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Properties of the FhuA Channel in the *Escherichia coli* Outer Membrane after Deletion of FhuA Portions within and outside the Predicted Gating Loop

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Escherichia coli **transports Fe^{3+} as a ferrichrome complex through the outer membrane in an energy-dependent process mediated by the FhuA protein. A FhuA deletion derivative lacking residues 322 to 355 (FhuA Δ322-355) forms a permanently open channel through which ferrichrome diffused. This finding led to the concept that the FhuA protein forms a closed channel that is opened by input of energy derived from the electrochemical potential across the cytoplasmic membrane, mediated by the Ton system. In this study, we constructed various FhuA derivatives containing deletions inside and outside the gating loop. FhuA Δ322-336 bound ferrichrome and displayed a residual Ton-dependent ferrichrome transport activity. FhuA Δ335-355 no longer bound ferrichrome but supported ferrichrome diffusion through the outer membrane in the absence of the Ton system. FhuA Δ335-355 rendered cells sensitive to sodium dodecyl sulfate and supported diffusion of maltotetraose and maltopentaose in a *lamB* mutant lacking the maltodextrin-specific channel in the outer membrane. Cells expressing FhuA Δ70-223, which has a large deletion outside the gating loop, were highly sensitive to sodium dodecyl sulfate and grew on maltodextrins but showed only weak ferrichrome uptake, suggesting formation of a nonspecific pore through the outer membrane. FhuA Δ457-479 supported Ton-dependent uptake of ferrichrome. None of these FhuA deletion derivatives formed pores in black lipid membranes with a stable single-channel conductance. Rather, the conductance displayed a high degree of current noise, indicating a substantial influence of the deletions on the conformation of the FhuA protein. FhuA also supports infection by the phages T1, T5, and ϕ80 and renders cells sensitive to albomycin and colicin M. Cells expressing FhuA Δ322-336 were sensitive to albomycin and colicin M but were only weakly sensitive to T5 and ϕ80 and insensitive to T1. Cells expressing FhuA Δ335-355 were resistant to all FhuA ligands. These results indicate different structural requirements within the gating loop for the various FhuA ligands. Cells expressing FhuA Δ457-479 displayed a strongly reduced sensitivity to all FhuA ligands, while cells expressing FhuA Δ70-223 were rather sensitive to all FhuA ligands except albomycin, to which they were nearly resistant. It is concluded that residues 335 to 355 mainly determine the properties of the gate with regard to FhuA permeability and ligand binding.**

Substrates cross the outer membrane of the gram-negative bacterium *Escherichia coli* by (i) diffusion through porins (2, 13, 36), (ii) facilitated diffusion, as characterized by maltodextrins through the LamB protein (6, 7) or nucleosides through the Tsx protein (5, 17, 26), and (iii) high-affinity active transport mechanisms, such as the uptake of ferric siderophores and vitamin B_{12}. Transport of ferric siderophores and vitamin B_{12} is mediated by ligand-specific receptor proteins and requires the electrochemical potential of the cytoplasmic membrane (9) and a protein complex consisting of the TonB, ExbB, and ExbD proteins (Ton system) (14, 15, 33). The latter are assumed to regulate outer membrane receptor activities in response to the energized state of the cytoplasmic membrane (10, 11, 16, 19, 30).

One of these receptor proteins is FhuA, through which Fe^{3+} as a ferrichrome complex, the structurally related antibiotic albomycin (10), and the bacterial toxin protein colicin M are transported through the outer membrane. FhuA serves also as a binding site for phages T5, T1, and ϕ80. The latter two phages recognize a certain conformation (10) that FhuA assumes in energized cells expressing the Ton system (10, 11, 15, 20, 22, 23, 25, 33), while T5 infects also unenergized cells and cells lacking the Ton system. It is likely that FhuA forms a channel in the outer membrane, which may be closed as long as it is not opened by the electrochemical potential of the cytoplasmic membrane through the action of the Ton system. A FhuA mutant strongly impaired in all TonB-dependent FhuA activities has a deletion of one residue, Asp-348, in the largest surface loop (22). This loop comprises residues 316 to 356, predicted by the FhuA transmembrane model (21, 25). Deletion of residues 322 to 355 in this loop converts FhuA into an open channel (21). Cells expressing FhuA Δ322-355 take up [$^{55}Fe^{3+}$]ferrichrome independent of the Ton system. The rate of uptake increases linearly with the concentration of added ferrichrome, whereas cells expressing FhuA wild type show saturation at about 5 μM ferrichrome (21). At low ferrichrome concentrations (up to 5 μM), uptake of ferrichrome into FhuA wild-type cells is faster than into FhuA Δ322-355 mutant cells. Cells expressing FhuA Δ322-355 become sensitive to sodium dodecyl sulfate (SDS) and bacitracin, showing that the FhuA deletion derivative renders the outer membranes permeable to these substances. Isolated FhuA Δ322-355 forms stable channels in black lipid membranes which display discrete single-channel conductance, in contrast to FhuA wild type, which fails to increase conductance. It has been concluded that the basic

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structure of FhuA in the outer membrane is a channel that is closed by loop 316-356 (gating loop) and that the gating loop is opened by the Ton system through induction of a conformational change in FhuA (21). Recently, it has been shown that binding of phage T5 to isolated FhuA inserted into planar lipid bilayers results in a high ion conductance (8). Conductance, kinetic properties, ion selectivity, partial reduction of the current by ferrichrome, and lack of gating to the closed state agree with the properties of FhuA Δ322-355 inserted into artificial bilayer membranes. The concordance of these results strongly supports the notion that FhuA forms a normally closed channel, in which the gating loop controls the permeability of the channel.

