Functional Organization of the Outer Membrane of Escherichia Coli: Phage and Colicin Receptors as Components of Iron Uptake Systems

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The functional interaction of outer membrane proteins of E. coli can be studied using phage and colicin receptors which are essential components of penetration systems. The uptake of ferric iron in the form of the ferrichrome complex requires the ton A and ton B functions in the outer membrane of E. coli. The ton A gene product is the receptor protein for phage T5 and is required together with the ton B function by the phages T1 and ϕ80 to infect cells and by colicin M and the antibiotic albomycin, a structural analogue of ferrichrome, to kill cells. The ton B function is necessary for the uptake of ferric iron complexed by citrate. Iron complexed by enterochelin is only transported in the presence of the ton B and feu functions. Cells which have lost the feu function are resistant to the colicins B, I or V while ton B mutants are resistant to all 3 colicins. The interaction of the ton A, ton B, and feu functions apparently permits quite different "substrates" to overcome the permeability barrier of the outer membrane.

It was shown for ferrichrome dependent iron uptake that the complexing agent was not altered and could be used repeatedly. Only very low amounts of ^{3}H-labeled ferrichrome were found in the cell. It is possible that the iron is mobilized in the membrane and that desferri-ferrichrome is released into the medium without having entered the cytoplasm.

Growth on ferrichrome as the sole iron source was used to select revertants of T5 resistant ton A mutants. All revertants exhibited wild-type properties with the exception of partial revertants. In these 4 strains, as in the ton A mutants, the ton A protein was not detectable by SDS polyacrylamide gel electrophoreses of outer membranes.

Albomycin resistant mutants were selected and shown to fall into 5 categories: 1) ton A; 2) ton B mutants; 3) mutants with no iron transport defects and normal ton A/ton B functions, which might be target site mutants; 4) mutants which were deficient in ferrichrome-mediated iron uptake but had normal ton A/ton B functions. We tentatively consider that the defect might be located in the active transport system of the cytoplasmic membrane; 5) a variety of mutants with the following general properties: most of them were resistant to colicin M, transported iron poorly, and, like ton B mutants, contained additional proteins in the outer membrane.

The outer membrane protein patterns of wild-type and ton B mutant strains were compared by slab gel electrophoresis in an attempt to identify a ton B protein. It was observed that under most growth conditions, ton B mutants overproduced 3 proteins of molecular weights 74,000–83,000. In extracted, iron-deficient medium, both the wild-type and ton B mutant strains had similar large amounts of these proteins in their outer membranes. The appearance of these proteins was suppressed by excess iron in both wild-type and mutant. From this evidence it is apparent that the proteins appear as a response to low intracellular iron rather than being controlled by the ton B gene. The nature of these proteins and their possible role in iron transport is discussed.

Key words: E. coli permeability barrier, phage receptors, iron uptake

INTRODUCTION

Concepts concerning the organization of proteins in the outer membrane of E. coli usually rely on data generated through indirect methods. Particles visualized in the fracture planes of frozen membranes were tentatively ascribed as proteins, without knowing which of the better characterized proteins they comprise (1–5). An exceptional case concerning the localization of an individual protein is provided by the lipoprotein which is covalently bound to the murein (peptidoglycan) (6–8). From electron micrographs of thin sections before and after degradation of the murein with lysozyme, it was deduced that the murein forms the innermost layer of the outer membrane (9, 10). Since the lipoprotein is fixed by the carboxyl-terminus of the polypeptide chain to the murein (6), one knows the location of that portion of protein exactly. Consideration of the arrangement of the rest of the lipoprotein within the outer membrane depends on assumptions about the overall organization of the membrane, in particular phospholipids, the lipopolysaccharide, and the other proteins. To obtain an indication of how far the lipoprotein penetrates into the outer membrane toward the cell surface we determined its accessibility from the outside of the cell by immunological methods. When wild-type cells of E. coli or Salmonella were injected into rabbits, only low antiserum titers against lipoprotein were obtained (8, 11, 12). The titers rose with mutant cells which lacked more and more sugar residues of the lipopolysaccharide at the cell surface. With the most rough mutants, the Re type, the anti-lipoprotein titers were even higher than the antilipopolysaccharide titers. Absorption studies of anti-lipoprotein antibodies to wild-type and mutant cells corroborate these results. Lipoprotein becomes increasingly immunogenic as well as antigenic as the number of sugar residues missing from the lipopolysaccharide increases. In wild-type cells lipoprotein is buried in the outer membrane. Its exposure in mutant cells is related to defects at the cell surface. Whether the immunogenicity and antigenicity of the lipoprotein reflects only its degree of penetration of the outer membrane is uncertain when one considers that some deep rough mutants of Salmonella typhimurium and E. coli K12 contain reduced amounts of major proteins (13, 14). Such mutants also become sensitive to some structurally and functionally unrelated antibiotics, dyes, and detergents pointing to a more general breakdown of the membrane's permeability barrier (7).

A further possibility for the localization of proteins within membranes comes from functional studies. Protein receptors for the large phages and colicins must be accessible from the outside, and therefore their binding regions must be close to the cell surface. As will be shown below, in some cases several proteins have to interact for receptor functions. It is unknown whether these proteins bind to each other permanently, thus forming functional and structural units, or if they move laterally in the membrane bilayer, as individual proteins and interact only temporarily.

The system which will be described in some detail below consists of products of two gene regions, called ton A and ton B (ton is derived from phage T one, T1). The products of either one or both gene regions are necessary for 11 different "substrates" to pass through the outer membrane. The substrates are the DNA of the phages T1, T5, ϕ80; the colicins B, I, V, M; the iron complexes ferric citrate, ferric enterochelin, and ferrichrome; and the antibiotic albomycin. In the last few years it has become apparent that the ton A and ton B functions, originally defined as phage and colicin receptors (15), are constituents of transport systems for the above iron complexes (16–21). Since the ton A gene product, a protein with a molecular weight of about 80,000 daltons (22), was

localized in the outer membrane (23) and since indirect evidence suggests a similar localization for the ton B function (24), it is likely that both interact to form specific pores through the outer membrane for the iron complexes. The outer membrane is a permeability barrier for substrates with a molecular weight greater than about 700 daltons (25). Since the iron complexes are in this molecular weight range they are probably unable to pass through the barrier quickly enough to serve the growth requirements without channels.

   We consider the interplay of the ton A / ton B functions a favorable system to study receptor-dependent processes. With phages, binding to the receptor triggers the release of the DNA of the phage and passage through the outer and cytoplasmic membrane. In the case of the colicins, binding to the receptor is an essential step in the process of killing the cell. After receptor binding the macromolecular deoxyribonucleic acids and colicins are probably channeled in different ways to their targets. For example, while bacteriophages T1, ϕ80, and T5 all require the ton A gene product as a receptor, this is the only requirement for T5 while bacteriophages T1 and ϕ80 additionally require the ton B product and the energized membrane state (26). As seen below, apart from the common ton B function, each iron complex has its own specific transport steps. There exist parallels in the coupling of different hormone receptors to the common adenylcyclase (27) and the triggering of lymphocytes by various antigens (28). The ease with which mutants and revertants of the ton A / ton B system can be obtained should assist in the understanding of its properties. It is conceivable that the system can be dissected by studying the uptake of the different substrates into wild-type cells, mutants, and partial revertants.

MATERIALS AND METHODS

Bacterial Strains

   The strains used have been described previously (17, 18). The E. coli K12 AB2847 aroB thi tsx λ^r was the parent strain from which all resistant mutants were derived, as described elsewhere (17, 18) or in this paper.

Bacteriophages and Colicins

   These have been described previously (17, 18, 22, 23, 29).

Media, Iron Uptake Measurements and Growth Conditions

   In addition to those media previously described (17, 18), the following were used. As rich media, 1% tryptone medium (Difco 0123-01), single strength [0.8% (w/v)] and double strength [1.6% (w/v)] nutrient broth (Difco 0003-01), and tryptone-yeast media [containing 0.8% (w/v) tryptone and 0.5% (w/v) yeast extract (Difco 0127-01)]. To each medium, 0.5% (w/v) NaCl was added before autoclaving, and for the making of solid medium for plates or soft agar for overlays, 1.5–2.0% and 0.7% agar was added respectively. For many experiments, the minimal medium used was CR medium supplemented as described (18). In addition, M9 minimal medium (22) was used for all experiments with ton B mutants. When iron-deficient medium was required the iron was extracted with 8-hydroxycholine and chloroform. The following supplements were used: tryptophan, tyrosine, phenylalanine, and thiamine, each at 20 μg/ml; 20 μM p-aminobenzoic acid; 20 μM p-hydroxybenzoic acid; and where indicated 0.5% glucose, 0.5% succinate, 1 mM citrate, 20 μM 2,3-dihydroxybenzoic acid (DHB), or 1% casamino acids. In the iron-deficient extracted media, cultures grew, even in the presence of 20 μM

DHB, to a final density 30% lower than that of a culture grown in unextracted medium. When required, iron was added as $FeCl_3$ or $FeSO_4$, the latter having been converted to Fe^{3+} by autoclaving.

