Molecular Mechanism of Regulation of Siderophore-Mediated Iron Assimilation

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INTRODUCTION

This review describes a molecular mechanism for regulation of one pathway of microbial assimilation of iron, an element which may well be a nutrient required by all living cells. Unlike organic compounds, minerals cannot be synthesized and must be acquired from the environment. In contrast to amino acids, fatty acids, and monosaccharides, all of which can be accumulated in the form of relatively innocuous polymers, living tissues in general seem to have little capacity to store metal ions and hence organisms prefer to limit the uptake at the membrane level. This concept, as it applies to iron, is firmly rooted in the pioneering work of McCance and Widdowson who, according to Underwood (55), concluded that the amount of the element "must be regulated by controlled absorption."

We have lately come to recognize that there are special reasons why aerobic and facultative anaerobic organisms should carefully meter the amount of iron taken up. This is because surplus iron may find its way into coordination sites where it can generate oxidizing radicals. While the following equation may not do full justice to all of the complications inherent in Haber-Weiss-Fenton chemistry, they do nonetheless illustrate the propensity of iron (18) to catalytically promote hydroxyl radical production from superoxide and peroxide, species which inevitably arise from partial reduction of molecular oxygen: \( O_2^- + Fe^{3+} = O_2 + Fe^{2+}; 2O_2^- + 2H^+ = H_2O_2 + O_2; H_2O_2 + Fe^{2+} = Fe^{3+} + OH^- + OH^- \).

The human body contains 4 to 5 g of iron, of which amount roughly three-quarters is in hemoglobin and virtually all of the remainder is stored in the liver as ferritin. This leaves only trace quantities for enzyme and transport forms of iron.

An internal cycle in which iron is salvaged from the spent erythrocytes, temporarily stored in ferritin, and called forth for a new round of hemoglobin synthesis operates with remarkable efficiency, only about 1 mg of iron being required per day to maintain homeostasis. For practical reasons, however, an intake of magnitude greater than this is needed to fend off anemia, now a prevalent nutritional ailment in the United States and one which extends across all socio-economic boundaries. Iron enters all cells of the body as transferrin, a glycoprotein normally about one-third saturated with Fe(III). Like the ovotransferrin of egg white, serum transferrin is in the first line of defense against sepsis. Transport of iron transferrin is dependent on a surface receptor consisting of two 93-kilodalton (kDa) disulfide-linked proteins. The level of synthesis of the receptor is inversely correlated with the iron supply, and there is some recent evidence to indicate that regulation occurs at the transcriptional stage (46). That there is no known mechanism for excretion of iron highlights the importance of regulation at the port of entry, which in the human is the upper portion of the small intestine.

The reader is referred to a current lucid and comprehensive review on iron transport and storage (12), to which we add a recent report on the structure of human lactoferrin at 0.32-nm resolution (1). Probably all three transferrins have similar Fe(II)-binding centers which, in the case of lactoferrin, consist of one histidine, two tyrosines, one aspartic acid, bicarbonate, and water.

Having just sketched the outlines of mammalian iron metabolism and its regulation, it may now be instructive to recount a few of the known salient roles of iron in microbial physiology. Iron is a constituent of all heme enzymes, which includes the cytochromes and hydroperoxidases. The common type of ribonucleotide reductase contains iron, as do the diverse forms of iron sulfur proteins. Nitrogenase, whether of the molybdenum or vanadium (48) variety, requires an iron protein in a complex carrying a complement of

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over 30 atoms of iron. Given these many crucial and very fundamental roles of iron, we can be sure that the element is needed by all microorganisms, save those with the most unusual nutritional requirements. Certain lactobacilli, species devoid of heme and containing the cobalt form of ribonucleotide reductase, grow well in milk. As a secretion, this fluid is low in total iron and the trace amount present is largely unavailable owing to the presence of lactoferrin. It has so far been impossible to demonstrate an iron requirement for certain lactobacilli (2).

Total iron is generally abundant in the environment, most soils containing several percent by weight. Much lower levels will be found in both fresh and salt water. In biological fluids the concentration is limited to ca. $10^{-18}$ M by the vanishingly low value of the solubility product constant of ferric hydroxide, $<10^{-38}$ (B. Schwy, Ph.D. dissertation, Eidgenossischen Technischen Hochschule, Zurich, Switzerland, 1983).

