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Iron Transport in *Escherichia coli* K-12

2,3-Dihydroxybenzoate-Promoted Iron Uptake

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Abstract. The study of iron uptake promoted by 2,3-dihydroxybenzoate (DHB) into *Escherichia coli* K-12 *aroB* mutants allowed some dissection of outer and cytoplasmic membrane functions. These strains are unable to produce the iron-transporting chelate enterochelin, unless fed with a precursor such as DHB. When added to the medium, enterochelin and its natural breakdown products, the linear dimer and trimer of 2,3-dihydroxybenzoylserine (DBS), efficiently transported iron via the *feuB*, *tonB* and *fep* gene products. Thus mutants in these genes were defective in transport of the above chelates. However, *feuB* and *tonB* mutants were able to take up iron when DHB was added to the medium. Thus DHB-promoted iron uptake bypassed two functions required for the transport of ferric-enterochelin from the medium. One of these functions, *feuB*, has been shown to be an outer membrane protein. In contrast to three other iron transport systems including ferric-enterochelin uptake, DHB-promoted iron uptake was little affected by the uncoupler 2,4-dinitrophenol. Dissipation of the energized state of the cytoplasmic membrane apparently only affects those iron transport systems which require an outer membrane protein. Since DHB-promoted iron uptake bypasses the *feuB* outer membrane protein and the *tonB* function, it is concluded that, in ferric-enterochelin transport, the *tonB* gene may function in coupling the energized state of the cytoplasmic membrane to the protein-dependent outer membrane permeability. DHB-promoted iron uptake required the synthesis and enzymatic breakdown of enterochelin as judged by the effects of the *entF* and *fesB* mutations. A *fep* mutant was not only deficient in the transport of the ferric chelates of enterochelin and its breakdown products, but was also deficient in DHB-promoted iron uptake. A scheme is presented in which iron diffuses as DHB-complex through the outer membrane, and is subsequently captured by enterochelin or DBS dimer or trimer and translocated across the cytoplasmic membrane.

Key words: Iron transport – 2,3-dihydroxybenzoate – *Escherichia coli* K-12 – Enterochelin – *tonB* gene.

Molecules taken up by gram-negative cells have to pass through both the outer and cytoplasmic membranes. The outer membrane forms a permeability barrier for compounds with molecular weights greater than 500–600 (Decad and Nikaido, 1976). Outer membrane proteins have been shown to be involved in the uptake of some substrates above the critical size limit. For example, three specific high affinity iron transport systems have been demonstrated in *Escherichia coli* K-12. They utilize as chelators of ferric iron, enterochelin (a cyclic trimer of DBS) (O'Brien and Gibson, 1970; Rosenberg and Young, 1974), deferriferrichrome (a siderochrome produced by certain fungi (Rogers and Neilands, 1974)) (Braun et al., 1976) or citrate (Frost and Rosenberg, 1973). From competition experiments and studies with mutants, it is known that ferrichrome shares a receptor with phages T1, ϕ80 and T5 and colicin M (see Braun et al., 1976 for review), while similar experiments indicate that colicin B and ferric-enterochelin also share a receptor (Guterman, 1973; Hancock et al., 1976; Pugsley and Reeves, 1976a; Wayne et al., 1976). These receptor proteins have been identified on polyacrylamide gels as the $tonA^+$ and $feuB^+$ gene products respectively, and both are outer membrane proteins (Braun et al., 1976; Hancock et al., 1976). We have also previously suggested (Hancock et al., 1976) that the "cit" pro-

List of Abbreviations. DHB = 2,3-dihydroxybenzoate; DBS = 2,3-dihydroxybenzoylserine; NTA = nitrilotriacetate; DNP = 2,4-dinitrophenol

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tein, an outer membrane protein specifically induced in cells grown in iron-deficient medium with 1 mM citrate present, is the receptor for the inducible citrate-dependent iron uptake system. Thus the initial binding receptors for the various transport systems have, to some extent, been characterized. However, although many other mutants in these transport systems have been isolated (e.g. Braun et al., 1976; Cox et al., 1970; Frost and Rosenberg, 1973; Hantke and Braun, 1975b; Langman et al., 1972; Pugsley and Reeves, 1976b; Wang and Newton, 1969) the post receptor transport mechanisms are not well understood.

We initially reported that both *feuA* and *feuB* mutants were partially deficient in ferric enterochelin transport (Hantke and Braun, 1975b). It was later discovered that *feuA* mutants lacked the colicin I receptor protein (Hancock and Braun, 1976a), while *feuB* mutants were missing the colicin B receptor protein (Hancock et al., 1976). The levels of these outer membrane proteins were controlled by intracellular iron levels (Braun et al., 1976), as were the levels of other proteins important in ferric-enterochelin transport (Rosenberg and Young, 1974). Ferric-enterochelin protected cells against colicin B (Guterman, 1973; Pugsley and Reeves, 1976b; Wayne et al., 1976) by competition for a common receptor. It also exhibited a protective effect against colicin I, but the mechanism appears to be different (Guterman, 1973; Pugsley and Reeves, 1976b; Wayne et al., 1976). In this paper we confirm that *feuB* mutants are totally deficient in ferric-enterochelin uptake, and extend this observation to the ferric-chelates of the linear dimer and trimer of DBS, two of the natural breakdown products of enterochelin. In contrast, *feuA* mutants were shown to be totally proficient in ferric-enterochelin uptake (Pugsley and Reeves, 1977a; see also this paper), which demonstrates that the colicin I receptor is not required for this process as previously suggested (Hancock and Braun, 1976a). Furthermore, new mapping data makes it probable that the *feuA* gene is identical to the *cir* gene (Cardelli and Konisky, 1974) and we propose to use the latter designation in future.

