MEMBRANE RECEPTOR DEPENDENT IRON TRANSPORT IN *ESCHERICHIA COLI*

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1. Introduction

We are interested in functions of the outer membrane of *Escherichia coli.* Of particular interest to us is the functional interplay between the tonA and the tonB gene products. For this reason we have isolated from *E. coli* B and *E. coli* K12 ROW the protein [1–4] specified by the tonA gene which serves as a receptor for the phages T5, T1 and ϕ80 and the colicin M [5]. This protein is a single polypeptide chain with a mol. wt of 85 000 [3] localized in the outer membrane of *E. coli* [2]. T5 irreversibly binds to the protein and releases its DNA. In whole cells the tonB function is additionally required for the productive infection by the phages T1 and ϕ80 and for murein dissolution by colicin M [6,7]. The phages and colicin M kill the cells. In a search for a function of the tonA protein besides that of being the receptor for killing agents, we obtained evidence that the tonA protein is involved in ferrichrome dependent iron uptake.

E. coli K12 has three high affinity systems for iron uptake. Ferric iron is taken up either as complex with citrate [8] or with enterochelin [9] or with ferrichrome [10–12]. A fourth low affinity system satisfies the iron requirement of the cell provided the medium contains a sufficiently high concentration of ferric iron [8]. In order to be able to differentiate between the four systems we used *E. coli* 83–24 (derived from *E. coli* W) which does not contain a citrate dependent system. This strain is also unable to synthesize enterochelin due to an early mutation in the aromatic amino acid pathway so that the enterochelin precursor 2,3-dihydroxybenzoate can not be made. In addition we supressed the low affinity iron uptake system by addition of 100 μM nitrilotriacetate [8]. Thus we could study the ferrichrome dependent iron uptake without interference by the other three iron uptake systems.

2. Materials and methods

2.1 *Strains and culture conditions*

E. coli K12ROW/V/22.1 M indicator strain and the M producing strain *E. coli* K12 32T19F/T1 were obtained from P. Fredericq, Liège. The strain *E. coli* 83-24 with a block in the shikimic acid synthesis pathway [13] was kindly supplied by H. Uesseler (University of Hohenheim) and is a derivative from *E. coli* W (ATCC 9637). It was grown in a CR minimal medium [14] supplemented with 0.4% glucose, 0.05% casamino acids and 20 μg per ml of the amino acids tryptophane, tyrosine and phenylalanine, in which the iron was extracted with 8-hydroxyquinoline and chloroform.

Albomycin resistant colonies were isolated from the inhibition-zones of filterpaper discs soaked with 0.01 mg albomycin per ml and placed on freshly seeded agar plates. Spontaneous phage resistant mutants were isolated from plates seeded with approx. 1×10^8 cells and 1×10^8 phages.

2.2 *Chemicals*

All chemicals used were analytical grade from Merck (Darmstadt). Highly purified δ_2 albomycin, ferrichrome and desferri-ferrichrome were a gift from H. Zähner, H. Diekmann and B. Krezdorn of this Institute.

2.3 *Iron uptake measurements*

Cells were grown to an optical density of 0.5 at 578 nm (corresponding to 0.19 mg dry weight per ml).

The cells were washed in the iron uptake medium adapted from [9]. After resuspension in this medium to an optical density of 2.0, the cells were incubated for 15 min at 37°C. They were then mixed at 37°C with an equal volume of uptake medium containing 0.18 μCi $^{55}Fe^{3+}$ per ml (Amersham Buchler 5.5 mCi/mg Fe). Samples of one ml were taken at two min intervals, applied to Selectron-filters (pore size 0.45 μm, Schleicher and Schüll) and washed twice with 4 ml uptake medium. Radioactivity was measured after adding 5 ml Aquasol (New England Nuclear Chicago Corp.) in the liquid scintillation counter Mark II from Nuclear Chicago Corp..

3. Results

Study of the tonB function alone is hampered by the fact that it acts only together with the tonA function. The only exception known is related to iron transport. Mutations in the ton B gene lead to a 10-fold increase of the K_m of ferric iron uptake without affecting the V_{max} [15]. We therefore investigated whether the ferrichrome dependent iron transport also depends on the tonB function. We used the antibiotic albomycin which is structurally closely related to ferrichrome [12]. We isolated 136 spontaneous albomycin resistant mutants from *E. coli* K12 ROW, the indicator strain for colicin M. From these mutants 33 were T1 and colicin M resistant, pointing to a deficiency in the adsorption of albomycin, T1 and colicin M to the cell. But unexpectedly 32 of the 33 mutants were also T5 resistant from which we had to conclude that these 32 mutants were deficient in the tonA function and only one in the tonB function. We then isolated 30 T5 resistant mutants from *E. coli* 83–24, of which all were albomycin resistant. In addition we selected for T1 resistant mutants. Only 5 out of 87 mutant colonies turned out to be mutants in the tonB gene; 82 were mutated in the tonA gene (resistance to T5). All mutants were resistant to albomycin and colicin M. This clearly shows that mutations in the tonA protein render *E. coli* albomycin resistant. Since the tonA protein functions as a receptor for the phages and colicin M it could also be a receptor for albomycin. If the ferrichrome is taken up by the *E. coli* cell through the same transport system as albomycin, the tonA protein could also function as an iron–ferrichrome receptor. We therefore studied ferrichrome-dependent iron transport.

