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Conversion of the FhuA transport protein into a diffusion channel through the outer membrane of *Escherichia coli*

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The FhuA receptor protein is involved in energy-coupled transport of Fe^{3+} via ferrichrome through the outer membrane of *Escherichia coli*. Since no energy source is known in the outer membrane it is assumed that energy is provided through the action of the TonB, ExbB and ExbD proteins, which are anchored to the cytoplasmic membrane. By deleting 34 amino acid residues of a putative cell surface exposed loop, FhuA was converted from a ligand specific transport protein into a TonB independent and nonspecific diffusion channel. The FhuA deletion derivative FhuA Δ322-355 formed stable channels in black lipid membranes, in contrast to wild-type FhuA which did not increase membrane conductance. The single-channel conductance of the FhuA mutant channels was at least three times larger than that of the general diffusion porins of *E.coli* outer membrane. It is proposed that the basic structure of FhuA in the outer membrane is a channel formed by β-barrels. Since the loop extending from residue 316 to 356 is part of the active site of FhuA, it probably controls the permeability of the channel. The transport-active conformation of FhuA is mediated by a TonB-induced conformational change in response to the energized cytoplasmic membrane. The ferrichrome transport rate into cells expressing FhuA Δ322-355 increased linearly with increasing substrate concentration (from 0.5 to 20 μM), in contrast to FhuA wild-type cells, which displayed saturation at 5 μM. This implies that in wild-type cells ferrichrome transport through the outer membrane is the rate-limiting step and that TonB, ExbB and ExbD are only required for outer membrane transport.

Key words: channel/*E.coli*/FhuA transport protein

Introduction

The permeability barrier of the outer membrane of *Escherichia coli* for substrates is overcome in three different ways: (i) diffusion through the porins which form water-filled channels, (ii) facilitated diffusion which involves stereospecific recognition between substrates and channel-forming proteins and (iii) energy-coupled transport. The least understood and most interesting process is the energy-coupled transport since no energy source is known to exist outside the cytoplasmic membrane. Substrates which are transported are ferric siderophores (Braun, 1985; Braun and Hantke, 1991) and vitamin B_{12} (Kadner, 1990). These are too large (ferric siderophores >700 daltons, vitamin B_{12} 1357 daltons) to diffuse through the porins with rates sufficiently high to support growth. Instead they bind to outer membrane receptor proteins from which they are translocated across the outer membrane into the periplasm. One of these receptors is the FhuA protein through which ferrichrome and the structurally similar antibiotic albomycin are taken up. FhuA also serves as the binding site of colicin M and of the phages T5, T1 and φ80. These multifunctional properties make FhuA a particularly attractive subject for the study of receptor-mediated transport through the outer membrane.

Ferrichrome, albomycin and colicin M remain bound to FhuA at the cell surface unless cells are energized (Hantke and Braun, 1978). Release of DNA from the heads of phage T1 and φ80 requires FhuA of an energized cell (Hancock and Braun, 1976). These data imply a conformational change of FhuA in response to cell energization. Only phage T5 infects unenergized cells. Related to energization is the requirement for the TonB, ExbB and ExbD proteins which are involved in all FhuA-dependent transport processes except infection by phage T5 (Braun, 1989). TonB (Postle and Skare, 1988; Hannavy *et al.*, 1990) and ExbD (Kampfenkel and Braun, 1992) are anchored by the N-terminal portion in the cytoplasmic membrane and extend with the remainder into the periplasmic space, ExbB crosses the cytoplasmic membrane three times and most of the protein is located in the cytoplasm (Kampfenkel and Braun, 1993). The interaction of TonB with FhuA has been shown by NMR (Brewer *et al.*, 1990), from physical stabilization of the TonB protein by FhuA (Günter and Braun, 1990) and has been inferred from suppression of certain *fhuA* mutants by *tonB* mutants (Schöffler and Braun, 1989). Since ExbB also stabilizes TonB (Fischer *et al.*, 1989; Skare and Postle, 1991) and ExbD (Fischer *et al.*, 1989), the three proteins apparently form a complex. Energized cytoplasmic membrane induces a conformational change of TonB, which in turn induces the transport-active conformation of FhuA. ExbB and ExbD are somehow involved in the induction of an active TonB conformation.

For the understanding of FhuA-mediated ferrichrome uptake it is essential to know the structure of the protein. If FhuA basically forms a channel one should be able to isolate FhuA derivatives that form open channels and no longer require the presence of TonB, ExbB and ExbD for the uptake of ferrichrome and albomycin. The channel of FhuA may be closed (gated) by a small portion of the polypeptide as has been found by three-dimensional X-ray analysis at the constriction site of the outer membrane porin of *Rhodobacter capsulatus* (Weiss *et al.*, 1991a,b; Weiss and Schultz, 1992) and of OmpF and PhoE of *E.coli* (Cowan *et al.*, 1992).

Recently, we have determined loops of FhuA at the cell surface and loops exposed to the periplasm by rendering FhuA susceptible to proteolysis through insertion of 4, 8,

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12, 16 and 22 amino acids at 34 different sites along the entire polypeptide (Koebnik and Braun, 1993). One such loop predicted to be at the cell surface extends from residue 316 to residue 356. Insertion of four or 16 amino acids after residue 321 conferred resistance to phage T1, a 10^5-fold reduction in phage ϕ80 and a 10^2-fold reduction in phage T5 sensitivity. Sensitivity to colicin M was hardly affected, cells were fully sensitive to albomycin and displayed a wild-type growth response to ferrichrome. In the same loop is located the Asp348 deletion mutation which rendered cells resistant to the phages T1 and ϕ80 and to albomycin, 1000-fold less sensitive to colicin M and 10- to 100-fold less sensitive to phage T5 (Killmann and Braun, 1992). Apparently, this loop is important for the activity of FhuA. If FhuA has a basic design similar to that of the porins, loop 316–356 may form, or be part of, the active site that controls ferrichrome permeation through FhuA. Therefore, deletion of this loop may convert FhuA into an open channel. We have undertaken such a study, which shows that FhuA is converted into an open channel by deleting residues 322–355. Uptake of ferrichrome into the periplasm through the FhuA derivative no longer required TonB, ExbB and ExbD, and the outer membrane no longer formed a permeability barrier for compounds unrelated to ferrichrome, such as SDS and bacitracin. Isolated FhuA Δ322–355 formed large stable channels in black lipid membranes in contrast to FhuA wild-type, which conferred only low, if any conductance.

Results

Construction and expression of FhuA deletion derivatives

Fragments of the predicted FhuA loop 316–356 (Figure 1) and larger fragments comprising this loop were excised by deleting DNA fragments from previously constructed plasmids using restriction endonucleases (Koebnik and Braun, 1993), as described in Materials and methods. The FhuA derivatives obtained (named according to the amino acid residues of mature wild-type FhuA that they lack) are FhuA Δ322–333 (lacking residues 322–333), FhuA Δ322–355, FhuA Δ322–405, FhuA Δ334–405 and FhuA Δ163–368. These FhuA derivatives contained three, four or 13 additional amino acid residues at the fusion sites (Table I) which had been deliberately inserted for the FhuA topology study (Koebnik and Braun, 1993). FhuA d322–405 and FhuA d334–417 contained tandem duplications of segment 322–405 and 334–417, respectively.

