Effect of Iron on Yields of Exotoxin A in Cultures of *Pseudomonas aeruginosa* PA-103

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The yields of exotoxin A in *Pseudomonas aeruginosa* cultures were influenced by the concentration of iron in the culture media. When the iron concentration of the culture media was increased from 0.05 to 1.5 μg/ml, there was at least a 90% decrease in exotoxin A (as measured both by enzymatic activity and by mouse lethality) and a slight increase in the growth of the bacteria. The addition of iron as late as 13 h after initiation of growth repressed further measurable increases of exotoxin A within 3 h. Intracellular toxin levels were also reduced by increasing the iron concentrations of the culture media. The addition of 3.0 μg of iron per ml did not significantly alter either the enzyme activity of preformed crude or purified exotoxin A or the mouse toxicity of the pure toxin. Thus it appears that either the rate of production or the rate of intracellular degradation of exotoxin A is regulated by the concentration of iron in the culture media.

Pseudomonas aeruginosa exotoxin A is a potentially important virulence factor (14, 16, 17, 24). Exotoxin A has been purified (4, 12, 18), and its effects have been characterized (8, 16, 17, 23, 24). Similar if not identical toxins have been found to be produced by approximately 90% of the strains of *P. aeruginosa* tested (3, 25). Exotoxin A, like diphtheria toxin, inhibits mammalian protein synthesis by catalyzing the transfer of the adenosine 5'-diphosphate-ribose (ADPR) from NAD$^+$ (nicotinamide adenine dinucleotide) onto mammalian elongation factor 2 (EF-2) (9-11).

Detectable quantities of exotoxin A were found in some but not all supernatants of media in which toxinogenic strains of *P. aeruginosa* were grown (14, 15, 17). Furthermore, individual strains of *P. aeruginosa* reportedly differ in their requirements for optimal toxin production (17). Since exotoxin A is not formed constitutively by toxinogenic strains of *P. aeruginosa*, it is likely that specific regulatory systems or factors control the synthesis and secretion of exotoxin A in a manner that is distinct from the regulation of bulk protein synthesis.

The present communication describes the effect of iron on the yields of exotoxin A and on the activity of preformed exotoxin A.

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MATERIALS AND METHODS

Reagents. NAD ([^{14}C]adenine) at 280 mCi/mmol was purchased from Amersham/Searle Corp., dithiothreitol (DTT) was purchased from Sigma Chemical Co., and lysozyme was purchased from Worthington Biochemicals Corp.

Microorganisms. *P. aeruginosa* strain PA-103, kindly provided by P. V. Liu (15), was used as a standard toxin-producing strain because it produces very little toxin-destroying protease (15, 31). Strain PA-103 was stored at −70°C in 12% glycerol.

Exotoxin A. Strain PA-103 was used to produce exotoxin A (15), which was purified as described previously (30). The purified toxin had a mouse 50% lethal dose (LD$_{50}$) of 0.25 μg per 22-g mouse when injected intraperitoneally, and migrated as a single homogeneous protein in sodium dodecyl sulfate-polyacrylamide gels with a molecular weight of 71,500. Exotoxin A was stored in small portions at −70°C.

The specific A antitoxin was prepared against highly purified toxin in rabbits as previously described (3).

Culture media. The culture medium used was that previously developed by Liu (15). This consisted of the dialysate from Trypticase soy broth, 0.05 M monosodium glutamate, and 1% glycerol. The medium was deferrated to obtain an iron concentration of 0.05 μg/ml by adding 2 ml of 50% CaCl$_2$·2H$_2$O per liter, boiling for 5 min in a water bath, and filtering through Whatman no. 1 filter paper to remove the precipitate (21). Residual iron in the medium was determined as described by Mueller and Miller (21). To obtain known concentrations of iron in the deferrated medium, standard sterile solutions of FeSO$_4$·7H$_2$O were added.