To combat the low solubility of external iron, those bacterial and fungal species obliged to spend all or part of their time in an aerobic environment have evolved a line of low-molecular-weight carriers genetically termed siderophores (34). These compounds are marked by an extreme affinity, and hence specificity, for the trivalent oxidation state of iron. Their efficient absorption at low concentrations is achieved by receptors in the cell envelope. Ferrichrome, which was isolated in 1952 (37) and for a variety of reasons, of which the principal one is the fact it has been consistently studied as an iron carrier, is generally regarded as the prototypical siderophore. It is commonly encountered in fungal species and is a product of all penicillia. Chemically, it is not the first natural product shown to contain the hydroxamic link and catechol-type siderophores (39). Ferrichrome, although a potent growth factor for certain fungal (Pilobolus) and bacterial (Arthrobacter) species, did not seem to have the properties expected of a redox coenzyme. Upon reduction with dithionite, iron was readily cleaved from the molecule. Ferrichrome was originally isolated from a strain of the smut fungus Ustilago sphaerogena which displayed the quirk of overproduction of cytochrome c, a low-molecular-weight protein relatively rich in iron. The nutritional defect in the natural auxotrophs requiring chelated iron could be overcome by various compounds, the only apparent common property of which was a propensity to bind Fe(III). Finally, the enhanced excretion of specific ligands for Fe(III) at low iron growth pointed strongly to a scavenger and transport role for ferrichrome. Three decades ago, one of us was sufficiently emboldened by the evidence to propose in a review scribbled for this journal that ferrichrome and related compounds could act as "transfer iron in microbial metabolism" (38). The iron carrier role of these compounds could be demonstrated genetically when mutants of Salmonella typhimurium LT2 defective in synthesis of enterobactin (enterochelin) could be made to grow on high-citrate media to which an excess of iron had been added (44). S. typhimurium, unlike Escherichia coli, lacks a system for uptake of ferric citrate, and hence a high-affinity carrier such as enterobactin or ferrichrome must be furnished when such mutants are cultivated on citrate as carbon source.

In 1973, Lankford (34) prepared a now classical review on bacterial iron nutrition in which he proposed the term siderophore to replace the earlier designations siderochrome, sideramine, and sideromycin. In the following year, the edited tome Microbial Iron Metabolism made its appearance (40). While these publications underlined the importance of high-affinity iron transport in microbial species, they emerged just prior to the development of the battery of powerful tools of molecular genetics now available for investigation of metabolic phenomena at a very basic level. We report here a molecular mechanism for mediated iron assimilation in K-12 strains of E. coli. Although the transport of ferric aerobactin is just one of many high-affinity iron transport systems available to E. coli, it is at present the best understood in terms of its regulation by iron. The proposed regulatory mechanism is probably shared by other transport systems in E. coli. The extent to which this regulatory circuit is retained, modified, or replaced in other bacterial and fungal species remains to be determined. Whether or not this model can be applied more generally to those plants and animals which have superficially similar high-affinity iron transport systems is still unknown.

The relevance of microbial iron assimilation to medical problems has been reviewed by Weinberg (60) and is the subject of a recent book (9). Agricultural aspects of the subject have been summarized in a recent monograph (52).

SIDEROPHORE SYSTEMS OF E. COLI

Multiple High-Affinity Iron Uptake Pathways

Figure 1 lists some of the better-characterized high-affinity iron uptake systems, and the genes required of which are known to exist in E. coli. For a more complete description of the bewildering variety of pathways for assimilation of iron in this organism, the reader is referred to a recent review (8). Substantial effort has been expended on the enterobactin system, and it is gradually yielding to analysis by the methodology of molecular genetics (16). E. coli, like most bacterial species, has a highly efficient transport system for ferrichrome, which, although apparently not synthesized by the organism, is a common siderophore among fungal species. Citrate is a relatively poor chelating agent for Fe(III), a concentration of 20-fold excess ligand being required to form the mononuclear complex. It is listed as a high-affinity carrier since an outer membrane receptor system is required, although, in contrast to what is the usual case, this receptor in E. coli must be induced by the chelate. Of the systems given in Fig. 1, the aerobactin cluster occurring on PColV-K30 is the one most thoroughly analyzed in terms of overall organization of the operon, identification and function of the gene products, and regulation of the complex (see below).

