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

by

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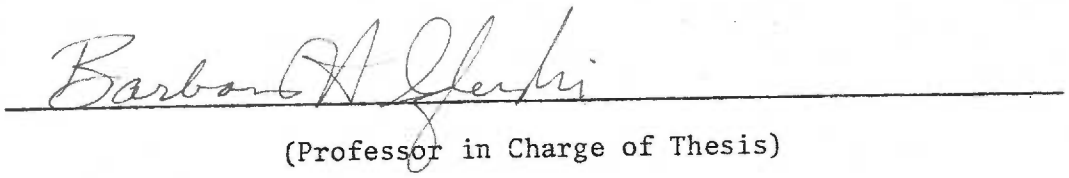

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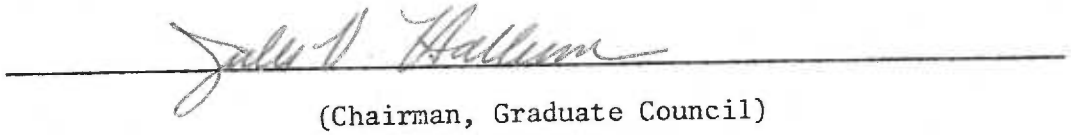

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I. Introduction and Statement of the Problem

*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 $2.1 \times 10^9$ 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.

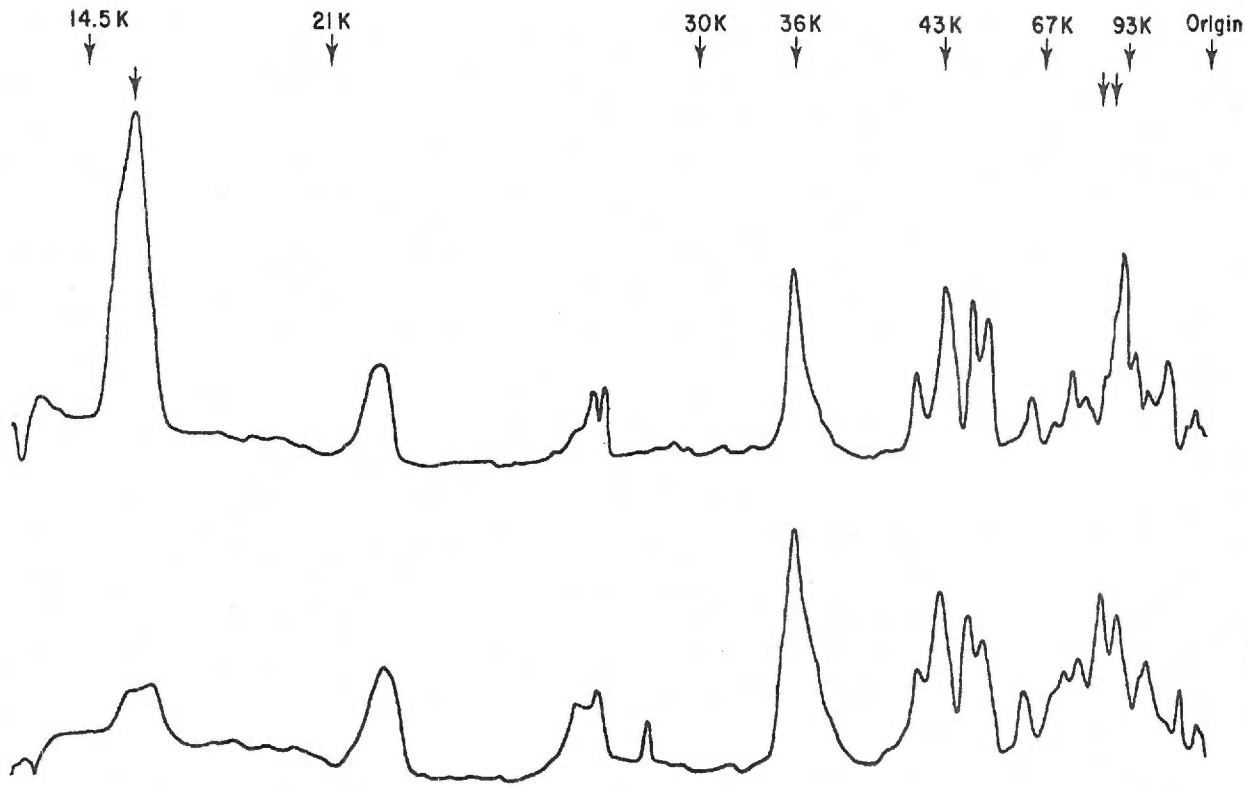
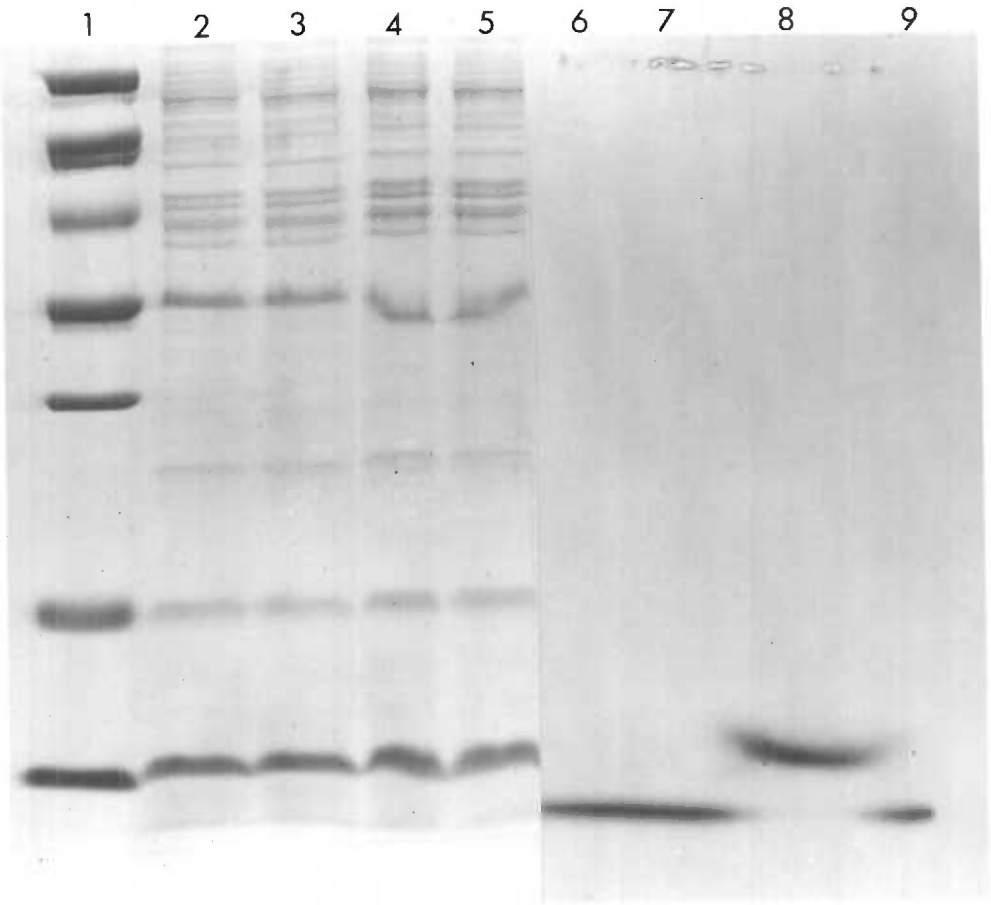

14.5K
21K
30K
36K
43K
67K
93K
Origin

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

Class II. This mutant produced only 1/3 the amount of pyoverdin as the parent strain (PAO-1) although production of pyochelin, uptake of $^{59}FeCl_3$, and utilization of ferripyochelin and ferripyoverdin appeared to be normal. This mutant was not further characterized.

Further characterization of Class II mutants. The Class II mutants were further characterized to determine if their altered sensitivity to the iron effect on either toxin yields (tox $^{FeR}$) or elastase yields (elas $^{FeR}$) extended to other extracellular products whose yields are known to be altered by the iron concentration of the medium. Yields of toxin A and alkaline protease in supernatants from cultures of PAO-elas$^{FeR-3}$ and elas $^{FeR-5}$ grown in medium with increasing iron concentrations were compared to yields of PAO-1. Toxin A yields in these elas$^{FeR}$ mutants were decreased as the iron concentration of the medium was increased in an identical manner to the parental strain PAO-1 (Figure 4a). Increased iron concentrations also decreased alkaline protease yields in these mutants to the same extent as in the parent (Figure 4b). Alkaline protease yields in medium containing 0.25 µg Fe per ml, were 14% or less of the yield obtained at 0.05 µg Fe per ml in both these mutants and the parental strain. Thus, although the PAO-elas$^{FeR}$ mutants continued to produce elastase in medium with high iron concentrations, yields of toxin and alkaline protease remain subject to regulation by iron in these mutants.

The PAO-tox$^{FeR}$ mutant, PAO-tox$^{FeR-18}$ was chosen for further study, since it most resembled the parent strain in the iron transport mechanisms examined. Alkaline protease and elastase yields in medium with increasing iron concentrations in culture supernatants from

PAO-1 and PAO-tox$^{FeR-18}$ were compared. PAO-tox$^{FeR-18}$ was equally sensitive to the iron effect on alkaline protease yields as PAO-1 (Figure 5a). In both strains alkaline protease yields were greatly reduced by iron concentrations of 0.25 µg or greater per ml. Elastase yields were also decreased to the same extent with PAO-tox$^{FeR-18}$ as with PAO-1 (Figure 5b). Although PAO-tox$^{FeR-18}$ produced less elastase than PAO-1 in the low iron medium, the elastase yields were reduced to the same levels in the medium with higher iron concentrations. These data indicate that while PAO-tox$^{FeR-18}$ was resistant to the effect of iron on toxin A yields, alkaline protease and elastase yields remained sensitive to iron regulation.

## Discussion

Previously, we reported that increasing the iron concentration of the growth medium decreased the yields of toxin A, elastase and total protease in several strains of *P. aeruginosa* (2). The iron effect on toxin A production was not strain dependent, and the magnitude of inhibition of toxin A yields was similar in all strains tested. The effect of the iron concentration of the growth medium on yields of total proteases and elastase was, however, shown to be strain dependent, suggesting the possibility that protease production is regulated by iron independently of its regulation of toxin A production (2). In the present study, we attempted to directly determine whether iron independently regulates yields of these extracellular products or coregulates these products by some common regulatory mechanism.

Mutants of strain *P. aeruginosa* PAO-1 were isolated that were resistant to the iron effect on yields of either toxin A or elastase.

These mutants varied in their level of sensitivity to the iron concentration of the medium. Two of the PAO-tox$^{FeR}$ mutants (PAO-tox$^{FeR-11}$ and tox$^{FeR-18}$) were hyperproducers of toxin A. The PAO-elas$^{FeR}$ mutants were less sensitive than the parent strain PAO-1 to the iron effect on elastase yields.

The altered sensitivity to iron concentration of toxin and/or elastase yields by these mutants could be explained in two ways. Mutants altered in genes involved in iron transport would be expected to take up less iron than the parent strain and therefore extracellular product yields would be less sensitive to a given iron concentration. Alternatively a mutation could have occurred in a regulatory gene(s) causing a specific extracellular product or products to be insensitive to regulation by iron. To distinguish between these possibilities, the iron uptake systems of the mutants were characterized. Mutants of both types were identified. PAO-tox$^{FeR-10}$ was found to be markedly defective in iron uptake, and is clearly a Class I mutant. PAO-tox$^{FeR-18}$, PAO-elas$^{FeR-3}$ and elas$^{FeR-5}$ appeared to have normal iron uptake mechansims and are Class II mutants.

Class II mutants which appeared to have mutations in iron regulatory genes but not iron transport genes were further characterized to determine if their resistance to the iron effect extended to extracellular products other than the one used in their initial identification. The use of specific radioimmunoassays for elastase and alkaline protease enabled us to quantitate these products individually and eliminated the need to measure total protease yields as done in our previous study (2). PAO-tox$^{FeR-18}$ although resistant to the iron effect on toxin A yields, remained sensitive to the iron effect on

both alkaline protease and elastase yields. In the elas$^{FeR}$ mutants, which continued to produce elastase at high iron concentrations, alkaline protease and toxin A yields were reduced by iron to the same extent as in the parent strain. These data indicate that iron independently regulates yields of these extracellular products in strain PAO.

The mechanism(s) by which toxin, elastase and alkaline protease yields are regulated by iron remains to be determined. A model for gene repression has been described for the trp operon (5). In this model a repressor is synthesized in an inactive state which cannot combine with the operator. In the presence of tryptophan, the repressor become activated and binds to the operator resulting in a repression of the genes involved in tryptophan biosynthesis. Based on the trp operon, Murphy et al. (18) have proposed that iron regulates diphtheria toxin yields by combining with and activating a toxin repressor. P. aeruginosa may synthesize a series of specific product repressors, which then complexed with iron, become active and bind to the specific product operator(s) thereby preventing synthesis of those extracellular products (Figure 6). Under conditions of iron starvation, the repressors remain inactive and these genes are derepressed. Since our data indicated that elastase and alkaline protease are regulated by iron independently from toxin A, it is reasonable to postulate individual repressors which are specific for each extracellular product regulated by iron. This model may be used to explain the mutations conferring resistance to the iron effect in the Class II tox$^{FeR}$ and elas$^{FeR}$ mutants described. The continued expression of

toxin or elastase may be due to the loss of repressor activity or an alteration in the operator. Both types of mutations would result in constitutive expression of the product involved. The locations of the structural genes for toxin, elastase and alkaline protease are presently unknown and no gene order is meant to be implicated in Figure 6.

The above model (Figure 6) proposes that iron regulation occurs at the level of transcription. Another possible explanation is that specific iron regulated events are involved in the translation of toxin, elastase and protease specific mRNAs. A third possibility is that iron regulates posttranslational processing of these extracellular proteins. Membrane proteins involved in transporting toxin, elastase or protease out of the cell may be specifically regulated by iron. These possibilities are consistent with the independent regulation of extracellular product yields by iron seen in the Class II mutants we have isolated.

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Table 1. Production of pyochelin and pyoverdin by PAO1-tox$^{FeR}$ and elas$^{FeR}$ mutants.

| Strain | Pyochelin (µg/ml) | Pyoverdin (µg/ml) |
|---|---|---|
| PAO-1 | 7.9 | 25.3 |
| PAO-tox$^{FeR}$-11 | 6.1 | 8.5 |
| PAO-tox$^{FeR}$-18 | 6.7 | 18.2 |
| PAO-elas$^{FeR}$-3 | 9.5 | 17.5 |
| PAO-elas$^{FeR}$-5 | 14.4 | 18.0 |

Cultures were grown in 0.5% Casamino acids, 0.2 mM $MgCl_2$ (pH 7.4) at 37$^{o}$C for 18 hours.

Figure 1. Effect of iron on toxin A yields in PAO-tox$^{FeR}$ mutants. ADP-ribosyl transferase activity was measured using partially purified EF-2 prepared from wheat germ extracts as a substrate. ● PAO-1, ▲ PAO-tox$^{FeR-10}$ O PAO-tox$^{FeR-11}$, Δ PAO-tox$^{FeR-18}$. Supernatants were obtained from cultures grown for 18 hours to the same optical density ($O.D._{540}$). This represents the average of two experiments.

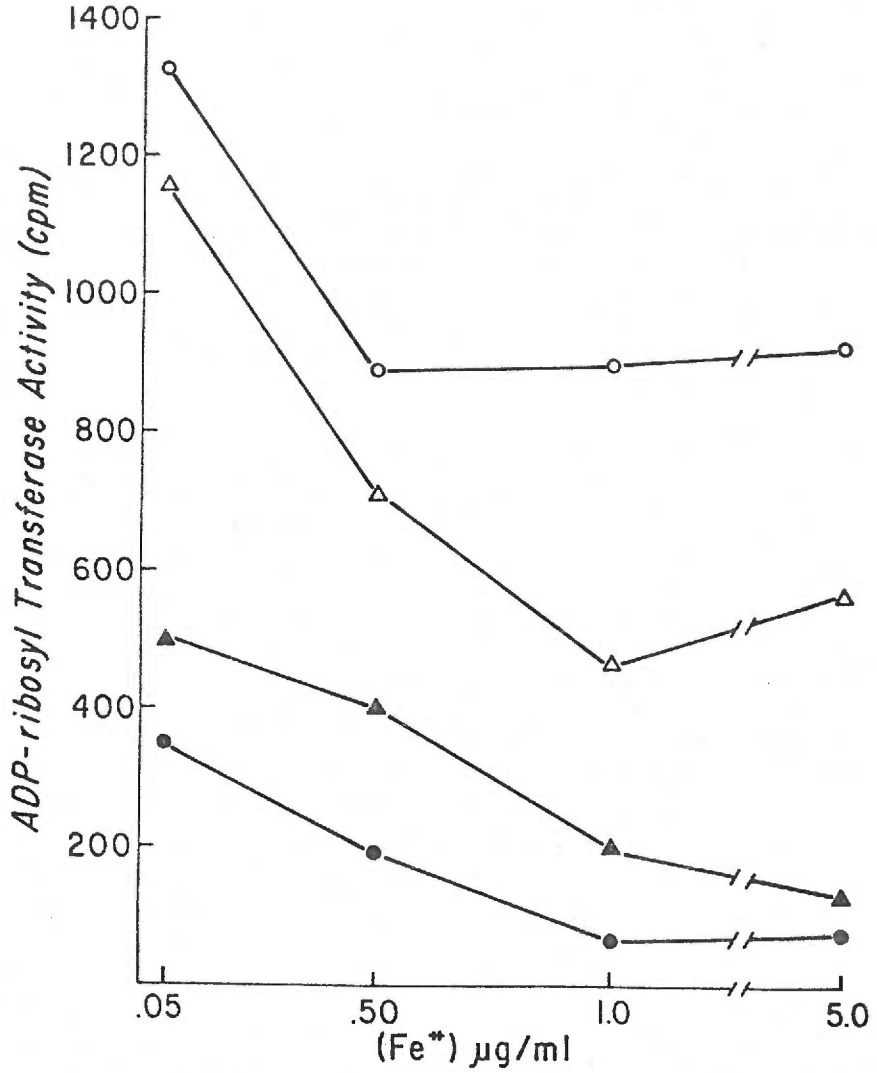

1400
1200
1000
800
600
400
200
ADP-ribosyl Transferase Activity (cpm)
.05
.50
1.0
5.0
(Fe**) µg/ml

Figure 2. Effect of iron on elastase yields in PAO-elas$^{FeR}$ mutants. Elastase was quantitated in supernatants obtained from 18 hr cultures grown to the same O.D$_{540}$. ● PAO-1, O PAO-elas$^{FeR-3}$, □ PAO-elas$^{FeR-5}$. This represents the average of two experiments.