Culture flasks were acid cleaned and rinsed with 20 changes of deionized water. Unless otherwise stated,

50 ml of medium was added to a 1-liter Erlenmeyer flask (flask-to-volume ratio of 20:1) and inoculated with 0.1 ml of a 15-h culture of PA-103. The flasks were incubated at 32°C on a reciprocating shaker (150 linear excursions/min) (Lab-line Instruments, Melrose, Ill.). Optical densities were obtained by aseptically removing a portion of the culture and reading the optical density at 540 nm in a Beckman spectrophotometer 20.

The culture supernatants were obtained by centrifugation at 10,000 × g for 20 min. The supernatants were filter sterilized, immediately frozen, and stored at −70°C. Both the animal toxicity and the enzymatic activity of toxin A were stable for at least 4 months when the toxin was stored at −70°C.

ADPR-transferase activity. Aminoacyl transferase-containing enzymes were prepared from crude extracts of rabbit reticulocytes as described by Allen and Schweet (1) and modified by Collier and Kandel (6). The ADPR-transferase activities of cultural supernatants and purified exotoxin A were measured according to the procedure of Collier and Kandel (6) modified exactly as described previously (3). Exotoxin A is found in culture supernatants as a proenzyme, and treatment with a denaturing agent and a reducing agent activates the ADPR-transferase activity of exotoxin A (12, 30). In this study, the ADPR-transferase activity was measured on culture supernatants both before and immediately after treatment with 4 M urea and 1% DTT as previously described (30).

Mouse lethality. Female Swiss-Webster mice (14 to 17 g) were used to determine the LD$_{50}$ of toxin preparations (27). These toxin preparations were diluted in sterile physiological saline, pH 7.8, and 1-ml portions were injected intraperitoneally into each of at least four mice.

Solid-phase radioimmune inhibition assay. Exotoxin A was quantified by a modification of a solid-phase radioimmune inhibition assay originally described by Zollinger and Mandrell (33). The wells of polyvinyl flexible U microtiter plates (Cooke Laboratory Products Inc., Alexandria, Va.) were coated with purified exotoxin A diluted in Dulbecco phosphate-buffered saline (PBS) (Grand Island Biological Co., Grand Island, N.Y.) to a concentration of 50 µg/ml by placing 25 µl of diluted antigen in the wells and allowing the antigen to bind for 1 h at 37°C. After aspiration of unbound antigen, the wells were rinsed with 50 µl of filler (10% fetal calf serum, 0.2% sodium azide, and 0.02% phenol red in PBS, pH 7.4) followed by addition of 100 µl of filler and incubation at 37°C for 1 h. The solution was then dumped out, and the wells were washed twice with PBS. Inhibition mixtures consisting of 50 µl of exotoxin A antisera diluted 1:8,000 in filler and 50 µl of various dilutions in filler of unknown or standard exotoxin A samples were placed in the well of a separate microtiter plate and incubated at 37°C for 1 h. The final concentration of exotoxin A antisera corresponded to approximately 50 ng of antitoxin per well as previously determined by a direct solid-phase binding assay (32). Controls consisted of filler only and filler plus antitoxin. After incubation, duplicate 25-µl samples of the mixture were placed in the antigen-coated wells of the flexible microtiter plate. Binding was allowed to proceed overnight at room tempera-

ture, the liquid was aspirated, and the wells were washed once with filler and five times with PBS. Goat anti-rabbit immunoglobulin (Antibodies Inc., Davis, Calif.) was further purified and labeled with ^{125}I (^{125}I-GARG) as described by Zollinger et al. (32). ^{125}I-GARG (25 µl) was placed in each well and allowed to bind overnight at room temperature. The liquid was aspirated, and the wells were washed once with filler and four times with PBS. The wells were then cut off into tubes and counted as previously described (32). The concentration of exotoxin A in each sample was determined from a standard curve obtained by using known amounts of pure exotoxin A. The percentage of inhibition was calculated by the formula:

percent inhibition = 100 −

$$\frac{\text{mean cpm bound with inhibitor} - \text{background} \times 100}{\text{mean cpm bound without inhibitor} - \text{background}}$$

Antigen-coated wells that received filler only were used as the background controls. The uninhibited controls received an inhibition mixture that contained filler instead of toxin samples. As shown in Fig. 1, the standard curve was linear when 0.04 to 0.2 µg of exotoxin A per ml was used in the competition mixtures.

