CHAPTER TWO

IRON AND INFECTION - AN OVERVIEW

2.1 The Importance of Iron in Biological Systems

Perhaps because of its abundance in nature, most living organisms have evolved an irreversible dependence on iron for various biological functions. The presence of iron as a cofactor is essential for enzymes such as ribonucleotide reductase (Reichard and Ehrenberg, 1983), RNA polymerase III (Shoji and Ozawa, 1985; Shoji and Ozawa 1986), and various amino acid hydroxylases and dioxygenases (Nozaki and Ishimura, 1974; Wrigglesworth and Baum, 1980). Moreover, enzymes involved in oxygen metabolism, mainly superoxide dismutase, catalase and peroxidase need iron as a cofactor (Halliwell and Gutteridge, 1985). Iron is also essential for the function of enzymes and proteins involved in electron transfer such as cytochromes, hydrogenases, ferridoxin and succinate dehydrogenase (Neilands, 1981a; Neilands, 1981b; Dallman, 1986). Several vertebrate cells such as neutrophils, T- and B- lymphocytes and natural killer cells are also dependent on iron as cofactor for immune damage of infective agents (Dallman, 1986; Brock and Mainou-Fowler, 1986; Bryan *et al.*, 1986).

This versatile biocatalytic role of iron is primarily due to the wide redox potential of the Fe^{3+}/Fe^{2+} couple, which is 0.76 volts (Byers, 1987). However this property also has its negative effects since iron reacts strongly with peroxide and

superoxide anions to produce the highly toxic hydroxide radicals (Flitter *et al.*, 1983) through the Haber-Weiss-Fenton reaction in which iron has an important catalytic role:

$$O_2 + Fe^{3+} = O_2 + Fe^{2+}$$

$$2O_2 + 2H^+ = H_2O_2 + O_2$$

$$H_2O_2 + Fe^{2+} = OH^- + OH^\bullet$$

These hydroxide radicals are the most potent oxidizing agents known and are implicated in the breakage of DNA and the destruction of biological membranes (Weinberg, 1989).

2.2 Restriction of Free Iron in Biological Fluids

Excessive free iron in organic fluids may have harmful effects and thus there is a need to restrict its level in biological systems. Most vertebrate organisms have evolved mechanisms to restrict the circulation of free iron in body fluids. Since iron is an essential growth factor for microbes, its control has an adverse effect on microbial growth during infection of the vertebrate host. A normal human body contains about four grams of iron, most of which is intracellular and about 70% of this intracellular iron is distributed in hemoglobin, 5% in special respiratory enzymes, and 20% stored in the bone marrow, liver, and spleen (Deis, 1983; Theil, 1987). Thus only a small percentage, about four milligrams, is found free which is not bound to hemoglobin in the bloodstream. The iron bound to heme or hemoglobin released during erythrocyte lysis are efficiently removed from circulation by an internal cycle in which serum proteins haptoglobin and to a lesser extent hemopexin and albumin play an important role (Muller-Eberhard, 1970). The body salvages iron from destroyed erythrocytes and

circulates it in the blood as free iron salts, which is recycled in the bone marrow to make new blood cells.

Extracellular iron is efficiently bound and removed from circulation by the glycoproteins transferrin and lactoferrin (Bezkorovainy, 1987; Weinberg, 1984; Crichton and Charloteaux-Wauters, 1987). Transferrin is a glycoprotein synthesized by the liver. It has a high affinity for iron, and its normal function is to store and deliver iron to host cells (Morgan, 1981). Since transferrin sequesters iron, it also plays an antibacterial role by depriving invading bacteria of the iron they need for growth. Lactoferrin is a protein that binds iron with high affinity and is found in the mucus and mother's milk. It also has an analogous function to that of transferrin since bacteria are unable to compete with lactoferrin for free iron. Thus, lactoferrin has a role in reducing the amount of iron available for bacterial growth. All these mechanisms ensure that free iron levels in the host body fluids are well below those required for microbial growth (Weinberg, 1992a; Weinberg, 1993).

In the presence of invading microbes, the already low levels of free iron in the biological fluids of the host are further diminished by a series of reactions collectively known as the 'hypoferraemic response' (Weinberg, 1978; Weinberg, 1984). This reduction is initiated by microbial proteins which stimulate the release of interleukin 1 by macrophages and monocytes (Westmacott *et al.*, 1986). The interleukin 1 stimulates synthesis and release of ferritin which results in ferritin bound iron to increase and thus iron available for serum transferrin to be reduced (Konijin and Hershko, 1989). Moreover, systemic bacterial infection causes a dramatic increase in

the concentration of transferrin in the blood which further helps to reduce even further the amount of iron available for bacterial growth. Reduced intestinal absorption of iron has also been considered as an agent of 'hypoferraemic response' (Cartwrite *et al.*, 1946).

While the hypoferraemic response is mainly concerned with the extracellular iron status, there are many intracellular pathogens with the ability to utilize intracellular iron. Thus other mechanisms are used to reduce intracellular iron levels. This process of reducing intracellular iron levels, generally termed as 'iron depletion' (Weinberg, 1992b), is known to be mediated by two mechanisms. One of these mechanisms involves the action of gamma-interferon (INF-γ) which reduces the expression of transferrin receptors of the host cells especially phagocytic cells such as monocytes and macrophages (Byrd and Horwitz, 1991). An activity of phagocytes that contributes to killing of bacteria is limitation of iron availability. Phagocytes actively ingesting bacteria reduce the number of transferrin receptors on their cell surface and decrease the concentration of intracellular ferritin (an intracellular iron storage protein). The combined effect of these changes is to decrease iron availability in the phagolysosome and thus reduce the amount of iron available for bacterial growth. The reduction in iron availability may be particularly important for control of bacteria that can escape the phagosome or otherwise avoid phagocyte killing mechanisms. This process has been shown to increase the resistance of the host against infection by *Legionella pneumophila* (Byrd and Horwitz, 1991,) and *Listeria* spp (Alford *et al.*, 1991). The second mechanism of intracellular iron depletion is achieved through the action of reactive nitrogen intermediates, nitric oxide, nitrate and nitrite formed during the

conversion of L-arginine to L-citrulline (Liew and Cox, 1990). These reactions, induced by the actions of gamma-interferon (INF-γ), tumor necrosis factor α (TNF-α) and interleukin 6 in various combinations, are capable of removing non-heme iron (mainly ferritin bound iron) from the intracellular 'iron pool'. The growth of intracellular pathogens, *Mycobacterium bovis* (Flesch and Kaufmann, 1991) and *Ehrlichia risticii* (Park and Rikihisa, 1992) has been shown to be inhibited by this mechanism.

2.3 Iron Acquisition Mechanisms of Bacteria

Bacterial colonization is the result of complex interactions between bacteria and the host. Humans have developed a variety of ways of interfering with bacterial colonization. At the same time bacteria have evolved ways to circumvent these host defences. A striking feature of bacterial pathogens is the multiplicity they have evolved for using host molecules and activities to their own advantage.

