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IRON ABSORPTION AND TRANSPORT IN MICROORGANISMS

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CONTENTS

INTRODUCTION AND SCOPE

The study of iron assimilation systems in microorganisms, particularly in enteric bacteria, has become an active field of research in recent years. There are several reasons for this state of affairs. One pathway of iron uptake involves special carriers, termed siderophores (1), and the matching membrane receptors. Since the latter are used by specific phages and bacteriocins as attachment sites (2), the mode of infection by these noxious agents can be studied via examination of the iron-uptake apparatus. The transport system is regulated by the iron nutritional status of the cell and several of the membrane proteins involved can be visualized in polyacrylamide gels. Thus, iron assimilation in procaryotic species such as *Escherichia coli* affords an ideal opportunity for application of the powerful techniques of modern molecular biology and extends beyond iron transport per se to more general transmembrane permeation processes and their regulation.

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Microbial iron assimilation has become popular in another context. It is linked rather directly to several practical applications. Because little free iron exists in the tissues of animals and plants, the capacity of invading pathogens to acquire enough of this "precious metal" to satisfy demands of growth may constitute one aspect of virulence and pathogenicity (3). Iron has been identified as a germination or sporulation factor within selected species (4). Finally, the systems used by microbes to gather iron have stimulated the search for substances that will deferrate human patients who suffer from iron overload (5) or actinide poisoning (6).

This chapter should be read in conjunction with a contemporary review that deals with the more chemical aspects of microbial iron assimilation (1). Historical aspects of the field have been covered by Lankford (7) in his encyclopedic review of bacterial iron assimilation. Emery (8) discussed iron transport in fungi, a subject to which he has made significant contributions. This account concentrates on bacterial systems and focuses in particular on the enteric bacteria. Such organisms lend themselves to genetic manipulation and hence considerable progress has been made in unraveling the diverse iron-uptake pathways in *E. coli* and closely related species.

ROLES OF IRON IN MICROBIAL METABOLISM

Iron is a nutrient probably universally required by living cells. The ubiquitous presence of cytochromes and non-heme iron in the respiratory chains of aerobic and facultative anaerobic species provides a central role for element No. 26 in the energy metabolism of microorganisms. With the dependency on O_2 as electron acceptor came the need to protect the cell against hydrogen peroxide, and once more iron is seen to be involved via the hydroperoxidases, catalase and peroxidase, and via certain forms of superoxide dismutase. During the past two decades iron sulfur proteins have assumed a status in microbial metabolism fully equivalent to that of the cytochromes. Other crucial roles for iron include the nitrogenase complex, thus far found only in procaryotic cells, and ribotide reductase (9), the enzyme responsible for synthesis of the deoxyribotides required for DNA formation.

If life without iron exists it is probably to be found among certain members of the lactobacilli (10). These species inhabit milk and other dairy products, the oral cavity, and specialized niches that are low, although not entirely lacking, in iron. Diligent efforts, so far unrewarded, have been made to demonstrate an iron requirement for true lactic acid bacteria. These species do not contain heme compounds of any type and use the cobalt-containing vitamin B12 form of ribotide reductase (9). They are apparently without siderophores, although growth in acid media, which they generate, could serve to solubilize iron.

Table 1 records only a few of the significant roles of iron in the microbial world. The list continues to expand—witness the recent demonstration that iron is required for aromatic biosynthesis in *E. coli* (11).

TYPES OF IRON ASSIMILATION SYSTEMS

Low Affinity

Iron is one of the most common elements on the surface of the earth, and in sheer abundance it is outranked only by aluminum, silicon, and oxygen. The average soil contains some 5% iron. However, the availability of iron stands in sharp contrast to its abundance. Both Fe(II) and Fe(III) exhibit an exceedingly high affinity for hydroxy ions, with which they form insoluble complexes. In the case of Fe(III), the predominant oxidation state prevailing in an aerobic environment, the solubility product may be less than 10^{-38} M (12).

The profound insolubility of Fe(III) notwithstanding, most microbes appear to have the power to utilize such polymeric forms of iron. This pathway is designated "low affinity," since it has been found that relatively high levels of iron, of the order of 10 μM, are required to achieve maximum growth rates. No specific solubilizing and transporting compounds (siderophores) or membrane receptors seem to be required. Lacking these entities, it is difficult to get a handle on the molecular mechanics of the system and hence very little is known about its basic features.

