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Influence of Iron on Yields of Extracellular Products in *Pseudomonas aeruginosa* Cultures

MICHAEL J. BJORN, PAMELA A. SOKOL, AND BARBARA H. IGLEWSKI*

Department of Microbiology and Immunology, University of Oregon Health Sciences Center, Portland, Oregon 97201

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The effect of the iron content of the medium on the yields of extracellular products by seven distinct strains of *Pseudomonas aeruginosa* was examined. All strains showed at least an 85% decrease in toxin A yields when grown in medium containing 5.0 μg of iron per ml (high iron) as compared to 0.05 μg/ml (low iron), whereas bacterial growth increased approximately twofold. During the course of examining extracellular products produced by *P. aeruginosa*, we found many strains that produced an extracellular factor which agglutinated erythrocytes. This hemagglutinin was nondialyzable, heat stable, and resistant to Pronase and trypsin. The effect of iron on extracellular yields of hemagglutinin was strain dependent; four of seven strains showed decreases in hemagglutinin yields in high-iron medium. Similarly, the effect of increasing the iron concentration of the growth medium on yields of total extracellular proteases or on elastase was strain dependent. The amount of total extracellular protein was decreased by at least 31% in the high-iron medium for all strains of *P. aeruginosa* examined. Detailed studies on one strain (WR-9) showed that, in the presence of increasing amounts of iron in the medium, the extracellular yields of toxin A, protease, and hemagglutinin were decreased in a similar manner. In addition, the kinetics of release of these extracellular products were similar at a given iron concentration. Thus it appears that the yields of other extracellular products of *P. aeruginosa* besides toxin A are influenced by the concentration of iron in the growth medium.

The presence of excess iron in the culture medium has been shown to inhibit yields of diphtheria toxin (16, 24, 26), *Shigella dysenteriae* type 1 toxin (5, 29), and *Pseudomonas aeruginosa* toxin A (1). These toxins are produced at maximal levels late in the bacterial growth cycle when iron is growth limiting (1, 6, 27).

Although the structural gene for diphtheria toxin is located on the DNA of toxin-positive corynebacteriophage, the physiological state of the host bacterium has been shown to influence toxin production (13, 22, 23). Thus Kanei et al. (13) isolated bacterial mutants that produced diphtheria toxin at the normal rate in medium containing excess iron. It is generally believed that in *Corynebacterium diphtheriae* the iron effect is specifically restricted to the toxin gene product because phage production continues in the presence of excess iron (11).

The location of the structural gene for *Pseudomonas* toxin A is presently unknown. However, the deleterious effect of iron on yields of extracellular products of *P. aeruginosa* is not restricted to toxin A. Yields of the non-proteinaceous pigments fluorescein and pyocyanine reportedly decreased with increasing concentrations of iron in the growth medium (3, 10, 14, 28).

Although the relationship between iron and the yields of bacterial toxins has been the subject of numerous studies (1, 5, 16, 24, 26, 29), the molecular mechanism(s) underlying iron regulation remains unknown. The current study was undertaken to extend our previous results on toxin A yields by strain PA-103 (1) to different strains of *P. aeruginosa* and to determine if the iron concentration of the growth medium influences the extracellular yields of other *P. aeruginosa* products such as proteases and hemagglutinin (HA).

MATERIALS AND METHODS

Microorganisms. *P. aeruginosa* PA-103, originally isolated by Lui (17), has been extensively characterized (25). The other *P. aeruginosa* strains utilized in this study (WR-4, WR-9, WR-27, WR-28, WR-35, and WR-56) were kind gifts of J. Sadoff, Walter Reed Army Institute of Research, Washington, D.C. The organisms were serotyped (8), using antisera prepared and kindly provided by M. Fisher, Parke Davis Co., Detroit, Mich. Pyocin typing was performed using the

indicator strains and method of Farmer and Herman (7).