Iron uptake experiments were as described previously (18). Growth on ferrichrome was tested as described before on solid medium (17) or in extracted, iron-deficient CR liquid medium with ferrichrome as the sole iron source.

For preparation of outer membranes, cells were grown overnight in the desired medium and diluted 1/50–1/100 into 500 ml of fresh medium in a 2,000 ml Erlenmeyer flask. When the growth medium used was iron-deficient extracted medium, the overnight culture was grown on nonextracted medium supplemented with 20 μM DHB and was pelleted by centrifugation and washed 2 times with extracted medium before innoculation of the culture vessel. Cultures were then grown with shaking to an OD_{578} of 0.5–0.6.

For the differential labeling of AB2847 and its ton B mutant BR158, two 2 liter Erlenmeyer flasks containing 1 liter of M9 media, all required supplements, 20 μM DHB, 0.5% glucose, 0.2% casamino acids (which is approximately equivalent to 20 μg/ml leucine), 1.8 μM $FeCl_3$, and either 50 μCi of ^{3}H-leucine (59 Ci/mMol, Amersham Lab., England) or ^{14}C-leucine (324 mCi/mMol, Amersham) were inoculated with 50 ml of an overnight culture ($OD_{578} \cong 2.0$) of AB2847 or BR158, respectively. The cultures were then grown at 37°C with shaking to a culture density of 1.4 before harvesting.

Preparation of Outer Membrane Fractions and Polyacrylamide Gel Electrophoresis

Outer membranes were prepared by two methods. The majority of outer membrane preparations consisted of the densest fraction (H-band) isolated after sucrose density centrifugation using the method of Osborn et al. (30). Where indicated the outer membrane protein preparations were prepared by disintegration of cells by shaking with glass beads in a Vibrogen Mickle-type disintegrator, (Bühler, Tübingen) followed by differential extraction of the cytoplasmic membrane and outer membrane proteins with Triton X-100 ± ethylenediamine tetraacetic acid (EDTA) according to the method of Schnaitman (31). Polyacrylamide gel electrophoresis and SDS solubilization of proteins at 100°C was performed using the techniques of Lugtenberg et al. (32).

Column Chromatography

Seventy-five milligrams of ^{3}H-labeled cell envelope derived from strain AB2847 (2×10^4 cpm/mg protein) were mixed with 115 mg of ^{14}C-labeled cell envelope derived from strain BR158 ton B (5×10^3 cpm/mg protein), and a Triton X-100 + EDTA soluble outer membrane fraction was isolated. This was precipitated with 2 vol of alcohol and resuspended in 2% Triton X-100 + 5 mM EDTA in 0.01 M Tris-HCl, pH 7.2 (column buffer) at a concentration of 5 mg/ml. This was then applied to a column of Whatman DE52, DEAE-cellulose (bed volume 50 ml) with the above-mentioned column buffer. The column was then eluted with 300 ml of column buffer followed by 2 salt gradients in 200 ml of column buffer, the first gradient 0–0.1 M NaCl and the second 0.1–0.5 M NaCl. Samples of 5 ml were collected from which 1 ml was mixed with 10 ml Aquasol (New England Nuclear, Boston) and shaken before determining the radioactivity in a Nuclear Chicago Mark II counter. The samples were pooled as described below, precipitated with alcohol and resuspended at a protein concentration of 2 mg/ml in distilled water prior to polyacrylamide gel electrophoresis.

Selection of Ton A Revertants

This was performed by plating 0.1 ml of a washed overnight culture on minimal agar plates with ferrichrome as the sole iron source. Revertants were those colonies which were largest after 3 days growth. Where indicated a crystal of N'-methyl-N'-nitro-N-nitrosoguanidine (NNMG) was placed in the middle of the plate to increase the frequency of revertants. To identify those albomycin resistant mutants, which map near ton A, P1, transduction into strain AT982 (dap D) was performed as summarized by Miller (33). The strains used are listed in Table I.

RESULTS

Ferrichrome Uptake by E. Coli

Cells generally have problems in satisfying their iron requirements, since under aerobic conditions at pH 7 iron is largely present in the ferric form as hydroxide with an

TABLE I. List of E. Coli K12 Strains Used

Strain	Relevant characteristics	Source
AB2847	aro B, thi, λ^r	(18)
AT982	dap D4, thi, rel-1	B. Bachmann
The following strains were:	derived from AB2847	
IR20	feu	(18)
VR42	feu	(18)
VR35	feu	(18)
BR158	ton B	
IR112	ton B	
K1 84	ton B	
P4	ton A	
P16	ton A	
P20	ton A	
P47	ton A	this
K1 21	ton A	
K1 63	ton A	study
4a	partial revertant of P4	
32f	partial revertant of P32	
45b	partial revertant of P45	
47a	partial revertant of P47	
16k	revertant of P16	
20b$_1$	revertant of P20	
K1 4	albomycin resistant group 3 (no ferrichrome-iron uptake)	
K1 8	albomycin resistant group 3 (no ferrichrome-iron uptake)	
K1 15	albomycin resistant group 3 (no ferrichrome-iron uptake)	
K1 1	albomycin resistant group 5 mutant (colicin M resistant with reduced iron uptake)	
K1 80	albomycin resistant group 5 mutant (colicin M resistant with reduced iron uptake)	

extremely low solubility constant of $10^{-36}-10^{-39}$ [summarized in (34)]. They have developed many kinds of compounds which form soluble complexes with ferric iron. In E. coli there exist four iron uptake systems. Ferric iron can be taken up in complex with citrate (21), enterochelin (21), or ferrichrome (17, 19). A fourth low-affinity system satisfies the iron requirement of the cell provided the medium contains a sufficiently high concentration of ferric iron. This last uptake system can be suppressed by the addition of 100 μM nitrilotriacetate (35).

The presence of an effective ferrichrome uptake system in E. coli is surprising since ferrichrome is only produced by certain moulds like Ustilago sphaerogena (36).

To characterize the ferrichrome uptake system we measured the uptake of radioactively labeled iron and tritium-labeled ferrichrome by E. coli K12 AB2847, which is an aro B$^-$ mutant unable to produce enterochelin. The cells took up iron rapidly, but we observed no uptake of the complexing agent (Fig. 1). The iron uptake rate was not significantly increased when equimolar amounts of iron and complexing agent (desferri-ferrichrome) were used (Fig. 1, upper curve) compared with conditions in which only 0.15 nmole desferri-ferrichrome per nmole ferric iron (Fig. 1, lower curve) was present. Under both conditions all the iron present in the medium was taken up by the cells within 30 min (3 nmole/mg cells dry weight). Repeated addition of iron without desferri-ferrichrome (indicated by the arrow in Fig. 1) led to further uptake of iron. This demonstrated that the complexing
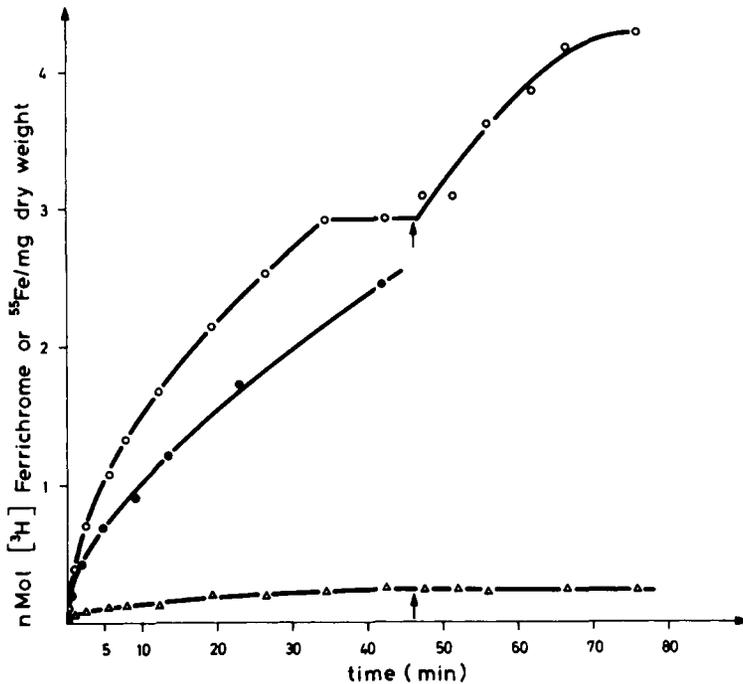

