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Ferric Uptake Regulation Protein Acts as a Repressor, Employing Iron(II) as a Cofactor To Bind the Operator of an Iron Transport Operon in *Escherichia coli*†

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Received November 4, 1986; Revised Manuscript Received March 13, 1987

ABSTRACT: The Fur (ferric uptake regulation) protein is a negative regulator of the aerobactin operon and of several other siderophore-mediated, high-affinity iron transport systems in *Escherichia coli*. The purified Fur protein and a plasmid containing a *lacZ* fusion to the aerobactin operon were used in conjunction with an in vitro coupled transcription/translation system to demonstrate that the Fur protein requires Fe(II) or certain other divalent metals as a cofactor to negatively regulate expression of the aerobactin operon. In a second set of experiments, using a restriction site protection assay, Fur was shown to bind to and block the aerobactin promoter in a metal-dependent fashion. It is concluded that Fur acts as a classical negative repressor that, under in vivo conditions, uses ionic Fe(II) as a corepressor. Our results support the hypothesis [Williams, R. J. P. (1982) *FEBS Lett. 140*, 3–10] that prokaryotic cells may contain a standing pool of free or loosely bound Fe(II) that is capable of acting in a regulatory capacity.

Iron is required for the growth of almost all living cells (Archibald, 1983). Among the significant roles of iron in biology are the transport and storage of oxygen, reduction of ribonucleotides and dinitrogen, activation and decomposition of peroxides, and electron transport via disparate carriers spanning a redox potential of essentially 1 V. Although abundant, iron is largely unavailable in an aerobic environment at physiological pH owing to the vanishingly small value for the solubility product constant of the hydroxide. However, in contrast to the biologically beneficial effects just enumer-

† This work has been supported in part by Grants A104156, PCM 78-12198, and CRCR-1-1633 from the U.S. Public Health Service, the National Science Foundation, and the Department of Agriculture, respectively.
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0006-2960/87/0426-5471$01.50/0

ated, iron may also play a noxious and deleterious role in vivo through the generation of oxidizing free radicals. Thus, the control of internal iron concentration is a biological event of considerable importance. A mechanism for the excretion of iron apparently does not exist, and as a consequence, uptake of the metal is regulated at the membrane level in all species studied to date.

Many microbial species garner iron through the elaboration of low molecular weight, virtually Fe(III)-specific ligands generically termed siderophores (Neilands, 1981). The biosynthesis of siderophores and the attendant uptake systems for their metal complexes constitute a high-affinity assimilation pathway that is strongly derepressed by growth of the microorganism at suboptimal levels of iron (Garibaldi & Neilands, 1956). This system may to some extent be paralleled by the process of entry of iron into animal cells. Thus, the level of transferrin receptor mRNA is regulated by an intracellular pool of chelatable iron (Rao et al., 1986). Studies of the mechanism of regulation of iron assimilation are most conveniently initiated in bacteria. *Escherichia coli* is the species of choice, since the organism is relatively well defined genetically.

Hospital isolates of *E. coli* commonly produce a particular siderophore, aerobactin, the presence of which correlates with virulence and invasiveness in the bacteria (Warner et al., 1981). The aerobactin determinants borne on V (virulence) plasmids have been studied in some detail (de Lorenzo & Neilands, 1986; Gross et al., 1985) and, in the case of pColVK30, shown to be organized in an operon, preceded by a strong promoter, containing the genes for synthesis and transport of the siderophore.

In 1978, Ernst et al. described a mutation in *Salmonella typhimurium*, designated *fur* (*f*erric *u*ptake *r*egulation), that resulted in constitutive expression of several high-affinity iron assimilation systems of the bacterium. Hantke (1982) subsequently generated a similar mutation in *E. coli*. Recently, Schaffer et al. (1985) sequenced the *fur* gene and deduced that its product is a 17-kDa polypeptide distinguished by an unusually high histidine content. Genetic evidence (Ernst et al., 1978; Hantke, 1982; Bagg & Neilands, 1985) has indicated that the *fur* gene product is a negative regulator of several *E. coli* operons involved in iron transport. In vivo quantitative S1 analysis (Bindereif & Neilands, 1985) has shown that the level of aerobactin operon mRNA is regulated by the availability of iron. The simplest model for the regulation of iron transport therefore places the Fur protein in the role of a classical repressor that binds iron as a corepressor. According to this model, when the internal concentration of iron is high, the Fur protein will complex iron and so become activated to bind to the putative aerobactin operator, thereby inhibiting the initiation of transcription. Because alternative mechanisms for the regulation of aerobactin by *fur* are also conceivable, the problem begs for experimental evidence.

In order to investigate the mechanism of action of the Fur protein, we have reconstituted the aerobactin regulatory system in vitro. We here present evidence that the purified Fur protein regulates aerobactin operon expression in vitro and requires Fe(II) [but not Fe(III)] to regulate aerobactin expression. We have employed a restriction site protection assay to demonstrate that Fur, in the presence of certain divalent metals, binds to the DNA of the gene it regulates, blocking the aerobactin promoter.