Reagents. NAD ([^{14}C]adenine) at 280 mCi/mmol was purchased from Amersham Corp. Dithiothreitol, elastin-Congo red, crystalline bovine serum albumin, and casein (technical grade) were purchased from Sigma Chemical Co., and bovine gamma globulin came from Bio-Rad Laboratories.

Medium and culture conditions. The culture medium consisted of Trypticase soy broth dialysate, 1% glycerol, and 0.05 M monosodium glutamate, as previously described by Lui (18). The medium was deferrated (21), and the residual iron concentration was determined by the procedure of Mueller and Miller (21) as previously described (1). To obtain known concentrations (0.05 to 5.0 μg of Fe per ml; 0.90 to 90 μM) of iron in the deferrated medium, standard sterile solutions of $FeSO_4 \cdot 7H_2O$ were added.

Culture flasks were acid cleaned and rinsed with 20 changes of deionized water. Unless otherwise stated, 10 ml of medium was added to a 125-ml Erlenmeyer flask and inoculated with 0.1 ml of a 15-h shaking culture of the appropriate *P. aeruginosa* strain. The flasks were incubated at 32°C in a reciprocating shaker (150 linear excursions per min) (Lab-line Instruments, Melrose, Ill.) for 22 h. Bacterial growth was measured by aseptically removing a portion of the culture and measuring the turbidity at 540 nm in a Beckman Spectrophotometer 20. The cultural supernatants were obtained by centrifugation at 10,000 × *g* for 20 min. The supernatants were dialyzed against 0.01 M Tris-hydrochloride buffer (pH 8.0) at 5°C for approximately 18 h, and then stored at −70°C.

ADP-ribosyl transferase activity. Partially purified EF-2 was prepared from extracts of wheat germ as described by Chung and Collier (4). The ADP-ribosyl transferase activity of activated (urea and dithiothreitol-treated) supernatants was measured as previously described (30). To quantitate toxin A based on its enzymatic activity, standard curves were obtained daily with pure toxin, and the amount of toxin A present in a crude supernatant was calculated from the standard curves (12).

Protease assays. Total proteolytic activity in the crude supernatants of cultures of *P. aeruginosa* was determined by the method of Kunitz (15) as modified by Wretlind and Wadstrom (31), using casein as the substrate. To minimize variation, a single batch of casein was purchased and used throughout this study. One unit of proteolytic activity was defined as a change of one optical density (280 nm) unit, by the assay method of Wretlind and Wadstrom (31).

Elastase activity was quantitated using elastin-Congo red as a substrate. The reaction mixture consisted of Tris-maleate buffer (0.1 M, pH 7.0) supplemented with $CaCl_2$ (1 mM) (buffer A). One milliliter of culture supernatant was added to 2 ml of buffer A containing 10 mg of elastin-Congo red. The reaction was carried out in stoppered 15-ml centrifuge tubes incubated for 2 h in a 37°C water bath with rapid shaking. The reaction was terminated by the addition of 2 ml of sodium phosphate buffer (0.7 M, pH 6.0). The precipitate was removed by centrifugation. The blank consisted of 3 ml of buffer A containing 10 mg of elastin-Congo red. Elastase activity was determined by reading absorbance of the supernatants at 495 nm in a Beckman Spectrophotometer 20.

