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Pseudomonas aeruginosa Mutants Altered in Their Sensitivity to the Effect of Iron on Toxin A or Elastase Yields

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Iron affects yields of toxin A, alkaline protease, elastase, pyochelin, and pyoverdin in *Pseudomonas aeruginosa*. Mutants of *P. aeruginosa* PAO1 resistant to the effect of iron on toxin (*toxC*) or elastase (*elaC*) yields were isolated. Two types of mutants were isolated: iron transport and iron regulatory mutants. The *toxC* regulatory mutants produced toxin A in medium containing iron; however, yields of elastase and alkaline protease remained sensitive to regulation by iron. The *elaC* regulatory mutants were resistant to the effect of iron on elastase yields, but toxin A and alkaline protease yields were decreased by iron, analogous to the parent strain. These data suggest that toxin A, elastase, and alkaline protease yields can be independently regulated by iron.

The iron concentration of culture medium significantly affects the yields of several extracellular products of *Pseudomonas aeruginosa*. Yields of toxin A, alkaline protease, elastase (1, 2), and the nonproteinaceous pigments fluorescein and pyocyanine (3, 8) are all reduced as the iron concentration of the medium is increased.

Previously, the effect of iron on toxin A yields was shown to be strain independent (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 shown to be strain dependent (2), suggesting the possibility that in some strains of *P. aeruginosa*, proteases and toxin A are regulated by iron independently. It is not known whether iron coregulates the yields of these extracellular products in strains in which toxin, protease, and elastase yields are all decreased by iron.

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

MATERIALS AND METHODS

Bacterial strain and culture conditions. *P. aeruginosa* PAO1, originally characterized by Holloway et al. (9), was used in this study. Trypticase (BBL Microbiology Systems) soy broth was deferrated with Chelex-100 (Bio-Rad Laboratories), dialyzed, and supplemented with 0.05 M monosodium glutamate and 1% glycerol (14).

Isolation of mutants. Cultures of PAO1 were mutagenized as described previously (14). To identify mutants which produced toxin A in high-iron medium, a modification of the agar well assay was used. $FeSO_4$ was added to the agar medium at a final concentration of 25 μg/ml after autoclaving. This iron concentration completely inhibited the formation of immunoprecipitin bands between colonies of the parent strain, PAO1, and adjacent antiserum wells containing antitoxin A immunoglobulin prepared from immune sheep serum (16). 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 for isolated colonies, retested in the high-iron agar well assay, and stored in sterile skim milk at −70°C.

To identify mutants which produced elastase in high-iron medium, cultures were plated on nutrient agar containing 0.3% elastin (Sigma Chemical Co.) and 375 μg of $FeSO_4$ per ml. This concentration of iron was necessary to inhibit the formation of zones of hydrolysis by the parent strain in this medium. Mutants were identified by their ability to produce zones of hydrolysis on this medium within 72 h at 37°C. Colonies which produced zones of hydrolysis were streaked, retested, and stored in sterile skim milk at −70°C.

ADP-ribosyl transferase activity. The ADP-ribosyl transferase activity of activated (urea and dithiothreitol treated) supernatants from cultures grown in deferrated Trypticase soy broth dialysate was measured as previously described (17) using partially purified elongation factor 2 (4).

Elastase and alkaline protease assays. Elastase and alkaline protease were quantitated in culture supernatants by radioimmunoassays as previously described

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(7, 13). Highly purified *P. aeruginosa* elastase and alkaline protease were obtained from K. Morihara (11). Specific anti elastase antiserum was the kind gift of B. Wretlind, Karolinska Hospital, Stockholm, Sweden. Alkaline protease antiserum was prepared as previously described (7).

Assay of iron uptake. Bacteria were grown to a density of 5×10^8 colony-forming units per ml at 32°C in a shaking water bath. Iron uptake assays in these cultures were initiated by the addition of $^{59}FeCl_3$ (1 mCi/100 μg of iron) (Amersham) to a final concentration of 50 ng/ml. At 10-min intervals, 1-ml samples of the culture were removed, placed on filters (pore size, 0.45 μm; Millipore Corp.), and vacuum filtered. The filters were washed with 10 ml of 0.5% thioglycolate (5), placed in vials, and counted in a Beckman Biogamma counter (Beckman Instruments, Inc.). Control reactions were run without cells to determine background levels of labeled iron associated with the filters. These values were subtracted from reactions with bacteria to yield values of $^{59}FeCl_3$ uptake by bacteria.