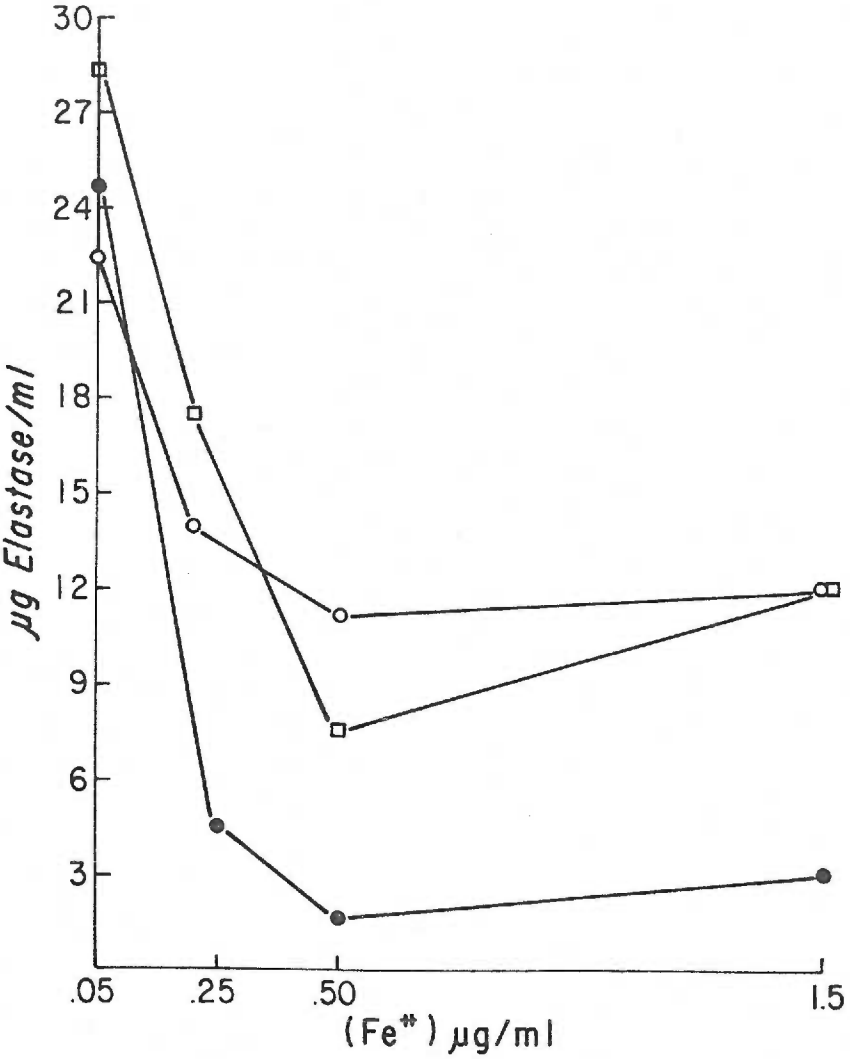

30
27
24
21
18
15
12
9
6
3
µg Elastase/ml
.05
.25
.50
1.5
(Fe**) µg/ml

Figure 3a. Uptake of $^{59}FeCl_3$ by PAO-tox$^{FeR}$ mutants. Cultures were grown in TSB-DC to a density of 5 x $10^8$ CFU/ml. Uptake assays were initiated by the addition of $^{59}FeCl_3$. One ml samples were removed at intervals and filtered. The filters were counted and the amount of $^{59}FeCl_3$ accumulated calculated from a standard curve of $^{59}FeCl_3$. ● PAO-1, ▲ PAO-tox$^{FeR-10}$, o PAO-tox$^{FeR-11}$, Δ PAO-tox$^{FeR-18}$. This represents the average of two experiments.

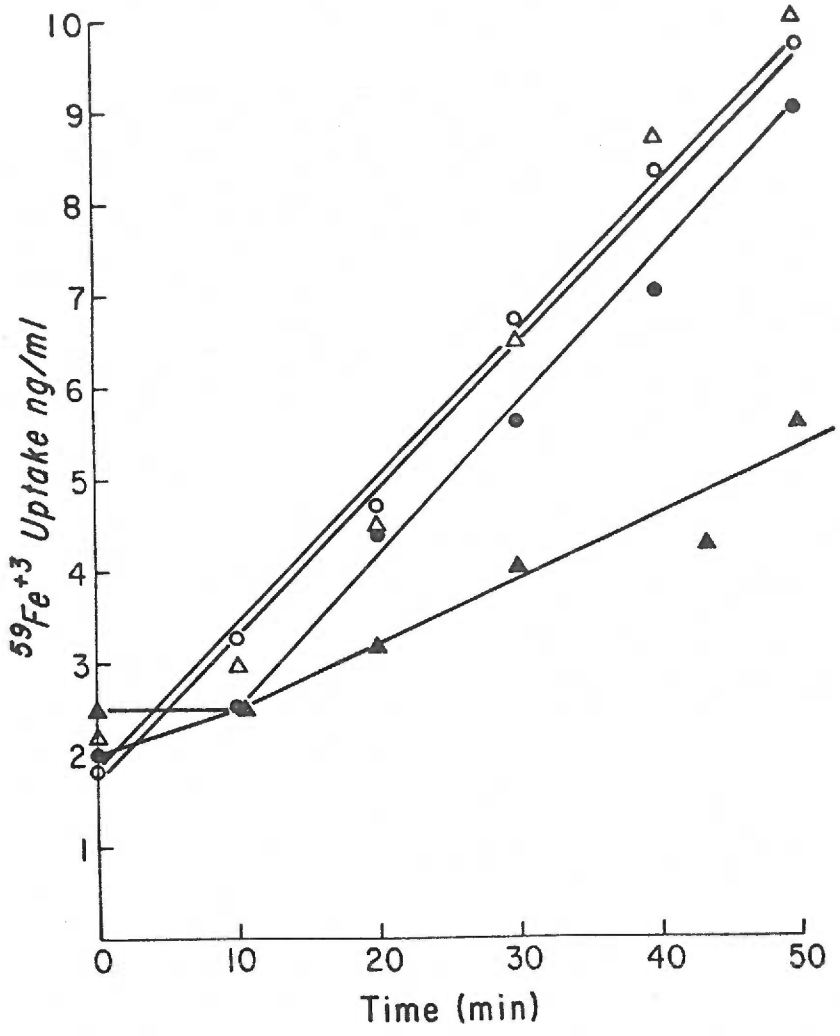

10
9
8
7
6
5
4
3
2
1
0
10
20
30
40
50
Time (min)
59Fe+3 Uptake ng/ml

Figure 3b. Uptake of $^{59}FeCl_3$ by PAO-elas$^{FeR}$ mutants. Iron uptake assay conditions were identical to those described in Figure 3a. ● PAO-1 o PAO-elas$^{FeR-3}$, ▫PAO-elas$^{FeR-5}$. This represents the average of two experiments.

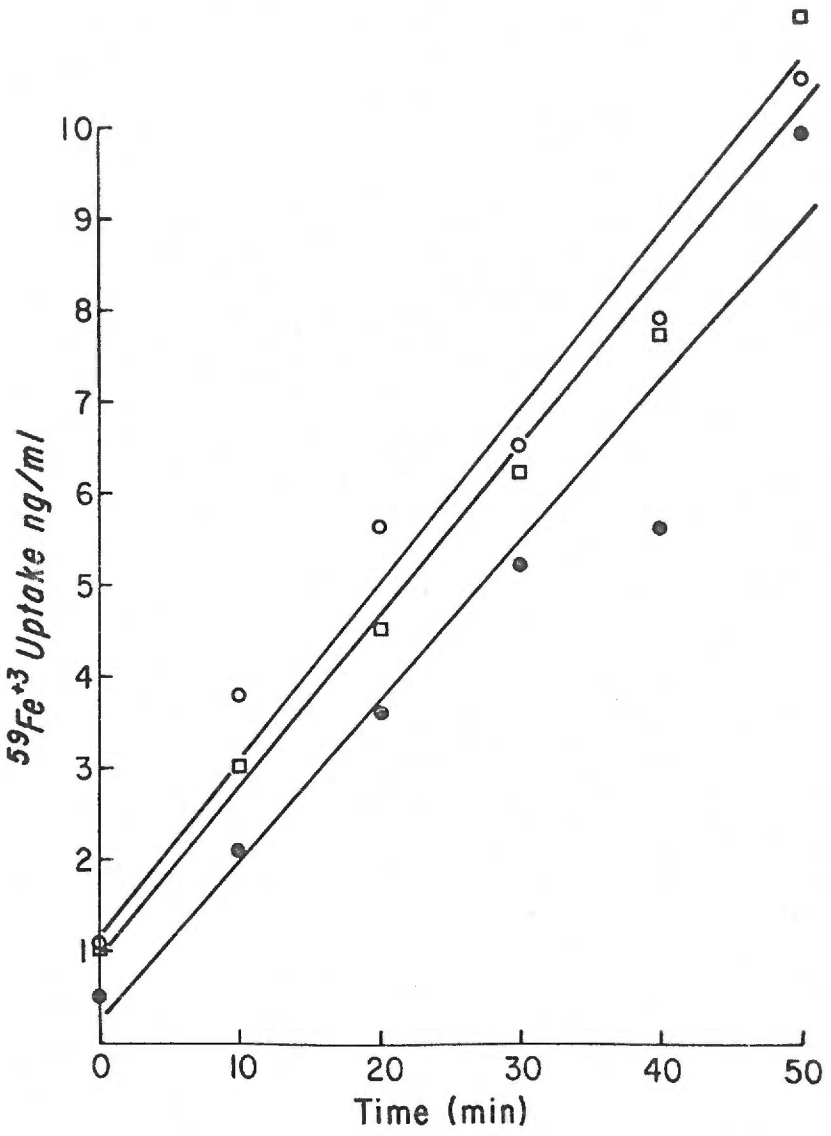

$^{59}Fe^{+3}$ Uptake ng/ml
10
9
8
7
6
5
4
3
2
1
0
10
20
30
40
50
Time (min)

Figure 4a. Effect of iron on toxin A yields of PAO-elas$^{FeR}$ mutants. Culture supernatants from 18 hr cultures grown to the same O.D.$_{540}$ were assayed for ADPR-transferase activity using partially purified EF-2 prepared from wheat germ extracts as a substrate. ● PAO-1, o PAO-elas$^{FeR-3}$, ▫ PAO-elas$^{FeR-5}$. This represents the average of two experiments.

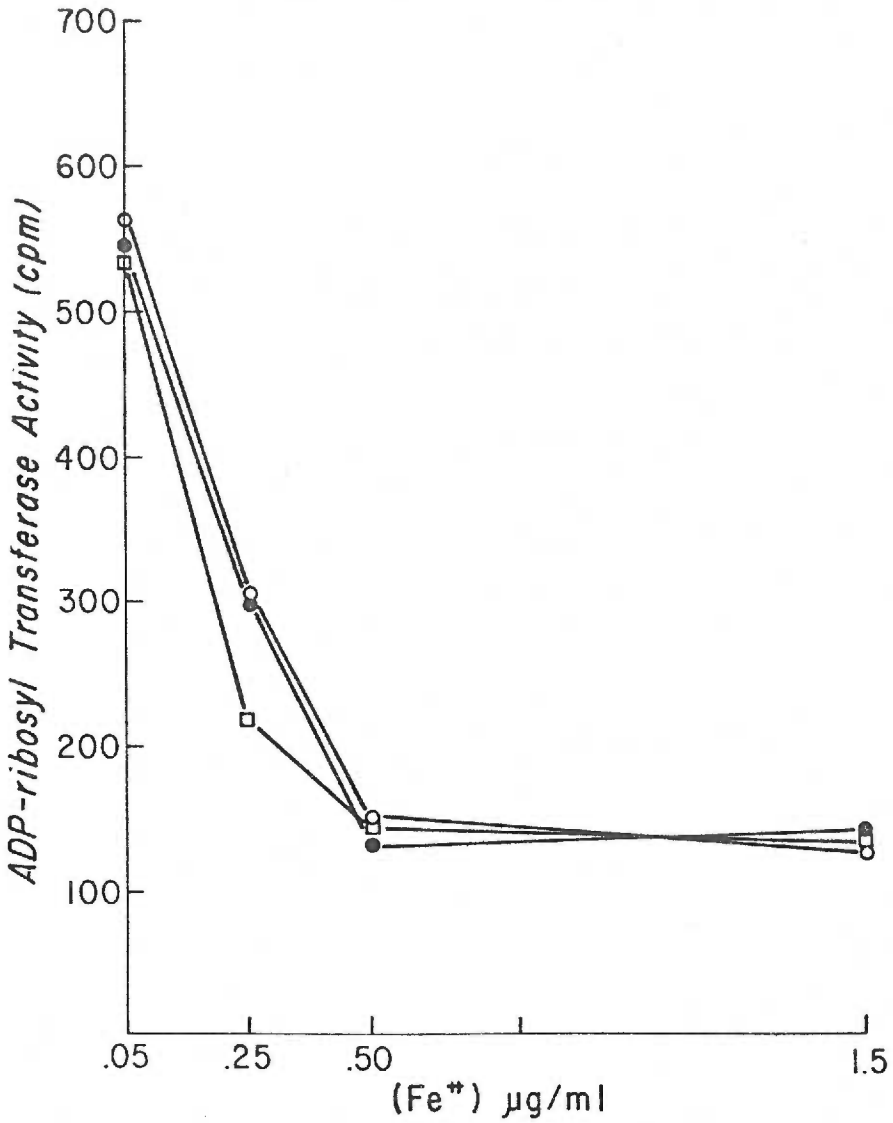

700
600
500
400
300
200
100
ADP-ribosyl Transferase Activity (cpm)
.05
.25
.50
1.5
(Fe++) µg/ml

Figure 4b. Effect on iron on alkaline protease yields of PAO-elas$^{FeR}$ mutants. Alkaline protease was quantitated in culture supernatants from 18 hr cultures grown to the same $O.D._{540}$ by radioimmunoassay. ● PAO-1, o PAO-elas$^{FeR-3}$, o PAO-elas$^{FeR-5}$. This represents the average of two experiments.

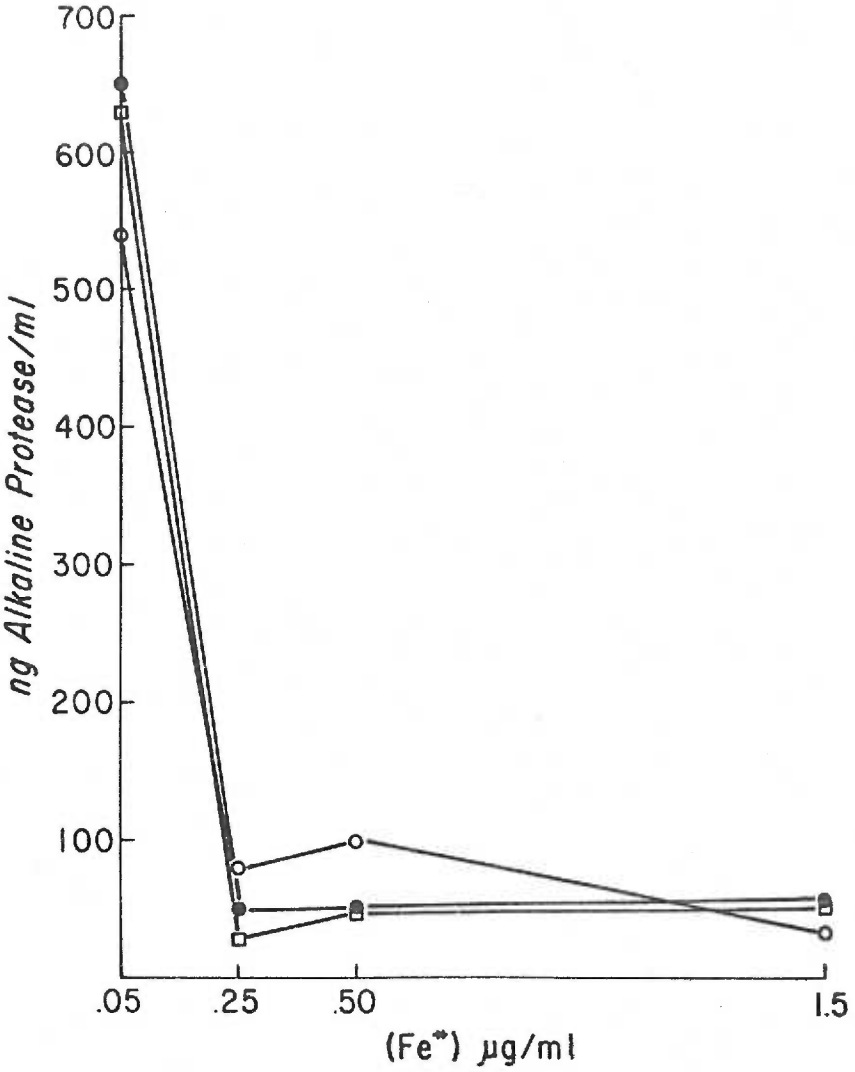

700
600
500
400
300
200
100
ng Alkaline Protease/ml
.05
.25
.50
1.5
(Fe**) µg/ml

Figure 5a. Effect of iron on alkaline protease yields of PAO-tox$^{FeR}$ mutants. Alkaline protease yields were quantitated in culture supernatants from 18 hr cultures grown to the same $O.D._{540}$ by radioimmunoassay. ● PAO-1, Δ PAO-tox$^{FeR-18}$. This represents the average of two experiments.

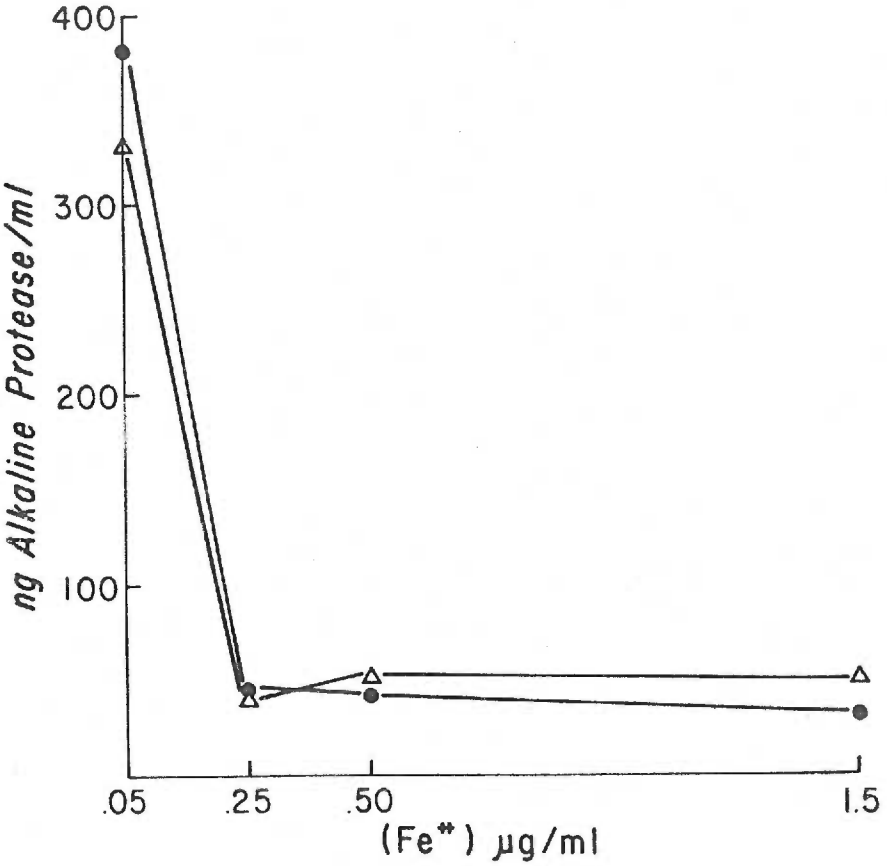

400
300
200
100
ng Alkaline Protease/ml
.05
.25
.50
1.5
(Fe**) µg/ml

Figure 5b. Effect of iron on elastase yields of PAO-tox$^{FeR}$ mutants. Alkaline protease was quantitated in culture supernatants from 18 hr cultures grown to the same O.D.$_{540}$ by radioimmunoassay. ● PAO-1, Δ PAO-tox$^{FeR-18}$. This represents the average of two experiments.