Experimental Procedures

Materials. In vitro transcription/translation reactions included CTP, GTP, and UTP (Calbiochem), ATP (P-L Biochemicals), and tRNA from *E. coli* grade XXI, PEG[1] (M_r ~8000), phosphoenolpyruvate trisodium salt, and pyruvate kinase, rabbit muscle type I (all from Sigma). Restriction enzymes were purchased from Bethesda Research Laboratories (*Hin*fI). Nuclease-free BSA was from Pharmacia, Inc. Acrylamide and bis(acrylamide) were purchased from Bio-Rad. The metal salts were of the highest grade commercially available.

Bacterial Strains. BN407, used for in vivo assays of aerobactin expresson, is a Δ(*lac*)U169 derivative of AB1157. The *lac* deletion, which spans the entire operon, was introduced by crossing AB1157 with the *lac*Δ, HfrH strain CA7027. In addition, BN407 carries the pColV::*lacZ* plasmid (Bagg & Neilands, 1985). BN4025, used in the preparation of transcription/translation extracts, is a *lac*Δ derivative of AB1157 and was constructed in the same way as BN407. It also carries the *fur*::Tn5 mutation described in Bagg and Neilands (1985). The *rna*$^-$19 mutation was introduced by P1 transduction, by use of an *rna*$^-$19, *zbe*::Tn*10* lysate provided by G. Storz of Dr. Bruce Ames's laboratory. Both AB1157 and CA7027 were gifts of Dr. Graham Walker's laboratory. JRB458, the host strain for the Fur-overproduction plasmid pMON2064, is *lac*ΔU169, *lon*Δ and was a gift of Dr. Jasper Rine's laboratory.

Plasmids. A diagram of pVC3, constructed by Victor de Lorenzo of this laboratory, is presented in Figure 5. It consists of a 410-bp fragment of the promoter region of the aerobactin operon inserted between the *Hin*dIII and *Bam*HI sites of pUC9 (Vieira & Messing, 1982). After purification over CsCl, this plasmid was dialyzed against 10 mM Tris, not TE, in order to eliminate the presence of EDTA, which would complex the metal ions used in the gel binding and restriction site protection assays. pMON2064, the Fur-overproduction vector constructed by Dr. Bruce Hemming's group at Monsanto, carries the *fur* gene 3′ to both the promoter/operator sequence of the *E. coli recA* gene and the translation initiation sequence of the coliphage T7 gene 10. The replication and ampicillin-resistance functions of this plasmid are derived from pBR327. Details of its construction, and of the cloning of the *fur* gene, will be presented in Wee et al. (unpublished results). The plasmids pABN5::lacZ and pJA1, used in the in vitro assay of the regulation of β-galactosidase production by Fur, are fully described in Bagg and Nielands (1985).

In Vivo Metal Ion Regulation. The procedure of Miller (1972) as modified by Putnam and Koch (1975) was used to assay β-galactosidase levels of BN407 harboring the plasmid pColVK30 carrying a *lacZ* fusion in the third *iuc* (*iron uptake chelate*) gene of the aerobactin operon (Bagg & Neilands, 1985). This construct places the expression of the *lacZ* gene under the control of the aerobactin promoter region. Three drops of an overnight culture were added to 1 mL of M9 medium plus required amino acids, Mg^{2+}, vitamin B1, 1% glucose, and the stated concentration of metal sulfate. Cells were lysed, and β-galactosidase levels were measured when the cultures reached an A_{600nm} of approximately 0.8 (Figure 1).

Preparation of Fur-Enriched Extract and Purified Fur Protein. A Fur protein overproduction vector (pMON2064) in which *fur* has been placed under the control of the *recA* promoter was kindly provided to us by Bruce Hemming of the Monsanto Corp. Strains carrying this vector overproduce the

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; TAE, Tris–acetate–EDTA; TE, 10 mM Tris–1 mM EDTA, pH 8.0; EDTA, ethylenediaminetetraacetic acid; PEG, poly(ethylene glycol); kDa, kilodalton(s); bp, base pair(s); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; LB, luria broth.