In this study, we wanted to determine whether the entire gating loop has to be removed in order to open the FhuA channel permanently, or whether FhuA derivatives with smaller deletions increase specifically the permeability of the outer membrane and of black lipid membranes. In addition, we examined the specificity of the gating loop by creating larger deletions outside the gating loop to determine whether other regions of FhuA contribute to FhuA permeability or render the outer membrane permeable nonspecifically. One might argue that if FhuA forms a β-barrel, like the porins, removal of any surface loop would open a hole in FhuA through which ferrichrome can diffuse. We found that the entire gating loop is involved in gating and binding of all of the agents that use FhuA as a binding site (henceforth designated FhuA ligands) but could differentiate various regions for different ligands. In addition, deletion of a loop predicted to be close to the gating loop influenced the gating loop activity. Deletions outside the gating loop influenced FhuA activities to various extents without opening FhuA for entering of ferrichrome by diffusion.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *E. coli* strains and plasmids used are listed in Table 1. Cells were grown in TY medium [10 g of Bacto Tryptone [Difco Laboratories], 5 g of yeast extract, and 5 g of NaCl per liter) or NB medium (8 g of nutrient broth and 5 g of NaCl per liter [pH 7]) at 37°C. To reduce the available iron of the NB medium, 2,2′-dipyridyl (0.2 mM) was added (NBD medium). When required, the antibiotics ampicillin (40 μg/ml) and neomycin (50 μg/ml) were added.

Plasmid pHK202 (FhuA Δ70-223) was constructed by ligating the *Bgl*II-*Xba*I fragment of pSKF223-04 into *Bgl*II-*Xba*I-cleaved pSKF069-04 (25). To construct plasmid pHK227 (FhuA Δ457-479), a *Pst*I site was introduced into the *fhuA* gene of plasmid pWK360 by using PCR and the primer 5′-GTTTACCTGCAGTGG TGG-3′ (mismatch underlined). To construct plasmids pHK234 (FhuA Δ322-336) and pHK237 (FhuA Δ335-355), a *Bgl*II site was introduced into the *fhuA* gene of plasmid pHK763 by using PCR and the primer 5′-TACGTGCCAGAT AATGGCCTTTAAGATCTGGCGCTAATGC-3′ (mismatch underlined). The primers of the complementary strand read 5′-AAGCGTGCGCCCGGGCACA CGAAAGGA-3′ (for pHK227) and 5′-CCGAAAGAAGACACTATCACCGT TACCGC-3′ (for pHK234 and pHK237). The amplified DNA fragments were purified by agarose gel electrophoresis and recovered from agarose by using Qiaex (Diagen, Düsseldorf, Germany). The DNA fragment with an introduced *Pst*I site was digested with *Pst*I-*Sau*I and ligated into *Pst*I-*Sau*I-cleaved pSKF456-12 (25), resulting in plasmid pHK227. The DNA fragment with an introduced *Bgl*II site was digested with *Van*91I and *Mlu*I and ligated into *Van*91I-*Mlu*I-cleaved pHK763, resulting in plasmid pHK334. Plasmid pHK334 was digested with *Bgl*II-*Bst*EII and *Bgl*II-*Mlu*I, and the resulting DNA fragments were ligated into the *Bgl*II-*BstE*II-cleaved pSKF321-04 (25) and into *Bgl*II-*Mlu*I-cleaved pHK226 (21), resulting in plasmids pHK234 and pHK237, respectively.

Recombinant DNA techniques. Standard techniques were used for isolation of plasmids, use of restriction enzymes, ligation, agarose gel electrophoresis, and transformation (32).

Isolation of the FhuA proteins. *E. coli* WM1576 transformed with the plasmids encoding the various FhuA derivatives was grown in TY medium at 27°C. When the cells reached an optical density at 578 nm of 1.0, T7 RNA polymerase synthesis was induced by shifting the temperature for 20 min to 42°C. Then incubation was continued for an additional 60 min at 27°C. Outer membranes were prepared by lysing cells with lysozyme-EDTA, followed by solubilization of the cytoplasmic membrane with 0.25% Triton X-100 and differential centrifugation, as described previously (14). The sedimented outer membranes were suspended in sample buffer (0.2 ml of 0.625 M Tris-HCl [pH 6.8], 0.4 ml of 10% SDS, 0.4 ml of 50% glycine, 0.4 ml of 0.01% bromophenol blue, 0.1 ml of 2-mercaptoethanol), and the proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) after the samples were heated for 3 min at 50°C. FhuA protein derivatives were isolated from the gel as described previously (21).

TABLE 1. *E. coli* strains and plasmids used

Strain or plasmid	Genotype or phenotype	Reference or source
E. coli K-12		
AB2847	*aroB malT tsx thi*	18
41/2	AB2847 *fhuA*	22
HK99	AB2847 *fhuA tonB*	21
WM1576	K38 HfrC pGP1-2	35
KB419	*lamB*	H. G. Krieger-Brauer
Plasmids		
pHK202	pBluescript SK+ *fhuA* Δ70-223 (A_{069} PDLA D_{224}[a])	This study
pHK226	pBluescript SK+ *fhuA* Δ322-355 (P_{321} PDL S_{356})	21
pHK227	pBluescript SK+ *fhuA* Δ457-479 (A_{456} DPSSSTCS G_{480})	This study
pHK234	pBluescript SK+ *fhuA* Δ322-336 (P_{321} PDL K_{337})	This study
pHK237	pBluescript SK+ *fhuA* Δ335-355 (P_{334} DL S_{356})	This study
pSKF069-04	pBluescript SK+ *fhuA* (A_{069} PDLA L_{070})	25
pSKF223-04	pBluescript SK+ *fhuA* (P_{223} PDLA D_{224})	25
pSKF456-12	pBluescript SK+ *fhuA* (A_{456} DPSSSTCRSSST D_{457})	25
pHK763	pT7-6 *fhuA* wild type	21
pWK360	pT7-5 *fhuACDB* wild type	W. Köster
pTO4	pBR322 *cma cmi*	29
pBluescript SK+	Amp^r	21
pT7-6	Amp^r	35
pT7-5	Amp^r	35

[a] The subscript numerals denote the start and the end of the deleted amino acids in FhuA, and the one-letter amino acid code between the numerals indicate amino acids inserted during cloning.

Phenotype assays. When the FhuA function was encoded on a plasmid, all phenotype tests were performed only with freshly transformed cells. Sensitivity of cells against the FhuA ligands (phages T1, T5, and ϕ80, colicin M, and albomycin) was tested by spotting 10-fold-diluted solutions (4 μl) on TY plates overlaid with 3 ml of TY soft agar that contained 10^8 cells of the strain to be tested. The most dilute solution that gave a clear zone of growth inhibition was taken as the phage, colicin, or antibiotic titer. The colicin M solution was a crude extract of a strain that carried plasmid pTO4 *cma cmi* (29). Sensitivity was also tested against 750 μg of SDS in 10 μl of water placed on filter paper disks (diameter, 6 mm).