The different iron-chelator complexes have different chemical structures as demonstrated by their requirements for different recognition proteins (outer membrane receptors); however, surprisingly, a single gene mutation, *tonB*, is capable of blocking uptake of all three iron-chelator complexes (Hantke and Braun, 1975b). The *tonB* gene also has a function in the irreversible adsorption and DNA injection of phages T1 and ϕ80 (Hancock and Braun, 1976b), and the killing of cells by colicins B, I, V and M and by the antibiotic albomycin (Davies and Reeves, 1975; Hantke and Braun, 1975a; Pugsley and Reeves, 1976b; Wayne and Neilands, 1975), in addition to a recently described function in vitamin B_{12} uptake (Bassford et al., 1976). Wang and Newton (1969) showed that *tonB* mutants required the addition of 5 µM iron to iron-deficient medium for good growth. Frost and Rosenberg (1975) subsequently showed that DHB (a precursor of enterochelin) was able to considerably enhance the growth of *tonB* mutants in iron-deficient medium, despite the fact that these mutants were unable to transport enterochelin or use it as a growth factor. We reported preliminary experiments showing that DHB itself was able to promote the uptake of iron in *tonB* mutants (Braun et al., 1976). In this paper, DHB-promoted iron uptake is further characterized.

MATERIALS AND METHODS

Bacterial Strains and Media. The bacterial strains used were mutants of *Escherichia coli* K-12 strains AB2847 and AN92: a description of their genotypes and relevant properties is given in Table 1. The media used and the method of iron extraction (with 8-hydroxychinoline) were as previously described (Braun et al., 1976).

Chemicals. DHB (99% pure form from EGA Chemie KG, Steinheim/Albuch, West Germany), DNP (Serva, Heidelberg, W.G.), NTA and disodium hydrogen arsenate (E. Merck, Darmstadt, W.G.) were of the highest purity commercially available.

Preparation of DBS-Containing Compounds (Enterochelin and Its Breakdown Products). Enterochelin was prepared as described by Young (1976). It was maintained in the solid form at $-20°$ C and solutions freshly prepared for use, since enterochelin broke down rapidly in solution. For the preparation of DBS, enterochelin-containing fractions after DE52 cellulose chromatography (Young, 1976) were acidified and extracted with ethyl acetate. The ethyl acetate extract was evaporated to dryness and the oily residue resuspended in 1 N NaOH and kept at room temperature under nitrogen for 1 h, after which it was acidified with 2 N H_2SO_4 and extracted with ethyl acetate. This ethyl acetate extract was evaporated to dryness, resuspended in a small volume of water, and DBS purified from oxidation products and small amounts of unhydrolyzed material by chromatography on a Biogel P2 column (0.8×15 cm) in 0.9% NaCl. The linear dimer and trimer of DBS were prepared according to Pugsley and Reeves (1976b), by gradient elution from a DE52 cellulose column. Since only incomplete separation of the compounds was achieved, the fractions were further purified by preparative thin layer chromatography on cellulose plates in the system benzene/acetic acid/water (125/72/3, v/v) (Luke and Gibson, 1971), followed by preparative thin layer chromatography in another system, 5% ammonium formate in 0.5% formic acid (O'Brien and Gibson, 1970). The two systems were also used for testing the identity of the compounds. Dihydroxybenzoylglycine was prepared as described by Ito and Neilands (1958).

Growth of the Cells and Iron Transport. Cells, grown overnight in unextracted M9 minimal medium with added 1 mM citrate (or in the case of *tonB* mutants, 10 µM DHB) and necessary growth factors, were centrifuged, washed and resuspended at an OD_{578} of 0.05 to 0.08 in extracted, iron-deficient M9 minimal medium, all necessary growth factors and 1 mM citrate (or 10 µM DHB). They were then grown for at least three generations, centrifuged down and washed three times in 0.01 M tris-(hydroxymethyl)-aminomethanehydrochloride pH 7.2 containing 100 µM NTA and 1 mM $MgCl_2$, and resuspended at an OD_{578} of 0.7 in the same buffer. The