The availability of iron is of crucial importance in the process of bacterial infection. The invading microbes have to overcome the iron-deficient conditions generally imposed by the presence of high-affinity iron-binding proteins in the body fluids of the host. The concentration of free iron is particularly low in human body because several proteins including lactoferrin, transferrin, ferritin and hemin bind most of the available iron. To survive in the human body, bacteria must have some mechanism for acquiring iron. Several strategies are employed by various microbes to acquire iron for growth and metabolism during infection of the host.

2.3.1 Abstinence From Using Iron

The only organism shown not to require iron for its functions is *Lactobacillus* spp (Archibald, 1983). These species substitute metals such as cobalt and manganese for enzymatic activities that usually require iron as the cofactor.

2.3.2 Reduction of Fe^{3+} and Utilization of Fe^{2+}

The microbes shown to exclusively depend on Fe^{2+} as iron source include *Bifidobacterium bifidum* (Bezkorovainy *et al.*, 1987), *Streptococcus mutans* (Evans *et al.*, 1986) and *Legionella pnemophila* (Johnson *et al.*, 1991, Poch and Johnson, 1993). *L. pneumophila* is known for its inability to produce siderophores (Liles and Cianciotto, 1996). Since two ferri-reductases have been identified in *L. pnemophila* (Poch and Johnson, 1993) and a membrane flavin reductase in *S. mutans* (Evans *et al.*, 1986), these two microorganisms are believed to reduce Fe^{3+} before transporting it. The reduction of Fe^{3+} to Fe^{2+} usually occurs at the cytoplasmic membrane and Fe^{2+} is transported into the cytoplasm. *Listeria monocytogenes* possesses a reductase specific for transferrin and lactoferrin bound iron (Cowart and Foster, 1985). Leukopyocyanin, the reduced form of pyocyanin, secreted by *Pseudomonas aeruginosa* is also believed to be able to reduce transferrin bound iron (Cox, 1986). *Bifidobacterium bifidus* possesses a cytoplasmic membrane associated ATPase activity as the ferrous iron acquiring mechanism (Bezkorovainy *et al.*, 1986). *Escherichia coli* has a ferrous iron transport system which becomes functional during anaerobic conditions (Hantke, 1987;

Kammler *et al.*, 1993). A ferrous iron transport system has also been identified in *Saccharomyces cerevisiae* (Lesuisse *et al.*, 1987; Dancis *et al.*, 1990; Dancis *et al.*, 1992; Georgatsou and Alexandraki, 1994).

2.3.3 Intracellular Infection

The intracellular environment, compared to the extracellular environment, appears relatively iron-rich. Intracellular parasites such as *Listeria* and *Shigella* penetrate into the cytoplasm of the cell where they can utilize the intracellular iron bound to heme and ferritin (Lawlor *et al.*, 1987; Weinberg, 1989). Other intracellular pathogens such as *Yersinia* and *Salmonella* remain within cell vacuoles during the course of infection, but are able to obtain sufficient iron for metabolism (Robins-Browne *et al.*, 1987; Benjamin *et al.*, 1985). How these microbes can overcome the host iron depletion mechanisms remains unknown.

2.3.4 Utilization of Host Iron Compounds

A number of pathogenic bacteria have been shown to use transferrin, lactoferrin, ferritin and hemin as a source of iron (Otto *et al.*, 1992). Transferrin is found in blood serum while lactoferrin is usually present in exocrine secretions, such as saliva, tears, and mucosal secretions. Ferritin is an intracellular iron storage protein which serves as an iron reserve for other cells and intracellular needs and also provides protection from iron overload. Generally these bacteria bind the host molecule that contains iron to their surfaces as part of their iron acquisition process. But the

mechanism of trapping the iron from the host proteins is not known. As more and more pathogens are studied, it is becoming apparent that acquisition of iron from transferrin and lactoferrin, once thought to be an unusual mechanism of iron acquisition, is much more common than previously suspected (Williams and Griffiths, 1992).

2.3.4.1 Heme and Hemoglobin

Escherichia coli (Griffiths, 1987) *Yersinia* spp (Perry, 1993; Perry and Brubaker, 1979) and *Shigella* spp. (Lawlor *et al.*, 1987) can utilize heme as their iron source while *Neisseria meningitidis*, *N. gonorrhoeae* (Dyer *et al.*, 1987), *Haemophilus influenzae* (Pidcock *et al.*, 1988), *Vibrio cholerae* (Stoebner and Payne, 1988) and *Campylobacter jejuni* ((Pickett *et al.*, 1992) has been demonstrated to use both heme and hemoglobin. Heme-binding membrane associated proteins has been identified in *H. influenzae* (Hanson and Hansen, 1991; Hanson *et al.*, 1992; Lee, 1992; Maciver *et al.*, 1996), *Yersinia enterocolitica* (Stojiljkovic and Hantke, 1992), *V. cholerae* (Henderson and Payne, 1993), *Bacteroides fragilis* (Otto *et al.*, 1996) and *Helicobacter pylori* (Worst *et al.*, 1995). A hemoglobin-binding outer membrane protein has also been identified in *Neisseria gonorrhoeae* (Chen *et al.*, 1996; Stojiljkovic *et al.*, 1996) and *Haemophilus ducreyi* (Stevens *et al.*, 1996).

The best studied bacteria for its ability to utilize iron bound to heme and hemoglobin are *Yersinia* spp (Perry, 1993; Lucier *et al.*, 1996). These bacteria have multiple iron acquisition systems. These include a siderophore based system that is

produced mainly at low temperatures and presumably helps the bacteria to obtain iron in the external environment. *Yersiniae* also have iron sequestration systems that are induced at 37°C. These allow the bacteria to obtain iron from hemin and transferrin, two abundant sources of iron in the human body. Not much is known about these iron acquisition systems except that acquisition of iron from hemin involves uptake of the entire heme molecule, not simply stripping of iron from hemin at the bacterial surface. In addition to being able to use hemin as an iron source, *Y. pestis* actively stores hemin and accumulates high enough concentrations to change the color of colonies on agar plates containing hemin. Hemin storage occurs maximally at 26°C, so that *Y. pestis* injected by flea bite would be hemin loaded. The stored hemin may serve as an iron reserve for growth inside the host.

