

Role of Iron in Regulation of Virulence Genes

CHRISTINE M. LITWIN¹ AND STEPHEN B. CALDERWOOD^{1,2*}

Infectious Disease Unit, Massachusetts General Hospital, Boston, Massachusetts 02114,^{1} and
Department of Microbiology and Molecular Genetics, Harvard Medical School,
Boston, Massachusetts 02115²*

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INTRODUCTION

Iron is an important element essential to the growth of almost all living cells (108). Iron is needed for important cellular functions, such as the transport and storage of oxygen, and as a catalyst in electron transport processes. Since iron has an extremely wide redox potential, spanning approximately 1 V, it is a very versatile biocatalyst. A variety of enzymes that require iron for activity have been characterized; these include ribotide reductase, nitrogenase, peroxidase, catalase, cytochromes, and succinic dehydrogenase. These enzymes serve important cellular roles, such as the reduction of ribonucleotides and dinitrogen and the activation and decomposition of peroxides.

Iron is abundant in the earth's crust, being the fourth most common element. Yet in the presence of oxygen, iron is oxidized to the ferric state and may form ferric hydroxide, which is insoluble in aqueous solution at neutral or alkaline

pH and thus is not readily accessible to bacteria (109). Because of iron's insolubility, microorganisms have evolved a number of mechanisms for the acquisition of adequate iron from the environment; these mechanisms are closely linked to bacterial virulence. The competitive growth and survival of bacteria in the host require a number of adaptive responses on the part of the bacteria; the acquisition of iron is one of the most important of these adaptive responses for bacterial pathogenesis.

ROLE OF IRON IN HOST-BACTERIUM INTERACTIONS

Iron Limitations in the Host

Free iron is extremely limited in the mammalian host, making acquisition of iron by invading pathogens especially difficult. The human body contains as much as 3 to 5 g of iron, but the majority of this iron is not easily accessible to bacteria. Iron is maintained in a soluble form by being complexed to carrier molecules or reduced to ferrous iron

* Corresponding author.

within cells. Most intracellular iron is found as hemoglobin, heme, ferritin, and hemosiderin. The trace quantities of iron present extracellularly are bound by the high-affinity iron-binding glycoproteins transferrin and lactoferrin (4). In normal human serum, the amount of free iron in equilibrium with transferrin-bound iron has been calculated by Bullen et al. (27) to be approximately 10^{-18} M. This is far below the level required to support bacterial growth (27). Unsaturated transferrin appears to contribute to the bacteriocidal activity of normal human plasma (152), while lactoferrin may serve a similar function on secretory surfaces and is also an important component of phagocytic cells (149). Extracellular hemoglobin and heme have been found to serve as iron sources for many bacteria; however, they are rapidly bound by two serum proteins, haptoglobin and hemopexin, respectively, and are removed from the circulation (49, 159).

During infection, iron availability is further limited by the induction of hypoferrremia, which has long been recognized as a component of the acute-phase response to infection. This acute-phase response, which can be induced by microbes and certain microbial products such as endotoxin, is mediated through the increased release of various cytokines, particularly interleukin-1 and tumor necrosis factor alpha (cachectin) (12, 45). The iron saturation in serum decreases with an accompanying increase in the level of transferrin, limiting iron availability for bacteria (105, 154). Lactoferrin levels are also increased in the blood during an inflammatory response. The release of apolactoferrin by leukocytes occurs during degranulation and phagocytosis, which additionally increases the local concentration of lactoferrin at sites of infection (88, 162).

A number of studies have shown enhanced resistance to infection in animals in which levels of iron in serum have been reduced by an iron-deficient diet. This enhanced resistance to infection is reversed if the iron-deficient mice are injected with sufficient iron to restore normal levels of iron in serum (73, 123).

If hypoferrremia limits infection, then chronic iron overload might be expected to increase susceptibility to infection. Numerous studies have demonstrated that injection of exogenous iron to produce iron overload in animals enhances susceptibility to bacterial infection (26, 56, 58, 125, 161). This effect of excessive iron has been noted in human diseases as well, such as in primary and secondary hemochromatosis. An association between iron overload and increased risk of bacteremia has been noted for several bacterial species, including *Vibrio vulnificus* (17), *Yersinia enterocolitica* (98), and *Escherichia coli* (8, 9). Excessive iron may enhance infection by reversing iron limitation and stimulating the growth of bacteria. Alternatively, others have suggested that iron overload may interfere with host defense mechanisms such as cell-mediated immunity (85) and the bacteriocidal activity of leukocytes (64). Thus, it may be difficult to separate the contribution of iron overload in stimulating bacterial growth from its contribution in interfering with host defense mechanisms.

Acquisition of Iron by Bacteria

For pathogenic bacteria to be able to establish an infection, they must be able to obtain iron from the host. Because iron is limiting in the host, a number of diverse mechanisms have been evolved by bacteria either to free iron from host sources or to utilize host iron-binding compounds directly. Many bacteria secrete low-molecular-weight, high-affinity

iron chelators termed siderophores in response to iron stress (109, 110). These siderophores can solubilize iron from mineral complexes in the environment and can compete effectively with the host iron-binding compounds transferrin and lactoferrin to mobilize iron for bacterial use. Subsequent transport of the iron-siderophore complexes into the bacterium requires cell surface receptors specific for each siderophore complex. Other pathogenic bacteria secrete reductants (38), which remove ferric iron from transferrin, or utilize citrate as a low-affinity iron carrier (62).

Some bacterial species utilize host iron complexes directly to enhance bacterial growth, without the production of siderophores. *Neisseria* spp., for example, possess specific receptors for ferric transferrin and can remove iron from transferrin at the cell surface (100). *Yersinia pestis* can use heme as a sole source of iron for growth (121), *V. vulnificus* can use the hemoglobin-haptoglobin complex (74), and *Haemophilus influenzae* can utilize hemoglobin, hemoglobin-haptoglobin, heme-hemopexin, and heme-albumin (140).

Another mechanism for iron acquisition by bacteria is the production of hemolysins, which release iron complexed to intracellular heme and hemoglobin. Synthesis of such hemolysins is frequently regulated by iron availability, with increased expression when iron is limiting (136, 150). Production of hemolysins is common among pathogenic bacteria but has been studied most thoroughly in *E. coli* (132).