Table 1. *Escherichia coli* K-12 strains used

Strain	Genotype and comments	References
AB2847	*aroB thi tsx malT;* parental, only produces enterochelin when supplied with the precursor DHB	Pittard and Wallace (1966)
IR20	AB2847 *feuB;* defective in ferric-enterochelin uptake, lacking the colicin B outer membrane protein receptor	Hancock et al. (1976) Hantke and Braun (1975b)
VR42	AB2847 *cir;* previously designated *feuA*, lacking the colicin Ia/Ib outer membrane protein receptor	Hancock and Braun (1976a) Hantke and Braun (1975b)
VR42/B9	AB2847 *cir feuB;* derived from strain VR42	Hancock et al. (1976)
BR158	AB2847 *tonB;* deficient in ferric-enterochelin, ferric-citrate and ferrichrome transport, resistant to phages T1 and ϕ80, colicins B, Ia, Ib, V, and M and the antibiotic albomycin	Hancock and Braun (1976b) Hantke and Braun (1975b)
IR112	AB2847 *tonB*; see above	Hantke and Braun (1975b)
BR128	AB2847 derivative; *tonB*-like, same iron transport deficiencies as strain BR158 but is phage T1 sensitive and partially sensitive to colicin Ia	Hancock et al. (1976) Hantke and Braun (1975b)
AN92	*aroB thi proA try argE pheA tyrA;* parental, see above	Langman et al. (1972)
AN92/B3	AN92 *feuB;* see above	
AN260	AN92 *fep;* defective in ferric-enterochelin transport, distinguished from *feuB* mutants by the fact that it is only very slightly resistant to colicin B (efficiency of plating = 10^{-1}) and has normal levels of colicin B receptor protein (unpublished results)	Cox et al. (1970) Langman et al. (1972)
AN441	AN92 *entF;* blocked in the conversion of DHB to enterochelin	Luke and Gibson (1971)
AN272	AN92 *fesB;* defective in ferric enterochelin esterase	O'Brien et al. (1971)
AN272/B4	AN92 *fesB feuB;* derived from AN272	

Genetic nomenclature is as described by Bachmann et al. (1976). Strains AN92, AN260, AN441, and AN272 were kindly provided by I. G. Young and H. Rosenberg. *FeuB* mutants were isolated by selection for colicin B resistance as previously described (Hancock et al., 1976). Using phage P1, the *feuB* mutations in strains IR20, VR42/B9, AN92/B3, and AN272/B4 were shown to be 5–9% cotransducible with the *purE* gene of strain PC1035 (*purE thi*) and 6–10% cotransducible with the lip gene from strain AT1325 (*lip-9 thi his pro purB*). This indicated that the *feuB* gene probably maps in the enterochelin gene cluster at 13 min (Bachmann et al., 1976), and is probably identical to the *cbr* gene (Pugsley and Reeves, 1976a, b, 1977a). We retain the mnemonic *feuB* which describes the function of the gene product (in *f*erric *e*nterochelin *u*ptake). The cir mutation in VR42 was approximately 5% cotransducible with the *his* locus of strain AT1371 (*proA lacY galK argE thi pan mtl tsx xyl supE*). All mapping strains were kindly supplied by B. Bachmann

cells were kept in an ice bath and before use shaken for 15 min at 37° C in the presence or absence of an inhibitor (1 mM arsenate or 1 mM DNP), then 0.5% glucose added and the cells incubated for a further 2 min at 37° C. Transport was started by the addition of a single solution to give a final concentration of 10 μM NTA (i.e. 110 μM NTA, in total was present during transport), 1 μM $^{55}Fe^{3+}$ (0.28 μCi/ml) and either 5–50 μM DHB or 2 μM enterochelin. Samples were taken at regular intervals, filtered onto membranes, washed and counted for radioactivity as previously described (Hantke and Braun, 1975a). Uptake of iron was linear over the first 3–5 min, and therefore the rates of transport were calculated by linear regression of the data points in this time range. For measuring the effects of inhibitors on glutamine or proline transport in cells grown under the conditions described above, transport was started by the addition of 1 mM ^{14}C-glutamine (5 μCi/ml) or ^{3}H-proline (10 μCi/ml) to an end concentration of 10 μM.

For measuring the uptake of the ferric chelates of the linear dimer or trimer of DBS, a slightly different method was used. Cells were grown in Tryptone-yeast broth to stationary phase, centrifuged and washed twice in Cohen-Rickenberg minimal medium, and resuspended at on OD_{578} of 0.03 in the same medium containing growth factors, 0.5% (w/v) glucose and usually 1 mM citrate as a growth factor promoting iron uptake (although citrate could be replaced by either 10 μM deferriferrichrome or 10 μM DHB). The cells were then grown to an OD_{578} of 0.8, washed twice in iron uptake medium (Langman et al., 1972), and resuspended at an OD_{578} of 2.0. Five milliliter of cell suspension was mixed with 5 ml of uptake medium containing the appropriate concentrations of iron and chelators, the two solutions having been first equilibrated to 37° C. The transport assay contained as final concentrations, 0.5 μM $^{55}Fe^{3+}$ (0.14 μCi/ml), 100 μM NTA, and either 1 μM enterochelin, 10 μM DHB, 10 μM dihydroxybenzoylglycine or 10 μM monomer, 5 μM dimer or 3.3 μM linear trimer of DBS.