Growth promotion by ferrichrome was tested by placing filter paper disks containing 10 μl of a 10 mM ferrichrome solution on NBD agar plates overlaid with 3 ml of NB top agar that contained 0.1 ml of an overnight culture of the strain to be tested. The diameter and the growth density around the filter paper disk were determined after incubation overnight.

Growth promotion by maltodextrins was tested with the *lamB* strain KB419 (as a control) and with strain KB419 transformed with the *fhuA* deletion plasmids. Overnight cultures (0.1 ml) were washed twice with 0.9% NaCl and dispersed in M9 minimal top agar on M9 minimal agar plates that contained no carbon source. Filter paper disks containing 10 μl of a 40% solution of maltodextrins (maltose up to maltohexaose) were placed onto the agar, and the plates were incubated overnight at 37°C. The diameter and the growth density around the filter paper disk were determined.

Transport assays. Cells grown overnight on TY plates were washed and suspended in transport buffer, which consisted of M9 salts (32) and 0.4% glucose, to an optical density at 578 nm of 0.5 at 37°C before [$^{55}Fe^{3+}$]ferrichrome was added, as described previously (1).

Black lipid bilayer membrane experiments. Membranes were formed from a 1% (wt/vol) solution of diphytanoyl phosphatidylcholine (Avanti Polar Lipids, Alabaster, Ala.) in *n*-decane in a Teflon cell consisting of two aqueous compartments connected by a circular hole with an area of 0.5 mm^2 (3, 4). The aqueous salt solutions (analytical grade; Merck, Darmstadt, Germany) were used unbuffered and had a pH of approximately 6. The temperature was kept at 20°C

TABLE 2. Properties of FhuA deletion mutants[a]

FhuA derivative on plasmid	Growth on Fc of:		Sensitivity of 41/2 to:						Growth of KB419 *lamB* on:		
	41/2	HK99	T1	T5	ϕ80	ColM	Albo	SDS	Maltotriose	Maltotetraose	Maltopentaose
FhuA WT	42 (+)	—	8	7	8	5	4	—	15 (+)	—	—
FhuA Δ322-336	38 (+)	15 (+/−)	—	(5)	(4)	4 (5)	3 (4)	11	15 (+)	—	—
FhuA Δ335-355	28 (+)	24 (+)	—	—	—	—	(0)	15	20 (+)	15 (+)	12 (+/−)
FhuA Δ322-355	26 (+)	26 (+)	—	—	—	—	—	12	19 (+)	14 (+/−)	12 (+/−)
FhuA Δ70-223	12 (+)	24 (−)	6	6	6	3	(0)	18	25 (+/−)	17 (+/−)	15 (−)
FhuA Δ457-479	40 (+)	20 (+/−)	(7)	(7)	(6)	(3)	(3)	9	14 (+/−)	—	—

[a] Growth promotion by ferrichrome (Fc) was determined with *E. coli* 41/2 *fhuA* and HK99 *fhuA tonB* expressing plasmid-encoded FhuA wild type (WT) or the FhuA derivatives listed. The growth zones were determined on iron-limited NBD plates around filter paper disks (6-mm diameter) that contained 10 μl of a 10 mM ferrichrome solution. The diameters of the growth zones (in millimeters) and the densities of growth, (high [+], medium [+/−], and low [−]) are presented. Sensitivities to phages T1, T5, and ϕ80, colicin M (ColM), and albomycin (Albo) are given as the final concentrations of a 10-fold dilution series that resulted in a clear zone (in parentheses, turbid zone) of growth inhibition. For example, 8 means that the phage T1 stock solution could be diluted up to 10^8-fold to give clear plaques. Sensitivity to SDS is represented by the diameters of the growth inhibition zones (in millimeters). The inhibitors were applied on filter paper disks at concentrations as given in Materials and Methods onto nutrient agar plates seeded with the strains to be tested. The growth zones with maltodextrins were determined by using *E. coli* KB419 *lamB* on minimal agar plates containing no carbon source around filter paper disks that contained 10 μl of a 40% solution of maltodextrins (only the results with maltotriose up to maltopentaose are presented). —, no inhibition or no growth promotion.

throughout the experiments. The membrane current was measured with a pair of calomel electrodes (with salt bridges) switched in series with a voltage source and an electrometer (Keithley 602). For single-channel recordings, the electrometer was replaced by a current amplifier (Keithley 427). The amplified signal was monitored with a storage oscilloscope and recorded with a strip chart recorder. Stock solutions containing FhuA and the FhuA mutants were added after the lipid membrane turned optically black to incident light.

RESULTS

Construction of FhuA deletion derivatives. Plasmids pHK202, pHK227, pHK234, and pHK237 were constructed as described in Materials and Methods and contained deletions in the *fhuA* gene that extended from residues 70 to 223 (termed FhuA Δ70-223), 457 to 479 (FhuA Δ457-479), 322 to 336 (FhuA Δ322-336), and 335 to 355 (FhuA Δ335-355), respectively. The deletions in FhuA Δ322-336, FhuA Δ335-355, FhuA Δ322-355, FhuA Δ70-223, and FhuA Δ457-479 comprised 15, 21, 34, 154, and 23 residues of FhuA, respectively, but the genetic technique used to construct the deletions introduced three (PDL), two (DL), three (PDL), four (PDLA), and eight (DPSSSTCS) residues, respectively (Table 1); therefore, the FhuA derivatives were in fact only 12, 19, 31, 150, and 15 residues shorter than FhuA wild type. Previously, a TAB linker insertion site was erroneously assigned due to a restriction site located at residue 333, which was actually at residue 301 so that pSKF333-04 should read pSKF301-04 (25). This site was previously used to construct FhuA Δ322-333 and FhuA Δ334-405 (21), which are incorrect. Cells expressing these FhuA derivatives show very weak ferrichrome uptake rates. The properties of the correct deletions named according to the deleted amino acids and proved by DNA sequencing are described in this report. The other described deletion derivatives were correct, including the most important one, FhuA Δ322-355, which was included in this study for comparison of its properties with those of the newly constructed derivatives (Table 2).