In the first instance, more information is required on the structure and stability of the Fe(III) oxy-hydroxide polymers, which have the general composition FeOOH. It is possible that some of the surface atoms of these polymers may be less firmly bound and hence available to the cell. An alternative possibility is that metal binding sites may be built into the cell envelope. Working with mutants of *Neurospora* defective in ornithine biosynthesis and hence totally lacking siderophores of the ferrichrome-coprogen series, Winkelmann (13) concluded that hydroxy acids could serve in the dissolution of adsorbed ferric polymers. Nitrilotriacetate, an effective

Table 1 Iron enzymes from microorganisms

Electron transfer proteins	Tricarboxylic acid cycle
Cytochromes	Aconitase
Hydrogenase	DNA biosynthesis
Iron-sulfur proteins (ferredoxin)	Ribotide reductase B2 subunit
Succinate dehydrogenase	N_2 fixation
H_2O_2 and O_2 metabolism	Nitrogenase
Catalase	
Peroxidase	
Superoxide dismutase	
Oxygenases	

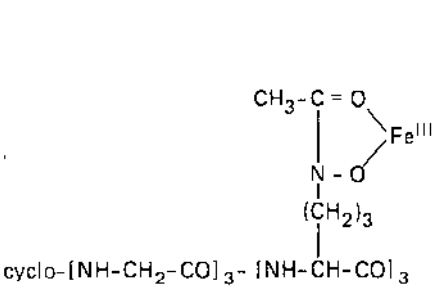
chelator for ferric ion, is often used to block low affinity uptake in *E. coli.*

The most compelling evidence for the presence of a low affinity system comes from mutational analysis. All vestiges of the "high affinity" system, described below, can be removed without impairing growth rate provided higher levels of iron are supplied.

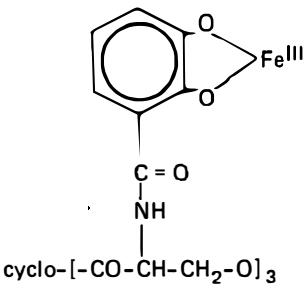
High Affinity

The iron assimilation system known as high affinity is comprised of two parts, namely, the siderophore and the cognate transport apparatus.

Siderophores are defined as relatively low-molecular-weight (500–1000), virtually ferric-specific ligands, the biosyntheses of which are tightly regulated by iron. Although considerable structural variation exists among the several dozen siderophores chemically characterized at the present time, most can be classed as either hydroxamates or catechols. The prototypical siderophores of these two classes are, respectively, ferrichrome and enterobactin. Ferrichrome was the first ferric trihydroxamate identified in nature and enterobactin was the first tri-catechol.



Ferrichrome



Ferric enterobactin

Receptors for both have been detected in the outer membrane of enteric bacteria.

During transport, the iron-laden form of the siderophore first contacts a surface receptor. In the case of gram-negative (double membrane) bacteria, the outer membrane constitutes a permeability barrier for water-soluble solutes larger than ca 500 daltons (14). In the evolution of siderophores, it was necessary to design a ligand that could contact all six octahedrally directed bonds of the Fe(III) atom. This cannot be readily achieved in a molecule smaller than 500–600 daltons—consequently the need for the surface receptor.

E. coli synthesizes enterobactin and maintains a receptor for this siderophore and for ferrichrome, the latter a heterologous siderophore commonly

found in fungi. Table 2 lists some of the properties of these receptors, which, as may be seen, also act as attachment loci for certain phages and colicins. The receptors are believed to behave as pores or channel formers in the sealed outer membrane, although this needs to be confirmed by experimentation.

The data in Table 2 would imply that common structural similarities might be present in the ferric siderophores, in the tips of phage tails, and in specific domains of the colicin molecules. This is not necessarily the case, since the receptor is undoubtedly a complex array of units and the binding at one site may affect the conformation of the entire pore. Competition among the various binding entities has been demonstrated in vitro for the solubilized outer membrane receptors for both ferrichrome and ferric enterobactin.

ENTERIC BACTERIA

Citrate It is not entirely clear that citrate should be regarded as a high affinity carrier. The substance itself is a relatively poor ligand for Fe(III), since the formation constant lies many orders of magnitude below those of the usual siderophore, which tend to cluster around 10^{30}. On the other hand, an outer membrane receptor exists for iron citrate in *E. coli* strains K12 and B/r (not w) (15). Unlike the one for ferric enterobactin, the ferric citrate receptor is induced only by growth of the organism in the presence of the substrate. The receptor has not been purified to any extent, but it has been detected on gels and the subunit size is about 80,000 daltons (16). The genes for ferric citrate transport, *fec,* are clustered at about 6 min on the chromosomal map.

Although *Salmonella typhimurium* LT-2 and *E. coli* K12 are closely related, they may be distinguished on the basis of their response to citrate and its iron complex. *S. typhimurum* uses citrate as its carbon source whereas *E. coli* does not; the organisms exhibit the inverse specificity for iron citrate (17).

Strains of *E. coli* are known to harbor plasmids that confer the ability to utilize free citrate (18, 19).

Table 2 Receptors for siderophores in the outer membrane of *E. coli* K12

Ferric siderophore	Gene	Locus	MW(SDS)	Phage or colicin	Antibiotic
Ferric enterobactin	*fep*A	13	81,000	colicin B	—
Ferrichrome	*ton*A	3	78,000	T1, T5, Φ80; colicin M	Albomycin

In a survey of meningococcal strains, Archibald & DeVoe (20) failed to find evidence for synthesis of conventional siderophores. However, carboxylic acids and phosphate esters were active in supplying iron to the cells. Citrate, which was especially effective, was declared a "functional siderophore."

Hydroxamates Although siderophores of the hydroxamate type are most common in fungi, they are also synthesized by some bacterial species and, as already indicated, enteric bacteria possess a well-characterized transport system for ferrichrome. This provides the opportunity for study of the transport process by means of genetic techniques.