HA assay. Fresh sheep erythrocytes stored in Alsevers solution for 3 to 5 days were washed three times with at least 20 cell volumes of the following buffer (buffer B): 0.082 M NaCl–0.043 M $Na_2HPO_4 \cdot 2H_2O$–0.0107 M KH_2PO_4 (pH 7.4). The washed cells were formalinized using the method of Galazka and Abgarowicz (9). Equal volumes of 10% (vol/vol) washed sheep cells (in buffer B) and 3% Formalin were mixed and stirred at 37°C for 18 h. The cells were centrifuged, washed five times in 2.5 volumes of buffer B, suspended to a 10% concentration in buffer B containing Merthiolate (1:10,000), and stored for up to 2 months at 4°C. Just beore use, the cells were washed and suspended in buffer B containing Merthiolate (1:10,000) to a final concentration of 0.2% (vol/vol). HA assays were performed in plastic microtiter plates with V-bottom wells (Linbro Chemical Co., Inc., New Haven, Conn.). Serial twofold dilutions (in buffer B containing 1% bovine serum albumin and 1:10,000 Merthiolate) of *P. aeruginosa* supernatants were made, and 50 μl of each dilution was added to the wells of a microtiter plate. Next, 50 μl of a 0.2% (vol/vol) suspension of formalinized sheep cells was added to each well. The plates were incubated at 25°C for 24 h and examined for HA activity. The reciprocal of the highest dilution that showed complete hemagglutination was considered the HA titer. Formalinized sheep erythrocytes were utilized to assay for the HA because they were much more stable than non-formalinized sheep erythrocytes and because they were resistant to the action of *P. aeruginosa* hemolysins (19), which may have otherwise made interpretation of the results difficult.

Protein determination. Protein was determined by the method of Bradford (2) modified by using a commercial reagent, Bio-Rad Protein Assay Dye Reagent Concentrate, purchased from Bio-Rad Laboratories, Richmond, Calif. Bovine gamma globulin was used as the standard.

RESULTS

Characterization of *P. aeruginosa* strains. The *P. aeruginosa* strains used in this study were originally isolated from human infections and differed from one another in serotype or pyocin type (Table 1). These isolates were chosen for this study because they represented seven distinct strains of *P. aeruginosa* which produced detectable quantities of toxin A in vitro. The decision to utilize these strains was made independent of their ability to produce proteases, elastase, or HA activity. However, all seven of the strains produced proteases and HA, and six of seven strains produced detectable elastase (Table 1).

Growth in high- and low-iron media. The *P. aeruginosa* strains were grown in deferrated medium containing 0.05 μg of iron per ml (low iron) and in deferrated medium to which iron was added to give a final concentration of 5.0

TABLE 1. *Characterization of P. aeruginosa strains*

Strain	Source	Serotype	Pyocin type	Toxin A	Protease	Elastase	HA
PA-103	Sputum	2	611 131	+	+	−	+
WR-4	Perineal ulcer	NR[a]	587 688	+	+	+	+
WR-9	Wound	2	617 161	+	+	+	+
WR-27	Blood	1	621 611	+	+	+	+
WR-28	Sputum	6	211 216	+	+	+	+
WR-35	Wound	5	111 214	+	+	+	+
WR-56	Sputum	3, 7	113 216	+	+	+	+

[a] NR, No reaction (agglutination) with any of the seven typing sera.

μg/ml (high iron). All seven of the strains grew to a higher cell density (approximately twofold) in the high-iron medium than in the low-iron medium (Table 2).

Toxin A yields in high- and low-iron media. The yields of toxin A in supernatants from cultures of the seven strains of *P. aeruginosa* grown in high- and low-iron media were examined. The quantity of toxin A in the supernatant fluids was calculated from standard curves obtained by assaying known nanogram amounts of pure toxin A (Fig. 1). Using our previously reported assay system, which included a 5-min incubation period (30), we were able to detect 4 ng of toxin A (400 ng/ml) (Fig. 1A). The sensitivity of the assay system was increased to 0.5 ng of toxin A (50 ng/ml) by extending the incubation period from 5 to 30 min (Fig. 1B). Because the quantity of toxin A varied from strain to strain and with the concentration of iron in the medium, it was necessary to assay portions of each sample under both incubation conditions.