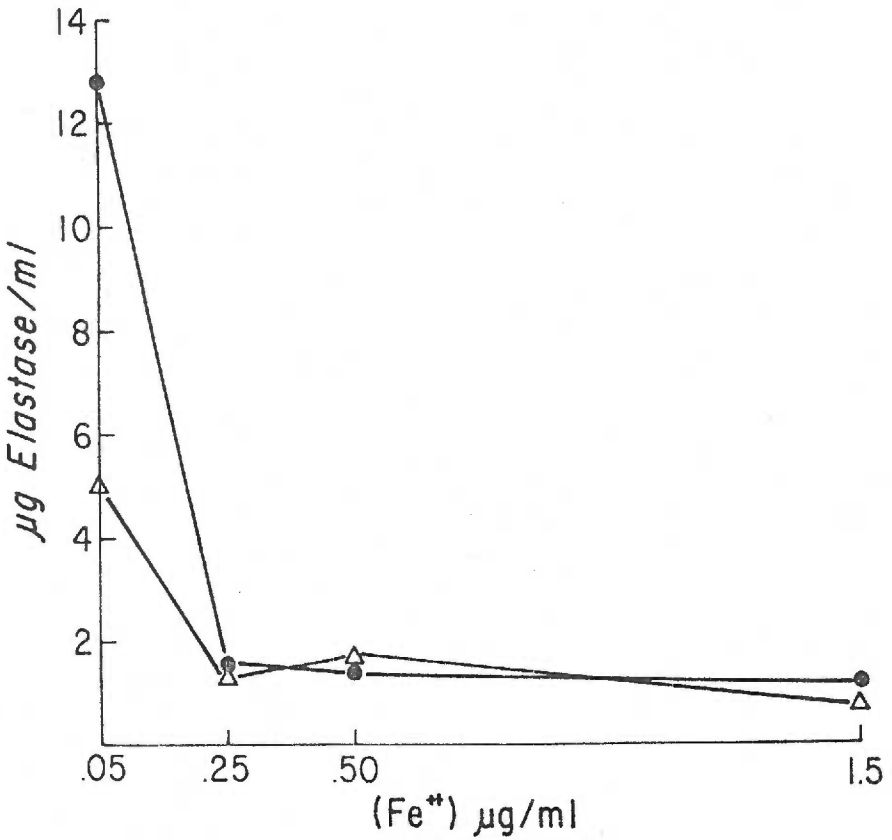
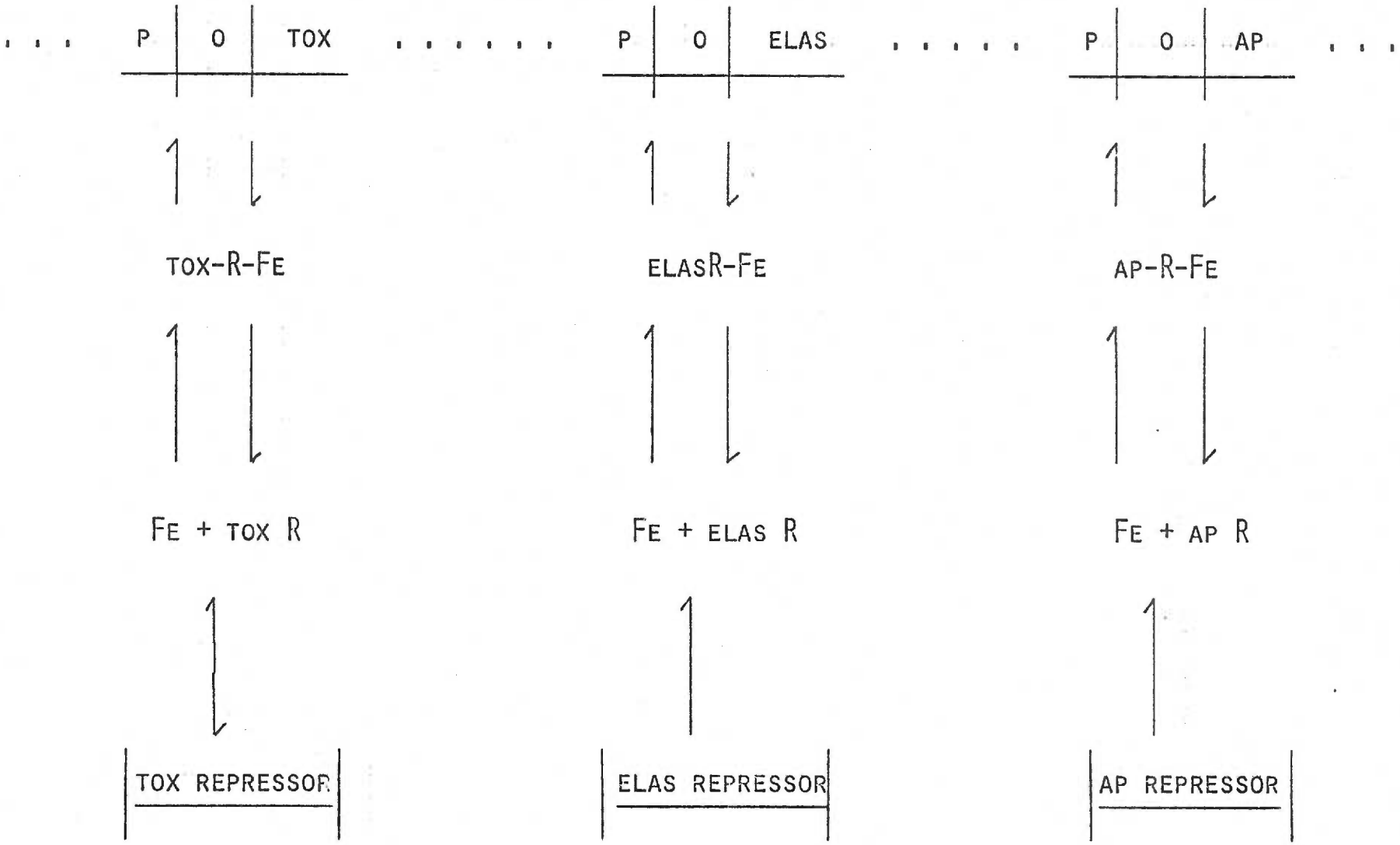

14
12
10
8
6
4
2
µg Elastase/ml
.05
.25
.50
1.5
(Fe++) µg/ml

Figure 6. Hypothetical model of extracellular gene product regulation by iron in P. aeruginosa. Specific product repressors are synthesized which are activated in the presence of iron. These repressors bind to the operator(s) preventing experssion of the specific extracellular product. Under conditions of iron starvation, the equilibrium shifts and the operon becomes derepressed.

FIGURE 6


P
O
TOX
P
O
ELAS
P
O
AP
TOX-R-FE
ELASR-FE
AP-R-FE
FE + TOX R
FE + ELAS R
FE + AP R
TOX REPRESSOR
ELAS REPRESSOR
AP REPRESSOR

Paper 3.

Characterization of an Iron Transport Mutant of *Pseudomonas aeruginosa*.

Abstract

A mutant of Pseudomonas aeruginosa PAO-1 was shown to be defective in all known P. aeruginosa systems of iron transport. This mutant, PAO-tox$^{FeR-10}$, produced parental levels of pyochelin and pyoverdin, but was unable to take up iron using the substrates ferripyochelin, ferripyoverdin, or ferric citrate. Outer membranes from PAO-tox$^{FeR-10}$ were defective in their ability to bind $^{59}$Fe-pyochelin. The binding of $^{59}$Fe-citrate and $^{59}FeCl_3$ by outer membranes prepared from the mutant PAO-tox$^{FeR-10}$ was similar to that of outer membranes from the parental strain PAO-1. The data presented suggest the possibility that there is a common iron uptake pathway utilized by these siderophores which has been altered in the mutant PAO-tox$^{FeR-10}$.

Introduction

Iron is an essential nutrient for microbial growth. Since iron is extremely insoluble at neutral pH, microorganisms must synthesize iron chelators, termed siderophores, which function in the solubilization and transport of iron into the cell (12,17). Three iron transport systems have been reported in Pseudomonas aeruginosa. Pyochelin is a phenolic siderophore (2,3) which promotes bacterial growth when added to iron-deficient medium (2). Pyoverdin, a hydroxamate, compound has also been shown to have siderophore activity (10). A mechanism for iron uptake from ferric citrate has also been described (3). These three compounds have been shown to act as substrates for iron transport (3,10). We have also identified an iron-siderophore binding protein in the outer membrane of P. aeruginosa (15).

Previously, we reported the isolation of a mutant from P. aeruginosa PAO-1 which was defective in the uptake of $^{59}FeCl_3$ (14). In the present study, we characterized this mutant (PAO-tox$^{FeR-10}$) in terms of production of siderophores, and the ability to utilize these compounds for iron transport. We also examined the ability of outer membranes isolated from PAO-tox$^{FeR-10}$ to bind ferripyochelin. The ferric citrate uptake system in P. aeruginosa was also further characterized by examining the ability of outer membranes isolated from PAO-1 and PAO-tox$^{FeR-10}$ to bind $^{59}Fe$ from ferric citrate.

## Materials and Methods

Bacterial strains. P. aeruginosa strain PAO-1 originally characterized by B.W. Holloway (8), and PAO-tox$^{FeR-10}$ (14), a nitrosoguanidine-induced mutant of PAO-1, were the strains used in this study.

Siderophore purification and assay. Pyochelin was purified for iron uptake assays by preparative thin-layer chromatography (3). Bacterial production of pyochelin in culture medium was assayed as previously described (3). Pyoverdin was purified from methanol solutions of spent medium which had been taken to dryness by rotary evaporation. The precipitate which formed after ethyl acetate addition to the methanol solution (20:1 ratio) was dissolved in water and subjected to paper electrophoresis at pH 8.5 in 0.005 M Tris-HCl buffer. The paper band containing fluorescence was eluted in methanol and the pyoverdin was precipitated by addition of ether (100:1 ratio). A filtrate of the water solution of this precipitate was applied to a 10 x 250 mm reversed phase (ODS2 Ultrasphere) high pressure column (Altex). A Beckman 322 MP solvent delivery

system was used to construct a 10 to 100% water gradient in acetonitrile for the elution of pyoverdin. The fractions containing pyoverdin were taken to dryness by vacuum, weighed, and stored at -20°C. Pyoverdin was measured fluorometrically in solutions of Tris-HCl buffer (0.01 M, pH 7.4). The samples were excited at 400 nm and fluorescence measured at 460 nm (3).

Assay of iron uptake. Bacteria were harvested by centrifugation at 5,900 x g for 10 min at 24°C from 0.5% casamino acids culture media (low-iron medium) containing 0.2 mM $MgCl_2$ (CAA) which had been incubated with shaking overnight at 37°C. The bacteria were washed three times with 40 ml volumes of 10 mM morpholinopropane sulfonate buffer (pH 7.4) containing 1 mM $MgCl_2$ (MOPS-Mg buffer), and finally suspended in the same buffer to yield 50 µg dry weight of bacteria per ml. These suspensions were made 0.04% with casamino acids and incubated for 10 min prior to assay. Uptake assays were initiated by addition of $^{55}$Fe-pyochelin, $^{55}$Fe-pyoverdin, or $^{55}$Fe-citrate to a final concentration of 50 nm. Samples of the reaction mixtures were taken at intervals and passed through filters with 0.45 µm pore size (Amicon). The filters were washed with water, dried, and the radioactivity on the filters was determined in a Prias 240 CL/D (Packard) counter using Scinti-Verse (Fisher) scintillation fluid. Values were corrected for quench and nonbacterial trapping of $^{55}$Fe on the filters.

Iron binding to outer membranes. Cultures were grown in sterile, acid-washed flasks (dilute $HNO_3$) containing 500 ml M-9 minimal salts medium (low-iron medium). In all cultures glucose (0.5%) was added as a carbon source. Where indicated $FeCl_3$ was added at a concentration of

100 μM (high-iron medium). Cultures were incubated for 48 hours in a shaking water bath at 37°C. Outer membranes were isolated as previously described (15) by a modification of the technique of Hancock and Nikaido (6). Iron binding to outer membranes was measured as previously described (15). Briefly, outer membranes were suspended in M-9 medium containing 100 μM nitriloacetic acid (NTA; Sigma Chemical Co., St. Louis, MO). Equal volumes of NTA (100 μM), pyochelin (20 μM) or citrate (50 μM) and $^{59}FeCl_3$ (10 μM, 1mCi/100 μg $Fe^{+3}$, Amersham, Arlington Heights, IL) were then added to 0.1 ml volumes of outer membrane suspensions containing 100 μg protein. To some samples $^{59}FeCl_3$ (10 μM) was added without chelators. Approximately 25,000 cpm of $^{59}Fe$ were added to each reaction mixture. 100 μg of bovine serum albumin (BSA) was used as a control. The reaction mixtures were incubated for 5 minutes at 25°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-Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed as described by Laemmli (9). The gels were cast in 1.5 mm thick slabs in a 10 cm electrophoresis apparatus from 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% bromophenol blue by heating at 100°C for 4 minutes. Electrophoresis was carried out at 25 mA per slab constant current for 4 hours. Following electrophoresis, the gels were fixed and stained for protein with Coo-

massie brilliant blue R250 in 50% methanol/10% acetic acid (vol/vol). 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. Gels were destained overnight at room temperature in 10% methanol/10% acetic acid (vol/vol).

## Results

Production of siderophores. PAO-tox$^{FeR-10}$ was previously shown to be defective in the uptake of $^{59}FeCl_3$ (14). One possible explanation for this deficiency is this mutant does not produce the siderophores pyochelin or pyoverdin and thus would not be able to take up iron other than by a low affinity system. Therefore, the quantities of pyochelin and pyoverdin produced by this mutant were compared to the amount of these siderophores produced by the parent strain PAO-1 (Table 1). PAO-tox$^{FeR-10}$ produced approximately the same amount of pyochelin and pyoverdin as the parent strain PAO-1.

Siderophore utilization. To determine whether or not PAO-tox$^{FeR-10}$ can effectively use the specific iron uptake systems described for P. aeruginosa, the ability of PAO-tox$^{FeR-10}$ to take up iron from pyochelin, pyoverdin, and citrate was examined. When iron was supplied to bacteria as ferripyochelin, $^{55}Fe$ was accumulated by the parental strain PAO-1 very rapidly (Figure 1). The accumulation of $^{55}Fe$ by PAO-tox$^{FeR-10}$ from ferripyochelin, however, was almost negligible (Figure 1). When iron was supplied as ferripyoverdin, PAO-tox$^{FeR-10}$ was also unable to take up iron from this substrate (Figure 2). PAO-1, however, accumulated $^{55}Fe$

from pyoverdin very efficiently. Similar results were obtained when $^{55}$Fe-ferric citrate was used as a substrate (Figure 3). PAO-tox$^{FeR-10}$ was unable to utilize ferric citrate as a source of iron, whereas the parental strain PAO-1 was able to take up $^{55}$Fe from this substrate.

Ability of outer membrane proteins to bind iron. We have previously described an outer membrane protein induced in low-iron medium which binds iron from ferripyochelin (15). Iron starvation also leads to the induction of some high molecular weight proteins in the outer membrane of P. aeruginosa which may function in iron transport (11,15). Since PAO-tox$^{FeR-10}$ produces parental levels of siderophores (Table 1), but is unable to utilize them for iron-uptake (Figures 1,2,3), the outer membranes of PAO-tox$^{FeR-10}$ were isolated and compared to membranes from the parent strain PAO-1 to determine if the mutation(s) in PAO-tox$^{FeR-10}$ caused an alteration in outer membrane proteins involved in iron transport. The outer membranes from cultures of PAO-1 and PAO-tox$^{FeR-10}$ grown in low-iron and high-iron medium were isolated and electrophoresed on 12.5% SDS-polyacrylamide slab gels (Figure 4). There was no significant difference in the electrophoretic profile of outer membrane proteins isolated from cells of PAO-tox$^{FeR-10}$ and the parent PAO-1 at either iron concentration. There were no proteins present in the outer membrane of PAO-1 which were not present in PAO-tox$^{FeR-10}$. The same proteins were induced in low-iron medium in both the mutant and the parent strains.

Although there was no apparent difference in the protein composition of the PAO-tox$^{FeR-10}$ outer membrane, it was possible that the ability of its membrane proteins to bind iron-siderophore complexes had been

altered. The ability of outer membranes prepared from low and high-iron cultures of PAO-1 and the mutant PAO-tox$^{FeR-10}$ to bind iron from $^{59}$Fe-pyochelin was compared (Table 2). PAO-1 outer membranes from cells grown in low-iron medium bound 22% of the total $^{59}$Fe added to the reaction mixture, whereas membranes from cells grown in high-iron medium bound only 10% of the total $^{59}$Fe added. These results agree with our previous data (15). The outer membranes from PAO-tox$^{FeR-10}$ cultures grown in low-iron medium, however, bound only 11.3% of the total $^{59}$Fe added to the reaction mixture. The outer membranes from PAO-tox$^{FeR-10}$ prepared from cultures grown in low-iron medium bound approximately the same amount of iron as outer membranes from PAO-1 grown in high-iron medium. The PAO-tox$^{FeR-10}$ membranes prepared from cultures grown in high-iron did not bind any more $^{59}$Fe than BSA, which is used as a non-specific iron binding protein control. The outer membranes from cultures of PAO-tox$^{FeR-10}$, therefore, were defective in the binding of iron from ferripyochelin as they bound only 50% of the amount of $^{59}$Fe bound by PAO-1 outer membranes from low-iron cultures.

The ability of outer membrane preparations from PAO-1 and PAO-tox$^{FeR-10}$ cultures to bind iron from $^{59}$Fe-citrate and $^{59}FeCl_3$ was also compared (Table 2). The outer membranes from the low-iron cultures bound more $^{59}$Fe from these substrates than the membranes prepared from the cultures grown in high-iron in both PAO-tox$^{FeR-10}$ and the parent strain PAO-1. There was no significant difference in the amount of $^{59}$Fe bound from either $^{59}FeCl_3$ or $^{59}$Fe-citrate by the outer membranes prepared from PAO-1 or PAO-tox$^{FeR-10}$. This indicates that outer membranes from PAO-tox$^{FeR-10}$, although defective in the binding of Fe-pyochelin, can

bind Fe-citrate and $FeCl_3$ as efficiently as the parent strain PAO-1.

## Discussion

We have previously reported the isolation of a mutant from P. aeruginosa PAO-1 which was defective in $^{59}FeCl_3$ uptake. In the present study we further characterized this mutant (PAO-tox$^{FeR-10}$) to determine the extent of its iron transport deficiency. Although PAO-tox$^{FeR-10}$ produced parental yields of the siderophores pyochelin and pyoverdin (Table 1), this mutant was defective in both types of siderophore-mediated iron uptake (Figures 1 and 2). These results confirm previous observations (3) that separate regulating mechanisms control siderophore synthesis and the formation of uptake mechanisms for siderophores in P. aeruginosa. The mutant PAO-tox$^{FeR-10}$ was also unable to take up iron utilizing ferric citrate as a substrate, whereas the parent strain PAO-1 was able to utilize iron in this form (Figure 3). This suggests the possibility that there is a common iron uptake pathway utilized by these three substrates which as been altered in the mutant PAO-tox$^{FeR-10}$.