An association between pathogenicity of *E. coli* and the production of α -hemolysin has been reported in a number of studies. Minshew et al. found that 80% of *E. coli* strains virulent in chicken embryos were hemolytic (103). Waalwijk et al. studied the role of hemolysins in *E. coli* by using a hematogenous pyelonephritis model in mice and found that a nonhemolytic avirulent *E. coli* strain was restored to virulence by simultaneous injection of the avirulent mutant with its hemolytic and virulent parent or with partially purified hemolysin (150). Linggood and Ingram similarly found that transfer of a plasmid encoding hemolysin to a nonhemolytic strain of *E. coli* increased the virulence of the recipient for mice. Injection of hemolysin, hemoglobin, iron, or manganese salts simulated the effect of the hemolysin plasmid (90). Various studies with clinical isolates of *E. coli* have demonstrated a statistical correlation between hemolysin production and recovery from blood and urine but not with recovery from stool. In a study of 223 *E. coli* isolates, Deboy et al. found that hemolytic strains were isolated more often from blood, urine, and wounds than from stools (41). In a study of 167 patients, Brooks et al. found that 58% of *E. coli* strains isolated from upper urinary tract infections produced hemolysin, but only 27% of strains isolated from the lower urinary tract did so (24). A collection of 115 *E. coli* strains isolated from women and children with acute nonobstructive pyelonephritis was compared with 96 fecal isolates; hemolysin synthesis, production of cytotoxic necrotizing factor, and aerobactin-mediated iron uptake occurred more often in strains from patients with pyelonephritis (145).

Hemolysins appear to promote infection by providing iron to the pathogen in vivo (90, 150). Opal et al. (117) compared the production of the siderophore, aerobactin, and hemolysin in *E. coli* strains isolated from blood, urine, and stool. The majority (55%) of blood isolates that lacked aerobactin production were hemolytic, suggesting that the hemolysin serves as an alternative mechanism of iron acquisition in the absence of aerobactin (117).

TABLE 1. Examples of iron-regulated bacterial virulence determinants

Organism	Virulence determinant(s)
<i>Escherichia coli</i>	Aerobactin SLT-I
<i>Shigella dysenteriae</i> type 1	α -Hemolysin Shiga toxin
<i>Serratia marcescens</i>	Hemolysin
<i>Vibrio cholerae</i>	Iron-regulated gene <i>A</i> (<i>irgA</i>)
<i>Vibrio anguillarum</i>	Anguibactin
<i>Yersinia</i> spp.	Iron-regulated outer membrane proteins
<i>Neisseria gonorrhoeae</i>	Transferrin-binding protein 1 (TBP1) Transferrin-binding protein 2 (TBP2)
<i>Pseudomonas aeruginosa</i>	Exotoxin A Elastase
<i>Corynebacterium diphtheriae</i>	Alkaline protease Diphtheria toxin

Iron as a Regulatory Signal

For effective bacterial pathogenesis, the organism must sense its environment appropriately and respond with coordinate alterations in the expression of virulence genes. A number of environmental factors, including pH, osmolarity, temperature, and amino acid concentration, coordinately regulate the expression of virulence genes (102). Since free iron is extremely limited in the mammalian host, a shift from a high- to a low-iron environment is an important environmental signal to bacteria for coordinate regulation of gene expression. As summarized above, hemolysins and siderophores have been implicated as virulence factors in several model systems and are regulated by the concentration of iron in the environment (24, 39, 41, 90, 150, 157). Other virulence factors regulated by the concentration of iron include Shiga toxin from *Shigella dysenteriae* I, Shiga-like toxin I (SLT-I) from enterohemorrhagic *E. coli*, diphtheria toxin from *Corynebacterium diphtheriae*, exotoxin A from *Pseudomonas aeruginosa*, and a number of iron-regulated outer membrane proteins. Examples of iron-regulated virulence determinants are provided in Table 1. These virulence determinants and the role of iron in their regulation will be discussed in greater detail below.

MECHANISMS OF REGULATION BY IRON

Isolation of *fur* Mutants

The molecular basis of coordinate regulation by iron has been most thoroughly studied in *E. coli*. In this organism, coordinate regulation of gene expression by iron depends on a single regulatory gene, *fur*. Mutations of *fur* were found first in *Salmonella typhimurium* by Ernst et al., who were studying unrelated periplasmic leaky (*lky*) mutants. A secondary mutation that led to the overproduction of iron-regulated outer membrane proteins and was termed *fur*, for ferric uptake regulation, was found (54). This mutation also resulted in constitutive production and transport of the siderophore enterobactin as well as constitutive uptake of the siderophore ferrichrome.

Hantke (71) first obtained *fur* mutants of *E. coli* by screening for constitutive expression of a Mu d(*Ap-lac*) fusion to the ferrichrome transport gene, *fhuA*, following use of the chemical mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. The *fur* mutants constitutively expressed all iron uptake systems, including the uptake of uncomplexed iron. The *fur* mutants were also unable to grow on several nonfermentable carbon sources, including acetate, succinate, and fumarate (6); the *fur* gene may be allelic to *dctB*, which is believed to be involved in succinate transport. Hantke (72) cloned the *fur* gene by screening an *E. coli* recombinant plasmid library for complementation of the *fur* mutation. Minicell analysis showed that the *fur* gene encoded an approximately 18-kDa protein. The gene was subsequently sequenced and shown to encode a 148-amino acid (17-kDa) protein with a high histidine content (129).

Regulation by Iron in *E. coli* Depends on the *fur* Product

Transcriptional regulation of genes in *E. coli* by iron is mediated by the protein product of the *fur* locus, which acts as a corepressor with ferrous iron via sequence-specific protein-DNA interactions at the promoter regions of *Fur*-regulated genes. Table 2 lists the functions of many of the iron-regulated genes in *E. coli*, most of which have also been shown to be *Fur* regulated. The aerobactin operon (*iucABCD*

TABLE 2. Iron-regulated genes in *E. coli*^a

Gene(s)	Location (min)	Function
<i>fhuA</i> (<i>tonA</i>)	3	Outer membrane receptor, ferric hydroxamate siderophores
<i>fhuBCD</i>	3	Transport, ferric hydroxamate siderophores
<i>fecA</i>	6	Outer membrane receptor, ferric dicitrate
<i>fecBCDE</i>	6	Ferric dicitrate transport
<i>entABCDEF</i>	13	Enterobactin biosynthesis
<i>fepA</i>	13	Outer membrane receptor, ferric enterobactin
<i>fepBCDEG</i>	13	Ferric enterobactin transport
<i>fes</i>	13	Ferric enterobactin esterase (reductase)
<i>fur</i>	16	Repressor, ferric uptake regulation
<i>fhuE</i>	16	Outer membrane receptor, coprogen and ferric rhodotorulate
<i>fiu</i>	18	Unknown
<i>tonB</i>	27	Transport of ferric siderophores, vitamin B ₁₂ , and lethal agents
<i>cir</i>	43	Colicin I transport
<i>exbBC</i>	64	Transport of ferric siderophores, vitamin B ₁₂ , and lethal agents
<i>iutA</i>	pColV	Outer membrane receptor, ferric aerobactin
<i>iucABCD</i> (<i>aerDBCA</i> , respectively)	pColV	Aerobactin biosynthesis
<i>slt-I</i>	Phage H-19B	Shiga-like toxin I

^a Adapted from reference 111 with permission.

iutA) has been used as a model for studying the regulation of gene expression in *E. coli* by iron.

