Biol Metals (1991) 4:7–13

BIOLOGY OF METALS

Iron and bacterial virulence – a brief overview

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Summary. Iron is now recognized as playing a vital role in infection. Not only does its restricted availability in tissue fluids present microbial pathogens with the problem of acquiring sufficient for multiplication in vivo, but it also constitutes a major environmental signal which co-ordinately regulates the expression of a number of virulence and metabolic genes. Progress in understanding the strategies used by pathogens for acquiring iron in vivo, and their responses to iron restriction, is providing a fresh insight into microbial pathogenicity.

Key words: Iron availability – Microbial pathogenicity – Iron-uptake system – Haem

Introduction

The ability of an invading pathogen to multiply successfully under the conditions found in the host is an essential factor in all infections. Here the pathogen must produce the full complement of virulence determinants required for pathogenicity. Some pathogens move through different environments once inside the host, for example from a mucosal surface into the blood stream or from an extracellular location to inside host cells. Others spend time both within and outside their hosts, or within two different types of hosts. In the past the effect of different host environments on bacterial pathogens has been largely ignored. It does however have important consequences not only for our understanding of bacterial pathogenicity and of the diseases caused, but also for the design of measures used to prevent or treat infections. This situation is now changing rapidly and we are beginning to appreciate that specialized determinants are induced only when a pathogen encounters its host (Griffiths 1989). We also see that pathogenicity generally depends on a variety of bacterial properties rather than on any one trait, and that the response to environmental signals involves coordinate alterations in the expression of sets of genes and operons encoding several factors (Miller et al. 1989).

One of the best understood properties of the environment encountered by pathogens in host tissues, and of its effects on bacterial characteristics and growth, concerns the availability of iron. Our understanding of the way the host normally restricts the availability of this metal has increased enormously in recent years and there is now a considerable literature on the relationship between iron and pathogenicity (Bullen and Griffiths 1987; Crosa 1989; Weinberg 1989; Martinez et al. 1990).

Availability of iron in vivo

Although there is a considerable amount of iron present in the body fluids of humans and animals, it is known that the amount of free iron which might be available to invading bacteria is normally extremely small. Most iron is found intracellularly in ferritin, haemosiderin or haem. Extracellular iron in normal body fluids such as plasma and mucosal secretions is attached to high-affinity iron-binding glycoproteins, transferrin or lactoferrin, or both. A related protein called ovotransferrin is found in avian egg white. These proteins have association constants for iron of about 10^{36} and thus bind iron extremely tightly. Indeed, it has been calculated that the amount of free iron in equilibrium with an iron-binding protein at neutral pH is of the order of 10^{-18} M which is far too low for bacterial growth. Nevertheless, pathogenic organisms can multiply successfully in these body fluids to establish extracellular infections. They must, therefore, be able to adapt to this iron-restricted environment and produce mechanisms for assimilating protein-bound iron, or for acquiring it from liberated haem. So far little is known about the availability of iron inside cells, although it seems that in some cells at least iron is readily available to support bacterial growth (Lawlor et al. 1987; Nassif et al. 1987; Byrd and Horwitz 1989). The nature of this 'available pool' of iron is unknown, although it is prob-

ably composed of iron complexed by relatively low-molecular-mass chelators. It is likely to represent only a very small amount of cellular iron in view of the toxicity of the loosely bound metal (Bullen and Griffiths 1987; Crichton and Charloteaux-Wauters 1987).

Siderophore-mediated iron-uptake systems

By far the best understood systems whereby bacterial pathogens assimilate iron from host iron-binding proteins are those which depend on the production of siderophores (Bullen and Griffiths 1987; Crosa 1989; Weinberg 1989). Some siderophores are able to remove iron from iron-binding proteins and the best characterized of these are those used by enteric organisms. For example, *Escherichia coli, Salmonella typhimurium* and *Klebsiella pneumoniae* produce a phenolate chelator called enterobactin (enterochelin) under conditions of iron restriction in vitro. This siderophore is synthesized only during iron restricted growth and it efficiently removes iron from iron-binding proteins and transports it into the bacterial cell. Enterobactin has also been shown to be produced in vivo during infection. However, in the case of *S. typhimurium*, the loss by mutation of the ability to make enterobactin had no overall effect on the virulence of the organism for several inbred strains of mice, although the Ent^- mutants were unable to multiply in mouse serum (Benjamin et al. 1985). Since *S. typhimurium* is an intracellular pathogen in mice it has been suggested that the failure of the enterobactin mutant to affect virulence occurs because the siderophore may not be required for growth once the pathogen has entered the cells of the reticuloendothelial system. The role of enterobactin in the virulence of *E. coli* which cause intestinal infections has not been tested.

