THE EFFECTS OF IRON ON THE VIRULENCE OF *Pseudomonas aeruginosa*

by

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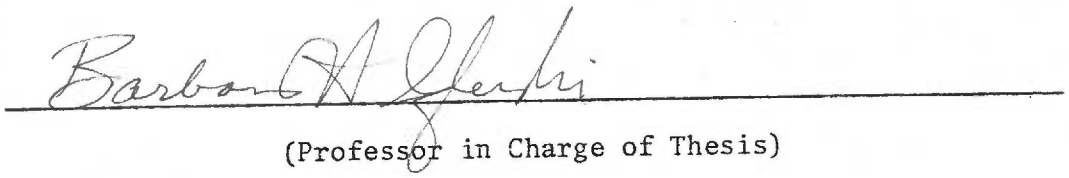

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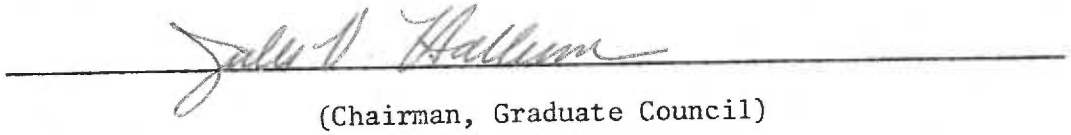

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I. Introduction and Statement of the Problem

Pseudomonas aeruginosa is an opportunistic pathogen which can cause severe infections in the compromised host. In particular, cystic fibrosis, burn and leukemia patients are highly susceptible to *P. aeruginosa* infections. *P. aeruginosa* has become one of the most common causes of severe corneal infections in individuals with previously traumatized corneas. *Pseudomonas* septicemia is particularly severe with a mortality rate ranging from 40-60% despite the use of antibiotics.

Many factors have been implicated in the pathogenesis of *P. aeruginosa* infections. *P. aeruginosa* produces a variety of extracellular products which have the potential to contribute to its pathogenicity. Little is known about the factors which regulate the production of these extracellular products. One factor which has been identified which influences elaboration of extracellular products is iron.

Iron appears to play a complex role in the virulence of *P. aeruginosa*. Iron is essential for microbial growth. In order to compete for iron with the hosts' iron-binding proteins, bacteria synthesize specific iron chelators termed siderophores, which function in the solubilization and transport of iron into the bacterial cell.

Iron also regulates yields of extracellular products of *P. aeruginosa in vitro*. As the iron concentration of the culture medium is increased, the yields of toxin A, elastase and alkaline protease are markedly decreased in culture supernatants. The molecular mechanism(s) of iron regulation of toxin A and protease production are currently unknown.

The objective of this study is to investigate the dual role of iron in the virulence of *P. aeruginosa*. The specific aims of this research

are as follows:

i. Isolate mutants altered in the iron regulation of extracellular product yields and/or altered in iron transport mechanisms.

ii. Determine if iron coregulates or independently regulates the elaboration of P. aeruginosa extracellular proteins.

iii. Further characterize siderophore mediated iron transport mechanisms.

iv. Investigate the relationship of both iron acquisition and regulation of toxin A to the virulence of P. aeruginosa in a mouse eye model.

Increased understanding of the role of iron in the physiology and virulence of P. aeruginosa may provide insight into the pathogenicity and potential prophylaxis of P. aeruginosa infections.

II. Literature Review.

A. General properties of Pseudomonas aeruginosa.

Pseudomonas aeruginosa is an ubiquitous organism, which can be found in soil, water, and in every part of the human habitat (77). It is a gram negative saprophytic rod ranging in size from 0.5 to 1.0 µm by 1.5 to 3.0 µm. Nearly all strains are motile with a single polar flagellum. Most have pili or fimbriae (23,77). P. aeruginosa is an aerobic organism, in contrast to most bacteria that cause disease in human beings, which are facultative anaerobes (77). In the absence of oxygen, however, nitrates can be utilized as the terminal electron acceptor resulting in the formation of N_2 gas (24).

Pseudomonas can metabolize a wide variety of substrates. It can grow on at least 80 different organic compounds, and can even multiply in water with atmospheric CO_2 as its source of carbon (75). Pseudomonas utilizes carbohydrates, proteins, amino acids, alcohols, amines, amides, and fatty acids and has been found to grow in antiseptics and disinfectant solutions (23,75,142).

P. aeruginosa is unique among human pathogens in that it infects not only other vertebrates, but lower animals, and insects (77). It also infects plants such as tobacco and sugarcane (77). Infection is rare in normal individuals. However, in compromised hosts P. aeruginosa can produce severe and often life threatening effects. Clinical infections with P. aeruginosa include local infections, i.e. of wounds, especially burns, urinary tract, respiratory tract, the intestine, the eye and ear, and generalized infections such as bacteremia (80,142). Conditions predisposing to P. aeruginosa infection include natural im-

munologic deficiency, as well as immunologic suppression found in cancer and transplant patients receiving chemotherapy treatments (142). A majority of patients with cystic fibrosis have chronic pulmonary infection with P. aeruginosa and its erradication by current therapy is virtually impossible (142).

Because P. aeruginosa is resistant to a wide range of antibiotics, the organism is difficult to treat (80). Since this organism is resistant to antibiotics which are usually effective against other pathogenic bacteria, it emerges by selection in hospitals where antibiotics are much used. The widespread use of antibiotics and the subsequent control of Streptococci, Pneumococci, and other organisms has increased the number of infections by resistant Staphlococci and P. aeruginosa (77,80). Development and spread of drug resistance appears partly due to plasmids containing R factors, which can be passed from strain to strain as well as from species to species (142). These plasmids may account for the resistance of P. aeruginosa to multiple antibiotics.

B. Potential virulence factors of P. aeruginosa.

P. aeruginosa produces a variety of extracellular products which have been implicated in the virulence of the organism. These include pigments, toxins, proteases, hemolysins, and slime (77,76). Several of these products have been purified and their biological activities determined.

1. Endotoxin. P. aeruginosa differs from most other gram negative rods in that most of its pathogenic activity appears to be due to extracellular products, rather than to endotoxins (80). Endotoxins are lipopolysaccharides associated with the cell wall which are released

when the bacteria die and disintegrate. In P. aeruginosa the endotoxin is much less potent than those isolated from the enteric bacteric. For example, it takes 2 to 3 mg of P. aeruginosa endotoxin to kill a 20 gm mouse (76,77). Furthermore, dead cells of P. aeruginosa injected into experimental animals produce no significant toxic response, in contrast to cells of many other gram negative organisms. Live P. aeruginosa, however, do produce a toxic response indicating the release of some other factor(s) (77).

2. Pigments. P. aeruginosa produces a number of pigments. The most common are pyocyanine, a blue phenazine pigment, and fluorescein, a yellow-green pigment. Others include phenazine alphacarboxylic acid, chloroaphine and a melanin-like brown pigment (76,80). Pyocyanine has been shown to have toxic effects on tissue cultures of fibroblasts and epithelial cells (27,120), but, injection of these pigments into animals does not cause deleterious effects (77). Evidence that these pigments are produced in quantities necessary for pathogenicity in vivo is lacking (77).

3. Hemolysins. P. aeruginosa produces two hemolytic substances. One is heat-labile and appears to be a phospholipase C that liberates phosphorylcholine from lecithin (34). The other is a heat resistant glycolipid which has low toxicity; it seems to act primarily as a detergent to solubilize phospholipids, thereby making them available to the action of phospholipase C (77,126). The two substances are usually produced concomitantly in an environment that is high in carbohydrates and low in phosphate content (72).

Phospholipase injected into the skin of animals produces a cen-

tral abscess surrounded by an area of redness and induration within 24 hours. When injected into the peritoneum phospholipase produces hepatic necrosis as well as edematous lungs (74).

Phospholipase may be involved in the pathogenesis of pneumonia due to P. aeruginosa. Alveolar membranes are usually covered with surfactant, a substance that functions to reduce the surface tension of the alveoli and prevent atelectasis (25). The main component of the surfactant is a lecithin, and therefore, production of phospholipase by an infecting organism may result in the destruction of pulmonary surfactant with consequent atelectasis (76). The phospholipase has been shown to cause necrosis of lung tissue and the combined effect of the destruction of the surfactant and the lung tissues may be of significance (76).

4. Enterotoxin. P. aeruginosa has been associated with diarrheal conditions, often described as five-day or Shanghai fever (76,77). An outpouring of fluid and electrolytes into the lumen of the intestines is the result of an enterotoxin (68). It is heat-labile and probably a protein, but it remains to be characterized.