The plasmids were introduced into *E.coli* HK96 *fhuA tonB* in which *fhuE* was also deleted to study receptor independent transport of ferrioxamine B which binds to FhuE. The transformants carried the mutated *fhuA* genes on the multicopy pBluescript SK+ vector downstream of the gene 10 promoter of phage T7. Formation of the FhuA derivatives and their incorporation into the outer membrane were examined in *E.coli* WM1576 which carried the temperature-inducible T7 RNA polymerase on plasmid pGP1-2 (Tabor

Fig. 1. Outer membrane topology of the FhuA protein as proposed by Koebnik and Braun (1993). The amino acid residues that indicate the deletions and tandem duplications constructed in this paper are numbered. The Asp348 (D) residue lacking in a mutant is boxed. Top, loops extending to the cell surface; bottom, loops exposed to the periplasmic side of the outer membrane.

and Richardson, 1985). So that only the *fhuA* genes were expressed, *E.coli* RNA polymerase was inhibited by rifampicin [the deletion derivatives exhibited an increased sensitivity to this antibiotic (see later) so only a quarter of the usual concentration was used]. In outer membrane preparations of transformants expressing FhuA d322−405 (Figure 2, lane 4), FhuA Δ322−355 (lane 6) and FhuA Δ163−368 (lane 7), the mutant proteins were found at levels similar to wild-type (lane 8). The size of these FhuA derivatives corresponded to their calculated molecular weights. In contrast, the amount of FhuA Δ322−405 was much less than wild-type (lane 2, indicated by arrow), as has been observed previously on immunoblots (Carmel and Coulton, 1991). Of the transformants carrying pHK210 (FhuA Δ322−333), pHK214 (FhuA Δ334−405), and pHK221 (FhuA d334−417) each contained a single major FhuA degradation product (indicated by an arrow). The proteins seen on the autoradiograph also reacted with anti-FhuA antibodies (immunoblots not shown).

FhuA Δ322−355 turned out to be the most interesting derivative so the amount exposed at the cell surface was compared with that of FhuA wild-type. Anti-FhuA serum raised in rabbits was incubated overnight with cells and the adsorbed antibodies determined enzymatically by hydrolysis of *p*-nitrophenylphosphate with alkaline phosphatase bound to anti-rabbit IgG. The supernatants of strains AB2847, HK96 pHK226 and HK96 yielded an OD_{405nm} of 2.6, 2.2 and 0.6 respectively, showing that FhuA Δ322−355 was not only associated with the outer membrane fraction but also integrated such that nearly as much FhuA Δ322−355 was exposed at the cell surface as wild-type FhuA. The value of 0.6 for HK96 represents nonspecific binding of proteins in the anti-IgG serum since the same optical density of 0.6 was obtained with all strains incubated with the anti-IgG serum in the absence of anti-FhuA serum.

Uptake of ferrichrome by the FhuA deletion derivatives no longer depends on TonB, ExbB and ExbD

E.coli HK96 (*fhuA tonB fhuE*) was used as the recipient of the plasmids carrying wild-type *fhuA* and the various *fhuA* deletion and duplication plasmids. In this strain FhuA-related activities depended on plasmid encoded FhuA and were independent of TonB activity. Growth was determined on

Table I. Bacterial strains and plasmids

Strain or plasmid	Genotype and phenotype	Reference or source
E.coli K12		
AB2847	*aroB malT thi tsx*	Hantke and Braun (1978)
UL3	AB2847 *fhuA recA* Tn*10*	F.Ullrich
MS172	F^- *ara D139 lacU169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR aroB thi fhuE*::*plac* Mu53	Sauer *et al.* (1987)
BR158	AB2847 *tonB*	Hoffmann *et al.* (1986)
H1388	AB2847 Δ(*lac*) *exbB*::Tn*10*	K.Hantke
H1020	*lac*(Am) *trp*(Am) *his metB tsx zad*::Tn*10*	K.Hantke
41/2	AB2847 *cir fepA fhuA*	Hantke and Braun (1978)
WM1576	K38 HfrC pGP1-2	Tabor and Richardson (1985)
HK96	MS172 *tonB fhuA*	This study
HK98	MS172 *tonB*	This study
HK99	BR158 *fhuA*	This study
Plasmids		
pHK210	pSK+ *fhuA* Δ322−333 (P_{321} PDLA P_{334})	This study
pHK211	pSK+ *fhuA* Δ322−405 (P_{321} PDLA V_{406})	This study
pHK214	pSK+ *fhuA* Δ334−405 (A_{333} PDLA V_{406})	This study
pHK220	pSK+ *fhuA* d322−405 (P_{405} PDLA A_{322})	This study
pHK221	pSK+ *fhuA* d334−417 (P_{417} PDLA P_{334})	This study
pHK226	pSK+ *fhuA* Δ322−355 (P_{321} PDL S_{356})	This study
pHK228	pSK+ *fhuA* Δ163−368 (K_{162} EGGTAELIPGTQ D_{369})	This study
pSKF0	pSK+ *fhuA* wild-type	Koebnik and Braun (1993)
pSKF162-16	pSK+ *fhuA* K_{162} EGGTAEFPWEFCGLPK E_{163}	Koebnik and Braun (1993)
pSKF321-04	pSK+ *fhuA* P_{321} PDLA A_{322}	Koebnik and Braun (1993)
pSKF333-04	pSK+ *fhuA* A_{333} PDLA P_{334}	Koebnik and Braun (1993)
pSKF369-16	pSK+ *fhuA* D_{369} LGTWNSRGSPGIPGTQ I_{370}	Koebnik and Braun (1993)
pSKF405-04	pSK+ *fhuA* P_{405} PDLA V_{406}	Koebnik and Braun (1993)
pSKF417-04	pSK+ *fhuA* P_{417} PDLA A_{418}	Koebnik and Braun (1993)
pHK763	pT7-6 *fhuA* wild-type	This study
pWK360	pT7-5 *fhuACDB*	W.Köster
pKE7	pUC18 *exbBD* wild-type	K.Eick-Helmerich
pIM91	pSU18 *tonB* wild-type	I.Traub
pTO4	pBR322 *cma cmi* wild-type	Ölschläger *et al.* (1984)
pT7-6	Amp^r	Tabor and Richardson (1985)
pT7-5	Amp^r	Tabor and Richardson (1985)
pSU18	Cm^r	Takeshita *et al.* (1987)
pUC18	Amp^r	Yanisch-Perron *et al.* (1985)
pBluescriptSK+	Amp^r	Short *et al.* (1988)

iron-limiting NBD agar plates around filter paper discs which contained 10 μl of a 10 mM ferrichrome solution. This is a very sensitive and reliable assay and also detects very low ferrichrome uptake which is almost undetectable by a [^{55}Fe]ferrichrome transport assay. The diameter of the growth zone is related to the logarithm of the ferrichrome concentration so that small differences in the diameter of the growth zones reflect large differences in ferrichrome concentration. Transformants expressing FhuA Δ322–355, FhuA Δ322–405 and FhuA Δ163–368 grew well. The diameter of the growth zone was large and cell density was high ('h' in Table II). FhuA Δ322–405 has been constructed previously in a study of the domains of FhuA that interact with ligands, and displayed properties as found in this paper (Carmel and Coulton, 1991). HK96 expressing FhuA wild-type encoded on pSKF0 displayed no growth on ferrichrome due to the lack of TonB activity. Ferrichrome uptake via the FhuA deletion derivatives was less efficient than uptake through wild-type FhuA and TonB as strain UL3 (*fhuA*$^-$ *tonB*$^+$ pSKF0: FhuA wild-type) exhibited a dense growth zone of 42 mm as compared with 26 mm for HK96 (*fhuA*$^-$ *fhuE*$^-$ *tonB*$^-$ pHK226) (FhuA Δ322–355) (Table II).