The yields of toxin A in the high-iron medium were decreased as compared to yields in the low-iron medium for all seven of the *P. aeruginosa* strains examined (Table 2). Strain PA-103 produced the highest yield of toxin A, which is not surprising since this strain was originally selected by Lui for this property (17). The other six strains tested produced less toxin A than PA-103 even when grown in the low-iron medium (Table 2). Thus, strain WR-9 produced 1/5 and WR-56 1/60 as much toxin A as did strain PA-103 (Table 2). However, like PA-103, the yields of toxin A by these six strains were decreased when the iron concentration of the growth medium was increased (Table 2). The extent of inhibition of toxin A yields in the high-iron medium appeared to be at least 85% for all seven of the *P. aeruginosa* strains tested. Results similar to these (Table 2) have been obtained using a reversed passive hemagglutination assay for toxin A which measured toxin A independent of its enzymatic activity (data not shown).

Effect of iron on protease yields. To determine if the concentration of iron in the growth

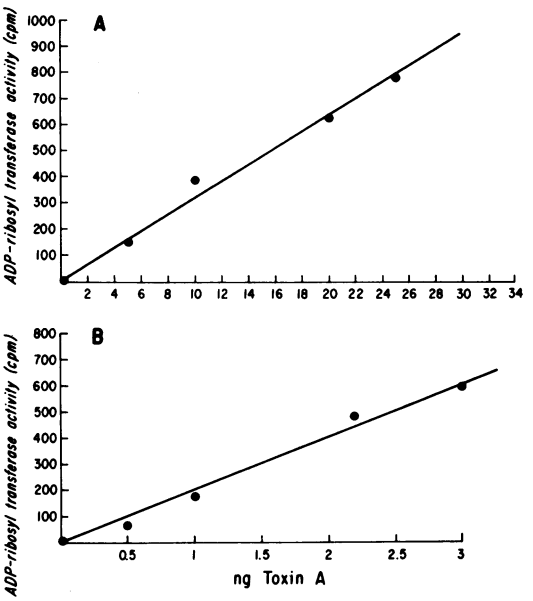

FIG. 1. *ADP-ribosyl transferase assay: standard curves obtained with pure toxin A. cpm, Acid-insoluble radioactive counts per minute from the ADP-ribosyl transferase assay of activated pure toxin A(12). (A) Standard curve obtained using a 5-min assay system. (B) Standard curve obtained using a 30-min assay system.*

medium affects the yields of other extracellular products of *P. aeruginosa,* we examined the effect of iron on total protease yields. The amount of extracellular protease produced in low-iron medium varied from strain to strain and was as high as 13.3 proteolytic units per ml for strain WR-4 and as low as 0.3 proteolytic units per ml for strain PA-103 (Table 3).

In contrast to the uniform decrease in toxin A yields (Table 2), the effect of increasing the iron content of the medium on protease yields varied from strain to strain (Table 3). Six of the seven strains examined showed a detectable decrease in protease yields when grown in high-iron medium as compared to low-iron medium (Table 3). These decreases varied over a range of 24 to

TABLE 2. *Effect of iron on bacterial growth and toxin A yields in cultures of P. aeruginosa*[a]

Strain	Bacterial growth (OD_{540})		Toxin A yield (μg/ml)	
	Low[b]	High[b]	Low	High
PA-103	8.1	18.7	25.5	2.9
WR-4	6.8	14.4	2.5	ND[c]
WR-9	8.3	16.5	4.5	0.05
WR-27	7.4	17.7	1.3	ND
WR-28	8.9	18.8	3.2	0.07
WR-35	6.3	14.7	1.5	ND
WR-56	6.9	15.5	0.4	ND

[a] This experiment has been repeated three times with comparable results.
[b] Low, low-iron (0.05 μg/ml) medium; High, high-iron (5.0 μg/ml) medium.
[c] ND, Not detectable.