Aerobactin Operon as a Model for Iron-Regulated Gene Expression

Strains of *E. coli* containing the plasmid pColV, which were characterized by their capacities to cause invasive disease in experimental animals, were the first strains described (by Williams [157]) as producing the bacteriocin colicin V as well as a novel siderophore. The genetic determinants for the bacteriocin and the siderophore were localized to this V (virulence) plasmid (157). Williams and Warner showed that the virulence characteristic was attributed to the plasmid-encoded siderophore system rather than to colicin V or to the chromosomally encoded enterobactin system (158). The siderophore produced in the presence of the V plasmid pColV-K30 was subsequently isolated and shown to be aerobactin (153). Genes encoding the production of aerobactin have subsequently been shown to be chromosomal in *Shigella* species (87), in some *Salmonella* species (96), and in certain *E. coli*, including *E. coli* K1 (147) and enteroinvasive strains (94). Unlike *E. coli* ColV strains, whose virulence is greatly enhanced by the presence of the aerobactin system, an aerobactin mutant of *Shigella flexneri* retained its virulence (86). This difference may be related to the fact that *E. coli* ColV strains are associated with septicemic infections, while *Shigella flexneri* strains produce a predominantly intracellular infection.

The entire high-affinity, aerobactin-mediated iron assimilation system of pColV-K30 was cloned by Bindereif et al. (14). The iron-regulated aerobactin gene cluster was shown to be organized in an operon consisting of four biosynthesis genes, *iucABCD*, and a transport gene, *iutA* (43).

Regulation by Iron Is at the Transcriptional Level

S1 nuclease protection assays specific to RNA from the aerobactin operon revealed the presence of major and minor transcriptional start sites immediately upstream from *iucA*, the first gene in the aerobactin cluster of pColV-K30. A quantitative S1 protection assay demonstrated that regulation of the aerobactin promoter by iron occurs directly at the transcriptional level. Following growth in high-iron conditions, no RNA initiating at the major promoter (P1) could be detected, while under iron starvation conditions, specific RNA was detected. The use of a *lacZ* transcriptional fusion to the aerobactin promoter also demonstrated that repression of aerobactin gene expression by iron occurs at the transcriptional level (13).

Fur Protein Acts as Repressor with Iron as Corepressor

Experiments performed in vivo to ascertain the effects of divalent manganese, iron, and cobalt on the expression of β -galactosidase in *E. coli* BN407 (pColV *iucC::lacZ*) showed that each of these divalent cations represses β -galactosidase activity expressed from the aerobactin promoter. Cloning of the *fur* gene in an overexpression vector allowed the purification of the Fur protein for in vitro studies (6). An in vitro transcription-translation system utilizing the *lacZ* fusion demonstrated specific regulation of transcription of the aerobactin operon by Mn(II) and Fe(II). In subsequent work, Mn(II) was used as an activator of Fur because of its stability in the presence of oxygen (7).

A Hill plot was generated from the repression of β -galac-

CONSENSUS	GAT AAT GAT A ATC ATT ATC
<i>iucA</i> promoter-primary	GAT AAT GAG A ATC ATT ATT
<i>iucA</i> promoter-secondary	GAT AAT TGT T ATT ATT TTA
<i>fepA</i> promoter	TAT TAT GAT A ACT ATT TGC
<i>slt-I</i> / <i>stxA</i> promoter	GAA TAT GAT T ATC ATT TTC
<i>irgA</i> promoter	GAA ATT AAG A ATA ATT ATC
<i>fhuA</i> promoter	CTT TAT AAT A ATC ATT CTC
<i>fur</i> promoter	TAT AAT GAT A CGC ATT ATC

FIG. 1. Homology between the dyad repeat sequences in various iron-regulated promoters and the Fur-binding consensus sequence of *E. coli*. Arrows indicate dyad symmetry in the consensus sequence.

tosidase activity as a function of Fur concentration. The slope of 2 was consistent with the hypothesis that the Fur repressor acts as a dimer (6). A negative complementation experiment, in which *E. coli* strains heterozygous for both the wild type and certain mutant alleles of *fur* retained a Fur⁻ phenotype, provided further evidence that Fur is active in vivo as a multimer (22).

Operator Sequences Bound by the Ferrous Fur Complex

In DNase I protection experiments, the Fur protein, activated by divalent heavy-metal ions including Mn(II), Fe(II), Co(II), and Cd(II), was found to bind and protect a specific nucleotide sequence overlapping the P1 promoter in the aerobactin operon of pColV-K30 (44). In the absence of divalent cations, Fur had very little capacity for binding the operator.

DeLorenzo et al. (44) performed DNA footprint experiments with the purified Fur protein with Mn(II) as corepressor. The Fur protein bound to two contiguous sites. With excess Mn(II), increasing the level of Fur first protected a sequence bracketing the -35 region of the P1 promoter, which was designated the primary binding site, and then an additional secondary binding site located just downstream of the -10 region. The primary and secondary binding sites had in common the sequence ATAATnnnnATnATT. The primary site extended over 31 bp and consisted of two overlapping symmetrical dyads sharing the sequence 5'-TCATT-3'.