5. Slime. The slimes of P. aeruginosa are polysaccharides on the surface of the cells and may function like the capsules of other gram negative organisms in resistance to phagocytosis (121,122). Purified polysaccharide fractions are usually nontoxic (1,2). A high frequency of strains isolated from cystic fibrosis patients produce large quantities of slime (142). These strains are referred to as mucoid. Fifty to sixty percent of cystic fibrosis patients infected with P. aeruginosa have the mucoid varient. Moreover, mucoid strains are almost exclusively confined to cystic fibrosis patients, who account for at

least 98% of the mucoid strains isolated (142). Mucoid strains may be more harmful in these patients than non-mucoid strains. Presence of the mucoid material in small airways may increase airway obstruction.

6. Proteases. Strains of P. aeruginosa produce three different proteases: an alkaline protease, a neutral protease with elastase activity, and an acidic protease which is produced in much smaller quantities than the other two (91). These proteases can be fractionated by diethylaminoethyl cellulose column chromatography (91). The alkaline protease and the elastase have been purified (90,93,143).

The alkaline protease is optimally active at pH 7 to 9. Its optimum temperature is 60°C (55). This protease acts on various protein substrates including casein, gelatin, egg albumin and hemoglobin. It does not degrade elastin (90). Molecular weights of 48,000 (54) and 20,000 (143) have been reported for alkaline protease; its isoelectric point is pH 4.08 (90). Most heavy metal ions inactivate the activity of this protease (90).

The elastase possesses optimal activity in the neutral pH range. Molecular weights of 39,500 (89) and at 20,000 (84) have been reported. Its isoelectric point is pH 5.9 (89). The elastase is a metal chelator-sensitive protease which requires Zn^{2+} for enzymatic activity (85). It has specificity for bulky or hydrophobic amino acid residues at the amino side of the splitting point (85).

The injection of either alkaline protease or elastase into the skin of animals induces hemorrhagic lesions (60,73). When these proteases were injected by various routes into mice, hemorrhagic lesions of the lungs and intestinal tracts were seen upon autopsy (59,86). Pa-

tients with P. aeruginosa septicemia commonly have edematous, hemorrhagic lungs and hemorrhagic lesions in the gastrointestinal tract (35). Both protease and elastase have been shown to cause corneal ulcers in mice and rabbits when dropped onto experimentally incised corneas (36, 57).

Using a burned mouse model, Holder et al. (46) demonstrated that non-lethal amounts of purified protease or elastase injected into the burn site would enhance mortality when a low protease producing strain of P. aeruginosa was used to challenge mice. When a_2-macroglobulin, a serum protein which inhibits protease activity, was injected into burned mice infected with a high protease producing strain, an increased survival time was observed.

7. Exotoxin A. Toxin A is produced by 90% of clinical isolates of P. aeruginosa (13,113). On a weight basis, toxin A is the most toxic product produced by P. aeruginosa (76). It is cytotoxic to cultured eukaryotic cells (105,134) and lethal for various animal species (5,18,78). The median lethal dose of pure toxin A when injected interperitoneally is 0.2 µg/22 gm mouse (52).

The mechanism of action of toxin A is identical to that of diphtheria toxin fragment A (49). Toxin A inhibits protein synthesis by catalyzing the transfer of the adenosine 5'-diphosphate ribosyl moiety of nicotinamide adenine dinucleotide (NAD) onto elongation factor 2 (EF-2) (49,50). The resultant ADP ribosyl-EF-2 complex is inactive in protein synthesis (49,50).

Toxin A has been shown to inhibit protein synthesis in vitro and in vivo (22,50,51,107,135). There is a decrease in functional EF-2

in the livers of burned mice infected with toxinogenic strains of P. aeruginosa (51).

Antitoxin has been shown to protect burned mice from subsequent challenge with toxinogenic strains of P. aeruginosa (106). Neutralizing antibody to toxin A has been detected in human sera in patients infected with P. aeruginosa as well as in normal control subjects (112). Survival from bacteremia with P. aeruginosa has been correlated with a higher antitoxin titer than is seen in those who died (112).

Toxin A has been purified in a number of laboratories (18,52, 70,78). It is a heat labile protein of approximately 70,000 daltons (135), with an isoelectric point of pH 5.0 (70). Toxin A is released by the organisms as a proenzyme which is toxic to animals and cultured cells but which is itself devoid or of very low enzymatic activity (135). The enzymatic activity of toxin A can be potentiated in two ways (22,71,135). Limited proteolysis yields an enzymatically active fragment, with a molecular weight of approximately 27,000 daltons (22, 135) and a non-enzymatically active fragment of approximately 43,000 daltons (135). Alternatively, the enzymatic activity of toxin A can be potentiated without altering the size of the toxin molecule by treating it concomitantly with denaturing and reducing agents such as urea and dithiothreitol (71,135). The enzymatically active form and fragment derived from toxin A are not toxic (135).

Toxin A is not produced constitutively in vitro by toxigenic strains of P. aeruginosa. The growth medium normally used for optimal toxin production consists of a dialysate of trypticase soy broth supplemented with 1% glycerol and 50 mM monosodium glutamate (72). A defined

medium for toxin production has recently been described (31). Toxin A production appears to be influenced by a number of factors. One of the most critical is iron. As the iron concentration of the medium is increased, the yield of toxin A in the culture supernatant is markedly decreased (10,12). Since toxin A is not produced constitutively, it seems likely that specific regulatory mechanisms or factors control the synthesis and secretion of toxin A in a manner that is distinct from the regulation of bulk protein synthesis.

8. Exoenzyme S. Exoenzyme S is an adenosine diphosphate ribosyl transferase that is produced by some strains of P. aeruginosa (53). It differs from toxin A in that it does not ADP-ribosylate EF-2, but rather catalyses the transfer of the ADP-ribose moiety of nicotinamide adenine dinucleotide to a number of substrate proteins in crude extracts of eukaryotic cells (53). Exoenzyme S also differs from toxin A in its heat stability and in its destruction, rather than potentiation by pretreatment with urea and dithiothreitol (DTT) (53). Furthermore, exoenzyme S is not precipitated or neutralized by antitoxin A (53).

Exoenzyme S requires growth medium and culture conditions for optimum production similar to that for toxin A (53,131). Common components are dialysed trypticase soy broth (TSBD) supplemented with monosodium glutamate and glycerol. Low iron concentrations are also necessary for optimum yields (131). Enzymatically active exoenzyme S production, however, requires the presence of a chelating agent, whereas toxin A production does not. The addition of 10 mM nitriloacetic acid (NTA) markedly enhances exoenzyme S yields (131).

Exoenzyme S has been shown to be produced in vivo in a burned

mouse model (11). Skin extracts and sera from burned mice infected with the exoenzyme S producing strain 388 contained ADPR transferase activity that was not found in skin or sera from uninfected mice. On the basis of immunological reactivity and enzymatic properties, this ADPR-transferase activity was identified as exoenzyme S (11). It was further demonstrated that strain 388 was virulent for burned mice and that this was not due to production of detectable levels of toxin A (11).

C. Diseases associated with P. aeruginosa.

P. aeruginosa is capable of causing a variety of diseases. Localized infection following surgery or thermal injury commonly results in a generalized and frequently fatal bacteremia (80,142). Urinary tract infections following introduction of P. aeruginosa on catheters or in irrigating solutions are not uncommon (80,142). As mentioned previously, most cystic fibrosis patients are chronically colonized with P. aeruginosa and may ultimately die of P. aeruginosa pneumonia (69). P. aeruginosa can produce severe corneal infections that usually result in ulceration and loss of vision in the infected eye (80). Although rare, P. aeruginosa may cause meningitis following lumbar puncture and endocarditis following cardiac surgery (80). It has also been associated with some diahrreal disease (77,124). P. aeruginosa corneal infections, burn wound infections and bacteremia will be discussed in more detail below.

1. Corneal infections. P. aeruginosa can cause severe ocular infections including corneal ulcers and postoperative endophthalmitis. The usual predisposing factor is preceding eye trauma involving a foreign body. Severe infections have been reported in premature infants,

burn patients, semi-comatose tracheostomized patients, wearers of contaminated soft contact lenses, and recipients of contaminated intraocular lens prostheses. Currently P. aeruginosa causes between 15-20% of bacterial corneal ulcers (65).

Corneal ulcers caused by P. aeruginosa usually evolve rapidly and frequently terminate in corneal rupture and loss of vision if untreated. Thus treatment must be begun as early in the course of the infection as possible. Gentamycin and carbenicillin are the drugs of choice (65).