Mutants of *S. typhimurium* LT-2 defective in enterobactin synthesis, designated *enb,* fail to grow on low iron media containing citrate (17). On such media, however, they do respond to heterologous siderophores such as ferrichrome, with a level of 0.1 μM providing the minimal optimal concentration for growth. A series of double mutants, *enb-sid,* were selected on the basis of resistance to albomycin, a close structural analogue of ferrichrome (21). Most of the *sid* mutations mapped close to *pan*C. On the *E. coli* chromosome *pan*C is located very close to *ton*A, a well-known genetic locus required for sensitivity to a group of phages and colicin M. Since phages and colicins often share the same receptor, and since transport of vitamin B12 had been defined as the biochemical function for the receptor for the E group colicins (22), it was suggested that the product of the *ton*A gene is the ferrichrome receptor (23–25). A further basis for this speculation was the demonstration that strains of *E. coli* that hyperexcrete enterobactin are resistant to colicin B—a phenomenon that could have been attributed to competition for the same receptor locus (26).

Siderophores were shown to protect sensitive cells against colicins by two distinct modes (27). The first, characterized by ferrichrome protection versus colicin M or by ferric enterobactin protection versus the B group colicins, involved competition for a common binding site on the surface receptor. The second mode of protection required utilization of siderophore iron, was nonspecific, and may represent inhibition of synthesis of receptor (28).

Ferrichrome transport in *E. coli* RW193, a mutant blocked in enterobactin biosynthesis, was postulated to involve re-utilization of the ligand (29). Recently, a modified form of deferriferrichrome, possibly bearing an acetyl group on one of the hydroxylamino functions, was demonstrated to occur in the course of ferrichrome transport (30). A mutational analysis has not yet been performed to see if any type of processing of ferrichrome is an obligatory step in the utilization of this siderophore.

Kaback-type vesicles from low-iron-grown cells of *E. coli* RW193 were shown to transport ferrichrome by a mechanism dependent on the ener-

gized state of the membrane, and possibly representing a non-proton symport process (31). Ferrichrome has been shown to bind alkaline earth cations such as Ca^{2+} or Mg^{2+}, but the biological significance of this event remains to be established (32). *E. coli* has not been demonstrated to need Ca^{2+} for growth.

Recently, a second genetic locus required for ferrichrome utilization has been identified in *E. coli* K12 and has been given the mnemonic *fhu*B (ferric hydroxamate uptake); *ton*A has been renamed *fhu*A (33). The gene order was shown by three point transduction crosses to be *pan-fhu*A-*fhu*B-*met*D at min 3.5 on the chromosome. Gene *fhu*B is believed to program the synthesis of a component necessary for transport of hydroxamate-type siderophores across the cytoplasmic membrane.

In *E. coli,* the *ton*A protein has been extracted from the cells and has been shown to have a subunit molecular weight of ca 78,000 and to be capable of releasing DNA from T5 (34). Ferrichrome competes in vitro with phage for the binding site on the isolated receptor. By means of this protection assay, the receptor was shown to be specific for siderophores structurally related to ferrichrome. Although ferrichrome transport is enhanced in iron-starved cells, the level of *ton*A does not seem to be affected drastically by iron nutritional status.

A second gene locus in *E. coli, ton*B, programs a membrane component required by phages T1, Φ80 (not T5), certain colicins, vitamin B12, and several iron compounds. The function is not required for ferrichrome-mediated ^{55}Fe uptake by spheroplasts (35). The *ton*B gene product has been suggested to be an inner (cytoplasmic) membrane protein, the function of which is to produce, via the proton motive force, a diffusible component that serves to release the adsorbed substrate from the outer membrane receptor (36). This suggestion has been put forth for vitamin B12 transport and has not been tested in the siderophore series.

An additional outer membrane protein, product of the *omp*A gene, affects ferrichrome transport (37). Polymer-bound ferricrocin was found to be weakly active in supplying iron to *E. coli* (38).

Enantio-ferrichrome, chemically synthesized from D-N^{δ}-hydroxyornithine, was shown to be taken up by fungi, but only at a rate substantially lower than that of the natural *cis,* Λ isomer (39).

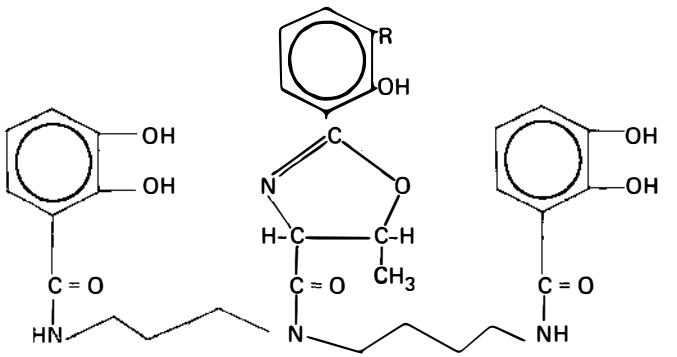
The biosynthesis of N^{ϵ}-hydroxylysine, the unique amino acid constituent of the hydroxamate-type siderophores mycobactin and aerobactin, has been studied in partially purified extracts of *Aerobacter aerogenes* 62–1 (40). The enzyme was found highly specific for lysine: L-ornithine, which occurs in hydroxylated form in a variety of siderophores, was a relatively poor substrate. The authors concluded, in harmony with findings for other siderophores (41, 42), that in aerobactin N-hydroxylation precedes acylation.