RESULTS

DHB-Promoted Iron Uptake by Mutants Deficient in Ferric-Enterochelin Transport

When *aroB* mutant strains are fed with the precursor DHB, they can make and excrete enterochelin (Luke and Gibson, 1971), which is then capable of chelating and transporting iron. As shown in Table 2, column 2, the *aroB* strains AB2847, VR42 *cir*, AN92, and AN272 *fesB* were all capable of transporting ferric-enterochelin. Thus under the conditions used to demonstrate DHB-promoted iron uptake, these strains could convert DHB to enterochelin, and the resulting enterochelin would stimulate iron uptake. We were mainly interested in the study of a system which could bypass some of the cell envelope requirements of the ferric-enterochelin transport system. Therefore, we intro-

Table 2. DHB-promoted and enterochelin-mediated iron transport into strains capable of transporting ferric enterochelin

Strain	Mutation[b]	Rate of transport[a]	
		DHB-promoted iron uptake	Enterochelin-mediated iron uptake
AB2847	–	4351	5404
VR42	*cir*	4066	5067
AN92	–	4320	3898
AN272	*fesB*	2800	2209
AN441	*entF*	573	1480

[a] Results are expressed as picograms of Fe^{3+} transported per mg cell dry weight per min, and are the average transport rates (3 to 9 separate determinations for each result) over the initial 3–5 min of transport. The average mean standard deviation of the above results was ± 13.6%. The level of transport after 15 min (cpm $^{55}Fe^{3+}$ transported) varied in a similar fashion to the above rates

[b] The various properties of the strains are described in Table 1

Table 3. DHB-promoted and enterochelin-mediated iron transport into strains incapable of transporting ferric-enterochelin

Strain	Mutation	Rate of transport[a]	
		DHB-promoted iron uptake	Enterochelin-mediated iron uptake
IR20	*feuB*	3232 (100%)	145 (3%)
BR158	*tonB*	1865 (58%)	108 (2%)
IR112	*tonB*	1875 (58%)	36 (1%)
BR128	–[b]	1951 (60%)	82 (2%)
VR42/B9	*cir feuB*	1801 (56%)	135 (2%)
AN92/B3	*feuB*	3806 (100%)	170 (3%)
AN260	*fep*	411 (11%)	151 (3%)
AN272/B4	*fesB feuB*	847 (22%)	110 (2%)
NTA[c]	control	110 (3%)	90 (2%)

[a] These experiments were performed and the results are expressed as described in the legend to Table 2. The percentage rates of DHB-promoted iron uptake are compared with the *feuB* mutants IR20 and AN92/B3 for mutants derived from strains AB2847 and AN92 respectively (see Table 1). The percentage rates of ferric-enterochelin transport are compared with the result obtained for strain AB2847 (see Table 2)

[b] This mutant is *tonB*-like (see Table 1)

[c] NTA was present in all transport experiments in order to suppress the "low affinity" iron transport system (Frost and Rosenberg, 1973). The control was performed in the presence of NTA as sole chelating agent, using a variety of the strains included above and in Table 2, although only the results for strain IR20 are presented here. Other results were essentially identical

duced *feuB* mutations into the above strains in order to specifically eliminate the contribution of ferric-enterochelin transport to DHB-promoted iron uptake. Each of the four resultant strains, IR20 *feuB*, VR42/B9 *feuB cir*, AN92/B3 *feuB* and AN272/B4 *feuB fesB*, had a concomitant loss of ability to transport ferric-enterochelin and reduction in the rate of DHB-promoted iron uptake (Table 3), when compared with their respective parent strains (Table 2). The difference in the rates of DHB-promoted iron uptake of parent and *feuB* mutant strains can be considered due to enterochelin excreted into the medium, trapping iron and subsequently transporting it into parent strains but not into *feuB* mutants. Other strains such as BR158 *tonB*, IR112 *tonB*, BR128 (*tonB*-like, see Table 1) and AN260 *fep* were already unable to transport ferric-enterochelin. Thus in all the strains described below, ferric-enterochelin transport was not possible under the assay conditions used for DHB-promoted iron uptake; the term DHB-promoted iron uptake, where used, excludes ferric-enterochelin transport.

The highest rates of DHB-promoted iron uptake (designated 100% in Table 3) were obtained for the *feuB* mutants IR20 and AN92/B3. The additional *cir* mutation in strain VR42/B9 led to a 44% reduced rate of transport, while the *tonB* mutants BR158, IR112 and BR128 (*tonB*-like, see Table 1), also underwent DHB-promoted iron uptake at a somewhat reduced rate (Table 3). Strain AN260 *fep* was highly defective in DHB-promoted iron uptake. All of the above strains have cell envelope defects (Braun et al., 1976; Frost and Rosenberg, 1975; Hancock and Braun, 1976a; Hancock et al., 1976; Rosenberg and Young, 1974), and the results were compatible with the ability of DHB to act as the sole iron transporting chelate. However, results with two other mutants negated this conclusion. The *entF* mutant AN441, although able to transport ferric-enterochelin (Table 2), is blocked in the conversion of DHB to enterochelin (Luke and Gibson, 1971), and thus under our assay conditions for DHB-promoted iron uptake cannot synthesize enterochelin. It was shown to be very defective in DHB-promoted iron uptake (Table 2) suggesting a requirement for the synthesis of enterochelin from DHB. Furthermore, the *fesB* mutant AN272/B4 was 78% defective in DHB-promoted iron uptake (Table 3), suggesting an additional requirement for the subsequent breakdown of enterochelin by the *fesB*-coded esterase.