Fig. 1. ^{3}H-Ferrichrome and ferrichrome-dependent iron uptake by E. coli K12 strain AB2847. Cells were pregrown on extracted, iron-deficient CR medium (18), in the presence of 1 mM citrate, to an E_{578} of 1 (corresponding to 0.38 mg dry weight/ml). They were washed twice and resuspended in uptake medium (17) at the same concentration. Transport was started by adding 0.2 μCi/ml^{55}FeCl$_3$ (final iron concentration was 1 μM) in the presence of 1 μM (O) or 0.15 μM desferri-ferrichrome (●). After 46 min, a further 1.5 μM ^{55}FeCl$_3$ was added as indicated by the arrow on the upper curve. Uptake of 1 μM (370 μCi/μmol) ^{3}H-ferrichrome (△) was studied using cells prepared as above; the lower arrow marks the addition of 1.5 μM nonradioactive FeCl$_3$.

agent was not depleted but could be used repeatedly in the uptake of iron. This finding is in contrast to the situation for enterochelin-iron uptake in which enterochelin is transported into the cell and hydrolyzed (21).

To corroborate the above results we measured the amount of $^{55}Fe^{3+}$ ions and tritium-labeled ferrichrome taken up by cells, compared with the amount remaining in the medium. Cells were incubated for various lengths of time with equimolar amounts of iron and desferri-ferrichrome and filtered through a Millipore filter. As the iron was taken up by the cells it disappeared from the filtrate, whereas the tritium-labeled complexing agent was not taken up by the cells and remained in the filtrate (Fig. 2). The iron could not be chased out of the cells by adding 10 times the amount of desferri-ferrichrome (marked by an arrow in Fig. 2). The low amount of tritium label, about 5% of the total added, remaining with the cells could also not be lowered by the chase. It is at present unknown whether this small percentage of ferrichrome is of physiological significance.

Mutants and Revertants in Ferrichrome Uptake

The isolation of revertants of a phage-resistant mutant is difficult since no simple selection method exists. However, it was recently shown that ton A mutants which are resistant to bacteriophages T1, T5, and ϕ80, and to colicin M were additionally unable to take up iron in complex with ferrichrome (17, 19). This provided an efficient selection for revertants of the ton A mutation, since strains which grow well on ferrichrome as the sole iron source (i.e. iron-deficient media in the absence of enterochelin synthesis), should not have the above-mentioned ton A defects.
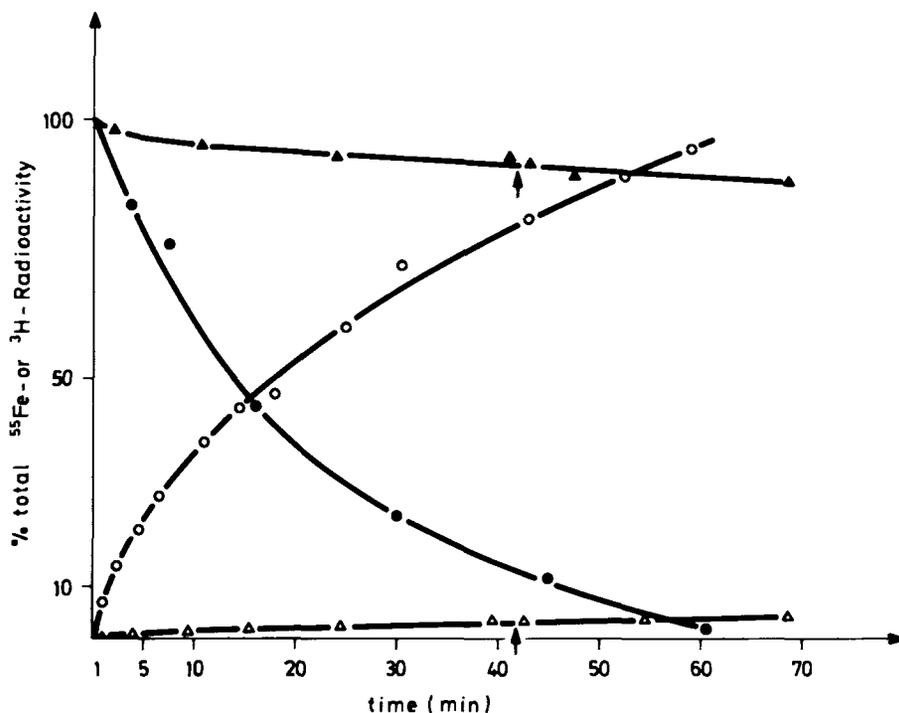

Fig. 2. Level of ^{3}H-ferrichrome and ferrichrome-dependent iron uptake by strain AB2847: ^{3}H-ferrichrome taken up by the cells ($\triangle$) and remaining in the filtrate after millipore filtration ($\blacktriangle$); ^{55}Fe taken up by the cells (O); and ^{55}Fe remaining in the filtrate ($\bullet$). Arrows indicate the addition of 10 μM unlabeled ferrichrome; all other conditions were as for Fig. 1.

Of 50 spontaneous T5-resistant mutants of the E. coli K12 strain AB2847, 28 yielded revertants to T5 sensitivity, both spontaneously or after mutagenesis, when the selection for growth on ferrichrome was used. Forty-six of these revertants were tested for growth in extracted, iron-deficient medium in the presence of added ferrichrome, and with only three exceptions (designated 32f, 45b and 47a) grew in a similar fashion to the wild-type strain AB2847 (Fig. 3). The three exceptions grew at a rate intermediate between those for the wild-type strain and ton A mutants (Fig. 3). These three strains were genuine partial revertants since they exhibited at best only a partial regaining of all ton A^+ properties tested: adsorption and plating efficiency of phage T5 and ϕ80, albomycin and colicin M sensitivity, and ferrichrome transport. In addition, two other revertants had wild-type properties with the exception that they were partially resistant to colicin M. About four times more colicin was required to inhibit these two strains (designated $20b_1$ and 39b) than to inhibit the wild-type or fully reverted strains.

The uptake of iron by the ferrichrome-iron transport system into the various types of revertants was studied. The five partial revertants mentioned above and one other strain (designated 4a) exhibited a low transport capacity. Results are shown in Fig. 4 for the wild-type strain AB2847, a ton A mutant (P16), a "normal" revertant (16k), and one of the revertants mentioned above with a low transport capacity (4a). Since the revertant 4a grew nearly as fast as the wild-type on ferrichrome as the sole iron source (Fig. 3), it was evident that the low level of transport for this mutant was sufficient to satisfy the growth requirements. The plating efficiency of T5 on the revertant 4a and colicin M
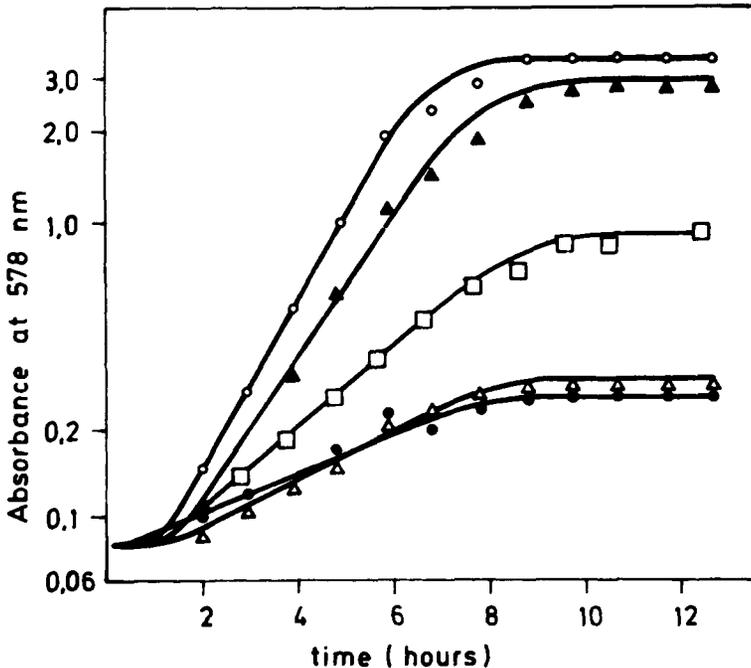