Although enterobactin seems to be the main endogenous siderophore made by *E. coli, Salmonella* and *Klebsiella* species during iron restriction, several clinical isolates of these organisms also possess a second high-affinity iron-uptake system mediated by the hydroxamate siderophore aerobactin. In particular, many of the strains which cause septicaemia or other extraintestinal infections produce aerobactin, the genes for the synthesis of which can be located in the chromosome or on a plasmid (Crosa 1989; Opal et al. 1990). The enteroinvasive pathogen *Shigella flexneri* also produces aerobactin and the genes in this case are located on the chromosome (Payne 1989). It is not entirely clear why acquiring the ability to make this second siderophore confers a selective advantage on bacteria that can already make enterobactin but there have been a number of hypotheses (see Bullen and Griffiths 1987; Crosa 1989). Roberts et al. (1989) have shown that the aerobactin iron-uptake system of plasmid ColV-K30, genetically isolated from other plasmid determinants by molecular cloning, is sufficient to restore to full virulence, in a mouse peritonitis model, a clinical *E. coli* isolate whose resident aerobactin-encoding ColV plasmid had been lost by curing. However, what is not clear is whether both enterobactin and aerobactin are required for full virulence. So far no well characterized Ent^- Aer^+ strains of *E. coli* have been reported amongst clinical isolates from septicaemic disease, although most strains of *S. flexneri* produce only aerobactin. The failure of *S. flexneri* to produce enterobactin does not reflect a lack of the genes involved in the production of the siderophore but indicates a defect in their expression (Schmitt and Payne 1988; Payne 1989; Crosa 1989). Studies have shown that the ability of *S. flexneri* to synthesize aerobactin has no effect on intracellular multiplication but it does give these invasive organisms a selective advantage for growth when located in extracellular compartments. *Shigella* have a relatively complex life-style alternating between growth within host cells and growth within the extracellular spaces, or outside the human host. Payne (1989) suggests that siderophores may be called into play when the *Shigella* are multiplying in the extracellular spaces of the host tissue, and also may be important for the survival of the pathogen in the environment outside the host.

An integral part of siderophore-mediated iron-uptake systems is the production of outer-membrane proteins which act as receptors for ferric siderophores, as well as mechanisms for the release of chelator-bound iron (see Bullen and Griffiths 1987; Crosa 1989). The enteric bacteria produce several new outer-membrane proteins under iron-restricted conditions, some of which have been identified as ferric-siderophore receptors. For example, an 81-kDa protein in *E. coli* functions as the receptor for ferric-enterobactin and a colicin V plasmid-encoded 74-kDa protein functions as the receptor for aerobactin. Most of the work on the iron-regulated outer-membrane proteins of *E. coli* has been carried out with laboratory strains, such as *E. coli* K12. However, pathogenic strains produce similar new proteins when grown in vitro in the presence of iron-binding proteins, or in vivo during infection. Results also show considerable qualitative and quantitative variation in the expression of iron-regulated membrane proteins by different strains of pathogenic *E. coli* (Chart et al. 1988). Some strains produce larger quantities of these proteins than *E. coli* K12 and some produce iron-regulated proteins not seen in laboratory strains. Naturally occurring antibodies against iron-regulated proteins have been detected in both man and animals (Griffiths et al. 1985).

Although much of the initial work was carried out in pathogenic Enterobacteria, siderophores and iron-regulated envelope proteins have now been found in many other pathogens (Bullen and Griffiths 1987) and the list continues to grow (Griffiths 1989). In some cases the connection between the ability to make a siderophore and to use it to acquire iron from host iron-binding proteins is clear and the role of such systems in virulence well established. For example, highly virulent strains of *Vibrio anguillarum,* which are responsible for a devastating septicaemic disease of fish, carry a plasmid which encodes a siderophore iron-transport system that allows the organism to grow under iron-restricted conditions. On losing the plasmid, *V. anguillarum* loses

its ability to grow under such conditions and also loses its virulence. The molecular analysis of this virulence factor, recently reviewed by Crosa (1989), has shown that the iron-uptake system is based on a phenolic siderophore, anguibactin, and a 86-kDa iron-regulated outer-membrane protein receptor, both of which are required for virulence.