TonB independent translocation across the outer membrane containing FhuA Δ322–355 implied also independence of ExbB and ExbD. This was tested with strain H1388 which carried a Tn*10* insertion in *exbB* that exerted a strong polar effect on *exbD* expression. Growth on NBD plates containing ferrichrome as an iron source was restored after transformation of H1388 with plasmid pHK226 *fhuA* Δ322–355, indicating ExbB and ExbD independence of ferrichrome entry through FhuA Δ322–355.

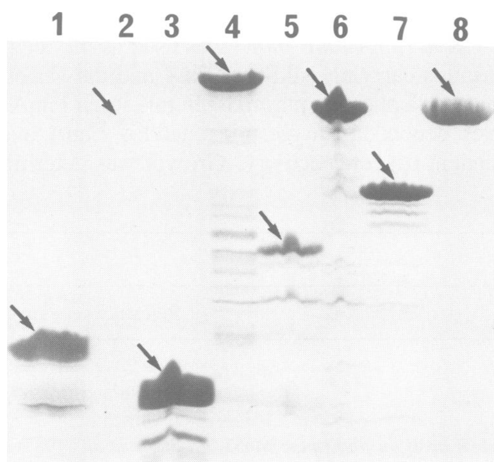

Fig. 2. Autoradiograph of outer membrane proteins after separation by SDS–PAGE. *E.coli* WM1576 pGP1-2 cells transformed with pHK210 (FhuA Δ322–333) (lane 1), pHK211 (FhuA Δ322–405) (lane 2), pHK214 (FhuA Δ334–405) (lane 3), pHK220 (FhuA d322–405) (lane 4), pHK221 (FhuA d334–417) (lane 5), pHK226 (FhuA Δ322–355) (lane 6), pHK228 (FhuA Δ163–368) (lane 7) and pSKFO (lane 8) were labelled with [^{35}S]methionine as described in Materials and methods. The arrows mark FhuA wild-type (lane 8) and the FhuA derivatives, in the case of lanes 1, 3 and 5, major degradation products.

FhuA Δ322–355 allows permeation of many ions

Growth stimulation was not restricted to ferrichrome since ferrioxamine B also provided the necessary iron (Table II). Ferrioxamine B had to enter HK96 via the FhuA derivatives since the ferrioxamine receptor FhuE was missing. Additional growth promotion assays were performed with a number of ferric siderophores derived from ferrichrome (listed in Killmann and Braun, 1992) and coprogen. They all stimulated growth of cells expressing FhuA Δ322–355, demonstrating the strong and nonspecific increase in outer membrane permeability with regard to solute structure.

The FhuA derivatives also conferred sensitivity to SDS and bacitracin against which transformants expressing wild-type FhuA, or cells expressing no FhuA were resistant. Bacitracin is too large (mol. wt 1421) to diffuse through the porins. SDS would be small enough (mol. wt 288) but is excluded by the outer membrane from entering the periplasm (Nikaido, 1979), and therefore does not reach the SDS sensitive cytoplasmic membrane.

HK96 transformants expressing FhuA Δ322–333 displayed a weak growth zone (Table II, small diameter and low density), suggesting that this deletion was probably too small to open the FhuA channel (see below). This was also the only derivative that still exhibited phage T5 sensitivity

Table II. Properties of FhuA derivatives

FhuA	Growth zone (mm) on		Sensitivity to					
	Fer	Fox	T5	T1	ϕ80	ColM	SDS	Bac
Δ322–333	11 l	16 l	6	–	–	–	–	–
Δ322–355	26 h	24 h	–	–	–	–	10	11
Δ322–405	24 h	22 l	–	–	–	–	(11)[a]	8
Δ334–405	8 l	14 l	–	–	–	–	–	–
Δ163–368	24 h	28 h	–	–	–	–	13	12
d322–405	16 l	22 l	7	8	8	4	–	–
d334–417	–	14 l	6	7	6	2	–	–
HK96 FhuA$^-$	–	–	–	–	–	–	–	–
HK96 pSKF0 FhuA$^+$	–	–	7	–	–	–	–	–
UL3 FhuA$^-$	–	16 l	–	–	–	–	–	–
UL3 pSKFO FhuA$^+$	42	14 l	7	8	8	5	–	–

Growth stimulation was tested with strain HK96 (*fhuA tonB fhuE*) and sensitivity was tested with strain UL3 (*fhuA tonB*$^+$), both strains transformed with plasmids encoding the FhuA derivatives listed (see also Table I). –, no sensitivity, no growth. h, high density of cells; l, low density of cells. The diameter of the paper disc (6 mm) was not subtracted from the diameter of the growth (inhibition) zone around the disc. d, duplication. Fer, ferrichrome. Fox, ferrioxamine B. ColM, colicin M. SDS, sodium dodecyl sulfate. Bac, bacitracin.
[a]Weak inhibition.

suggesting that the remaining portion of the loop closed the channel and served as a phage binding site. Unexpectedly, HK96 expressing FhuA Δ334–405 (and FhuA Δ334–416, not listed) also showed only a very weak growth stimulation as if the region between residues 321 and 334 has to be removed to open the channel.

The derivatives FhuA d322–405 and FhuA d334–417 carrying tandem duplications of 85 amino acid residues were properly inserted into the outer membrane since they were highly sensitive to the phages and to colicin M. Less FhuA d334–417 was observed in cell lysates (data not shown) which explains its lower activity. According to the model (Figure 1) both duplication derivatives contained the loop 316–356 and four consecutive transmembrane segments twice, without impairment of FhuA activity. This result supports the high specificity in the complete alteration of the transport properties displayed by the FhuA deletion derivatives.

Iron transport into cells expressing FhuA Δ322–355 is TonB independent

Time dependent transport of [$^{55}Fe^{3+}$]ferrichrome (0.5 μM $^{55}Fe^{3+}$ in the assay) was determined with strain HK99 *fhuA tonB* lacking both the FhuA protein and the TonB function, and with the transformant HK99 pHK226 containing FhuA Δ322–355. The transformant took up iron with linear kinetics during the assay (90 min) while no uptake was observed in the mutant (Figure 3B). The transport rate into the strain expressing the FhuA deletion protein was 20% of the rate into the wild-type parent strain AB2847 *fhuA*$^+$ *tonB*$^+$ (Figure 3A).