Fig. 3. Growth of revertants of ton A mutant on extracted, iron-deficient M9 minimal medium with 5 μM desferri-ferrichrome providing the only high-affinity iron uptake system. Revertant $20b_1$ (O), growth is as for the wild-type (strain AB2847 ton A^+; not shown); partial revertant 4a (▲) growth is as for the wild-type although the ton A protein is not visible (see Fig. 5, gel f); partial revertant 47a (□), growth is intermediate between that for the wild-type and the ton A mutants P4 (△) and P20 (●).
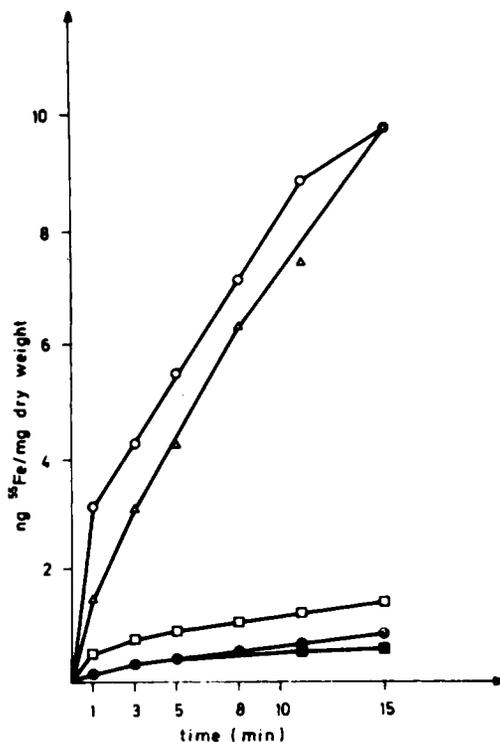
Fig. 4. Ferrichrome-dependent iron uptake in strain AB2847 (△); the ton A mutant P16 (●); strain 16k, a full revertant of P16 ton A (○); and 4a, a partial revertant of the ton A mutant P4 (□). As a control, iron uptake of strain AB2847 in the presence of 100 μM nitrilotriacetate was measured (■). Experimental details were as for Fig. 1 except that only 0.17 μM ^{55}Fe was added.

sensitivity was indistinguishable from the wild-type response. However, the adsorption rate of phage T5 to whole cells or isolated outer membrane of strain 4a was greatly reduced (data not shown).

With the exception of the partial revertants 32f, 45b, and 47a, all revertants tested had identical plating efficiencies for the bacteriophage T5. Plaques could only be observed on these partial revertants with higher than normal input phage number. For example, 360 phages gave rise to no observable plaques while 3.6×10^4 input phages resulted in many minute turbid plaques. Even with a concentration of T5 phages which resulted in confluent lysis (3.6×10^6) of normal sensitive strains, we observed individual minute plaques on these partial revertants. Under conditions where full revertants and the wild-type strain adsorbed 87% of the input phage, adsorption by the partial revertants did not exceed 5%. Interestingly, the plating efficiency of phage ϕ80 vir on strains 32f, 45b, and 47a was nearly as high as on the wild-type strain, e.g. 60–70 clear plaques on the wild-type, 18–30 small turbid plaques on strains 32f, 45b, and 47a.

There is a close correlation between the presence of the ton A protein, a band of MW 78K (K = kilo = times 1,000, e.g. 78,000 daltons molecular weight), and its functions. Of 19 spontaneous ton A mutants of E. coli K12 AB2847 studied, only one had not lost this protein (Fig. 5, gels a–c, n). In all full revertants, the ton A protein was present at wild-type levels (Fig. 5, gels d and e). In the partial revertants 32f, 45b, and 4a, which as
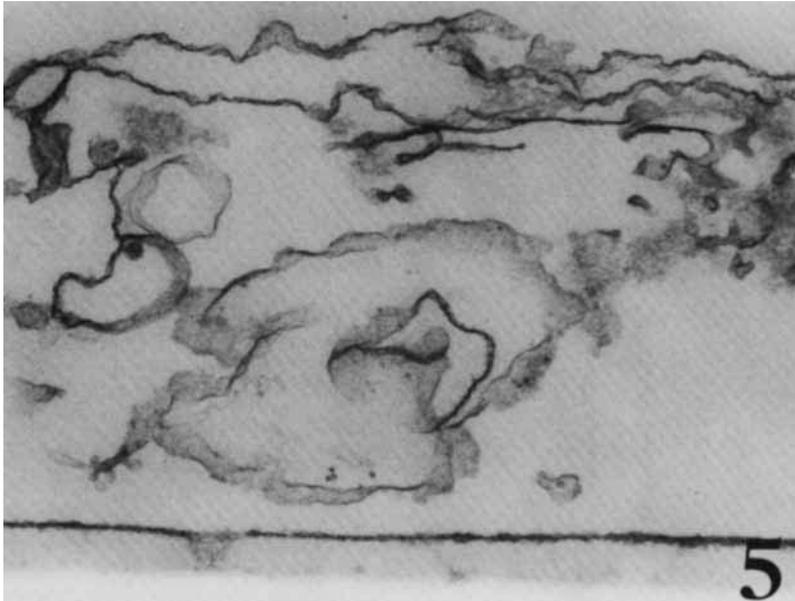
Fig. 5. SDS polyacrylamide gel electrophoresis of the outer membrane proteins of derivatives of strain AB2847. Gels a, b, and c are the ton A mutants P20, P16, and P4, respectively (a band of 78K, the ton A protein, is missing); gel d, revertant 20b$_1$ (a band of approximately 96K is reduced and a new band has appeared at 55K: the ton A protein and all other proteins are present in normal amounts in the original gel pattern although this revertant is still colicin M resistant); gel e, the full revertant 16k which is indistinguishable from the wild-type strain AB2847; gels g and h, the ton A mutant P47 and its derivative, the partial revertant 47a, respectively. Gels i—o are albomycin resistant mutants: gel i, strain K1 1 (group 5) showing a typical ton B-like pattern; gel k, K1 4 (group 3) an exceptional mutant of this group showing a ton B-like pattern; gels l and m, K1 8 and K1 15, respectively (both group 3), showing wild-type patterns; gel n, K1 21 (group 1) a typical ton A mutant; gel o, K1 9 (group 4) showing a wild-type pattern. The running position of the protein standards used for molecular weight determination is indicated by arrows: 1, bovine serum albumin (67 K); 2, ovalbumin (45 K); 3, chymotrypsinogen A (25 K); 4, horse myoglobin (17 K).

discussed above adsorb phage T5 very poorly, no ton A protein was observed (shown in Fig. 5. for strains 47a, in gel h and for 4a in gel f). However, because phage T5 can produce plaques on all of these revertants, it is likely that there is a small level of ton A protein in the outer membranes of these strains, undetectable due to the limited amount of protein which one can apply to the gel. An additional possibility is the presence in these strains of an altered receptor protein with a different electrophoretic mobility, although we have as yet found no evidence to support this hypothesis. In fact we could not distinguish between the protein pattern of the ton A mutant P47 (Fig. 5, gel g), and its partial revertant 47a (Fig. 5, gel h). We are at present fractionating the outer membrane proteins of these partial revertants in an attempt to find a phage-binding protein and thus to distinguish between the above two possibilities.

Albomycin Resistant Mutants

The antibiotic albomycin is a structural analogue of ferrichrome (34, 36). There-
fore, the isolation of albomycin resistant mutants might yield mutants defective in the
ferrichrome-mediated uptake of iron which are different from the ton A and ton B
mutants described previously (17). It was noted in our previous study that many of the
albomycin resistant mutants did not fit into the ton A / ton B groups (17). We hoped that
these might include transport mutants defective in the energy-dependent translocation
step through the cytoplasmic membrane and mutants unable to mobilize the iron from
the complex.