Properties of the FhuA deletion derivatives: receptor activity and ferrichrome diffusion through the outer membrane. The phenotype of each of the plasmid-encoded *fhuA* mutants was tested by using the *E. coli* RNA polymerase and the natural promoter upstream of the *fhuA* genes. Similar amounts of FhuA wild type (cloned on pT7-6) and FhuA deletion derivatives (cloned on pBluescript SK+) were present in cells (Fig. 1). Sensitivity to the FhuA-specific phages T1, ϕ80, and T5, colicin M, albomycin, and SDS was examined with *E. coli* 41/2 *tonB*$^+$. This strain carries a number of point mutations in the *fhuA* gene, among them a deletion of Asp-348 in the gating loop (22). The mutations exerted no low polar effect on the expression of the downstream *fhuCDB* genes, which are required for transport of ferrichrome and albomycin through the cytoplasmic membrane (10). Strain 41/2 was resistant to the phages and to colicin M, albomycin, and SDS. SDS was suitable for identifying an increased nonspecific permeability of the outer membrane since wild-type cells were fully resistant to the concentrations used in this study (Table 2).

Growth on ferrichrome was tested with derivatives of *E. coli* AB2847, *E. coli* 41/2 *fhuA* and *E. coli* HK99 *fhuA tonB*; the latter strain contains the mutated *fhuA* gene of *E. coli* 41/2 and was used to test TonB-independent diffusion of ferrichrome through the outer membrane into the periplasm and subsequent TonB-independent transport across the cytoplasmic

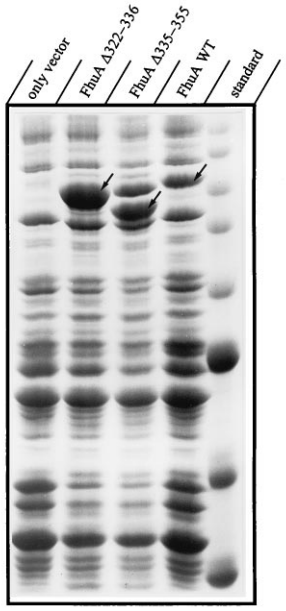

FIG. 1. SDS-PAGE of whole cells of *E. coli* 41/2 after growth in NB medium without T7 induction. The strains contained chromosomally encoded mutated FhuA (lane 1), plasmid-encoded FhuA Δ322-336 (lane 2), FhuA Δ335-355 (lane 3), or FhuA wild type (WT) (lane 4). The gel was stained with Serva Blue G. The arrows mark FhuA wild type (lane 4) and the FhuA derivatives (lanes 2 and 3). Lane 5 shows a 10-kDa ladder of standard proteins ranging from 30 kDa (bottom) to 100 kDa (top).

membrane. Response to ferrichrome as sole iron source was tested on nutrient agar plates in which added dipyridyl reduced the available iron to an extent that cells grew only when they took up ferrichrome. Strains 41/2 and HK99 do not produce a siderophore; therefore, growth under iron-limiting conditions depended on added ferrichrome, which was supplied on a filter paper disk from which it diffused into the agar and supported growth. No growth zones were formed by strains 41/2, HK99, and HK99 transformed with *fhuA* wild type (Table 2).

Growth of the *E. coli* 41/2 transformant expressing FhuA Δ322-336 was nearly as strongly stimulated by ferrichrome as growth of strain 41/2 expressing plasmid-encoded FhuA wild type (Table 2) and growth of *E. coli* AB2847 expressing chromosomally encoded FhuA wild type (data not shown). Growth stimulation required TonB, since strain HK99 *tonB* FhuA Δ322-336 grew very poorly on ferrichrome as the sole iron source (Table 2). *E. coli* 41/2 FhuA Δ322-336 still bound ferrichrome (see below), showing that the remaining portion of the gating loop was operating for ferrichrome transport, but it displayed greater SDS sensitivity than *E. coli* 41/2 FhuA wild type.

E. coli 41/2 FhuA Δ322-336 showed a greatly reduced sensitivity to T5 and ϕ80 (only turbid plaques formed, even with undiluted phage stock solutions) and was resistant to T1 (Table 2). Sensitivity to the phages demonstrated a correct insertion of FhuA Δ322-336 into the outer membrane. This conclusion is supported by the high colicin M and albomycin sensitivity of strain 41/2 FhuA Δ322-336 (Table 2).

Deletion of the segment adjacent to Δ322-336 had a profound effect on all FhuA-related activities. Growth of *E. coli* 41/2 FhuA Δ335-355 on ferrichrome was weak, and the cells were fully resistant to all FhuA-specific phages and to colicin M and only very weakly sensitive to albomycin (Table 2). Growth on ferrichrome was independent of TonB, as *E. coli* HK99 FhuA Δ335-355 formed a growth zone similar to that of *E. coli* 41/2 FhuA Δ335-355 (Table 2). *E. coli* 41/2 FhuA Δ335-355 was highly sensitive to SDS, and *E. coli* KB419 *lamB* expressing plasmid-encoded FhuA Δ335-355 in addition to FhuA wild type could grow on maltotetraose and maltopentaose (Table 2), demonstrating an open channel in the outer membrane through which SDS and the maltodextrins diffused into the periplasm.

Excision of the surface loop in proximity to the gating loop (residues 457 to 479) resulted in a FhuA derivative that supported TonB-dependent ferrichrome uptake but rendered cells only weakly sensitive to all FhuA ligands (Table 2). Removal of these residues only slightly increased sensitivity to SDS and failed to facilitate diffusion of the maltodextrins. These results indicated that this loop influences the ligand binding activity of the gating loop without determining directly the gating properties.

FhuA Δ70-223 was the largest deletion studied; the deletion was located outside the gating loop and comprised eight transmembrane segments with the corresponding loops, including the second largest surface loop of FhuA. *E. coli* 41/2 FhuA Δ70-223 displayed a reduced but still pronounced sensitivity to all FhuA-specific ligands except albomycin, demonstrating that FhuA Δ70-223 was properly inserted into the outer membrane (Table 2). The amount of FhuA Δ70-223 in the outer membrane fraction was lower than the amount of wild-type FhuA and of the other FhuA deletion derivatives (see Fig. 4A). This, however, does not account for the lower sensitivity since cells expressing much lower amounts of chromosomally encoded FhuA are fully sensitive. The results obtained with FhuA Δ70-223 indicated that deletions that did not extend into the gating loop conferred no resistance to the FhuA-specific ligands but reduced sensitivity to all FhuA ligands 10- to 100-fold. FhuA Δ70-223 rendered strain 41/2 strongly sensitive to SDS and rendered the outer membrane permeable to maltodextrins. Growth on ferrichrome was weak, and the residual ferrichrome uptake activity was TonB dependent. Apparently, this large deletion nonspecifically increased the permeability of the outer membrane and affected to some extent the function of the gating loop.