Animal models for P. aeruginosa keratitis have been developed in which bacteria are applied to traumatized corneas or are injected intracorneally. The virulence of P. aeruginosa for the cornea has been attributed to the production of extracellular proteases and toxin A by the organism. P. aeruginosa protease preparations have produced gross corneal damage similar to that observed during severe experimental and human keratitis produced by P. aeruginosa (15,66,67). When different strains were compared, two low-protease producing strains were reported to cause less severe corneal disease than highly proteolytic strains (58). Kruger et al. (66,67) compared the structural alterations to the eye during experimental P. aeruginosa keratitis in rabbits with the intracorneal injection of P. aeruginosa proteases. The protease preparation used was a mixture of alkaline protease and elastase. Light and electron microscope examination of corneal lesions were made 4 to 6 h after injection of the proteases. These observations showed (1) degeneration of epithelium, endothelium, and keratocytes; and (2) degradation of corneal and stromal proteoglycan ground substance with the dispersal

of collagen fibrils (67). These alterations were very similar to those observed during experimental keratitis (68). Kawaharajo et al. (57) examined the individual effects of alkaline protease and elastase on corneal tissue. The corneas of mice were incised, purified alkaline protease or elastase were topically applied, and the course of damage was followed. Alkaline protease or elastase caused the same histological changes in the cornea, with 0.8 to 2 μg of either enzyme causing opacity, and 4 to 50 μg resulting in ulceration. The above studies suggest that extracellular proteases are at least partly responsible for the pathogenesis of P. aeruginosa keratitis.

Toxin A has also been shown to play a role in P. aeruginosa keratitis. Iglewski et al. (48) reported that the injection of purified toxin A into the corneas of rabbits produced opacity at the injection site. Toxin A killed the epithelial, endothelial and stromal cells of the cornea within 24 h. PMN infiltration was also apparent. Control eyes injected with toxin A neutralized by antitoxin showed only slight stromal opacity.

A genetic approach to determine the role of toxin A and elastase in corneal infections was reported by Ohman et al. (101). Mutant strains deficient in the production or activity of those enzymes were compared with their virulent parental strains in experimental mouse corneal infections. Infections produced by toxin A-deficient mutants (Tox^-) were less severe than infections by the parental strains. The Tox^- mutants did not persist in the eyes as long as their parental strains. Addition of subdamaging doses of exogenous toxin A to eyes infected with the Tox^- mutant PA103-29 significantly increased its viru-

lence. The course of infection and the resulting corneal damage produced by the mutant deficient in elastase activity were indistinguishable from those of its parental strain.

2. Bacteremic infections. As early as 1897, P. aeruginosa was recognized as a pathogen in bacteremic infections (6). Bacteremia with gram-negative organisms remains a major cause of mortality among hospital patients, particularly those with serious underlying diseases (6,37, 129). P. aeruginosa is the causative agent in 11 to 18% of bacteremias. The mortality rate associated with P. aeruginosa bacteremia is reported between 40-60% (82,129,140). The fact that this far exceeds the mortality rate from bacteremia with other gram-negative organism implies that P. aeruginosa has special virulence factors not shared by the Enterobacteriaceae (144).

The primary sources of the origin of P. aeruginosa bacteremia are the respiratory tract, followed by the urinary tract (37,129,140). The skin and the gastrointestinal tract are also occasional sources of primary infection (37,129,140).

There has been little change in the outcome associated with P. aeruginosa bacteremia in the past twenty-five years, despite the availability of antimicrobial drugs with considerable in vitro activity against P. aeruginosa (37). Since the introduction of gentamicin and carbenicillin, the chemistry, toxicity and in vitro antimicrobial activity of these drugs have been extensively studied. Unfortunately, their in vitro effectiveness does not correlate with their activity in vivo (37). Treatment with gentamicin and carbenicillin alone or in combination improved bacteriologic cure or survival rates in bacteremic pa-

tients, but were no more effective than treatment with antibiotics having little or no effect upon P. aeruginosa in vitro (37).

Successful control of underlying disease may be necessary before the mortality associated with P. aeruginosa bacteremia can be expected to decrease. Strict asepsis, prevention of nosocomial infection, and better control of patients' underlying diseases may make the greatest contribution to reversing the trend in P. aeruginosa bacteremia (37).

An animal model has been developed to study vasculitis and bacteremia in immunosuppressed hosts (144). Virulent strains of P. aeruginosa when instilled into the conjunctival sac of agranulocytic rabbits first invade the eyelid, then the bloodstream, causing death in a high proportion of animals in 24-130 hours. Neither the lid lesion nor death follows similar inoculation with Escherichia coli or Klebsiella pneumoniae (144). Because invasion of the conjunctiva by P. aeruginosa must occur before bacteremia develops, this experimental infection provides a means of measuring the invasive as well as the disseminated phase of infection. The close resemblance of this model to infection in neutropenic patients makes it particularly suitable for evaluating the efficacy of antibiotics and immunotherapeutic agents in the compromised host.

3. P. aeruginosa burn infections. Bacterial infection is a major cause of death following burn injury (3,30,89). Although death from invasive wound sepsis has been significantly reduced with the introduction of topical antimicrobial therapy, systemic and pneumonic infection, specifically with P. aeruginosa, persists as the primary killer

in this clinical condition (83).

Until the early 1940's, group A hemolytic Streptococci were a significant pathogen for burned patients. But with the development and widespread use of penicillin, species of Staphlococcus became the predominant life-threatening organisms of the 1950's. Thereafter the use of antibiotics effective against Staphlococci led to the emergence of Pseudomonas as the bacterial species most often associated with the fatal infection of the burn wound (115).

P. aeruginosa can be transmitted to burn wounds from a variety of sources in the hospital environment (115). Considerable controversy has prevailed over the relative importance of endogenous and exogenous contamination of the burn wound by P. aeruginosa (81). Although airborne spread of P. aeruginosa appears to be of little importance, spread is possible from hospital personnel to patient (115). When strict isolation procedures are utilized, the number and kinds of bacteria infecting burn wounds can be reduced. However, these procedures do not control rapid colonization by bacteria derived from endogenous sources. P. aeruginosa is present in the gastrointestinal tracts of some patients and may spread from the perineal area to colonize burns and other wounds (128).

Several animal models have been developed to study the pathogenesis of P. aeruginosa in burns and the roles of various extracellular factors in its virulence. A number of groups describe an experimental burned rat model for studying P. aeruginosa infection (85,130,136,137). The most recently described is that developed by Walker et al. (137). Animals are subjected to a dorsal burn by immersion in boiling water for

30 seconds producing a uniform full thickness burn covering 18 to 22% of the total body surfaces. Organisms are seeded topically over the entire burned area. Since rats are highly resistant to toxin A, 8 times less sensitive than mice on a body weight basis (137), this model is not practical for studying the pathogenesis of toxin A.

Steiritz and Holder (127) described a burned mouse model which is clinically relevant to burn wound sepsis caused by P. aeruginosa. In this model enhanced susceptibility to infection appears to be restricted to P. aeruginosa (127). Anesthesized mice receive a 10 second burn by ethanol flame on the back involving approximately 30% of the total body surface. Immediately afterwards the mice are given injections of organisms s.c. The LD_{50} in the burned animals decreased dramatically as compared to that of normal animals (127).

D. Genetics of Pseudomonas aeruginosa.

The genome in a bacterium is usually divided into two main structural entities, the chromosome and the plasmids. The chromosome of P. aeruginosa consists of a single circular, double-stranded DNA molecule with a molecular mass of 2.1×10^9 daltons (109). Plasmids have varying degrees of stability and are not essential to the replicative survival of the bacterium. They may be important sources of versatility in adapting to different environments. Plasmids are usually recognized by some specialized property they impart to their host cells. Some plasmids have the ability to facilitate chromosome transfer during bacterial conjugation. These are known as fertility (F) plasmids. Resistance or R plasmids determine resistance to various antibiotics. Some species of Pseudomonas harbor metabolic or degradative plasmids that

allow growth on unusual carbon sources such as camphor, octane, napthalene, toluene and xylene. Over 100 plasmids have been described in Pseudomonas.

In P. aeruginosa there are three processes of genetic recombination that can be used in the genetic investigation of this organism. These are conjugation, transduction, and transformation. A conjugation system depends on a plasmid which has the ability to mobilize the host chromosome and transfer it to a recipient cell. Most of the genetic studies in P. aeruginosa have involved the sex factor FP2 (47). FP2 has one site of integration into the chromosome. This has been arbitrarily denoted 0 minutes on the chromosome map. Much of the existing chromosome map has been established using this plasmid. The frequency of recovery of markers situated more than 40 minutes from the FP2 origin, however, is too low to provide accuracy for mapping the so-called late region (more than 40 minutes from the FP2 origin). There has been a continual search for other plasmids to solve the problem of mapping in the late region. FP39 is thought to have an origin 10 minutes proximal to that of FP2, but the absence of markers in this region has prevented proof of this. FP110 has a site of origin about 25 minutes distal to the FP origin and transfers chromosome in the opposite direction as FP2. Most sex factors show only one major integration site from which they can transfer the chromosome.

Stanish and Holloway (126) first showed that certain R plasmids acted as sex factors. Included were the IncP-1 plasmids, such as R68, which are distinguished by their ability to transfer to a wide variety of bacterial genera. Several R plasmids have been shown to promote

chromosome transfer at a high frequency in a variety of strains of P. aeruginosa. Unlike FP2 and the other sex factors that have been studied R68.45 transfers chromosome from a range of sites around the chromosome. Recently the chromosome of P. aeruginosa PAO has been shown to be circular (119). This was demonstrated using R68.45 and recently isolated late gene markers involved in degradative processes.