Extraction of intracellular toxin. Two methods, sonic treatment and lysis of spheroplasts, were used to extract intracellular toxin from strain PA-103. In both procedures, the cells from 22-h cultures were extensively washed with 0.01 M tris(hydroxymethyl)-aminomethane (Tris) buffer, pH 8.0 (Sigma). Washed cells were resuspended in 10 ml of Tris buffer and disrupted by sonic treatment with ten 30-s bursts (50% maximal output), using a Biosonik IV (Bronwill) sonic oscillator. Each burst was followed by a 2-min incubation in an ice bath. The above treatment resulted in the destruction of greater than 90% of the cells as determined by light microscopy. The cellular debris was removed by centrifugation at 10,000 × g for 45 min, and the resulting intracellular extract was filter sterilized and stored at −70°C.

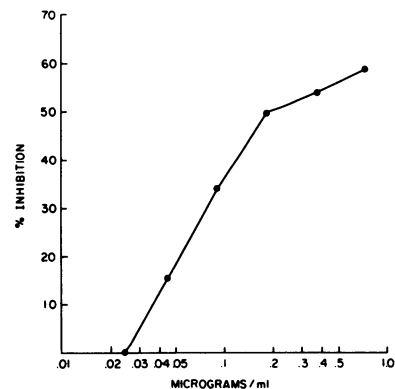FIG. 1. *Solid-phase radioimmune competition assay: standard curve obtained with exotoxin A.* (●) *Percentage of inhibition of binding of* ^{125}I-GARG *in the presence of increasing concentrations of pure exotoxin A.*

Ethylenediaminetetraacetate-lysozyme sphero-plasts of PA-103 were obtained by using the procedure of Birdsell and Cota-Robles (2) with the following modifications: lysozyme was added to a concentration of 40 $\mu g/ml$, and the cells were incubated at room temperature for 15 min. The osmotically fragile spher-oplasts were lysed by the addition of cold distilled-deionized water. The resulting intracellular extract was clarified by centrifuging at $10,000 \times g$ for 45 min to remove cellular debris, filter sterilized, and stored at $-70°C$.

Protein determinations. Protein was determined by a modification of the Lowry method (13).

RESULTS

Effect of iron on yields of exotoxin A. Strain PA-103 was grown for 22 h in media containing various iron (ferrous sulfate) concen-trations. The yield of exotoxin A as measured by its enzymatic activity was maximal in cultures containing 0.05 μg of iron per ml. Increasing the iron concentration from 0.05 to 0.1 $\mu g/ml$ de-creased the exotoxin A yield (Fig. 2). The toxin yield in a culture containing 1.5 $\mu g/ml$ was less than 10% of that in a culture containing 0.05 $\mu g/ml$. The inhibitory effect of iron on exotoxin A yields began to level off at iron concentrations greater than 1.0 $\mu g/ml$, and small amounts of toxin activity could still be detected in superna-tants of cultures containing 10 μg of iron per ml.