Time-dependent transport. To obtain quantitative data on the permeability of the FhuA deletion derivatives, we determined the rate of [$^{55}Fe^{3+}$]ferrichrome uptake of *fhuA* transformants of *E. coli* 41/2 *fhuA*. [$^{55}Fe^{3+}$]ferrichrome supplied at an initial concentration of 1 μM rapidly bound to *E. coli* 41/2 FhuA Δ322-336, followed by an uptake with time that was slower than the uptake rate of 41/2 transformed with *fhuA* wild type (Fig. 2A). In contrast, *E. coli* 41/2 FhuA Δ335-355 displayed a much weaker ferrichrome binding (Fig. 2A and B [expanded scale]) and took up iron with slow linear kinetics. Untransformed *E. coli* 41/2 *fhuA* showed no ferrichrome binding and no iron uptake (Fig. 2A and B). At the low ferrichrome concentration used (1 μM), receptor-dependent, TonB- and energy-dependent transport into *E. coli* 41/2 FhuA wild type and 41/2 FhuA Δ322-336 occurs faster than diffusion into 41/2 Δ335-355.

E. coli 41/2 FhuA Δ457-479 displayed a ferrichrome transport rate similar to that of strain 41/2 FhuA wild type (Fig. 2C). In this mutant, the gating loop was still present; therefore, ferrichrome entered the periplasm mainly by TonB-dependent transport.

E. coli 41/2 FhuA Δ70-223 did not bind ferrichrome, and uptake was below the detection limit (Fig. 2C). All of the transport rates correlated with the growth zones on iron-limited nutrient agar plates (Table 2).

Concentration-dependent transport and diffusion. Diffusion into cells of *E. coli* HK99 *fhuA tonB* transformed with the mutated *fhuA* genes was measured. As shown previously (21), the overall transport rate is determined by the rate of diffusion through the outer membrane and not by the active transport through the cytoplasmic membrane. Since diffusion is the rate-limiting step, determination of transport into the cytoplasm measures the diffusion rate through the channels formed by the FhuA deletion mutants.

Iron uptake was determined for 30 min at [$^{55}Fe^{3+}$]ferrichrome concentrations of 1, 2.5, 5, 10, and 17.5 μM. Strain HK99 took up iron very slowly and depended on the [$^{55}Fe^{3+}$] ferrichrome concentrations supplied in the transport assay (Fig. 3A). *E. coli* HK99 FhuA Δ322-336 contained the highest amount of iron at the lower [$^{55}Fe^{3+}$]ferrichrome concentrations supplied in the assay, which presumably reflected absorption of ferrichrome to FhuA Δ322-336. Iron uptake into *E. coli* HK99 FhuA Δ335-355 reached the highest values at the higher [$^{55}Fe^{3+}$]ferrichrome concentrations. *E. coli* HK99 FhuA Δ322-336 and HK99 FhuA Δ335-355 contained at the higher ferrichrome concentrations more iron than *E. coli* AB2847 *fhuA*$^+$ *tonB*$^+$ because diffusion through the FhuA deletion derivatives within the gating loop became most effective at the higher ferrichrome concentrations. Iron taken up into *E. coli* HK99 FhuA Δ457-479 remained below the level of that reached by strain AB2847 (Fig. 3B), and HK99 FhuA Δ70-223 took up little iron but more than HK99 (Fig. 3).

The FhuA deletion derivatives do not form stable channels in black lipid membranes. FhuA proteins containing deletions were prepared from cells that contained the *fhuA* mutant genes downstream of a phage T7 promoter. After transcription with the T7 RNA polymerase, cells were disrupted, the membrane fraction was prepared, and the cytoplasmic membrane was

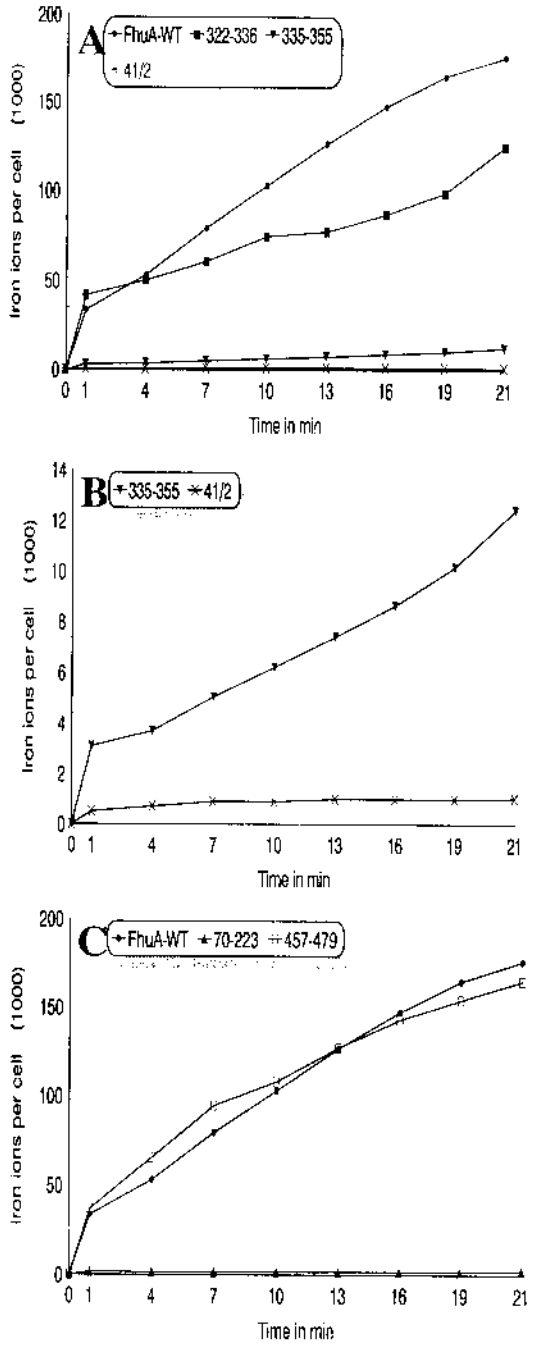

FIG. 2. Time-dependent transport of [$^{55}Fe^{3+}$]ferrichrome (1 μM) into *E. coli* 41/2 *fhuA tonB*$^{+}$ and into *E. coli* 41/2 expressing plasmid-encoded FhuA wild type (FhuA-WT), FhuA Δ322-336, or FhuA Δ335-355 (A), *E. coli* 41/2 and *E. coli* 41/2 FhuA Δ335-355 on an expanded scale (B), and FhuA Δ70-223 and FhuA Δ457-479 (C).

