

Modulatory Effect of Iron on the Pathogenesis of *Pseudomonas aeruginosa* Mouse Corneal Infections

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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 PAO1 was cultured in high-iron medium (5 μg of Fe per ml), it was less virulent than when it was cultured in low-iron medium (0.05 μg of Fe per ml). The iron concentration 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.

Iron plays a complex role in the pathogenesis of *Pseudomonas aeruginosa* infections. Iron is essential for microbial growth (10). In competing for iron with the iron-binding proteins of host organisms, 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 (9, 13). *P. aeruginosa* synthesizes two siderophores, pyochelin and pyoverdine, which have been shown to facilitate iron uptake (3, 4). Siderophore production and the synthesis of membrane proteins responsible for binding the siderophore-iron complex are maximally induced under conditions of iron starvation (13).

Iron also regulates yields of extracellular products of *P. aeruginosa*, including toxin A and proteases (1, 2). As the concentration of iron in 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* (6, 11). No data, however, are available on the role of iron in these infections. In the present studies, mutants were used 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* PAO1, originally isolated by B. Holloway (5), was the parental strain of the mutants used in this study. PAO-tox^{FeR-18} and PAO-tox^{FeR-10} are independently isolated mutants which have been previously de-

scribed (P. A. Sokol, C. D. Cox, and B. H. Iglewski, submitted for publication). The characteristics of the bacterial strains are summarized in Table 1. PAO-tox^{FeR-10} produces toxin at slightly higher levels than does the parent PAO1 in both low- and high-iron media. This mutant is severely deficient in the ability to transport iron from FeCl₃, ferripyochelin, and ferrityoverdine. Outer membranes isolated from PAO-tox^{FeR-10} exhibit reduced binding of ⁵⁹Fe-labeled 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 does the parent strain PAO1 in both low- and high-iron media.

Cultures were grown in Trypticase soy broth dialysate (BBL Microbiology Systems, Cockeysville, Md.) treated with Chelex-100 (TSB-DC) as previously described (12). The iron concentration of this low-iron medium was 0.05 $\mu\text{g}/\text{ml}$. FeCl₃ was added to TSB-DC to a concentration of 5 μg of Fe per ml (high-iron medium). Medium 10 ml was added to 250-ml Erlenmeyer flasks 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 the late log phase of growth (optical density at 540 nm 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 (colony-forming units) 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 to 22 g, were anesthetized with methoxyflurane (Pitman-Moore, Washington Crossing, N.J.). The mice were subjected to three 1-mm corneal incisions with a 27-gauge needle while viewed through a 50 \times power stereoscopic microscope. Bacteria, at a concentration of 10⁶ colony-forming units suspended in 5 μl of 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 μ l of 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 signifies 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. Eyes from which *P. aeruginosa* could not be recovered for 2 days in a row were considered culture negative.

RESULTS

Previous studies have demonstrated the virulence of *P. aeruginosa* PAO1 in a mouse corneal infection model (11). Those experiments were done with inocula prepared from cultures grown in low-iron medium. For an examination of 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 (0.05 μ g of Fe per ml) and high-iron (5.0 μ g of Fe per ml) media, and the degree of damage resulting from the inoculation of these bacteria into traumatized mouse corneas was compared.

Effect of iron concentration of the medium on the virulence of parental strain PAO1. When PAO1 was cultured in low-iron medium and applied to incised mouse corneas, extensive damage was observed in all eyes (Fig. 1). Opacity, especially in the central portion of the cornea, was apparent in the majority of infected eyes by 2 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 PAO1 (11) indicated that by 15 days postinfection all

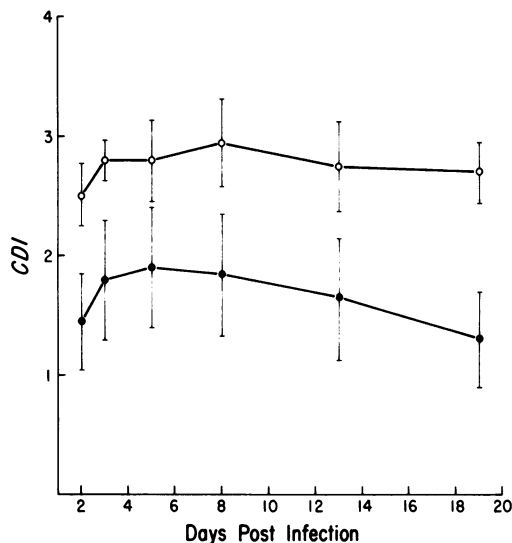


FIG. 1. Average corneal damage \pm standard error of the mean resulting from the inoculation of 10^6 colony-forming units of *P. aeruginosa* PAO1 grown in low-iron medium (○) and high-iron medium (●) into incised corneas of 10 mice.

eyes were sterile and the amount of damage remained unchanged.

When PAO1 was cultured in high-iron medium and applied to the scratched corneas, the amount of corneal damage was considerably reduced (Fig. 1). Although corneal opacity was observed by 2 days postinfection, 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, PAO1, 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}.