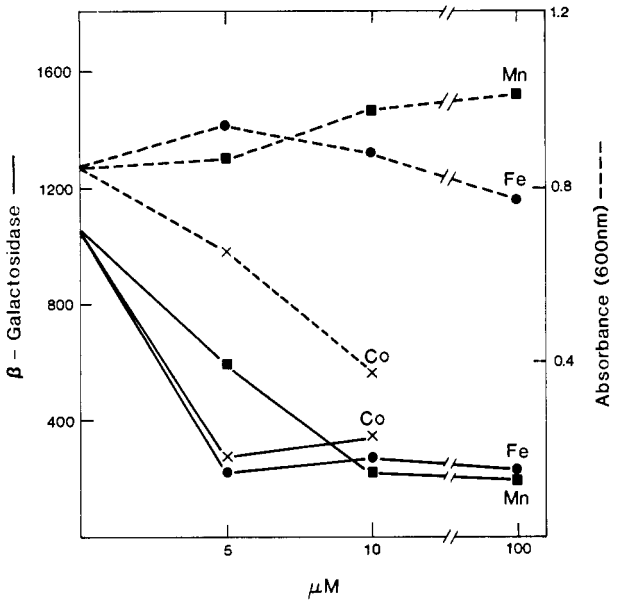

FIGURE 1: Growth and β-galactosidase production by *E. coli* carrying a *lacZ* operon fusion to the aerobactin operon. *E. coli* BN407 pColV iucC::lacZ was grown in M9 minimal medium supplemented with different levels of metal ions. β-Galactosidase activity is expressed as units/mg of dry weight. Values were subject to a variation of about 10%.

Fur protein when induced with naladixic acid. Fur protein purified to a single band on Coomassie-stained SDS–PAGE was kindly donated by Dr. S. Wee of this laboratory (S. Wee et al., unpublished results).

In Vitro Coupled Transcription/Translation System. Preparation of cell extracts and the transcription/translation reaction were performed according to the procedure of Rebecca Reynolds and Michael J. Chamberlin (unpublished results), with the modification that EDTA was not added to the extract, as millimolar amounts of EDTA would chelate the micromolar amounts of metal added to the system in our assays. The extract was prepared from the *lac*Δ, *fur*::Tn*5*, *rnaseI*⁻ strain BN4025.

Ms. Reynold's procedure for the preparation of cell extracts for transcription/translation is essentially that developed by Fuller et al. (1981), although T4 lysozyme is not added. This procedure differs from that described by Zubay et al. (1970) in that there is no incubation of the extract at 37 °C and the extract is not dialyzed. The extracts are stored in liquid nitrogen and then quickly thawed at 30 °C immediately before use. Excess extract is discarded, rather than refrozen. Ms. Reynolds modified the reaction conditions for coupled transcription/translation developed by Zubay et al. by leaving out pyridoxine, triphosphopyridine nucleotide, flavine adenine nucleotide, *p*-aminobenzoic acid, and $CaCl_2$, while adding PEG to 2%, 0.8 mM spermidine, and 6 units/mL pyruvate kinase.

One microliter of plasmid DNA in 10 mM Tris, pH 8.0–1 mM EDTA (TE) was added to each 100-μL transcription/translation assay. DNA was aliquoted from a 167 ng/μL stock solution of the 10.8-kb plasmid pABN5::lacZ (1 μL = 2.35×10^{-14} mol) or a 110 ng/μL stock of the 6.0-kb plasmid pJA1 (1 μL = 2.75×10^{-14} mol).

Working under Anaerobic Conditions. In order to assay the effect of Fe(II) on Fur protein activity, it was necessary to make up our $FeSO_4$ solution and perform all assays in the absence of oxygen. Fur protein and transcription/translation extracts were degassed by flowing H_2O-saturated argon over a thin layer of these solutions. Argon was bubbled through the transcription/translation "mix" (which contains nucleotides, amino acids, and other cofactors). Solid $FeSO_4$ was flushed with argon and then dissolved in H_2O, which had been degassed by boiling. All reactions were carried out in argon-saturated test tubes capped with rubber septa. Solutions were added to these tubes through the septa with hypodermic syringes.

HinfI Protection Assay. One microgram of purified Fur protein was mixed with 500 ng of pVC3 in Fur buffer (44 mM Tris, pH 8.2, 14 mM $MgSO_4$, 2.6 mM ammonium acetate, 5.5 mM potassium acetate) plus nuclease-free BSA (100 μg/mL) to a final volume of 9 μL. Any metals or metal chelators were added to a final concentration of 100 μM. After the mixture was preincubated at 37 °C for 3 min, 2 units of *Hin*fI (diluted in Fur buffer) was added, and the digestion was carried out at 37 °C. After 1 h the vials were placed on ice, and EDTA was added to a final concentration of 25 mM. A 5-μL aliquot of each reaction was run on a 1.5% agarose gel in TAE buffer (Maniatis et al., 1982) at 60 V for 30 min. A *Hin*fI digest of pBR322 was included as a molecular weight marker. DNA bands were visualized by staining with ethidium bromide and photographing under UV light. This assay is fairly sensitive to the amounts of Fur protein and restriction enzyme added, as well as to the duration of digestion. For this reason, controls (Fur protein plus manganese, Fur without additional metal, and Fur plus EDTA) were performed with every assay.