Growth Studies

The ability of the various mutants to grow in extracted, iron-deficient medium with DHB present as the sole compound supporting iron uptake, was studied. All strains with a low level of DHB-promoted iron uptake grew extremely poorly in such medium, although they grew at a similar rate to the wild type when 1 mM citrate was present (data not shown). The *feuB* mutants IR20, VR42/B9 and AN92/B3 and the *tonB* mutants BR158 and IR112, which could synthesize

but not transport enterochelin, were all able to grow on DHB-containing medium at a good rate. Thus the growth tests reflected the ability of these strains to undergo DHB-promoted iron uptake. NTA (100 μM) was present in all transport experiments to suppress the "low affinity" iron uptake system (Frost and Rosenberg, 1973). The above growth experiments, in which NTA was absent, suggested that ferric-NTA chelates did not function in DHB-promoted iron uptake. This was directly confirmed by replacing 100 μM NTA with 1 mM citrate in iron uptake experiments involving either the *tonB* mutants BR158 and IR112 or uninduced (i.e. grown in the absence of citrate) cells of the *feuB* strains IR20 or AN82/B3. In the presence of 1 mM citrate and 1μ M iron, none of the above strains could transport iron. However, 20 μM DHB stimulated iron transport in these strains, the rate of iron uptake being more than half of that achieved in the presence of 100 μM NTA. This was compatible with the observation that the addition of 1 mM citrate to iron deficient medium had no effect on the growth of *tonB* and *feuB* mutants in the presence of 10 μM DHB. In contrast to the above, when 2 μM enterochelin replaced 100 μM NTA in iron transport experiments, 20 μM DHB did not stimulate iron uptake.

Transport of Iron with Enterochelin Breakdown Products

The enterochelin esterase breaks down enterochelin, a cyclic trimer of DBS, in three steps yielding the linear trimer, dimer and monomer of DBS as products (O'Brien et al., 1971). These breakdown products cannot act as precursors of enterochelin synthesis (Bryce and Brot, 1972; O'Brien et al., 1971). The *fesB feuB* mutant AN272/B4 is derived from the *fesB* mutant AN272 which is defective in the enzymatic breakdown of enterochelin (Langman et a., 1972). Since strain AN272/B4 was also defective in DHB-promoted iron uptake when compared to the *feuB* single mutant AN92/B3 (Table 3), we decided to test the breakdown products of enterochelin for their ability to mediate in the transport of iron.

There was essentially no stimulation of iron uptake with 10 μM monomeric DBS or a similar monomeric compound from *Bacillus subtilis*, 2,3-dihydrobenzoylglycine (Ito and Neilands, 1958), in any of the tested strains. Addition of the ferric chelates of the linear dimer and trimer of DBS, resulted in good levels of iron uptake in the wild type strain AB2847 and its *cir* mutant VR42 (Fig. 1). In fact, the rate of iron uptake was higher than that promoted by DHB. However, in the *feuB* mutants of these strains, IR20 *feuB* and VR42/B9 *cir feuB*, transport of the ferric chelates of DBS linear dimer and trimer was strongly reduced.

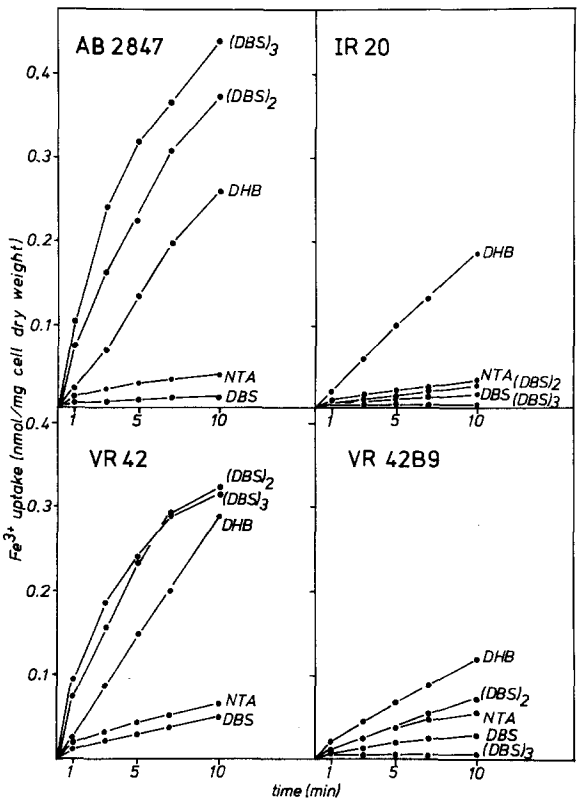

Fig. 1. Iron uptake by strain AB2847 and its derivatives IR20 *feuB*, VR42 *cir*, and VR42/B9 *cir feuB* in the presence of 0.5 μM $^{55}Fe^{3+}$ and 100 μM NTA, and as a specific chelator either 10 μM DHB, 10 μM DBS, 5 μM DBS dimer [$(DBS)_2$], 3.3 μM DBS trimer [$(DBS)_3$], or no additional chelator (NTA). The levels of chelators used were the maximum possible that could be produced from 10 μM DHB, if the DHB was totally converted through enterochelin to the specific breakdown product

The *tonB* mutant IR112 and the *fep* mutant AN260 were also unable to transport iron under these conditions (results not shown). Since both *feuB* and *tonB* mutants exhibited a high level of DHB-promoted iron uptake (Table 3), it is unlikely that the two types of transport are identical. These results, however, do not eliminate the possibility that breakdown products have a role in one step of DHB-promoted iron uptake.