Since free heme is present only in trace quantities in serum, some microbes are able to increase the availability of free heme and hemoglobin in blood by secreting hemolysins capable of lysing erythrocytes. Hemolysins belong to a class of toxins known as membrane disrupting toxins, which are basically proteins that insert into the host cell membrane and forms channels (or pores), thus allowing cytoplasmic contents to leak out and water to enter. Since the osmotic strength of the cytoplasm is much higher than that of the surrounding environment, damage in the host cell membrane lead to a sudden in-rush of water into the cell, which causes the cell to swell and rupture. This kind of membrane disrupting activity produces its toxic effect simply by inserting into membranes (Ikigai *et al.*, 1996; Song *et al.*, 1996; Menzl *et al.*, 1996). Most of these toxins are referred to as "hemolysins" because erythrocytes are a convenient cell type for assaying the activity of such toxins. However, such toxins are

toxic for many cells because their targets (membrane lipids) are found on all types of host cell. Such toxins are more appropriately designated cytotoxins. The invasive strains of *E. coli* (Waalwijk *et al.*, 1983; Gruenig *et al.*, 1987), *V. cholerae* O1 biovar eltor (Stoebner and Payne, 1988) and *Aeromonas sobria* (Goebel *et al.*, 1988) have been shown to produce increased amounts of hemolysins under iron-limited conditions and a role for hemolysins in iron acquisition has also been recognized in these species.

2.3.4.2 Transferrin and Lactoferrin

N. meningitidis and *N. gonorrhoeae* were also able to acquire iron from lactoferrin and transferrin (Mickelsen and Sparling, 1981; Simonson *et al.*, 1982; Tsai *et al.*, 1988; Retzer *et al.*, 1996). These bacteria express outer membrane receptors that binds directly to lactoferrin and transferrin (Schryvers and Lee, 1989) and the iron is transported across the cellular membrane into the cytoplasm by an active transport mechanism (Lee and Bryan, 1989; Blanton *et al.*, 1990; Chen *et al.*, 1993; Irwin *et al.*, 1993). The transferrin receptor is specific for human transferrin and similarly the lactoferrin receptor is also specific for human lactoferrin (Schryvers and Morris, 1988). Virtually nothing is known about the process by which iron is removed from transferrin and lactoferrin except that it involves an outer membrane protein that is a TonB homologue (Cornelissen *et al.*, 1992). In *E. coli* TonB also interacts with siderophore receptors to internalize siderophore bound iron. Although acquisition of iron from transferrin and lactoferrin does not involve siderophores, the mechanism of iron removal and internalization has at least some features in common with the siderophore based systems. Like the siderophore receptor proteins, transferrin and lactoferrin

receptor proteins are produced at highest levels when bacteria are grown in low iron medium. The genes encoding the two outer membrane associated transferrin binding proteins have been cloned from *N. meningitidis* (Wilton *et al.*, 1993; Palmer *et al.*, 1993). *N. gonorrhoeae* can also acquire iron from heme and hemoglobin, but little is known about the proteins that mediate this type of acquisition.

Haemophilus influenzae was also found to be able to utilize iron bound to transferrin but not lactoferrin (Herrington and Sparling, 1985; Morton and Williams, 1989; Morton and Williams, 1990; Holland *et al.*, 1991). Outer membrane receptors with putative role in binding transferrin has been identified in this species (Schryvers, 1989; Holland *et al.*, 1992; Stevenson *et al.*, 1992; Gray-Owen and Schryvers, 1995). *Helicobacter pylori*, the causative agent of gastritis and peptic ulcer, derives its iron from lactoferrin present in stomach mucosal secretions (Husson *et al.*, 1993). Similarly, *Bordetella pertussis*, the causative agent of whooping cough also scavenges sufficient iron from transferrin and lactoferrin (Redhead *et al.*, 1987). At least one case has been reported on the ability of *Aeromonas salmonicida* to acquire iron from transferrin and lactoferrin (Chart and Trust, 1983) but further studies are needed to confirm the observation. *Bacteroides* spp were also shown to use transferrin as the iron source (Verweij-Van Vught *et al.*, 1988). A transferrin-binding protein has also been isolated from *Borrelia burgdorferi* (Carroll *et al.*, 1996).

Listeria monocytogenes has been shown to produce a 10-kDa protein that can remove iron from transferrin. This process requires NADH and Mg^{2+} but little is known about the mechanism of iron removal in this process. Moreover, it is not known

whether the protein is essential for virulence. Recently it was shown that shigellae can bind lactoferrin (Tigyi *et al.*, 1992). This could indicate that *Shigellae* are able to acquire iron from lactoferrin and other host iron-binding proteins such as the intracellular iron storage protein, ferritin. *Moraxella catarrhalis* also produce an outer membrane protein with putative role in acquisition of iron from transferrin and lactoferrin (Aebi *et al.*, 1996).

2.3.5 Siderophore mediated iron acquisition.

The best studied type of bacterial iron acquisition mechanism involves siderophores. Siderophores are low-molecular mass extracellular proteins enzymatically synthesized by many microbes in iron-limiting conditions to obtain iron for the growth of the organism. Siderophores have a high affinity for ferric-iron which enters the microbial cell as a ferric-siderophore complex through the outer membrane receptors specific for the siderophore. The majority of the siderophores can be broadly classified into hydroxamates and catecholates although the presence of other classes such as polyhydroxycarboxylates and α-keto-/α-hydroxyacids have been recognized.

Once synthesized, the siderophore must be rapidly exported from the cell to prevent the sequestering of the intracellular iron in an unavailable form. The siderophore is secreted into the external environment and binds to Fe^{3+}. The siderophore bound with iron, known as ferric-siderophore, then binds to its outer membrane receptor protein specific for that siderophore. The ferric-siderophore is then transferred from its outer membrane receptor complex to a carrier protein. This step is

mediated by TonB, a periplasmic protein anchored to the cytoplasmic membrane which is also needed for uptake of vitamin B_{12}. This protein is involved in energy-coupled transport across the outer membrane (Bitter *et al.*, 1993; Skare *et al.*, 1993; Braun *et al.*, 1996;). Two other cytoplasmic membrane associated proteins, ExbB (Kempfenkel and Braun, 1993) and ExbD (Kempfenkel and Braun, 1992) are considered necessary for the recycling of TonB between an active and inactive form (Karlsson *et al.*, 1993). Three recent detailed reviews are available on the role of TonB and related membrane-associated proteins in iron transport (Klebba *et al.*, 1993; Postle, 1993; Braun, 1995). The next step in this process is the release of the ferric-siderophore from its carrier protein and its transportation across the inner membrane which requires the presence of several inner membrane bound proteins (Nikaido and Saier, 1992). The iron bound to the siderophore is released either through cleavage of the siderophore or reduction of iron. In the case of enterobactin (a catecholate siderophore produced by *E. coli*), an esterase activity cleaves the ester backbone and thus facilitates the dissociation of iron (O'Brien *et al.*, 1971; Brickman and McIntosh, 1992). Several microbes, including *E. coli*, can reduce ferric-siderophore complexes (through the action of ferri-reductases) which promote the release of iron (Fontecave *et al.*, 1994). Since siderophores have little affinity for Fe^{2+}, iron is released. Siderophore reduction can either occur at the cytoplasmic membrane, or in the cytoplasm itself. If it occurs in the cytoplasm, the deferrated siderophore must be rapidly excreted or modified to or stored in an inactive form to avoid interference with the intracellular utilization of iron. Some bacteria not only produce their own siderophores but also produce receptors capable of binding siderophores produced by other organisms (utilization of exogenous siderophores).