Identification of a Fur DNA-Binding Consensus Sequence

Using the data from the defined aerobactin promoter and genetic data from the iron-regulated promoter of SLT-I (see below), several investigators independently proposed a consensus "iron box" for binding of the Fur repressor (29, 42, 44). This proposed iron box is a perfect 19-bp hyphenated dyad repeat. The promoters of *slt-IA*, *iucA*, *fepA*, *irgA*, and *fhuA* show homology with this consensus sequence that ranges from 13 to 17 base-pair matches (Fig. 1). Homology to the iron box is also found in the promoter region of the *E. coli fur* gene itself, raising the possibility that there may be autoregulation of *fur* by iron (44). Bases in the iron boxes shown in Fig. 1 which are invariant from the consensus sequence include the T at position 6 and ATT at positions 14 to 16. The remaining bases in the iron boxes match the consensus sequence in four to six of the promoters shown in Fig. 1.

On the basis of the evidence presented above, the mech-

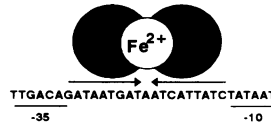
Inactive Fur Repressor**Active Fur Repressor**

FIG. 2. Schematic representation of iron regulation by Fur in *E. coli*. The Fur-binding site overlaps the promoters of iron-regulated genes. Fe^{2+} acts as a corepressor with Fur at sufficient intracellular concentrations of iron. The active Fur-iron complex binds to the Fur-binding site and prevents transcription.

anism by which iron concentration regulates gene expression in *E. coli* can be schematically represented by the model depicted in Fig. 2. The consensus Fur-binding site is located near or within the promoter of iron-regulated genes. Fe^{2+} is a corepressor such that at sufficient intracellular concentrations of iron the Fur-iron complex binds DNA at the iron box and prevents transcription of the iron-regulated gene.

REGULATION OF TOXIN EXPRESSION BY IRON**Shiga Toxin and SLTs**

Background. Shiga toxin from *S. dysenteriae*, *E. coli* SLT-I, SLT-II, and the *E. coli* pig edema disease toxin SLT-IIv constitute a group of closely related proteins that have similar biological activities and homologous amino acid sequences. Within this group, only Shiga toxin and SLT-I are regulated by iron. The biological activities of these toxins include cytotoxicity for certain tissue culture cell lines, neurotoxicity and lethality after injection into mice, and enterotoxicity in the ligated rabbit ileal loop assay (50, 113).

Shiga toxin produced by *S. dysenteriae* serotype 1 was discovered in 1903 by Conradi (36). In 1982, O'Brien et al. reported that certain strains of enterohemorrhagic *E. coli* produced similar toxins that were neutralized by antisera to Shiga toxin and named such toxins Shiga-like toxins (SLTs) (114). SLT-producing *E. coli* have been epidemiologically associated with hemorrhagic colitis (81, 115) and the hemolytic uremic syndrome in humans (84). Subsequently, the SLT family has been divided into SLT-I (neutralized by anti-Shiga toxin antibody) and SLT-II (not neutralized) (139); the related pig edema disease toxin has been defined as a variant of SLT-II (95).

The deduced amino acid sequences of Shiga toxin and SLT-I are highly homologous (28, 138), while those of SLT-II and SLT-IIv are more divergent (95). Mature Shiga toxin is composed of a single 31-kDa A subunit and multiple 7.7-kDa B subunits (116) with a stoichiometry of 1:5 (46). Mild trypsin cleavage of the A subunit results in a 27-kDa A1 fragment that is catalytically active and inhibits protein synthesis by inactivation of the 60S ribosomal subunit (116, 124). Shiga toxin, SLT-I, SLT-II, and SLT-IIv all have the same mechanism of action as the A chain of ricin, i.e., cleaving a specific adenine residue from the 28S rRNA component of the 60S eukaryotic ribosomal subunit (51–53, 128). This RNA *N*-glycosidase activity rapidly inhibits protein synthesis.

Regulation of expression of Shiga toxin and SLT-I by iron. Dubos and Geiger demonstrated that Shiga toxin from

S. dysenteriae was produced in lesser amounts when an excess of iron was present in the culture medium (47). Van Heyningen and Gladstone also studied the effects of iron on Shiga toxin production and showed that iron deficiency enhanced production of the toxin (148).

The molecular mechanism of regulation by iron has been examined in detail for the SLT-I of *E. coli*. Calderwood and Mekalanos used gene fusions between the promoter proximal *slt-IA* gene and *TnphoA* to study regulation of SLT-I expression by iron (29). Using the alkaline phosphatase activity of the gene fusion as a recorder, they demonstrated that iron represses SLT-I expression. When the gene fusion was transferred into a *fur* mutant strain of *E. coli*, iron no longer decreased SLT-I expression, suggesting that the Fur protein served as a repressor for the SLT-I operon. Deletion analysis of the *slt-I* promoter localized the region necessary for regulation of *slt-I* expression by iron and Fur. A 21-bp dyad repeat identified in this region was homologous to similar dyads in the promoter regions of three other iron-regulated genes in *E. coli* (29) (Fig. 1). Primer extension with RNA from a strain carrying the SLT-I operon cloned on a multicopy plasmid identified a transcriptional start site (42) compatible with the promoter proposed by Calderwood and Mekalanos. Semiquantitative primer extension experiments showed that this RNA transcript was greatly increased under conditions of iron starvation (42). These data led three groups of investigators (29, 42, 44) to propose a common consensus sequence for the binding of Fur to iron-regulated promoters (Fig. 1) Calderwood and Mekalanos subsequently confirmed this proposed consensus sequence by introducing it as a synthetic oligonucleotide into a heterologous promoter and showing that the oligonucleotide was sufficient to confer iron regulation (30). Since the sequence of the Shiga toxin operon (138) is nearly identical to that of SLT-I, it is likely that regulation of Shiga toxin expression by iron occurs by the same mechanism as regulation of SLT-I expression. In contrast, genes that encode expression of SLT-II and SLT-IIv are not regulated by iron and do not contain the Fur-binding consensus sequence in their promoter regions (141).

Diphtheria Toxin

Background. Diphtheria toxin from *C. diphtheriae* belongs to a class of secreted bacterial toxins that transfer the ADP-ribose moiety of NAD^+ onto specific target proteins within mammalian cells (35, 79, 80). *Pseudomonas* exotoxin A is another member of this class, and both of these toxins are regulated by the concentration of iron in the environment. Despite the similar intracellular mechanisms of action of diphtheria toxin and *Pseudomonas* exotoxin A, these toxins are associated with different disease processes, probably because of different target cell specificities (101).

Regulation of expression of diphtheria toxin by iron. Diphtheria toxin is synthesized by strains of *C. diphtheriae* that are lysogenic for one of a family of corynebacteriophages carrying the structural gene for the toxin, *tox* (25, 146). Pappenheimer and Johnson first observed in 1936 that addition of iron to the growth medium inhibited the production of diphtheria toxin by *C. diphtheriae* (119). More recently, a siderophore of *C. diphtheriae* that is coordinately regulated with diphtheria toxin by iron has been described (126, 143).