In other cases the contribution of siderophore-mediated iron-uptake systems to virulence is less clear. For example, the production of the *Vibrio cholerae* siderophore, vibriobactin, seems not to be essential for infection of intestinal mucosae by this organism, although there is evidence to suggest that at least some of the iron-regulated membrane proteins are switched on in vivo (Sigel et al. 1985; Jonson et al. 1989; Richardson et al. 1989). Part of the difficulties in assessing the role of siderophores in natural infection are often related to the type of animal model used in experimental work. Of course other iron-uptake systems may be operating in vivo and it has been suggested that traces of haemin or haemoglobin could serve as a source of iron for *V. cholerae* (Stoebner and Payne 1988). Further studies are required to clarify this situation. The relative roles in infection of two siderophores produced by *Pseudomonas* species, pyochelin and pyoverdin, also remain unclear (Sokol 1987; Sokol and Woods 1988; Ankenbauer et al. 1988; Döring et al. 1988; Meyer et al. 1990). It may well be, of course, that each plays a part in different types of *Pseudomonas* infections.

An increasingly reported phenomenon is the ability of pathogens to use siderophores produced by other microorganisms, but which they themselves are unable to synthesize. It has been known for a long time that enteric bacteria express protein receptors not only for their own siderophores but also for those of other organisms. Thus, *E. coli* is capable of using the fungal iron chelators ferrichrome and coprogen (Bullen and Griffiths 1987), whilst *Neisseria gonorrhoeae* can use aerobactin (West and Sparling 1987). Another study has shown that a high percentage of enterotoxigenic *E. coli* strains isolated from children with diarrhoeal disease in Chile had an aerobactin receptor, but were unable to make this chelator (Fernandez-Beros et al. 1988). Some strains of *K. pneumoniae* also produce a novel aerobactin receptor without producing aerobactin itself (Williams et al. 1989). Recently it has been shown that *Haemophilus parainfluenzae* and *H. paraphrophilus,* but not *H. influenzae* type b, were able to use enterobactin but not aerobactin or ferrioxamine (Williams et al. 1990). Ferrioxamine is, however, able to supply iron to certain other organisms, such as *Klebsiella, Salmonella* and *Yersinia enterocolitica* (Bullen and Griffiths 1987). Since the methane sulphonate salt of desferrioxamine B is available commercially as Desferal (Ciba-Geigy) and is widely used clinically in deferration therapy, the possibility should be borne in mind that it might increase the susceptibility of treated individuals to bacterial infections by making iron more readily available to the invading pathogen. Indeed, cases of septicaemia due to *Y. enterocolitica* in individuals treated with Desferal have been reported (Melby et al. 1982). Whether the ability to use exogenous siderophores plays any part in natural infections, possibly mixed infections, remains to be seen.

Siderophore-independent receptor-mediated iron-uptake systems

The fact that siderophore-dependent systems are the best understood at present does not mean that they are necessarily the most common or indeed the most effective systems for sequestering iron in vivo. Some pathogens, like the gonococcus, meningococcus and *H. influenzae,* use a mechanism which depends on the direct interaction between the bacterial cell surface and the iron-binding protein in a manner analogous to the reaction between transferrin and the mammalian cell (Crichton and Charloteaux-Wauters 1987) and these systems are now attracting considerable attention; no siderophores are involved.

Perhaps the most significant feature which distinguishes them from the known siderophore-mediated mechanisms is the highly specific nature of the process (Bullen and Griffiths 1987; Schryvers and Morris 1988a, b; Morton and Williams 1989). For example, the iron-uptake system of *N. meningitidis* is highly specific for human transferrin and human lactoferrin and discriminates against lactoferrin and transferrin from other species. This has obvious implications for explanations of host specificity of *N. meningitidis* and for the development of an animal model (Schryvers and Gonzalez 1989).

Although the details of the molecular mechanism of iron-uptake in such organisms are not understood, there is considerable evidence to show that saturable membrane receptors specific for either transferrin or lactoferrin are involved (McKenna et al. 1988; Tsai et al. 1988; Schryvers and Morris 1988a; Niven et al. 1989; Blanton et al. 1990). These transferrin and lactoferrin receptors are distinct entities and their expression has been shown to be regulated by iron. However, the biochemical identities of the receptors remain unclear although lactoferrin- and transferrin-binding proteins have been identified amongst the iron-regulated proteins found in the envelope of *N. meningitidis, N. gonorrhoeae* and *H. influenzae* (Schryvers and Morris 1988a, b; Schryvers and Lee 1989; Schryvers 1989; Banerjee-Bhatnager and Frasch 1990; Morton and Williams 1990). Of particular interest is the considerable molecular and antigenic heterogeneity of the meningococcal transferrin-binding protein in the many different clinical isolates of *N. meningitidis* (Griffiths et al. 1990; Ala'Aldeen et al. 1990a). Most strains have a transferrin-binding protein with a molecular mass between 78–83 kDa and a few have one of about 68 kDa. The size of the protein appears unrelated to the serogroup or serotype of the organism. Conserved antigenic regions have also been found amongst these proteins (Griffiths and Stevenson, unpublished data). The precise nature of the functional receptor in the bacterial cell membrane, and of the part played by the transferrin- or lac-