RESULTS

Divalent Metal Ion Regulation in Vivo. Figure 1 shows the influence of the presence of different levels of iron, cobalt, and manganese, all added as divalent ions, on cell growth and expression of β-galactosidase from an *E. coli* strain harboring the aerobactin::*lacZ* fusion. Cobalt eliminated cell growth at 100 μM concentration, while iron and manganese had no effect on cell proliferation at any of the levels tested. All three metal ions, even at 5 μM concentration, repressed the expression of β-galactosidase from the aerobactin promoter. Control experiments (data not shown) proved that under the assay conditions used the metal ions did not inhibit the activity of preformed β-galactosidase. A second control with *E. coli* 294, a *lac*⁺ strain, indicated that the highest levels of iron, cobalt, and manganese employed did not affect the β-galactosidase levels produced by cells grown in M9–lactose. Certain other metals tested, including zinc, apparently had no effect on expression of β-galactosidase from the aerobactin promoter.

Regulation of Aerobactin Expression in Vitro. Our in vitro assay for Fur activity consisted of three components: a crude extract for coupled transcription/translation prepared from a *fur*::Tn*5*, *lac*Δ strain, Fur protein preparations from an overproducing strain, and purified DNA of the plasmid pABN5::lacZ (Bagg & Neilands 1985), which carries a *lacZ* fusion in the *iucC* gene of the aerobactin operon (de Lorenzo & Neilands, 1986). The production of β-galactosidase by this particular fusion is regulated by iron (and Fur) in vivo. As a control the plasmid pJA1 (Bagg & Neilands 1985) was employed, which carries the *lacZ* gene downstream of the *lac* promoter. The addition of iron in the presence of oxygen depressed expression of β-galactosidase from both the *lac* and *iuc* promoters in a non-Fur dependent fashion (data not shown). Apparently the addition of iron under these conditions inhibits some step of the transcription/translation reaction.

Because the addition of manganese to living cultures mimics the effect of iron, repressing aerobactin expression, we investigated the effect of manganese on the expression of the aerobactin operon in vitro. The addition of $MnSO_4$ produced an effect similar, though less drastic, to that of iron, reducing

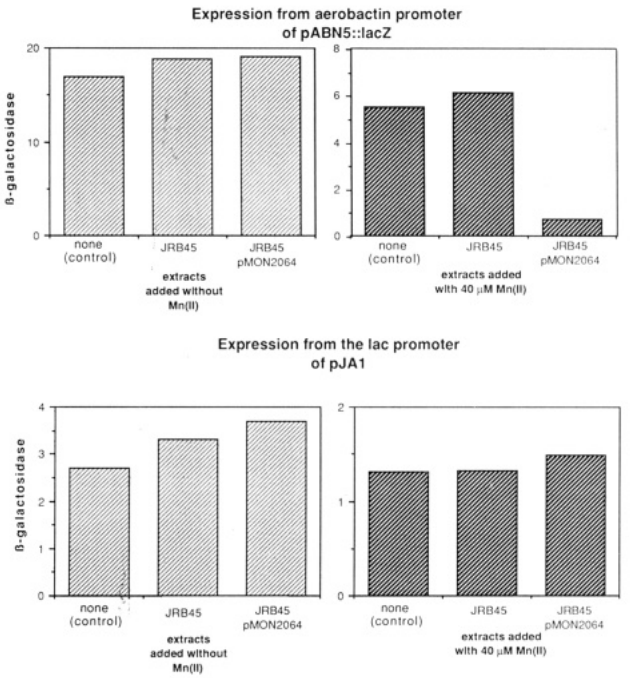

FIGURE 2: Fur requires manganese to regulate the expression of an aerobactin::*lacZ* operon fusion in vitro. One microgram of protein (from either a wild-type or a Fur-overproducing strain, as indicated) was added to each 100-μL transcription/translation reaction. Cell extract was prepared from JRB45, a wild-type Fur-producing strain. Fur-enriched extract was prepared from JRB45 carrying pMON2064, a fur-overproduction plasmid, as described under Experimental Procedures. "Aerobactin promoter" mixes contained 167 ng of the plasmid pABN5::lacZ, while "lac promoter" tubes had 57 ng of pJA1 (Bagg & Neilands, 1985). The mixes were at 10 μM EDTA. $MnSO_4$ at 1 mM was added to bring the Mn^{2+} concentration to 40 μM. The reaction was allowed to proceed at 37 °C for 1.5 h, after which the total β-galactosidase activity was assayed according to Miller (1972). The assays were performed at 30 °C and typically required 10–200 min to develop color. All transcription/translation reactions were performed in duplicate; the average values are presented. Values are accurate to within 20%.