Outer membranes isolated from PAO-tox$^{FeR-10}$ grown in low-iron medium were capable of binding $^{59}Fe$ from $^{59}Fe$-pyochelin, but at a much lower level than outer membranes prepared from PAO-1, and when PAO-tox$^{FeR-10}$ was grown in high-iron medium the outer membranes did not bind $^{59}Fe$-pyochelin above background levels. Although PAO-tox$^{FeR-10}$ had the 18,000 dalton iron-pyochelin binding protein present in its outer membrane, this protein may be altered in its ability to bind ferripyochelin, or may require the activity of some other outer membrane protein which has been altered.

In Escherichia coli it has been shown that a functional ton B gene is required for enterochelin-, citrate-, ferrichrome-, and rhodotorulic acid-mediated iron uptake (5,7,13). It has been proposed that the ton B gene or some product regulated by this gene is an outer membrane component that functions in iron transport (16). The inability of ton B mutants to transport iron is not due to a loss of proteins in the outer membrane (1,4). Outer membranes prepared from ton B mutants retain the ability to bind ferric-enterochelin (13) and these mutants are able to synthesize enterochelin (5,13). It is possible that a gene analogous to the E. coli ton B gene exists in P. aeruginosa and that PAO-tox$^{FeR-10}$ is analogous to an E. coli ton B mutant. PAO-tox$^{FeR-10}$ produces siderophores and its outer membranes can bind ferripyochelin, although at lower levels than the parent PAO-1. This mutant is severely deficient in the transport of iron from either pyochelin, pyoverdin, or citrate complexes. PAO-tox$^{FeR-10}$, therefore, appears to be similar to an E. coli ton B mutant and may be altered in one gene which is required for the three known P. aeruginosa iron transport systems.

The possibility that PAO-tox$^{FeR-10}$ contains two mutations must also be considered. Since PAO-tox$^{FeR-10}$ outer membranes bind $^{59}$Fe-pyochelin less efficiently than the parent PAO-1, there may be one mutation affecting this ferripyochelin binding protein and a second mutation affecting general transport. This mutant (PAO-tox$^{FeR-10}$) was nitrosoguanidine (NTG) induced and NTG has been shown to cause multiple mutations (8). Further studies are needed to determine whether a single or multiple mutation(s) have occurred in PAO-tox$^{FeR-10}$.

The ability of P. aeruginosa outer membranes to bind $^{59}$Fe-citrate

and $^{59}FeCl_3$ was also examined. Although there was a difference in the amount of $^{59}Fe$ bound from these substrates by outer membranes prepared from low-iron versus high-iron cultures, there was almost no difference in the binding whether the substrate was $^{59}Fe$-citrate or $^{59}FeCl_3$. Significantly less $^{59}Fe$ was bound from either $^{59}Fe$-citrate or $^{59}FeCl_3$ than from $^{59}Fe$-pyochelin. Outer membranes prepared from the mutant PAO-tox$^{FeR-10}$ bound approximately the same amount of $^{59}Fe$-citrate and $^{59}FeCl_3$ as the membranes from the parent strain PAO-1 at either iron concentration. Previous studies indicated that $^{59}FeCl_3$ does not bind specifically to any outer membrane proteins in P. aeruginosa (15). It is unknown whether Fe-citrate has a specific outer membrane receptor. Free citrate has been shown to compete with cell-associated $^{55}Fe$-citrate, and little variation exists in the amount of $^{55}Fe$-citrate associated with cells grown in different media (3). Since outer membranes from P. aeruginosa bind both $^{59}Fe$-citrate and $^{59}FeCl_3$ with a much lower affinity than $^{59}Fe$-pyochelin, it is possible that Fe-citrate and $FeCl_3$ are low affinity non-specific iron uptake systems.

Outer membranes isolated from PAO-tox$^{FeR-10}$ bind $^{59}Fe$-citrate and $^{59}FeCl_3$ with the same affinity as PAO-1 outer membranes, suggesting that PAO-tox$^{FeR-10}$ may have a functioning low-affinity iron uptake system which enables it to grow *in vitro* although it is defective in the high affinity iron-uptake systems. PAO-tox$^{FeR-10}$ does not grow *in vivo* (18) indicating high-affinity iron-uptake systems are required for *in vivo* growth.

Further studies are needed to localize and characterize the altered gene in PAO-tox$^{FeR-10}$ and to determine the extend of the similarity be-

tween iron-uptake mechanisms of P. aeruginosa and E. coli.

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Table 1. Production of siderophores by PAO-1 and PAO-tox$^{FeR-10}$.[a]

| Strain | Pyochelin (µg/ml) | Pyoverdin (µg/ml) |
|---|---|---|
| PAO-1 | 7.9 | 25.3 |
| PAO-tox$^{FeR-10}$ | 10.8 | 22.4 |

[a]Cultures were grown in 0.5% casamino acids, 0.2 mM $MgCl_2$ (pH 7.4) at 37°C for 18 hours.

Table 2. Comparison of $^{59}Fe$ Binding to Outer Membrane Preparations of Pseudomonas aeruginosa PAO-1 and PAO-tox$^{FeR-10}$.

| Strain | $FeCl_3$ added to culture medium | $^{59}Fe$ Bound (cpm/100 µg protein)[1,3] Substrate[2] | | |
|---|---|---|---|---|
| | | $^{59}Fe$-pyochelin | $^{59}Fe$-citrate | $^{59}FeCl_3$ |
| PAO-1 | 100 µM | 2598 | 1000 | 424 |
| PAO-1 | 0 | 5807 | 1681 | 1512 |
| PAO-tox$^{FeR-10}$ | 100 µM | 0 | 1259 | 724 |
| PAO-tox$^{FeR-10}$ | 0 | 2964 | 2018 | 1176 |

[1]Minus background (BSA, 1124 cpm bound).

[2]Form of $^{59}Fe$ (10 µM) added to reaction mixtures.

[3]Mean values of two experiments.

Figure 1. $^{55}Fe$ uptake by cells from ferripyochelin. Reaction mixtures contained 50 nM $^{55}Fe$-ferripyochelin. ● PAO-1, ○ PAO-tox$^{FeR-10}$.

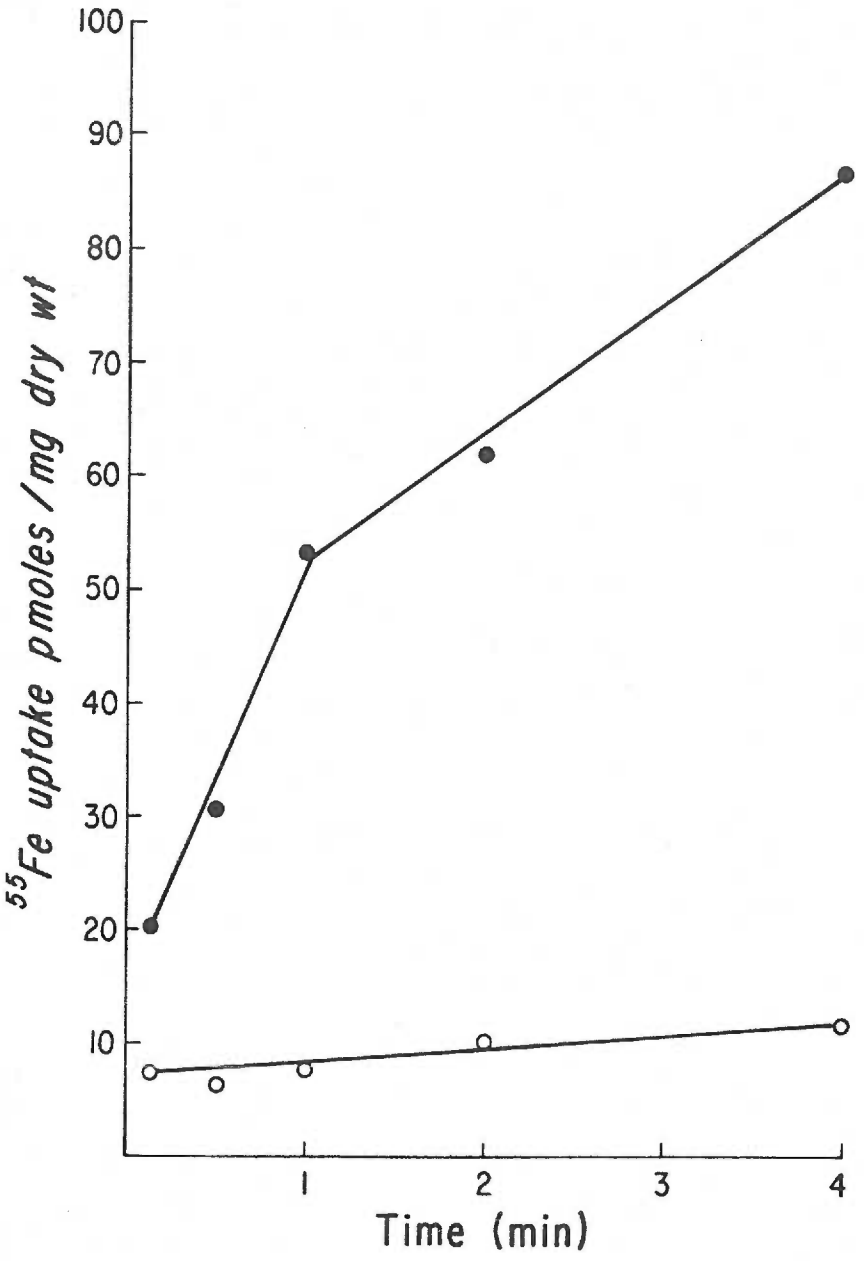

100
90
80
70
60
50
40
30
20
10
$^{55}Fe$ uptake pmoles /mg dry wt
1
2
3
4
Time (min)

Figure 2. $^{55}Fe$ uptake by cells from ferripyoverdin. Reaction mixtures contained 50 nM $^{55}Fe$-ferripyoverdin. ● PAO-1, ○ PAO-tox$^{FeR-10}$.

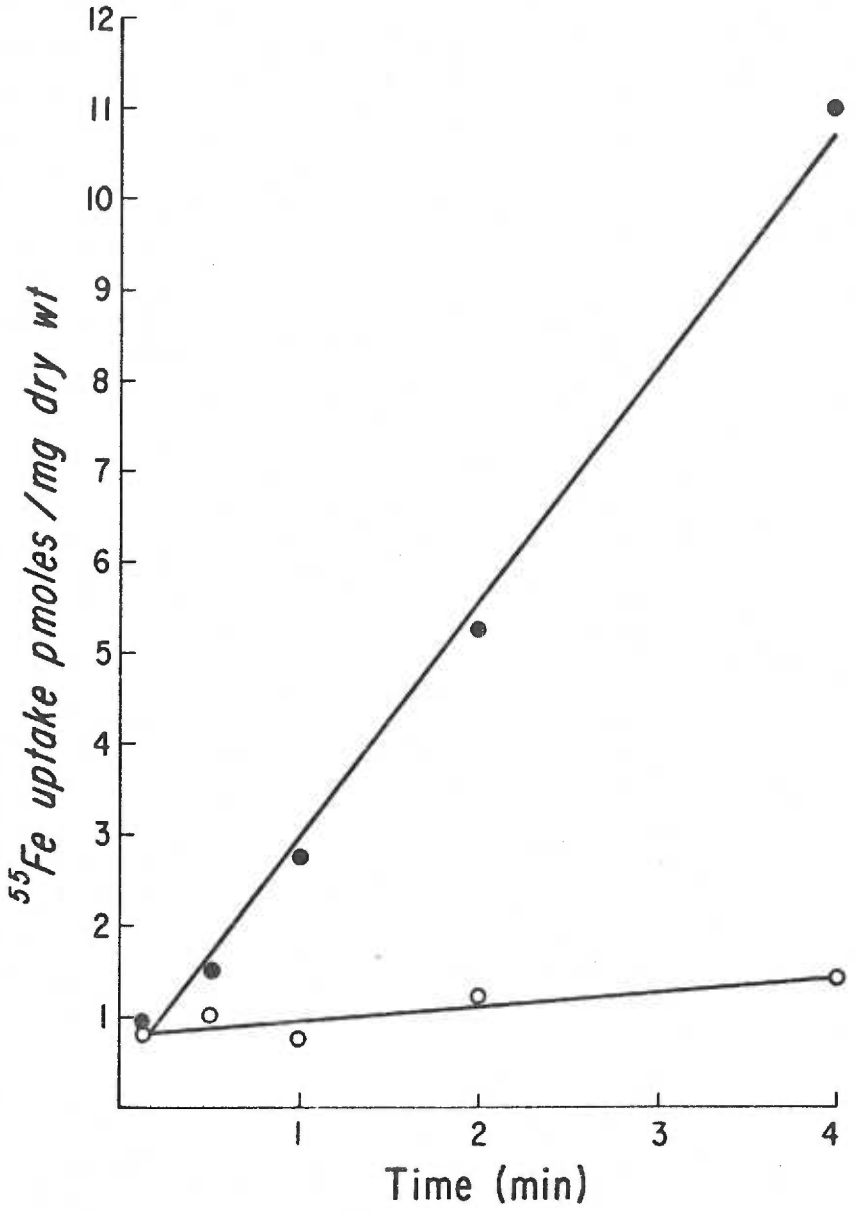

12
11
10
9
8
7
6
5
4
3
2
1
$^{55}Fe$ uptake pmoles /mg dry wt
1
2
3
4
Time (min)

Figure 3. $^{55}Fe$ uptake by cells from ferric citrate. Reaction mixtures contained 50 nM $^{55}Fe$-ferric citrate. ● PAO-1, ○ PAO-tox$^{FeR-10}$.

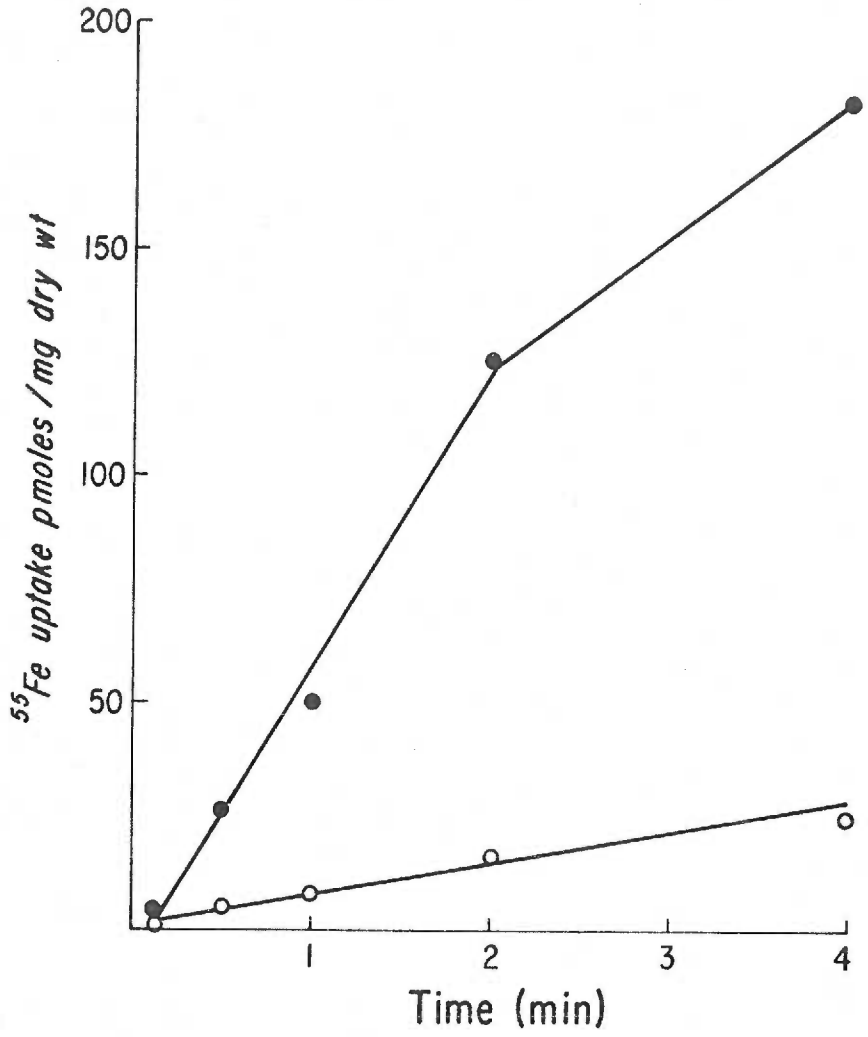
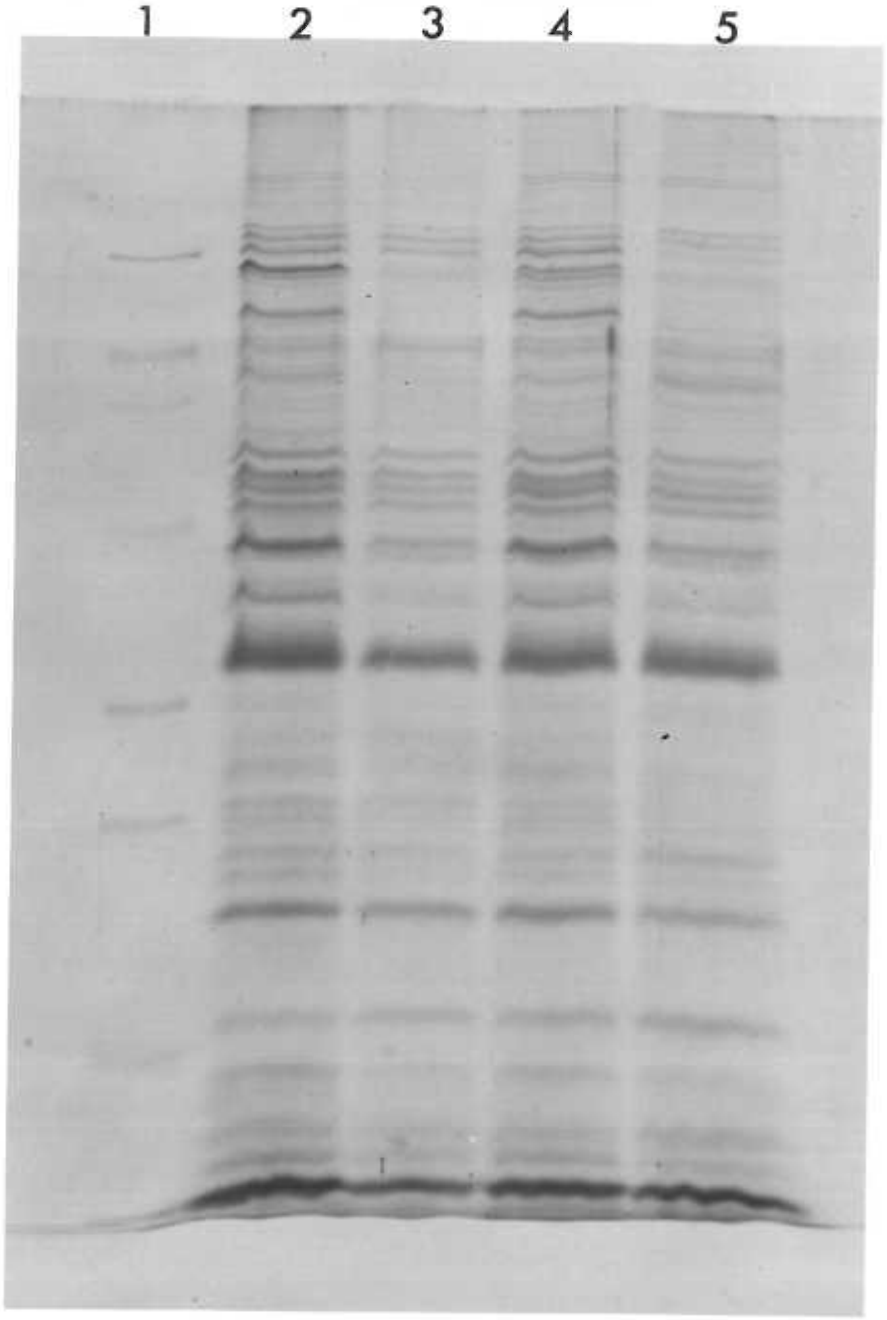

200
150
100
50
$^{55}Fe$ uptake pmoles / mg dry wt
1
2
3
4
Time (min)

Figure 4. SDS-polyacrylamide gel electrophoresis of outer membrane (OM) proteins from Pseudomonas aeruginosa PAO-1 and PAO-tox$^{FeR-10}$. The gel was stained with Coomassie blue. Well 1: Molecular weight standards described in the text. Well 2: OM preparations from PAO-tox$^{FeR-10}$ grown in low-iron medium. Well 3: OM preparations from PAO-tox$^{FeR-10}$ grown in high-iron medium. Well 4: OM preparations from PAO-1 grown in low-iron medium. Well 5: OM preparations from PAO-1 grown in high-iron medium.