It must be stressed that all siderophore systems, citrate not excepted, require participation of membrane components in addition to specific receptors. These include the tonB gene, which, however, is also needed for uptake of vitamin B12 and sensitivity to various lethal agents. In some siderophore systems the intact chelate apparently penetrates to the cytoplasm, while in others the iron is merely delivered to the cell envelope. In all cases, the Fe(III) is believed to be released by a still poorly understood reductive step (41).
Aerobactin as a Virulence Determinant

In 1979, Williams (61) reported the presence of a novel hydroxamate type of siderophore in certain pColV-containing clinical isolates of E. coli. The isolates were characterized by their capacity to cause disseminating infections in experimental animals. These workers also showed by genetic techniques that the virulence attribute could be assigned to the unusual siderophore rather than to the bacteriocin colicin V or to enterobactin, the siderophore indigenous to practically all enteric bacteria. Although citrate-hydroxamate-type siderophores had been isolated from Arthrobacter species (terregens factor, arthrobactin) as well as from Bacillus megaterium and Anabaena sp. (schizokinen), the only enteric bacterium known to form a hydroxamate of any type was Aerobacter aerogenes 62-I, from which Gibson and Magrath (22) had isolated aerobactin in 1969. The hydroxamate of E. coli pColV-K30 was hence suspected to be aerobactin, and this was proven to be the case through use of field desorption mass spectroscopy and other techniques (58).

Comparison of the stability constants of aerobactin and enterobactin (Fig. 2) for Fe(III), $10^{23}$ and $10^{52}$, respectively, posed a question as to why a siderophore with a relatively feeble affinity for ferric iron should have been selected for coding of pColV. First, we should stress that those constants refer to the completely deprotonated ligands (47). The $pK_a$ of the ring hydroxyls of enterobactin are difficult to measure accurately because of oxidative and hydrolytic instability of the ligand, but the value for the 2-hydroxyl is probably between 7.5 and 8.0, while that for the 3-hydroxyl is substantially higher. Thus, like pyrocatechol itself, enterobactin loses much of its affinity for ferric iron as the pH approaches neutrality and fails to complex the metal in faintly acid solution, where the coordinated metal tends to be reduced by the ligand (27). At neutral pH enterobactin is only marginally superior to aerobactin as regards its thermodynamic stability with ferric iron, and the latter siderophore does have the ability to remove the metal when it is complexed to transferrin (30). Enterobactin deferrates iron transferrin at a faster rate than aerobactin when the reaction is run in HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid) buffer; in the presence of serum albumin, however, the rates are reversed. Clearly, the aromatic character of enterobactin causes it to adhere to proteins, and this is probably a major reason why it has not been adopted as part of the virulence armamentarium of E. coli (31). However, the presence of enterobactin is doubtless to be preferred over the complete absence of all siderophore systems.

At the time this work was done our Australian colleagues had already performed a preliminary analysis of the enterobactin gene complex mapping at min 13 on the E. coli chromosome (32, 33). From their data it was apparent that the complex extends over more than 20 kilobases (kb) of

FIG. 1. Relatively well-characterized envelope components of some high-affinity iron transport systems in E. coli K-12 (8). The organism is equipped to utilize an array of ferric hydroxamate-type siderophores such as ferrichrome, coprogen, and ferric rhodotorulate, all of which are products of other microbial species.

FIG. 2. Structures and thermodynamic stability constants of the iron(III) complexes of enterobactin and aerobactin.
deoxyribonucleic acid (DNA) and is organized into several transcriptional units. We therefore turned to a study of the aerobactin system of pColV-K30 in the expectation that it would be simpler in constitution and thus more readily lend itself to genetic analysis. The main objective was a deeper understanding of the intimate mode of regulation by iron.

**AEROBACTIN OPERON OF pCOLV-K30**

Cloning of the Aerobactin Operon

Following the demonstration that the ability of *E. coli* to transport ferrichrome and its sensitivity to a group of lethal agents including colicin B (23), with the establishment of a similar correlation between aerobactin transport and sensitivity to colicin (56), a bacteriocin produced by *Enterobacter cloacae*, the way was paved for cloning the aerobactin operon by screening for acquired sensitivity to colicin (4). Ironically, the tonA mutant of *E. coli* used (35) to demonstrate spontaneous mutations in bacteria we now know to lack the 78-kDa outer membrane ferrichrome receptor.