TABLE 1. Comparison of toxin A production and iron transport mechanisms in *P. aeruginosa* PAO1 and mutants PAO-tox^{FeR-10} and PAO-tox^{FeR-18}

| Strain | Toxin A (μ g/ml) ^a | | Rate of uptake (pg/min per mg of dry wt) ^b | | | ⁵⁹ Fe-labeled pyochelin binding to outer membranes (low-iron medium) (cpm/100 μ g of protein) ^c |
|---------------------------|------------------------------------|-----------|---|------------------------------------|-------------------------------------|---|
| | Low iron | High iron | ⁵⁵ FeCl ₃ | ⁵⁵ Fe-labeled pyochelin | ⁵⁵ Fe-labeled pyoverdine | |
| PAO1 | 1.0 | 0.2 | 14.3 | 14.3 | 5.7 | 3,353 |
| PAO-tox ^{FeR-10} | 1.3 | 0.4 | 2.33 | 0.7 | 0.15 | 1,581 |
| PAO-tox ^{FeR-18} | 3.0 | 1.5 | 15.2 | 20.0 | 2.7 | 3,318 |

^a Toxin A was measured as previously described (12).

^b Rates of ⁵⁵FeCl₃ and ⁵⁵Fe-labeled siderophore uptake were measured as previously described (3).

^c ⁵⁹Fe binding to outer membranes was measured as previously described (Sokol, Woods, Cox, and Iglewski, submitted for publication).

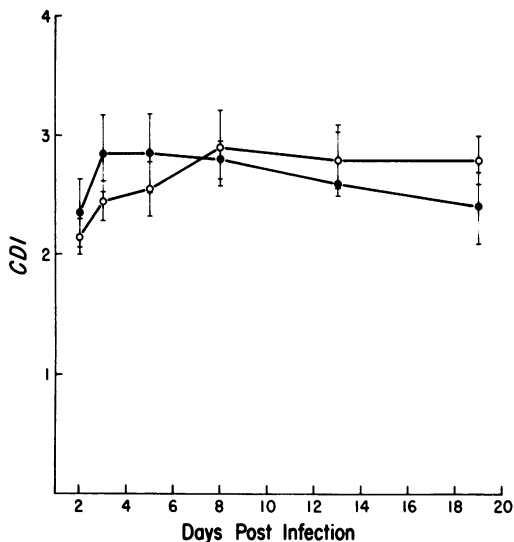


FIG. 2. Average corneal damage \pm standard error of the mean resulting from the inoculation of 10^6 colony-forming units of PAO-tox^{FeR-18} grown in low-iron medium (○) and high-iron medium (●) into incised corneas of 10 mice.

One explanation for the decrease in virulence of PAO1 when grown in high-iron medium 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 media 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 that caused by the parent strain PAO1 (Fig. 2). Extensive corneal opacity was observed by 2 days after inoculation. The extent of corneal damage increased 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 (Fig. 2). The iron concentration in the growth medium had no effect on the progression of pathology in mouse eye infections produced by PAO-tox^{FeR-18}. Thus mutant, which is resistant to the iron effect on toxin A yields, was equally virulent when grown in low- and high-iron media. 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; P. A. Sokol, D. E. Woods, C. D. Cox, and B. H. Iglewski, submitted for publication). A mutant deficient in the ability to transport iron (PAO-tox^{FeR-10}) was grown in low- and high-iron media and inoculated into traumatized mouse eyes for a determination of 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* (P. A. Sokol, C. D. Cox, D. E. Woods, and B. H. Iglewski, submitted for publication). PAO-tox^{FeR-10} has a markedly reduced rate of iron uptake from pyochelin, pyoverdine, and ⁵⁵FeCl₃ when compared with the parent strain PAO1. PAO-tox^{FeR-10}, however, has the same growth rate in complex medium as does PAO1 (35-min generation time [Sokol, Cox, Woods, and Iglewski, submitted for publication]) so the organism can obtain iron in vitro presumably by a low-affinity iron uptake system. The mutant PAO-tox^{FeR-10} was avirulent in the mouse corneal infection model regardless of whether the infecting inoculum was grown in low- or high-iron medium (Fig. 3).

PAO-tox^{FeR-10} was significantly less capable of establishing corneal infections than either the parent PAO1 or the mutant PAO-tox^{FeR-18}. At least 70% of the eyes infected with 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 were cleared by day 11. In contrast, only 20% of the eyes infected with PAO-tox^{FeR-10} were culture positive on

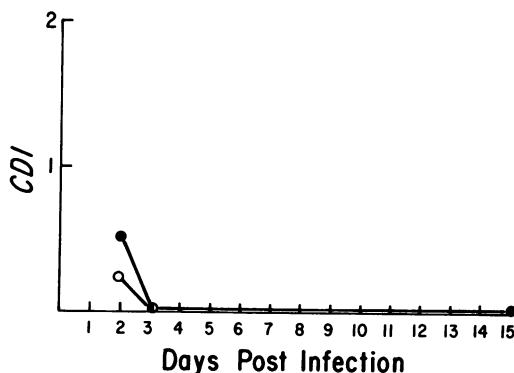


FIG. 3. Average corneal damage resulting from the inoculation of 10^6 colony-forming units of PAO-tox^{FeR-10} grown in low-iron medium (○) and high-iron medium (●) into incised corneas of 10 mice.

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 in which corneal infection models were used 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} (Fig. 2).

If toxin A production is markedly reduced at the time of initial infection, as is the case with PAO1 when the inoculum has been 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 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* (Fig. 3). Therefore, not only is iron important in regulating

toxin A production, but the acquisition of iron by *P. aeruginosa* is a requirement for virulence.

Our results indicate that particular attention must be paid to the medium in which bacteria are cultured before 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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