1
2
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4
5

Paper 4

The modulatory effect of iron on the pathogenesis of *Pseudomonas aeruginosa* mouse corneal infections

Abstract

The iron concentration of the culture medium used to prepare the inocula influenced the pathogenesis of mouse corneal infections by Pseudomonas aeruginosa. When the parental strain PAO-1 was cultured in high-iron medium (5 µg Fe/ml), it was less virulent than when it was cultured in low-iron medium (.05 µg Fe/ml). Iron concentration (Fe/ml) of the growth medium had no effect on the virulence of a P. aeruginosa mutant which was resistant to the iron regulation of toxin A yields (PAO-tox$^{FeR-18}$). A severely defective iron transport mutant, PAO-tox$^{FeR-10}$, was avirulent regardless of the iron concentration of the growth medium. These studies indicate that both iron acquisition and iron regulation of toxin production are important factors in the determination of P. aeruginosa virulence.

Introduction

Iron plays a complex role in the pathogenesis of Pseudomonas aeruginosa infections. Iron is essential for microbial growth (10). In order to compete for iron with the hosts' iron binding proteins, bacteria synthesize specific iron chelators termed siderophores. The siderophore-iron complex binds to outer membrane proteins on the bacterium and the iron is then transported into the intracellular milieu (16). P. aeruginosa synthesizes two siderophores, pyochelin and pyoverdin which have been shown to facilitate iron uptake (3,4). Siderophore production as well as the synthesis of membrane proteins responsible for binding the siderophore-iron complex are maximally induced under conditions of iron staravtion (16).

Iron also regulates yields of extracellular products of P. aeruginosa, including toxin A and proteases (1,2). As the concentration of the culture medium is increased, the yields of toxin A, elastase and alkaline protease are markedly decreased (1,2).

Previous studies have suggested that toxin A contributes to the pathogenesis of mouse corneal infections with P. aeruginosa (11,6). No data, however, is available on the role of iron in these infections. In the present studies mutants were employed to examine the relationship of both iron acquisition and iron regulation of toxin production to virulence of P. aeruginosa.

## Materials and Methods

Bacterial strains and culture conditions. P. aeruginosa strain PAO-1 originally isolated by B. Holloway (5) was the parental strain of the mutants used in this study. PAO-tox$^{FeR-18}$ (13) and PAO-tox$^{FeR-10}$ (13) are indepently isolated mutants which have been previously described. The characteristics of the bacterial strains are summarized in Table 1. PAO-tox$^{FeR-10}$ produces toxin at slightly higher levels than the parent PAO-1 in both low and high iron medium. This mutant is severely deficient in the ability to transport iron from $FeCl_3$, ferripyochelin and ferripyoverdin. Outer membranes isolated from PAO-tox$^{FeR-10}$ exhibit reduced binding of $^{59}$Fe-pyochelin. PAO-tox$^{FeR-18}$ is normal in all mechanisms of iron transport examined. This strain is resistant to the effect of iron on toxin A yields. PAO-tox$^{FeR-18}$ produces markedly more toxin A than the parent strain PAO-1 in both low and high iron medium.

Cultures were grown in chelexed trypticase soy broth dialysate (TSB-DC) as previously described (12). The iron concentration of this low iron medium was .05 µg Fe/ml. $FeCl_3$ was added to TSB-DC to a concentration of 5 µg Fe/ml (high iron medium). Ten ml of medium was added to a 250 ml Erlenmeyer flask and inoculated with 0.1 ml of an overnight culture of the appropriate strain. The flasks were incubated at 32°C with maximum aeration until the cultures reached late log phase of growth ($OD_{540}$ of 0.70). The organisms were washed twice with sterile phosphate-buffered saline (PBS), pH 7.2, to remove extracellular products and then resuspended in PBS. Viable numbers of bacteria (cfu) were obtained by plating dilutions on nutrient agar.

Mouse corneal infections. Corneal infections were established in mice as previously described (11). Briefly, female Swiss-Webster mice, weighing 20-22 g, were anesthetized with methoxyflurane (Pitman-Moore, Washington Crossing, NJ). The mice were subjected to three 1 mm corneal incisions with a 27 gauge needle while viewed through a 50 x power stereoscopic microscope. Bacteria, at a concentration of $10^6$ cfu suspended in 5 µl PBS, were immediately applied to the traumatized corneas. Each strain was tested in 10 mice. Controls were eyes which were traumatized and treated with 5 µl PBS.

Eyes were examined microscopically at intervals during each experiment. The level of corneal damage was quantitated according to a corneal damage index (CDI). As previously defined (11) a CDI of 1.0 indicates light or partial opacity whereas 4.0 consists of perforation of the cornea.

Bacteria were recovered from the eyes by swabbing the eyes with a cotton swab moistened with PBS and streaking onto TSB-DC agar.

Characteristic colonies of P. aeruginosa were identified after incubation at 37°C. When P. aeruginosa could not be recovered from eyes on two consecutive days, the eyes were considered culture negative.

## Results

Previous studies have demonstrated the virulence of P. aeruginosa strain PAO-1 in a mouse corneal infection model (11). Those experiments were done with inocula prepared from cultures grown in low iron medium. In order to examine the relationship between iron regulation of toxin production and virulence of P. aeruginosa in mouse corneal infections, cultures in the present study were grown in both low iron (.05 µg Fe/ml) and high iron (5.0 µg Fe/ml) medium, and the degree of damage resulting from the inoculation of these bacteria into traumatized mouse corneas were compared.

Effect of iron concentration of the medium on the virulence of parental strain PAO-1. When PAO-1 was cultured in low iron medium and applied to incised mouse corneas extensive damage was observed in all eyes (Figure 1). Opacity, especially in the central portion of the cornea, was apparent in the majority of infected eyes by two days after inoculation. The extent of corneal damage increased until 8 days after infection with most eyes exhibiting some central necrosis. The corneal damage then began to clear but even at day 19 when the experiment was terminated, extensive opacity (CDI = 2.70) was still present. Previous studies with strain PAO-1 (11) indicated that by 15 days post infection all eyes were sterile and the amount of damage remained unchanged.

When PAO-1 was cultured in high iron medium and applied to the

scratched corneas, the amount of corneal damage was considerably reduced (Figure 1). Although corneal opacity was observed by 2 days post infection, the iris was still visible in these mice (CDI = 1.45). The pathology increased slightly until day 5 and then began to decrease. Extensive central opacity and necrosis were not observed in any of these mice as had been the case when the parental strain, PAO-1, was grown in low iron medium. The final CDI at day 19 was 1.25.

Effect of iron concentration on the virulence of a deregulated toxin A mutant, PAO-tox$^{FeR-18}$. One explanation for the decrease in virulence of PAO-1 when grown in high iron is that the bacteria were not producing toxin A at the time of inoculation (Table 1) and therefore, did not cause the same extent of damage in the eyes. A mutant deregulated with respect to the iron effect on toxin A yields (PAO-tox$^{FeR-18}$) was cultured in high and low iron medium and inoculated into mouse eyes to further examine this phenomenon.

PAO-tox$^{FeR-18}$, when grown in low iron medium, established an infection similar to the parent strain PAO-1 (Figure 2). Extensive corneal opacity was observed by 2 days after inoculation. The extent of corneal damage increases until day 8 (CDI = 2.85) and then remained relatively constant until the experiment was terminated. In contrast to the parent, PAO-tox$^{FeR-18}$ produced the same amount of corneal damage when cultured in high iron medium as when cultured in low iron medium (Figure 2). The iron concentration in the growth medium had no effect on the progression of pathology observed in mouse eye infections produced by PAO-tox$^{FeR-18}$. This mutant which is resistant to the iron effect on toxin A yields, was therefore equally virulent when grown in either low iron or high iron medium. These data suggest that the

ability of an organism to produce toxin A at the time of inoculation is a determining factor in the degree of corneal damage observed in these mouse infections.

Effect of iron on the virulence of an iron transport mutant PAO-tox$^{FeR-10}$. The above results suggest a relationship between iron regulation of toxin production and the virulence of P. aeruginosa. Iron is also an essential requirement for microbial growth (10,15). A mutant deficient in the ability to transport iron (PAO-tox$^{FeR-10}$) was grown in low and high iron medium and inoculated into traumatized mouse eyes to determine the importance of iron acquisition in this corneal infection model.

PAO-tox$^{FeR-10}$ is severely deficient in all known mechanisms of iron transport in P. aeruginosa (14). PAO-tox$^{FeR-10}$ has a markedly reduced rate of iron uptake from pyochelin, pyoverdin, and $^{59}FeCl_3$ when compared to the parent strain PAO-1. PAO-tox$^{FeR-10}$ however, has the same growth rate in complex medium as PAO-1 (35 min generation time, 14) so the organism can obtain iron in vitro presumably by a low affinity iron uptake system. The mutant PAO-tox$^{FeR-10}$ was totally avirulent in the mouse corneal infection model regardless of whether the infecting inoculum was grown in low or high iron medium (Figure 4).

PAO-tox$^{FeR-10}$ was significantly less capable of establishing corneal infections than either the parent PAO-1 or the mutant PAO-tox$^{FeR-18}$. At least 70% of the eyes from mice infected with PAO-1 and PAO-tox$^{FeR-18}$ were culture positive on day 2 regardless of whether the inocula were grown in low or high iron medium. The eyes cleared at approximately the same rate in each case and all eyes were cleared by day 11. In contrast, only 20% of the eyes infected with PAO-tox$^{FeR-10}$ were culture

positive on day 2 and all were culture negative by day 4. This indicates that the ability of an organism to acquire iron by a high affinity system is essential for establishing infection. These data also indicate that the ability of a strain to produce toxin A is not sufficient for virulence. Other factors including those which affect growth in vivo are important in the pathogenesis of P. aeruginosa in mouse corneal infections.

## Discussion

Previous studies utilizing corneal infection models have suggested that the virulence of P. aeruginosa is multifactorial. Using toxin A deficient mutants in the mouse model, Ohman et al. demonstrated that toxin A contributes to the corneal damage observed in these infections (11). A role of toxin A in corneal tissue destruction has been shown directly by its killing of epithelial and stromal cells after its injection into rabbit corneas (6). Other studies have suggested that the production of proteases may contribute to the pathogenesis of P. aeruginosa in corneal infections (7,8).

Our data also suggest that several factors are involved in the virulence of P. aeruginosa in mouse eyes. Although toxin A production is not sufficient for establishing infection, toxin A does appear to be required for maximum virulence in this animal model. Not only must the organisms have the ability to produce toxin A, but the organisms must be actively synthesizing toxin A at the initial time of inoculation for maximum virulence. Since the mutant PAO-tox$^{FeR-18}$ continues to produce significant amounts of toxin A when grown in high iron medium, the inocula prepared from cultures grown in either low or high iron

medium are actively synthesizing and processing toxin A at the time of infection. The iron concentration of the growth media had no effect on the virulence of PAO-tox$^{FeR-18}$ (Figure 2).

If toxin A production is markedly reduced at the time of initial infection, as is the case with PAO-1 when the inoculum was grown in high iron medium, or if a toxin-deficient mutant is inoculated into the incised mouse corneas (11), there is still a modest amount of corneal damage observed in this infection model. This suggests that other factors are involved in the production of some of the corneal damage in infected mouse eyes. Certainly, the ability of the organisms to acquire iron is important in establishing an infection. Although PAO-tox$^{FeR-10}$ produces toxin, it was avirulent in the eyes, apparently due to its inability to acquire iron *in vivo* (Figure 3). Iron, therefore, is not only important in regulating toxin A production, but also the acquisition of iron by *P. aeruginosa* is a requirement for virulence.

Our results indicate that particular attention must be made to the medium in which bacteria are cultured prior to inoculation in animals. Certainly, the amount of iron available *in vitro* influences the virulence of *P. aeruginosa*. The iron concentration of the medium used to prepare the inocula may also be an important consideration in animal models for other organisms whose extracellular products or surface characteristics are regulated by iron.

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Table 1. Comparison of toxin A production and iron transport mechanisms in Pseudomonas aeruginosa PAO-1 and mutants PAO-tox$^{FeR-10}$ and PAO-tox$^{FeR-18}$.

| | Strain | | |
|---|---|---|---|
| Property | PAO-1 | PAO-tox$^{FeR-10}$ | PAO-tox$^{FeR-18}$ |
| Toxin A (μg/ml)[a] | | | |
| Low Iron | 1.0 | 1.3 | 3.0 |
| High Iron | 0.2 | 0.4 | 1.5 |
| Rate of $^{55}FeCl_3$ uptake[b] | 14.3 | 2.33 | 15.2 |
| Rate of $^{55}$Fe-pyochelin uptake[b] | 14.3 | 0.7 | 20.0 |
| Rate of $^{55}$Fe-pyoverdin uptake[b] | 5.7 | 0.15 | 2.7 |
| $^{59}$Fe-pyochelin binding to outer membranes[c] (low iron medium) (cpm/100 μg protein) | 3353 | 1581 | 3318 |

[a] Toxin A was measured as previously described (13).

[b] Rates of $^{55}$Fe-siderophore uptake were measured as described (4,14). Values are expressed as pg/min/mg dry weight.

[c] $^{59}$Fe binding to outer membranes was measured as described (15).

Figure 1. Average corneal damage ± SEM resulting from the inoculation of $10^6$ cfu of Pseudomonas aeruginosa PAO-1 grown in low iron medium ( o ) and high iron medium ( • ) into incised corneas of 10 mice.

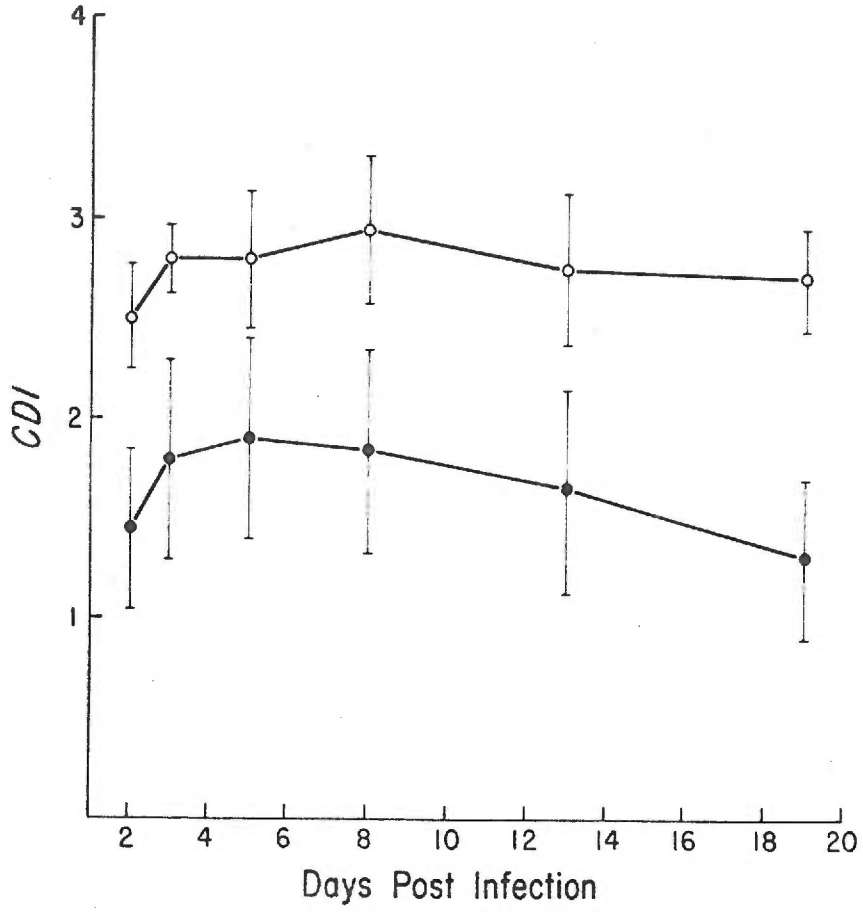

4
3
2
1
CDI
2
4
6
8
10
12
14
16
18
20
Days Post Infection

Figure 2. Average corneal damage ± SEM resulting from the inoculation of $10^6$ cfu of PAO-tox$^{FeR-18}$ grown in low iron medium ( o ) and high iron medium ( ● ) into incised corneas of 10 mice.