If FhuA Δ322–355 forms an open channel one would expect a linear relationship between the transport rate and the concentration of [$^{55}Fe^{3+}$]ferrichrome as long as permeation through the outer membrane and not transport through the cytoplasmic membrane is the rate-limiting step. We therefore determined uptake of iron at iron concentrations from 0.5 to 20 μM after 30 min of incubation. The transformant expressing FhuA Δ322–355 showed a linear increase of the iron uptake rate with increasing ferrichrome concentrations (Figure 4). In contrast, wild-type AB2847 displayed saturation kinetics in that the maximum uptake rate was reached at 5 μM ferrichrome, and higher concentations increased only slightly the iron transport rate (Figure 4).The value of 5 μM was not determined exactly, and is, for unknown reasons, certainly too high since previous studies determined a Michaelis–Menten constant (K_M) in the order of 0.1 μM (Wookey *et al.*, 1981; Rutz *et al.*, 1992). At low ferrichrome concentrations (0.5 μM) the TonB dependent transport through the outer membrane was five times faster than the diffusion rate. At higher ferrichrome concentrations the diffusion rate through the FhuA Δ322–355 channel became higher so that the overall transport rate was more than double the transport rate into the wild-type at 20 μM ferrichrome, and 5-fold the transport rate into the wild-type at 0.5 μM ferrichrome. The time and concentration dependence indicates that the permeability rate through the outer membrane was the rate-limiting step in iron uptake of FhuA wild-type cells via ferrichrome.

FhuA Δ332–355 forms a large stable channel in black lipid membranes

The *in vivo* experiments revealed a substantial change in the permeability properties of the outer membranes caused by

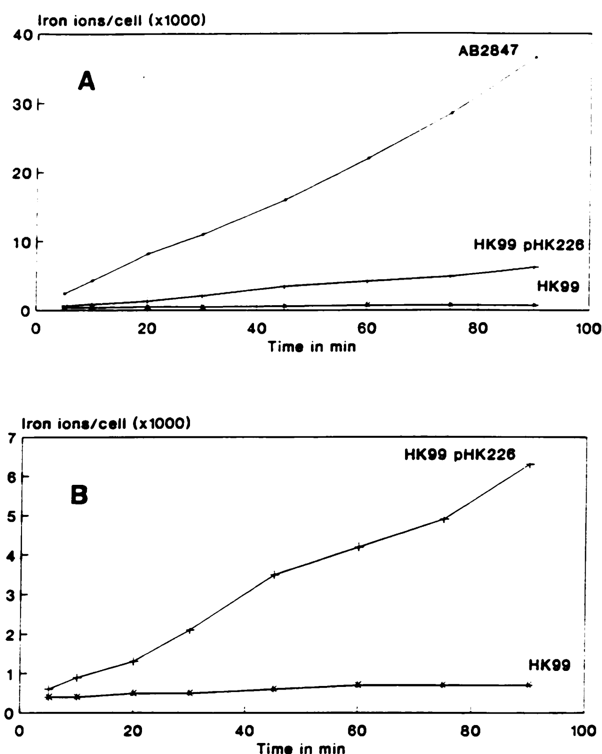

Fig. 3. Transport of [$^{55}Fe^{3+}$]ferrichrome (0.5 μM) into cells of *E.coli* AB2847, HK99 pHK226 (FhuA Δ322–355) and HK99 FhuA$^-$. Panel B shows the same data as panel A with an extended scale on the *y*-axis to demonstrate the difference in transport between HK99 lacking FhuA and HK99 containing FhuA Δ322–355.

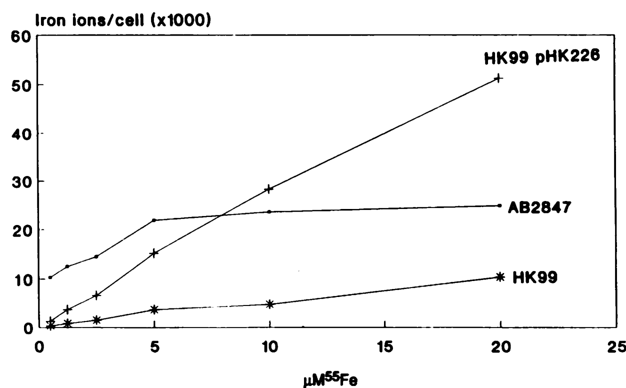

Fig. 4. Dependence of [$^{55}Fe^{3+}$]ferrichrome transport into cells of AB2847, HK99 pHK226 and HK99 on ferrichrome concentration.

FhuA Δ322–355. To study whether FhuA Δ322–355 formed channels in lipid bilayer membranes, reconstitution experiments with FhuA Δ322–355 were performed. To avoid irreversible denaturation of FhuA Δ322–355, the protein was solubilized from the outer membrane with 1% octylglucoside and purified by SDS–PAGE. The fraction of FhuA Δ322–355 that entered the gel was low (Figure 5, lane 3, Coomassie-stained gel) unless the sample was heated to 50°C for 3 min prior to electrophoresis (Figure 5, lane 2; sample in lane 1 was heated to 95°C for 3 min). The 50°C sample was eluted with buffer from the gel and was added to a solution of 0.1 M KCl (final protein concentration 10 ng/ml) on both sides of a black lipid bilayer membrane with no difference in channel formation. Conductance steps were

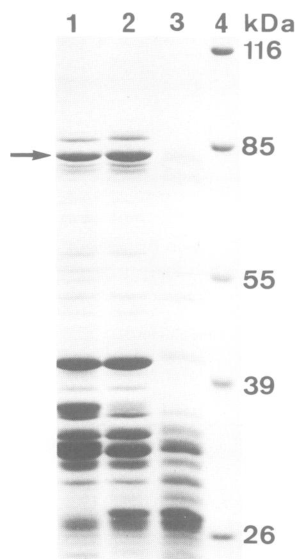

Fig. 5. Coomassie blue stained gel after SDS–PAGE (11% acrylamide) of outer membranes prepared from *E.coli* UL3 pHK226 boiled for 3 min in a water bath (lane 1), heated for 3 min to 50°C (lane 2) or unheated (lane 3). FhuA Δ322–355 is marked by an arrow. Standard proteins and their molecular weights are indicated in lane 4.

observed which appeared similar to those described previously for general diffusion pores from the outer membrane of Gram-negative bacteria (Benz *et al.*, 1978, 1980). Figure 6A shows a typical conductance recording in the presence of FhuA Δ322–355, where each step corresponds to the incorporation of one channel-forming unit into the lipid bilayer membrane. The channels had a very long lifetime in the order of 10 min or longer. In recordings with different salts and at various concentrations only upward conductance steps and no terminating ones were observed. Minor changes in the current of the open channels occurred (indicated by arrows in Figure 6A), suggesting some molecular fluctuations inside the channel. Addition of the detergents SDS or Genapol X-80 without FhuA Δ322–355 did not lead to any increase in the membrane conductance, which means that the current fluctuations of Figure 6A were specific for the presence of FhuA Δ322–355.

FhuA wild-type isolated in exactly the same way as FhuA Δ322–355 formed no large ion-permeable channels in black lipid bilayer membranes (Figure 6B). Only minor current fluctuations were observed which, under the conditions used, were <100 pS. This result also demonstrated that the large channels formed by FhuA Δ322–355 were not caused by contaminating proteins, for example porin trimers which under the conditions used (1% octylglucoside, 50°C) may not completely dissociate into monomers. However, the difference in the electrophoretic mobility of FhuA Δ322–355 (mol. wt 76 kDa) is sufficient to separate FhuA from porin trimers (mol. wt 111 kDa).