Siderophore purification and assay. Pyochelin was purified and quantitated in culture supernatants as previously described (5). Pyoverdin was purified from methanol solutions of spent media 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-hydrochloride buffer. The paper band containing fluorescence was eluted in methanol, and the pyoverdin was precipitated by the addition of ether (100:1 ratio). A filtrate of the water solution of this precipitate was applied to a reverse-phase (ODS2 Ultrasphere) high-pressure column (10 by 25 mm; 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-hydrochloride buffer (0.01 M, pH 7.4). Samples were excited at 400 nm, and fluorescence was measured at 460 nm.

RESULTS

Isolation of mutants. *P. aeruginosa* PAO1 mutants resistant to the effect of iron on toxin A yields (*toxC* mutants) were identified by their ability to produce immunoprecipitin bands in an agar well assay with medium supplemented with $FeSO_4$. Approximately 8,000 colonies were screened in two independent experiments. Eight colonies produced toxin in the high-iron agar well assay, for a mutation frequency of approximately 10^{-3}. Mutants resistant to the iron effect on elastase yields (*elaC*) were identified by their ability to produce zones of hydrolysis on elastin nutrient agar with added iron. A total of 45,250 colonies were examined in four independent experiments. Six mutants were identified which produced elastase on high-iron plates, for a mutation frequency of 1.3×10^{-4}. All mutants isolated were cultured in deferrated Trypticase soy broth dialysate for quantitation of toxin A or elastase yields.

The effect of iron on toxin A yields in three of the *toxC* mutants was compared with that in the parent strain, PAO1 (Fig. 1A). These mutants demonstrated a decrease in toxin A yields as the iron concentration of the medium was markedly increased. Fe10 produced approximately 1.5 to 2 times the amount of toxin A as did the parental strain at all iron concentrations tested. Fe11 and Fe18 produced much greater amounts of toxin A than did either the parent strain or mutant Fe10 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 of iron per ml than the parent strain did even when grown at its optimal (1) iron concentration (0.05 μg/ml). In low-iron medium, these mutants produced approximately four times the amount of toxin A as did PAO1. Thus, Fe11 and Fe18 ae hypertoxin A producers.

Of the six *elaC* mutants isolated, the two (Fe3 and Fe5) which produced the largest zones of hydrolysis on the iron-supplemented elastin agar were selected for further characterization. Fe3 and Fe5 produced approximately the same amount of elastase as did the parent strain, PAO1, at 0.05 μg of iron per ml (Fig. 1B). As the iron concentration of the medium was increased to 0.25 μg/ml or greater, Fe3 and Fe5 produced approximately three times the amount of elastase as did the parent strain. All of the *toxC* and *elaC* mutants had the same generation time (35 min) as the parent strain.

Characterization of *toxC* and *elaC* mutants. To determine whether these mutants were deficient in iron transport, their ability to take up $^{59}FeCl_3$ was examined. Fe11 and Fe18 accumulated $^{59}FeCl_3$ at the same rate as PAO1 (Fig. 2). Fe10, however, had a much slower rate of $^{59}FeCl_3$ uptake than did PAO1 (Fig. 2). Fe3 and Fe5 took up $^{59}FeCl_3$ at approximately the same rate as did the parent strain (data not shown).

Mutants were further characterized for specific siderophore production and uptake systems. *P. aeruginosa* produces two siderophores which are involved in iron transport: pyochelin, a phenolic compound; and pyoverdin, a hydroxamate compound (5, 6, 10). Yields of pyochelin and pyoverdin in culture supernatants varied only slightly among the mutants (Table 1). The only mutant deficient in synthesis of a siderophore was Fe11, which produced markedly less pyoverdin than did the parent strain. Separate mechanisms appear to control pyochelin synthesis and the formation of the uptake mechanism for pyochelin (6). The ability of the mutants to take up iron with either [^{55}Fe]pyoverdin or [^{55}Fe]pyochelin was therefore investigated. Fe3,