Effect of Energy Inhibitors

In an attempt to show that DHB-promoted iron uptake was energized, the effects of two different energy inhibitors were studied. DNP is an uncoupler of oxidative phosphorylation and is thought to facilitate proton movement across the cytoplasmic membrane and thus to dissipate the energized membrane state (Cunarro and Weiner, 1975). Therefore, it inhibits those transport systems which require the energized membrane state for energization, e.g. proline transport. Control experiments were performed to demonstrate

Table 4. Inhibition of DHB-promoted iron uptake by DNP and disodium hydrogen arsenate in strains IR20 *feuB* and BR158 *tonB*

Inhibitor added	Rate of transport[a, b]	
	IR20 *feuB*	BR158 *tonB*
No inhibitor	3095 (100%)	2339 (100%)
1 mM DNP	2657 (86%)	2214 (95%)
1 mM Arsenate	381 (12%)	542 (23%)

[a] Results are expressed as picograms of Fe^{3+} transported per mg cell dry weight per min, and are the average transport rates (of 3 to 6 separate determinations) over the initial 5 min of transport. The numbers in brackets are the percentage rates compared to the control in the absence of inhibitor

[b] At the inhibitor concentrations shown and using the same medium and cells as used for the above experiments, controls were performed which showed that DNP reduced the initial rate of ^{3}H-proline transport to 22% of the uninhibited level while glutamine transport remained at 85% in the presence of DNP. Arsenate lowered the initial rate of glutamine transport to 5% and proline to 72% of the uninhibited level

that in strain IR20 *feuB* under conditions of iron starvation, proline transport was reduced 78% by 1 mM DNP, although glutamine transport was 86% functional in the presence of this inhibitor, which agrees with results obtained by other workers in cells grown under iron-proficient conditions (Berger, 1973; Berger and Heppe, 1974). Under the above conditions, 1 mM DNP had little effect on DHB-promoted iron uptake in either strain IR20 *feuB* or BR158 *tonB* (Table 4). This is in contrast to ferric-enterochelin transport, which is strongly inhibited by DNP (Pugsley and Reeves, 1977b; Hancock, unpublished results).

Another inhibitor, sodium hydrogen arsenate, depresses cellular ATP levels (Klein and Boyer, 1972). It inhibits transport systems which are energized, either directly or indirectly, by ATP, e.g. the shock sensitive amino acid transport systems (Berger and Heppel, 1974). Indeed the shock sensitive glutamine transport system was 95% inhibited by 1 mM arsenate. However, in strains which have a functional electron transport chain, arsenate does not fully discharge the energized membrane state. Accordingly, the initial rate of proline transport was inhibited only 28%. DHB-promoted iron uptake was 88% inhibited by arsenate in IR20 *feuB* and 77% inhibited in BR158 *tonB* (Table 4); inhibition was in fact observed in all other strains tested. This suggests that ATP is involved in some step of DHB-promoted iron uptake. However, ATP is required for the synthesis of enterochelin from DHB and serine (O'Brien et al., 1971) and as shown above, enterochelin synthesis was required for DHB-promoted iron uptake. This makes it impossible, at present, to conclude with any conviction, that ATP, or in fact any form of energization is required for the actual transport process.

Kinetics of DHB-Promoted Iron Uptake

Frost and Rosenberg (1973) demonstrated that both ferric-enterochelin and ferric-citrate uptake obeyed simple Michaelis-Menten saturation kinetics. We have confirmed this for strains IR20 (ferric-citrate uptake) and AB2847 (ferric-citrate and ferric-enterochelin uptake), and extended the observation to ferrichrome uptake, which was shown to have an apparent K_m of 0.12–0.2 μM Fe^{3+} and an apparent V_{max} of 50–90 patoms iron transported/mg cell dry weight per min. The above experiments were performed using a fixed concentration of chelator (1.0 μM deferriferrichrome, 1 mM citrate, or 1.25 μM enterochelin) and varying concentrations of iron. In each case, raising the concentration of chelator four-fold did not stimulate transport at any of the five iron concentrations (0.2–1.0 μM) used in these studies. This demonstrated that these transport systems are monoreactant in nature, in that the ferric-chelator complexes interact as single substrates.

In contrast, DHB-promoted iron uptake differed from the above transport systems. Although altering the iron concentration at a fixed DHB concentration gave a linear double reciprocal plot, both the slope and Y axis intercept of this plot varied considerably at different DHB concentrations (Fig. 2). This suggested that the mechanism of transport was of the bireactant type. In analogy to enzyme kinetics (Cleland, 1970), a replot of the slopes and intercepts of the various double reciprocal plots against the reciprocal concentration of DHB, yielded straight lines (Fig. 2, inset) which permitted calculation of the following apparent kinetic constants: $K_{Fe} = 0.4$ μM, $K_{DHB} = 26$ μM, $V_{max} = 172$ patoms Fe^{3+}/mg dry weight of cells per min. For another strain VR42/B9 *feuB cir*, the apparent Michaelis constants were shown to be similar to the above, although the apparent V_{max} was reduced to 107 patoms/mg dry weight per min. A bireactant mechanism implies that the iron and DHB (or a product derived from DHB in a relatively fast reaction) interact with the carrier individually rather than as a complex.