The absence of contaminating protein was also examined with a sample eluted after SDS–PAGE from a gel loaded with the outer membrane of *E.coli* UL3 *fhuA* pSK+ (vector only) lacking FhuA protein. No current was measured when the probe was added to black lipid bilayer membranes, excluding contaminating protein as the cause of FhuA Δ322–355 conductivity.

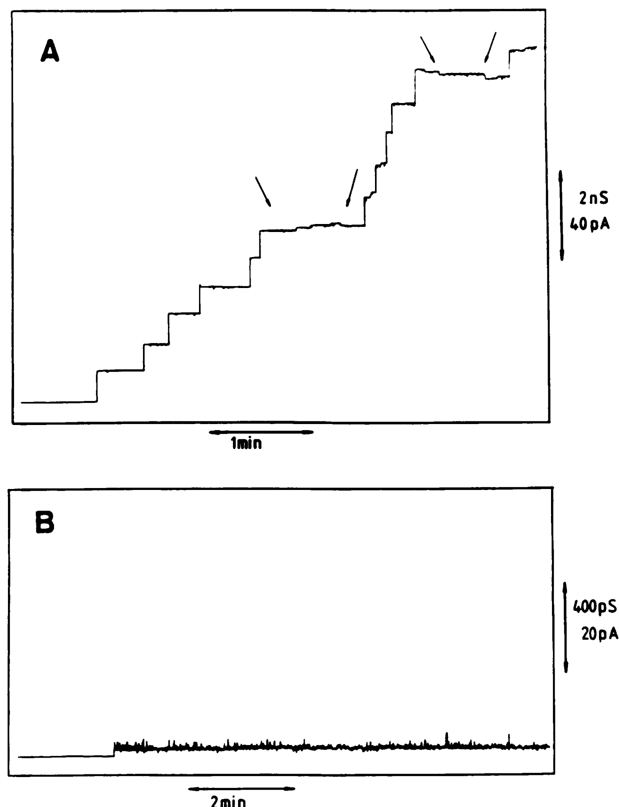

Fig. 6. Single-channel recording of a diphytanoyl phosphatidylcholine membrane in the presence of 5 ng/ml FhuA Δ322–355 (A) and 10 ng/ml FhuA wild-type (B) of *E.coli* K12. The aqueous phase contained 0.1 M KCl (A) and 1 M KCl (B). The applied membrane potential was 20 mV and the temperature 20°C. Note that the resolution of the lipid bilayer instrumentation was lower in (A) than in (B). The arrows indicate the small current fluctuations described in the text.

Single-channel analysis

The conductance steps observed with FhuA Δ322–355 were found to be fairly uniform in size. This is demonstrated in Figure 7A which shows the statistics of the conductance fluctuations in 0.1 M KCl. The single-channel conductance of FhuA Δ322–355 was ~0.6 nS, which is at least 3-fold higher than the single-channel conductance of OmpF under otherwise identical conditions (Figure 7B). This result represents another control to show that the conductance steps of Figure 6A were caused by FhuA Δ322–355 and did not represent an artefact caused by contaminating porin such as OmpF.

The high single-channel conductance observed in the presence of FhuA Δ322–355 suggested that it forms a general diffusion pore, i.e. a wide, water-filled channel. Measurements with other salts supported this assumption, since the single-channel conductances in 1 M LiCl and 1 M potassium acetate (Table III) were smaller than in 1 M KCl, by approximately the same amount as the bulk aqueous conductivities of the different salts differ (Benz *et al.*, 1985). The single-channel data of Table III suggested also that the channel of FhuA Δ322–355 displayed a certain preference for cations since the single-channel conductance was somewhat larger in potassium acetate than in LiCl. Li^+ and the acetate anion have the same aqueous mobility (Benz *et al.*, 1985) but the single-channel conductance in potassium acetate is approximately twice that in LiCl which means that the channel conducts cations preferentially without being

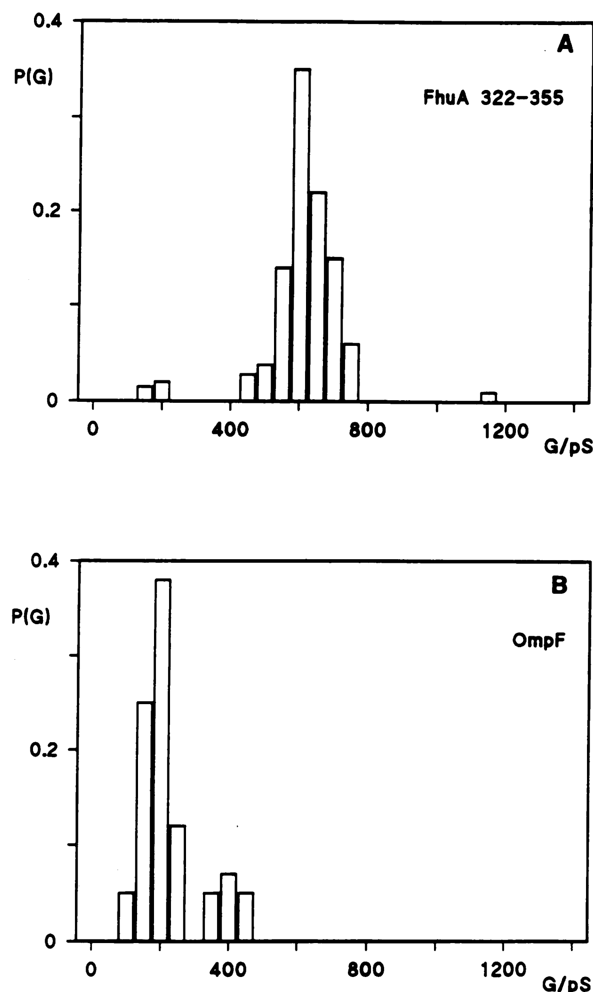

Fig. 7. (A) Histogram of the probability of the occurrence of certain conductivity steps observed with membranes formed of diphytanoyl phosphatidylcholine/*n*-decane in the presence of 5 ng/ml FhuA Δ322–355. The aqueous phase contained 0.1 M KCl. The applied membrane potential was 20 mV and the temperature was 20°C. The average single-channel conductance, G, for the maximum was 610 pS for 171 single-channel events. (B) Histogram of the probability of the occurrence of certain conductivity steps observed with membranes formed of diphytanoyl phosphatidylcholine/*n*-decane in the presence of 10 ng/ml OmpF from *E.coli* K12. The other conditions were identical to those in (A). The average single-channel conductance, G, for the maximum was 180 pS for 137 single-channel events. The right-hand maximum at 400 pS probably represents the simultaneous reconstitution of two OmpF trimers.

fully selective for cations. Probably, the channel contains both negatively and positively charged groups and the excess of negative charges makes it cation-selective, as is similarly the case with the OmpF cation-selective general diffusion pores of *E.coli* (Benz *et al.*, 1985; Cowan *et al.*, 1992) and of *Rhodobacter capsulatus* (Benz *et al.*, 1987; Weiss *et al.*, 1991a,b), i.e. both anions and cations can enter the channel and influence one another.

