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Functions in Outer and Inner Membranes of *Escherichia coli* for Ferrichrome Transport

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Mutants of the *fhuA* gene of *Escherichia coli* K-12, which encodes a receptor protein in the outer membrane, took up ferrichrome after exposure to pronase, whereas *fhuB* mutants remained transport negative. The latter finding supports our previous proposal that *fhuB* mutants are defective in a function that resides in the cytoplasmic membrane. Cells remained completely viable after treatment with pronase, although they became sensitive to the antibiotic actinomycin.

The uptake of the iron siderophore ferrichrome and the structurally related antibiotic albomycin into *Escherichia coli* requires at least two genes, designated *fhuA* and *fhuB* (7). The *fhuA* (formerly *tonA*) gene product could be ascribed to an outer membrane receptor protein since *fhuA* mutants lack a protein in the membrane and are resistant to the phages T1, T5, and φ80 and to colicin M (7). Proof for the existence of a function in the cytoplasmic membrane usually depends on genetic and functional studies. In the case of transport systems in which the outer membrane is already a permeability barrier that is overcome by highly specific mechanisms, it is difficult to correlate functions with each of the two membranes. To locate the *fhuB* gene product in the cytoplasmic membrane, it was therefore important to make the outer membrane permeable without affecting functions in the cytoplasmic membrane. We tested pronase P since Reithmeir and Bragg (9) showed that pronase cleaves primarily only one protein in the outer membrane (now called the OmpA protein). Under the conditions we used for pronase treatment, the cells remained completely viable. However, they became sensitive to actinomycin (Fig. 1), whose access to the interior of the cell is prevented by the outer membrane (8). *fhuA* mutants deficient in ferrichrome uptake regained the ability to transport ferrichrome after pronase treatment. The increase in the initial rate of ferrichrome uptake largely followed the time course required for cells to become sensitive to actinomycin (Fig. 2).

In Fig. 3 the uptake of ferrichrome is shown for the parent and *fhuA* and *fhuB* mutant strains with and without prior exposure to pronase (1 mg/ml). It is clear from these results that whereas the uptake rates of the parent and, particularly, the *fhuA* strains were greatly stimulated by pronase treatment, no such increase was observed in a *fhuB* strain. The *fhuA* (RK3944) and *fhuB* (RK3931) strains shown in Fig. 1 have previously been distinguished by genetic means, including recombination and complementation studies (7).

A number of other mutants, selected for their resistance to albomycin, have been described previously (2). These strains were divided into classes on the basis of colicin M and phage resistance patterns and growth responses to and the uptake of ferrichrome (2) (Table 1). As expected, only the mutants resistant to phages T5 and φ80 (*fhuA*) transported ferrichrome after pronase treatment. This indicates that the other mutants had lesions in the cytoplasmic membrane, and, hence, were of the *fhuB* type.

The nature of the ferrichrome uptake was further characterized in the parent and *fhuA* strains by measuring the K_m and V_{max} with and without pronase treatment (Fig. 4). It should be noted that whereas the V_{max} value derived for the *fhuA* treated strains was about 25% of that from the treated parent strain, the K_m value was almost identical, about 0.5 μM; the K_m value measured for the untreated parent was about one order of magnitude lower, 0.06 μM. The saturation kinetics support the view that the uptake, after the bypass of the *fhuA* receptor in the outer membrane, requires an additional function.

The affinity of the receptor in the outer membrane as revealed by the K_m values indicates that it has a high affinity for ferrichrome but a relatively low maximal velocity of uptake. The affinity of the uptake step in the cytoplasmic membrane is given by the K_m measured after pronase treatment in both the parent and *fhuA* strains. It is reasonable to assume that this affinity can be associated with the uptake function in the inner membrane for two reasons: firstly, the FhuA receptor was destroyed by treatment

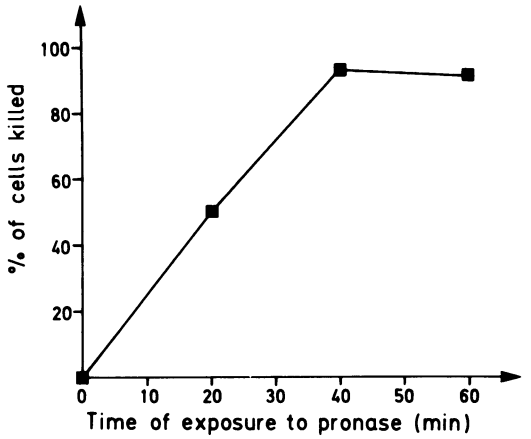

FIG. 1. *Effect of incubation time with pronase on the extent of killing with actinomycin in strain P49 (fhuA). Cells were grown in a phosphate buffer (12) containing glucose, the required amino acids and vitamins (Table 1), and 1 mM citrate to an optical density at 578 nm of 0.5, washed once in phosphate uptake buffer (12) with glucose, and suspended in uptake buffer to an optical density at 578 nm of 5.0. The suspension was incubated with 1 mg of pronase per ml in a shaking water bath (37°C), and samples were removed at time intervals shown on the abscissa. Samples were processed by centrifugation, washed once, and suspended in uptake buffer with glucose to an optical density at 578 nm of 1.5 (ready for use). The percentage of cells killed by actinomycin was expressed as a function of those killed after zero time exposure to pronase. Sensitivity to actinomycin was determined by incubating 0.5 ml of suspensions with all growth factors, Casamino Acids (Difco Laboratories), and 50 μg of actinomycin per ml in a shaking water bath at 37°C for 1 h. Suspensions were diluted 1:8 × 10^6, and 50 μl was plated out onto tryptone-yeast agar plates. Survivors were counted after overnight incubation.*