Several investigators have shown that regulation of diphtheria toxin expression by elevated levels of iron in the growth medium occurs at the level of transcription (82, 89). Evidence for both *cis*- and *trans*-acting regulatory elements

responsive to iron has been presented. Evidence for a *cis*-acting (operator) site in the *tox* gene itself was suggested by the isolation of corynebacteriophage mutants in which *tox* expression was insensitive to iron (107, 155). Evidence that iron regulation of *tox* expression also requires a negative *trans*-acting bacterial factor was suggested by the isolation of bacterial mutants in which *tox* expression from a number of different corynebacteriophages was also insensitive to iron (83). These biochemical and genetic observations supported the hypothesis proposed by Murphy and Bacha that the corynebacteriophage *tox* gene is regulated by an iron-binding repressor encoded by the bacterial host (106).

The putative *tox* operator locus has been identified as a 27-bp region that contains inverted repeated sequences separated by 9 bp (20). This putative *tox* operator locus shares some homology with the *E. coli* Fur-binding consensus sequence. Fourel et al. identified an iron-binding protein in extracts of *C. diphtheriae*, which they named DtoxR, that could bind to the presumptive *tox* operator locus and protect it from DNase I digestion (59). Tai and Holmes reported that the *E. coli* Fur protein partially regulated expression of a *tox::galk* transcriptional fusion in which the *tox* regulatory region and the coding sequence for fragment A and a portion of fragment B of diphtheria toxin were cloned upstream of a promoterless *galk* gene (142). In *E. coli*, the expression of this *tox::galk* fusion was repressed approximately fivefold under iron-sufficient conditions, and this repression appeared to depend on the *E. coli fur* gene (142).

Boyd et al. cloned the *trans*-acting *tox* regulatory element from *C. diphtheriae* by using a *tox* promoter-operator (*toxPO*)-*lacZ* transcriptional fusion introduced into the chromosome of *E. coli* (21). This strain was used to screen a genomic library of nontoxigenic, nonlysogenic *C. diphtheriae* for clones that would repress *lacZ* expression in the presence of iron. The diphtheria *tox* iron-regulatory gene *dtxR* was cloned in this fashion and shown to regulate the expression of β -galactosidase from the *toxPO::lacZ* fusion in an iron-dependent manner. They found that *dtxR* was homologous to the *E. coli fur* gene at both the nucleotide (66%) and amino acid sequence (25%) levels. Schmitt and Holmes, however, performed an independent analysis of nucleotide and deduced amino acid homologies between *dtxR* and *fur* with the Genetics Computer Group sequence analysis program and found less homology, only 39% identity at the nucleotide level and 18% identity at the amino acid level, under optimal sequence alignment (130). Boyd et al. found that the expression of the *toxPO::lacZ* fusion was not repressed by *E. coli fur* in *trans*, and the presence of *dtxR* did not inhibit expression of the iron-regulated outer membrane proteins of *E. coli* (21). The limited homology between the deduced amino acid sequences of DtxR and Fur is consistent with the finding that complementation between these proteins is weak (142) or absent (21). These data suggest that, despite similar mechanisms of action by both DtxR and Fur, their iron-regulatory activities are restricted to the specific operator-binding sites characteristic of their species of origin.

P. aeruginosa Exotoxin A

Background. Of the numerous extracellular proteins produced by *P. aeruginosa*, including exoenzyme S, elastase, alkaline protease, phospholipase C, and leukocidin, exotoxin A is the most toxic and appears to be an important virulence factor in this opportunistic pathogen (160). The expression of exotoxin A is regulated in a temporal manner

and by numerous environmental factors. Toxin production is enhanced late in the growth cycle and by aeration, glycerol, or growth at 32°C (92). The divalent cations Fe²⁺, Co²⁺, Cu²⁺, and Mn²⁺ inhibit exotoxin A expression, while Mg²⁺ and Zn²⁺ have no effect (19). Ca²⁺ increases exotoxin A yields (19). The regulation of exotoxin A production is thus complex and appears to require the participation of a number of gene products in response to environmental stimuli.

Regulation of exotoxin A expression by iron. In *P. aeruginosa*, iron limitation derepresses the synthesis of not only the siderophores pyochelin and pyoverdinin but also a number of virulence factors, including exotoxin A, elastase, and alkaline protease (15, 16). Exotoxin A production usually decreases 10-fold when clinical isolates of *P. aeruginosa* are grown in iron-containing medium (15, 16). Bjorn et al. (15) postulated that iron either decreased the rate of synthesis of exotoxin A or increased the rate of its degradation.

Sokol et al. identified several iron-regulatory mutants of *P. aeruginosa* PAO1 by selecting for strains that still produced elastase or exotoxin A when grown in medium supplemented with iron (133). Mutants insensitive to the effect of iron on the production of exotoxin A (*toxC*) or of elastase (*elaC*) fell into two classes: mutants with mutations in iron uptake and mutants with gene expression altered in response to iron. In the first class of mutants, one strain, PAO-Fe10, produced normal amounts of pyochelin and pyoverdinin but was deficient in the transport of these siderophores and inorganic iron. Another mutant, PAO-Fe11, overproduced exotoxin A but was deficient in the production of pyoverdinin. These mutants suggested that defects in iron acquisition by *P. aeruginosa* could result in the overproduction of gene products that are normally repressed under high-iron conditions. In the second class of mutants, some produced exotoxin A in high-iron conditions (*toxC*), but alkaline protease and elastase expression remained sensitive to iron. Other mutants produced elastase in high-iron conditions (*elaC*), but expression of exotoxin A and alkaline protease remained iron regulated. These data suggested that exotoxin A, elastase, and alkaline protease expression could be independently regulated by iron.

Several studies have suggested that regulation of exotoxin A expression by iron occurs at the level of transcription (60, 61, 69, 93). In addition, transcription of *toxA* requires the product of a positive regulatory gene, *regA*, which is itself negatively regulated by iron (76). The *regA* transcript appears earlier in the growth cycle than the *toxA* transcript, and RegA is required for maximal *toxA* transcription. When transcription of *regA* is inhibited by high-iron conditions, transcription of *toxA* is similarly reduced (76).