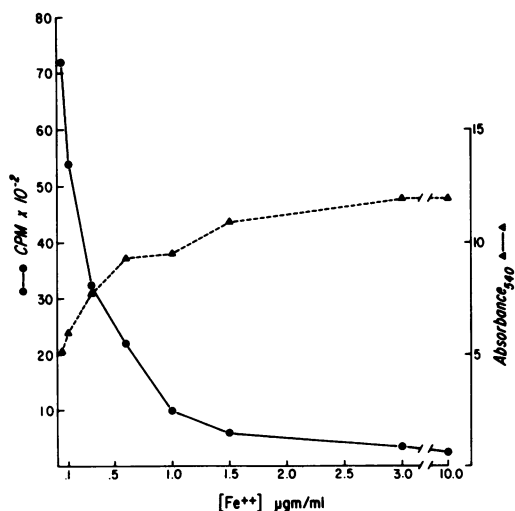Increasing the iron concentration of the growth media also decreased the mouse lethality of the supernatants of such cultures. The mouse LD_{50} of a culture supernatant containing 0.05 μg of iron per ml was contained in 1 ml of a 1:75 dilution, whereas the LD_{50} of a culture contain-ing 3.0 $\mu g/ml$ was contained in 1 ml of a 1:15 dilution (Table 1). That the toxicity of the cul-ture supernatants was due to exotoxin A was shown by the ability of specific antitoxin to neutralize this toxicity (Table 1). The small amount of residual toxicity of the antitoxin-treated crude supernatant was probably due to other extracellular products known to be pro-duced by *P. aeruginosa* (16).

Effect of iron on the kinetics of growth and exotoxin A release. Strain PA-103 was grown in medium under two different iron (fer-rous sulfate) concentrations, 0.05 and 1.5 $\mu g/ml$ (Fig. 3). Increasing the iron concentration of the medium had no significant effect on the growth rate of PA-103 through the first 19 h of culture. The culture grown in medium containing 0.05 μg of iron per ml entered maximum stationary phase at 19 h, whereas the culture grown in medium containing 1.5 $\mu g/ml$ continued to in-crease through 24 h, resulting in a slightly larger final yield of bacteria.

The earliest time at which exotoxin A could be detected in the culture grown in medium containing 0.05 μg of iron per ml was 9 h (Fig. 3). The concentration of exotoxin A continued to increase in this culture through 19 h. Exotoxin A was not detectable until 11 h in the culture grown in medium containing 1.5 μg of iron per ml, and both the rate of release and the final yield of exotoxin A were markedly decreased as compared with the culture grown in medium containing 0.05 $\mu g/ml$ (Fig. 3).

Kinetics of toxin release after the addi-tion of iron to low-iron growth media. Strain PA-103 was grown in low-iron (0.05

FIG. 2. *Effect of increasing concentrations of iron on the yield of exotoxin A in cultures of P. aeruginosa PA-103. CPM $\times$ 10^{-2} is the acid-insoluble radioactiv-ity counts per minute from the ADPR-transferase assay of activated (urea + DTT-treated) samples. Symbols: (●) ADPR-transferase activity (cpm $\times$ 10^{-2}); (▲) bacterial growth as measured by optical density at 540 nm.*

TABLE 1. LD_{50} *values of supernatants from cultures grown in media containing 0.05 and 1.5 μg of iron per ml*

Concn of iron in medium ($\mu g/ml$)	Treatment[a]	LD_{50} (dilution of supernatant)
0.05	None (saline)	1:75
	NRS[b]	>1:100
	Antitoxin	1:4
1.5	None	1:15

[a] One part of supernatant was mixed with one part of saline, normal rabbit serum, or antitoxin and incu-bated for 5 min at 37°C before being injected into mice.