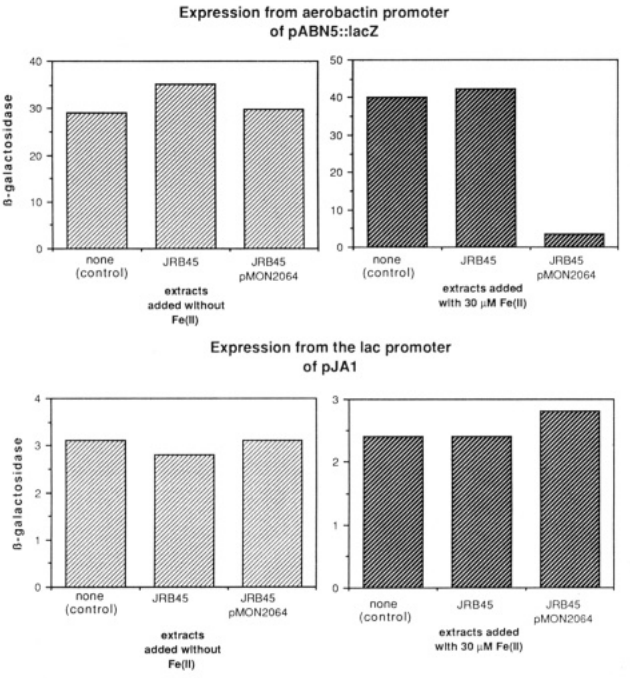

FIGURE 3: In the absence of oxygen, Fur utilizes Fe(II) to regulate an aerobactin::*lacZ* fusion in vitro. The reactions were performed as described in the legend to Figure 2 with the following modifications: (1) all reaction mixes were at 35 μM EDTA; (2) $FeSO_4$ was added to a final concentration of 30 μM; (3) transcription/translation reactions were performed under argon as described under Experimental Procedures.

the overall efficiency of transcription/translation to about one-third of its metal-free level. In addition, when $MnSO_4$ was added to the transcription/translation system, we observed that our Fur-enriched extract regulates the production of β-galactosidase from the *iuc* but not the *lac* promoter in a metal-dependent fashion (Figure 2). The addition of an identically prepared extract from JRB45, a wild-type producer of Fur protein (where the level of Fur protein is at least 100-fold lower than that observed for the overproducer), has no aerobactin-specific regulatory effect.

When we performed the same experiments in the absence of oxygen (see Experimental Procedures), we found that the addition of 30 μM $FeSO_4$ has no general depressive effect on in vitro transcription/translation. Moreover, under anaerobic conditions, addition of Fur without any additional iron results in some repression of the aerobactin, but not *lac*, promoter (data not shown). Because the transcription/translation extract probably contains some free iron that is available to Fur, all anaerobic experiments were run at 35 μM EDTA, while experiments in the presence of oxygen were 10 μM in EDTA. When we performed the assay under these conditions, we found iron to be at least as effective as manganese in activating Fur-dependent regulation of the aerobactin operon (Figure 3). Our transcription/translation extract is heavily contaminated with iron (around 200 μM), and it is difficult to determine what percentage of this iron is available to the Fur protein (rather than being covalently bound up in heme or iron–sulfide proteins, or present as insoluble ferric hydroxide, or coordinated

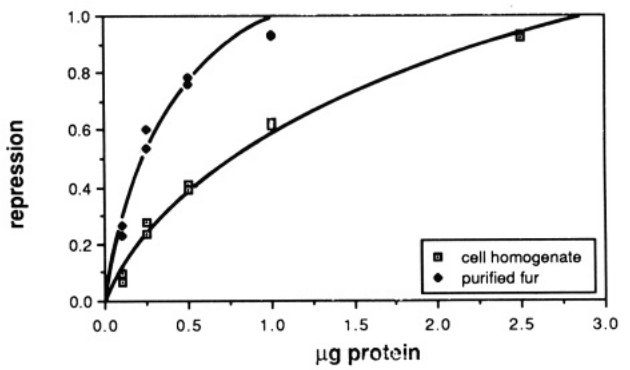

FIGURE 4: Purification of Fur protein from crude extract increases its specific activity as a repressor. Reactions were carried out as described in the legend to Figure 2. "Repression" was calculated as [(level of β-galactosidase observed in the absence of Fur) − (level of β-galactosidase observed at a specific concentration of Fur)]/(unrepressed level − completely repressed level). For this set of experiments, 50% repression was achieved with 0.72 μg of unpurified Fur, vs. 0.21 μg of purified Fur.

to metal-activated proteins). We estimate the "loosely bound", cold acid-soluble (Brumby et al., 1967) iron content of our transcription/translation mix to be approximately 25 μM, though not all of even this iron may be available to the Fur protein.

It is possible that the aerobactin-specific, metal-dependent effect of our Fur-enriched extract is not due to the Fur protein itself but rather due to some other factor present in our extract that is induced by overproduction of the Fur protein, or as a result of the addition of nalidixic acid. To find out whether the Fur protein itself acts to repress aerobactin expression, we tested a specimen that had been purified by Dr. S. Wee of this laboratory (unpublished results). Purified Fur protein was found to retain its repressor activity, and 3.5-fold purification of the Fur band (as assayed by densitometer scan of a Coo-