Frank and Iglewski have performed more detailed experiments on *regA* expression in response to iron (60). They reported two *regA* transcripts, one (called T1) occurring early in the growth phase and not tightly regulated by iron and the other (called T2) occurring later in the growth phase and tightly regulated by iron. Northern (RNA) blot analysis suggested that the two *regA* transcripts, T1 and T2, originated from different start sites. Analysis of *toxA* transcription showed a similar pattern of growth phase and iron regulation, providing further evidence that regulation of *toxA* transcription by iron reflects regulation of the positive transcriptional activator *regA*.

Regulation of *toxA* and *regA* transcription was studied further by using *P. aeruginosa* PA103 containing the *regA* gene on a multicopy plasmid (61). There were still two phases of *regA* transcript accumulation, a T1 (early phase) and a T2 (late phase) transcript. However, no effect of iron

on the accumulation of the T1 transcript was seen, while accumulation of the T2 transcript was still completely inhibited in high-iron conditions. Primer extension analysis confirmed that *regA* transcription is controlled by two separate promoters, P1 and P2, which correspond to transcripts T1 and T2, respectively. Transcription from the P1 promoter is not tightly regulated by iron, while transcription from P2 is. Regulation of transcription from the P2 promoter by iron late in the growth phase is responsible for iron regulation of exotoxin A expression.

Storey et al. have shown that the *regB* gene, which is downstream of the *regA* gene in an operon, is required for transcription from the P1 promoter (137). Transcription from the *regAB* operon, therefore, is autoregulated, and production of RegB from the T1 transcript early in the growth cycle is required for maximal synthesis of exotoxin A, while later in the growth cycle, transcription from the P2 promoter (T2 transcript) is RegB independent and iron regulated.

Analysis of the sequences upstream of the two start sites for *regA* transcription reveals little homology between the proposed P1 and P2 promoters. The P2 promoter contains a region (TTGATT) 24 bp upstream of the T2 start site with limited homology to the consensus -35 box of *E. coli* (TTGACA) but with perfect homology to the -35 region of the iron-regulated diphtheria toxin promoter. Similarly, the -10 region of the P2 promoter (TAGGGT) is highly homologous to the -10 region of the diphtheria toxin promoter (TAGGAT). On the basis of these observations, a common mechanism for iron regulation of exotoxin A and diphtheria toxin can be postulated (60).

Recent evidence suggests that negative regulation of gene expression in *P. aeruginosa* by iron is mediated by a Fur homolog. Prince et al. (122) introduced a multicopy plasmid containing the *E. coli fur* gene into *P. aeruginosa* PA103C, which contains a *toxA::lacZ* fusion integrated into the chromosome. Normally, β -galactosidase expression in strain PA103C is regulated by iron, but this activity remained repressed even under low-iron conditions in the presence of multiple copies of the *E. coli fur* gene. Transcription of *regA* was similarly repressed under low-iron conditions when multiple copies of the *E. coli fur* gene were present. A polyclonal rabbit antiserum to *E. coli* Fur recognized a similarly sized protein in whole-cell extracts of *P. aeruginosa*, thus providing direct evidence of a Fur homolog (122).

Prince et al. (122) also found by Northern (RNA) blot analysis that multiple copies of *E. coli fur* inhibited transcription from the P1 promoter, which presumably leads to expression of RegA and RegB, but did not repress transcription from the P2 promoter. This contrasts with the findings by Frank and Iglewski (60) and Storey et al. (137), who found that the P1 promoter of *regA* was not tightly regulated by iron and the P2 promoter was active later in the growth cycle and tightly regulated by iron. The data by Prince et al. suggest that iron regulation of transcription from P2 is not regulated by *E. coli* Fur. The mechanism by which *E. coli* Fur affects *regA* transcription is unclear, and further clarification will require the cloned *P. aeruginosa fur* gene.

IRON-REGULATED OUTER MEMBRANE PROTEINS ASSOCIATED WITH VIRULENCE IN *VIBRIO* SPECIES

Vibrio cholerae

IrgA is an outer membrane protein described recently as an iron-regulated virulence determinant in *V. cholerae*.

Identification of IrgA followed *TnphoA* mutagenesis to obtain insertion mutations in several iron-regulated genes of *V. cholerae* O395 and subsequent assay of these transposon mutant strains for virulence defects (68). One such mutant strain had lost the 77-kDa major iron-regulated outer membrane protein of *V. cholerae* and was attenuated for virulence; the 50% lethal dose of this mutant in suckling mice was 3×10^5 bacteria compared with 4×10^3 bacteria for the wild-type parent. An in vivo competitive assay between the mutant and the parent suggested a possible colonization defect.

To define the mechanism of regulation of *irgA* expression by iron, the 5'-proximal portion of *irgA* was cloned and sequenced, and the promoter region was defined by primer extension (66). Regulation of *irgA* expression by iron occurred at the transcriptional level. An interrupted dyad symmetric nucleotide sequence was identified in the vicinity of the promoter that was homologous to the consensus Fur-binding site in *E. coli* (Fig. 1). Unlike transcription of iron-regulated genes in *E. coli*, however, transcription of *irgA* required an additional 900 bp of upstream DNA that contained an open reading frame in inverse orientation to *irgA*.

Transcription of this upstream open reading frame was also negatively regulated in *V. cholerae* by iron (67), and this gene was designated *irgB*. Insertional inactivation of *irgB* on the *V. cholerae* chromosome led to loss of expression of an *irgA::TnphoA* fusion; expression of the fusion was restored to normal when *irgB* was present on a plasmid in *trans*. The predicted protein product of *irgB* (IrgB) is homologous to the LysR family of positive transcriptional activators (75). The promoters of *irgB* and *irgA* are divergent but overlap each other and the dyad sequence homologous to the *E. coli* Fur-binding site. A model for iron regulation of *irgA* expression was proposed on the basis of these studies. In the presence of sufficient iron, transcription of both *irgA* and *irgB* is repressed by the Fur protein of *V. cholerae*. When iron becomes limiting, negative regulation of transcription is removed and the production of IrgB leads to positive transcriptional activation of *irgA*.

The complete sequence of *irgA* is now available and demonstrates that IrgA is homologous to the TonB-dependent outer membrane transport proteins of *E. coli* (65). Despite this homology, however, a mutant of *irgA* is not defective in the transport of any known iron source for *V. cholerae*, including vibriobactin, ferrichrome, ferric citrate, hemin, or hemoglobin. Gene sequences hybridizing at high stringency to an *irgA* probe are found in several *Vibrio* species, including non-O1 *V. cholerae*, *Vibrio parahaemolyticus*, *Vibrio fluvialis*, and *Vibrio alginolyticus*. Whether *irgA* is a virulence gene in these other species is unknown, as is the role of IrgA in the pathogenesis of *V. cholerae* infection.