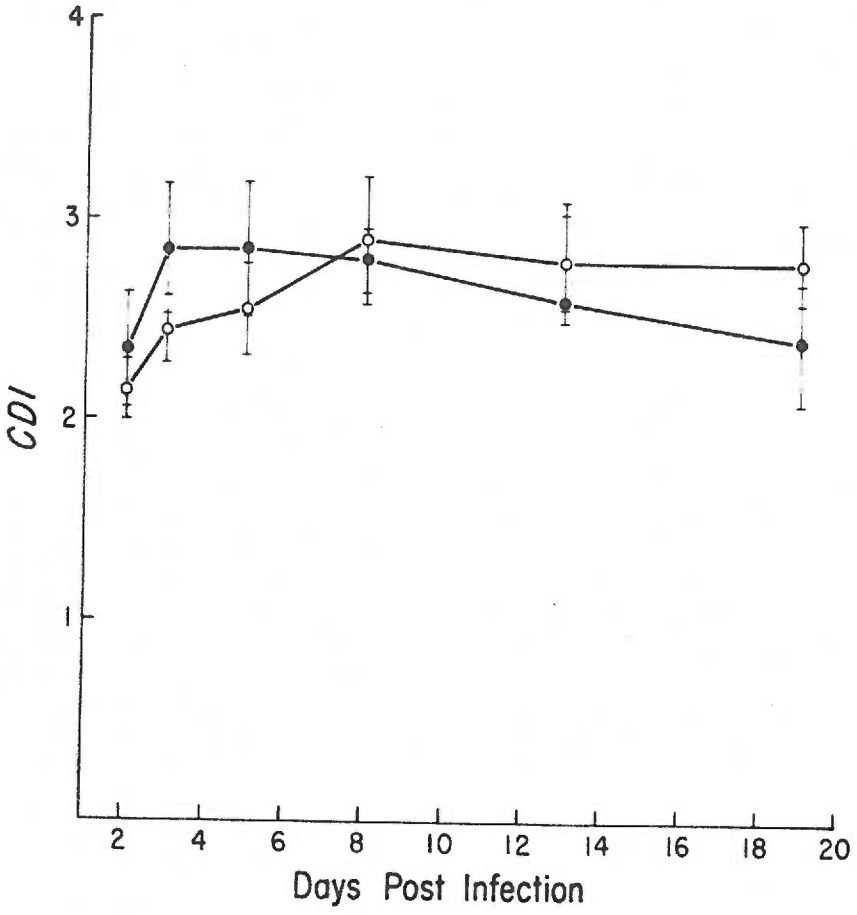
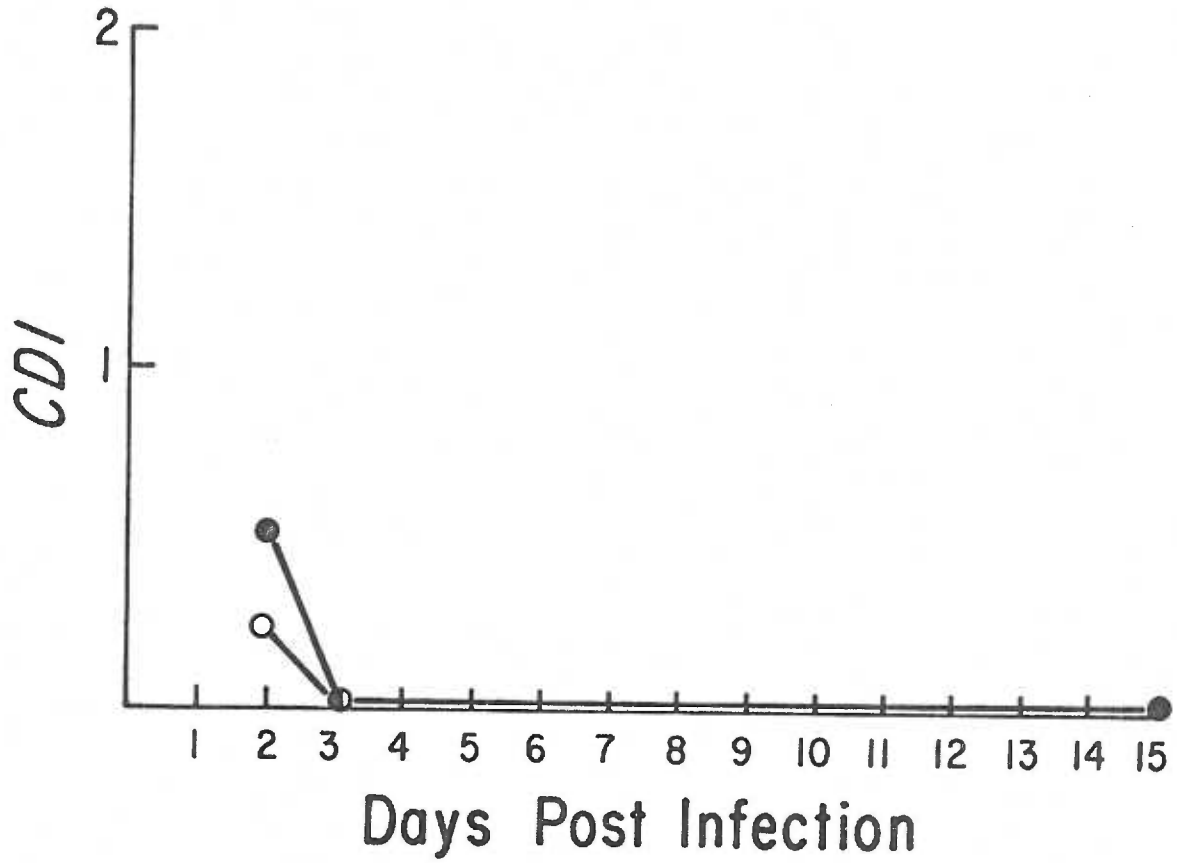

4
3
2
1
CDI
2
4
6
8
10
12
14
16
18
20
Days Post Infection

Figure 3. Average corneal damage ± SEM resulting from the inoculation of $10^6$ cfu of PAO-tox$^{FeR-10}$ grown in low iron medium ( o ) and high iron medium ( ● ) into incised corneas of 10 mice.


2
1
CDI
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days Post Infection

IV. Discussion and Summary

Iron plays a complex role in the virulence of Pseudomonas aeruginosa. Iron is an essential requirement for microbial growth (10). The acquisition of iron by bacteria is correlated with the ability of a variety of pathogenic bacteria to establish and maintain infections (1). Because of the extreme insolubility of ferric iron at neutral pH, bacteria must synthesize specific iron chelators termed siderophores, which function in the solubilization and transport of iron into the cell (10).

Three iron transport systems have been reported in P. aeruginosa (4). P. aeruginosa produces two compounds with siderophore activity, pyochelin and pyoverdin. Citrate can also be used as an iron chelator (4). Although these compounds have been shown to promote iron uptake in P. aeruginosa, little is known about the actual transport of iron into the cell.

Outer membranes have been implicated to be involved in iron-uptake in a variety of organisms (8). Iron starvation leads to the induction of certain proteins in outer membranes of many organisms (8). Mutants deficient in iron-transport have been isolated lacking outer membrane proteins implicated in siderophore-mediated iron transport (8).

Iron also regulates the production of extracellular proteins by P. aeruginosa in vitro (1,2). Several of these extracellular products are implicated in the pathogenesis of P. aeruginosa infections, including toxin A, alkaline protease and elastase. 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 (1,2). The molecular mechanism(s) of iron regulation of toxin A and protease

production are poorly understood.

The present study has examined both aspects of the involvement of iron in P. aeruginosa virulence; iron acquisition and iron regulation of extracellular products. The specific binding of an iron siderophore complex to the outer membranes of P. aeruginosa was examined (Paper 1). Isolated outer membranes from P. aeruginosa were shown to bind $^{59}$Fe-pyochelin. Ferripyochelin was found to specifically bind to an outer membrane protein with a molecular of approximately 18,000. This protein is induced in conditions of iron deprivation where it becomes a major outer membrane protein. Although binding studies and genetic evidence have suggested the involvement of high molecular weight outer membrane proteins in iron uptake of other bacterial species, no direct evidence has been obtained that these proteins are involved in the actual binding. The current study is the first direct demonstration of binding of an iron-siderophore complex to any outer membrane protein (Paper 1).

A mutant severly defective in iron transport mechanisms was also characterized (Paper 3). PAO-tox$^{FeR-10}$ was found to be deficient in all high affinity iron uptake systems. Although this mutant produced siderophores, PAO-tox$^{FeR-10}$ was unable to utilize any of the currently recognized siderophore mediated iron transport systems. Outer membranes isolated from this mutant were defective in the ability to bind ferripyochelin. These data suggested the possibility that there is a common iron uptake pathway utilized by the three known iron chelating systems of P. aeruginosa, and that PAO-tox$^{FeR-10}$ has a mutation in a gene which is required for all three systems. In this regard, PAO-tox$^{FeR-10}$ may be analogous to an E. coli ton B mutant. A functional ton B gene is

required for all high-affinity iron uptake systems in E. coli (5).

Although this mutant (PAO-tox$^{FeR-10}$) presumably has a functioning low affinity iron uptake system enabling it to grow in vitro, it was unable to grow in mouse eyes (Paper 4). PAO-tox$^{FeR-10}$ was therefore avirulent in a mouse corneal infection model (Paper 4). This suggests that high affinity iron uptake mechanisms are required for growth in vivo where the bacteria must compete for iron with the host's iron-binding proteins.

The acquisition of iron, therefore is a major requirement for the virulence of P. aeruginosa. Further understanding of iron acquisition systems will increase our knowledge of this organism's pathogenic mechanisms. Purification and characterization of the ferripyochelin binding protein from the outer membrane (Paper 1) may have potential as a vaccine or vaccine component to prevent P. aeruginosa infections. Similarly, antibodies to this outer membrane protein, administered passively, may be protective against P. aeruginosa in immuno-compromised patients. The vaccine potential of this iron-siderophore binding protein should therefore be explored.

In addition to being essential for bacterial growth, iron also regulates the production of extracellular products by P. aeruginosa (1,2). It has previously been shown that increasing the iron concentration of the growth medium decreased the yields of toxin A and proteases in several strains of P. aeruginosa. This iron effect on toxin A yields was not strain dependent. All strains tested showed the same decrease in magnitude of toxin production by iron. The effect of iron on protease and elastase yields was, however, shown to be strain dependent suggest-

ing the possibility that protease yields are regulated by iron independently from toxin A. In the present study mutants were isolated that were resistant to the iron effect on yields of either toxin or elastase to directly determine whether iron independently regulates yields of extracellular products or coordinately regulates these products by some common regulatory mechanism (Paper 2).

The mutants isolated varied in their level of resistance to the iron concentration of the medium. Two of the $tox^{FeR}$ mutants were hyperproducers of toxin A, whereas the $elas^{FeR}$ mutants were less sensitive than the parent strain PAO-1 to the iron effect on elastase yields, but none of these later mutants hyperproduced elastase.

Two classes of mutants were identified. Class I mutants were defined as having alterations in genes involved in iron transport. These mutants take up less iron than the parent strain and therefore extracellular product yields would be less sensitive to a given iron concentration. Class II mutants were defined as having normal iron transport mechanisms but having a mutation in a gene causing a specific product (or products) to be insensitive to regulation by iron.

Class II mutants were further characterized to determine if their resistance to the iron effect extended to extracellular products other than the one used in their initial identification. The $tox^{FeR}$ mutant characterized, remained sensitive to the iron effect on both alkaline protease and elastase yields. The $elas^{FeR}$ mutants, which were resistant to the iron effect on elastase, remained sensitive to the effect of iron on toxin A and alkaline protease yields. Iron, therefore, can independently regulate yields of toxin A, elastase and alkaline protease *in vitro*.

The molecular mechanism(s) by which extracellular product yields are regulated by iron remains to be determined. Iron may have an effect at the level of transcription, translation, or in the processing and transport of extracellular products. A model for the iron regulation of extracellular products at the transcriptional level was proposed (Paper 2, Figure 6) based on the model for gene expression described for the trp operon. P. aeruginosa may synthesize a series of specific product repressors. These repressors may be synthesized in an inactive state, but in the presence of iron, become activated and bind to the specific product operator thereby repressing synthesis of these extracellular products. Under conditions of iron deprivation, the repressors remain inactive and these genes are derepressed. The continued expression of toxin or elastase in the class II mutants may be due to a loss or decrease in repressor activity or an alteration in the operator. Both types of mutations would result in constitutive expression of the product involved.

Other possible models include the iron regulation of specific events involved in the translation of toxin A, protease and elastase mRNA's. Also the iron regulation of posttranslational processing of extracellular proteins must be considered. Membrane proteins involved in transporting toxin, elastase, or protease out of the cell may be specifically regulated by iron. Alternative models of iron regulation of extracellular products may also be proposed.

The relationship of iron regulation of toxin A production to the virulence of P. aeruginosa was explored in a mouse corneal infection model (Paper 4). Previous studies have implicated a role of toxin A in

the pathogenesis of P. aeruginosa corneal infections (6,7). Toxin A deficient mutants were much less virulent than their parental strains in mouse corneal infections (7). Toxin A has been shown to kill epithelial and stromal cells after injection into rabbit corneas (6).

In order to investigate the relationship of iron regulation of toxin A to virulence of P. aeruginosa, cultures were grown in both low iron and high iron medium, and the degree of damage resulting from the inoculation of these bacteria into traumatized mouse corneas was compared (Paper 4). The iron concentration of the growth medium used to prepare the inocula influenced the pathogenesis of mouse corneal infections. When the parent strain PAO-1 was cultured in high iron medium it was considerably less virulent in the mouse eye model than when the inoculum was grown in low iron medium. When mutant PAO-tox$^{FeR-18}$, which is resistant to the iron effect on toxin A production, was grown in high and low iron medium and inoculated into traumatized mouse corneas, no difference in the amount of corneal damage was seen. The iron concentration in the growth medium, therefore, had no effect on the progression of pathology observed in mouse eye infections produced by this mutant. These data suggested that the bacteria must be actively synthesizing toxin A at the initial time of inoculation for maximum virulence.

The idea that the medium used to grow organisms prior to inoculation in animals can influence the virulence of the organism is an important concept. The iron concentration of the growth medium may also be an important consideration in other animal models employed with P. aeruginosa as well as for other organisms whose extracellular products or surface characteristics are regulated by iron. It was recently reported

(3) that the combined effects of iron starvation and low pH during growth of N. meningitidis greatly enhanced the virulence of this organism for mice. The data presented here and those reported by Brenner et al. (3) indicate that particular attention must be made to the medium bacteria are cultured in prior to inoculation in animals.

This research underscores the fact that iron plays a complex role in the pathogenesis of P. aeruginosa infections. Iron is not only important in regulating the production of extracellular proteins, but also the acquisition of iron by P. aeruginosa is a requirement for virulence. This thesis provides further evidence that the virulence of P. aeruginosa is multifactorial. Many unanswered questions remain regarding the effect of iron on the virulence of P. aeruginosa. The molecular level at which iron regulates extracellular protein production (i.e. transcription, translation, or processing) is only speculative at this time. The importance of the ferripyochelin binding protein in virulence is unknown. Further studies are needed to determine if antibodies to this iron-binding protein would protect against P. aeruginosa disease. It also must be determined if there are other specific siderophore binding proteins in the outer membrane or if all iron-siderophore uptake is mediated through the 18,000 dalton protein. Answers to these questions will increase our understanding of the role of iron in the physiology and virulence of P. aeruginosa, and may provide insight into the future treatment and prophylaxis of P. aeruginosa infections.

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V. Appendices

Paper 1.

Production of Exoenzyme S by Clinical Isolates of *Pseudomonas aeruginosa*.

Abstract

Exoenzyme S differs from toxin A and diphtheria toxin in that it does not ADP-ribosylate EF-2, but rather catalyzes the transfer of the ADP-ribose moiety of nicotinamide adenine dinucleotide to a number of different proteins in extracts of eukaryotic cells. Polyoma transformed BHK-21 cells were isolated which were resistant to diphtheria toxin and toxin A. Extracts from these cells are ADP-ribosylated by exoenzyme S but not toxin A or diphtheria toxin providing an assay which distinguishes between S and A activity. A total of 124 clinical isolates of P. aeruginosa were analyzed for production of toxin A and exoenzyme S. Exoenzyme S production was detected in 38% of the strains, whereas 80% of the strains produced toxin A.

## Introduction

Pseudomonas aeruginosa is an opportunistic pathogen which can cause serious and lethal infection in compromised patients (19,20). P. aeruginosa produces a variety of extracellular products that may contribute to its pathogenicity (17). Toxin A has been shown to be the most toxic extracellular product of P. aeruginosa on a weight basis (19). The mechanism of action of toxin A is identical to that of diphtheria toxin in that it inhibits eukaryotic protein synthesis by catalyzing the transfer of the adenosine 5'-diphosphate ribosyl (ADPR) moiety of nicotinamide adenine dinucleotide (NAD) to elongation factor-2 (6,10,11,12). The resultant ADPR-elongation factor-2 is inactive in protein synthesis (10,11).

Some strains of P. aeruginosa produce a second extracellular pro-

tein (exoenzyme S) that has been shown to have ADPR transferase activity (14). Exoenzyme S differs from toxin A in that it does not ADP-ribosylate EF-2, but rather catalyzes the transfer of the ADPR moiety of NAD to a number of substrate proteins in crude extracts of eukaryotic cells (14). Exoenzyme S also differs from toxin A in its heat stability and its inactivation, rather than potentiation, by pretreatment with urea and dithiothreitol (DTT) (14). In vitro production of S is increased by the addition of 10 mm nitriloacetic acid (NTA) to the culture medium, whereas toxin A yields either remain unchanged or are slightly reduced in the presence of NTA. Furthermore, exoenzyme S is not precipitated or neutralized by antitoxin A (14).

While exoenzyme S has been shown to be produced in vivo in a burned mouse model (2), its role in human P. aeruginosa infections has not yet been determined. Preliminary studies on the production of S by clincial isolates of P. aeruginosa involved growing each strain in trypticase soy broth dialysate (TSBD) with and without NTA, then determining the ADPR transferase activity of both supernatants in a wheat germ extract with and without prior incubation with 4 M urea + 1% dithiothreitol (DTT). Confirmation of enzyme phenotype was obtained by neutralization utilizing antisera prepared against pure toxin A or partially purified S (29). While this method was reasonably accurate, it was extremely slow for screening large numbers of isolates. In some strains that produced low amounts of both A and S, this procedure sometimes yielded ambiguous results depending on the relative amounts of A and S produced. Therefore, a specific assay was needed to detect S.

This study was undertaken to determine the percentage of clinical isolates of P. aeruginosa that produce exoenzyme S and to evaluate the possibility of a correlation between exoenzyme S and/or toxin A production and patient mortality. This report describes the development of an assay specific for S utilizing polyoma transformed baby hamster kidney cells (PyBHKR) resistant to both diphtheria toxin and Pseudomonas toxin A. This assay was used to determine the ADPR transferase enzyme phenotype of 124 clinical isolates of P. aeruginosa.

## Materials and Methods

Cell and Culture Conditions: Stocks of baby hamster kidney (BHK-21) and polyoma virus transformed baby hamster kidney (PyBHK-21) cells were provided by Dr. Jules V. Hallum (Univ. of Ore. Health Sci. Center, Portland, OR). All cells were grown in Eagle's Minimum Essential Medium (MEM) with Hank's Salts, supplemented with 10% fetal calf serum, and 50 µg/ml gentamicin. Cells were incubated at 37 C in a 5% $CO_2$ atmosphere. Cells were transferred by removing the growth medium and washing the cells twice with 0.25% trypsin in versene (0.5 mM EDTA, 140 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4$, 1 mM glucose, 1.5 mM $KH_2HPO_4$), then incubating them at 37 C until all cells are lifted.

Toxins: Diphtheria toxin and Pseudomonas aeruginosa toxin A were purified as previously described (13,16). Partially pure S was kindly provided by Dr. Michael R. Thompson. Exoenzyme S was purified from culture supernatants as previously described except that all buffers contained 10 mM DTT (29). The addition of DTT was found to stabilize the enzymatic activity of S.

Radioactive Reagents: $^{14}$C-NAD labeled in the adenine moiety (25 μCi/ml, spec. act. 302 mCi/mmole) and $^{3}$H-labeled mixture of amino acids (1.0 mCi/ml) were obtained from the Amersham Corporation (Arlington Heights, Illinois).