TABLE 3. *Effect of iron on protease yields in cultures of P. aeruginosa*[a]

Protease	Strain	Protease yield (units[b]/ml)		% Decrease
		Low[c]	High[c]	
Total extracellular proteases	PA-103	0.3	0.02	93
	WR-4	13.3	10.1	34
	WR-9	7.8	3.0	62
	WR-27	9.2	12.1	0
	WR-28	2.3	0.1	96
	WR-35	11.6	6.1	47
	WR-56	12.7	9.6	24
Extracellular elastase	WR-9	0.28	0.04	86
	WR-27	0.31	0.37	0
	WR-28	0.36	0.18	50

[a] These experiments were repeated twice with comparable results.
[b] Total extracellular proteases are expressed as proteolytic units; elastase yields are expressed as elastolytic units.
[c] Low, Low-iron (0.05 μg/ml) medium; High, high-iron (5.0 μg/ml) medium.

96%. The other strain, WR-27, showed an actual increase in the yield of extracellular protease when grown in medium containing the higher concentration of iron (Table 3). Thus, *P. aeruginosa* strains differed in their response to iron with regard to yields of extracellular proteases.

P. aeruginosa produces at least three separate proteases: I, II (elastase), and III (20, 31). The results obtained when total extracellular protease yields were quantitated (Table 3) might be due to different levels of sensitivity of the individual proteases to the iron concentration of the growth medium. Therefore, we examined the effect of the iron concentration of the growth medium on the yields of one of the individual proteases, elastase. We studied strains WR-9 and WR-28, because they represented two strains of *P. aeruginosa* whose total extracellular protease yields were markedly decreased when grown in high-iron medium, and strain WR-27, because it was the only strain tested whose yield of total extracellular protease actually increased when grown in high-iron medium (Table 3). Although not identical to the effect seen on total protease yields, the yields of elastase by strains WR-9 and WR-28 were markedly decreased in cultures grown in high-iron medium. On the other hand, the yield of elastase by strain WR-27 was increased in the high-iron medium, as was the yield of total protease (Table 3).

HA yields in high- and low-iron media. During the course of developing a reversed passive hemagglutination assay for toxin A, we noticed that many strains of *P. aeruginosa* produced an extracellular factor that caused the agglutination of formalinized sheep red blood cells. We termed this factor an HA. To our knowledge, the identification of an extracellular *P. aeruginosa* product that has HA activity has not previously been reported. The HA as it was found in crude supernatant fluid was nondialyzable, heat stable (100°C for 15 min), and trypsin and Pronase resistant.

The effect of increasing the concentration of iron in the growth medium on the HA yields was investigated. Five of the seven strains showed a dramatic decrease in HA yields in medium supplemented with 5 μg of iron per ml (Table 4). With these five strains the relationship between iron and HA yields resembled those seen with toxin A (Table 2). However, strain PA-103 produced very small amounts of HA, and the yields were not altered by increasing the iron concentration of the growth medium. With strain WR-28, HA was not detectable in the low-iron medium but was detectable when the organism was grown in the high-iron medium (Table 4). Thus,

TABLE 4. *Effect of iron on HA yields in P. aeruginosa cultures*[a]

Strain	HA titer	
	Low[b]	High[b]
PA-103	2	2
WR-4	64	0
WR-9	256	8
WR-27	64	8
WR-28	0	16
WR-35	512	64
WR-56	16	0

[a] This experiment has been repeated three times with comparable results.
[b] Low, Low-iron (0.05 μg/ml) medium; High, high-iron (5.0 μg/ml) medium.

as was found with total extracellular proteases, the effect of iron on HA yields was strain dependent.

We have used extensively dialyzed supernatants in these studies to remove any effects that iron might have on the activities of preformed toxin A, proteases, or HA. Furthermore, we have previously shown that iron at concentrations used in this study had no effect on crude or pure toxin A activity (1). We have also found that 5 μg of iron per ml had no effect on the activity of preformed *P. aeruginosa* proteases or HA (data not shown). Thus the data (Tables 2 to 4) on the effect of iron on the yields of toxin A, protease, or HA cannot be accounted for by the effect of iron on the activities of these extracellular products.

