binding protein involved in the recognition of the membrane complex of the histidine transport system of *Salmonella typhimurium*. J. Biol. Chem. **266:**9673–9677.
30. **Sanderman, H., and J. L. Strominger.** 1972. Purification and properties of C_{55}-isoprenoid alcohol phosphokinase from *Staphylococcus aureus*. J. Biol. Chem. **247:**5123–5131.
31. **Speiser, D. M., and G. F.-L. Ames.** 1991. *Salmonella typhimurium* histidine permease mutations that allow transport in the absence of histidine-binding proteins. J. Bacteriol. **173:**1444–1451.
32. **Staudenmaier, H., B. Van Hove, Z. Yaraghi, and V. Braun.** 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.
33. **Strauch, K. L., and J. Beckwith.** 1988. An E. coli mutant preventing degradation of abnormal periplasmic proteins. Proc. Natl. Acad. Sci. USA **85:**1576–1580.
34. **Studier, F. W., and B. Moffat.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189:**113–130.
35. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7-RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82:**1074–1078.