[b] Normal rabbit serum. We have consistently found that the addition of normal serum or bovine serum albumin enhances the toxicity of toxin A (probably by stabilizing the toxin molecule).
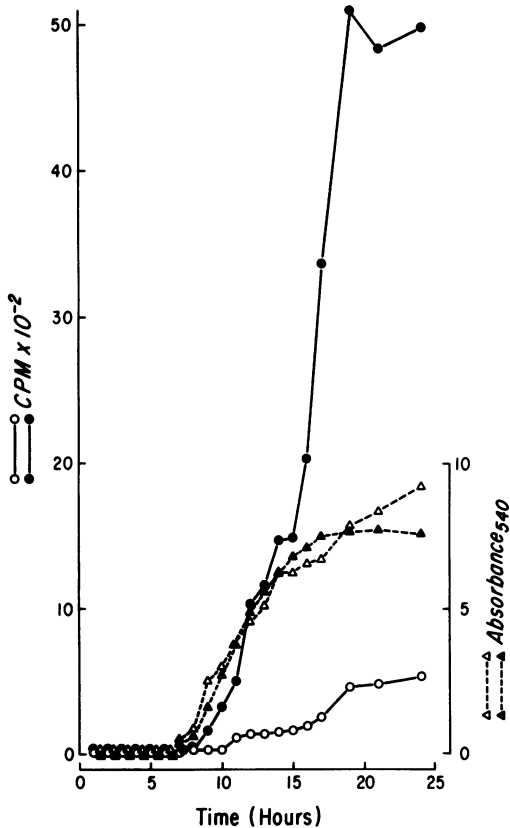
FIG. 3. *Effect of iron on the kinetics of growth and exotoxin A release. Symbols: (●) ADPR-transferase activity (cpm × 10^{-2}) of PA-103 supernatants in low-iron (0.05 μg/ml) media; (○) ADPR-transferase activity in high-iron (1.5 μg/ml) media. Bacterial growth as measured by optical density at 540 nm in (▲) low- and (△) high-iron media.*

μg/ml) growth medium. After 13 h of growth, the culture was split into two separate flasks. To one flask enough ferrous sulfate was added to result in a final concentration of 1.5 μg of iron per ml. No additional iron was added to the second flask. The cultures were returned to the shaker, and equal portions were removed at hourly intervals and assayed for exotoxin A enzyme activity. There was a lag period of 3 h before a significant difference in the enzyme activity of the two cultures was observed (Fig. 4). At this point the concentration of exotoxin A leveled off in the 1.5-μg/ml culture but continued to increase in the 0.05-μg/ml culture. It should be noted that the enzyme activity of the culture grown in medium containing 1.5 μg of iron per ml, although not increasing after 15 h, did remain constant through 24 h and did not decrease as might have occurred if protease activity was increasing in the presence of added iron.

Effect of iron on the biological activity of preformed exotoxin A. While the stability of the enzyme activity of exotoxin A released into the medium containing 1.5 μg of iron per ml (Fig. 3 and 4) suggested that iron was not affecting the biological activity of preformed exotoxin A, this possibility was tested further by adding iron to preformed exotoxin A. The addition of 3 μg of iron per ml had no significant effect on the enzymatic activity of either crude or purified toxin (Table 2). The enzymatic activity of exotoxin A has been shown to be potentiated by treatment with a combination of denaturing and reducing agents (12, 30). Iron had no effect on the ability of urea and DTT to potentiate the enzymatic activity of exotoxin A, nor did the addition of iron effect the mouse lethality of preformed exotoxin A (Table 2).
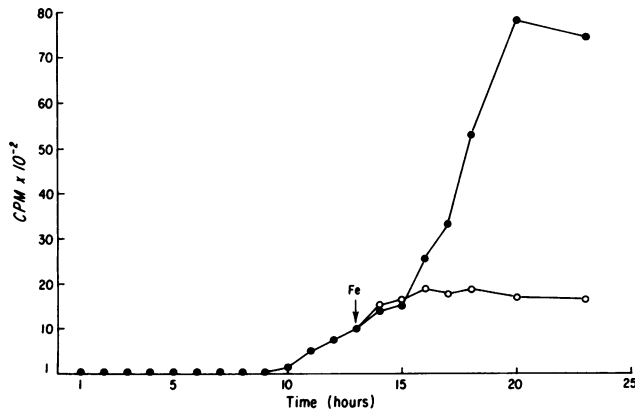