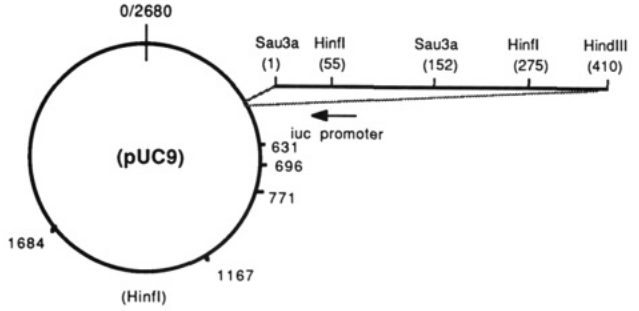

FIGURE 5: Structure of plasmid pVC3. A 410-bp *Sau*3a to *Hin*dIII fragment of the aerobactin operon was inserted between the *Bam*HI (421) and *Hin*dIII (441) sites of the pUC9 polylinker to create pVC3 (de Lorenzo and Neilands, unpublished results). *Hin*fI sites are indicated within the plasmid.

massie-stained gel) enhanced the specific activity 3.4-fold (Figure 4 and Table I).

Metal-Dependent DNA Binding by Fur. Classical repressors act by binding to a specific "operator" site near the genes they regulate, thereby inhibiting binding (Gilbert & Maxam, 1973; Majors, 1975; Reznikoff, 1976) or progress (Deuschle et al., 1986) of RNA polymerase holoenzyme. We hypothesize that the Fur protein, once activated by an increase in the level of free intracellular iron, inhibits the expression of iron-regulated genes by binding to their operator regions. Our restriction site protection assay shows that Fur protects a *Hin*fI site at the aerobactin promoter from digestion and that Fur requires the presence of micromolar amounts of free divalent metal to achieve this effect.

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 *Sau*3a fragment of the aerobactin operon is sufficient to provide regulation by iron of a downstream *iucA*::*lacZ* protein fusion (Bindereif & Neilands, 1985). The aerobactin promoter has been defined by in vivo and in vitro S1 mapping and the transcription start site mapped by RNA sequencing (Bindereif & Neilands, 1985). A *Hin*fI site is located between the −10 and −35 regions of the main promoter. If the Fur protein acts as a classical repressor, blocking access of RNA polymerase to the aerobactin promoter, it might also block the binding of *Hin*fI to this same region. A restriction site protection assay has previously been used to assay the activity of the Trp repressor (Joachimiak et al., 1983). The following experiments show that Fur does indeed inhibit the digestion of a cloned aerobactin regulatory region at the promoter's *Hin*fI site but not

Table I: Repressor Activity of Purified Fur Protein vs. Crude Extract from the Overproducing Strain JRB45 pMON2064[a]

μg of protein added	units of β-Gal produced	% repression (=38 units of β-Gal/36)
	Crude Extract	
0.1	35.6	6.7
0.1	34.6	9.4
0.25	28.1	27.5
0.25	29.5	23.6
0.5	23.2	41.1
0.5	23.8	39.4
1.0	15.4	62.8
1.0	15.9	61.4
2.5	4.4	93.3
2.5	4.9	91.9
	Purified Fur Protein	
0.1	28.4	26.7
0.1	29.8	22.8
0.25	16.4	60.0
0.25	18.6	53.9
0.5	10.6	76.1
0.5	9.8	78.3
1.0	4.5	93.1
1.0	4.6	92.8
2.5	2.3	100
2.5	2.2	100
0	38.0	0

[a] These data are represented in Figure 4. The method by which repressor activity was calculated is described in the legend to Figure 4.

at the other seven available *Hin*fI sites of pVC3 (see Figure 5). The presence of any one of a number of divalent cationic metals, including Fe(II), is required for this site-specific inhibition of digestion.

In a typical experiment, 500 ng of pVC3 supercoiled DNA was incubated with 1 μg of purified Fur protein in the presence of manganese or EDTA. The mixture was then subjected to digestion with 2 units of *Hin*fI for 1 h at 37 °C. When pVC3 is completely digested by *Hin*fI, the fragments produced include the 1448- and 220-bp fragments that make up the promoter region. If the *Hin*fI site at the aerobactin promoter is protected, these two fragments merge to produce a single 1668-bp band. Figure 6A demonstrates this effect. Lane 1 shows the lack of effect of Fur on the mobility of pVC3 DNA *pre*digested with *Hin*fI. Lanes 2 and 3 show that, in the absence of Fur, neither 0.1 M manganese nor 0.1 M EDTA has an effect on *Hin*fI activity or gel mobility. In lane 4 we see that Fur, in the presence of manganese, inhibits *Hin*fI digestion at the aerobactin promoter. This results in the fusion of the 1448- and 220-bp fragments to yield a new band mi-