pColV-K30, a plasmid approximately 100 kb in length, was isolated and digested with HindIII. The resulting fragments were ligated into the HindIII site of the multicopy vector pGL102 and used to transform *E. coli* strain 294 to ampicillin resistance. Colonies were then screened for sensitivity to colicin to identify clones carrying the gene for the 74-kDa ferric aerobactin receptor. Fortuitously, the same 16-kb fragment that encodes the receptor was also found to carry the aerobactin biosynthetic genes and their cis-acting regulatory elements. A subclone, carrying approximately the left half of the 16-kb insert, expressed only the determinants for aerobactin biosynthesis. It seemed likely from these findings that the *EcoRI* site used to split the larger insert occurred in the receptor gene and that the entire aerobactin biosynthesis and transport complex was packaged on not more than 7-8 kb of DNA. Considering the structure of aerobactin, a minimum of three proteins should be required for its synthesis, namely, an oxygenase, an acetylase, and a synthetase. Assuming 2 kb of DNA needed to encode the 74-kDa receptor, an absolute minimum of 5 kb would be anticipated for an operon. A preliminary analysis with minicells suggested the presence of about five polypeptides in the aerobactin gene cluster (7).

**Organization of the Aerobactin Operon**

Several years of experimentation had to be invested in sorting out the various biosynthetic genes, assigning a function to each, and determining the *M* on gels (13). The work was plagued by two major difficulties. Deletions early in the complex led to strong polar effects, thus rendering it difficult to detect expression of downstream transcription. The second difficulty centered on the product of the third gene, which proved very elusive on gels. This protein, one subunit of the synthetase, moves with a mobility almost identical to that of the other subunit (13). We have retained the original abbreviation for the aerobactin genes, namely, *iuc* (iron uptake chelate) and *iut* (iron uptake transport). The molecular weights shown in Fig. 3 refer to sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and the values are hence only approximate. The *iucA* protein has been isolated (42) but not further studied. The product of *iucB*, the acetylase, has been purified to homogeneity. The protein is strongly expressed in our clones and displays a strong affinity for Reactive Blue Sepharose, a property which greatly facilitated its isolation (11). The enzyme is not entirely specific for *N*-hydroxylysine but will acetylate a number of other hydroxylamines including, to some degree, organic hydroxyamine. It does not, however, react with free lysine. The third gene, *iucC*, codes for the second subunit of the synthetase. This protein has not been isolated. The final biosynthetic gene, *iucD*, encodes an oxygenase. The gene has been sequenced (M. Herrero, personal communication), from which it can be inferred that the product has several hydrophobic regions. The enzyme has been obtained in a cell-free particulate state but has not been further characterized and no cofactors have been positively identified (57). Enzymes of this type generally require heme, iron, flavin, or copper. Evidence obtained by use of inhibitors of thiamine pyrophosphate-dependent decarboxylation reactions suggests that pyruvate both acts as a source of reducing equivalents for hydroxylation of lysine and provides the actyl moiety of the product (53). This enzyme is definitely the most interesting one in the pathway and should be the target for any nostrum or “magic bullet” designed to knock out aerobactin synthesis. Hydroxylamines have not been found in animal tissues, and hence a nontoxic specific inhibitor of their biosynthesis might have considerable therapeutic value.

Although most workers agree that the reaction sequence should be *iucD*, - *B*, - *A*, - *C* (Fig. 3), one report claims that acetylation precedes oxidation (19). This is difficult to reconcile with the observation that the isolated acetylase uses only *N*-hydroxylysine and not lysine as substrate (11) and with the finding that a plasmid containing as insert only gene *iucD* produces *N*-hydroxylysine (Herrero, personal communication). Similarly, a hydroxyamine derivative, presumably *N*-hydroxylysine, arises from insertional mutagenesis in acetylase gene *iucB* of the closely related ColV plasmid K311 (8).

The biosynthetic pathway from lysine and citrate to aerobactin is shown in Fig. 4.

**REGULATION OF THE AEROBACTIN OPERON OF pCOLV-K30**

**Characterization of the Promoter**

The promoter was located by S1 mapping and shown to have two start sites, a strong one detected both in vivo and in vitro and labeled P1 and a weaker one, designated P2, only

![FIG. 3. Organization of the aerobactin operon of pColV-K30. Transcription proceeds from left to right from a promoter (P) and operator (O) sequence. Four *iuc* (iron uptake chelate) genes are required for biosynthesis of aerobactin. The outer membrane receptor for ferric aerobactin is coded by gene *iutA* (iron uptake transport), an integral member of the operon.](http://mmbr.asm.org/Downloadedfrom"http://mmbr.asm.org")

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**References:**

detected in vitro and located about 50 base pairs (bp) further upstream (Fig. 5) (6). P1 is a typical strong, near-consensus *E. coli* promoter in which the −10 and −35 boxes are separated by the optimum 17 bp. The start site for the transcript of P1 was established by sequencing the messenger ribonucleic acid (RNA). The putative ribosome-binding sequence AGGAGCTG is separated from the first codon of *iucA* by 6 bp. Inspection of Fig. 5 shows that several inverted repeat and palindromic sequences, analyzed in more detail elsewhere (A. Bindereif, Ph.D. dissertation, University of California, Berkeley, 1984), are especially prevalent between the −10 and −35 regions of P1.