Clinical Isolates of P. aeruginosa: Clinical isolates of P. aeruginosa were obtained from Walter Reed Army Institute of Research, U.S. Army Institute of Surgical Research, and the University of Virginia School of Medicine. These isolates were determined to be different strains on the basis of their Fisher-Devlin-Gnabasik serotype (8), colonial morphology, pigment production and protease production. Seventy-one of the clinical isolates were from (nonburn) bacteremia patients and 53 of the strains were from burn patients.

Bacteriological Medium and Growth Conditions: The culture medium consisted of a dialysate of trypticase soy broth (TSBD) 1% glycerol, and 0.05 M monosodium glutamate (18) deferrated with Chelex-100 (Bio-Rad). Where indicated the medium was supplemented with 10 mM NTA (2). Ten ml of medium were added to 250 ml Erlenmeyer flasks and inoculated with 1.0 ml of a 15 hr shaking culture of the appropriate strain. The flasks were incubated at 32 C for 20 hr in a reciprocating shaker (150 linear excursions per min) (Lab-line Instruments, Melrose, IL). Culture supernatants were obtained by centrifugation at 10,000 x g for 15 min.

Isolation of diphtheria toxin resistant cell lines: PyBHK-21 cells were seeded in 25 cm$^2$ culture flasks at a concentration of 1 x 10$^6$ cells per flask, and allowed to adhere overnight. The medium was replaced with 10 ml fresh medium containing diphtheria toxin. Toxin

was removed after four hours, the cells washed and incubated at 37°C. Dead cells were washed off every other day and the medium replaced. After 5-7 days growth, flasks containing 1-20 surviving colonies were kept. The surviving cells were grown in toxin free medium and then re-exposed to higher concentrations of diphtheria toxin. The diphtheria toxin concentrations used were: first passage ($1.2 \times 10^{-1}$ µg toxin/ml); second passage (1.2 µg toxin/ml); third passage (12 µg toxin/ml); and fourth passage (120 µg toxin/ml). The final passaged cells, PyBKHR were grown from a single cell by cloning in 96-well plates.

Protein Synthesis Inhibition Assay: Cells were seeded in Linbro 6-well plates (30 mm wells) at a concentration of $5 \times 10^5$ cells/well, and allowed to attach overnight. The medium was then removed and the cells washed with medium containing 1/10 the normal concentration of amino acids. This was followed by incubation at 37 C, 5% $CO_2$, for three hrs in a mixture of 0.8 ml of the 1/10 amino acid medium and 0.1 ml of toxin diluted in Tris-Glu buffer (25 mM Tris-HCL pH 7.2, 140 mM NaCl, 5 mM KCl, 0.7 mM $Na_2HPO_4$, 6 mM glucose, pH adjusted to 7.4 with 1 N HCl). Control plates received no toxin. At the end of this time period 0.1 ml $^3H$ amino acid mixture diluted to 4 µCi/ml in 1/10 amino acid medium was added to each plate, and incubation continued for two hrs. The medium was then decanted and 1.0 ml 0.25% trypsin in $H_2O$ was added per plate. After lifting, the cells were lysed by freezing at -20 C overnight, and thawing the next day. Proteins in the lysate were precipitated by the addition of trichloroacetic acid to a final concentration of 10%, heating at 90 C for 15 mins, and cooling in an ice bath. The precipitated proteins were collected on 0.45 µM Milli-

pore filters and the incorporated radioactivity measured in a Beckman LA-200B scintillation counter. The triplicate samples were averaged and expressed in graphs as the percent of control protein synthesis.

Tumor Induction and Preparation of Cell Extracts. Three week old male golden hamsters were inoculated subcutaneously and intrascapulary with $10^6$ diphtheria toxin resistant PyBHK cells (PyBHKR) or toxin sensitive (parental) PyBHK cells in growth medium. Five weeks post-inoculation, large palpable tumors were found on all the animal's backs. Animals were sacrificed, tumors removed aseptically and weighed. Care was taken to remove only the well encapsulated tumor mass. The tumors were minced, then homogenized in 4 volumes of 0.25M sucrose at $4^oC$ using a rotary homogenizer. To each ml of homogenate, 0.16 ml of diluent (4 M NaCl and 20 mM DTT) and 0.25 g of prewashed activated charcoal was added and the mixture was shaken at $5^oC$ for 15 min to remove endogenous NAD (9). The extract was centrifuged at 27,000 RPM (Type 30 rotor) for 75 mins, the supernatant removed and the protein concentration adjusted to 7.2 mg/ml with 200 mM sodium acetate buffer pH 6.0 (Buffer II).

Cell-free ADP-ribosylation of EF-2. Cellular EF-2 was measured by the method of Gill and Dinius (9). Fifty µl of cell extract was added to 300 µl histamine buffer (0.11 M histamine; 90 mM Tris-HCl, pH 8.0; 70 mM DTT; 0.017 mM EDTA). Diphtheria toxin fragment A or Pseudomonas toxin A was added at a concentration of 6.0 µg/ml. The reaction mixture was equilibrated at $37^oC$ after which 0.125 µCi of $^{14}C$-NAD in 5 µl were added to each tube. The reaction mixtures were incubated at $37^oC$ for 10 min, then terminated by the addition of 5% TCA. The precipitates

were collected by filtration on 0.45 μ filters, and counted.

ADPR transferase activity. Toxin A production was identified by a significant increase in ADPR transferase activity in TSBD (no NTA) culture supernatants, following preincubation with 4 M urea - 1% DTT (31). Partially purified EF-2 prepared from extracts of wheat germ was used as a substrate (5). The reaction mixture consisted of 10 μl of culture supernatant, 25 μl of wheat germ extract, 25 μl of buffer I (125 mM Tris-HCl, pH 7.0; 100 mM Dithiothreitol) and 5 μl of $^{14}$C-labeled NAD.

Exoenzyme S production was detected in supernatants from cultures grown in TSBD with 10 mM NTA, utilizing the PyBHKR extracts as a substrate. The reaction mixture consisted of 10 μl of buffer II (200 mM sodium acetate, pH 6.0), 10 μl of PyBHKR extract and 5 μl of $^{14}$C-labeled NAD.

The reaction mixtures for both toxin A and exoenzyme S assays were incubated at 25°C for 30 mins. The reaction was terminated with the addition of 10% TCA. The precipitates were collected and counted as previously described (31).

Protein determination. Protein was determined by the method of Bradford (4) modified by using a commercial reagent, Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories). Lysozyme was used as the standard.

## Results

Isolation of Diphtheria toxin and Pseudomonas toxin A resistant cell lines. Polyoma virus transformed baby hamster kidney cells (PyBHK) are quite sensitive to diphtheria toxin. Diphtheria toxin at a concen-

tration of $3 \times 10^{-3}$ µg/ml is sufficient to cause 50% inhibition of protein synthesis in a standard five hour assay system. PyBHK cells were exposed to medium containing diphtheria toxin in varying concentrations for four hours. The surviving cells were grown in toxin free medium and then re-exposed to greater concentrations of toxin.

Cells (PyBHK) surviving prior exposure to $1.2 \times 10^{-1}$ µg/ml toxin (first passage) showed little increase in their subsequent resistance to inhibition of protein synthesis by diphtheria toxin (Figure 1). However, exposure of these cells to 1.2 µg/ml toxin yielded survivors with a greatly increased resistance to diphtheria toxin. Subsequent exposure of these diphtheria toxin resistant PyBHK cells to 12 and 120 µg/ml toxin further increased this resistance. Many survivors resulted from the final toxin exposure of PyBHK resistant cells. These cells when plated for isolation of single cell clones varied in their resistance to intoxication by diphtheria toxin. The clone with the highest resistance to toxin was termed PyBHKR (Figure 1) and used in further experiments. Exposure of these PyBHKR cells to diphtheria toxin concentrations of up to 750 µg/ml for up to 24 hours had no discernible effect on the cells.

Pseudomonas aeruginosa toxin A has been shown to catalyze the same intra-cellular ADP-ribosylation of EF-2 as diphtheria toxin (10,12). PyBHK cells are sensitive to toxin A, with a concentration of $2 \times 10^{-2}$ µg/ml sufficient to inhibit protein synthesis by 75% (Figure 2). PyBHKR cells on the other hand were completely unaffected by 20 µg/ml toxin A.

The cellular attachment and/or uptake of toxin A appears to be different than that of diphtheria toxin with various cell types displaying different sensitivity to the two toxins (21,22,30). Since these two

toxins utilize different receptors, our results (Figures 1 and 2) suggest that the toxin resistance of the PyBHKR cells may be mediated at a point common to both toxins, the ADP-ribosylation of EF-2.

A cell free assay was used to compare the realtive abilities of the EF-2 from the sensitive and resistant cells (PyBHK and PyBHKR) to be ADP-ribosylated by diphtheria toxin fragment A and toxin A of P. aeruginosa. The results shown in Table 1 indicate that PyBHK EF-2 is readily ADP-ribosylated by both fragment A and toxin A, while PyBHKR EF-2 is not labeled by either toxin. The possibility that the resistant cell extracts contained a factor that inhibited the transfer of ADPR to EF-2 was tested. Resistant cell extracts were mixed with an EF-2 preparation from normal PyBHK cells. The addition of resistant cell extracts did not interfere with the transfer of labeled ADPR to the PyBHK EF-2 (data not shown). These results indicate that the mutation is cytoplasmic in nature and may be due to an altered EF-2 which is incapable of accepting ADPR in the toxin catalyzed reaction (Table 1).

Characterization of PyBHKR cells. The PyBHK and PyBHKR cells had similar morphology. They both appeared as squamous or somewhat rounded, short fibroblasts with a tendency to overlap and form piles. The cloning efficiency at various serum levels (2-10%) of the PyBHK and PyBHKR cells was indistinguishable. Both PyBHK and PyBHKR cells had similar rates of cell division with an average doubling time of 13.2 and 12.5 hours respectively in medium containing 10% fetal calf serum. Karyotypic analysis showed that both the PyBHK and PyBHKR cells contained 84 chromosomes in a similar distribution and both cell lines induced tumors in golden hamsters. Cells recovered from tumors remained totally resistant to

diphtheria toxin, indicating the mutation conferring toxin resistance was stable during in vivo culture of the cells. The resistance of PyBHKR cells to diphtheria toxin has remained stable during two years of passage in cell culture.

Comparison of ADPR transferase activities of toxin A and exoenzyme S in PyBHK or PyBHKR extracts. Tumors were induced in hamsters with PyBHKR or PyBHK cells and extracts were prepared from the tumors as described in the methods. In these extracts enzyme activities of toxin A, partially purified S, and culture supernatants from various strains of P. aeruginosa were compared. Controls contained wheat germ extracts in place of the tumor cell extracts. The results of these experiments (Table 2) showed that EF-2 in the PyBHKR cell extracts is not ADP-ribosylated by either pure toxin A or culture supernatants from $A^+S^-$ strains of P. aeruginosa. Identical results were obtained with purified fragment A of diphtheria toxin (data not shown). On the other hand, proteins in the PyBHKR extracts are ADP-ribosylated by partially purified S and by culture supernatants of $S^+$ strains of P. aeruginosa. Thus these extracts provided a simple assay which distinguished S from A.

Production of S by clinical isolates of Pseudomonas aeruginosa. A total of 124 clinical isolates of P. aeruginosa were analyzed for production of the ADPR transferases A and S. Production of S was assayed using the PyBHKR assay described above. Toxin A production was identified by at least a 2-fold increase in ADPR transferase activity in TSBD (no NTA) culture supernatants following preincubation with 4 M urea-1% DTT (13). The results (Table 3) showed that 48 (38%) of all strains tested produced S. There was no significant difference in the percentage

of S producers between the bacteremia and burn patients. The majority of the strains (34/48) that produce S also produce A. The percentage of toxin A producing strains (80%) agrees with that previously reported (3, 25,27).

The relationship between S and/or A production and patient mortality is shown in Table 4. There are no differences between the bacteremic and the burn isolates in the mortality rates for each phenotype. Although the numbers are small, there is an increased mortality rate associated with patients infected with strains of P. aeruginosa which produce both S and A. Using a one tailed T-test for proportions, when the mortality rate associated with the $A^+S^+$ strains is compared to the mortality rates of the other three phenotypes, a value of 1.66 is obtained. A value of 1.65 or greater is considered significant ($p < .05$) (15).

## Discussion

Although there is increasing evidence that toxin A is a major virulence factor of P. aeruginosa, little is known about the role of exoenzyme S in P. aeruginosa infections. In vivo production of S has been demonstrated in a burned mouse model (2). However, the prevalence of exoenzyme S production by strains of P. aeruginosa was unknown prior to this report.

To determine the incidence of S production by clinical isolates it was necessary to develop specific methods to detect S and to distinguish it from toxin A. Moehring and Moehring isolated mutants of Chinese hamster ovary cells resistant to diphtheria toxin that were termed presumptive translational mutants (23). The mutation conferring resistance was

shown to be in EF-2 (24). More recently similar mutants have been isolated following a single exposure of normal or mutagenized cells to diphtheria toxin (24). Utilizing the original methods of Moehring and Moehring (23), PyBHKR cells were isolated which also appear to have an altered EF-2. These cells were resistant to protein synthesis inhibition by both diphtheria toxin and Pseudomonas toxin A. The toxin resistant cells were isolated from polyoma virus transformed cells that were capable of inducing tumors in hamsters, thus providing a convenient source of cells to prepare extracts. Extracts from these cells are ADP-ribosylated by exoenzyme S, but not toxin A or diphtheria toxin, thus providing an assay highly specific for S. When coupled with the ADPR transferase assay for toxin A, the S specific assay makes it possible to determine the ADPR transferase enzyme phenotype of strains of P. aeruginosa.

Clinical isolates of P. aeruginosa were obtained from two groups of patients, those with burn wound infections and bacteremia (non-burn). These types of infections were chosen because of their severity. The mortality rate associated with Pseudomonas bacteremia is reported between 43-68% (1,7,28) and Pseudomonas is also associated with fatal infections of the burn wound (26). Of the 124 strains tested, 38% produced exoenzyme S. Production of S by clinical isolates of P. aeruginosa is therefore not a rare event as was recently suggested (32). There was no difference in the incidence of S production between the two patient populations. It is interesting to note that out of 53 burn isolates, there were no $A^-S^-$ strains suggesting that the ability of a strain to produce A or S may provide a selective advantage for the organism in this type of infection. The mortality rate associated with P. aeruginosa

strains producing both A and S is greater than the mortality rate of patients infected with strains producing either one of these enzymes alone. Although more strains should be analyzed, these data suggest that a patient infected with a strain which produces both S and A has less of a chance of survival than a patient infected with a strain which produces only one or neither of these enzymes.

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Table 1. ADP-ribosylation of EF-2 in PyBHK and PyBHKR cell extracts by diphtheria toxin fragment A and Pseudomonas toxin A[a].

| Cell Extract | Toxin | |
|---|---|---|
| | Diphtheria Fragment A | Pseudomonas Toxin A |
| PyBHK | 15,714[b] | 6,205 |
| PyBHKR | 278 | 214 |

[a] These experiments have been repeated three times with comparable results.

[b] Numbers are expressed in counts per minute per mg added EF-2 extract. Cell extract protein concentrations in the reation mixture were PyBHK, 590 µg/ml; and PyBHKR, 300 µg/ml.

Table 2. Comparison of P. aeruginosa A and S enzyme activities in extracts of wheat germ, PyBHK and PyBHKR cells[a].

| | pmoles ADPR transferred[b] / Mg Protein / Source of Cell Extracts | | |
|---|---|---|---|
| | PyBHK | PyBHKR | Wheat Germ |
| Toxin A (.05 µg) | 19.4 | 0 | 42.4 |
| S (.06 µg) | 757 | 660 | 309.3 |

| Strain[c] | ADPR Transferase Phenotype | | | |
|---|---|---|---|---|
| PA103 | $A^+S^-$ | 18.8 | 0 | 38.1 |
| PS388 | $A^-S^+$ | 539 | 432 | 181.2 |
| PA16 | $A^+S^+$ | 49.8 | 64 | 19.9 |
| WR5 | $A^-S^-$ | 0 | 0 | 0 |

[a]Enzyme activity was measured by incubating 10 µl of enzyme or culture supernatant with 10 µl of buffer, 10 µl of cell extract and 5 µl of $^{14}C$-labeled NAD for 30 minutes at 25°C.

[b]These experiments were repeated three times with comparable results.

[c]Supernatants from 20 hour cultures.

Table 3. Production of A and S by clinical isolates of *Pseudomonas aeruginosa*.

| Toxin Phenotype | Number Positive[a] Patient Categories | | |
|---|---|---|---|
| | Bacteremia | Burn | Total |
| $A^+S^-$ | 32 (45) | 33 (62) | 65 (53) |
| $A^+S^+$ | 21 (29) | 13 (25) | 34 (27) |
| $A^-S^+$ | 7 (10) | 7 (13) | 14 (11) |
| $A^-S^-$ | 11 (16) | 0 (0) | 11 (9) |
| $A^+$ | 53 (74) | 46 (87) | 99 (80) |
| $S^+$ | 28 (39) | 20 (38) | 48 (38) |

[a]Numbers in parentheses are percentages.

Table 4. Association of the production of enzyme A or S with patient mortality.

| | Patient Category | | | | | |
|---|---|---|---|---|---|---|
| | Bacteremia | | Burn | | Total | |
| Enzyme Phenotype | S[a] | D[b] | S | D | S | D |
| $A^+S^-$ | 19(59)[c] | 13(41) | 20(60) | 13(40) | 39(60) | 26(40) |
| $A^+S^+$ | 9(43) | 12(57) | 5(38) | 8(62) | 14(41) | 20(59) |
| $A^-S^+$ | 4(57) | 3(43) | 3(43) | 4(57) | 7(50) | 7(50) |
| $A^-S^-$ | 6(55) | 5(45) | 0 (0) | 0(0) | 6(55) | 5(45) |

[a]S = number of patients surviving.

[b]D = number of patients dying.

[c]Numbers in parentheses are percentages.

Figure 1. Increasing resistance of PyBHK cells to diphtheria toxin. Inhibition of protein synthesis by diphtheria toxin in: ● , PyBHK parent cells; Δ , first passage cells surviving prior exposure to $1.2 \times 10^{-1}$ µg/ml toxin; ○ , second passage cells surviving exposure to 1.2 µg/ml toxin; □ , third passage cells surviving exposure to 12 µg/ml toxin; ▲ , a single clone from fourth passage cells surviving prior exposure to 120 µg/ml toxin (PyBHKR cells).