DISCUSSION

In Figure 3A, a scheme is presented for the transport of the ferric chelates of enterochelin and the linear dimer and trimer of DBS, in order to facilitate discussion of the results. The precise location of the various gene products has been definitely established in the case of the *feuB* outer membrane protein (Hancock et al., 1976), but has not yet been ascertained

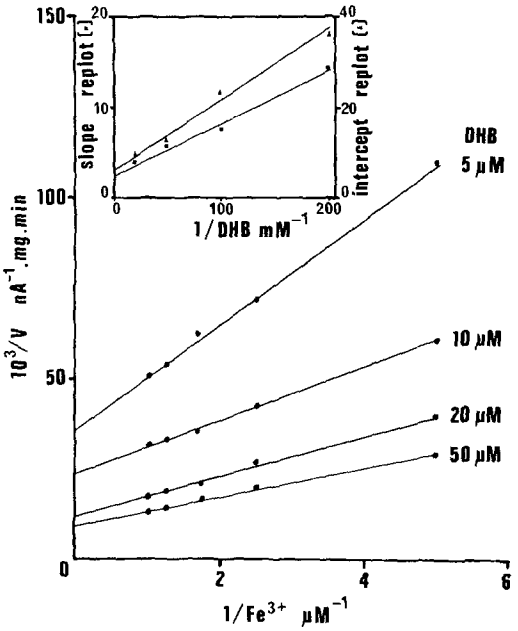

Fig. 2. Kinetics of DHB-promoted iron uptake into strain IR20 *feuB*. Iron uptake rates (V) were measured at various concentrations in the presence of the indicated amounts of DHB. Each point is the mean calculated from at least 5 separate experiments. The slopes and intercepts of the various double plots, as calculated by linear regression analysis of the data, were replotted against the reciprocal concentration of DHB, yielding a straight line as shown in the inset

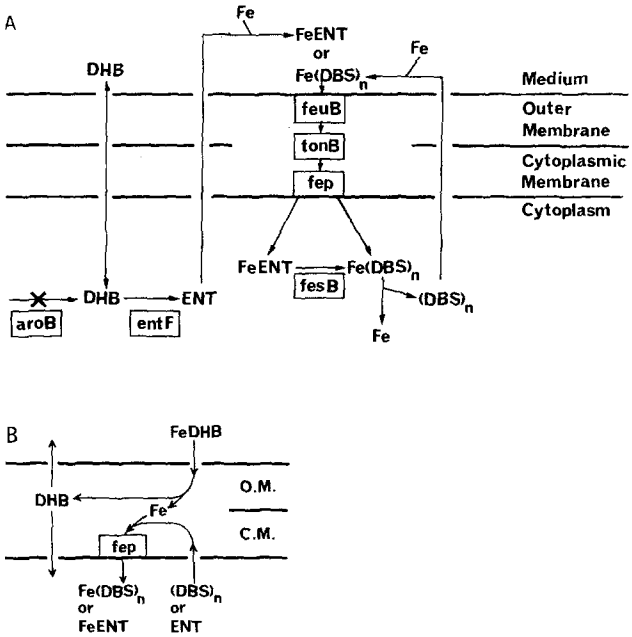

Fig. 3 A and B. Schemes showing possible mechanisms for ferric-enterochelin and DHB-promoted iron uptake. The proposed sites of action of the various gene products are indicated by the inclusion of the gene mnemonic in a box. Abbreviations: *ENT* enterochelin; $(DBS)_n$ linear dimer ($n = 2$) and trimer ($n = 3$) of DBS. To keep the scheme relatively simple, it has been divided into two parts and only relevant pathways are included. (A) Scheme for ferric-enterochelin and ferric-$(DBS)_n$ transport. (B) Possible mechanism of DHB-promoted iron uptake. It should be stressed that the only possible source of enterochelin or $(DBS)_n$ in *aroB* mutants is synthesis from added DHB

for the *tonB* or *fep* gene products. However, *tonB* mutants were highly defective in ferric-enterochelin and ferric-DBS dimer and linear trimer transport but relatively proficient in DHB-promoted iron uptake, while the *fep* mutant was defective in all of these transport systems. We therefore consider that the products probably interact with ferric-enterochelin transport in the order *feuB*, *tonB*, *fep*, and have followed the convention of placing the *fep* gene product in the cytoplasmic membrane (Frost and Rosenberg, 1975; Langman et al., 1972; O'Brien et al., 1970). Since DHB-promoted iron transport is functional in both *feuB* and *tonB* mutants (which are both unable to transport ferric-enterochelin), then this system must bypass these gene products. As postulated by Frost and Rosenberg (1975), the bypass might involve the diffusion of iron as a complex with DHB across the outer membrane. We have eliminated the possibility that the ferric chelates of enterochelin (Table 3) or its individual breakdown products (Fig. 1) carry out this first outer membrane step, since *feuB* mutants cannot transport these ferric chelates. However, a mechanism involving a mixture of the breakdown products still remains a possibility. It is unlikely that iron alone can overcome the outer membrane permeability barrier under our transport assay conditions, since NTA, which effectively suppresses low affinity iron uptake (Frost and Rosenberg, 1973), was present in all transport assays. We have also demonstrated that ferric chelates of NTA are not important in DHB-promoted iron uptake.