Cells of *E. coli* took up $^{55}Fe^{3+}$ rapidly without addition of any complexing agent by the low affinity system as shown by suppression of uptake with 100 μM nitrilotriacetate in the medium (fig.1a). Cells incubated for 15 min with 20 μM 2,3-dihydroxybenzoate prior to the uptake experiment took up $^{55}Fe^{3+}$ also in the presence of 100 μM nitrilotriacetate (fig.1a). This shows that enterochelin synthesis and enterochelin-dependent transport took place again when the enterochelin precursor was supplied. The ferrichrome complex was taken up also in the presence of nitrilotriacetate (fig.1a).

Several tonA mutants of *E. coli* 83-24 were tested for iron uptake in the same manner. As shown in fig.1b these mutants were only impaired in the ferrichrome dependent iron uptake. Omission of nitrilotriacetate from the uptake medium did not result in iron uptake by the low affinity system since the iron is strongly complexed by ferrichrome. Iron uptake was also studied with tonB mutants of *E. coli* 83–24. Iron uptake was strongly reduced in the enterochelin as well as in the ferrichrome dependent system (fig.1c).

If the binding requirements for the iron–ferrichrome complex at the tonA/tonB functional unit in the outer membrane are the same as for colicin M, the iron–ferrichrome complex should inhibit killing of the cells by colicin M. When ferrichrome and various concentrations of purified colicin M were cross-streaked over a nutrient agar plate, killing of the indicator strain by colicin M was inhibited at those regions where both substances came into contact (fig.2). When a solution of 1 mM ferric chloride was used instead of 1mM ferrichrome no inhibition was observed.

4. Discussion

The very high incidence of tonA mutants in albomycin resistant *E. coli* mutants was the first hint that the ferrichrome uptake system may contain a constituent which is identical to the tonA receptor protein. Since all tonA mutants were albomycin resistant and all which have been studied were specifically impaired in ferrichrome uptake, the tonA receptor protein is a component of the ferrichrome transport system. Active transport is most probably