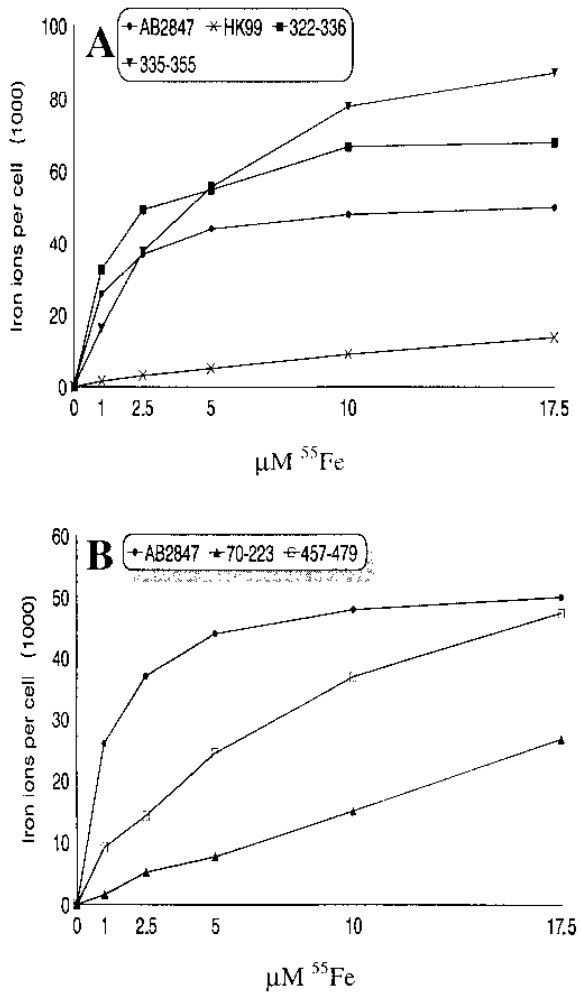

FIG. 3. Concentration-dependent transport (in 30 min) of [$^{55}Fe^{3+}$]ferrichrome into *E. coli* HK99 *fhuA tonB* expressing the plasmid-encoded FhuA deletion derivatives FhuA Δ322-336 and FhuA Δ335-355 (A) and FhuA Δ70-223 and FhuA Δ457-479 (B). Wild-type *E. coli* AB2847 and *E. coli* HK99 (lacking a plasmid) served as controls.

dissolved in 0.25% Triton X-100 in the presence of protease inhibitors to avoid proteolytic degradation of the FhuA derivatives. Under these conditions, the FhuA deletion derivatives became the most prominent proteins in the outer membrane fraction (Fig. 4). The amounts of the FhuA derivatives exceeded those of the porins OmpC/F and OmpA (Fig. 4), each of which amounts to about 100,000 copies per cell. The proteins of the outer membrane were dissolved in SDS buffer, heated at 50°C instead of the usual boiling to avoid irreversible denaturation of FhuA, then separated by SDS-PAGE, and stained with $ZnCl_2$ to visualize the protein bands. The FhuA-containing gel slice was precisely excised and, after removal of $ZnCl_2$, incubated overnight in Tris-EDTA buffer on ice to solubilize the FhuA derivatives. Gel slices of outer membranes containing FhuA wild type or no FhuA were treated exactly in the same way and served as controls.

The FhuA derivatives were added to the aqueous-phase bathing lipid bilayer membranes formed of diphytanoyl phosphatidylcholine–*n*-decane. Single-conductance steps of a constant size and a lifetime of more than 2 min were not observed with any of the FhuA deletion derivatives. Instead, the FhuA deletion derivatives FhuA Δ322-336 and FhuA Δ335-355 displayed conductance units with a high degree of current noise (Fig. 5). In the open state, the conductance (2 nS in 1 M KCl) was similar to the conductance of the stable pores formed by FhuA Δ322-355 (Fig. 5) (21). Other FhuA deletion mutants, FhuA Δ163-368, FhuA Δ322-405, FhuA Δ70-223, and FhuA Δ457-479, also formed no stable channels (data not shown).

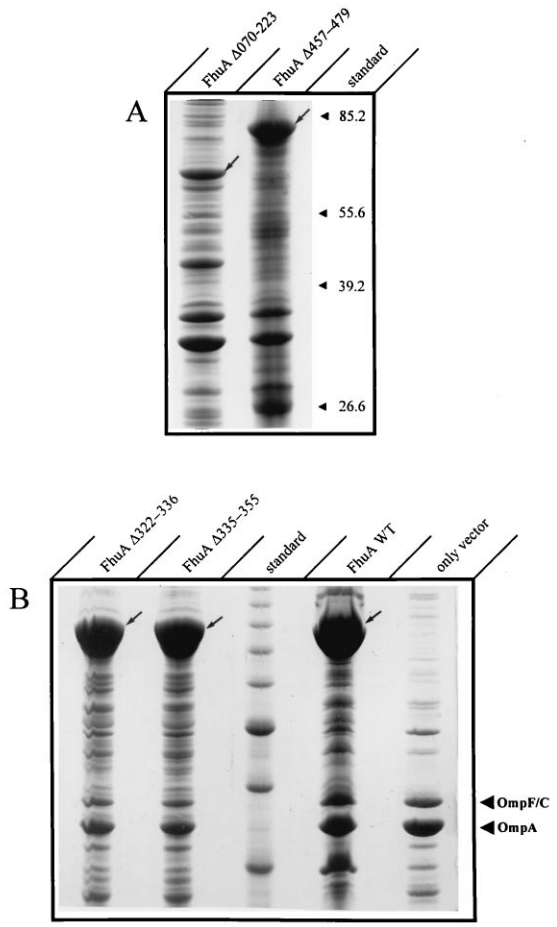

FIG. 4. SDS-PAGE of isolated outer membranes of *E. coli* WM1576 expressing FhuA wild type (WT) and FhuA deletion derivatives as indicated. Lane 3 in panel B shows a 10-kDa ladder of standard proteins ranging from 30 kDa (bottom) to 100 kDa (top). The gels were stained with Serva Blue G. The arrows mark FhuA wild type and the FhuA derivatives. The proteins OmpF/C and OmpA are indicated in panel B.