with pronase in the parent strain (11), and, secondly, the *fhuA* strain had no detectable amount of the FhuA protein. Thus, the affinity for the function(s) in the cytoplasmic membrane was about an order of magnitude lower than that calculated for the outer membrane, although the V_{max} for iron transport across the cytoplasmic membrane was somewhat higher.

Experiments were also performed to establish the sensitivity of the ferrichrome transport, after

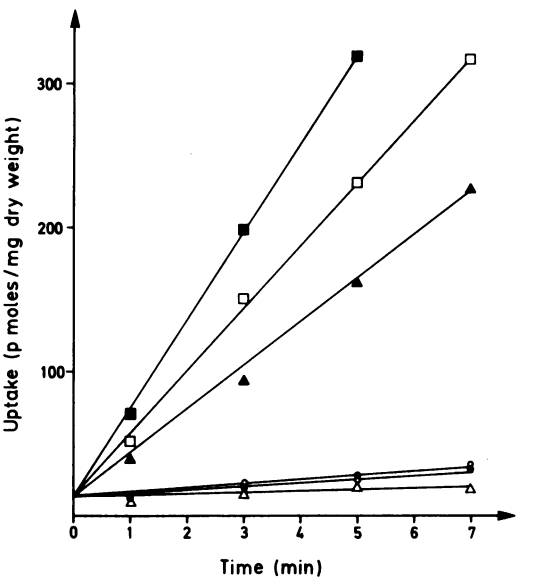

FIG. 3. *Ferrichrome uptake. Uptake of 1 μM ferrichrome was measured in treated (■) and untreated (□) parent, treated (▲) and untreated (△) RK3944 fhuA, and treated (●) and untreated (○) RK3931 fhuB strains. Strains were grown and treated with pronase as described in the legend to Fig. 1.*

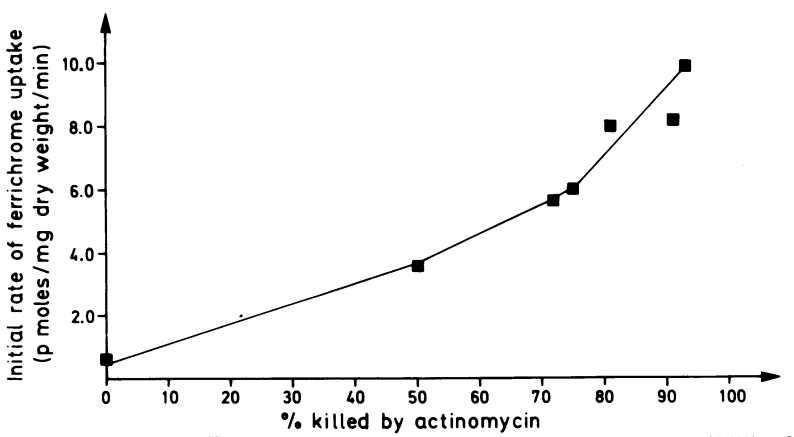

FIG. 2. *Initial rate of uptake of [^{55}Fe]ferrichrome (1 μM) correlated with the extent of killing by actinomycin in strain P49 (fhuA) after treatment with pronase for various time intervals (Fig. 1). [^{3}H]proline (4 μM) uptake remained unaltered by pronase treatment (data not shown). The techniques and conditions have been described elsewhere (12). Uptakes were assayed over the first 7 min as shown in Fig. 3, and from these curves the initial rates were calculated.*

pronase treatment, to the uncoupler 2,4-dinitrophenol. It was found that ferrichrome transport in both the untreated and treated suspensions was inhibited by 2 mM 2,4-dinitrophenol (data not shown).

Three *tonB* strains were also treated with pronase, and they behaved like the *fhuB* mutants (data not shown); there was no significant uptake of ferrichrome in untreated or treated suspensions of *tonB* cells. The susceptibility of the pronase-treated *tonB* to actinomycin was, however, lower (60% killed) than that of the parent strains. The reason for this is believed to be related to the lower growth rate of *tonB* strains during exposure to actinomycin.