2.4 Regulation of Siderophore Production

The regulation of siderophore biosynthesis here will be discussed with particular emphasis on the siderophore systems of *E. coli*, an extensively studied organism in these aspects. All the genes involved in siderophore biosynthesis and transport in *E. coli* are negatively regulated by the Fur (*F*erric *u*ptake *r*epressor) repressor protein encoded by the *fur* gene (Braun *et al.*, 1990, Griggs *et al.*, 1989, Hantke, 1981) which has been cloned and sequenced (Hantke, 1984; Schaffer *et al.*, 1985). At low concentrations of Fe^{2+} the Fur repressor has a weak affinity for operator DNA and with the operator region unoccupied transcription occurs from the promoter site of the relevant gene(s). The Fur binding site of operator DNA is known as the 'iron box' or 'Fur box'. At high cytoplasmic concentrations of Fe^{2+}, the Fur protein binds tightly to the 'iron box' thus the transcription of Fur regulated genes are blocked (Bagg and Neilands, 1987). The fur gene itself is autoregulated by Fur in a metal-dependent manner and also by the catabolite activator protein (CAP) (De Lorenzo *et al.*, 1987; De Lorenzo *et al.*, 1988a; De Lorenzo *et al.*, 1988b) . Regulatory genes homologous to *E. coli fur* have been cloned from *Yersinia pestis* (Staggs and Perry, 1991), *Pseudomonas aeruginosa* (Prince *et al.*, 1993), *Vibrio cholerae* (Litwin *et al.*, 1992), *V. vulnificus* (Litwin *et al.*, 1993), *V. anguillarum* (Waldbesser *et al.*, 1993; Tolmasky *et al.*, 1994), *Neisseria gonorrhoeae* (Berish *et al.*, 1993) and *N. meningitidis* (Thomas and Sparling, 1994).

Fur repressor also regulates genes other than those directly involved in siderophore mediated iron uptake. Various toxins, enzymes and virulence determinants

has been shown to be regulated by iron and/or Fur. Furthermore, the genes encoding iron- and manganese dependent superoxide dismutase in *E. coli* are also under Fur regulation (Niederhoffer *et al.*, 1990). In *Salmonella typhimurium*, 36 proteins were found to be regulated by iron, of which 34 were regulated by Fur (Foster and Hall, 1992; Tsolis *et al.*, 1995). Similarly, in *Y. pestis* synthesis of 6 proteins were controlled by iron (Staggs and Perry, 1991), while in *V. cholerae* 56 proteins have been identified to be regulated by iron and/or *fur* (Litwin and Calderwood, 1994). From these observations, it has been proposed that an iron-deficient environment may serve as a signal for the microbe that it has entered a host and various toxins or enzymes that promote its survival inside the host should be produced.

In some bacteria, metabolic status of the organism is also seem to be controlled by *fur*. In *V. cholerae*, utilization of carbon sources, particularly glycerol, acetate, succinate, lactate and fumarate are probably under regulation of *fur* (Litwin and Calderwood, 1994). Similar control mechanism has also been recognized in *E. coli* in the utilization of succinate, fumarate and acetate (Hantke, 1987).

2.5 Siderophores and Virulence

Since iron is one of the essential nutrients required by microbes, it appears natural that the iron uptake systems which promote the survival of microbial pathogens within the vertebrate host play an important role in bacterial virulence. Although siderophore based iron acquisition has been shown to contribute to the virulence of some bacterial pathogens, mutations that eliminate siderophore production or uptake

by a bacterial pathogen do not always decrease virulence. This is because the bacteria often have more than one iron-sequestering system, and thus a mutant deficient in one system may still be able to rely on the remaining mechanisms for survival. Nonetheless, it is also possible that siderophore based iron acquisition systems are adaptations for survival of free-living bacteria in soil and water, and other complex strategies of iron acquisition are needed in the human body. In soil and water, bacteria more often scavenge free iron in the form of iron salts, but in the human body virtually all of the iron is bound to proteins such as hemin, transferrin, lactoferrin or ferritin and iron extraction requires other techniques.

The aerobactin siderophore system encoded by pColV-K30 plasmid of *E. coli* has been convincingly demonstrated to enhance the virulence of this species (Williams, 1979). The aerobactin system is also found to be chromosome-mediated in pathogenic bacteria such as invasive strains of *E. coli*, *Shigella flexneri* and enteroinvasive *E. coli* (Valvano and Crosa, 1984; Valvano and Crosa, 1988; Valvano *et al.*, 1986). The possession of an aerobactin system is found to enhance the pathogenic properties of *Klebsiella pneumoniae* (Nassif and Sansonetti, 1986). *Shigella* spp. also produce the siderophore aerobactin, but mutants that no longer produce aerobactin grow as rapidly as the wild type and are similarly virulent in animals (Lawlor *et al.*, 1987; Nassif *et al.*, 1987). The observation does not imply that aerobactin is dispensible but suggests that *Shigellae* have other ways of obtaining iron from host iron stores for their growth in the host tissues.

The anguibactin-mediated plasmid-encoded iron scavenging system of *Vibrio anguillarum* is another important siderophore system with a recognized role in the virulence of this marine fish pathogen (Crosa *et al.*, 1977; Crosa *et al.*, 1980; Crosa, 1979; Crosa, 1984; Crosa, 1989). This catechol siderophore system is encoded by the 65 kbp pJM1 plasmid (Crosa, 1980). The presence of the anguibactin system correlates with high virulence in these bacteria, and is absent in the avirulent strains.

The role of other siderophore systems in the virulence of bacteria has not been sufficiently demonstrated. Conflicting evidence exists for the role of enterobactin in virulence. Investigations carried out so far on *Salmonella typhimurium* and *E. coli* have not been able to demonstrate sufficiently the role of enterobactin in virulence (Yancey *et al.*, 1979; Benjamin *et al.*, 1985; Griffiths and Humphreys, 1980). The importance of vibriobactin, a catecholamine siderophore produced by *V. cholerae*, in the pathogenicity of this species also remains controversial in view of the conflicting reports available (Sigel *et al.*, 1985; Goldberg *et al.*, 1990a; Goldberg *et al.*, 1990b). The presence of pyoverdin and pyochelin, endogenous siderophores produced by *Pseudomonas aeruginosa* has been shown to influence successful corneal infections in mouse (Woods *et al.*, 1982). A putative role in virulence has also been considered for an uncharacterized siderophore secreted by *Yersinia* in the establishment of successful mouse infections (Heesemann *et al.*, 1993).