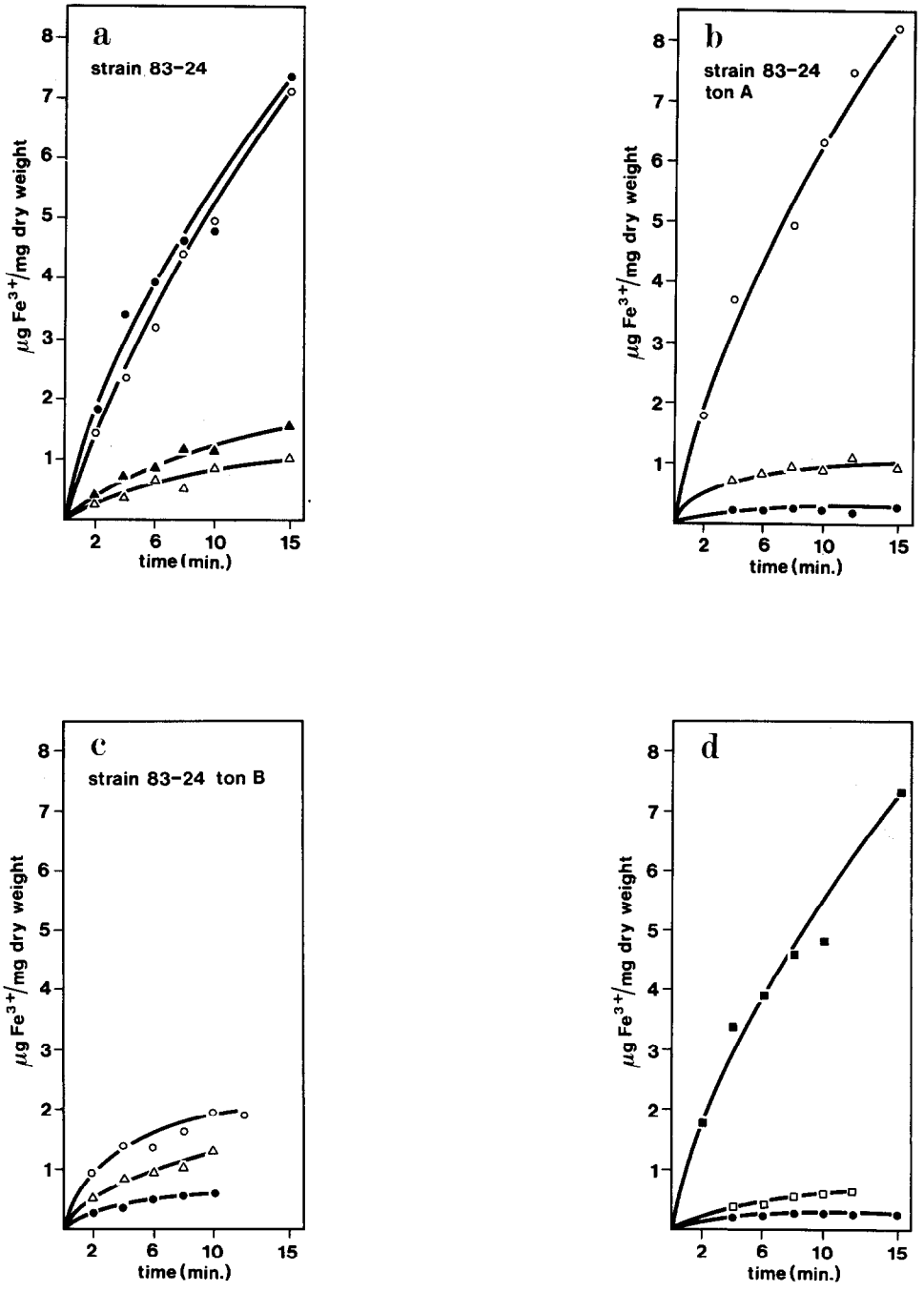

Fig.1. Iron uptake by *E. coli* 83-24 and derived strains. All measurements were made in the presence of 100 µM nitrilotriacetate. a) Iron uptake by *E. coli* 83-24 incubated with $^{55}FeCl_3$ (△△△), with $^{55}FeCl_3$, cells preincubated for 15 min with 20 µM 2,3-dihydroxybenzoate (○○○), with $^{55}Fe^{3+}$– ferrichrome (●●●), with $^{55}Fe^{3+}$-citrate (100 µM citrate) cells pregrown in 1 mM citrate (▲▲▲). b) Iron uptake by *E. coli* 83-24/97 (tonA mutant), symbols as in a). c) Iron uptake by *E. coli* 83-24/91 (tonB mutant), symbols as in a). d) Ferrichrome uptake by *E. coli* 83-24 (■■■), by *E. coli* 83-24/97 (tonA mutant) (●●●), by *E. coli* 83-24/91 (tonB mutant) (□□□).