toferrin-binding protein in iron uptake now needs to be determined; evidence suggests that the transferrin receptor contains at least two proteins (Schryvers and Morris 1988b; Padda and Schryvers 1990). The parts of the transferrin and lactoferrin molecules recognised by their receptors and responsible for species specificity also need to be elucidated. Recent results by Padda and Schryvers (1990) indicate that the N-linked oligosaccharides of human transferrin are not required for binding to the meningococcal transferrin receptor.

Use of haem iron

In addition to obtaining iron from iron-binding proteins, some pathogens might be able to obtain sufficient iron in vivo from cell-free haem or haemoglobin; numerous pathogens are able to use haem or haemoglobin as a source of iron (Bullen and Griffiths 1987). Normally, however, there is only a trace of free haem in plasma and this is bound to haemopexin or serum albumin; likewise, traces of haemoglobin are bound to haptoglobin. Thus, if organisms are to use this limited source of iron they must aquire it from these complexes. Some organisms, such as *E. coli* (Eaton et al. 1982), are unable to use haemoglobin bound to haptoglobin, but others, like *V. vulnificus,* can do so (Helms et al. 1984). Meningococci are also able to use bound haemoglobin, but not haem bound to haemopexin (Dyer et al. 1987). It has been suggested that increasing the level of haem or haemoglobin by haemolysin-induced haemolysis of erythrocytes is a way of increasing the available iron pool in vivo. The production of haemolysins is a virulence factor for many organisms (Martinez et al. 1990) and in the case of *V. cholerae* and *Serratia marcescens* haemolysin production has been shown to be iron-regulated (Stoebner and Payne 1988; Poole and Braun 1988). Of course, haemolysins not only lyse erythocytes but other mammalian cells as well. In the case of the enteric pathogen *V. cholerae* it has been suggested that the haemolysin liberates iron compounds from intestinal cells which then form an available iron pool to be taken up by the vibrios (Stoebner and Payne 1988). The mechanisms used by bacteria for obtaining iron from haem or haemoglobin are unclear.

Regulation by iron

The co-ordinated responses of many bacteria to low iron levels involves not only the production of siderophores and/or new membrane proteins, but also the synthesis of a number of factors unrelated to iron metabolism, such as bacterial toxins (Bullen and Griffiths 1987; Stoebner and Payne 1988; Frank et al. 1989; Poole and Braun 1988; Calderwood and Mekalanos 1987; Fourel et al. 1989). Some of these systems are chromosomally encoded whereas other genes are carried by plasmids and transposons. In *E. coli*, expression of these iron-controlled systems, including the bacteriophage-encoded Shiga-like toxin I (SLT-I), are negatively regulated via a global repressor protein, Fur (17 kDa), which uses ferrous iron as a co-repressor (Bagg and Neilands 1987). Fur is the product of the regulatory gene *fur*. Binding sites for the Fur repressor protein, now called the 'iron-box' have been identified in several iron-regulated promoters in *E. coli* (Bagg and Neilands 1987; Griggs and Konisky 1989; Postle 1990; Niederhoffer et al. 1990; Elkins and Earhart 1989). The consensus sequence contains a highly A + T-rich palindrome and deletions that disrupt the palindromic structure make the promoter unresponsive to regulation by iron (Calderwood and Mekalanos 1988). It has also been shown that the presence of the 'iron-box' is sufficient in itself to allow *fur*-mediated iron regulation of a gene to occur. A *fur*-like system operates in some other pathogens including *Corynebacterium diphtheriae* where it controls the synthesis of diphtheria toxin as well as other iron-responsive determinants (Fourel et al. 1989; Boyd et al. 1990).