An indirect role in virulence has been proposed for pyochelin, one of the siderophores synthesized by *P. aeruginosa* (Cox, 1982; Britigan *et al.*, 1992). Pyocyanin, a pigment produced by these bacteria can catalyze the conversion of

oxygen to superoxide and hydrogen peroxide. Free iron and iron bound to certain chelators can catalyze the conversion of superoxide radical and peroxide into hydroxide radicals, which is well known to cause tissue damage. Iron bound to transferrin, one of the primary forms of iron found extracellularly in the lung, does not participate in this type of reaction, but iron bound to the *P. aeruginosa* siderophore, pyochelin, does catalyze hydroxyl radical production. Thus, a combination of pyocyanin and pyochelin, both of which are produced by bacteria growing in the host, could make a significant contribution to tissue damage, especially in an oxygen-rich area like the lung (Miller *et al.*, 1996). Recently, it has been reported that pyoverdin, another siderophore produced by *P. aeruginosa* is a virulence factor in this species (Meyer *et al.*, 1996).

2.6 Regulation of Toxin Production by Iron and/or Fur

Another type of iron acquisition strategy of pathogenic bacteria is the production of toxic factors that kill host cells. Some toxic proteins of bacteria (exotoxin) are produced only when iron levels are low (Mekalanos, 1992). Since these toxic proteins kill host cells, they might be part of an iron acquisition strategy in which host cells are killed by the toxin to release their iron stores (primarily ferritin- or heme-bound iron), which can then be trapped by ferritin- or heme-binding bacterial proteins.

Many virulence genes, especially toxin genes are regulated by iron. High levels of gene expression are seen only when iron concentrations are low. So far, all iron-regulated genes have proved to be regulated by repressors that are able to bind DNA

only when they are complexed with an iron molecule. Thus, in the presence of high iron concentrations, most repressor molecules will be in the iron-bound active form and will bind to the operator site, shutting off transcription of the gene. In the absence of iron, repressor molecules do not complex with iron and hence leave the operator free and this situation allows the gene to be transcribed.

Production of diphtheria toxin by lysogenized *C. diphtheriae* is enhanced considerably when the bacteria are grown in low iron medium (Cryz *et al.*, 1983). Initial studies of diphtheria toxin gene expression in *E. coli* showed that expression was repressed by Fur (Tai and Holmes, 1988). This suggested that a Fur-like repressor was produced by *C. diphtheriae* and was responsible for regulation of toxin production by iron levels. Recently, the gene encoding this repressor, *dtxR* (*d*iphtheria *t*oxin *r*egulation), has been cloned and characterized (Boyd *et al.*, 1990; Schmitt and Holmes, 1991). Like Fur, DtxR functions as an iron dependent repressor protein. When iron levels are high, both DtxR and Fur are in the iron-bound form, and this is the form that can bind DNA. Binding to a region of DNA that overlaps the promoter blocks binding of RNA polymerase to the promoter. In low iron concentrations, the repressor is no longer in the iron-bound form. This iron-free form of the protein does not bind DNA, thus allowing the toxin gene to be transcribed. The fact that synthesis of the toxin is enhanced when iron levels are low has led to the suggestion that the bacteria produce diphtheria toxin to kill host cells and release their iron stores. Thus, diphtheria toxin appears to be part of the iron acquisition system of the bacteria.

V. cholerae produces two types of iron-sequestering systems, siderophores (Sigel and Payne, 1982; Sigel *et al.*, 1985; Stoebner *et al.*, 1992) and a surface protein that binds hemin and hemoglobin (Stoebner and Payne, 1988; Henderson and Payne, 1993). Not surprisingly, the genes involved in iron sequestration are regulated and are produced at highest level in low iron medium. Other genes regulated by iron include *irgA* (*i*ron *r*egulated *g*ene), a gene of unknown function that is required for virulence of newborn mice and a gene that encodes a hemolysin. It is tempting to speculate that the hemolysin acts to free iron by lysing host cells and thus acts in concert with the iron sequestering systems. In the case of *V. cholerae*, a Fur-like repressor appears to be responsible for repressing transcription of *irgA* and the hemolysin gene and may also regulate other genes involved in iron acquisition.

Early studies of the regulation of *B. pertussis* virulence genes identified temperature, magnesium and nicotinic acid as the signals sensed by the *bvg* (*b*ordetella *v*irulence *g*enes) system but did not note any evidence for gene regulation. A recent report (Gross and Carbonetti, 1993) suggests that iron levels should be added to the list of signals recognized by the *bvg* system. Inspection of the DNA sequence upstream of *bvgA* has revealed a Fur binding site. Thus, Fur could be one of the factors controlling BvgA expression. If so, the effect of Fur is complex because iron starvation led to enhanced levels of Ptx (*P*ertussis *t*oxin) subunits but reduced levels of Fha (*F*ilamentous *h*em*a*gglutinin). Thus the role of Fur, if any, is not simply repression of *bvgA* expression, because both *ptx* and *fha* genes seem to be similarly regulated by BvgA.

In *Shigella dysentriae*, the expression of *stxA* and *stxB* genes which encode the Shiga toxin seem to be regulated by iron concentration. This regulation is mediated by a Fur-like repressor which is chromosomally encoded. Moreover, regulation of the Shiga-like toxin of *E. coli* was also shown to be mediated by the *fur* gene (Calderwood and Mekalanos, 1987)

Survival in an acidic environment is important to *Salmonella* spp. at two stages during the infection process: transiting the stomach to reach the small intestine and surviving in the acidic environment of the phagocyte phagosome. This phenomenon is known as the 'acid tolerance response' (Foster, 1993; Wilmes-Riesenberg *et al.*, 1996). Little is known about the mechanism of the acid tolerance response or its role in virulence but evidence suggests that Fur may be a regulator of this response (Hall and Foster, 1996). Since iron-binding by Fur is pH dependent, it appears possible that Fur may have a dual role in both iron regulation and acid tolerance response.

Pseudomonas aeruginosa produces an exotoxin, called exotoxin A, that has exactly the same mechanism as diphtheria toxin although biochemically different. Another similarity to diphtheria toxin is that exotoxin A synthesis is regulated by iron, with maximal expression seen when iron levels are low (Bjorn *et al.*, 1978; Bjorn *et al.*, 1979; Sokol *et al.*, 1982; Prince *et al.*, 1993; Ochsner *et al.*, 1995). Rather than being regulated directly by a Fur-like repressor, however, the exotoxin A gene (*toxA*) is regulated by a transcriptional activator (RegA), which is in turn, controlled by *P. aeruginosa* analog of Fur (Prince *et al.*, 1991). Most clinical isolates of *P. aeruginosa* produce exotoxin A, and mutants deficient in exotoxin A production exhibit reduced

virulence in animal models. Thus, exotoxin A appears to have a direct role in virulence, perhaps acting to cause tissue damage and to diminish the activity of phagocytes. *P. aeruginosa* also has an elastolytic activity (Sokol *et al.*, 1982) that appears to be due to the concerted activity of two enzymes: LasA and LasB. Production of LasA and LasB is affected by a number of factors, including the concentration of iron and zinc in the medium. Iron and zinc probably act at a postranscriptional level.