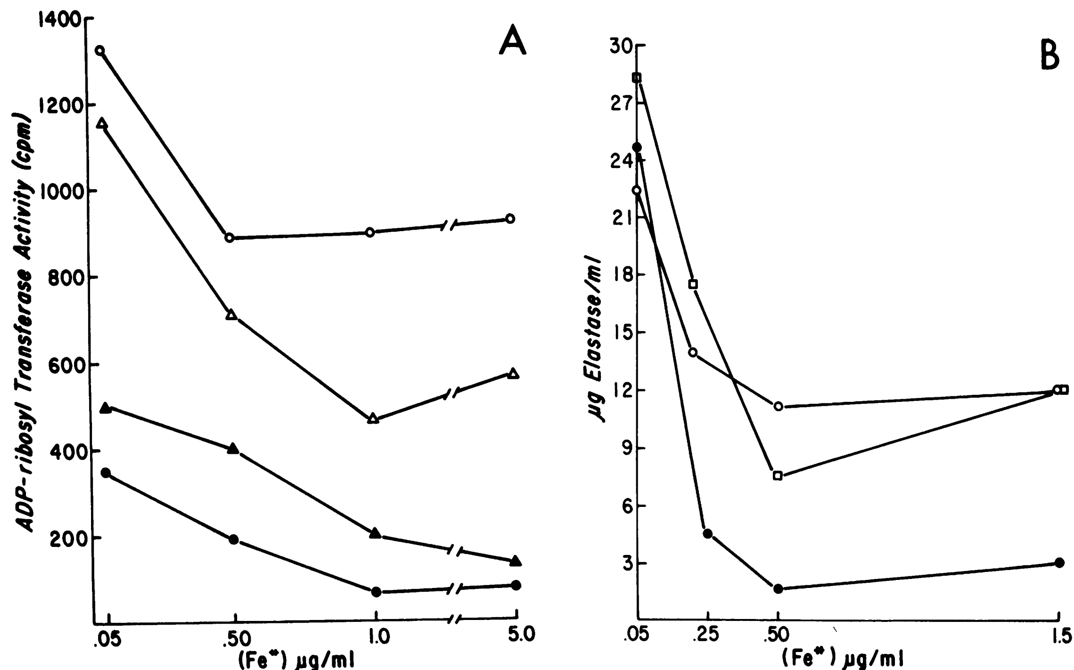

FIG. 1. Effect of iron on toxin A or elastase yields. (A) Toxin A yields in: ●, PAO1; ▲, Fe10; ○, Fe11; △, Fe18. (B) Elastase yields in: ●, PAO1; ○, Fe3; □, Fe5.

Fe5, Fe11, and Fe18 were all similar to the parent strain in the uptake of iron from either of these substrates. The rate of [^{55}Fe]pyoverdin uptake in these strains was in the range of 2.7 to 8 pg/min per mg (dry weight), whereas the rate of [^{55}Fe]pyochelin uptake ranged from 12 to 20 pg/min per mg (dry weight). Mutant Fe10, however, took up pyoverdin and pyochelin at rates of 0.15 and 0.7 pg/min per mg (dry weight), respectively, indicating that it is defective in iron transport.

Those mutants (Fe3, Fe5, and Fe18) which produced parental levels of siderophores and had normal iron transport systems were further analyzed to determine whether their altered sensitivity to the iron effect on either toxin A yields or elastase yields 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 of Fe3 and Fe5 grown in medium with increasing iron concentrations were compared with yields of PAO1 over a range of iron concentrations. Alkaline protease yields in the *elaC* mutants were decreased as the iron concentration of the medium was increased in an identical manner to the parent strain, PAO1 (Fig. 3A). Increased iron concentrations also decreased toxin A yields in the *elaC* mutants to the same extent as in the parent strain (data not shown). Fe18, the *toxC* mutant, was equally sensitive to the iron effect on alkaline protease yields as was the parent strain (Fig. 3B). Elastase yields were also decreased to the same extent with Fe18 as with PAO1 (data not shown).

DISCUSSION

Previously, we reported that increasing the iron concentration of the growth medium decreases the yields of toxin A, elastase, and total protease in *P. aeruginosa* (2). In the present study, we attempted to determine whether iron independently regulates yields of these extracellular products or coregulates these products by

TABLE 1. Production of pyochelin and pyoverdin by *toxC* and *elaC* mutants[a]

Strain	Pyochelin (μg/ml)	Pyoverdin (μg/ml)
PAO1	7.9	25.3
Fe10	10.8	22.4
Fe11	6.1	8.5
Fe18	6.7	18.2
Fe3	9.5	17.5
Fe5	14.4	18.0

[a] Cultures were grown in 0.5% Casamino Acids–0.2 mM $MgCl_2$ (pH 7.4) at 37°C for 18 h.