The cell-free synthesis of an hydroxamate-type siderophore has not yet been reported. Obviously, the properties of the enzymes involved would be of considerable interest.

Ferrichrome transport in *S. typhimurium* LT-2 proceeds by a mechanism analogous to that which occurs in *E. coli.* Albomycin-resistant mutants were found to be also resistant to ES18 phage, and it is believed the latter uses the *ton*A equivalent in *S. typhimurium* (43).

Substances extracted from Western red cedar and found to be active in supporting growth of siderophore-defective mutants of *S. typhimurium* were identified as the α-hydroxyketones α-, β-, and γ-thujaplicin (44).

Catechols Two types of catechol-containing siderophores are known, namely, the 2,3-di-hydroxybenzoyl derivatives of L-serine and spermidine. The former was independently isolated in 1970 from *S. typhimurium* (45) and *E. coli* (46) and was assigned, respectively, the trivial names enterobactin and enterochelin. Two specific spermidine-derived siderophores, agrobactin (47) and parabactin (48, 49), are obtained from *Agrobacterium tumefaciens* and *Micrococcus* (now *Paracoccus*) *denitrificans,* respectively. Nothing is known yet about the mode of iron transport performed by these linear catechols and they are not discussed further here. It should be noted, however, that



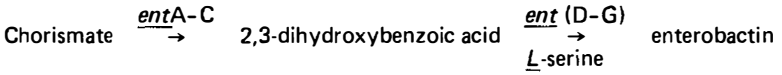
Agrobactin, R = OH, from *Agrobacterium tumefaciens*, and parabactin, R = H, from *Paracoccus denitrificans*

parabactin has been synthesized in cell-free extracts from iron-deficient *P. denitrificans* from N^1,N^8-*bis*-2,3-dihydroxybenzoylspermidine, L-threonine, and 2-hydroxybenzoic acid (48).

During the past decade, a considerable literature has accumulated on the genetics and metabolism of enterobactin (50). This is because it is the native siderophore of *E. coli* and probably of most other enteric bacteria. It has

been shown by genetic analysis to be the means whereby these organisms scavenge iron when the element is either limiting in absolute concentration or is present in some poorly available form. The chemistry and biophysics of ferric enterobactin have been explored in some detail (1); suffice it to note that it has the largest known stability constant of any iron chelate (51).

Substantial progress has been achieved in defining the pathway of enterobactin formation from chorismate, a main branching point in aromatic biosynthesis. The genes for these enzymes are clustered in one or more



operons between *pur*E and *lip* at 13 min on the *E. coli* chromosomal map. Although the products of genes A, B, and C are present as soluble enzymes, those of genes D-G are believed to exist in a multienzyme complex bound to the cytoplasmic membrane. Complementation studies with a series of *ent* mutants of *E. coli* by use of extracts from *S. typhimurium* and *Klebsiella pneumoniae* (formerly *A. aerogenes*) afforded further evidence for physical association of *ent* biosynthetic gene products (52).

Apart from the biosynthetic genes for *ent,* two additional genes are known to occur at min 13 on the chromosome of *E. coli.* These are *fep* and *fes*B (53). The former, also known as *cbr* and *feu*B, is considered the structural gene for the outer membrane ferric enterobactin receptor, historically known as the colicin B receptor. The other gene, *fes,* was reported to program synthesis of two components of an esterase that degrades ferric enterobactin to release the iron. The enzyme was thought to be comprised of component A, MW 140,000, and component B, MW 22,000. Only *fes*B mutants have been isolated thus far.

There is still some confusion in the literature concerning the actual substrate and mode of action of the *fes* gene products. Bryce & Brot (54) reported the enzyme to act only on the ligand, whereas the Australian group (53) found the iron complex to be a superior substrate. The specificity was reexamined and it was concluded that the esterase is a single component that acts on both free enterobactin and ferric enterobactin, the former, inexplicably, at 2.5 times the rate of the latter (55). To make good biological sense, the complex should be the exclusive, or at least preferred, substrate. The ligand and complex exhibit vast differences in conformation and charge. An absolute requirement for an esterase for ferric enterobactin is difficult to reconcile with the finding that certain synthetic analogues, devoid of ester linkages, faithfully follow the enterobactin transport system (56, 57). This does not seem to amount to degradation to 2,3-dihydroxybenzoic acid followed by resynthesis of enterobactin, since class I mutants of

S. typhimurium, unable to convert 2,3-dihydroxybenzoic acid to enterobactin, respond to the analogues. It has been suggested that the esterase is in reality the reductase, or a part thereof, and that in the process of iron release ester bonds, if present, are hydrolyzed (56). Although enterobactin esterase remains somewhat of a mystery, the genetic lesion is real. Mutants designated *fes* accumulate ferric enterobactin and cells or colonies assume a definite pink coloration.