To confirm that this promoter region is the site of regulation by iron, a protein fusion was prepared in which lacZ, minus its promoter, was ligated in frame through a three-codon linker to the sixth base of *iucA*. The resulting protein fusion was found to induce the expression of β-galactosidase to a level of about 1,200 U per absorbancy unit at 600 nm when the cells were deprived by addition of excess bipyridyl to scavenge iron. The relatively great strength of the aerobactin promoter enabled application of a novel quantitative S1 protection assay to prove that regulation occurs at the transcriptional level. Protection of the labeled probe at low, intermediate, and high iron gave a radiographic band of decreasing intensity, as would be expected for a progressive loss of a specific RNA as iron is added (6). Thus, transcriptional regulation of the level of specific RNA, which had previously been inferred for the iron-regulated *cir* gene (63), was established for the aerobactin operon by the most rigorous techniques available. Because the aerobactin::lacZ fusion, whose transcript includes only six bases of aerobactin sequence, is also regulated by iron, we can safely assume that the level of aerobactin RNA is regulated at the level of transcription and not by specific degradation in the presence of high concentrations of iron (6).

**Regulation of Aerobactin Gene Expression by a Chromosomally Encoded Factor**

The regulation of chelate-mediated iron transport in bacteria and fungi by the availability of iron was first suggested in 1956 (21); subsequently, it was proposed that there might exist an iron-binding repressor protein which, when intracellular concentrations of the element are high, inhibits the expression of gene clusters required for siderophore synthesis (39). The first indications of the viability of this hypothesis were not to arise until 1978, when Ernst et al. (17) in a study of periplasmic leaky (lky) mutants of *S. typhimurium* fortuitously isolated an unrelated secondary mutation which constitutively overexpressed iron-regulated outer membrane proteins. The mutation, termed *fur* (ferric uptake regula-
FIG. 6. Relative linkage of genes in E. coli K-12 which are negatively regulated in global fashion by a combination of the product of the fur gene and iron. The aerobactin determinants are here shown on pCoLV-K30 but may also occur on the chromosome (9). An additional gene mapping at min 99 appears to be required for uptake of ferrioxamine-type siderophores (8).

characteristic, namely, an inability to grow on certain nonfermentable carbon sources. The growth characteristics of a fur+ and fur isogenic pair in an E. coli JS053 background under aerobic and anaerobic conditions are shown in Table 1. At least in the case of succinate, the defect appears to be localized to transport of the substrate. By using the O2 electrode, it could be shown that broken cell preparations, unlike whole cells, are able to oxidize succinate. The fur gene may occupy the same locus as dciB, which has been loosely mapped to 17 min and which is believed to be involved in succinate transport.

fur Gene Product

To study the mechanism of action of the product of the fur gene, it was considered necessary to acquire the pure protein so that studies could be performed in vitro. While Fur could have been assumed to act as a classical repressor, binding iron as corepressor, a number of additional scenarios are possible. The complex could bind to RNA polymerase and alter its specificity for interaction with operators of the iron-affected genes and operons. It could be a positive regulator of a de facto repressor or a negative regulator of some type of activator protein. All of these factors would require additional genetic elements, and so far only the single fur locus has been described. However, it is also possible that the mutation of additional genes involved in iron transport could be lethal.

Access to the Fur protein would enable us to determine both the oxidation state and the specificity of the activating metal ions, the need for additional cofactors, if any, and the identification of the operator sequences of the aerobactin promoter.

To produce a Fur overexpression vector, the gene was recloned in our laboratory (A. Bagg, Ph.D. dissertation, University of California, Berkeley, 1986). We constructed a cosmids bank of 800 E. coli (DH1) colonies each carrying an approximately 23-kb insertion in pLAFR (20) of chromosomal DNA isolated from the fur::Tn5 strain BN4020. Colonies which carried the mutant locus were identified as kanamycin resistant, and the wild-type fur gene was recovered by homologous recombination after mating the cosmids into a recA+ fur+ background.