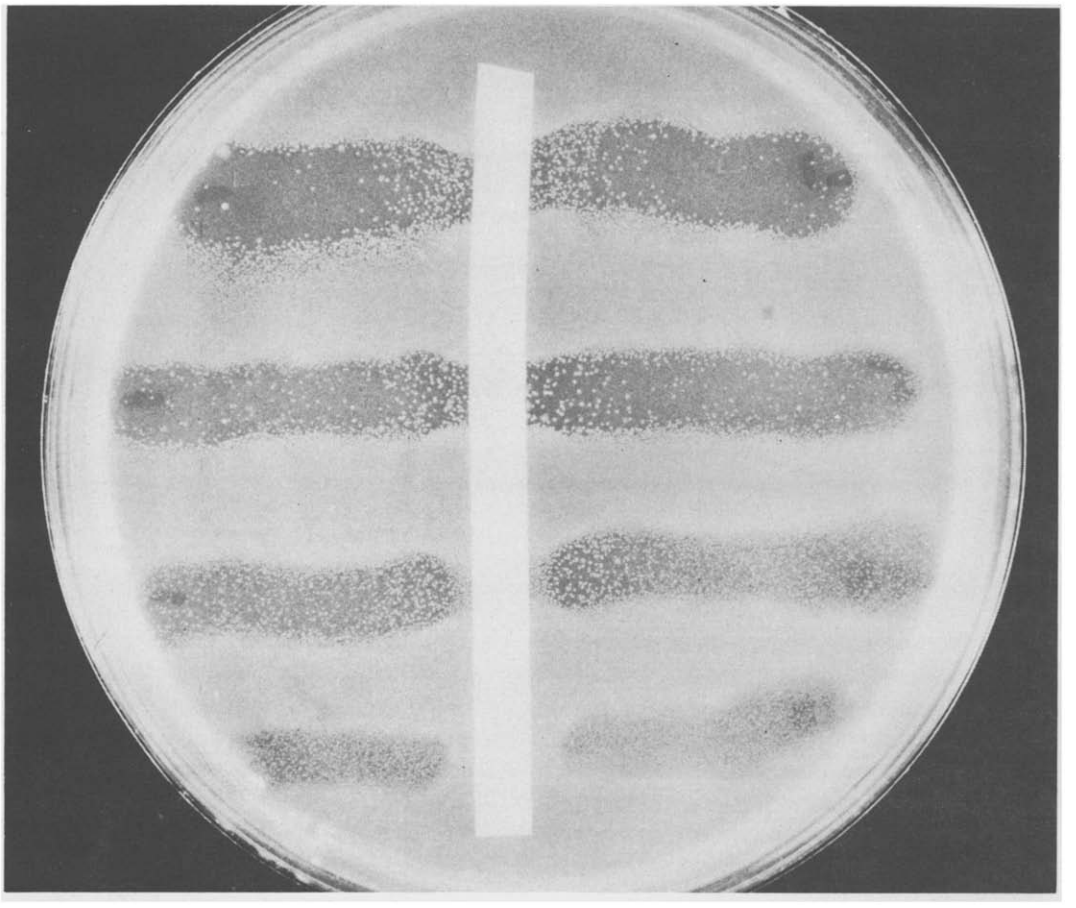

Fig. 2. Colicin M [13] was diluted 1:1, 1:5, 1:10 and 1:20 with CR medium and streaked on a nutrient agar plate (from top to bottom). After it had dried down 2.5 ml of soft agar with approx. 1×10^8 bacteria was poured on the plate and a strip of filter-paper soaked with 1 mM ferrichrome was layed on top and incubated for 18 hr at 37°C.

taking place since the amount of ferrichrome in the cells exceeds the concentration in the medium at least by a factor of 200. In the context of the tonA receptor protein for ferrichrome, it is also of interest that uptake of the sideromycin antibiotic A22,765 is inhibited in *Staphylococcus aureus* SG511 by ferrioxamine B despite the fact that the latter is only very poorly taken up by the cell [17]. This can be explained by competition of the two iron complexes at a peripheral binding protein at the cell membrane. Ferrichrome uptake in wild type and tonA and tonB mutant cells is summarized in fig.1d. Both tonA and tonB mutants are deficient in ferrichrome uptake. An interesting similarity exists between the tonA gene mutants of *E. coli* and *Salmonella typhimurium* mutants impaired in ferrichrome (siderochrome) uptake [11]. Various genes called 'sid' map near 7 min on the Salmonella linkage map, close to other genes which surround the tonA locus at 3 min on the *E. coli* linkage map.

The interplay of the tonA and tonB functions for the four iron uptake systems, infection by the phages T1, T5 and ϕ80 and killing by colicin M is shown in fig.3. T5 infection needs the tonA receptor but is independent of the tonB function. For ferrichrome uptake, infection by T1 and ϕ80 and killing by colicin M the tonA and the tonB functions are necessary. The tonB function but not the tonA function

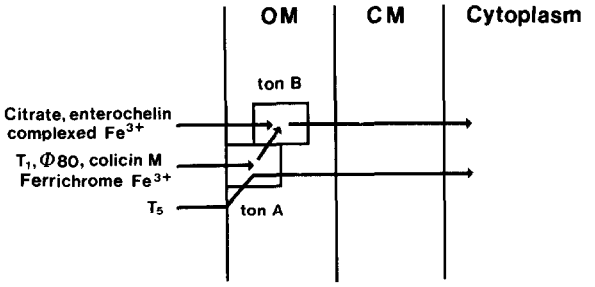

Fig.3. Present view of the functional interplay of the tonA and tonB gene products. The figure should only represent the functional but not the spatial correlations between the tonA receptor protein in the outer membrane and the tonB gene product which until now is neither identified biochemically nor localized in the membrane system of *E. coli*. OM = outer membrane, CM = cytoplasmic membrane. The arrow pointing into the cytoplasm should also not imply that there is a common transport system for all iron chelates in the cytoplasmic membrane.

is involved in the uptake of citrate and enterochelin iron complex. We assume that the tonA protein which is the receptor for T5, also functions as receptor for ferrichrome. The specific inhibition of colicin M killing by ferrichrome is explained by competitive binding at the receptor protein. The tonB function, so far not identified biochemically could be a 'binding protein' [18] for all three types of iron complexes serving as shuttle between the outer membrane and the energy dependent iron transport system in the cytoplasmic membrane. Two other receptor dependent high affinity transport systems emerged recently. These are the vitamin B12 transport system which uses the receptor protein for the E-colicins and the phage BF23 [19] and the maltose transport system in which the λ receptor serves as a high affinity binding protein in the outer membrane [20]. Both systems are also dependent on an additional 'binding protein'. It seems that receptor proteins evolved as high affinity binding components of transport systems for the uptake of substrates present in very low amounts in the cell environment. The receptor proteins were then employed by toxic agents like phages and colicins for entering the cell.

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