2.7 *Vibrio cholerae*

The genus Vibrio consists of Gram-negative straight or curved rods, motile by means of polar flagella. Vibrios are capable of both respiratory and fermentative metabolism. Most species are oxidase-positive. Most vibrios have relatively simple growth factor requirements and will grow in synthetic media with glucose as a sole source of carbon and energy. However, most species require 2 to 3 % NaCl or a sea water base for optimal growth. Vibrios are one of the most common organisms in surface waters of the world. They occur in both marine and freshwater habitats and in associations with aquatic animals. Some species are pathogenic for fish, eels, and frogs, as well as other vertebrates and invertebrates. Both *V. cholerae* and *V. parahaemolyticus* are human pathogens and cause diarrhea, but in ways that are entirely different. *V. parahaemolyticus* is an invasive organism affecting primarily the colon but *V. cholerae* is noninvasive, affecting the small intestine through secretion of an exotoxin (enterotoxin).

2.7.1 The Epidemiology of Cholera

Cholera (frequently called epidemic cholera) is a severe diarrheal disease caused by the bacterium *Vibrio cholerae* (Finkelstein, 1973). It has been long assumed that only *V. cholerae* serovar O1 (biovars El Tor and classical) causes cholera in humans (Feeley, 1965; Sen, 1969) but recently this distinction has been blurred by the isolation of a non-O1 strain (known as *V. cholerae* O139 "Bengal") capable of causing epidemic cholera (Albert, 1996). Transmission to humans is by water or food. The

natural reservoir of the organism is still not known but may be the aquatic environment, although originally it was assumed to be humans.

Cholera is an ancient disease and there are references to deaths due to dehydrating diarrhea dating back to Hippocrates and Susruta (Sanskrit). Epidemics of cholera on the Indian subcontinent date back to the late 15th century. The first long-distance spread of cholera to Europe and Americas began in 1817 and by the early 20th century, six waves of cholera had spread across the world in devastating epidemic fashion. Since then, until the 1960s, the disease contracted remaining present only in southern Asia. In 1961, the El Tor biotype (originally distinguished from classic biotype by the production of hemolysins) emerged and produced a major epidemic in Indonesia which spread rapidly to China, Hong Kong, Taiwan, and Malaysia. It then reached India and Bangladesh, and spread to Pakistan, Afghanistan, Iran, Bahrain and Israel. In 1970 it reached North Africa and in 1971 a major outbreak occurred in West Africa which spread to include most of central and East Africa. Outbreaks were also reported from Japan, Guam, Kirbati, the Philippines, Sri Lanka, Italy and Portugal.

In December, 1992, an epidemic of cholera began in Bangladesh, and large numbers of people were involved (Albert, 1996). The organism has been characterized as *V. cholerae* O139 "Bengal" (a non-O1 strain). It is derived genetically from the El Tor pandemic strain (Waldor *et al.*, 1994; Comstock *et al.*, 1996) but it has changed its antigenic structure resulting in lack of immunity at all ages, even in endemic areas.

Antigenic variation plays an important role in the epidemiology and virulence of cholera as demonstrated by the emergence of the Bengal strain. The flagellar antigens (H antigens) of *V. cholerae* are shared with many vibrios isolated from aquatic environment and therefore are of no use in distinguishing strains causing epidemic cholera. The O antigens, however, do distinguish strains of *V. cholerae* into 139 known serotypes. Almost all of these strains of *V. cholera* are non-virulent. Until the emergence of the Bengal strain, a single serotype, designated O1, has been responsible for epidemic cholera. However, there are three distinct O1 biotypes, named Ogawa, Inaba and Hikojima, and each biotype may display the "classical" or "El Tor" phenotype. The Bengal strain is a new serological strain with a unique O-antigen which partly explains the lack of residual immunity.

2.7.2 The Pathogenicity of Cholera

Cholera toxin activates the adenylate cyclase enzyme in cells of the intestinal mucosa leading to increased levels of intracellular cAMP, and the secretion of water, Na^+, K^+, Cl^- and HCO_3^- into the lumen of the small intestine. The effect is dependent on a specific receptor, monosialosyl ganglioside (GM1 ganglioside) present on the surface of the intestinal mucosal cells. The bacterium produces an invasin, which is a neuraminidase, during the colonization stage which has the interesting property of degrading gangliosides to the monosialosyl form, which is the specific receptor for the toxin.

The toxin has been characterized and contains 5 binding (B) subunits of 11,500 daltons, an active A1 subunit of 23,500 daltons, and a bridging piece (A2) of 5,500 daltons that link A1 to the 5B subunits. Once it has entered the cell, the A1 subunit enzymatically transfers ADP ribose from NAD to a protein (called Gs or Ns), that regulates the adenylate cyclase system which is located on the inside of the plasma membrane of mammalian cells.

Enzymatically, fragment A1 catalyzes the transfer of the ADP-ribosyl moiety of NAD to a component of the adenylate cyclase system. Adenylate cyclase is activated normally by a regulatory protein (Gs) and GTP; however activation is normally brief because another regulatory protein (Gi), hydrolyzes GTP. But in the presence of the cholera toxin, the A1 fragment catalyzes the attachment of ADP-Ribose (ADPR) to the regulatory protein forming Gs-ADPR from which GTP cannot be hydrolyzed. Since GTP hydrolysis is the event that inactivates the adenylate cyclase, the enzyme remains continually activated.

Thus the net effect of the toxin is to cause cAMP to be produced at an abnormally high rate which stimulates mucosal cells to pump large amounts of Cl^- into the intestinal contents. Water, Na^+ and other electrolytes follow due to the osmotic and electrical gradients caused by the loss of Cl^-. The lost water and electrolytes in mucosal cells are replaced from the blood. Thus, the toxin-damaged cells become pumps for water and electrolytes causing diarrhea, loss of electrolytes and dehydration that are characteristics of cholera. A recent detailed review (Spangler, 1992) is available on the nature of cholera enterotoxin.

Recently, the structural genes necessary for the cholera toxin production were shown to be encoded by a filamentous lysogenic bacteriophage (designated CTXΦ). This bacteriophage uses the toxin-coregulated pili (TCP) as its receptor. Thus, *Vibrio cholerae* requires two coordinately regulated factors to be virulent: cholera toxin (an enterotoxin) and TCP, the surface organelles required to colonize the intestines (Waldor and Mekalanos, 1996).