The effect of iron on yields of total extracellular protein. Each of the seven strains showed decreased yields of one or more extracellular products besides toxin A when the concentration of iron in the growth medium was increased. Therefore, we examined the effect of the iron content of the medium on the yield of total extracellular protein. The amount of protein was expressed as a function of bacterial growth (micrograms of extracellular protein per milliliter per unit of optical density at 540 nm [OD_{540}]) to compensate for the differences in growth between the cultures grown in high- and low-iron media (Table 2). The amount of extracellular protein per milliliter per OD_{540} unit was dramatically decreased in the high-iron medium for all strains of *P. aeruginosa* examined (Table 5). The magnitude of the decrease was most apparent with strain PA-103, where the yield of total extracellular protein decreased 87% when the iron content of the medium was increased from 0.05 to 5.0 μg/ml. Yields of extracellular protein decreased from 31 to 67% with the other strains (Table 5). Results similar to those shown have been found using viable bacterial counts (colony-forming units) as a measure of bacterial growth instead of OD_{540} (data not shown). Similar to results shown earlier (Table 2), the yields of toxin A were markedly decreased when the iron content of the medium was increased (Table 5). Comparisons between low-iron and high-iron growth media showed that the percent decrease in yields of toxin A was greater for each strain tested than was the percent decrease in extracellular protein. However, the decrease in the total extracellular protein was greater than could be accounted for simply by the decrease in toxin A yields (Tables 2 and 5). Thus, if one subtracts the amount of toxin A found in cultural supernatants of strain PA-103 grown in low-iron medium from the total amount of protein produced, one gets 55.3 μg of protein or a decrease of only 5%. Yet when strain PA-103 was grown in high-iron medium the total extracellular protein was reduced to 7.6 μg/ml per OD_{540} unit or an 87% decrease (Table 5). These data are consistent with our finding that the yields of other extracellular products besides toxin A are decreased as the iron concentration of the growth medium increases.

TABLE 5. *Effect of iron on yields of extracellular protein and toxin A in P. aeruginosa cultures*[a]

Strain	Extracellular protein yield (μg/ml per OD_{540} unit)		Toxin yield (μg/ml per OD_{540} unit)	
	Low[b]	High[b] (% decrease[c])	Low	High (% decrease[c])
PA-103	58.4	7.6 (87)	3.1	0.16 (95)
WR-4	42.7	29.2 (32)	0.37	≤0.003 (≥99)
WR-9	40.4	15.4 (62)	0.54	0.003 (99)
WR-27	29.3	16.0 (45)	0.18	≤0.003 (≥98)
WR-28	27.0	15.6 (42)	0.36	0.004 (99)
WR-35	44.4	30.6 (31)	0.24	≤0.003 (≥98)
WR-56	55.1	18.1 (67)	0.06	≤0.003 (≥95)

[a] This experiment has been repeated twice with comparable results.

[b] Low, Low-iron (0.05 μg/ml) medium; High, high-iron (5.0 μg/ml) medium.

[c] Numbers in parentheses represent percent decrease in yields of total extracellular protein or toxin A in the high-iron medium as compared to low-iron medium.

Effect of varying concentrations of iron in the medium on growth and yields of extracellular products by *P. aeruginosa* WR-9. The yields of toxin A, total proteases, and HA were all markedly decreased in culture supernatants of strain WR-9 when the iron concentration of the growth medium was increased from 0.05 to 5 μg/ml (Tables 2 to 5). To further evaluate the relationship between iron and yields of these products, we examined their yields in growth medium containing various concentrations of iron (Fig. 2). The final yield of bacteria increased as the concentration of iron in the medium increased until at concentrations greater than 0.5 μg of iron per ml was no longer limiting bacterial growth (Fig. 2). The yields of all three extracellular products tested (toxin A, total proteases, and HA) were dramatically decreased as the iron concentration of the medium was increased from 0.05 to 0.2 μg/ml (Fig. 2). The inhibitory effect of iron began to level off at concentrations greater than 0.5 μg/ml, but small amounts of these extracellular products could still be detected in cultures containing 5 μg of iron per ml (Fig. 2). The three curves showing the effect of iron on the yields of these products were similar (Fig. 2).