Certain bacteriophages can promote transfer of bacterial genetic material. In P. aeruginosa the most commonly used phage for transduction analysis is F116 (4). F116 is not integrated into the chromosome but is located extrachromosomally, like a plasmid (47). All of the transducing phages identified display general transduction and no example of prophage-linked specialized transduction has been reported. F116 and G101 are capable of transducing 1-2% of the bacterial chromosome, and are particularly useful for linkage studies by cotransduction (47).

Transformation has been described for various species of Pseudomonas (61,62) but has not been used in the genetic analysis of this genus.

The analysis of gene arrangement in E. coli and S. typhimurium has shown that associations of genes may have significance in terms of the regulation of gene activity. Although the mapping of the P. aeruginosa chromosome has not proceeded to the extend of E. coli, the arrangement of genes has been shown to differ (47). With biosynthetic functions in P. aeruginosa, the degree of clustering of genes controlling related functions is much less than that found in E. coli or S. typhimurium. However genes of catabolic pathways show a high degree of clustering (47). It is presently unknown why the gene arrangements of

these two metabolic systems have evolved differently.

E. Role of iron in bacterial pathogenesis.

Iron is believed to be an essential element for all forms of life. It is widely distributed over the earth in rocks and soil and is thought to be the fourth most abundant element in the earth's crust (118). However, certain of its physicochemical properties, especially the extreme insolubility of ferric iron (K_{sp} of $Fe(OH)_3$ at 25°C = $10^{-38.7}$ M) at neutral and alklaine pH, impose severe restrictions on iron availability for biological assimilation (125). Thus, despite its abundance, the bulk of iron is sequestered in rocks and soil as insoluble aggregates and precipitates. The relatively small quantities of iron essential to plant and microbial life may be solubilized by acid metabolites excreted by microorganisms (4) or by specific microbial iron chelators (97).

Iron in the human body is primarily intracellular. The small quantity of iron in the body fluids is bound to high affinity iron-binding glycoproteins located in the blood and secretions of mammals and birds (9,108,139). Transferrin is the primary non-heme iron-binding protein of the plasma, whereas transferrin and lactoferrin are present in secretions depending on the host species (9).

In addition to iron-binding proteins there are other mechanisms by which hosts withhold iron from microorganisms during infection. Decreased intestinal absorption of iron has been observed in humans who were febrile because of natural infections (139). This is not a nonspecific depression of intestinal adsorption of all nutrients as the adsorption of zinc is significantly increased in rats during experimental in-

fections (139). Evidence also exists that infected animals shift iron from plasma to storage sites in the liver during infection. Parenteral administration of exogenous iron to infected persons does not restore the normal level in the plasma, instead the metal accumulates in reticuloendothelial cells of the liver and spleen (7,41).

Exogenous iron has been found to promote infection in a variety of experimental systems. When iron is given to animals in sufficient concentration to increase saturation of iron binding proteins, the ability of many strains of microorganisms to grow in host cells and tissues is increased (108,139). There is also evidence that excess iron or increased serum iron saturation predisposes humans to infection (19). The incidence of bacterial infections is significantly increased in diseases associated with high serum iron saturation such as Kwashiorkor (84) and acute myelogenous leukemia (20). It has been suggested that a mild nutritional deficiency of iron can suppress infections in humans (94,95). Of 181 persons who were shifted abruptly from a "famine" diet to a "hospital" diet, 41% developed clinical malaria in 5 days; their plasma iron and transferrin iron saturation had risen dramatically within 2 days of onset of refeeding (96). In a similar study, recrudescence of brucellosis, tuberculosis, as well as malaria was observed (95). It was proposed that mobilization of iron from stores after resumption of an adequate diet temporarily exceeded the rate of host utilization of the metal (95,96).

Several studies in which experimental animals were rendered hypoferremic resulted in an increased resistance to infection (139). Iron deficiency can be induced by administering various hypoferremic

agents just prior to or at the time of infection, including endotoxin, attenuated microorganisms, transferrin or siderophores that could not be utilized by the pathogen under consideration. Hypoferremia may also be induced by prior administration of an iron deficient diet.

Microorganisms have evolved specific high affinity systems to acquire iron. Microbial iron transport compounds are referred to as siderophores or siderochromes, and belong to at least two chemical families; secondary hydroxymates and phenolic acids. The affinity constant of siderophores for iron is comparable to that of transferrin. Therefore, the siderophores are capable of competing with host transferrin molecules for iron. The quantity of iron available to the organisms is influenced by factors such as the extent of iron saturation of transferrin, the pH of the environment, the presence of other chelating agents (111), and the molar ratio of siderophore to host transferrin (64).

Microbial siderophores are released by cells into the environment and subsequently reassimilated as ferric chelates. Special receptors and transport components at or within the cell envelope are required for uptake of the chelates. These receptors then serve as an intracellular source of iron. The metal is withdrawn from the siderophores by reducing it to the ferrous state (139). Microorganisms can often utilize siderophores produced by cells of other microbial genera (17). Also various siderophores may be bound and transported by independent systems within a single organism (17).

Four iron transport systems have been described for E. coli. There are three specific systems which transport iron in complex with enterochelin (100), citrate (38), or ferrichrome (45). The fourth sys-

tem is a low-affinity system for which no such complexing agent has been described (38). The outer membrane of E. coli contains several protein receptors which play a role in high affinity uptake of certain iron chelators. These include the feu B gene product (the 81,000 dalton protein postualted to be the ferri-enterochelin receptor) (44), the ton A protein (a 79,000 dalton protein thought to be the receptor for ferri-ferrichrome) (45), and the proposed receptor protein for ferri-citrate uptake (an 80,000 dalton protein induced in medium supplemented with 1 mM citrate (43). Some of these proteins have also been characterized as receptors for phages and colicins. For example, the ton B protein is required for all the iron transport systems in E. coli. The product of the ton B locus has been proposed to be an outer membrane component which provides the energy for active transport through the primary chelator receptors, since mutations at the ton B locus have pleiotropic effects on a variety of iron uptake systems (44,138).

When E. coli is grown under iron stress conditions, certain outer membrane proteins believed to be siderophore receptors are induced (44,116). The production of these proteins is suppressed in medium supplemented with iron. Whole cells and outer membranes prepared from cells grown under iron stress bind more ferri-enterochelin than similar preparations from cells grown in the presence of available iron (116,117). This increase in ferri-enterochelin binding and uptake activities coincides with the induction of outer membrane proteins observed in conditions of iron stress (116).

Outer membrane proteins of similar molecular weights as found in E. coli are induced in other bacteria when these organisms are grown

in iron-starved conditions. These proteins have been reported in S. typhimurium (8), Neisseria gonorrhoeae (99), N. meningitidis (124), Pseudomonas spp. (88) and Vibrio anguillarum (28).

Plasmid-mediated iron uptake systems have been demonstrated in E. coli (141) and V. anguillarum (28). These plasmids are associated with the virulence of the organisms. Strains with the plasmids grow well in the presence of iron chelators such as transferrin. Growth of isogenic plasmidless derivatives is inhibited by the presence of iron chelators (28,141). In both cases, radioactive iron uptake experiments indicated that the ability to grow under conditions of iron limitation was due to an efficient iron uptake system mediated by the virulence plasmid. In addition, two novel outer membrane proteins are induced in V. anguillarum cells grown under conditions of iron stress. One of them, an 86,000 dalton protein, is inducible only when the plasmid is present (28). It is unknown whether this protein is actually coded for by the plasmid and induced in response to a decrease in the iron concentration or is a chromosomal product that is regulated by a plasmid-specific substance (28).

F. Iron metabolism in P. aeruginosa.

The iron chelating systems of P. aeruginosa have not been extensively characterized although three iron uptake systems have been reported. Pyoverdin, a yellow-green fluorescent pigment produced by Pseudomonas has been described as a siderophore. Pyoverdin is produced in vitro in many different media, but is inhibited by increased iron concentrations (102). Pyoverdin is a hydroxamate compound but its structure appears to vary among different species of Pseudomonas (21,40,

98). Iron binding abilities of this pigment were demonstrated by the activation of several iron-containing enzymes (98). Although pyoverdin does not support iron uptake in Bacillus subtilis (110) it has been shown to stimulate iron transport in P. fluorescens (87).

Pyochelin is an iron-binding compound isolated from ethyl acetate extracts of culture supernatants of P. aeruginosa (26). This compound has been purified by successive paper and thin-layer chromatographic procedures. On the basis of UV, visible, infrared, and fluorescence spectroscopy, it was determined that the compound possessed phenolic characteristics, with little or no similarity to dihydroxybenzoate, and no indication of a hydroxymate group (26). P. aeruginosa synthesized this compound during growth in culture media containing less than 5 x 10^{-6} M added $FeCl_3$. When pyochelin was added to iron deficient cultures of P. aeruginosa, it promoted the growth of the bacterium and also reversed growth inhibition by the iron chelator EDDA (ethylenediamine-di-(o-hydroxyphenyl-acetic acid)) (26).