2.7.3 Siderophores

V. cholerae strains produce a phenolate/catecholate type siderophore known as vibriobactin (Sigel and Finkelstein, 1978; Griffiths *et al.*, 1984), which is similar to, but distinct from, enterobactin, the siderophore synthesized by *Escherichia coli* K-12 and *Salmonella typhimurium*. No hydroxamate type siderophores have been detected in *V. cholerae* (Sigel and Payne, 1982). Clinical isolates of *V. cholerae* synthesize high-efficiency iron-chelating siderophores when grown in iron-deficient medium. But the role of vibriobactin in virulence remains to be established and it has been reported vibriobactin does not contribute to virulence in *V. cholerae* (Sigel *et al.*, 1985). *V. cholerae* is not an invasive pathogen and remains in the gut during the course of infection, where the concentration of iron may be sufficient enough to suppress the siderophore-mediated iron acquisition system. Siderophores could play an important role in the survival of *V. cholerae* in salt and brackish water environments where the availability of iron is much reduced (Sigel and Payne, 1982). The siderophore system of *V. cholerae* is negatively regulated (repressible system) by the *V. cholerae fur* gene.

This gene has been cloned and sequenced and shown to be very similar to the *E. coli fur* gene (Litwin *et al.*, 1992). The gene encoding the receptor protein for vibriobactin, *viuA*, also has been cloned and sequenced (Butterton *et al.*, 1992).

2.7.4 Hemolysins

Hemolysin production was initially considered as a differentiating factor between the classical and El Tor biotypes of *V. cholerae*. The El Tor strains were originally identified as being hemolytic, whereas classical strains were not (Honda and Finkelstein, 1979). However, weakly hemolytic El Tor strains have been isolated (Roy and Mukerjee, 1962) as well as strains that produce no hemolysin but have other biochemical properties characteristic of the El Tor biotype (de Moor, 1963). However, the gene for a hemolysin determinant from *V. cholerae* El Tor has been cloned and characterized (Goldberg and Murphy, 1984; Goldberg and Murphy, 1985; Manning *et al.*, 1984; Manning *et al.*, 1986).

Stoebner and Payne (1988) have reported that increased levels of hemolytic activity were found in iron-starved cultures of El Tor and non-O1 strains of *V. cholerae*, suggesting that iron has a role in the regulation of hemolysin production in these strains. It has also been noted that hemolysin-deficient mutants of El Tor strains which occur at a high rate also failed to produce the *V. cholerae* siderophore, namely, vibriobactin. The production of both hemolysin and vibriobactin were regained when a cloned *fur* gene was inserted into these mutants.

2.7.5 Proteases

V. cholerae strains produce a zinc- and calcium- dependent protease which was originally named mucinase, based on its ability to degrade mucin (Finkelstein *et al.*, 1983; Crowthers *et al.*, 1987). It also degrades different types of proteins including fibronectin, lactoferrin, and cholera toxin itself (Booth *et al.*, 1984). This protease also has an hemagglutinating activity and thus also called hemagglutinin (Booth *et al.*, 1983; Finkelstein and Hanne, 1983). Although it can nick cholera toxin and thus activate it, mutants lacking this protease were also shown to be fully virulent. It has been suggested the hemagglutinin/protease might contribute to detachment from intestinal mucosal cells (Finkelstein *et al.*, 1992) rather than attachment. Possibly, the vibrios need to detach from cells that are being sloughed off the mucosa in order to reattach to newly formed mucosal cells and also may enable them to find another human host via fecal transfer.

The gene encoding this protease has been cloned (Van-Dongen *et al.*, 1986; Franzen and Manning, 1986; Hase and Finkelstein, 1991) and was designated *hap* (for *hem*agglutinin-*p*rotease). This *V. cholerae* protease was also found to be functionally similar to *Pseudomonas aeruginosa* elastase (Hase and Finkelstein, 1990). These two enzymes have been considered as therapeutic agents in clearing airways obstruction as in cystic fibrosis. In *Vibrio vulnificus,* protease deficient mutants were also unable to utilize heme as an iron source, indicating that the protease may be involved in iron-acquisition (Nishina *et al.*, 1992).

2.8 *Aeromonas hydrophila*

Aeromonas hydrophila is a short gram-negative rod which occurs singly or in pairs and is motile by a single polar flagellum. They are generally β-hemolytic on blood agar. They are differentiated from members of entrobacteriaceae by a positive oxidase reaction. The organisms are not halophilic and do not grow on 7.5% NaCl. (von Graevenitz and Altwegg, 1985; Khardouri and Fainstein, 1988).

A. hydrophila are mostly water-borne and occur widely in fresh and estuarine waters (Hazen *et al.*, 1978) and has been found in stagnating water, streaming surface water and also from wells (Park, 1961; Meeks, 1963). This species are also present in polluted and unpolluted fresh waters, in sewage and in drinking water (Rippey and Cabelli, 1980; Burke *et al.*, 1984; Monfort and Baleux, 1990). Some strains of *A. hydrophila* are capable of causing illness in fish (Dixon *et al.*, 1990; Allan and Stevenson, 1981; Trust, 1986) and amphibians (Carlton and Hunt, 1978; Rigney *et al.*, 1978) as well as in humans (Kuijper *et al.*, 1989; Altwegg and Geiss, 1989) who may acquire infections through open wounds or by ingestion of a sufficient number of the organism in food or water.

2.8.1 *A. hydrophila* Infections in Humans

A. hydrophila may cause gastroenteritis in healthy individuals (Lautrop, 1961; Sanyal *et al.*, 1975; Agger *et al.*, 1985) or septicemia (Trust and Chapman, 1979; Harris *et al.*, 1985) in individuals with impaired immune system or various

malignancies. At the present time, there is controversy as to whether *A. hydrophila* is a cause of human gastroenteritis (Figura *et al.*, 1986). The organism possesses several attributes which could make it pathogenic for humans, but with volunteer human ingestion studies, even enormous number of bacterial cells (i.e. 10^{11}), have failed to elicit human illness suggesting that all isolates may not have the potential to cause disease. Its presence in the stools of individuals with diarrhea, in the absence of other known enteric pathogens, suggests that it has some role in disease (Burke *et al.*, 1983; Janda *et al.*, 1983; Agger *et al.*, 1985).

Two distinct types of gastroenteritis have been associated with *A. hydrophila*: namely a cholera-like illness with a watery diarrhea and a dysenteric illness characterized by loose stools containing blood and mucus. Non-invasive strains usually produce watery diarrhea, whereas invasive strains produce dysenteric symptoms resulting in blood and mucus (Watson *et al.*, 1985). The infectious dose of this organism is unknown, but scuba divers who have ingested small amounts of water have become ill, and *A. hydrophila* have been isolated from their stools.

A general infection in which the organisms spread throughout the body (septicemia) has been observed in individuals with underlying illness. This is a rare condition usually observed only in immunorepressed or debilitated patients. Symptoms of *A. hydrophila* septicemia include rheumatic fever-like syndrome and flu-like illness, similar to other types of gram-negative septicemia. Hypotension and abdominal pain is common, and occasional nausea and vomiting are present (Trust and Chapman, 1979).