The effect of iron on the kinetics of re-

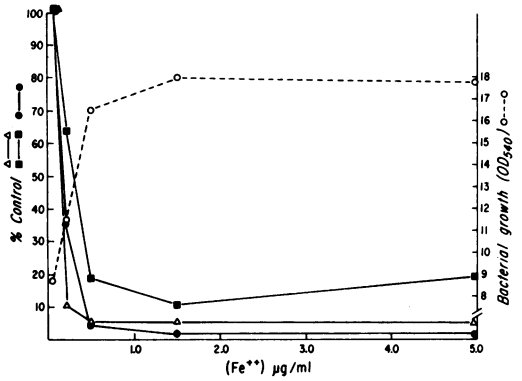

FIG. 2. *Effect of increasing concentrations of iron on the yields of bacterial growth, toxin A, proteases, and HA in cultures of P. aeruginosa WR-9. Yields of the extracellular products in the low-iron (0.05 μg/ml) medium were considered 100% and were: toxin A, 4.2 μg/ml; proteases, 8.2 proteolytic units per ml; and HA, 0.5120 HA units per ml. Symbols:* (○) *bacterial growth;* (●) *toxin A yields;* (■) *protease yields;* (△) *HA yields.*

lease of extracellular products by *P. aeruginosa* WR-9. We determined the times that toxin A, total proteases, and HA were released into the supernatant fluid and the effect of iron on these kinetics. The yield of bacteria was greater in the high-iron medium than in the low-iron medium and began to level off at about 20 h (Fig. 3A). Toxin A in the low-iron medium was first detectable at about 10 h and continued to increase through 22 h. In contrast, the yield of toxin A was dramatically decreased in the high-iron medium (Fig. 3A). Toxin A in the high-iron medium was first detectable at 16 h, and even at 22 h the levels of toxin A were barely detectable in our transferase assay (Fig. 3A). Proteolytic activity in the low-iron medium was first detectable at 10 h and continued to increase through 20 h (Fig. 3B). In the high-iron medium the yield of protease was markedly depressed and was first detectable at 12 h (Fig. 3B). The kinetics of HA release followed the same basic pattern as toxin A and protease release. HA (8 HA units/50 μl) was first detectable at 12 h in the low-iron medium and increased through 18 h, at which time it leveled off at a titer of 640 HA units/ml. The maximum yield of HA in the high-iron medium was 16 HA units/50 μl (Fig. 3B).

We also examined the kinetics of release of total extracellular protein using strain WR-9. In agreement with our preceding data (Table 5), the amount of extracellular protein per milliliter per OD_{540} unit was decreased when the organism was grown in the high-iron medium. This decrease in extracellular protein yields in high-iron medium was evident at all times tested (13, 15,

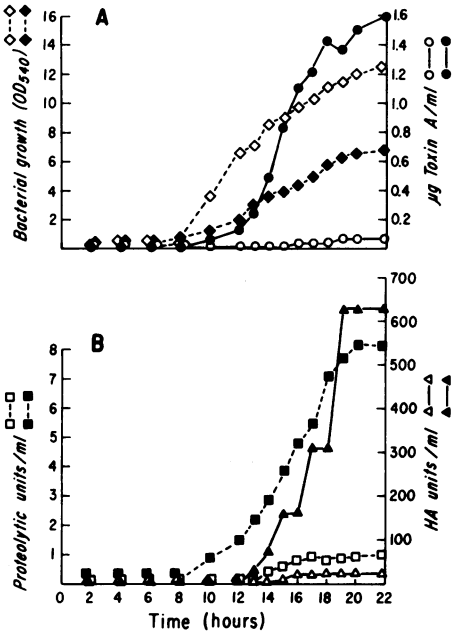

FIG. 3. *Effect of low (0.05 μg/ml) and high (5.0 μg/ml) iron concentrations on (A) bacterial growth and toxin A release and (B) protease and HA release in cultures of P. aeruginosa WR-9. Symbols:* (◇, ◆) *bacterial growth;* (○, ●) *toxin A;* (□, ■) *protease;* (△, ▲) *HA. Open symbols represent yields in high-iron medium; closed symbols indicate yields in low-iron medium.*