A mechanism for iron uptake from ferric citrate has also been described (26). Iron complexed with citrate was accumulated by P. aeruginosa in an energy-dependent process. The mechanisms for iron uptake from ferric citrate were present in cells grown in a variety of media but were lowest in cells grown in citrate medium. The ferric citrate uptake system in P. aeruginosa is different than that reported in E. coli in that it is not induced by citrate (26).

G. Regulation of extracellular products by iron.

The presence of excess iron in the culture medium has been shown to decrease yields of diphtheria toxin (79,104,114), Shigella

dysenteriae type 1 toxin (32,133) and P. aeruginosa toxin A (10,12). These toxins are produced at maximal levels late in the bacterial growth cycle (10,33,104).

In contrast to Corynebacterium diphtheriae and S. dysenteriae, iron has been shown to have a deleterious effect on yields of extracellular products of P. aeruginosa other than toxin A. Iron decreases the yields of elastase, hemagglutinin, total extracellular protease (12), and exoenzyme S (131) in some strains of P. aeruginosa. Furthermore, this iron effect is not restricted to extracellular proteins but includes other extracellular products. Several investigators have shown that the yields of the nonproteinaceous pigments fluorescein and pyocyanine decreased with increasing concentrations of iron in the growth medium (16,39,63,132).

The molecular mechanism(s) underlying iron regulation of extracellular products in P. aeruginosa are unknown. The effect of iron on yields of extracellular products is not the same in all strains that have been tested (12). Although the iron effect on toxin A appears to be strain independent, the iron effect on yields of hemagglutinin, total protease or elastase was strain dependent (12), suggesting that at least in some strains of P. aeruginosa the yields of these products are regulated by iron independently of its regulation of toxin A.

In two separate studies, mutants of C. diphtheriae have been isolated that produce high yields of diphtheria toxin in medium containing excess iron (29,56). In one study four mutants were independently isolated that produced more toxin than the wild type C7 (β) in medium with increased iron concentrations, but which differed in their maximal

yields and susceptibility to the inhibitory effect of iron on toxigenesis (29). All four of these mutants were found to be defective in the transport of ferric iron (29). In an independent study by Kanei et al. (56) five mutants were isolated from C7 (β) that produced toxin at the normal rate in medium containing excess iron. The nature of these mutants have not been extensively characterized. It is unknown whether these mutants are defective in iron transport (56).

At present there are no reports of mutants isolated from P. aeruginosa altered in their susceptibility to the iron effects on yields of extracellular products. Mutants of this type are necessary for investigating the molecular mechanism(s) of iron regulation of extracellular products in P. aeruginosa.

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III. Manuscripts

Paper 1.

Demonstration of Iron-Siderophore Binding Protein in the Outer Membrane of *Pseudomonas aeruginosa*

Abstract

Outer membranes prepared from Pseudomonas aeruginosa grown in low iron medium bind three times as much ^{59}Fe-pyochelin as membranes from cells grown in high iron medium. The deletion of pyochelin reduced ^{59}Fe binding to background levels. Autoradiographic analysis of sodium dodecyl sulfate polyacrylamide gel electrophoretograms of outer membrane preparations previously incubated with ^{59}Fe-pyochelin revealed that the iron-siderophore complex bound to a protein with an apparent molecular weight of 18,000.

Manuscript (Note format)

Iron is an essential nutrient for all forms of life (11,15). The acquisition of iron by microorganisms is correlated with the ability of a variety of pathogenic bacteria to establish and maintain infections (16). Due to the extreme insolubility of ferric ion at neutral pH, aerobic and facultative microorganisms must synthesize iron chelators, termed siderophores, which are taken up by cells in complex with Fe(III) iron (19).

Three iron transport systems have been reported in Pseudomonas. Pyochelin is a phenolic iron-binding compound produced by P. aeruginosa which has been shown to have siderophore activity (3). Pyochelin promotes bacterial growth when added to iron-deficient medium (2) and ferripyochelin acts as a substrate for iron transport (3). A mechanism for iron uptake from ferric citrate has also been described in P. aeruginosa (3). A third iron uptake system involving pyoverdine, a hydroxamate siderophore, has been reported in P. fluorescens (13).

Outer membrane proteins have been implicated to be involved in the

uptake of iron by a variety of organisms (1,9,12,18). Iron starvation leads to the induction of several high molecular weight proteins in the outer membranes of several species of Pseudomonas including P. aeruginosa (14). Isolated outer membranes have been shown to be capable of siderophore mediated iron uptake in Escherichia coli and Salmonella typhimurium (1). Mutants deficient in iron transport have been isolated that lack the outer membrane proteins implicated in siderophore mediated iron transport (11,12). However, the direct binding of an iron-siderophore complex to any of these outer membrane proteins has not yet been demonstrated. In the present study, we identify by direct means an iron-siderophore binding outer membrane protein of P. aeruginosa.

Cultures of P. aeruginosa strain PAO-1 (8) were grown in sterile, acid-washed flasks (dilute HNO_3) containing 500 ml M-9 minimal salts medium (17). Glucose (0.5%) was added as a carbon source in all cultures and $FeCl_3$ was added where indicated (high-iron medium) at a concentration of 100 μM. Cultures were incubated for 48 hours in a shaking water bath at 37°C.

Outer membranes were isolated by a modification of the technique of Hancock and Nikaido (6). Briefly, cells grown in 500 mls of medium were harvested by centrifugation (10,000 x g, 20 minutes). The cells were washed with 30 mM Tris-HCl, pH 8.0 (Tris buffer) and resuspended in 20 ml of 20% (wt/vol) sucrose in Tris buffer containing 1 mg of pancreatic deoxyribonuclease and 1 mg of pancreatic ribonuclease. The cells were then sonicated for 2 minutes in a Biosonik IV (Bronwill, Rochester, NY) at 50% maximal output. After sonication, 2 ml of hen egg white lysozyme (1 mg/ml) were added. Ten minutes later, phenylmethyl sulfonyl-fluoride was added to 1 mM. Cell debris were removed by centrifugation at 1,000

x g for 15 minutes. The supernatant was decanted and diluted by the addition of 14 ml of Tris buffer. The diluted supernatant was then layered onto a two-step sucrose gradient with 4 ml of 70% (wt/vol) sucrose in the bottom later and 4 ml of 60% (wt/vol) sucrose in the top layer. This was centrifuged at 183,000 x g for 3 hours in a Beckman SW 41 Ti rotor (Beckman Instruments, Inc., Irvine, CA). The lower band containing outer membrane was collected and washed free of sucrose. The isolated membranes were analyzed for D-lactate dehydrogenase enzymatic activity to assess the degree of purity (5).

Pyochelin was purified by preparative thin-layer chromatography as previously described (3). Purified pyochelin was stored at $4^{o}C$ and reconstituted in ethanol immediately before use.

Ferripyochelin binding to outer membranes was measured by a modification of the method of Pugsley and Reeves (17). Outer membranes were suspended in M-9 medium containing 100 µM nitrilotriacetic acid (NTA; Sigma Chemical Co., St. Louis, MO). Equal volumes of NTA (100 µM) and $^{59}FeCl_3$ (10 µM, 1 MCi/100 g Fe^{+3}, Amersham, Arlington Heights, IL) were then added to 0.1 ml volumes of outer membrane suspensions containing 100 µg protein. 100 µg of bovine serum albumin (BSA) was used as a control. The reaction mixtures were incubated for 5 minutes at either $4^{o}C$ or $25^{o}C$. They were then filtered through cellulose nitrate filters (0.2 µm pore diameter, Sartorius, Brinkman Instruments Inc., Westbury, NY) and washed with 10 mls of 0.9% saline. The $^{59}Fe^{+3}$ retained by the filters was counted in a Beckman Bio-Gamma counter (Beckman Instruments, Inc., Irvine, CA).

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed as described by Laemmli (10). The gels were cast in 1.5 mm

thick slabs in a 10 cm electrophoresis apparatus (Aqueboque Machine and Repair Shop, Aqueboque, NY). Samples were dissolved in a solution containing 0.05 M Tris-HCl at pH 6.8, 2% SDS, 10% (vol/vol) glycerol, and 0.001% bromphenol blue by heating at 100°C for 4 minutes. Where indicated, samples also contained 1% (vol/vol) 2 mercaptoethanol. Electrophoresis was carried out at a constant current of 25 mA per slab for 4 hours. Following electrophoresis, the gels were fixed and stained for protein with Coomassie brilliant blue R250 in 50% methanol/10% acetic acid (vol/vol). Stained gels were scanned with a Transidyne General Scanner (Madison, WI) at 580 nm. Molecular weight standards (phosphorylase b, bovine serum albumin, pyruvate kinase, ovalbumin, lactic dehydrogenase, carbonic anhydrase, serum trypsin inhibitor, and lysozyme) were run on the same gel as the sample.