The genes just enumerated certainly do not represent all of those in the iron operon(s). Growth of enteric bacteria at low iron, i.e. <0.1 μM, leads to insertion of several proteins in enhanced amounts in the outer membrane. Thus, iron-starved K12 strains upon polyacrylamide gel electrophoretic analyses display bands for components with molecular weights 83,000, 81,000, and 74,000 (58–60). The first of these is of unknown function, the 81,000 component is the *fep* gene product, and the last is the colicin I receptor, the biochemical function of which is also unknown. A similar set of iron-regulated outer membrane proteins is found in *S. typhimurium* whereas in other strains of *E. coli,* such as B and B/r, the 83,000 band is missing.

Experiments with ^{14}C-labeled enterobactin indicate that the intact coordination compound enters the cell, or at least penetrates to the inner leaf of the cytoplasmic membrane (50). The iron-laden siderophore first contracts the outer membrane (*fep*) receptor, the probable function of which is to provide a specific transport channel. As all genes in the iron operon(s) become derepressed at low iron growth, a unique opportunity is thus provided for collection of reasonable quantities of the ferric enterobactin receptor protein. Hollifield & Neilands (56) devised an assay for this receptor based on filtration of ^{55}Fe enterobactin, which at neutral pH is a trivalent anion, through a column of DEAE-cellulose. The binding assay was applied in vitro to extracts containing EDTA-Triton X-100-solubilized membrane and was correlated with the band at 81,000 MW in polyacrylamide gel electrophoresis. Ferric enterobactin and colicin B were found to compete for the solubilized receptor. In a subsequent paper Hollifield et al (61) reported that incubation of the extracted receptor prior to gel analysis revealed a processing activity that converted the 81,000-MW protein to an inactive component that behaved as though it had a gel molecular weight of about 75,000 (81,000*). The conversion had all of the earmarks of an enzymatic process and was inhibited by benzamidine. Fiss et al (62) employed a commercially available column with benzamidine covalently linked to sepharose to filter out the processing activity. The latter was eluted with free benzamidine and was identified by sensitivity to heat and by mutational analysis as outer membrane protein *a.* This affords a biochemical activity, if not function, for protein *a.* The nature of the processing,

probably a proteolysis, has not been clarified and the biological rationale for the activity remains obscure.

A by-pass for iron uptake in *E. coli* has been described in which *fep* and *ton* B are not required (63). In this system iron is believed to cross the outer membrane as a complex with 2,3-dihydroxybenzoic acid and to exchange into enterobactin in the periplasmic space.

As with ferrichrome, a synthetic retro-isomer of enterobactin has been prepared, in this case from D-serine (64). However, unlike the ferrichrome analogue, the enantio-enterobactin, which forms the *cis*, Λ coordination isomer, proved to be not only totally inactive in *E. coli* RW193 but to actually deny iron to the cells (J. B. Neilands, T. Erickson, and W. H. Rastetter, unpublished data). The relationship of the Λ and Δ isomers of ferric siderophores is illustrated in Figure 1. These are, respectively, left- and right-handed coordination propellers and they exist by virtue of a preferred chirality specified by the asymmetric α-carbon of the amino acid residues. Although it is not possible to change the screw sense around the iron without simultaneously altering the directional sense of the 12-membered ring in the enterobactin platform, all evidence favors the view that the receptor recognizes the iron side of the molecule. Thus, the carbocyclic analogue of ferric enterobactin, which must be optically inactive, was found to be just half as potent as the natural product in competition with colicin B for the extracted receptor. Similarly, ferric agrobactin and parabactin, which are *cis*, Λ, are inactive for *E. coli* RW193 but acquire significant

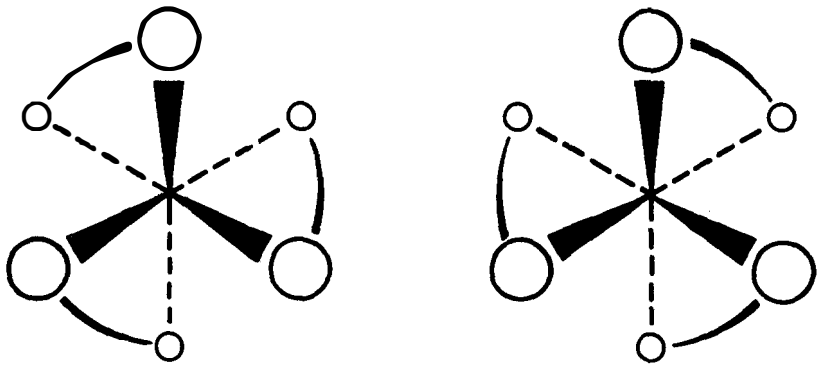


Figure 1 Enantiomeric pair of complexes formed by clustering of three identical pairs of bidentate, all-oxygen ligands around a six-coordinate metal ion. On the left is the left-handed coordination propeller (Λ); on the right is the right-handed coordination propeller (Δ). Siderophores that lack optical activity form racemic iron complexes, whereas those with asymmetric centers form complexes that are mainly, or completely, Λ, i.e. ferrichrome, or Δ, i.e. ferric enterobactin. There is substantial evidence that particular transport systems can accommodate only siderophores with specified chirality at the iron center.