TABLE 1. *Bypass of the outer membrane receptor protein by treatment with pronase*[a]

E. coli K-12 strains	Genetic markers	Initial rate of ferrichrome uptake (pmol/mg (dry weight) per min)		% Killed by actinomycin
		Untreated	Treated	
AB2847	*thi aroB tsx malT*	44.3	62.3	99
P1	*fhuA*	4.4	31.7	NT[b]
P8	*fhuA*	1.9	62.3	NT
P48	*fhuA*	2.8	69.2	NT
P49	*fhuA*	6.0	67.9	99
KL6	*fhuA*	2.9	50.0	60
RK3944	*fhuA*	2.1	29.8	83
KL13	*fhuB*	2.8	12.7	90
KL79	*fhuB*	6.5	6.0	98
KL80	*fhuB*	2.4	3.4	90
RK3931	*fhuB*	3.5	3.5	72

[a] The strains listed are derivatives of the strain AB2847. They have been described previously (2, 7). Their relevant properties are as follows. *E. coli* AB2847 grows with ferrichrome as the sole iron source, whereas all the derivatives are deficient in ferrichrome uptake. The strains P1, P8, P48, P49, RK3944 and KL6 are resistant to phages T5 and ϕ80 and to colicin M, in contrast to the strains KL13, KL79, and RK3931 which are sensitive. The strain KL80 is also insensitive to colicin M (2).

[b] NT, Not tested.

The data presented here support the following points. Firstly, a functional receptor in the outer membrane (FhuA) and a function in the cytoplasmic membrane specified by the *fhuB* gene are integral components of the ferrichrome transport system, as suggested previously on the basis of genetic data (7). It is worth noting at this point that no significant ferrichrome uptake occurs in the *fhuB* strains whether treated with pronase or not, therefore excluding the possibility of any significant accumulation into the periplasmic space (untreated) as has been proposed for the vitamin B12 system, another *tonB*-dependent system (10). Secondly, the kinetic properties (K_m *and* V_{max}) of the outer membrane receptor, as determined by the difference between untreated and treated cells of the parent, imply that the kinetic properties of the total intact ferrichrome system are largely determined by the outer membrane receptor. The pronase-treated *fhuA* mutants exhibited lower V_{max} values than those of the treated parent strain. It is reasonable to assume that mutations leading to deletions in the *fhuA* gene (all mutants were devoid of the FhuA protein) decreased somewhat the expression of the closely linked *fhuB* gene.

In this paper we interpret the kinetic data as if there would be two simple translocation steps

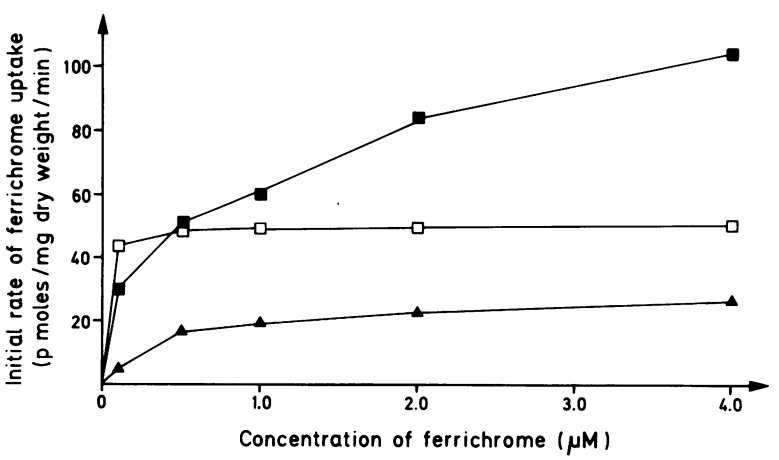

FIG. 4. *Determination of kinetic characteristics (K_m and V_{max}) of the ferrichrome transport system. Determinations were made in treated (■) and untreated (□) parent (K_m = 0.7 μM, V_{max} = 110 pmol/mg [dry weight] per min; and K_m = 0.06 μM, V_{max} = 50 pmol/mg [dry weight] per min, respectively) and treated (▲) P49 strains (K_m = 0.45 μM, V_{max} = 30 pmol/mg [dry weight] per min).*

for ferrichrome, one across the outer membrane and one across the cytoplasmic membrane. However, we are aware of the complexity of the iron transport systems. For ferrichrome we have shown that the ligand is inactivated by acetylation and excreted (5). Now we have found with a cell-free system that the iron is released in an NADH-dependent reaction and that subsequently deferri-ferrichrome is acetylated (R. Schneider, A. Hartmann, and V. Braun, FEMS Lett., in press). Both reactions occurred in the membrane fraction and not in the cytoplasm. They were independent of the *fhuB* gene. Furthermore, acetylated deferri-ferrichrome is taken up in the absence of ferrichrome, and the structurally analogous antibiotic albomycin enters the cells via the same transport system (6). In addition, we have shown that the activity of the FhuA protein is regulated by the *tonB* gene product and the energy state of the cell (1, 3, 4). The kinetic constants are therefore derived from a rather complex situation. The arguments, however, have little bearing on the major finding in this paper that the requirement for the FhuA protein for ferrichrome transport across the outer membrane can be bypassed by treatment with pronase. Since the pronase action is confined to the outer membrane and renders it permeable to actinomycin and since the requirement for the FhuA protein in the outer membrane but not the function determined by the *fhuB* gene was overcome, it is concluded that the latter function resides in the cytoplasmic membrane.

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