The conductance of the channel formed by FhuA Δ322–355 was measured at KCl concentrations between 0.1 and 3 M. The single-channel conductance showed a minor saturation since it increased ~10 times over this concentration range instead of 30 times. Nevertheless, it was approximately a linear function of the bulk aqueous conductivity, which is consistent with the assumption that FhuA Δ322–355 forms a wide, water-filled channel.

Table III. Average single-channel conductance, G, of the FhuA Δ322–355 channel as a function of different salt solutions

Salt	Concentration (M)	V_m (mV)	G (nS)
LiCl	1.0	20	0.93
KCl	0.1	20	0.61
	0.3	20	1.1
	1.0	20	3.0
	3.0	20	8.0
KCH_3COO (pH 7)	1.0	20	1.8

The membranes were formed from 1% diphytanoyl phosphatidylcholine dissolved in *n*-decane; T = 20°C. The pH of the aqueous salt solutions was around 6 unless otherwise indicated. G is given as the mean of at least 100 single steps.

Effects of ferrichrome on the conductance of the FhuA Δ322–355 channel

In preliminary experiments we examined whether FhuA Δ322–355, incorporated into a black lipid bilayer membrane, binds ferrichrome as FhuA does in outer membranes and in isolated form (Hantke and Braun, 1978; Hoffmann *et al.* 1986). Indeed, multi-channel experiments showed a decrease in membrane conductance with increasing concentrations of ferrichrome similar to the inhibition of the LamB channel conductance by maltodextrins (Benz *et al.*, 1986). Single-channel conductance also decreased with increasing concentrations of ferrichrome but the FhuA Δ322–355 channels did not close completely. From a formalism similar to that described for LamB (Benz *et al.*, 1986) a preliminary stability constant for ferrichrome binding of ~700 l/mol (half saturation constant ≈1.4 mM) could be calculated. However, one has to remember that this stability constant reflects binding to the Δ322–355 derivative of FhuA and not to wild-type.

Discussion

The FhuA protein is essential for Fe^{3+} uptake by ferrichrome. Deletion mutants and certain point mutants in the *fhuA* gene render cells inactive in the uptake of ferrichrome (Schöffler and Braun, 1989; Killmann and Braun, 1992). In unenergized cells, or in TonB, ExbB or ExbD mutants, ferrichrome binds to FhuA and prevents binding of phage T5 (Hantke and Braun, 1978) and of colicin M (Braun *et al.*, 1980). However, uptake of ferrichrome and colicin M requires TonB, ExbB and ExbD activity. Based on these observations we have proposed that FhuA can assume both an unenergized and an energized conformation. The latter is triggered by an energized TonB conformation which in turn is induced by the action of ExbB and ExbD (Eick-Helmerich and Braun, 1989).

Our recent FhuA membrane topology model suggested regions of FhuA that might be important for controlling FhuA-mediated transport through the outer membrane. Of special interest was loop 316–356 in which deletion of Asp348 strongly impaired all FhuA-related activities (Killmann and Braun, 1992). In this paper we show that excision of residues 322–355 converted FhuA into an open channel. Cells expressing FhuA Δ322–355 were able to grow on ferrichrome as the sole iron source in the absence of TonB. The FhuA Δ322–355 channel was also used by ferrioxamine B to enter the periplasm of a *fhuE* mutant

lacking both the ferrioxamine B receptor protein FhuE and TonB. We also tested other ferric siderophores, which could hardly, or not at all deliver iron to wild-type cells due to the lack of outer membrane receptors or the lack of TonB activity. These ferric siderophores stimulated growth of cells expressing FhuA Δ322−355, presumably by diffusing through FhuA Δ322−355 into the periplasm. The FhuB,C,D inner membrane transport system into the cytoplasm accepts a large variety of ferric siderophores of the hydroxamate type, as has been clearly demonstrated for ferrichrome, ferrichrysin, ferricrocin, albomycin, aerobactin, coprogen and ferrioxamine B (Braun and Hantke, 1991; Killmann and Braun, 1992).

Cells expressing FhuA Δ322−355 also became sensitive to SDS and bacitracin in contrast to cells expressing FhuA wild-type or no FhuA which were protected by the permeability barrier of the outer membrane. In addition to the components listed in Table II, novobiocin, erythromycin, rifampicin and vancomycin increased the sensitivity of FhuA Δ322−355 cells compared with that of $FhuA^+$ or $FhuA^-$ cells. These data clearly show that the outer membrane permeability was strongly increased in cells expressing FhuA Δ322−355, that permeation of the substances did not require TonB activity and that the channel did not discriminate between compounds of very different structures.

The results of the transport assays were consistent with the above plate assays. FhuA Δ322−355 $TonB^-$ cells transported ferrichrome at a concentration of 0.5 μM in the assay medium with 20% the rate of $FhuA^+$ $TonB^+$ wild-type cells. The rate of transport increased linearly with the concentration of ferrichrome from 0.5 to 20 μM in the assay medium, in contrast to wild-type cells, which exhibited saturation kinetics in that the transport rate did not increase above 5 μM ferrichrome. This finding indicates that FhuA Δ322−355 functions as a channel and that in wild-type cells the transport system through the outer membrane is saturated by much lower ferrichrome concentrations than the transport system through the cytoplasmic membrane. Apparently, translocation through the outer membrane is the rate-limiting step in ferrichrome transport.

The most important result of this study was channel formation of FhuA Δ322−355 in black lipid membranes. In contrast to FhuA wild-type, FhuA Δ322−355 formed stable diffusion channels with an unusually long lifetime of more than 10 min. That these channels were genuinely formed by FhuA Δ322−355 and did not represent an artefact was supported by their large size and high stability, as well as by the lack of channel formation with FhuA wild-type.

Besides FhuA Δ322−355, FhuA Δ322−405 and FhuA Δ163−368 displayed a strong TonB-independent growth promotion by ferrichrome and ferrioxamine B and sensitivity to SDS and bacitracin while FhuA Δ322−333 formed no channel. The latter derivative still exhibited rather high phage T5 sensitivity but had lost phage T1, φ80 and colicin M sensitivity similar to the *fhuA* mutant which carries the Asp348 deletion (Killmann and Braun, 1992). It seems that the loop that controls FhuA permeability also forms part of the phage and colicin binding sites. This loop is particularly exposed at the cell surface since of 33 tetrapeptide insertions along the entire FhuA protein only the insertion at site 321 was cleaved by subtilisin (Koebnik and Braun, 1993). All the other sites required larger inserted peptides to become protease susceptible. With this loop we have identified a short segment (34 residues out of 714 FhuA residues) that controls FhuA permeability and contains FhuA receptor determinants.