potency after hydrolysis to their opened or "A" forms. The latter, still containing L-threonine, appear to form ferric complexes that belong to the *cis,* Δ series. In sum, these data support the concept that microbes have constructed siderophores from optically active fragments in an attempt to package iron in a form recognizable only to their own receptors. This is a confirmation of the idea expressed by O'Brien & Gibson (46), who wrote: "If the metal chelating compounds excreted are effective only for the cells of a particular species, they may have a high survival value for cells growing in a mixed population in an environment deficient in metals." The rate of exchange and thermodynamics of binding would then become critical factors in the availability of siderophore-complexed iron.

Iron proteins that resemble ferritin have recently been found in bacteria, including *E. coli* (65), but proof that these substances act as iron storage depots is still lacking.

NONENTERIC BACTERIA *Pseudomonas spp.* The *Pseudomonas* are known to produce siderophores of the hydroxamate class. Depending on the source, these have been named ferribactin, pyoverdine, pyochelin, and pseudobactin; none has yet been completely characterized in a chemical sense (66).

Bacillus spp. Bacillus subtilis was the source of the first 2,3-dihydroxybenzoyl-containing substance from microbial species. As the glycine conjugate, this has been shown to stimulate uptake of ^{59}Fe. Schizokinen was isolated and characterized from *Bacillus megaterium.* The siderophore presents iron to the cells via a shuttle mechanism (66).

Mycobacteria A large family of closely related siderophores, designated mycobactins, are derived from the mycobacteria (66). As these substances, and their iron complexes, are only lipid soluble they dwell in the waxy envelope of the cell. Water-soluble siderophores, called exochelins, are thought to be excreted and to exchange iron to the mycobactins (66).

Cyanobacteria A siderophore isolated from *Anabaena* sp. proved to be identical with one obtained earlier from *B. megaterium,* namely, schizokinen. A coccoid cyanobacterium, *Agmenellum quadruplicatum* PR-6, has been shown to form an hydroxamate-type siderophore, and synthesis of such iron scavengers may be a general characteristic of the cyanobacteria (66).

Neisseriae spp. Certain members of the *Neisseriae* include several important human pathogens. A description of the iron-gathering mechanisms of

these species was unaccountably omitted in a recent review of microbial iron metabolism (66). The field has been quite active in recent times. Norrod & Williams could not detect siderophores in iron-deficient cultures of *Neisseriae gonorrhoeae* (67). A study of 20 meningococcal strains did not reveal the presence of siderophores, and salicylhydroxamate was the only siderophore-like substance with iron feeding activity (20). However, a series of carboxylic acids and phosphate esters were shown to play a role as "functional siderophores." Other workers have found evidence for siderophore production among the *Neisseriae* (R. Finkelstein, personal communication).

Vibrio spp. Payne & Finkelstein (68) found evidence for siderophore production in *Vibrio cholerae,* and Crosa (69) has described a very interesting plasmid-programmed siderophore transport system in *Vibrio anguillarum,* a fish pathogen.

Yersiniae spp. An examination of a number of *Yersiniae* species led to the conclusion that the organisms do not excrete siderophores but that they can use hemin and possess a very efficient, cell-bound system for transport of Fe(III) (66).

Bacteriodes spp. In a study of 17 strains of *Bacteriodes,* a differential inhibitory effect of ethylenediamine-di-(*o*-hydroxyphenylacetic acid) was noted and the results were ascribed to production of chelators of unknown type (J. H. Marcelis, personal communication). Hemin has been identified as a growth requirement in *Bacteriodes* spp. There is still a dearth of information on hemin assimilation by microbes, a subject that should be of considerable interest in medical microbiology.

Staphylococci Production of siderophores was reported in a number of *Staphylococci* (70).

FUNGI The smut fungus, *Ustilago sphaerogena,* grows readily in its spodial stage in laboratory media and was the original source of ferrichrome. Emery (71) demonstrated that the siderophore supplies iron to the cells via a shuttle mechanism. When thoroughly starved for iron, *U. sphaerogena* produces more deferriferrichrome A than deferriferrichrome. Although the former is inactive, the fact that its binding constant is only three times higher than that of deferriferrichrome assures a supply of the transportable chelate via iron exchange.

The siderophores coprogen and ferricrocin, and possibly ferrichrome C, have been found in *Neurospora crassa* where they have been shown to serve as germination factors (4).

All members of the *Penicillia,* including those used to ripen cheese (72), probably synthesize ferrichromes or hydroxamate siderophores of some type.

Ferrichrome has been crystallized from *Aspergillus niger* (J. A. Garibaldi, personal communication) and is a product of *Aspergillus quadricinctus.* In the latter organism, enantio-ferrichrome penètrated the cells at a rate substantially slower than does the natural product (73). In *Aspergillus nidulans,* a role for iron in sporulation competence has been postulated (74).