Subcloning of the fur gene from a 22 to a 2.6-kb fragment was straightforward since comparison of the restriction maps of the fur::Tn5 and fur+ plasmids revealed the location of the fur gene. Fine restriction mapping indicated that a 2.6-kb clone, designated pF3, overlapped the fur clone previously isolated by Schaffer et al. (49). In maxicell analysis, pF3 was observed to encode two polypeptides, a

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* Growth was measured by the appearance of colonies on plates after 3 days at 37°C. GasPak jars (BBL Microbiology Systems, Cockeysville, Md.) were used to create an aerobic environment (Bagg, Ph.D. dissertation).
highly expressed 21-kDa component and a weakly expressed 17-kDa polypeptide. Mutational and maxicell analysis revealed that the two proteins were coded by separate genes and that only the 17-kDa member was required for complementation of fur::Tn5.

Following the demonstration that pF3 codes for Fur, the plasmid was made available to our colleagues Bruce Hemming and Michael Bittner at the Monsanto Chemical Co. in St. Louis, Mo., who ligated the fur gene to the inducible recA promoter to afford pMON2064. This construction was returned to us by the Monsanto workers and has served as a highly effective source of pure Fur protein. Sechan Wee of this laboratory has worked out a one-step affinity column purification procedure for Fur in which the protein is bound to cleavage-iminodiacetate-agarose and subsequently eluted with histidine.

Identification of the Fur Protein as a Repressor

That the fur mutant, which leads to constitutive derepression of the iron-regulated systems of E. coli, is a loss-of-function, i.e., recessive, mutation indicates that the wild-type gene product should be a negative regulator of gene expression. One obvious possibility is that Fur binds iron, or some derivative of iron, which then attaches reversibly to the operator sequences to effect negative regulation of transcription. To reconstruct this system in vitro it was deemed convenient to test Fur for acceptance of some metal ion other than iron. This is because ferrous salts are rapidly oxidized and precipitated at neutral pH while, as already noted, ferric ion is quantitatively insoluble under these conditions.

To survey the metal ion specificity of Fur, a preliminary experiment was performed in vivo. The lacZ operon fusions were grown up in minimal medium and then treated with increasing levels of a series of divalent metal ions (3a). The results with Mn, Fe, and Co, all added in the divalent state, were clear-cut and indicated that any of these could repress the expression of the β-galactosidase gene placed under control of the iron-sensing aerobactin promoter. Other metals gave negative or ambiguous results. The activity with Mn was gratifyingly similar to the divalent form of this ion is the most common and most stable oxidation state.

As an in vitro extension of this work, we set up a transcription-translation system, using template DNA from the operon fusion, and measured the level of β-galactosidase generated after addition of Fur and metal ions. Specific regulation of the aerobactin operon by Mn(II) was observed, but the addition of iron salts completely and nonspecifically inactivated in vitro transcription-translation from both has and aerobactin promoters. Eventually, it was found that rigorous exclusion of oxygen was necessary when iron salts were introduced. This suggested that the biologically active metal is Fe(II). For routine measurements of repressor activity Mn(II) is a more convenient activator, since in this case anaerobic conditions are not required. In this experimental arrangement the isolated, homogeneous Fur protein, purified 3.5-fold from a crude extract of an E. coli strain carrying pMON2064, was found to be 3.4-fold more active than the unpurified extract. Thus, it does appear that it is the fur gene product itself, and not some other protein induced by Fur (or coincidentally with overproduction of Fur), that represses aerobactin expression in our in vitro transcription-translation system. The Hill plot generated from the in vitro data and shown in Fig. 7 suggests that the Fur protein is acting as a dimer in its interaction with iron or the aerobactin promoter. This same cooperative interaction has been demonstrated by Braun et al. (8), who have observed that E. coli strains heterozygous for both the wild type and certain mutant alleles of fur exhibit a Fur" phenotype.