The observations that *irgA* is iron regulated in an *E. coli* background and that the *E. coli* gene *slt-1A* is reciprocally iron regulated in a *V. cholerae* background suggest that the mechanism of regulation by iron in *V. cholerae* is similar to the *E. coli* Fur system (66). Another study supporting this hypothesis was provided by Stoebner and Payne, who described a mutant strain of *V. cholerae* in which iron regulation of the soluble hemolysin and synthesis of the siderophore vibriobactin had been lost (136). Regulation of these genes by iron was restored when the *E. coli fur* gene was introduced into the mutant on a plasmid.

The *fur* gene of *E. coli* has been used recently as a probe to recover the intact *fur* gene of *V. cholerae*. Southern

hybridization of *V. cholerae* chromosomal DNA probed with a DNA fragment internal to the *E. coli fur* gene under conditions of low stringency showed single hybridizing bands with a number of restriction enzymes that allowed development of a restriction map around the hybridizing region in *V. cholerae* (91). The *V. cholerae fur* gene was recovered, and the intact cloned gene was able to complement an *E. coli fur* mutation. Primer extension analysis localized two promoters for the *V. cholerae fur* gene; no significant homology to the *E. coli Fur*-binding site was identified in either promoter. According to Northern blot analysis, the two *fur* transcripts were not strongly regulated by iron. DNA sequencing revealed a single open reading frame of 150 amino acids that was 76% homologous to the *E. coli Fur* protein.

Vibrio anguillarum

V. anguillarum is the causative agent of vibriosis, a widespread hemorrhagic septicemic disease in salmonid fishes. A major virulence factor for this bacterium is the presence of a specific plasmid, such as the 65-kbp plasmid pJM1, that encodes a siderophore-mediated iron acquisition system (39). This system includes the outer membrane receptor protein OM2 (2) and the catechol siderophore anguibactin (1). Virulence of *V. anguillarum* is attenuated by mutations in this plasmid-mediated system that abolish anguibactin biosynthesis or OM2 production (151) or by absence of the entire plasmid (40).

Genetic analysis of plasmid pJM1 by insertion mutagenesis and *lacZ* fusions identified six distinct genetic units that were required for the efficient uptake of iron. Five of these genetic units were essential for biosynthesis of the siderophore anguibactin (units I, III, IV, V, and VI). Bacteria containing mutations in the remaining genetic unit, unit II, were deficient in iron transport and produced reduced levels of anguibactin (144). Cloning, mutagenesis, and sequencing of genetic unit II localized the gene for OM2, the outer membrane protein essential for transport of iron by anguibactin, (144) and a gene encoding a 40-kDa polypeptide, designated p40 (3).

Cloning and analysis of an area noncontiguous to the iron uptake region also identified genetic determinants for a *trans*-acting factor (*taf*) required for full expression of the anguibactin uptake system. Analysis of siderophore production and β -galactosidase activity of *lacZ* fusions suggested that Taf was a transcriptional activator of the siderophore biosynthetic genes (144). More recent evidence suggests that Taf may also activate the genes involved in transport of the ferric anguibactin complex into the cell (127).

In a survey of *V. anguillarum* isolates from different parts of the world, certain strains were identified as producing higher levels of the siderophore anguibactin. This increased siderophore production (Isp) was encoded by plasmids similar to pJM1, such as plasmid pJHC1. The gene responsible for the Isp phenotype was cloned from pJHC1 and designated *angR*; this gene encoded a 110-kDa polypeptide product. Both the gene *angR* and its protein product were also found in strains containing pJM1, and *angR* mapped to the previously identified genetic unit III. The differences in *angR* that lead to the Isp phenotype in strains containing pJHC1 rather than pJM1 are not clear (127).

Analysis of specific RNA transcripts demonstrates that AngR and Taf regulate the production of anguibactin by activating transcription of the biosynthetic genes under iron-limiting conditions. Both AngR and Taf can act inde-

pendently, but the factors together activate anguibactin biosynthesis in a synergistic manner (127).

Transcriptional analysis of *angR* reveals that this gene itself is regulated by iron. The cloned *angR* gene was sequenced, and a region upstream of *angR* was found to have weak homology to the *E. coli Fur* consensus binding sequence. This suggests that a Fur-like protein is responsible for regulation of *angR* transcription in the presence of iron. The deduced amino acid sequence of AngR predicts a helix-turn-helix motif in the protein that has homology to prokaryotic DNA-binding proteins (55), which is consistent with its role as a transcriptional activator.

ROLE OF IRON IN THE VIRULENCE OF OTHER BACTERIA

Neisseria Species

The genus *Neisseria* includes two species that cause infections in humans, *Neisseria meningitidis* and *Neisseria gonorrhoeae*. The importance of iron in infections with *Neisseria* spp. has been demonstrated in a variety of animal models. Studies by Holbein (77, 78) demonstrated that pretreatment of mice with iron dextran or ferric transferrin increased the risk of lethal septicemia following experimental infection with meningococci. Addition of iron to inocula of gonococci increased lethality for chicken embryos (120), while injection of the iron chelator desferrioxamine protected chicken embryos from infection (57). Recently, Genco et al. isolated iron uptake mutants of *N. gonorrhoeae* that could not utilize transferrin or hemoglobin as the sole source of iron and showed that these mutants were avirulent in a mouse subcutaneous chamber model (63).

N. gonorrhoeae and *N. meningitidis* do not appear to produce siderophores (5, 112, 156) but instead efficiently utilize a variety of host iron compounds. Meningococci and many gonococci utilize lactoferrin-bound iron (99). Both species can obtain sufficient iron for growth in vitro from transferrin (5, 97) as well as from hemoglobin (100), heme (100, 163), heme, and haptoglobin-bound hemoglobin (48). Iron acquisition from transferrin requires direct contact between transferrin and the organism (5) as well as iron-regulated outer membrane proteins, one or more of which may function as specific receptors (70, 131).