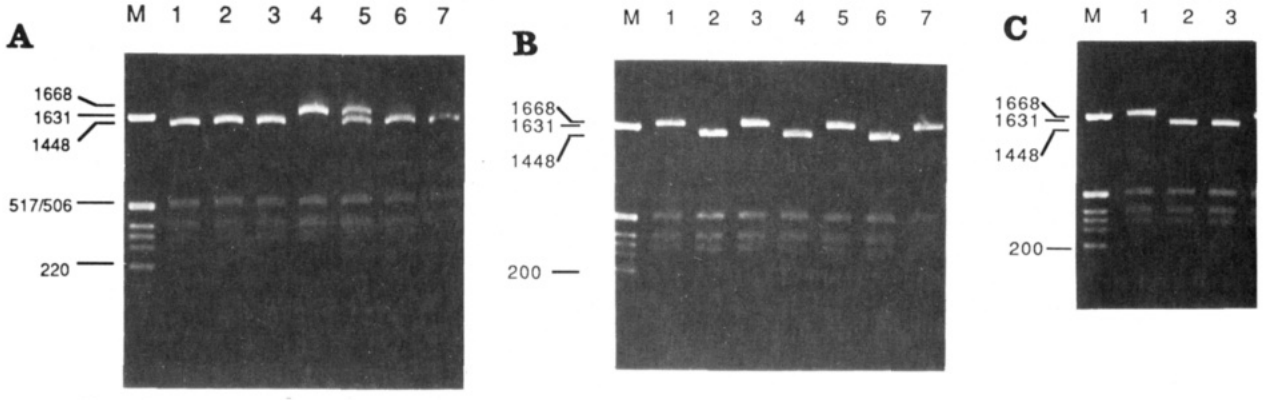

FIGURE 6: Effect of the availability of metals on the ability of Fur to specifically protect the *Hin*fI site at the aerobactin promoter. (A) (Lane 1) Fur plus 100 μM $MnSO_4$ plus *pre*digested pVC3; (lane 2) 100 μM $MnSO_4$, no Fur; (lane 3) 100 μM EDTA, no Fur; (lane 4) 100 μM $MnSO_4$, plus Fur; (lane 5) Fur, no additional metal; (lane 6) Fur, 50 μM EDTA; (lane 7) Fur, 100 μM EDTA. (B) (Lane 1) 100 μM $FeSO_4$ plus Fur; (lane 2) FeSO4 only; (lane 3) 100 μM $CoCl_2$ plus Fur; (lane 4) 100 μM $CoCl_2$ only; (lane 5) 100 μM $CdCl_2$ plus Fur; (lane 6) 100 μM $CdCl_2$ only; (lane 7) 100 μM $MnSO_4$ plus Fur. (C) (Lane 1) 100 μM $MnSO_4$ plus Fur; (lane 2) no supplemental metal, plus Fur; (lane 3) 100 μM $AlCl_3$ plus Fur.

grating at 1668 bp. Lane 5 shows the intermediate effect of Fur in the absence of any additional metal. This partial protection is probably due to metals contaminating the restriction enzyme preparation. Lane 6 shows the elimination of this effect by the addition of 50 μM EDTA, which chelates any contaminating metals. Figure 6B demonstrates the effect of other metals on the ability of Fur to protect the promoter's *Hin*fI site. Certain metals that are added as divalent cations, including iron, cobalt, cadmium, copper, and zinc (not shown), can act as cofactors of the Fur protein. Fur requires Mg^{2+} in millimolar concentrations in conjunction with one of the above metals to protect the aerobactin promoter from *Hin*fI digestion; Mg^{2+} alone cannot activate Fur to bind DNA (data not shown). Aluminum, a trivalent cation, did not enhance binding of Fur to the promoter (Figure 6C).

Discussion

About one-third of all known enzymes contain or require a metal ion, the specific coordination of which depends on the nature of both the inorganic element and the electron-donating atoms of the ligand. In order to sense precisely the prevailing metal ion concentration, Fur should be a metal-binding protein rather than a metalloprotein, and its affinity for iron should be somewhat lower than that of certain essential metal-activated enzymes. A number of lines of evidence indicate that the oxidation state of the regulatory iron should be Fe(II) rather than Fe(III). The latter is quantitatively insoluble at biological pH unless coordinated to a special ligand such as a porphyrin or siderophore, and there is evidence that both of these are excluded from the regulatory circuit (Klebba et al., 1982). Fe(II) has a faster exchange rate than Fe(III) and is soluble, at least to the extent of 100 μM, at biological pH in the absence of oxygen. These properties of ferrous iron would enable the cell to accumulate a standing pool of "loosely coordinated" Fe(II) sufficient to saturate the iron-activated enzymes (Williams, 1982) and, at the same time, to regulate the uptake machinery for the metal.