Although the exact sequence of the aerobactin operator (or indeed any operator of iron-controlled genes) has not been genetically defined, we do know that a 152-bp Sau3A1 fragment of the aerobactin operon (Fig. 5) is sufficient to provide regulation by iron of a downstream iucC::lacZ protein fusion. A Hinfl site is located between the −10 and −35 regions of the aerobactin promoter (Fig. 5). We reasoned that the Fur protein, if it is acting as a repressor which blocks access of RNA polymerase to the promoter, might also inhibit the binding of Hinfl to this same region. Restriction site protections has been used previously to assay Trp repressor activity (28). In a typical experiment, a plasmid constructed by V. de Lorenzo of this laboratory and carrying the aerobactin operator is precipitated with purified Fur in the presence of a divalent metal "activator" or the chelating agent ethylenediaminetetraacetic acid. This mixture is then subjected to digestion with Hinfl. When this plasmid is completely digested with Hinfl, the fragments produced include the 1,448- and 220-bp fragments which make up the promoter region. In the presence of Fur plus activator, these fragments remain fused to produce a 1,648-bp band, while all other Hinfl sites are digested to completion. With this simple assay we were able to show that a freshly prepared FeSO₄ solution, as well as solutions of the divalent salts of Mn, Co, and Cd, activated Fur to specifically protect the Hinfl site at the aerobactin promoter from digestion (3a). Fur also requires Mg²⁺ in millimolar concentrations, in conjunction with micromolar amounts of one of the above metals, to protect the aerobactin promoter from digestion; Mg²⁺ alone cannot activate Fur to bind DNA. Aluminum, a trivalent cation, did not enhance binding of Fur to the promoter.

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**FIG. 7.** Hill plot of activity of the Fur protein in repression of in vitro synthesis of β-galactosidase from a lacZ fusion in the aerobactin operon as a function of concentration of the repressor protein (Bagg, Ph.D. dissertation). The slope of 2 is consistent with the hypothesis that the repressor acts as a dimer.
Operator Sequences Bound by the Ferrous Fur Complex

The experiments just described demonstrate convincingly that Fur is a DNA-binding protein and that it requires a divalent heavy-metal ion as activator. Moreover, evidence was adduced to show that the repressor contacts the pro-
moter of the aerobactin operon at a site close to the −35 box. These data did not allow a more precise definition of the operator.

It will be recalled that in vitro transcription of the aerob-
bactin promoter indicated the presence of major and minor start sites, P1 and P2, respectively. To rule out any role of P2 in the regulatory mechanism, de Lorenzo et al. (14), working from the published sequence through this region (6), con-
structed a series of fusions with lacZ which contained the entire P1 through P2 region down to a small insert carrying only the −10 and −35 sequences of P1. Maximum expres-
sion of β-galactosase was found to be vested in this region. A fragment carrying this region was end labeled, and the metal ion-dependent ability of Fur to protect against deoxyribonu-
clease I was measured (14). The results showed that in the absence of an activating metal ion Fur has, at least in these in vitro studies, very little capacity to bind the operator. The active metals included Mn(II), Fe(II), Co(II), and Cd(II); Zn(II) was only partially effective and at higher concentra-
tions interfered with the activity of Mn(II).

This study also provided evidence regarding the operator sequences binding the metal ion-activated Fur repressor. At excess Mn(II) concentration, increasing the level of Fur first protected a set of sequences bracketing the −35 region, designated a primary binding site, and then an additional, or secondary, binding site located just downstream of the −10 region. The primary and secondary binding sites in common the sequence ATAAATTnnnnATTA. With only two mismatches, a corresponding sequence occurs in the segment immediately upstream of the fur gene itself. A closely related array of bases is present in the putative promoter regions of the published sequences of two other iron-repressor genes, fhuA and fepA, which code for the ferrichrome and ferric enterobactin receptors, respectively. From these data a palindromic “iron box,” 5'-GATAAT
GATAATTCATTAC, was suggested as the sequence recog-
nized by the ferrous Fur complex.

CONCLUDING REMARKS

This laboratory is interested in the mechanism of iron assimilation, particularly the regulation of this process, in all life forms. We use microorganisms as experimental subjects because of the plasticity of their metabolism and the ease of application of genetic techniques. The extent to which the molecular mechanism for regulation of the particular path-
way of bacterial iron absorption described here can be applied to other species can only be speculated. It may be significant that cobalt and manganese poisoning mimics iron deficiency in Neurospora crassa (26). Excessive concentra-
tions of manganese and other salts precipitate iron deficiency in oat plants (54). Finally, rabbits fed a diet high in manga-
nese were observed to develop anemia, the latter apparently a consequence of impaired uptake of iron (36).

It is apparent from the data summarized in this review that Fur is a negative regulator of expression of siderophore-
mediated iron assimilation and that it is a repressor which binds Fe(II) as corepressor. To monitor fluctuations in the iron level of the cell, Fur should have properties of a metal-activated rather than a metalloprotein. These two classes of metal-binding proteins are distinguished on the basis of their affinities and specificities for metal ions. Although intermediate types are known, such as β-
aminovalinate dehydratase (10), the former are generally isolated in the metal-free state and will accept a range of metal ions as activators, while the latter are obtained bearing a stoichiometric content of a specific metal ion. The metals we have found to activate Fur in vitro are unlikely to be present in E. coli in sufficient quantity to interfere with iron in vivo. The exception is Zn(II), which represses generally with Fur. Within the cell, zinc is probably mainly present as a structural element in metalloproteins.