The spontaneous albomycin resistant mutants of E. coli K12 strain AB2847 which we
obtained are listed in Table II. The 74 mutants were shown to fit into 5 categories. The 15
mutants of group 1 were typical ton A mutants, resistant to phages T1, T5, and ϕ80 and
to colicin M. The outer membranes of 7 of them were analyzed by polyacrylamide gel
electrophoresis. With one exception, they were lacking the ton A protein (Fig. 5, gel n).
This exception (designated strain K1 63) had a wild-type protein pattern (compare, for
example, with Fig. 5, gels e, l, and m), and it was interesting that the strain was able to
grow weakly on ferrichrome as the sole iron source. Thus, this strain might have an altera-
tion to, rather than a deletion of its ton A protein, so that it cannot bind phages or
colicins and can only bind ferrichrome weakly. Group 2 mutants were typical ton B
strain showing all of the properties of these mutants, as summarized later in this paper.
Analysis of the outer membrane proteins of these strains revealed a typical ton B-like
pattern (Fig. 5, gel k). The mutants of group 3 grew well on ferrichrome, showed normal
sensitivity against phages T5 and ϕ80 and colicin M, and in most cases had normal levels
of ton A protein (Fig. 5, gels k, l, and m). Thus, it is possible that these are target-site
mutants. The target site of albomycin is presently thought to be protein synthesis, with

TABLE II. Albomycin Resistant Mutants

Group	No. of mutants isolated	Sensitivity to T5	ϕ80	colicin M	Growth on ferrichrome	Iron transport	Genetic locus	ton A protein
1	15	–	–	–	–	–(2)	ton A	–(5) +(1)
2	4	+	–	–	±	–(2)	ton B	+(3)
3	21	+	+	+	+	n.d.	near to dapD (4)	+(11) –(1)
4	22	+	+	+	–	–(6)	near to dapD (3)	+(13)
5	13	+	+	–	+	–(4) +(1)	near to dapD (1)	–(1) +(8)

The figures in parentheses indicate the number of mutants studied in each case. + denotes wild-type
response, i.e. phages T5 and ϕ80 and colicin M sensitivity, growth on ferrichrome as the sole iron
source, normal transport of iron by all 3 systems mentioned in the text and presence of the ton A
protein (phage T5 receptor) in the outer membrane; ± denotes intermediate level of growth on
ferrichrome as the sole iron source: – denotes an alteration to the wild-type response; n.d.,
not determined.

the albomycin causing inhibition due to the uracil derivative bound to the ferrichrome-like portion of the molecule. However, it has been shown that the inhibitory concentrations are at least 10 times higher in vitro (10 μM) than in vivo (D. Dengler and H. Wolf, private communication). Furthermore, we were able to demonstrate only very poor transport of albomycin complexed to $^{55}Fe^{3+}$ into albomycin-sensitive cells. Thus further studies on the transport and mode of action of albomycin must be undertaken before we can properly characterize the group 3 mutants.

The group 4 mutants differed from those of group 3 by their inability to transport and grown on ferrichrome (Table II). Since these mutants are sensitive to phages and colicins, it is possible that the defects reside at the translocation step in the cytoplasmic membrane, where the mobilization of iron from the ferrichrome-iron complex may occur. If so this system must be highly specific for the ferrichrome-mediated iron uptake system, since the mutants transported iron in complex with enterochelin or citrate as efficiently as the wild-type. Preliminary mapping studies of the mutations in 3 group 4 mutants indicated that they were cotransducible with the dap D locus which lies close to the ton A gene. However, in addition to the differences in phage and colicin sensitivity of the 2 groups of mutants, the outer membrane of all 13 group 4 mutants studied contained wild-type levels of the ton A protein (Fig. 5, gel o, compare with the ton A mutants Fig. 5, gel n). Thus it is unlikely that the group 4 mutants map in the ton A gene, but there is a possibility that the various genes involved in ferrichrome-mediated iron supply are clustered and may form on operon. We are unable as yet to interpret the significance of the finding that the 4 group 3 mutants studied also carried defects which were contransducible with dap D.

Group 5 of the albomycin resistant mutants were originally characterized by their resistance to colicin M and sensitivity to phages T5 and ϕ80. On further examination, 4 of them were found to be sensitive to high concentrations of colicin M. Most of the mutants studied showed almost no uptake of iron in complex with either ferrichrome or citrate, while the enterochelin-mediated iron uptake was reduced but not as much as in ton B mutants in the presence of DHB (but see later Fig. 11). However, the mutants generally showed unimpaired growth on ferrichrome. The outer membrane proteins of 9 mutants were studied and 6 demonstrated a ton B-like pattern (Fig. 5, gel i). It is interesting that one of these mutants (K1 80) with a ton B-like protein pattern, mapped at a locus contransducible with dap D, i.e. near the ton A region. Again nearly all the mutants contained in their outer membranes the ton A protein. We were able to find in this group of mutants, individual strains which had a single property differing from those general properties described above. However, with the exception of the result for colicin M, only one strain differed from the rest in any given property, and no strain had more than a single property differing from the general properties of the group. Thus we define group 5 mutants as a phenotypic group. Characterization of these interesting mutants is proceeding.

Outer Membrane Functions Involved in Iron Uptake

Recent work has added much to our understanding of the outer membrane functions involved in iron transport. This work is summarized below together with additional, previously unpublished data from our laboratory.

The wild-type strain E. coli K12 AB2847 (aro B) is able to transport iron in complex with ferrichrome, enterochelin, or citrate (Fig. 6) or by a low-affinity transport system which can be suppressed by 100 μM nitrilotriacetate (NTA). Transport of iron with enterochelin in this strain will occur when enterochelin is either added to the medium or

WT

ng ^{55}Fe / mg dry weight

8

6

4
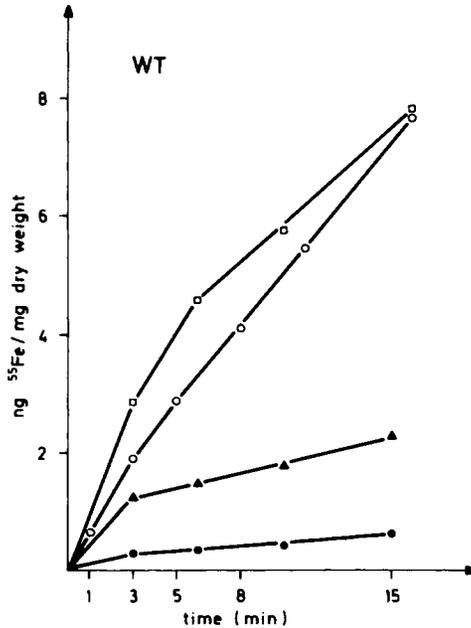
2

1 3 5 8   15

time (min)

Fig. 6. Iron uptake into strain AB2847 mediated by ferrichrome (○), citrate (▲), or enterochelin (□). The low-affinity uptake system was suppressed by the addition of 100 μM nitrolotriacetate (●). Ferrichrome-mediated uptake was studied after the addition of 0.20 μM desferrichrome, citrate-mediated uptake after addition of 20 μM sodium citrate, and enterochelin-mediated uptake by the preincubation of cells for 15 min with 10 μM DHB before uptake was started by the addition of ^{55}FeCl$_3$. Otherwise experimental procedure was as described for Fig. 1, except that only 0.17μM ^{55}FeCl$_3$ was used.