To visualize the binding of ^{59}Fe-pyochelin to outer membranes, aliquots of the incubation mixtures (each containing 15,000 cpm of ^{59}Fe-labeled membranes) were dissolved in sample buffer and electrophoresed on 12.5% acrylamide-0.17% bisacrylamide SDS slab gels. The gels were dried onto filter paper by heating under reduced pressure for 3 hours. ^{59}Fe was visualized by autoradiography for 12 hours on Kodak RP/R2 x-ray film (Kodak, Rochester NY).

Binding of ^{59}Fe to outer membranes prepared from cells grown in low and high iron was performed under a variety of experimental conditions. Membrane preparations from cells grown in low iron medium exhibited the highest ^{59}Fe binding activity (Table 1). When incubated with 20 μM pyochelin, these preparations bound 15.4% of the total ^{59}Fe added. In contrast, membrane preparations from cells grown in high iron (100 μM $FeCl_3$) medium bound only 5.0% of the total ^{59}Fe added. The deletion of

pyochelin from the incubation mixture abolished ^{59}Fe binding to outer membranes (Table 1). Temperature of incubation (4°C, 25°C, 37°C) had no effect on binding while the addition of 2-mercaptoethanol reduced ^{59}Fe binding to background levels. Outer membrane preparations were relatively free from contaminating inner membrane materials as less than 1 μmole of dimethylthiazolyl-diphenyltetrazolium bromide was reduced per minute per mg outer membrane protein.

A number of outer membrane proteins varied dependent upon the iron concentration of the growth medium (Figure 1). For the most part, these differences were limited to quantitative changes. There does, however, seem to be an induction of a small molecular weight protein of about 18,000 daltons and to a lesser extent large molecular weight proteins of about 75,000 and 80,000 daltons in outer membranes from cells grown in low iron medium as compared to outer membranes from cells grown in high iron medium.

To further characterize the binding of ^{59}Fe-pyochelin to outer membrane proteins, preparations from cells grown in low iron medium were electrophoresed on 12.5% polyacrylamide gels and subjected to autoradiographic analysis (Figure 2). ^{59}Fe-pyochelin bound to a protein with an apparent molecular weight of 18,000. The addition of 2-mercaptoethanol to the sample buffer abolished the binding of ^{59}Fe-pyochelin to this protein. Deletion of pyochelin from the reaction mixture also eliminated ^{59}Fe binding to this protein, and no other outer membrane protein bound ^{59}Fe in the absence of pyochelin.

The induction of high molecular weight proteins in the outer membrane of P. aeruginosa grown in low iron conditions is in agreement with the results of Meyer, et al. (14). These proteins, however, do not

appear to be involved in the binding of ferripyochelin. Radioactively labeled ferripyochelin is associated with a single protein band with an apparent molecular weight of 18,000 on SDS-polyacrylamide gels. This low molecular weight protein is present in a higher concentration in outer membranes from cells grown in low iron medium. This protein accounts for 21 per cent of the total protein in membranes from cells grown in low iron medium as compared to 8 per cent of the total membrane protein from cells grown in high iron medium (Figure 1). Further, these membrane preparations from cells grown in low iron medium bind approximately three times more ^{59}Fe-pyochelin than outer membranes from cells grown in medium supplemented with iron. The increased concentration of this 18,000 molecular weight protein in the outer membrane preparation from cells grown in low iron is therefore sufficient to account for all of the increased binding of ^{59}Fe-pyochelin observed with these membrane preparations. This suggests that there is not a change in the affinity of this protein for iron, but a difference in the amount of this protein present in the outer membrane depending on the iron concentration in the medium.

Low affinity, siderophore independent iron utilization systems have been reported for a number of organisms (15). However, the attainment of background levels of ^{59}Fe binding to outer membrane preparations in the absence of pyochelin indicates that we are examining a specific siderophore-mediated response in P. aeruginosa PAO-1. Additional studies are required to determine if P. aeruginosa utilizes the same outer membrane protein receptor for iron uptake from ferripyoverdine or ferric citrate complexes.

The finding that 2-mercaptoethanol abolishes the binding of ferri-

pyochelin to outer membranes could be explained in several ways. Pyochelin is known to contain sulfulhydryl groups which may be involved in chelation of iron (4). Reduction of these could result in release of bound iron. Alternatively, the outer membrane protein responsible for binding of the ferripyochelin might be susceptible to reduction by 2-mercaptoethanol resulting in the inability to bind the complex. Certainly, a number of outer membrane proteins of P. aeruginosa have been shown to be sensitive to 2-mercaptoethanol (7).

Although binding studies and genetic evidence have suggested the involvement of high molecular weight outer membrane proteins in iron uptake, no direct evidence has been obtained that these proteins are involved in the actual binding. As pointed out previously by Simonsen et al. (18), these proteins may be involved in the removal of iron from protein complexes. The present studies indicate that high molecular weight outer membrane proteins from P. aeruginosa induced during iron limitation are not involved with binding of ferripyochelin. Further, the present data would suggest that a reexamination of iron binding mechanisms in other organisms by direct means is warranted.

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Table 1. ^{59}Fe binding to outer membrane preparations of *Pseudomonas aeruginosa* strain PAO-1

Iron added to culture medium	Pyochelin added to reaction mixtures[a]	^{59}Fe bound (cpm/100 µg protein)[c]
100 µM	20 µM	1980[b] (5.0)[c]
0	20 µM	6117 (15.4)
100 µM	0	113 (0)
0	0	68 (0)

[a]Reaction mixtures contained 100 µg outer membrane protein in 0.1 mls M-9 medium with 100 µM NTA and equal volumes of NTA (100 µM) and $^{59}FeCl_3$ (10 µM).

[b]Minus background (BSA, 1313 cpm bound)

[c]Numbers in parentheses represent percent total ^{59}Fe added (39,651 cpm)

Figure 1. Outer membrane preparations of Pseudomonas aeruginosa PAO-1 grown in low iron (top portion) and high iron medium (low portion) electrophoresed on a 12.5 percent SDS-polyacrylamide gel. The gel was stained with Coomassie blue and scanned at 580 nm.