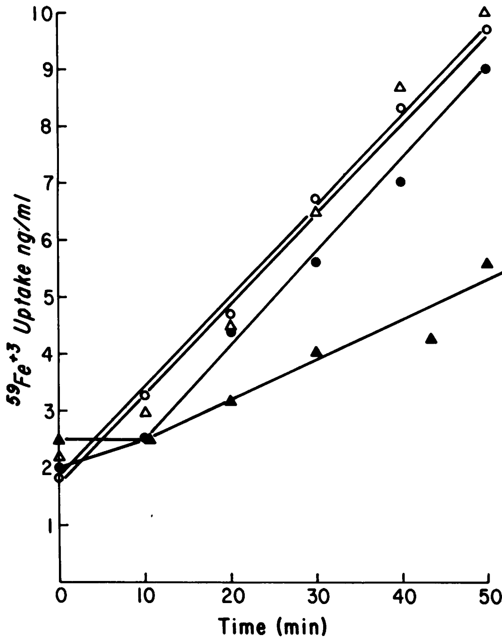

FIG. 2. Uptake of $^{59}FeCl_3$ by *toxC* mutants. Cultures were grown in deferrated Trypticase soy broth dialysate to a density of 5×10^8 colony-forming units per ml. Uptake assays were initiated by the addition of 50 ng of $^{59}FeCl_3$ per ml. One-milliliter samples were removed at intervals and filtered. The filters were counted, and the amount of $^{59}FeCl_3$ accumulated was calculated from a standard curve of $^{59}FeCl_3$. Symbols: ●, PAO1; ▲, Fe10; ○, Fe11; △, Fe18.

some common regulatory mechanism. Mutants of *P. aeruginosa* PAO1 were isolated that were resistant to the iron effect on yields of either toxin A or elastase. The altered sensitivity to iron concentration of toxin 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 would the parent strain; 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. Mutants of both types were identified.

Mutants which appeared to have mutations in iron regulatory genes but not in iron transport genes were further characterized to determine whether 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. Fe18, although resistant to the iron effect of toxin A yields, remained sensitive to the iron effect on both alkaline protease and elastase yields. The *elaC* mutants continued to produce elastase at high iron concentrations, but alkaline protease and toxin A yields were reduced by the same iron concentrations as in the

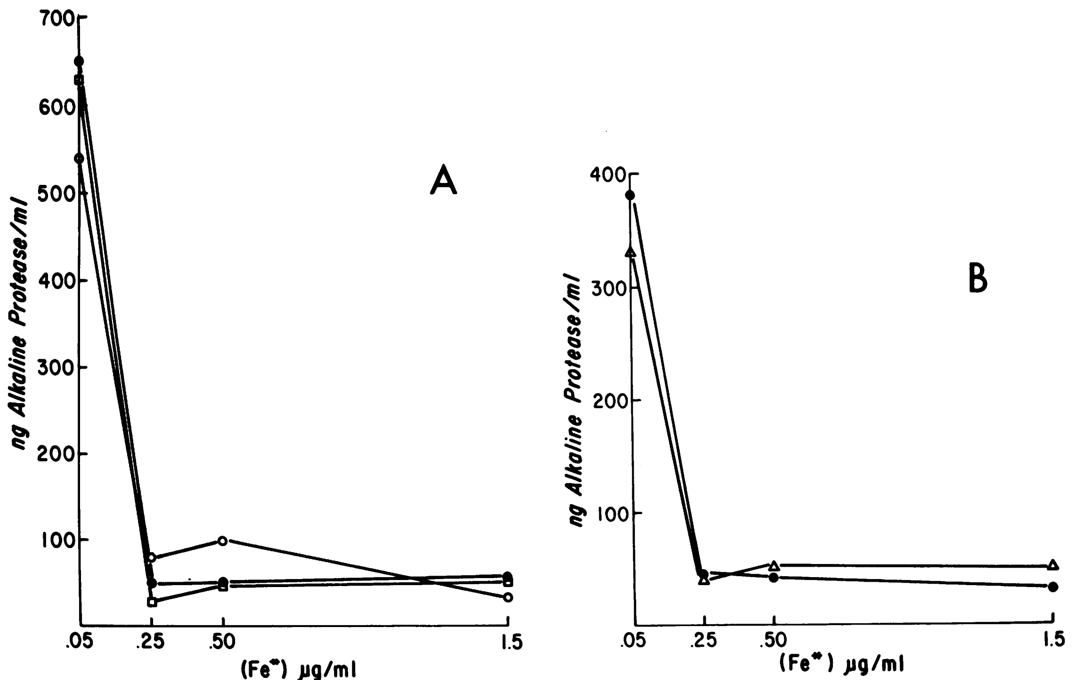

FIG. 3. Effect of iron on alkaline protease yields. Symbols: (A) ●, PAO1; ○, Fe3; □, Fe5. (B) Symbols: ●, PAO1; △, Fe18.

parent strain. These data indicate that iron independently regulates yields of these extracellular products in strain PAO1.

The mechanism(s) by which toxin, elastase, and alkaline protease yields are regulated by iron remains to be determined. Murphy et al. (12) 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, when complexed with iron, become active and bind to the specific product operator(s), thereby preventing synthesis of those extracellular products. Under conditions of iron starvation, the repressors remain inactive and these genes are derepressed. The continued expression of toxin or elastase may be due to the loss of repressor activity or to an alteration in the operator, resulting in the constitutive expression of the product involved. 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. Any of these possibilities is consistent with the independent regulation of extracellular product yields by iron seen in the mutants we have isolated.

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