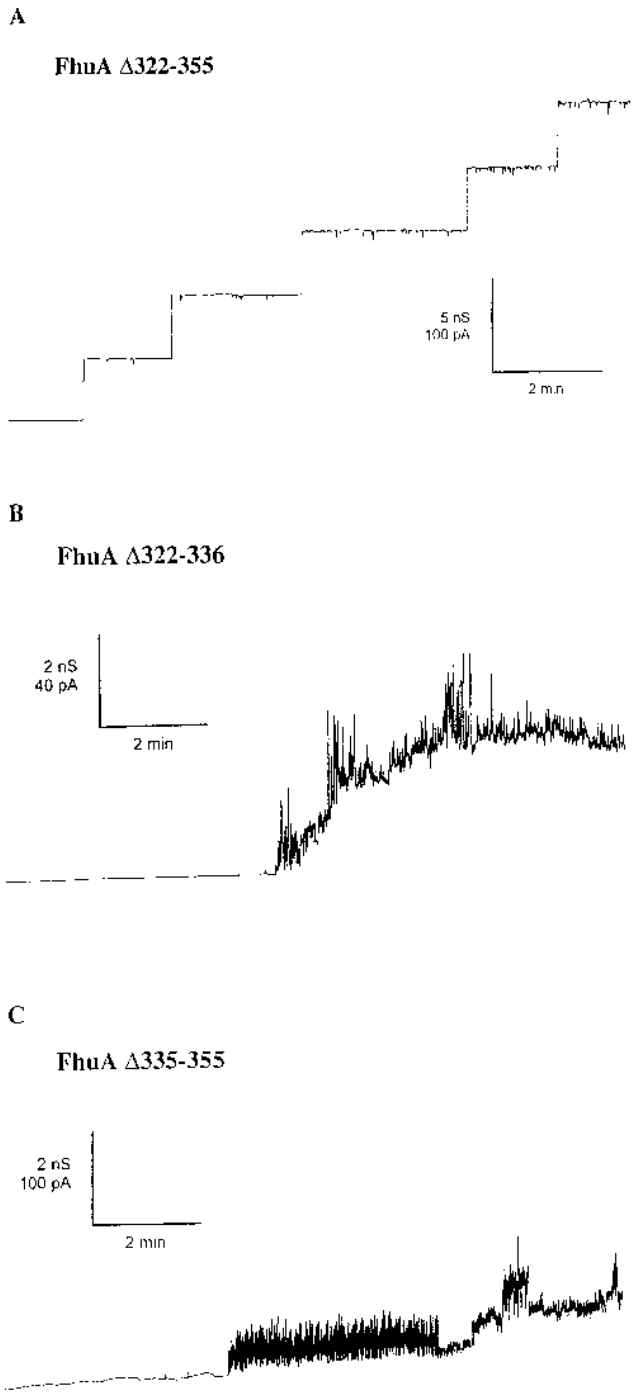

FIG. 5. Single-channel conductance of a diphytanoyl phosphatidylcholine membrane separating two aqueous compartments containing 0.1 M KCl (A) or 1 M KCl (B and C) to which was added 5 ng of FhuA Δ322-355 (A), FhuA Δ322-336 (B), or FhuA Δ335-355 (C) per ml. The membrane voltages were 20 mV (A and B) and 50 mV (C).

DISCUSSION

Excision of residues 322 to 355 from the predicted surface loop (21) created an open FhuA channel through which ferrichrome, SDS, and bacitracin diffuse into the periplasm and through which KCl, potassium acetate, and LiCl cross artificial black lipid membranes (21). Larger deletions including the gating loop extending from residues 163 to 368 and 322 to 405 of FhuA display in cells properties similar to those of FhuA Δ322-355 (21). Cells lacking the gating loop were resistant to phages T1, T5, and ϕ80 and to colicin M. Fine mapping of the presumed phage binding sites at the gating loop has been performed with hexapeptides representing the entire gating loop (24). This approach has revealed three main regions, around residues P-321, D-336, and Q-353, that are involved in phage infection. A peptide comprising D-336 triggers the release of the DNA from the phage T5 heads and causes the release of the phage tail from the phage head, which is not observed with isolated FhuA or FhuA integrated in the outer membrane (24).

In this study, we excised portions of the FhuA gating loop to identify regions important for its channel properties. The deletion in FhuA Δ322-336 did not reduce binding of ferrichrome to FhuA. Ferrichrome adsorbed as strongly to FhuA Δ322-336 as to FhuA wild-type, and FhuA Δ322-336 supported growth nearly to the same extent as FhuA wild type. Growth promotion was Ton dependent; therefore, this deletion had little effect on FhuA ferrichrome transport activity. In contrast, FhuA Δ322-336 conferred only a very low sensitivity to all FhuA-related phages. This finding agrees with the results ob-

tained by competitive peptide mapping since the deletion comprises one phage binding site and half of a second site. Colicin M sensitivity of cells expressing FhuA Δ322-336 was only 10-fold reduced, which agrees with the 10-fold reduction of colicin M sensitivity by hexapeptides around D-336. Peptides around P-321, in contrast, have no effect (24). The deletion derivative identified a subdomain in the gating loop that is essential for phage binding, of some importance for transport of albomycin and colicin M and nearly dispensable for ferrichrome transport. Removal of this FhuA segment increased only slightly the nonspecific permeability of FhuA since cells expressing FhuA Δ322-336 were only weakly sensitive to SDS and did not take up maltotetraose and maltopentaose in the absence of LamB.