synthesized by the cells from added 2,3-dihydroxybenzoate (DHB). The citrate-iron uptake system, which is induced by the growth of cells in medium containing citrate, can be as much as two-fold more efficient than shown in Fig. 6 (see for example Ref. 18). In ton A mutants (Fig. 7) only the ferrichrome uptake system is impaired, while ton B mutants are defective in all 3 high-affinity systems (Fig. 8). Another genetic locus named "feu" (for ferric enterochelin uptake) (18) also has a function important in the uptake of iron complexed to enterochelin and is genetically distinguishable from the above two loci, being located at 65 min (18) on the Taylor and Trotter E. coli K12 genetic map (37). The ton B and feu mutants are additionally resistant to colicins B, I and V. It has been shown previously that enterochelin protects sensitive cells against colicins B, I (38) and V (18) by competition for a membrane-binding site (18). In feu mutants only enterochelin-iron uptake was substantially reduced, while ferrichrome- and citrate-iron transport were equivalent to the wild-type levels (Fig. 9). The feu function could be considered most simply to be a receptor protein for enterochelin and for at least some of the above-mentioned colicins, analogous to the ton A protein which is the receptor for the phages T5, T1, φ80; colicin M; ferrichrome; and albomycin. However, Cardelli and Konisky (39) isolated colicin I resistant mutants which mapped at approximately 41 min and which had lost the ability to adsorb colicin I but not colicin B (39) [they appear also to be tolerant to colicin V (40)]. They suggested that the so-called cir locus is a structural gene for the colicin I receptor (39). However, these adsorption studies do not necessarily mean that the cir function is the only function required for colicin I adsorption, as it has been shown
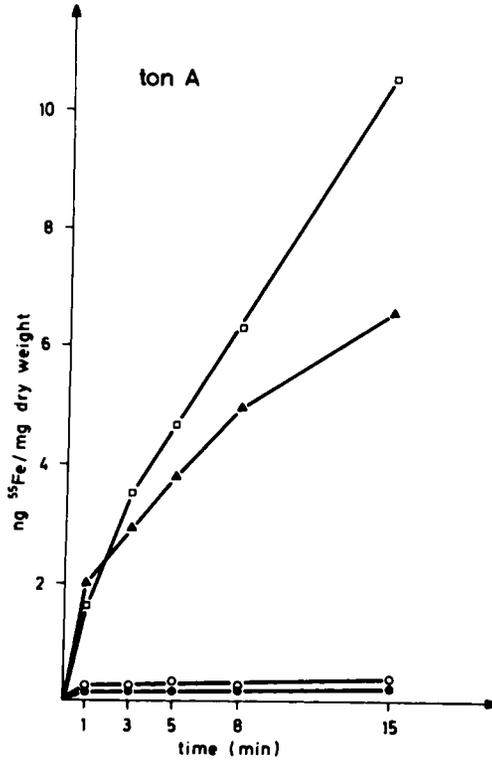
Fig. 7. Iron uptake into the ton A mutant P4. Symbols and conditions as in Fig. 6 except that 100 μM citrate was used.
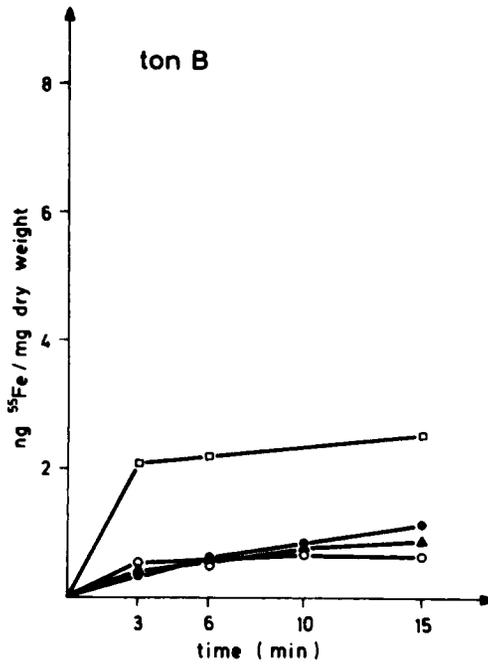

Fig. 8. Iron uptake into the ton B mutant IR122, symbols and conditions as in Fig. 6.
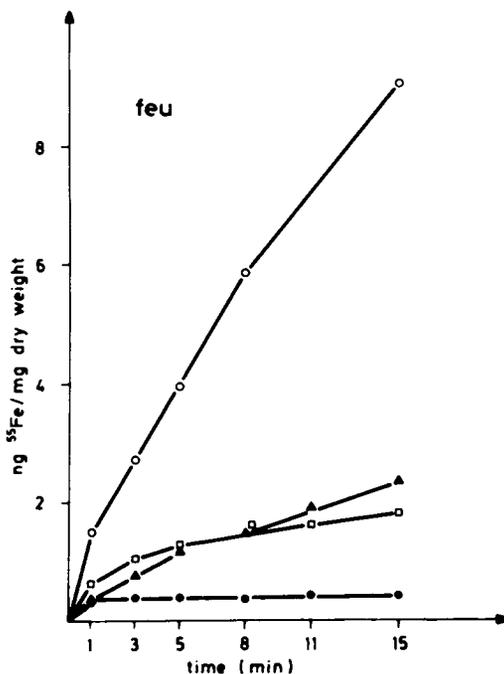
Fig. 9. Iron uptake into the feu mutant VR35, symbols and conditions as in Fig. 6.

that the functions of two genes are required for irreversible adsorption of bacteriophages T1 and ϕ80 (see below). Further studies are needed to unravel the relationship between the feu and cir functions.

The functional relationships between the gene products of ton A, ton B, and feu gene regions are summarized in Fig. 10. The sequence of reactions and the localization of the functional units have not in all cases been proven, and this should only be considered as a working hypothesis.

The Ton B Function: The Common Element of Iron Uptake

The ton B function is known to be necessary for the irreversible adsorption of phage T1 to E. coli cells (41). Ton B mutants bind the phage reversibly without becoming infected. We found that the bacteriophages T1 and ϕ80 also bind reversibly to the isolated ton A protein (26). It was shown by Garen and Puck (41) and Christensen and Tolmach (42) that phage T1 requires cellular energy for the irreversible step of adsorption. Our studies have shown that irreversible adsorption could be energized both through the electron-transport chain and from ATP via the Ca^{++}, Mg^{++} ATPase, indicating the involvement of the energized membrane state, which has been shown to exist in the cytoplasmic membrane. However, the relationship between the requirement for the ton B function and the requirement for the energized membrane state of the cytoplasmic membrane for irreversible adsorption remains obscure. The ton B product has not been identified biochemically so that its location in the inner or outer membrane is also unknown. Attempts to find a protein corresponding to the ton B gene product by the comparison of wild-type strains with ton B mutants were unsuccessful, although this does not rule out the existence of one. However, indirect evidence suggests that the ton B function may be located in the outer membrane.
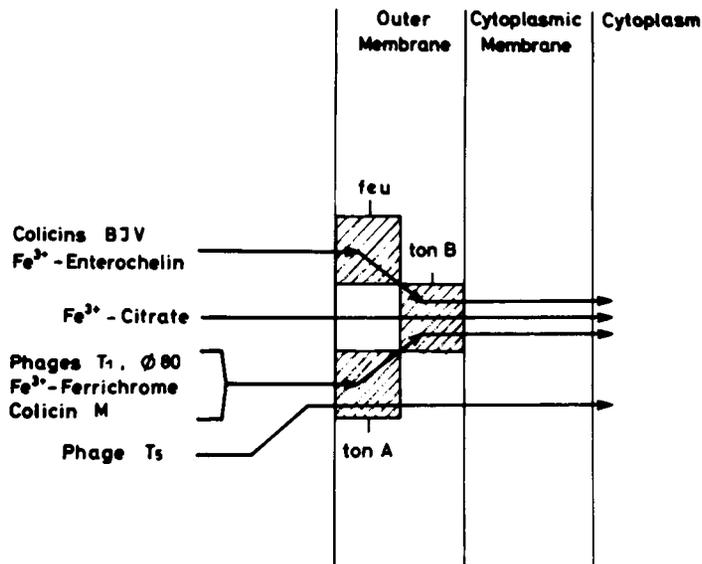
Fig. 10. Schematic presentation of the requirements of the different agents for getting access to the cell.

Frost and Rosenberg (24) noted that the requirement for the ton B function for growth on extracted, iron-deficient medium could be overcome by added 2,3-dihydroxy-benzoate (DHB) or shikimate but not by added enterochelin. There was a further requirement for enterochelin synthesis from these substrates since ent F mutants, which were blocked in enterochelin synthesis from DHB or shikimate, were unable to overcome the requirement for the ton B product for growth on iron-deficient medium. The authors presented a model suggesting that DHB could complex iron and transport it some distance into the cell envelope, thus bypassing the ton B function. Thereafter, the iron was transferred to enterochelin and taken up into the cells using the enterochelin-iron uptake system. If the cells were given too much DHB then they overproduced enterochelin which was released and complexed much of the iron in the medium. Thus ton B mutants which cannot take up enterochelin-iron complexes would suffer from an iron shortage in the presence of excess DHB.

We measured transport of $^{55}Fe^{3+}$ into E. coli K12 IR112 (ton B aro B) in the presence of DHB added 15 min prior to uptake experiments. As can be seen in Fig. 11 the presence of DHB under these conditions leads to iron uptake in the presence of NTA, while excess DHB (200 μM) suppresses this uptake to some extent. Thus, these uptake studies support the conclusions of Frost and Rosenberg based on growth curves.