One of the iron-regulated proteins in *Neisseria* spp. is synthesized in significantly greater amounts than the others and has been called the major iron-regulated protein, recently changed to Fbp (for iron-binding protein). The gene for Fbp has been cloned, characterized, and sequenced (10, 11). Fbp has a molecular mass of approximately 37 kDa and is antigenically conserved among pathogenic species of *Neisseria*. The observation that iron copurifies with Fbp suggests that Fbp may play a role in iron acquisition; the presence of antibodies to Fbp in sera of patients with gonococcal infection suggests that Fbp is expressed in vivo and may play a role in neisserial pathogenicity (104).

Brener et al. (23) have reported a 69-kDa iron-regulated protein in the outer membrane of *N. meningitidis*, while *N. gonorrhoeae* strains have been found to synthesize a number of outer membrane proteins in response to iron deprivation (156). Schryvers and Morris (131) identified a 71-kDa, iron-regulated, transferrin-binding protein in *N. meningitidis*. A mutant of *N. meningitidis* that failed to utilize iron bound to transferrin or lactoferrin lacked a 70-kDa iron-regulated protein (48).

Genetic evidence that *N. gonorrhoeae* produces specific

receptors for transferrin and lactoferrin was provided by Blanton et al. (18), who isolated three phenotypic classes of mutants: those unable to utilize transferrin-bound iron (*trf* [transferrin receptor function]), those unable to utilize lactoferrin-bound iron (*lrf* [lactoferrin receptor function]), and those unable to utilize either (*tlu* [transferrin and lactoferrin utilization]). Each of these mutations appeared to be in a separate linkage group.

Two different meningococcal iron-repressible proteins (Frps) have been identified by their ability to bind human transferrin and have been designated TBP1 (transferrin-binding protein 1) and TBP2 (transferrin-binding protein 2) (118). Cornelissen et al. used polyclonal antiserum raised against gonococcal TBP1 to screen an expression library for fragments of the gene encoding TBP1, *tbpA* (37). Simultaneously, part of a wild-type gene that repaired the defect in a *trf* mutant was cloned, and DNA sequencing revealed that the two clones overlapped. Transposon mutagenesis of both clones and recombination of mutagenized fragments into the gonococcal chromosome generated mutants that were incapable of growth on transferrin. No TBP1 was produced in these mutants, but TBP2 expression was normal. The complete amino acid sequence of TbpA was homologous to the class of TonB-dependent proteins in *E. coli*, suggesting a possible role for a meningococcal TonB-like protein in the utilization of transferrin.

Yersinia Species

In vitro growth of *Yersinia* species in iron-deficient medium results in the expression of several iron-regulated outer membrane proteins. Carniel et al. found that the degree of virulence of various *Yersinia* species correlated with the presence of two high-molecular-weight, iron-regulated membrane proteins (33). These two polypeptides were found to have common epitopes and to be antigenically related. Less pathogenic or nonpathogenic strains of *Yersinia* spp. did not exhibit cross-reacting material, suggesting that these strains did not synthesize altered forms of the high-molecular-weight proteins (HMWP) (30). A portion of the gene encoding the smaller 190-kDa HMWP was cloned and used to probe various *Yersinia* species. Less pathogenic and nonpathogenic strains did not hybridize to the HMWP probe, suggesting that the absence of the gene rather than the lack of its expression was responsible for the failure of strains to synthesize HMWP (34).

Regulation of gene expression in *Yersinia* spp. by iron is mediated by a Fur protein homologous to that in *E. coli*. Carniel et al. found that the promoter for the 190-kDa HMWP (*irp2*) is iron and Fur regulated in an *E. coli* background (32). Staggs and Perry (134) found that an *E. coli* iron-regulated β -galactosidase reporter construct was also iron regulated in *Y. pestis*. Chromosomal DNA of *Y. pestis* hybridized to an *E. coli fur* probe, and a cloned fragment of *Y. pestis* DNA complemented a Fur⁻ phenotype in *E. coli* (134). The DNA sequence of the *Y. pestis fur* gene was determined recently (135); the gene exhibits 75% homology to the *E. coli* gene at the nucleotide level and 84% homology at the predicted amino acid level.

CONCLUSIONS AND FUTURE DIRECTIONS

The competitive growth and survival of bacteria in the host require a number of adaptive responses on the part of the bacteria; the acquisition of iron is one of the most important of these adaptive responses for bacterial patho-

genesis. A shift from a high- to a low-iron environment is an important environmental signal to bacteria that they have entered a host. Bacteria respond to the low-iron environment by increasing expression of iron acquisition systems as well as of virulence determinants.

The molecular basis of coordinate regulation by iron has been most thoroughly studied in *E. coli*. In this organism, coordinate regulation of gene expression by iron depends on the repressor gene, *fur*. Fur-binding sites homologous to a 19-bp consensus sequence overlap the promoters of iron-regulated genes. Fe²⁺ is a corepressor such that at sufficient intracellular concentrations of iron the Fur-iron complex binds to Fur-binding sites to prevent transcription. The work with *E. coli* provides a model of gene regulation that can be used to study the molecular mechanisms of regulation by iron in other bacterial species.

Most of the research on the mechanisms of regulation of virulence determinants by iron has been in gram-negative bacteria. Regulation of gene expression by iron in *V. cholerae* and *Y. pestis* is coordinated by proteins highly homologous to the Fur protein of *E. coli*. The *fur* genes of these two pathogens have been cloned and sequenced. Recent evidence also suggests that negative regulation of gene expression in *P. aeruginosa* by iron is mediated by a Fur homolog.

Relatively little is known concerning iron regulation in gram-positive bacteria. In this group, iron regulation has been primarily studied in *C. diphtheriae*. The gene *dtxR* of *C. diphtheriae* has been cloned and shown to be responsible for the regulation of diphtheria toxin expression by iron. Homology between the deduced amino acid sequences of DtxR and Fur is quite limited, consistent with the finding that complementation between these two gene products is weak or absent. Despite similar mechanisms of action for DtxR and Fur, their binding sites may be limited to their species of origin. Further studies on iron regulation in gram-positive bacteria are needed.

Regulation of a number of virulence determinants by iron appears to be complex and requires more than one regulatory factor. Examples of this complex regulation include exotoxin A of *P. aeruginosa*, iron-regulated gene A (*irgA*) of *V. cholerae*, and anguibactin of *V. anguillarum*. Additional regulatory proteins may be needed to provide the fine-tuning necessary for the precise regulation of individual virulence genes in response to iron and other environmental signals. Further studies of the interactions of iron and other environmental signals on virulence gene expression should lead to a better understanding of the adaptive responses of bacteria to the complex environment of their host.

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