Deletion of residues 335 to 355 destroyed the function of the gating loop. Cells containing FhuA Δ335-355 took up ferrichrome independent of the Ton system, they transported maltotetraose and maltopentaose in the absence of LamB, and they were highly sensitive to SDS, indicating that the FhuA channel was permanently open. They did not bind ferrichrome, and they were resistant to all FhuA ligands. Despite properties similar to those of the larger FhuA Δ322-355 channel, isolated FhuA Δ335-355 showed no uniform single-channel conductance in artificial membranes. Rather, the conductance increased and decreased rapidly, indicating that the remaining loop was still able to close the FhuA channels partially. The frequent on-and-off switching suggests that these are unstable FhuA Δ335-355 channels within the back lipid membrane or, much less likely, that FhuA Δ335-355 enters and leaves the artificial membrane frequently. FhuA, like some other outer membrane proteins, is probably composed of β-barrels. X-ray crystallography of porins of *Rhodobacter capsulatus* (36) and *E. coli* (6, 13) has shown that the β-barrels in these porins are stabilized by hydrogen bonds. The interruption of one or more of these bonds may destabilize the channel structure. Insertion of additional amino acids into the "eyelet" of the PhoE surface loop that restricts the size of the channel leads to an increase of the current noise (34). FhuA Δ322-355 was the only derivative that formed stable channels in black lipid membranes that displayed single-channel conductance of rather uniform size, suggesting that the gating loop of FhuA was precisely deleted and that this loop does not contribute much to the overall structure of FhuA.

E. coli 41/2 FhuA Δ457-479, which lacks a surface loop proximal to the gating loop, took up ferrichrome much better in the presence than in the absence of the Ton system (Table 2). The much larger and denser growth zone of the *tonB* wild-type strain than of the *tonB* mutant strain indicated TonB-dependent transport and slow TonB-independent diffusion of ferrichrome via FhuA Δ457-479 through the outer membrane. *E. coli* 41/2 FhuA Δ457-479 was weakly sensitive to SDS, in contrast to *E. coli* 41/2 FhuA wild type, which was fully SDS resistant at the concentrations used. The weak SDS sensitivity agrees with the failure of *E. coli* KB419 *lamB* FhuA Δ457-479 to grow on maltotetraose and maltopentaose. Removal of this segment increased the outer membrane permeability only slightly. However, it strongly reduced sensitivity of cells to the FhuA-related phages, colicin M, and albomycin. The zones of growth inhibition were not clear but were turbid even at the highest concentrations of the ligands. Ferrichrome uptake was less affected. This loop contributes to the permeability of the FhuA channel by directly influencing the gating loop and/or the structure of the channel. The failure of FhuA Δ457-479 to exert a defined single-channel conductance (data not shown) makes it more likely that excision of the loop destabilizes FhuA and opens FhuA slightly and nonspecifically.

FhuA Δ70-223, despite the removal of 154 residues, formed a stable protein that was secreted across the cytoplasmic membrane and integrated into the outer membrane. Cells expressing FhuA Δ70-223 formed clear zones of growth inhibition with all FhuA ligands except albomycin, which, like ferrichrome, was only weakly taken up. Although the deletion did not cut into the gating loop, Ton-dependent ferrichrome uptake was very low. This could be caused by a disturbance in the interaction of FhuA Δ70-223 with the Ton system since sensitivity to the Ton-dependent phages T1 and ϕ80 and to colicin M was reduced 100-fold, while sensitivity to the Ton-independent phage T5 was reduced only 10-fold. *E. coli* 41/2 FhuA Δ70-223 displayed the highest sensitivity to SDS among the deletion mutants studied, and growth of *E. coli* KB419 *lamB* FhuA Δ70-223 was supported well by maltotetraose and maltopentaose. The TonB-independent diffusion rate of ferrichrome at the concentration used (which was 50-fold lower than the concentration of the maltodextrins) was not high enough to support growth well. The lack of single-channel conductance in black lipid membranes (data not shown) suggests that FhuA Δ70-223 confers a nonspecific increase of outer membrane permeability.

The FhuA protein tolerates at many sites the insertion of heterologous peptides in that FhuA inserts faithfully into the outer membrane and displays undisturbed activity (12, 25, 28). Within the gating loop, the addition of foreign peptides after residue 321 reduced FhuA activity to various extents, depending on the inserted peptide. Insertion of the dipeptide Ser-Ser abolishes ferrichrome uptake but does not alter sensitivity to phages T5, T1, ϕ80, and UC-1 and to colicin M (12). Insertion of the tetrapeptide PDLA does not impair ferrichrome uptake but strongly reduces phage sensitivity and weakly affects colicin M sensitivity (25). Insertion of the hexadecapeptide PDPSSST CRSSSTDLA alters the properties of FhuA less than the tetrapeptide insertion in that cells are fully sensitive to colicin M, sensitivity to phage ϕ80 is 10-fold higher, and sensitivity to phage T5 is 100-fold higher (25). Insertion of the hexadecapeptide SSDNNPASTTNKDKSS reduces the sensitivity to phage T1 1,000-fold but leaves the other FhuA activities unaffected (28). These results agree qualitatively with the results presented in this report, since removal of this segment of the gating loop did not entirely inactivate FhuA activity. FhuA Δ322-336 retained full activity with colicin M, displayed only a 10-fold-reduced sensitivity to albomycin, but had largely lost activity with the phages.

In FepA, the receptor of ferric enterobactin and of colicins B and D, segments of 135 (residues 205 to 339) and 139 (residues 202 to 340) amino acids have been excised, a deletion which, according to the proposed transmembrane topology, comprises 2.5 transmembrane regions and 3 loops. Cells expressing the FepA deletion derivatives transport ferric enterobactin and ferrichrome independently of TonB and are sensitive to a number of antibiotics (31). Incorporation of a FepA deletion derivative renders liposomes permeable to saccharides (27). From the dependence of the swelling rate of the liposomes on solute size, a pore diameter about twice as large as the OmpF diameter has been estimated. The diameter of the FhuA Δ322-355 channel, as derived from the black lipid experiments, is at least three times as large as in OmpF (21).

The data presented in this report identified a segment of only 21 of 741 total amino acids as essential for controlling the permeability of FhuA. The same segment determines largely the ligand binding properties of FhuA. Ligand binding but not the control of the permeability is influenced by the surface loop 457-479 located close to the gating loop. The specificity of the increase of ferrichrome permeability and resistance to the FhuA ligands upon removal of segment 335-355 is supported

by the properties of the large deletion FhuA Δ70-223, which only weakly supports diffusion of ferrichrome and only slightly affects ligand binding although it allows diffusion of maltodextrins and SDS. These data together with previous results (21–24) strongly indicate that the surface loop 322-355, and in particular region 333-355, regulates the permeability of FhuA.

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