Outer Membrane Protein Alterations Related to Cellular Iron Supply

The protein compositions of wild-type strains were compared with ton B mutants in an attempt to identify a ton B protein. The outer membrane proteins of the ^{14}C-leucine-labeled E. coli K12 strain AB2847 ton B[+] and the ^{3}H-leucine-labeled ton B mutant BR158 were mixed and solubilized with Triton X-100 + EDTA as described by Schnaitman (31). This extract was run on a DE52 DEAE-cellulose column (Fig. 12). An increase in the amount of protein derived from the ton B mutant occurred in fractions 44– 46, corresponding to 4% of the total protein solubilized from this strain.
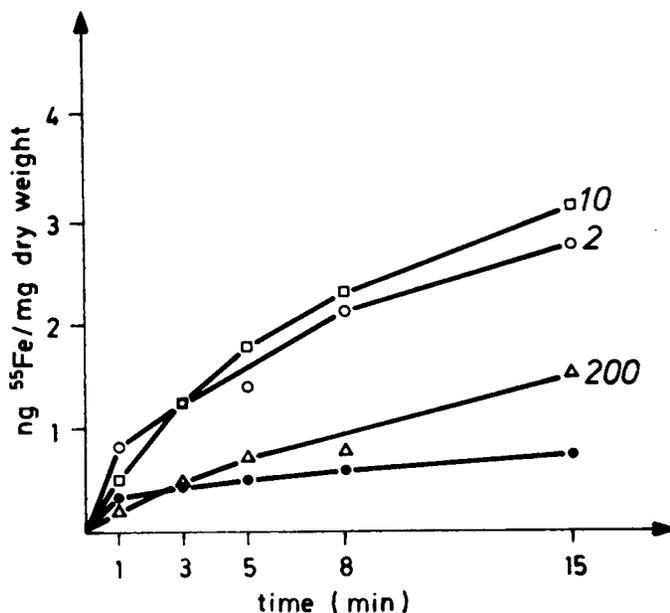
Fig. 11. Iron uptake into strain IR112 (ton B mutant) preincubated for 15 min with 2 (O), (□), and 200 μM (△) DHB and as a control uptake in the presence of 100 μM nitrilotriacetate only (●).

Fractions were pooled into seven samples, as described in Fig. 12, and run on poly-acrylamide gels. As can be seen in the inset to Fig. 12, there was at least partial separation of all major bands, and samples 3 and 5 were almost pure preparations of proteins d (also known as B, 3a or II*) and b (also called the matrix protein, protein A_1, or I), respectively [for the protein designation see (43)]. Although several reports on the purification of these proteins have been made, this is the mildest since it does not utilize SDS. A similar experiment suggested that protein d was, after the above treatments, still able to adsorb phage K3 (Hancock and Reeves, unpublished results).

By comparing sample 4 (which has an excess of proteins derived from the ton B mutant) with the neighboring samples 3 and 5, it can be seen that 2 sets of proteins are present only in sample 4; 5 proteins of MW 70K-90K and 3 proteins of 50K-60K. This is evidence that one or more of these proteins is increased in quantity in ton B mutants. It was further shown that both the colicin I and phage T5 binding activities were uniquely present in sample 4. Davies and Reeves (40) previously noted the presence of two additional proteins of undefined but high molecular weight in their ton B and exb B mutants.

The production of excess proteins in ton B mutants grown on various well-defined and rich media was investigated. Changes in the amounts of some proteins were not always reproducibly observed; however, three proteins of MW 74K, 81K, and 83K were found to be consistently increased in the outer membranes of ton B mutants grown under all growth conditions. They were most strongly produced when the ton B mutant BR158 was grown in M9 minimal medium. The presence of casamino acids, or succinate instead of glucose, made little difference to the level of these proteins. Strain AB2847 ton B$^+$, grown under the same conditions, always produced a low level of proteins with similar mobilities to the above proteins. The level of these proteins was at least 5–10-fold lower than in the ton B mutant, and in fact was often only visible for ton B$^+$ strains when the gels were quite
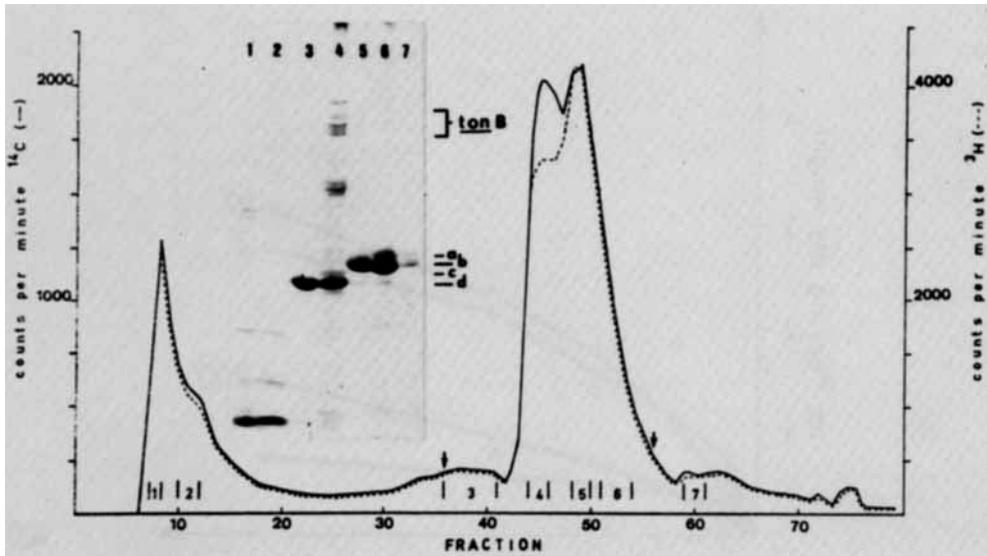
Fig. 12. Separation by DE 52 cellulose chromatography of a mixture of Triton X-100 + EDTA soluble, outer membrane proteins from strain AB2847 (^{3}H-labeled, dashed line) and its ton B mutant BR158 (^{14}C-labeled, solid line). The column was eluted as described in Materials and Methods. The starting points of the salt gradients are indicated by arrows. Five ml fractions were collected and pooled into 7 samples as indicated (e.g. sample 1 was fractions 7 + 8, etc.). These samples were run on SDS polyacrylamide gels. Results are shown in the inset. The positions of the major proteins a, b, c, and d are indicated to the right of the gels, as are the proteins found subsequently to be always overproduced in ton B mutants.

heavily loaded with protein. This suggested that the high level of the 74K, 81K, and 83K proteins in ton B mutants might result from overproduction rather than synthesis of new protein.

The ton B mutant strain BR158 also overproduced these proteins when grown on rich media, although the magnitude of the increase was generally lower than observed after growth on defined media. Growth on tryptone or nutrient broth [the latter being the media used by Davies and Reeves (40)] resulted in an increase in the three proteins; however, when ton B mutants were grown on tryptone-yeast medium, the increase in the 74K, 81K, and 83K proteins was at best minimal.

Outer membrane proteins of a number of strains with deficiencies in iron uptake were isolated by two different methods; those of Schnaitman (31) and Osborn et al. (30). While a larger number of proteins was noted using the techniques of Schnaitman, no additional proteins were consistently altered, and the 74K, 81K, and 83K proteins were always seen to be increased in ton B mutants. Strain BR128 of similar phenotype to the exb B mutants of Davies and Reeves (40) and differing only from the ton B mutant BR158 by its sensitivity to phage T1 (18) also had an increased amount of the three proteins in its outer membrane. However, neither the feu mutants VR42 or IR20 (18) nor the ton A mutant P16 showed an increase in these proteins. Furthermore, from these experiments we concluded that the ton A protein is not one of the above-mentioned proteins. No Triton X-100 soluble "cytoplasmic membrane" proteins were shown to be altered in the ton B strain or, in fact, in any of the above strains.