It is apparent that manganese and cobalt mimic iron in their power to effect control of the operon fusion in vivo. This may explain in part why cobalt poisoning to some extent mimics iron deficiency (Healy et al., 1955). These three elements, atomic numbers 25, 26, and 27, are all adjacent on the periodic table. In the divalent state they have identical charges and similar radii. The affinity constants of manganous and cobaltous ions bracket those of the ferrous ion on the Irving–Williams plot relating affinity of divalent metal ions for nitrogen ligands (Williams, 1982). Although both Mn(II) and Co(II) may have higher oxidation states, it is most likely that they are acting here as the divalent metal ions. Mn(II) is the most common and stable oxidation state of manganese; higher oxidation forms tend to be oxides. Six-coordinate, octahedral complexes are common with all three ions. Both manganese and cobalt are micro trace elements, and it is improbable that they could compete effectively in vivo for regulatory molecules designed for iron. The array of histidine residues [including the sequence His-His-His-Asp-His in the Fur protein (Schaffer et al., 1985)] affords a likely site for coordination of transition metal ions, although this remains to be demonstrated by biophysical and chemical methods. Failure to detect regulation in vivo by other metal ions such as Zn(II) might be attributed to lack of uptake of a sufficient level of the metal or to its inability to contact the regulatory site in the cell. In summary, work in vivo pointed to Fe(II) as the regulatory species of the metal. This was confirmed by the in vitro transcription/translation experiments, which showed that anaerobic conditions were necessary to demonstrate regulation of the operon fusion by iron while the presence of oxygen could be tolerated with manganese. The hypothesis is also supported by data indicating that the Fur protein can utilize divalent transition metals to bind to the aerobactin operator. The adventitious iron present in the transcription/translation assay system, as determined by cold acid treatment, was found to be about 25 μM. This level of iron is sufficient to result in 50% repression of aerobactin expression (data not shown). Assuming that half this iron is available to the Fur protein, we can estimate a dissociation constant of about 10^{-5} M for the Fe(II)–Fur complex.

Repressors generally work by hindering the binding of RNA polymerase to specific promoters. We reasoned that if Fur can inhibit binding of polymerase to the aerobactin promoter, it might also inhibit binding of the restriction enzyme *Hin*fI to a specific site between the −35 and −10 regions of that promoter. As demonstrated in Figure 6A, Fur specifically blocks this *Hin*fI site (leaving the other seven sites in the plasmid unprotected) in a metal-dependent fashion. This suggests that the sequence between the −35 and −10 regions of the promoter makes up at least part of the operator. Because the assay, performed with highly purified Fur protein, DNA, and *Hin*fI restriction enzyme (which may contain unknown contaminants from *Haemophilus influenzae*), revealed Fur activity, it follows that the Fur protein probably requires only certain divalent metals, and no additional cofactors, for its site-specific DNA binding activity.

The restriction site protection assay is simpler than the in vitro transcription/translation system, requiring only the activities of *Hin*fI and Fur. Thus, it was possible to use this system to test qualitatively the effect of a number of other metals that nonspecificly inhibit transcription/translation. Fur was found to be activated to bind to the aerobactin promoter region by a number of different divalent metals, including manganese, zinc, cobalt, cadmium, and copper. A freshly prepared iron sulfate solution, even in the presence of oxygen, also activated Fur. The trivalent cation Al(III) did not activate Fur. As a metal-activated protein Fur would not be expected to be entirely specific for a single metallic cation, as we observe in both the in vivo and in vitro Fur activity assays. However, it is likely that, in *E. coli*'s natural environment, Fe(II) is the sole species present in sufficient quantity and with the requisite coordination chemistry to form a biologically active complex with the protein. Quantitative data are needed regarding the intracellular concentrations of free metal ions and the ability of these ions to effect Fur-dependent protection of the *Hin*fI site of the operator. In addition, a study of the stabilities of different metal complexes of the pure protein is in order.

The simplest explanation to account for our results is that Fur behaves as a divalent metal ion binding protein that, in the metal complex form, acts as a transcriptional repressor by binding at the aerobactin operator site. It is possible that there are trans-acting components in addition to the Fur protein involved in the regulation of iron transport. Genetic studies in our laboratory and others have yielded several mutants that are constitutive for the expression of iron-regulated operons. These mutations all map to the same locus, but complementation analysis will be required to determine whether all of these mutations are the result of lesions in the same gene. The possibility that factors may exist which act positively to promote gene expression in the absence of iron should also be investigated.

Further elucidation of the nature and position of Fur binding sites, as well as of the relationship between Fur/operator binding and aerobactin regulation, will require footprinting

and, more importantly, genetic analysis of the operator region. These experiments are currently under way in our laboratory. Investigation of the nature of the operator regions of other iron-regulated genes in *E. coli* and comparative studies of their sequences should yield new insights into the mechanism of Fur-dependent regulation of gene expression by iron.

ACKNOWLEDGMENTS

We are indebted to Dr. Kayoko Nakamura, who initiated the transcription/translation experiments, and Rebecca Reynolds, of this Department, who kindly provided us with her unpublished coupled transcription/translation procedure and invaluable advice. We thank Michael Bittner and Bruce Hemming of Monsanto Corp. for creating and generously providing us with the Fur-overproduction vector. We are obligated to the following individuals of this Department: Dr. Victor de Lorenzo for making available the plasmid pVC3, Dr. H. A. Barker for advice on working under argon, and Dr. Sechan Wee for purified Fur protein.

Registry No. Fe, 7439-89-6.

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