Fusaria sp. produce siderophores that belong to the fusarinine and ferrichrome classes. Extracts of *Fusarium roseum* ATCC 12822 contain an enzyme that hydrolyzes the ester bonds in fusarinine C; the iron complex is totally resistant. An esterase in *Penicillium* extracts, on the other hand, hydrolyzes ester links in both ligand and the Fe(III) complex, but not in the Al(III) chelate (75).

In *Rhodotorula pilimanae,* the source of rhodotorulic acid, siderophore iron appears to be exchanged into the cell surface (76).

REGULATORY MECHANISMS

Although a detailed understanding of the mechanism of regulation of microbial iron assimilation is lacking, some progress has been made in understanding the process as it operates in the high affinity system in *E. coli.* Several laboratories have observed alterations in tRNA modification under iron deficiency (77, 78). Specifically, lack of iron results in failure to thiomethylate the hypermodified nucleoside 2-methyl-thio-N^6-(Δ^2-isopentenyl) adenosine situated next to the 3' end of the anticodon. The accumulation of isopentenyl-adenosine instead of the thiomethylated product has been noted in $tRNA^{phe}$, $tRNA^{tyr}$, $tRNA^{trp}$, and in few other tRNA's. The degree of thiomethylation is thought to regulate aromatic amino acid biosynthesis via a transcription attenuation mechanism (M. Buck, B. D. McLennan, and E. Griffith, personal communication).

Regulation cannot be dependent on enterobactin synthesis, as mutants unable to make the siderophore fail to express products of the operons when cells are cultured at ca 10 μM iron. There is substantial evidence that the three iron-related outer membrane proteins of *E. coli* K12 are coordinately regulated (59). An *E. coli* strain deficient in enterobactin syntheses and iron starved with deferriferrichrome A was shown to rapidly reduce synthesis of all three proteins upon supplementation with iron (P. Klebba, personal communication). Even at very high iron concentration, it is certain that constitutive levels of the high affinity system are synthesized. *E. coli* is always sensitive to phages and colicins irrespective of cultural conditions,

and ferrichrome can be crystallized from the cells of fungi grown on yeast-extract medium.

In *E. coli* K12, the genes for the enterobactin system are on the chromosome, but in other bacterial species, the genetic program for synthesis of certain hydroxamate-type siderophores may be plasmid-borne.

IRON AND INFECTION

A possible link between iron and infection was indicated when Schade & Caroline (79) demonstrated that iron could overcome the bacteriostatic effect of egg white, i.e. ovotransferrin. Along with the understanding that siderophore production is a common trait among pathogenic microorganisms has come the speculation that iron may be a virulence factor in animal (reviewed in 3, 80, 81) and plant (47) disease. The concentration of free iron in human plasma is of the order of 10^{-12} μM, and it has been assumed that the production of siderophores of either the hydroxamate or catechol types might facilitate microbial growth by removal of iron from transferrin. The ability of pathogenic species to produce siderophores ranges over a number of families and genera and includes some of those known to cause the most serious infections. The unambiguous correlation of iron assimilation with virulence remains to be established, although certain recent experiments tend to support the theory.

Yancey et al (82) showed that inability to synthesize enterobactin diminished both the virulence of *S. typhimurium* in laboratory animals and the capacity of the organism to grow in human serum. Moore et al (83) reported preliminary evidence for the presence of enterobactin-specific immunoglobulin in normal human serum, and Griffiths & Humphreys (84) published chromatographic evidence for the presence of enterobactin in peritoneal washings of guinea pigs injected with a lethal dose, some 10^8 cells, of *E. coli* O111K58HZ. The suffix bactin was incorporated in the trivial name, enterobactin, because the siderophore is the product of a major endosymbiont of man. If the data of Moore et al (83) can be confirmed, then this would tend to favor enterobactin as a more suitable trivial name than enterochelin for the *E. coli* siderophore.

A new dimension was added by Williams (85), who discovered that *E. coli* carrying colicin V plasmids possessed the ability to make an iron sequestering system distinct from that of enterobactin. It had been observed previously that most of the so-called invasive strains of *E. coli* isolated from human and animal infections harbor Col V plasmids. Colicin V itself does not appear to be a virulence factor (86, 87). Recently, an hydroxamate-mediated iron-uptake system was reported to be present in cells and supernatants of Col V^+, but not cured, strains of *E. coli* (88). It has been shown

that *A. aerogenes* and several other enteric bacteria producing aerobactin-type siderophores harbor a plasmid (A. Bindereif, personal communication).

Montgomerie et al (89) found no correlation between virulence (renal infection) and enterobactin production in 20 clinical isolates of *E. coli.* There was an inverse relation between enterobactin production and serum resistance, which could be correlated with rough and smooth strains, with the latter yielding significantly less siderophore.