Since ton B mutant cells are defective in the high-affinity, iron-uptake systems (16–21) they probably have lower than normal internal iron pools when grown in media with low amounts of iron. Furthermore, the ton B mutant BR158 produced the 74K, 81K, 83K proteins most strongly in defined media and hardly at all in the iron-rich, tryptone-yeast medium. Thus, it was decided to investigate the effect of varying the iron levels of the growth medium on the production of these proteins in the outer membranes of both ton B mutants and wild-type strains. Results are presented in Fig. 13. Strain AB2847 ton B^+ aro B grown in extracted, iron-deficient medium supplemented with required amino acids and glucose and, in addition, DHB, citrate, or a combination of the two overproduced the 74K, 81K, and 83K proteins (Fig. 13, gel a). The mutant strain BR158 ton B aro B grown on the same media supplemented with 5 μM $FeCl_3$ produced approximately the same levels of the three proteins (Fig. 13, gels b, d), as indeed it does in unextracted M9 minimal media. However, the appearance of all three of these proteins was suppressed completely by 20 μM $FeSO_4$ in the wild-type strain (Fig. 13, gels c, e, and f) and by 50
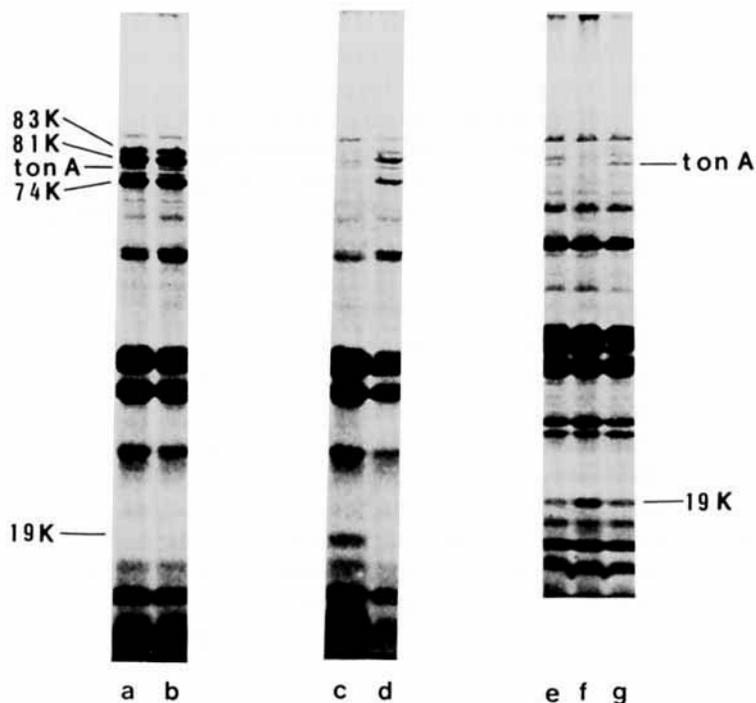

Fig. 13. SDS polyacrylamide gel electrophoresis of the outer membrane proteins of strain AB2847 ton B^+ and its ton B mutant BR158, grown in extracted, iron-deficient M9 medium in the presence or absence of added iron. Gels a, c, e, and f are outer membrane proteins of strain AB2847 grown without added iron (a), or with 20 μM (e), or 50 μM $FeSO_4$ (c, f) as a supplement. Gels b, d, and g are outer membrane proteins of the ton B mutant BR158 grown with 5 μM $FeCl_3$ (b, d) or 50 μM $FeSO_4$ (g) as supplements. In order to calculate the molecular weight of the various bands indicated, a series of standard proteins were run at the same time (see Fig. 5). Gels e, f, and g are slightly shorter, but the ton A protein has been aligned for all gels. The position of a 19K band which appears only when cells are grown in iron-rich medium (20 or 50 μM $FeSO_4$: gels c, e, f, and g) has been indicated for gels a–d (on the left) and for gels e–g (right). All gels were loaded with 105 μg of protein with the exception of gel d (50 μg).

μM FeSO$_4$ in the ton B mutant (Fig. 13, gel g). The actual level of iron leading to sup-
pression of the appearance of these proteins has not as yet been quantitated, since it seems
to depend on a number of factors. In addition we noted in these experiments that a band
of MW 19K appeared only in those strains grown in medium with enough iron to suppress
the appearance of the 74K, 81K, and 83K proteins (Fig. 13, gels c, e, f, and g). The signi-
ficance of this observation is as yet unknown.

DISCUSSION

The results presented in this paper demonstrate the complex interaction of various
outer membrane functions by means of which iron complexes are transported from the
medium into the cell. The passage through the outer membrane of ferric iron in complex
with ferrichrome, citrate, and enterochelin is only possible when the ton A, and ton B,
and feu functions are present (see scheme Fig. 10). These functions are also required by
other substrates to traverse the membrane. In addition, specific requirements have been
demonstrated. For example, the feu function is involved in enterochelin uptake and killing
of cells by colicins B, I, V, but the fep function is highly specific for enterochelin transport
(21). Similarly the ton A protein (ton A function) is the receptor for the phages T5, T1,
and ϕ80, colicin M, and ferrichrome, while the group 4 albomycin resistant mutants
(Table II), in analogy with fep mutants, define a function which is required only in
ferrichrome-mediated iron uptake. The ton A protein has been located in the outer mem-
brane (23). By analogy, we consider the feu function to be a protein located somewhere
between the outer surface of the cytoplasmic membrane and the cell surface.

The components of iron transport may be constituents of specific pores through
the outer membrane, or they could act like the components of multienzyme complexes
in which the substrates move from one subunit to another. The latter model would imply
that the outer membrane proteins physically interact with the cytoplasmic membrane
permeases. There is presently no evidence for a direct physical linkage. Although, as is
clear from the data presented in this paper, the different iron transport systems have a
number of unique requirements, they also have common requirements for the ton B$^+$
function and for cellular energy [(21) and unpublished results]. During ferrichrome-
mediated iron transport, tritium-labeled ferrichrome does not become associated with the
cell and can be used repeatedly to translocate ferric iron from the medium into the
cytoplasm (Figs. 1 and 2). It is unknown at which stage the iron is mobilized from the
complex, but this could well occur at the cytoplasmic membrane or in the periplasmic
space. Enterochelin is, in contrast, taken up by the cell (21). However, it is not certain
where the hydrolysis of the complex and reduction of the iron to the ferrous form occurs.
The respective enzymes have been found in the soluble protein of a cell homogenate (21),
but they could be of periplasmic origin.

An interesting problem arises when one considers the specificity of the outer mem-
brane functions and specifically the ton A function. Extremely varied substrates interact
with the ton A protein. Ferrichrome, the tails of the phages T5, T1, and ϕ80, and colicin
M have, as far as we know, nothing in common structurally. Thus, concepts deriving from
the study of enzyme-substrate interactions are hardly applicable, since these interactions
are of narrow specificity. It seems more appropriate to think along the lines of antigen-
antibody reactions which feature overlapping binding regions and the structural flexibility
of antibody molecules. In this context, it would be interesting to know if the various sub-

strates described above bind to the same or different regions of the ton A polypeptide chain. We have to date identified only one ton A mutant (K1 63) in which a 78K protein is present, and this mutant has retained none of the ton A functions. All other ton A mutants lack the ton A protein. This is not caused by the ton A region being a deletion "hot spot" similar to the ton B region (16), since from many of these mutants we were able to select revertants which had regained the ton A protein and associated properties. Therefore, either there exists an unusually high incidence of spontaneous mutations leading to termination codons, or a receptor protein which is structurally altered by amino acid exchanges cannot be integrated into the outer membrane.

Cotransfer of the albomycin resistant mutants of groups 3–5 (Table II) with the dap D locus by P1 transduction shows that additional ferrichrome-mediated iron transport functions map near the ton A gene. Most of the so-called sid mutants of Salmonella typhimurium map in an equivalent region of the genetic map of this organism (44). Thus genes for ferrichrome-mediated transport are apparently clustered in both organisms.

In this paper we have shown that one can induce the appearance of three proteins (the 74K, 81K, and 83K proteins) in the wild-type strain by lowering the iron content of the growth medium. Ton B mutants [(40) and this paper] overproduce these proteins in normal medium. However, the fact that these proteins are suppressible in both ton B^+ strains and ton B mutants by the addition of sufficient iron to the growth medium (Fig.13), suggests that these proteins are produced as a response to low levels of intracellular iron. Thus their production is only linked to the ton B gene in the sense that mutants in this gene lack high-affinity iron uptake systems (see Fig. 8) and thus in most growth media have low intracellular iron levels.

It has been shown by Wang and Newton (16) and also observed by us (unpublished results) that the presence of high amounts of iron in the growth medium suppresses high-affinity iron uptake. Thus under conditions of low iron in the growth medium there is a high level of active iron uptake and also a high level of 74K, 81K, and 83K proteins appearing in the outer membrane;conversely, high iron in the growth medium suppresses both high-affinity iron uptake and the appearance of the three proteins. This is highly suggestive that one or more of these proteins has a role in iron transport. Since there is a large increase in the level of colicin I and B receptors in ton B mutants (18), it is extremely likely that one of these proteins is the colicin I receptor and another the colicin B receptor (since these are distinct, see Ref. 39). Further evidence that the colicin I receptor is one of these proteins was proved by the fact that after partial purification of the proteins (Fig. 12) the colicin I binding activity was found in the same column fraction.

Although our knowledge of receptor-dependent processes in bacteria has advanced considerably, there are still many unanswered questions. Studies of iron transport and its interaction with other receptor-dependent substrates have shown it to be a highly complex process. For the understanding of outer membrane functions and of membrane surface interactions in general it is worthwhile to study the ton system further.

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