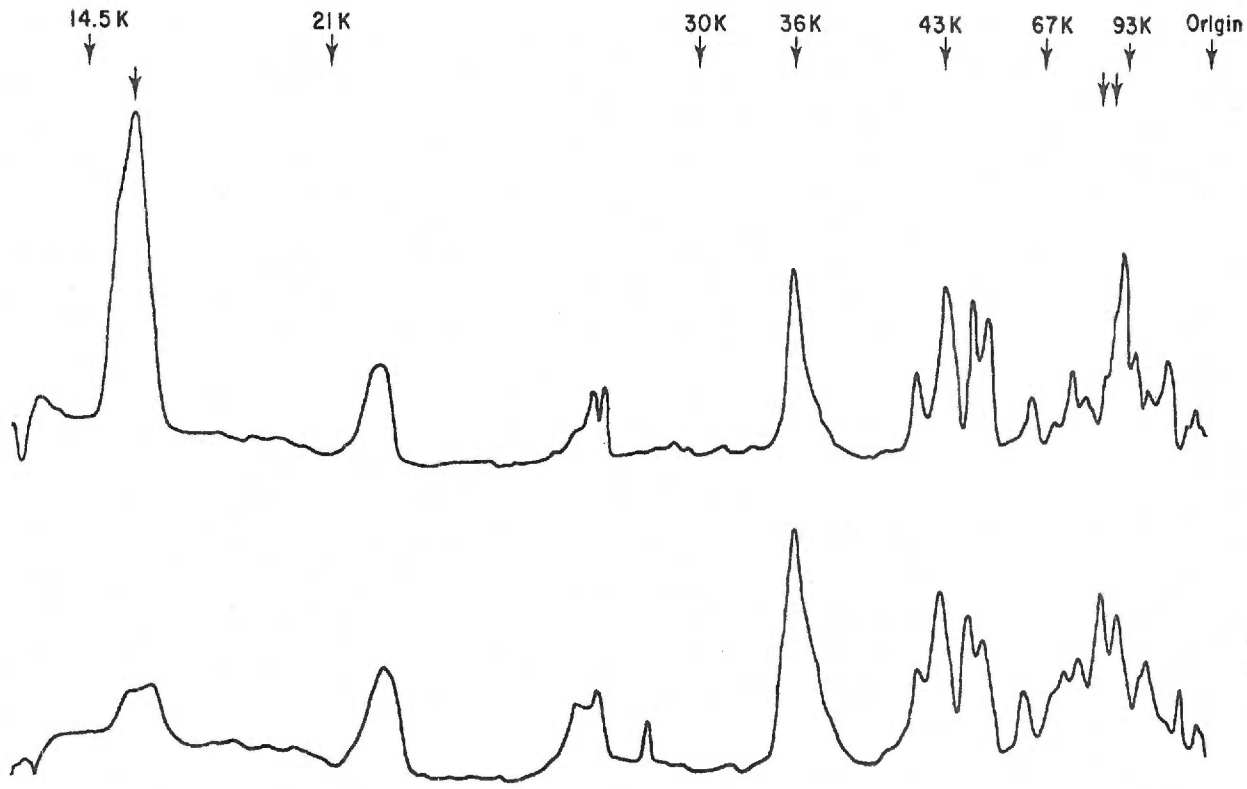
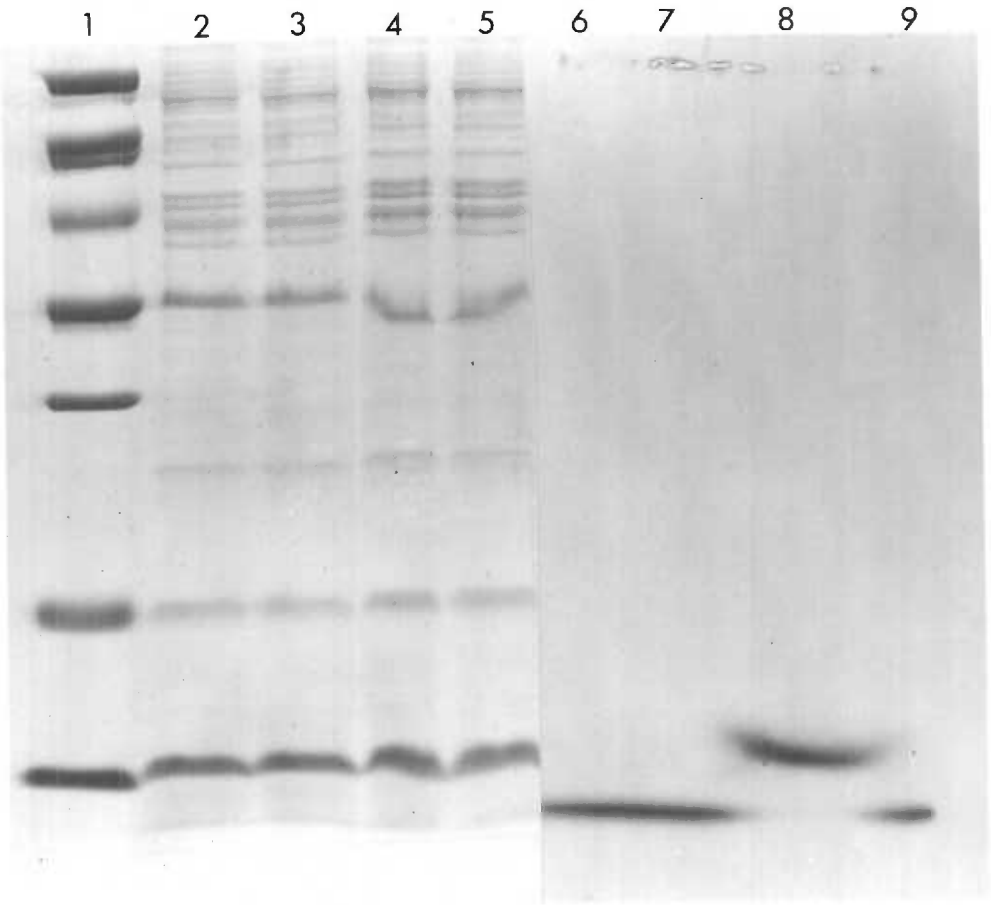

14.5K
21K
30K
36K
43K
67K
93K
Origin

Figure 2. SDS-polyacrylamide gel electrophoresis of outer membrane (OM) proteins from Pseudomonas aeruginosa PAO-1 grown in low iron medium. Wells 1-5 are Coomassie blue-stained and wells 6-9 are autoradiograms of unstained preparations corresponding to wells 2-5. Well 1: molecular weight standards described in text. Wells 2 and 6: OM preparations incubated with ^{59}Fe-pyochelin plus 2-mercaptoethanol (2-ME). Wells 3 and 7: OM preparations incubated with $^{59}FeCl_3$ plus 2-ME. Wells 4 and 8: OM preparations incubated with ^{59}Fe-pyochelin. Wells 5 and 9: OM preparations incubated with $^{59}FeCl_3$.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Paper 2.

Isolation and Characterization of Pseudomonas aeruginosa Mutants Altered in Their Sensitivity to the Effect of Iron on Toxin or Elastase Yields.

Abstract

Iron has been shown to affect yields of toxin A, alkaline protease, elastase, pyochelin and pyoverdin in P. aeruginosa during growth in vitro. Mutants of P. aeruginosa strain PAO-1 resistant to the effect of iron on toxin A (tox^{FeR} mutants) or elastase yields ($elas^{FeR}$ mutants) were isolated following NTG mutagenesis. The iron transport systems of the mutants were characterized by uptake of $^{59}FeCl_3$, and production and utilization of pyochelin and pyoverdin. The mutants fell into two classes; Class I mutants were defective in iron transport. Class II mutants were normal in iron transport and appeared to have mutations in genes regulating extracellular proteins. Class II mutants were further characterized to determine the specificity of their altered sensitivity to iron. PAO-tox^{FeR} mutants produced toxin A in medium containing high iron concentrations, however, yields of elastase and alkaline protease remained sensitive to regulation by iron. The $elas^{FeR}$ mutants were resistant to the effect of iron on elastase yields, but yields of toxin A and alkaline protease were decreased by iron analogous to the parental strain. These data suggest that toxin, elastase and alkaline protease yields can be independently regulated by iron.

Introduction

Iron has been shown to inhibit yields of diphtheria toxin (14,21, 22), Shigella dysenteriae type 1 toxin (9,25), and Pseudomonas aeruginosa toxin A (1,2) in a concentration dependent fashion. The structural gene for diphtheria toxin is coded for by toxin positive corynebacteriophage. In Corynebacterium diphtheriae the iron effect is believed to be

specific for the toxin gene product because phage production continues in the presence of excess iron (18). In contrast to C. diphtheriae and Shigella, the deleterious effect of iron on yields of extracellular products of P. aeruginosa is not restricted to toxin A. Protease and elastase production are also decreased in medium with excess iron (18). In contrast to C. diphtheriae and Shigella, the deleterious effect of iron on yields of extracellular products of P. aeruginosa is not restricted to toxin A. Protease and elastase production are also decreased in medium with excess iron as are yields of the non-proteinaceous pigments fluorescein and pyocyanine (2,3,11,13,24).

The effect of iron on toxin A yields is not strain dependent (2). The magnitude of inhibition of toxin A yields in the presence of iron was similar in all strains tested. However, the effect of excess iron in the culture medium on yields of protease and elastase was strain dependent (2) suggesting the possibility that proteases and toxin A are regulated by iron independently in some strains of P. aeruginosa. It is not known if iron co-regulates yields of these extracellular products in strains where toxin, protease, and elastase yields are all decreased by iron.

The current study was undertaker to isolate mutants which were resistant to the iron effect on yields of either toxin A or elastase and to determine if their resistance to iron extended to other extracellular products. Such mutants make it possible to determine if iron co-regulates or independently regulates yields of extracellular products in P. aeruginosa.

Materials and Methods

Bacterial strain. P. aeruginosa strain PAO-1, originally characterized by B.W. Holloway (12), was used in this study. Strain PAO-1 was chosen since its yields of toxin A, elastase and alkaline protease are all sensitive to the iron effect.

Culture conditions. Chelexed trypticase soy broth dialysate (TSB-DC) was prepared as described (20) and supplemented with 0.05 M monosodium glutamate and 1% glycerol. The residual iron concentration was determined by the method of Mueller and Miller (17). Standard sterile solutions of $FeSO_4 \cdot 7\ H_2O$ were added to obtain known concentrations of iron per ml.

Culture flasks were cleaned with dilute nitric acid and rinsed with deionized water. Ten ml of medium was added to a 250 ml Erlenmeyer flask and inoculated with 0.1 ml of a 15h shaking culture. The flasks were incubated at 32°C with maximum aeration for 18 h. Bacterial growth was measured by an increase in turbidity (optical density at 540 nm). Supernatants obtained following centrifugation at 10,000 x g for 15 min were stored at -70°C.

Isolation of mutants. Log phase cells of P. aeruginosa PAO-1 grown in nutrient broth with 0.5% yeast extract (Difco) (NYB) were mutagenized with 50 µg of N-methyl-N'-nitrosoguanidine (Sigma Chemical Co.) per ml at 37°C for 30 minutes by the method of Finkelstein et al. (10). The cells were washed twice, resuspended in NYB and incubated with shaking at 37°C overnight. The mutagenized overnight cultures were inoculated into fresh NYB and incubated at 37°C with shaking until reaching a cell density of 10^8 cells per ml. These cultures were diluted in saline, and

plated on the appropriate agar medium for mutant selection. Approximately 50 colonies were grown on each plate.