The finding that our fur mutant cannot be cultivated on certain nonfermentable carbon substrates was quite unex-
pected and remains completely unexplained at the present time (Bagg, Ph.D. dissertation).

We have not yet identified the Fe(II)-binding sites in Fur or the precise nature of the contacts between the Fur-Fe(II) complex and the operator. These data are most accurately obtained by an X-ray diffraction study. However, consider-
ing the particular metals which are active it seems likely that they fit into an octahedral coordination site formed by disposition in space of six O, N, or S donor atoms. The affinity of Fur for Fe(II) has to be large enough to confer specificity without, at the same time, resulting in a denial of iron to other essential ferrous iron-activated enzymes and proteins within the cell.

A computer analysis of the Fur sequence by use of various programs reveals the potential for a substantial degree of alpha-helical structure. This is to be expected for DNA-
binders repressors, which typically exhibit a helix-turn-helix motif (45). There is no obvious Ala-x-x-x-Gly-x-x-x-x-x-
Val/Ile array for the proposed consensus between helic regions of repressors, although it may be that the metal can substitute for this sequence. There is in Fur a sequence around the single methionine residue which is chemically (size, hydrophobicity, charge) related to a consensus se-
quence in sigma factors. The significance of this homology, if any, is as yet unclear.

An important conclusion from this work is that the interior of E. coli must be highly reducing in character and should contain a fluctuating level of free hexaaquo-ferrous ion or loosely coordinated Fe(II). In striking contrast to the case with Fe(III), ferrous salts are soluble to the extent of 100 mM at pH 7 in the absence of oxygen. Significantly, the oxygenase enzyme coded by the aerobactin operon appears to be associated with the membrane. The data predict that the respiratory enzymes of the cytoplasmic membrane operate with substantial efficiency, resulting in a constant deficit of oxygen in the cytosol. Our results support the views advanced by Williams (62), who predicted that free Mn(II) and Fe(II) could act as intracellular controls. While we have demonstrated that only the Fur repressor plus ferrous iron are required for regulatory activity, there may be some factor(s) which renders the system more efficient in vivo.

Such factors, if they exist, have so far eluded detection by the methodology of genetics.

More work needs to be done on the forms and content of iron in E. coli. The data of Archibald (2) suggest that the level of total iron can vary between 77 and 890 μM, depending on the conditions of growth, which corresponds to 109 and 1010 atoms per cell. Obviously, we are in most acute need of an effective method to monitor the level of free or loosely bound ferrous iron. Heme- and siderophore-
bound iron has previously been eliminated from the regula-
tory circuit (29). Bacteria can store iron and accumulate the
element in a protein with the Mossbauer spectrum of ferritin (51). This protein, however, contains heme and has enzymatic activity. Its role in iron metabolism is unclear at present.

We do not know the extent to which the regulatory model offered here for the combination of the aerobactin operon of pColV-K30 in E. coli K-12 can be applied to other strains and species. The aerobactin cluster in clinical isolates of E. coli is not confined to large plasmids but occurs commonly on the chromosome (5). The cloned aerobactin elements of pColV-K30 appear to be poorly regulated by iron when the cluster is transformed into Shigella flexneri (43). Apart from the product of gene iucC, which could not be detected, the structure and organization of the aerobactin operon of pColV-K311 have been shown to be very similar to those found in pColV-K30 (8).

In regard to the level of Fur, it is noteworthy that the putative promoter region of its gene exhibits a significant degree of homology with sequences in promoters of genes negatively regulated by the Fur repressor. Thus, the unrealistically simple regulatory circuit we describe here, which varies but little from the hypothetical version proposed many years ago (39), may be complicated substantially by factors regulating expression of the fur gene, a subject about which we know nothing at the present time.

SUMMARY

The aerobactin-promoted iron assimilation system coded on plasmid ColV-K30 of E. coli has been shown through application of standard techniques of molecular genetics and biochemistry to be regulated by a repressor which uses ferrous iron as corepressor. The same repressor negatively regulates several other siderophore-related genes in E. coli, apparently by binding an "iron box" operator sharing the common recognition site 5'-GATAATGATAATCATTAC.

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LITERATURE CITED