Crosa (69) has described a plasmid-specified iron-harvesting system in the fist pathogen *V. anguillarum.* Curing experiments established a link between this plasmid, utilization of complexed iron in vitro, and virulence in *V. anguillarum.*

Garibaldi (90) was the first to observe the critical temperature sensitivity of siderophore production, and he suggested that fever might be a host defense mechanism designed to deprive the pathogen of iron. This novel idea seems to have been confirmed by Kluger & Rothenberg (91) with the rabbit pathogen *Pasteurella multocida.* Injection of live organisms was followed by a dramatic fall in plasma iron and elevated body temperature. Febrile temperatures were required for the expression of the inhibitory effect of low iron.

Virulence is undoubtedly dependent upon a multitude of interactions between host and pathogen. The cumulative evidence points to iron as one, if only one, of these factors: In specific circumstances iron assimilation may emerge as the critical element in the host-pathogen interplay. What competitive advantage would be associated with synthesis of an hydroxamate-type siderophore in organisms empowered to form enterobactin, the latter a thermodynamically more potent chelator? Enterobactin is chemically unstable, suffering both oxidation and hydrolysis. Its affinity for Fe(III) fades rapidly as the pH is lowered below neutrality. Enterobactin is not very water soluble, and its aromatic character causes it to adhere to proteins and to act as a haptene for generation of antibodies. Antibodies may also be raised to the surface receptor for ferric enterobactin. An hydroxamate-type siderophore such as aerobactin suffers from few of these defects, and its capacity to complex Fe(III), although inferior to that of enterobactin in the pH range 7.0–7.5, is nonetheless still within striking distance of that of iron transferrin.

CONCLUSION

Although a relatively primitive organism in the genetic sense, *E. coli* is equipped with multiple pathways for the assimilation of iron. This emphasizes once more the absolutely crucial role played by iron in biology. In

E. coli and related enteric bacteria, the iron uptake pathways can be divided into low affinity and high affinity, the latter involving specific carriers, the siderophores, and an obligatory, membrane-bound transport apparatus. The relatively insensitive, nonspecific, low affinity system occurs side-by-side in *E. coli* with the high affinity systems for uptake of the endogenous siderophore, enterobactin, for ferrichrome, which is a fungal siderophore, and for iron citrate.

The ease of generation and isolation of mutants of *E. coli* has resulted in substantial knowledge of the mechanism of action of the high affinity iron transport process in this organism. At low iron growth (ca <1.0 μM), the genetic complement of the cell senses the iron deficiency and responds by enhanced synthesis of both siderophore (enterobactin) and surface receptor for ferric enterobactin. The complexed Fe(III) is taken into the cell and the metal ion is liberated by a process that involves reduction and/or hydrolysis of the ligand. Although separate receptors exist for ferric enterobactin and ferrichrome, transport of these two siderophores in *E. coli* has much in common. The siderophore receptors serve as binding sites for an array of specific bacteriophages and bacteriocins.

In procaryotic and eucaryotic microbes, as well as in plants and animals, the assimilation of iron is regulated at the membrane level, since no biological mechanism exists for the excretion of this vital element. Among the procaryotes, *E. coli* is the organism of choice and the model for studies of the regulation of iron transport. Synthesis of the catechol-type siderophores in *E. coli* (enterobactin) and in *A. tumefaciens* (agrobactin) is programmed on the chromosome. Although hydroxamate-type siderophores occur most commonly in fungi, they are also produced by a few bacteria, but not by *E. coli* K12 or *S. typhimurium* LT-2, and it may be the case that plasmids are required for hydroxamate synthesis in bacteria. The number and order of genes in the iron operon of *E. coli* centered at min 13 on the chromosome needs to be established with greater precision. Ferric enterobactin itself can be excluded as a regulatory substance. Although a certain constitutive level of the high affinity system is produced at even saturating concentrations of iron, mutants lacking all of the system and dependent only on the low affinity uptake pathway are not impaired in growth on complex media.

There is now substantial evidence that the chirality around the siderophore complexed Fe(III) determines recognition of the complex by the specific surface receptor of individual bacterial species. Microorganisms have thus employed a characteristic steriochemistry within the siderophore ligand in an attempt to solubilize and monopolize environmental Fe(III), which tends to be abundant but nonetheless biologically unavailable.

The high affinity pathway of iron assimilation appears to be unique for Fe(III) and has not been found for other essential metal ions. Possibly this

is because of several factors, such as the intense insolubility of Fe(III) at biological pH, the significantly higher requirements for iron as opposed, for example, to copper, and the fact that the usual divalent metal ions require nothing more sophisticated than an α-amino carboxyl group to act as chelation center.

Additional evidence has been adduced in support of the theory that iron is a virulence factor in bacterial diseases of animals. The most rational interpretation of the data is that the capacity of the invading pathogen to obtain iron from living tissue is one of a great many factors involved in the host-parasite relation.

Iron assimilation routes in microbes, plants, and animals share several common features in addition to the repression-regulated mode of initial uptake. It is the fond, if perhaps naive, hope of those working with bacteria that experiments here will afford at least an outline of the path of iron in all cells.

Iron may be solubilized by acidification and reduction, as well as by chelation. The amazing versatility of bacterial and fungal metabolism suggests that additional novel iron harvesting mechanisms remain to be discovered in the microbial world.

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