A mutant defective in the enzyme which converts DHB to enterochelin, the *entF* mutant AN441, was also defective in DHB-promoted iron uptake (Table 2). Thus DHB must be converted to enterochelin for DHB-promoted iron uptake to occur. This requirement for enterochelin synthesis suggests that iron cannot be transported through the entire cell envelope as a chelate of DHB, but that at some stage the iron must be transferred to enterochelin or one of its breakdown products (Fig. 3B). The *fesB feuB* mutant was 78% deficient in DHB-promoted iron uptake, suggesting a requirement for the enzymatic breakdown of enterochelin by the *fes*-esterase. This indicates that a large portion of the transport observed in *feuB* mutants probably involves one or more of the breakdown products of enterochelin, the linear trimer, dimer or monomer of DBS. However, none of these products

alone can efficiently stimulate iron uptake into *feuB* mutants (Fig. 1). Therefore, much of the observed DHB-promoted iron uptake in *feuB* mutants may result from the capture of iron from its DHB-chelate by one or other of the breakdown products (Fig. 3B). The bireactant kinetics of this transport system are consistent with this scheme.

The *fep* mutant AN260 was unable to undergo DHB-promoted iron, ferric-enterochelin or ferric-DBS dimer or linear trimer transport. The hypothesis that the *fep* gene product functions in the translocation of the ferric chelates of enterochelin and its breakdown products across the cytoplasmic membrane, is consistent with this evidence. As pictured in the scheme (Fig. 3B), it could have the same function in DHB-promoted iron uptake. The scheme fails to explain why the *cir feuB* double mutant VR42/B9 was only half as proficient in DHB-promoted iron uptake as the *feuB* single mutant IR20. Both the *cir* (*feuA*) and *feuB* mutations lead to the loss of different outer membrane proteins, which in the wild type strain, under conditions of iron deprivation, are present in large amounts (Hancock and Braun, 1976a; Hancock et al., 1976). The combination of outer membrane defects in *cir feuB* double mutants (Hancock et al., 1976), might have had a nonspecific effect on ferric-DHB diffusion through the outer membrane. A further possibility is that DHB-promoted iron uptake consists of two independent systems, one of which requires the *cir* outer membrane protein.

It is interesting that *tonB* mutants are 60% proficient in DHB-promoted iron uptake, since they are extremely deficient in three high affinity iron uptake systems, the enterochelin-, citrate-, and ferrichrome-mediated iron transport systems (Hantke and Braun, 1975b). Based on the observation that the irreversible adsorption of phages ϕ80 and T1 to cells, had only one known cellular function requirement, for energy from the energized membrane state, and one known bacterial gene requirement, for the *tonB* gene, we previously postulated that the *tonB* function mediated in the energy-requiring process (Hancock and Braun, 1976b). In support of this view, it has been recently shown that vitamin B_{12} transport also requires the energized membrane state (Bradbeer and Woodrow, 1976) as well as the *tonB* function for the second (i.e. post-receptor binding), energy-dependent phase of transport (Bassford et al., 1976). Our preliminary results would suggest that ferrichrome, ferric-citrate and ferric-enterochelin uptake are all relatively sensitive to uncouplers (unpublished results). Thus it is possible that these *tonB*-dependent iron transport systems also require the energized membrane state. This in fact has been very recently confirmed by Pugsley and Reeves (1977b) for ferric-enterochelin transport. In the case of DHB-promoted iron uptake, where, in contrast to the above transport systems, the outer membrane receptor requiring step was bypassed, uptake was largely independent of the *tonB* function (Table 3) and resistant to the uncoupler DNP (Table 4). This makes it likely that our previous postulate (Hancock and Braun, 1976b) that the *tonB* function couples the energized membrane to the above transport processes and phage adsorption events, is correct. However, this does not imply that the *tonB* function is a general energy mediator for systems with an outer membrane protein requirement, since maltose transport into *tonB* mutants is normal (Bassford et al., 1976). In addition, *tonB* mutants are not altered in such outer membrane receptor independent, nutrient transport systems as serine and proline (Bassford et al., 1976; Frost and Rosenberg, 1975), which are strongly inhibited by DNP and rely on the energized membrane state for energization (Berger, 1973; Berger and Heppel, 1974). Wang and Newton (1971) previously presented kinetic evidence that the *tonB* function was not involved in energization, by comparing the kinetic constants for iron transport into wild type and *tonB* mutant strains in the presence or absence of inhibitors. However, under the conditions which they employed, DHB-promoted and citrate- and enterochelin-mediated iron transport would all occur simultaneously. Since both the initial uptake rates and the affinities for iron of the three systems differ (Frost and Rosenberg, 1975; Schmid and Hancock, unpublished results), the conclusions made are not warranted by the new data.

Whether DHB-promoted iron transport is important to wild type *Escherichia coli* is disputable, especially when one takes into consideration the strong iron chelator enterochelin. In the present study it allowed dissection of the sequence of outer and cytoplasmic membrane translocations. When the outer membrane protein was bypassed, there was also no requirement for the *tonB* function and no need for the DNP-sensitive energized state of the cytoplasmic membrane. It is therefore possible that the permeability of the presumed outer membrane pore, or the release of the iron complex from its first binding site, is controlled by the energy state of the cytoplasmic membrane, and that the *tonB* function serves as a coupling device between outer and cytoplasmic membrane translocations.

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