FIG. 4. *Kinetics of exotoxin A release after the addition of iron to low-iron (0.05 μg/ml) media. The culture was split into two separate cultures at 13 h, and iron was added to one flask to give a concentration of 1.5 μg/ml. Symbols: ADPR-transferase activity (cpm × 10^{-2}) in cultures grown in (●) low-iron (0.05 μg/ml) and (○) high-iron (1.5 μg/ml) media.*

Effect of increasing iron concentrations in the culture media on the intracellular toxin concentration. It is possible that the effects of iron are due to changes in the transport of toxin out of the cell. In this case, it might be expected that the intracellular concentration of toxin would either remain essentially constant or increase as the iron concentration in the growth medium was increased. To test this, we sonically treated washed cells of PA-103 that were grown in medium supplemented with various concentrations of iron and measured the enzyme activity of the cell lysate. Increasing the concentration of iron in the growth medium resulted in a decrease in the intracellular exotoxin A activity (Table 3).

An unexpected result of this experiment was that the intracellular toxin appeared to be in an altered enzymatic form. Thus the activity of the intracellular toxin was not increased by treatment with urea and DTT (Table 3). As previously reported (30), the enzyme activity of culture supernatants of PA-103 was increased by treatment with a denaturant (4 M urea) and a reducing agent (1% DTT). In contrast, the enzyme activity of the intracellular toxin obtained by sonic treatment was actually decreased by treatment with these agents. Furthermore, when the intracellular toxin was obtained by a completely different method (lysis of spheroplasts), it again was in the active form (data not shown). The enzymatic activity of the intracellular toxin, like that of extracellular toxin, was completely neutralized by preincubation with specific anti-A toxin antibody (data not shown). To quanti-

TABLE 2. *Effect of iron on the activity of preformed toxin*

	Enzyme activity[b]	Mouse LD$_{50}$ (μg)
Crude supernatant prepn[a]		
Untreated	1.0	
Supernatant + Fe	1.05	
Supernatant + urea, DTT	8.6	
Supernatant + Fe, urea, DTT	8.3	
Pure toxin prepn[a]		
Untreated	1.0	0.25
Toxin + Fe	0.9	0.25
Toxin + urea, DTT	4.0	
Toxin + Fe, urea, DTT	3.7	

[a] Iron was added to give a final concentration of 3.0 μg/ml.
[b] Enzyme activities are based on the acid-insoluble radioactivity counts per minute from the ADPR-transferase assay, where counts per minute from the assay of the untreated toxin or supernatant preparation are equivalent to 1.0 activity units.

TABLE 3. *Effect of increasing concentrations of iron in the culture media on the intracellular toxin A concentration*[a]

Concn of iron in medium (μg/ml)	ADPR-transferase activity		Exotoxin A (μg/ml)[b]
	Untreated[c]	Treated[d]	
0.05	236	41	0.16
0.5	89	0	0.08
2.5	87	0	
5.0			ND[e]
10.0	0	0	

[a] The lysates were adjusted to a protein concentration of 260 μg/ml.
[b] Obtained by using the solid-phase radioimmune inhibition assay described in the text.
[c] Counts per minute of untreated portion.
[d] Counts per minute of portion treated with urea and DTT.
[e] ND, Not detectable.

tate intracellular toxin A independent of its enzymatic activity, a solid-phase radioimmune inhibition assay as described in Materials and Methods was utilized. Increasing the iron concentration of the culture medium from 0.05 to 0.5 μg/ml decreased the intracellular toxin from 0.16 to 0.08 μg/ml, and when the iron concentration was 5.0 μg/ml, exotoxin A protein could not be detected in the cell sonic fluid (Table 3).

DISCUSSION

Liu (15) reported that a number of factors, such as aeration, 32°C, and the presence of glycerol, were required for maximal yields of exotoxin A. He also reported that nucleic acids inhibited production of exotoxin A but enhanced the growth of *P. aeruginosa* strain PA-103 (15). Furthermore, individual strains of *P. aeruginosa* differed in their requirements for optimal toxin production (17). Thus exotoxin A is not formed constitutively by toxinogenic strains of *P. aeruginosa*.