In a recent paper, Rutz *et al.* (1992) deleted 139 (residues 202−340) and 135 (residues 205−339) amino acid residues from the ferric enterobactin receptor FepA. According to their model for the outer membrane topology of FepA, the excised region forms two large surface loops (36 and 82 residues), one very short periplasmic loop (3 residues) and two and a half transmembrane segments. They measured the TonB independent uptake of [$^{59}Fe^{3+}$]enterobactin and [$^{59}Fe^{3+}$]ferrichrome into cells expressing the FepA deletion derivatives. Uptake rates were proportional to the concentrations of the ferric siderophores (between 5 and 100 μM). The deletion derivatives also became sensitive to SDS, bacitracin, erythromycin and rifampicin. Cells expressing the FepA derivatives remained resistant to EDTA and deoxycholate, indicating that the outer membrane integrity was not grossly disturbed, becoming leaky to all kinds of detrimental agents. Monoclonal antibodies reacted with surface epitopes still present in the FepA derivatives, showing that the derivatives were properly integrated into the outer membrane. Two epitopes (residues 100−142) became accessible in the FepA derivatives suggesting that removal of the major surface loops uncovered additional epitopes located close to the cell surface. In that study the FepA derivatives were not isolated and examined in reconstituted lipid bilayers as we did for FhuA and that we consider as the final proof of channel formation. In contrast to the large deletions in FepA including loops and transmembrane regions, we have removed only 34 residues in a single surface loop and did not affect transmembrane segments. The latter are probably most important in channel formation so that we created no artificial hole in the transmembrane region of the FhuA protein but opened only a pre-existing central channel. It is also excluded that the small deletion in the loop distorted the outer membrane so that it became leaky to many compounds. We propose that all ferric siderophore receptors and the vitamin B_{12} receptor form closed channels that are opened through interaction with the proposed TonB-ExbB-ExbD energy transduction complex.

The mechanism of ferrichrome transport through FhuA is unknown. FhuA could function as a gated channel, which is opened in both directions through the action of TonB-ExbB-ExbD and exhibits its binding site, contained in loop 316−356 to both sides of the outer membrane. However, it is also possible that FhuA is a transporter in which loop 316−356 forms the gate that opens towards the periplasmic side and closes after release of ferrichrome. For gated channels of nerve and muscle membranes high transport rates in the order of 10^7 solute molecules per second were measured. Similarly, the substrate-specific channels Tsx and LamB of the *E.coli* outer membrane have a high turnover number (Benz, 1988). In contrast, FhuA wild-type does not transport more than 0.1 molecule of ferrichrome per second since 10^5 ferrichrome molecules are taken up by a single cell containing 10^3 FhuA receptors during a 20 min generation time. At saturating ferrichrome concentrations V_{max} is ~10 pmol/min per 10^9 cells (Wookey *et al.*, 1981; Rutz *et al.*, 1992) so that ~10 ferrichrome molecules are transported per cell per second. Since, as stated above, the overall transport rate is largely determined by the transport rate through the outer membrane, the low ferrichrome

transport rates suggest a transporter mechanism rather than a gated channel mechanism. The strong binding of ferrichrome to FhuA and the high specificity of FhuA for ferrichrome and structurally closely related ferrichrome derivatives also make a simple gated channel mechanism more unlikely. However, FhuA is able to channel ferrichrome in and out as found in experiments designed to measure binding of ferrichrome to FhuD. The cells employed were devoid of ferrichrome transport through the cytoplasmic membrane (Köster and Braun, 1990). To measure periplasmic ferrichrome, FhuD had to be overproduced suggesting that ferrichrome was not accumulated in the periplasm unless bound to FhuD. Radioactive ferrichrome could be released from the cells by a 500-fold excess of nonradioactive ferrichrome, indicating that ferrichrome could also be exported through wild-type FhuA out of the cell.

The analysis of the substrate-specific channels Tsx and LamB of the *E.coli* outer membrane revealed that the substrate bound to the binding site has two possibilities. It can move outwards or inwards and the probability for the outward movement is 50% (Benz, 1988). Even if FhuA in the open state is a channel similar to the substrate specific channels, binding of ferrichrome to FhuD makes the periplasmic space a sink for ferrichrome. Therefore, ferrichrome uptake through the outer membrane becomes essentially unidirectional. We conceive that binding of ferrichrome to FhuA and cyclic opening and closing of FhuA results in ferrichrome translocation through the outer membrane. Opening of the FhuA channel through movement of loop 316–356 consumes conformational energy of TonB which after each step has to be reenergized by the electrochemical potential of the cytoplasmic membrane whereby ExbB and ExbD play a role which is mechanistically not understood.

Materials and methods

Bacterial strains and growth conditions

The *E.coli* strains and plasmids used in this study are listed in Table I. *E.coli* HK96 was obtained by isolating *tonB* mutants of strain MS172 *fhuE* with colicin B and phage ϕ80 used simultaneously as selecting agents. Retention of phage T5 sensitivity was tested and mutation in *tonB* as opposed to mutation in *exbB exbD* was tested by transformation with plasmids pIM91 *tonB* and pKE7 *exbB exbD*. The resulting strain HK98 *fhuE tonB* was made *fhuA* by selecting phage T5 resistant mutants (HK96). Transformants of HK96 carrying plasmid pSKFO *fhuA* were again T5 sensitive. Transformants carrying plasmids pIM91 and pSKFO were sensitive to T5, ϕ80, T1, colicin M and albomycin and grew on ferrichrome as sole iron source showing that HK96 was a *fhuA tonB* mutant.

E.coli HK99 *fhuA tonB* was obtained by phage P1 transduction of *zad*::Tn*10* of strain H1020 into strain 41/2, selecting for tetracycline resistance and FhuA$^-$ phenotype. From the resulting strain HK9, the mutated *fhuA* gene was cotransduced with *zad*::Tn*10* into strain BR158 *tonB*.

Cells were grown at 37°C in TY medium [10 g/l Bactotryptone (Difco Laboratories), 5 g/l yeast extract, 5 g/l NaCl, at pH 7] or NB medium (8 g/l nutrient broth, 5 g/l NaCl, at pH 7). To limit the available iron, 2,2′-dipyridyl (0.2 mM) was added to nutrient broth (NBD). Ampicillin (40 μg/ml), chloramphenicol (25 μg/ml) and neomycin (50 μg/ml) were added when required.

Plasmids used

To construct pHK226, a *Bgl*II site was introduced into the *fhuA* gene of plasmid pWK360 using PCR and the primer 5′-GCTGCAAGATCTCTCCGTTG-3′ (mismatch bases underlined; bp 1691–1710, Coulton *et al.*, 1986). The primer of the complementary strand reads 5′-AAGCGTGCGCCCGGGCACACGAAAGGA-3′ (bp 2911–2885). The amplified DNA fragment was purified by agarose gel electrophoresis, recovered using Qiaex (Diagen, Düsseldorf), digested with *Bgl*II and *Bst*EII, and ligated with *Bgl*II- and *Bst*EII-cleaved plasmid pSKF321-04. Plasmid pHK228 was constructed by ligating the *Eco*RI–*Xba*I fragment of pSKF369-16 with *Eco*RI- and *Xba*I-cleaved pSKF162-16. The reading frame of resulting plasmid was restored by cleavage with *Eco*RI, filling-in with Klenow polymerase and religation. Plasmids pHK210 and 221 were constructed by ligating the *Bgl*II–*Xba*I fragment of pSKF 333-04 into *Bgl*II- and *Xba*I-cleaved pSKF321-04 and 417-04, respectively. Plasmids pHK211 and 214 were constructed by ligating the *Bgl*II–*Xba*I fragment of pSKF405-04 into *Bgl*II–*Xba*I cleaved pSKF321-04 and 333-04, respectively. Plasmid pHK220 was constructed by ligating the *Bgl*II–*Xba*I fragment of pSKF321-04 into *Bgl*II- and *Xba*I-cleaved pSKF405-04.