An additional iron-controlled global regulatory system based on the under-modification of several transfer RNAs (tRNAs) also appears to operate in *E. coli* and *S. typhimurium*. Undermodification of *E. coli* tRNAs containing 2-methylthio-N^6-(Δ^2-isopentenyl)-adenosine (ms^2i^6A) located adjacent to the anticodon (A37) to give isopentenyl-adenosine (i^6A) occurs during iron-restricted growth both in vitro and in vivo during infection (Bullen and Griffiths 1987): similar changes occur in the tRNA of *S. typhimurium* which contains a hydroxylated form of ms^2i^6A (ms^2io^6A). Variations in the degree of modification of A37 in tRNA induce strong pleiotropic effects on cell physiology, in some instances by effects on the translational efficiency of the tRNA, in others by altering codon context effects, and in others by unknown mechanisms (Buck and Griffiths 1981, 1982; Ericson and Björk 1986; Bouadloun et al. 1986; Wilson and Roe 1989; Blum 1988; Petrullo and Elseviers 1989). There is evidence that this modification also acts to increase the frequency of spontaneous mutations when cells need to adapt to environmental stress (Connolly and Winkler 1989). Recent studies have shown that under-modification of tRNA in *E. coli* during iron restriction is independent of *fur* regulation (Griffiths and Stevenson, unpublished data).

Final comments

Without doubt, current developments are rapidly increasing our understanding of the critical role of iron in infection and producing a fresh insight into the capability of bacteria to multiply in vivo and to cause disease. A significant development has been the recognition that the low availability of iron in mammalian tissues constitutes a major environmental signal which controls the co-ordinate expression of sets of genes during infection. Analysis of pathogens grown in iron-restricted environments is giving a considerably clearer

insight into characteristics associated with their virulence and with different disease specificities (Chart et al. 1988; Carniel et al. 1989; Goldberg et al. 1990). More and more subtle iron-regulated determinants, unconnected with the acquisition of iron itself, continue to come to light, for example the 'mini pilin' of *Aeromonas hydrophila* (Ho et al. 1990). Often environmentally regulated systems are controlled by two or more signals (Griffiths 1989). Thus, the 'mini-pilin' of *A. hydrophila* is produced not only during iron restriction but also when the organism grows at 22° C instead of 37° C. It is suggested that the pathogenic and ecological roles of this pilus are related to the organism's existence as a free-living organism in aquatic environments and its ability to cause infection in both cold-blooded vertebrates and the human intestine. Iron is also involved in the regulation of swarmer cell differentiation of *V. parahaemolyticus,* when the organism becomes covered with numerous lateral flagella which allow it to spread and colonize surfaces (McCarter and Silverman 1989). There is in addition increasing evidence that iron-regulated proteins are expressed in vivo during infection (Fernandez-Beros et al. 1989; Ward et al. 1988; Donachie and Gilmour 1988; Ala' Aldeen et al. 1990b) and the diagnostic potential of these host-induced proteins is being explored by some (Williams et al. 1988) and their use as vaccine antigens by others (Ward et al. 1988; Donachie and Gilmour 1988; Petterson et al. 1990; Ala' Aldeen et al. 1990b; Banerjee-Bhatnagar and Frasch 1990; Griffiths et al. 1990). This new knowledge is also being applied to the construction of live attenuated bacterial mutants for evaluation as vaccines. For example, a double mutant of *S. flexneri* has recently been constructed which is impaired in its ability to spread intracellularly, and from cell to cell, due to the presence of a Tn*pho*A insertion in the plasmid *icsA* gene, and in its capacity to grow extracellularly in the host due to the presence of a Tn10 insertion in the chromosomal *iuc* gene which encodes aerobactin production (Sansonetti and Arondel 1989).

Much remains to be learnt about the ways pathogens acquire iron in vivo during infection, about the role of the metal in the co-ordinate regulation of virulence genes, and about the molelcular mechanisms involved in these processes. Partially understood mechanisms for acquiring iron from host iron-binding proteins, such as those involving a reduction of the ferric iron to ferrous in transferrin (Cowart and Foster 1985) need further investigation, as do the iron-uptake mechanisms used by commensal species such as bifidobacteria (Bezkorovainy et al. 1987). Significantly, there is a growing interest in the mechanisms used by non-bacterial pathogens to sequester iron in vivo and about the effects of iron restriction on their properties. Two areas of particular interest are the iron-regulated antigens of fungal pathogens (Paul et al. 1989) and the role of transferrin receptors in the pathogenicity of the protozoan pathogen *Trypanosoma cruzi* (Lima and Villalta 1990).

Acknowledgements. I thank Pauline Stevenson for helpful discussion.

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