To identify mutants which produced toxin A in high iron medium, a modification of the agar-well assay of Ohman et al. (20) was used. TSB-DC agar medium was prepared as described (20) except that $FeSO_4$ was added to a final concentration of 25 μg per ml after autoclaving. This iron concentration completely inhibited the formation of immunoprecipitation bands between colonies of the parental strain PAO-1 and adjacent antiserum wells containing antitoxin A immunoglobulin prepared from sheep immune serum (23). Mutants producing toxin under high-iron conditions were identified by their ability to produce toxin-antitoxin precipitin bands in this agar-well assay. Those colonies which produced precipitin bands were streaked to obtain isolated colonies, retested in the high-iron agar-well assay, and stored in sterile skim milk at -70°C.

ADP-ribosyl transferase activity. Partially purified EF-2 was prepared from extracts of wheat germ (4). The ADP-ribosyl transferase activity of activated (urea and dithiothreitol treated) supernatants from cultures grown in TSB-DC was measured as previously described (26).

Elastase and alkaline protease assays. Elastase and alkaline protease were quantitated in TSB-DC culture supernatants by radioimmunoassays as previously described (8,19). Highly purified P. aeruginosa elastase and alkaline protease were obtained from K. Morihara (16). Specific anti-elastase antiserum was the kind gift of B. Wretlind, Karolinski Hospital, Stockholm, Sweden. Alkaline protease antiserum was prepared as previously described (8).

Assay of iron uptake. Bacteria were grown in TSB-DC to a density

of 5 x 10^8 CFU/ml at 32°C in a shaking water bath. Iron uptake assays in these cultures were initiated by addition of $^{59}FeCl_3$ (1 m Ci/100 µg Fe) (Amersham) to a final concentration of 50 ng/ml. At ten minute intervals one ml samples of the culture were removed, placed on 0.45 µm filters (Millipore) and vacuum filtered. The filters were washed with 10 ml of 0.5% thioglycolate (7), placed in vials and counted in a Beckman Biogamma counter. A control assay without cells was used to determine the background levels of labeled iron non-specifically associated with the filters. These values were subtracted from reactions run with bacteria to yield values of $^{59}FeCl_3$ uptake by bacteria. Quantities of $^{59}FeCl_3$ accumulated were determined from a standard curve reflecting cpm $^{59}FeCl_3$ versus ng iron concentration.

Siderophores. Purified pyochelin and pyoverdin were a kind gift of Dr. Charles D. Cox, University of Iowa, Iowa City, Iowa.

Results

Isolation of mutants. Following NTG mutagenesis of P. aeruginosa strain PAO-1, mutants resistant to the effect of iron on toxin A yields (tox^{FeR} mutants) were identified by the production of immunoprecipitin bands in an agar-well assay using medium supplemented with $FeSO_4$. Approximately 8,000 colonies were screened after 2 independent mutageneses. Seven colonies produced toxin in the high-iron agar-well assay for a mutation frequency of approximately 10^{-3}. Mutants which were resistant to the iron effect on elastase yields ($elas^{FeR}$ mutants) were identified by their ability to produce zones of hydrolysis on elastin nutrient agar plates with added iron. A total of 45,250 colonies were examined follow-

ing 4 independent NTG mutageneses. Six mutants were identified which produced elastase on high-iron plates for a mutation frequency of 7 x 10^{-3}. All mutants isolated were recloned, subcultured, and cultivated in TSB-DC broth from quantitation of toxin A or elastase.

The effect of iron on toxin A yields in three of the tox^{FeR} mutants was compared to the parent strain by assaying for ADP-ribosyl transferase activity in supernatants obtained from cultures grown in TSB-DC with iron concentrations ranging from .05 to 5.0 µg/ml (Figure 1). These mutants varied in resistance to the iron effect on toxin A yields. All mutants demonstrated a decrease in toxin A yields as the iron concentration of the medium was markedly increased, PAO-tox^{FeR-10} produced approximately 1.5 to 2 times the amount of toxin A as the parental strain PAO-1 at all concentrations of iron tested. PAO-tox^{FeR-11} and PAO-tox^{FeR-18} produced much greater amounts of toxin A than either the parent PAO-1 or mutant PAO-tox^{FeR-10} at all iron concentrations tested. Although there was still a decrease in toxin A yields at high iron concentrations, these latter two mutants produced more toxin A at 5.0 µg/ml of iron than the parental strain did even when grown at its optimal (17) iron concentration for toxin production (.05 µg/ml). In low-iron medium these mutants produced approximately 4 times the amount of toxin A as PAO-1. Thus, PAO-tox^{FeR-11} and PAO-tox^{FeR-18} are hypertoxin A producers.

Of the 6 $elas^{FeR}$ mutants isolated, the two which produced the largest zones of hydrolysis on the iron supplemented elastin agar were selected for further characterization. PAO-$elas^{FeR-3}$ and PAO-$elas^{FeR-5}$ produced zones of 2.0 and 3.0 mm respectively after 72 hours incubation on iron supplemented elastin agar. The parental strain, PAO-1 produced

a zone of 3.0 mm on elastin agar without added iron and no zone on iron supplemented elastin agar following 72 hours incubation at 37°C. Elastase yields in culture supernatants from elas^{FeR-3} and elas^{FeR-5} grown in TSB-DC containing iron .05 to 1.5 µg Fe/ml were compared with the parental strain (Figure 2). PAO-elas^{FeR-3} and elas^{FeR-5} produced approximately the same amount of elastase at the lowest iron concentration (0.05 µg/ml) as the parent strain PAO-1. As the iron concentration of the medium was increased to 0.25 µg/ml or greater, PAO-elas^{FeR-3} and elas^{FeR-5} produced approximately three times the amount of elastase as the parent strain PAO-1. Mutants PAO-elas^{FeR-3} and elas^{FeR-5} therefore have a much greater resistance to the effect of iron on elastase yields than the parental strain. All of the tox^{FeR} and the elas^{FeR} mutants discussed above had the same generation time (35 minutes) in low and high-iron medium as the parent strain, PAO-1.

Characterization of iron transport in the tox^{FeR} and elas^{FeR} mutants. To determine if these mutants were deficient in iron transport, their ability to take up $^{59}FeCl_3$ was examined. PAO-tox^{FeR-11} and PAO-tox^{FeR-18} had rates of $^{59}FeCl_3$ uptake in TSB-DC medium similar to PAO-1 (Figure 3a). PAO-tox^{FeR-10}, however, had a much slower rate of $^{59}FeCl_3$ uptake than PAO-1 (Figure 3a). The rate of $^{59}FeCl_3$ uptake by PAO-tox^{FeR-10} was 80 pg/min/ml as compared to 150 pg/min/ml by PAO-1. Since PAO-tox^{FeR-10} was obviously an iron transport mutant it was not further characterized for this study. PAO-elas^{FeR-3} and elas^{FeR-5} took up $^{59}FeCl_3$ at approximately the same rate in TSB-DC as the parent strain (Figure 3b).

Although the mutants other than PAO-tox^{FeR-10} appeared to take up

iron normally they were further characterized for specific siderophore production and uptake systems. P. aeruginosa produces two siderophores which are involved in iron transport, pyochelin and pyoverdin (7,15). The amount of pyochelin and pyoverdin produced by the mutants was quantitated in 0.5% CAA (Table 1). Pyochelin and pyoverdin yields varied only slightly among the mutants. The only mutant deficient in synthesis of a siderophore was PAO-tox^{FeR-11} which produced markedly less (1/3) pyoverdin than the parent strain PAO-1. Separate mechanisms appear to control pyochelin synthesis and the formation of the uptake mechanism for pyochelin (7). The ability of the mutants to take up iron utilizing either [^{55}Fe] ferripyoverdin or ferripyochelin was investigated PAO-tox^{FeR-11}, tox^{FeR-18}, elas^{FeR-3} and elas^{FeR-5} were all similar to the parent strain PAO-1 in the uptake of iron from either of these substrates (data not shown).

The data in Figures 3a and b suggest that two distinct classes of mutants were isolated; those deficient in mechanisms of iron transport (PAO-tox^{FeR-10}), and those mutants which had normal iron transport mechanisms of iron transport [(PAO-tox^{FeR-10}), and those mutants which had normal iron transport mechanisms] but appeared to be altered in a regulatory gene(s). These mutants were designated Class I and Class II mutants respectively. PAO-tox^{FeR-10} was designated a Class I mutant since it was markedly defective in the uptake of $^{59}FeCl_3$ from culture medium. Mutants PAO-tox^{FeR-18}, elas^{FeR-3} and elas^{FeR-5} were designated as Class II mutants since they produced pyochelin and pyoverdin, and were able to take up iron from the medium in a manner similar to the parent strain. The mutant PAO-tox^{FeR-11} does not fit easily into either Class I or