Recombinant DNA techniques

Isolation of plasmid DNA, use of restriction enzymes, ligation and agarose gel electrophoresis were performed by standard techniques. DNA was sequenced according to the dideoxy-chain termination method (Sanger *et al.*, 1977) using the sequencing kit of United States Biochemicals (Cleveland, OH) and [^{35}S]dATP for labelling.

Phenotype assays

All tests were performed with freshly transformed cells if the functions were encoded on plasmids. Growth stimulation by siderophores was tested by placing filter paper discs (diameter 6 mm) containing 10 μl of a 1 mM and 10 mM siderophore solution on NBD agar plates seeded with 0.1 ml of an overnight culture of the strain to be tested. The diameter and the growth density around the filter paper disc was determined. Sensitivity to albomycin, colicin M and phages was tested by spotting series of 10-fold diluted solutions (4 μl) on TY plates seeded with the indicator bacteria. The most dilute solution that gave a clear zone of growth inhibition was taken as antibiotic, colicin and phage titre. For example the stock solution of phage T1 could be diluted 10^8-fold to yield clear plaques on the wild-type strain (titre 8). The colicin M solution was a crude extract of a strain which carried plasmid pTO4 *cma cmi* (Ölschläger *et al.*, 1984).

Sensitivity was tested to the following agents placed on filter paper discs (in μg per 10 μl water): SDS 750, EDTA 1.5, sodium deoxycholate 750, gentamycin 10, neomycin 10, novobiocin 30, erythromycin 15, rifampicin 5, vancomycin 20, bacitracin 30 and lysozyme 100.

Transport assays

Cells grown overnight on TY plates were suspended in M9 salts (Miller, 1972), 0.4% glucose to an OD_{578nm} of 0.5 ($\sim 5 \times 10^8$ cells per ml). They were shaken for 30 min at 37°C after which [$^{55}Fe^{3+}$]ferrichrome was added for determination of iron transport, as described previously (Bäumler and Hantke, 1992).

Isolation of the FhuA protein

Outer membranes were prepared by lysis of cells with EDTA–lysozyme followed by solubilization of the cytoplasmic membrane with Triton X-100 and differential centrifugation, as has been described previously (Eick-Helmerich and Braun, 1989). The sedimented outer membrane was suspended in buffer containing 50 mM Tris–HCl, 1 mM EDTA, 1% octylglucoside, pH 8 and kept for 30 min on ice, interrupted by occasional vortexing. The suspension was centrifuged for 10 min at 40 000 *g*, the supernatant containing the solubilized FhuA protein was mixed with an equal volume of sample buffer [0.2 ml 0.625 M Tris–HCl (pH 6.8); 0.4 ml 10% SDS; 0.4 ml 50% glycine; 0.4 ml 0.01% bromophenol blue; 0.1 ml 2-mercaptoethanol] and then heated for 3 min at 50°C prior to separation of the proteins by SDS–PAGE. The proteins were stained with 0.3 M $ZnCl_2$, the FhuA band was excised from the gel and treated for 15 min with 30 ml of 0.25 M Tris–HCl, 0.25 M EDTA, pH 9 to remove $ZnCl_2$. FhuA was eluted by pressing the gel slice through the needle of a 1 ml syringe into an Eppendorf cup on ice. The smashed gel was suspended in 0.2 ml of 10 mM Tris–HCl, 1 mM EDTA, pH 8 and kept overnight on ice. More buffer (0.4 ml) was added prior to centrifugation for 1 h at 40 000 *g*. This sample was used for the measurements on black lipid membranes. FhuA wild-type and FhuA Δ322–355 were isolated from *E.coli* UL3 transformed with plasmid pSKFO and pHK226, repectively. A control sample of UL3 transformed with the vector pBluescript SK+ lacking FhuA was isolated by exactly the same procedure.

Radiolabelling of proteins

Logarithmically growing cells of *E.coli* WM1576 pGP1-2 (5 ml), transformed with plasmids encoding FhuA wild-type and the various FhuA derivatives, were collected by centrifugation at an absorbance of 0.4 at 578 nm. They were suspended in 1 ml of M9 salt medium (Miller, 1972) supplemented with 0.4% glucose, 0.01% methionine assay medium (Difco Laboratories), 0.01% thiamine, 1 mM phenylmethylsulfonyl fluoride and 0.1% *p*-aminobenzamidine. After shaking for 1 h at 27°C, T7 RNA

polymerase synthesis was induced by shifting the temperature for 15 min to 42°C. Rifampicin (10 μl of 5 mg/ml methanol) was added and incubation continued for another 10 min at 42°C followed by 20 min at 27°C. [^{35}S]methionine (370 kBq) was added and the suspension incubated for 20 min. Cells were collected and the outer membranes were prepared by differential extraction of the cytoplasmic membrane with 0.1% Triton X-100 as described previously (Hantke and Braun, 1978). The outer membrane preparation was suspended in 25 μl sample buffer, boiled for 3 min and 25 μl subjected to SDS-PAGE.

Determination of cell surface exposed FhuA protein

Polyclonal rabbit anti-FhuA antiserum (Hoffmann *et al.*, 1986) was diluted in TNT buffer (20 mM Tris-HCl, 0.5 mM NaCl, 0.01% Tween-20, pH 7.5) and then incubated at 4°C for 2 days with *E.coli* UL3 *fhuA* (1 × 10^9 cells/ml) to remove proteins which bind nonspecifically to the cell surface. The antiserum was then incubated overnight with cells (1 × 10^9/ml) to be tested. The controls contained TNT buffer without antiserum. Cells were harvested by centrifugation and washed three times with TNT buffer. They were then incubated for 1 h with anti-rabbit IgG alkaline phosphatase conjugate (1 μg/ml, Sigma). Cells were washed three times with TNT buffer after which they were still fully viable as determined by plating. They were incubated in 1 ml 10% diethanolamine, pH 9.8, containing 1 mg/ml *p*-nitrophenylphosphate. Cells were removed by centrifugation and the absorbance of the supernatant was measured at 405 nm, which under the conditions used was linearly related to the concentration of *p*-nitrophenolate.

Black lipid bilayer membrane experiments

Insertion of FhuA wild-type and FhuA Δ322-355 into artifical lipid bilayer membranes was done as described previously (Benz *et al.*, 1978). In brief, membranes were formed from a 1% (w/v) solution of diphytanoyl phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) in *n*-decane in a Teflon cell consisting of two aqueous compartments connected by a small circular hole. The area of the hole was 0.5 mm^2 for the experiments performed in this study. Membranes were formed across the hole and between 25 and 50 ng of FhuA wild-type or FhuA Δ322-355 were added to 5 ml of the aqueous salt solution on one side or on both sides of the membrane. The aqueous salt solutions (analytical grade, Merck, Darmstadt, Germany) were used unbuffered and had a pH of ~6.0. The temperature was kept at 20°C throughout. The membrane current was measured with a pair of calomel electrodes switched in series with a voltage source and a current amplifier (Keithley 427). The amplified signal was monitored with a storage oscilloscope and recorded with a strip chart recorder.

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