This report describes another factor that influences the yield of exotoxin A in cultures of *P. aeruginosa* PA-103. We have shown that the yield of exotoxin A is influenced by the concentration of iron in the culture media. The toxin yield was greatest under conditions of low iron (approximately 0.05 μg/ml) in the culture medium (Fig. 2). Strain PA-103 grown in media supplemented with increasing concentrations of iron yielded proportionally less toxin (Fig. 2 and 3; Table 1). The rate of release of exotoxin A into the culture medium was also greater in cultures grown in low-iron media as compared with those grown in media containing added iron (Fig. 3). Furthermore, adding iron to a culture

of PA-103 already producing toxin inhibited further increase in extracellular toxin levels within 3 h (Fig. 4).

Intracellular levels of exotoxin A as measured in a solid-phase radioimmune competition assay were also reduced by growing strain PA-103 in culture medium containing increasing concentrations of iron (Table 3). This assay was particularly useful since it detected small amounts of toxin (0.04 µg/ml), and the standard curve obtained with pure toxin was the same regardless of the enzymatic form of the toxin. Thus the standard curve was unaltered when pure exotoxin A was or was not treated with urea and DTT to convert it to its enzymatically active form (J. Sadoff, unpublished observation). This proved important in the current study since the biological activity of the intracellular toxin was different from that of the extracellular toxin. Thus the intracellular toxin was enzymatically active (Table 3), whereas the extracellular toxin (as previously reported [12, 30]) required treatment with urea and DTT to convert it from its proenzyme form to its enzymatically active form. Since the intracellular extract was obtained by sonic treatment, it was possible that the sonic treatment in some way "activated" the intracellular toxin. Although this possibility cannot be totally ruled out, two lines of evidence tend to discount it. First, the enzyme activity of supernatants that were sonically treated in the same manner as the cells could still be increased by treatment with urea and DTT, and second, the intracellular toxin obtained by a completely different method (lysis of spheroplasts) was in the active form (data not shown). The possibility that the bulk of intracellular toxin A is in the enzymatically active, nonlethal form (12, 30) suggests that the structure of toxin A may somehow be altered during or after secretion from the bacterial cell.

The addition of iron to preformed exotoxin A did not alter its mouse toxicity, reduce its enzymatic activity, or convert the toxin from its proenzyme form to its active enzyme form (Table 2). Thus it appears likely that increasing the iron content of the culture medium reduced the yield of exotoxin A by either decreasing the rate of production or increasing the rate of intracellular degradation of toxin.

The final yield of bacteria was slightly increased in high-iron compared with low-iron cultures (Fig. 2 and 3). However, no significant effect of iron could be seen on the rate of bacterial growth through 19 h of culture (Fig. 3). Furthermore, although the iron effect on exotoxin A yields was quite reproducible, the effect on bacterial growth varied somewhat from day to day. Although the studies reported here were carried out using the ferrous ion (in the form of $FeSO_4 \cdot 7H_2O$), we have obtained similar results using the ferric ion.

Diphtheria toxin and exotoxin A from *P. aeruginosa* have similar if not identical modes of action. Both toxins catalyze the transfer of the adenosine 5'-diphosphate-ribosyl moiety of NAD^+ onto EF-2 (5, 9–11). This covalent modification of EF-2 renders it unable to cause translocation of ribosomes along messenger ribonucleic acid during protein synthesis (5). It has long been known that the production of diphtheria toxin (7, 19, 20, 22, 26, 28) is regulated by the concentration of iron in the growth media. It has also been reported that the production of *Shigella dysenteriae* toxin is regulated by the concentration of iron in the culture media (29). This study extends the effect of iron on toxin yields to *P. aeruginosa* exotoxin A.

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