A few cases of *A. hydrophila* meningitis have been reported some of which were fatal to babies (Yadava, 1979; Freij, 1984; Parras *et al.*, 1993). *A. hydrophila* has also been isolated from open wound infections (Joseph *et al.*, 1979). This species were also known to cause rare cases of urinary tract infections and bacteremia (McCracken and Barkley, 1972). Other reported cases of *A. hydrophila* infections include osteomyelitis (Davis *et al.*, 1978; Karam, 1983), septic arthritis (Dean, 1967), endocarditis (Davis *et al.*, 1978; Ong *et al.*, 1991) and peritonitis (Slotnick, 1970; McCracken and Barkley, 1972).

2.8.2 Siderophores

Under iron-limiting conditions, most of the *Aeromonas hydrophila* isolates produce the phenolate/catecholate siderophores, either amonabactin or enterobactin, but not both (Barghouthi *et al.*, 1989; Zwyno *et al.*, 1992). Amonabactin production is more prevalent among the *A. hydrophila* strains. An amonabactin biosynthetic gene from *A. hydrophila* (*amoA*) has been cloned and sequenced (Barghouthi *et al.*, 1991). Amonabactin producing strains were able to acquire iron from the serum iron-binding protein Fe-transferrin, while the enterobactin producing strains were not capable of this. The amonabactin producing strains also grew in heat inactivated serum whereas the enterobactin producing strains did not (Massad *et al.*, 1991). These observations indicate that amonabactin is essential for the survival of bacteria in heat inactivated serum, and thus can be considered a virulence factor. The amonabactin producing strains were also more resistant towards lysis by complement than the enterobactin producing strains (Massad *et al.*, 1991).

2.8.3 Hemolysins

Two hemolysins, α- and β- hemolysin are produced by *A. hydrophila* (Ljungh and Wadstrom, 1982; Ljungh and Wadstrom, 1983) Most of the clinical isolates are β-hemolytic. Both the α- and β- hemolysins were also cytotoxic to several cell lines. The cytotoxic effect of β-hemolysin (aerolysin, cytolytic factor) is irreversible, but the effect of α-hemolysin is reversed when the cells are resuspended in fresh medium.

The hemolysins of *A. hydrophila* has been shown to be regulated by iron and zinc. While the synthesis of hemolysin decreased in the presence of iron in the growth media, the presence of zinc stimulated the accumulation of the toxin (Riddle *et al.*, 1981). The addition of iron or zinc to preformed hemolysin did not alter the enzymatic activity of the toxin. In view of this negative regulation of hemolysin production by iron, it is tempting to speculate that hemolysins are produced in order to lyse the erythrocytes (and other cells) to release the iron bound to heme compounds. It has also been demonstrated that both the amonabactin and enterobactin producing strains were able to acquire iron from heme compounds through a siderophore independent process (Massad *et al.*, 1991). The structural gene encoding the hemolysin (aerolysin) has been cloned (Howard and Buckley, 1986) and the nucleotide sequence is known (Howard *et al.*, 1987).

2.8.4 Proteases

The number and types of proteases secreted by *A. hydrophila* are still under dispute. Most strains of *A. hydrophila* secrete a heat-stable metalloprotease and some other strains also secrete an unrelated heat-labile serine protease (Leung and Stevenson, 1988a; Nieto and Ellis, 1986). The metalloprotease was shown to possess an esterase activity but not cytotoxic activity, and also lethal to fish (Rodriguez *et al.*, 1992). So far, the research on the role of proteases in *A. hydrophila* seems to be focused on mainly on fish isolates rather than human isolates (Leung and Stevenson, 1988b). The gene for a heat-stable metalloprotease (Rivero *et al.*, 1990) and a temperature labile serine protease (Rivero *et al.*, 1991) from *A. hydrophila* has been cloned.

The proteases of *A. hydrophila* were also shown to be regulated by iron and zinc (Riddle *et al.*, 1981). Iron exerted an inhibitory effect, whereas zinc stimulated the accumulation of protease. The addition of iron or zinc to preformed protease extract did not alter the activity of this enzyme. Howard and Buckley (1985) showed that the protease has a role in the activation of hemolysin and thus could be involved indirectly in iron-acquisition.

2.9 Practical Applications of Iron Acquisition Systems

Microbes growing under the iron-limited conditions of the vertebrate host, express various iron-repressible outer membrane proteins which include the receptors for siderophores and other host iron compounds. Expression of such iron-repressible outer membrane receptors has been noted in *Pseudomonas aeruginosa* (Brown *et al.*, 1984), *E. coli* (Griffiths *et al.*, 1983) and *V. cholerae* (Sciortinio and Finkelstein, 1983). It was seen that sera from patients recovering from infections often contain antibodies against iron-repressible outer membrane proteins. Consequently, these iron-repressible outer membrane proteins have considered as potential immunogens for immunoprophylactic therapy (Williams and Griffiths, 1992; Griffiths *et al.*, 1985). Sera from patients with gonococcal and meningococcal infections were found to contain antibodies against these proteins (Black *et al.*, 1986; McKenna *et al.*, 1988). Antibodies against iron-repressible outer membrane proteins of *Salmonella typhi* were also found in patients with typhoid fever (Gonzalez *et al.*, 1987; Fernandez-Beroz *et al.*, 1987). Antibodies against the transferrin binding protein of *Haemophilus influenzae* and *Neisseria meningitidis* were noticed in the convalescent sera of infected patients (Griffiths *et al.*, 1993a; Griffiths *et al.*, 1993b; Rokbi *et al.*, 1997). Antibodies raised against the receptor for pyochelin, a siderophore produced by *P. aeruginosa* was found to inhibit the binding of pyochelin to the receptor and also aids in phagocytosis of these bacteria (Sokol and Woods, 1984; Sokol and Woods, 1986). In *E. coli*, antibodies against the ferrichrome receptor decreased ferrichrome binding to

its receptor (Coulton, 1982) and similarly, antibodies against the aerobactin receptor blocked the binding of the siderophore to the receptor (Roberts *et al.*, 1989).

The clinical applications of siderophores and other iron-binding proteins of bacteria has been extensively reviewed in a recent publication (Bergeron and Brittenham, 1994). Siderophores have been proven to be very useful in the treatment of iron overload diseases. Desferal®, the commercialized name for desferrioxamine B (a siderophore produced by *Streptomyces pylosus*) has found wide use in the treatment of iron overload diseases. Siderophores are also being considered as a part of synthetic antibiotics. Antibiotics attached to the siderophore can be delivered into the cells more effectively through the siderophore receptors of the bacteria (Watanabe *et al.*, 1987). Other possible medical applications of siderophores include their use as biomimetic carriers for use in malaria therapy and also as anticancer drugs. Recently, siderophores have been used as selective growth factors and the growth of *Salmonella* spp. was greatly enhanced by adding ferrioxamine E to the growth media. Another siderophore, desferrioxamine B has been found to be a useful diagnostic tool for differentiating *Staphylococcus* spp.