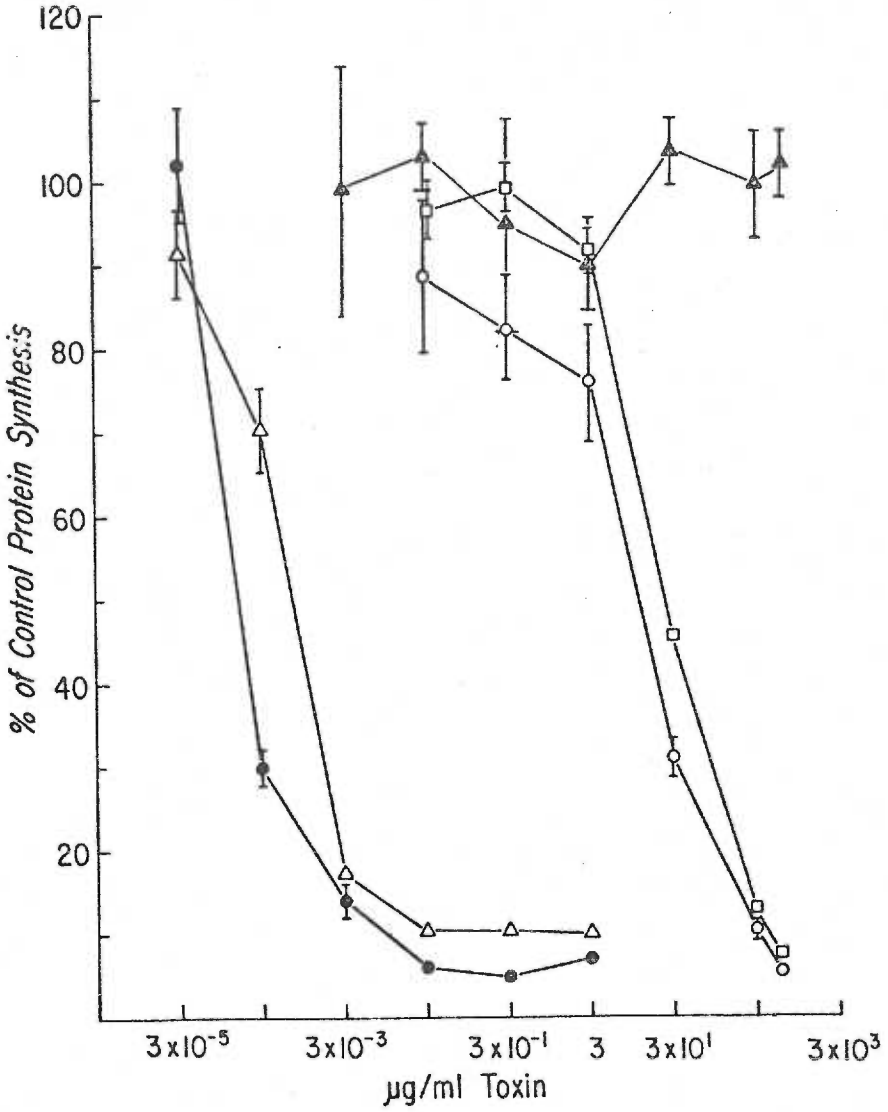
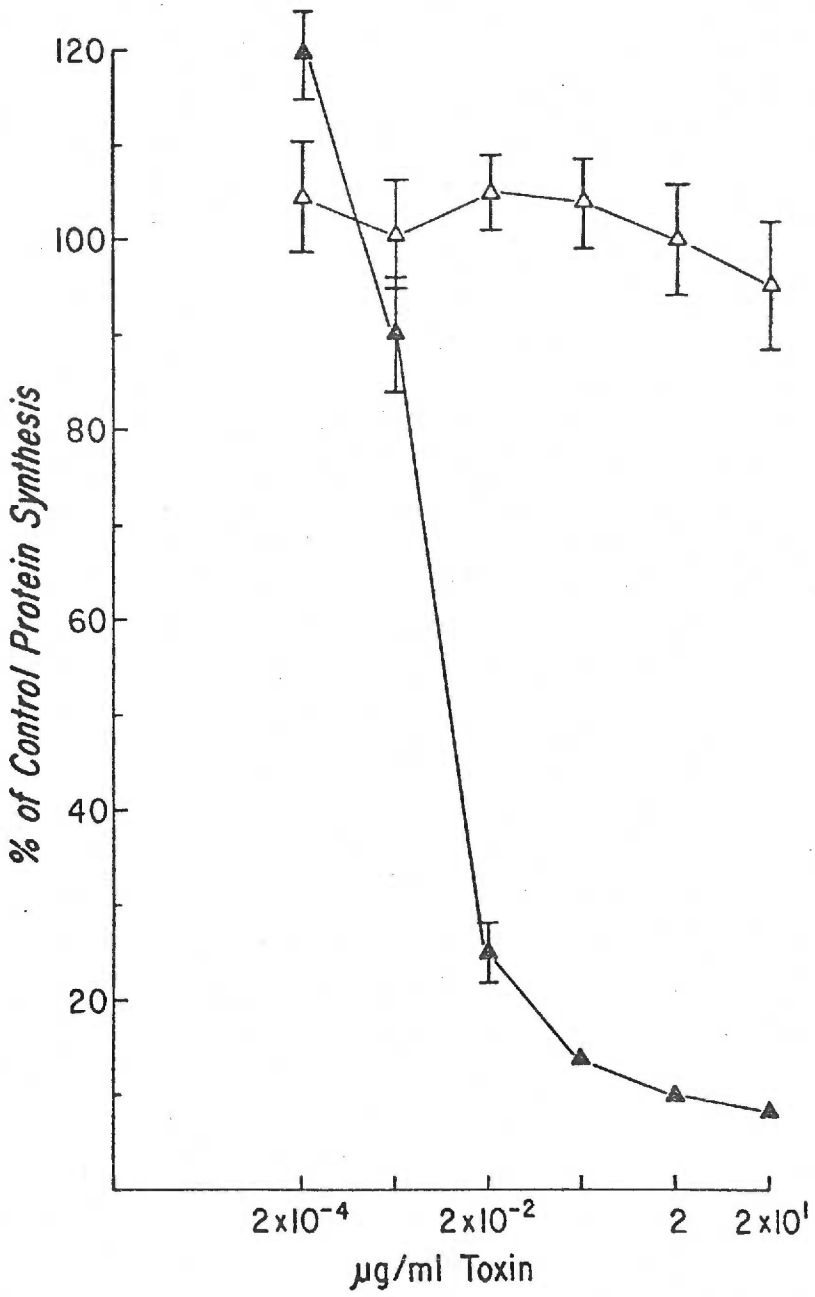

120
100
80
60
40
20
% of Control Protein Synthesis
3x10⁻⁵
3x10⁻³
3x10⁻¹
3
3x10¹
3x10³
µg/ml Toxin

Figure 2. Inhibition of protein synthesis by Pseudomonas aeruginosa toxin A in ▲ , PyBHK cells; and △ , PyBHKR cells.


120
100
80
60
40
20
% of Control Protein Synthesis
2x10⁻⁴
2x10⁻²
2
2x10¹
µg/ml Toxin

Paper 2.

A more sensitive plate assay for detection of protease production by Pseudomonas aeruginosa

Abstract

A plate assay to measure protease production by Pseudomonas aeruginosa is described. This assay is more sensitive and rapid than established methods.

Manuscript (Note format)

Proteases are thought to play a role in the pathogenesis of some Pseudomonas aeruginosa infections (2-5 . Most strains of P. aeruginosa produce three proteases which have different pH optima, isoelectric points, and substrate specificities (6,7). All three proteases, however, are capable of degrading casein. Therefore, skin milk agar is used for identifying P. aeruginosa protease producers and for differentiating P. aeruginosa from other species of Pseudomonas (1,8). These established assays generally require a 48 h incubation period and fail to identify weak protease producing strains of P. aeruginosa. We developed a medium which permits detection of protease production in 24 h or less and which detects protease production by P. aeruginosa strains that give negative reactions on previously described media.

Dialyzed brain heart infusion milk medium (D-BHI milk medium) was prepared by dissolving 18.5 g brain heart infusion broth (BHI, Difco) in 50 ml water and dialyzing it against 1 L of water for 18 h at 4°C. Bacto agar (Difco) was added to the dialysate to a concentration of 3%. A 3% (w/v) solution of skim milk (Difco) was prepared and the solutions autoclaved separately. Equal volumes of the 2 sterile solutions were mixed at 60°C. The skim milk media described by Brown et al. (1, Brown's medium) and that described by Wretlind et al. (8, Wretlind's medium) were prepared as they reported. Ten ml of each medium was dispensed into

petri plates.

Protease production of P. aeruginosa was compared on D-BHI milk medium, Wretlind's and Brown's media. The strains tested included one high protease producer (PAKS-1), six low protease producers (PA103, 10, 3, 6, PAKS-10, and PAKS-17), and two intermediate producers (PAO-1 and PAT-964). Strains PAKS-1, PAKS-10, and PAKS-17 were kindly provided by B. Wretlind, Karolinska Hospital, Stockholm, Sweden; PAT-964 and PAO1 by B. Holloway, Monash Univ., Clayton Victoria, Australia; strains 10, 3, 6 by E. Ziegler, Univ. of California at San Diego, San Diego, CA. and PA103 by P. Liu, Univ., of Louisville School of Medicine, Louisville, KY. An appropriate dilution of a mid log phase broth culture was spread on each type of plate.

The D-BHI milk plates were considerably more sensitive than either of the other two methods (Table 1, Fig. 1 and 2). Strains which produced no detectable protease on Brown's or Wretlind's media had readily apparent zones of hydrolysis on the D-BHI milk medium after 24 h incubation (Table 1). Other strains which produced detectable protease on all three media had 2.5 to 10 times larger zones of hydrolysis on D-BHI milk medium. Figures 1 and 2 compare PA103 (a low protease producer) and PAKS-1 (a high protease producer) on the three types of media. Within 24 h zones of hydrolysis around colonies of PA103 were visible on the D-BHI milk plates, whereas with this strain we were unable to detect protease production on either Brown's or Wretlind's medium (Fig. 1 and Table 1).

After 48 h of incubation small zones ($\leq$ 0.5 mm) of hydrolysis surrounded colonies of PA103 grown on Brown's or Wretlind's medium whereas the zones had increased to 2.0 mm around colonies on D-BHI milk medium (Fig. 2). With the high protease producing strain (PAKS-1) protease

activity was detectable within 24 h incubation on all 3 media. The zones of hydrolysis surrounding colonies of PAKS-1 were much larger on the D-BHI milk medium than on the other media (Fig. 1 and Table 1). Furthermore, using D-BHI milk medium we can detect protease production with PAKS-1 within 10 h (data not shown).

The major difference between D-BHI milk medium and Wretlind's medium (8) is that the former contains a lower concentration of BHI and it is dialyzed. In comparison to Brown's medium (1), D-BHI milk medium contains a lower concentration of skim milk. These alterations result in a more sensitive assay than those (1,8) previously described. This increased sensitivity is particularly useful for the detection of weak protease producers since it permits the detection of protease production by P. aeruginosa strains which are negative on the other media. It is also a more rapid method for detecting protease production with many strains of P. aeruginosa. This medium should expediate the routine screening of strains and may be useful in genetic studies for the isolation of protease deficient mutants. Furthermore, casein can be degraded by many different proteases, and so D-BHI medium may prove useful for studying protease production by other bacterial species. In a preliminary experiment we found D-BHI medium more sensitive than Brown's or Wretlind's medium for detecting protease production by Bacillus subtilis ATCC 9466.

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Table 1. Zones of hydrolysis around colonies of *P. aeruginosa* grown on D-BHI milk, Brown's and Wretlind's media after 24 h.

| | Zones of Hydrolysis[a] | | |
|---|---|---|---|
| Strain | D-BHI | Brown | Wretlind |
| PAKS-1 | 2.5 | 1.0 | 1.0 |
| PAT-964 | 1.0 | 0.1 | 0.2 |
| PAO1 | 1.0 | 0.1 | 0.2 |
| PA103 | 0.5 | 0 | 0 |
| PAKS-10 | 1.0 | 0.1 | 0.2 |
| PAKS-17 | 1.2 | 0 | 0.3 |
| 3 | 1.0 | 0 | 0.2 |
| 6 | 0.5 | 0 | 0.1 |
| 10 | 0.8 | 0 | 0 |

[a]Zones were measured from the edge of the colony to edge of clearing. Results were expressed in mm.

Figure 1. Colonies of P. aeruginosa strains PA103 (top row across) and PAKS-1 (bottom row across) on (from left to right) D-BHI milk, Brown's and Wretlind's media following 24 h incubation at 37°C.

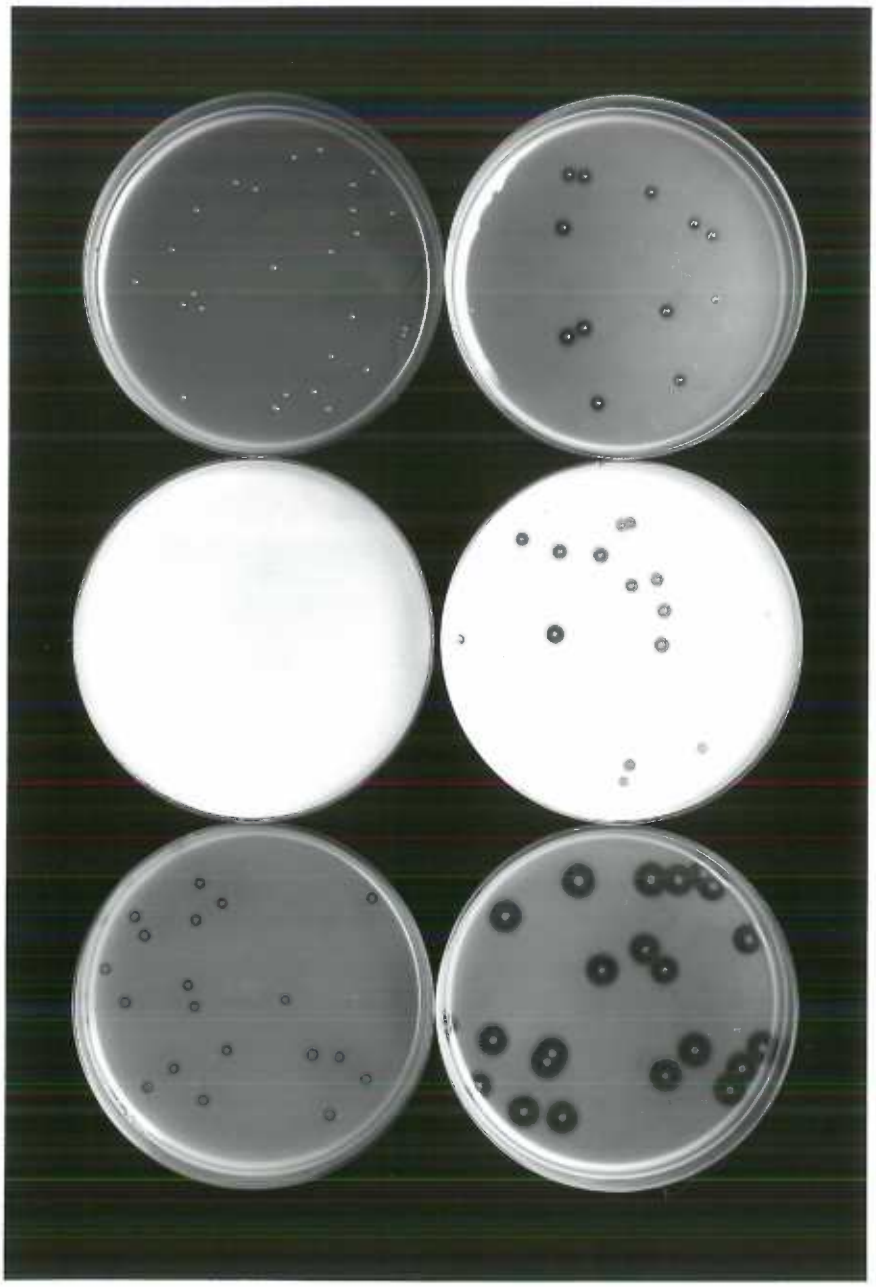
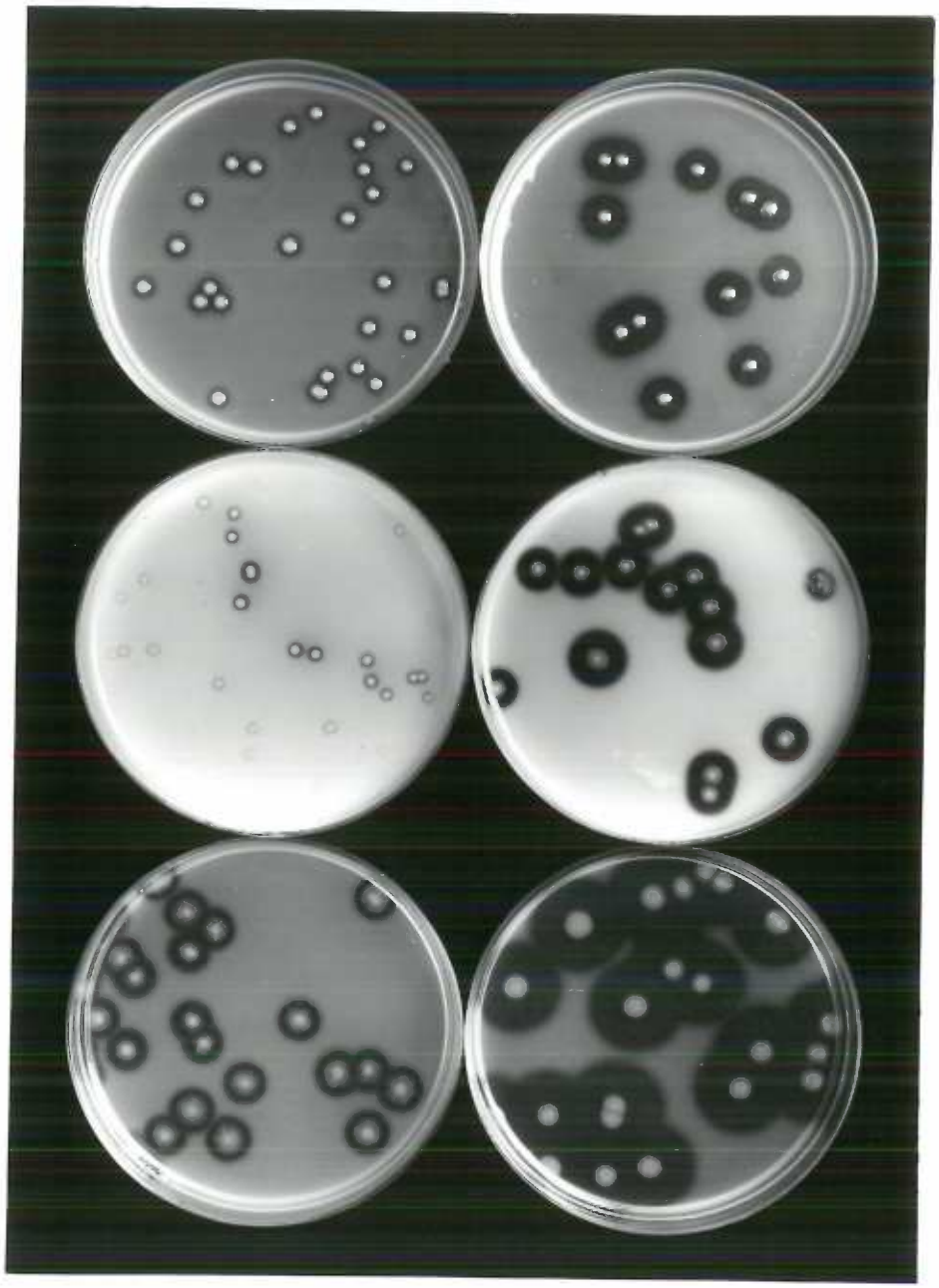


Figure 2. Colonies of P. aeruginosa strains PA103 (top row across) and PAKS-1 (bottom row across) on (from left to right) D-BHI milk, Brown's and Wretlind's media following 48 h incubation at 37°C.



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