and 22 h). Most of the extracellular protein was released into the supernatant fluid between 15 and 22 h regardless of whether the cultures were grown in high- or low-iron medium (data not shown).

DISCUSSION

Previously, we reported that increasing the iron concentration of the growth medium decreased the yields of toxin A, using *P. aeruginosa* PA-103 (1). That study was limited to only one strain (PA-103) of *P. aeruginosa,* and only one extracellular product (toxin A) was examined. The present study utilized seven different strains of *P. aeruginosa.* We examined the effect of the iron concentration of the medium on bacterial growth, yields of four extracellular products (toxin A, total proteases, elastase, and HA), and total extracellular protein. To our knowledge the identification of a *P. aeruginosa* factor with hemagglutinating activity has not previously been described. The biological significance of the HA is not known at this time.

The final yield of bacteria (22-h cultures) in the high-iron medium was about twice that found in the low-iron medium for all seven

strains of *P. aeruginosa* tested (Table 2). In contrast, yields of the individual extracellular products generally decreased with increasing concentrations of iron in the growth medium (Tables 2 to 4). The effect of increasing the concentration of iron in the medium on toxin A yields was strain independent. The magnitude of inhibition of toxin A yields in the presence of increasing concentrations of iron was similar in all seven strains (Table 2) and consistent with our previous report (1). On the other hand, the effect of increasing the iron concentration of the growth medium on yields of total proteases, elastase, and HA was strain dependent (Tables 3 and 4). Increasing the iron concentration of the growth medium resulted in decreased extracellular yields of total proteases in six of seven strains, elastase in two of three strains, and HA in five of seven strains. Yields of total proteases and elastase were increased slightly with strain WR-27, and yields of HA increased with strain WR-28 and remained unaltered (but very low) with strain PA-103 when the iron concentration of the medium was increased from 0.05 to 5 μg/ml (Table 4). These results suggest that in some strains of *P. aeruginosa* (i.e., WR-27 and WR-28) protease and/or HA yields are regulated by iron independently from its regulation of toxin A.

The amount of total extracellular protein, when normalized for bacterial growth, was markedly decreased in all strains tested by increasing the concentration of iron in the growth medium (Table 5). The yields of total extracellular protein could not be accounted for simply by the decreased toxin A yields (Table 5). This is consistent with our finding that the yields of other *P. aeruginosa* extracellular proteins (total proteases and elastase) were generally decreased as the concentration of iron in the growth medium was increased. Furthermore, this effect was not restricted to extracellular proteins but includes other extracellular products. Several investigators have shown that iron inhibits the yields of the fluorescein and pyocyanine pigments produced by *P. aeruginosa* (3, 10, 14, 28). In the current study, we observed that yields of HA were generally reduced by increased concentrations of iron in the growth medium (Table 4). Based on our observations with toxin A, total extracellular proteases, elastase, HA, and total extracellular protein (Tables 2 to 5) and those reported earlier for *P. aeruginosa* pigments, we expect that the yields of still other extracellular products will be similarly influenced by the iron concentration of the medium.

When grown in high-iron medium, four of the seven *P. aeruginosa* strains that we examined (WR-4, -9, -35, and -56) showed significant decreases in the yields of three extracellular products examined (toxin A, total proteases, and HA). A more extensive examination employing strain WR-9 indicated that, in the presence of increasing amounts of iron in the medium, the yields of these three products were decreased in a similar manner (Fig. 2). As little as 0.5 μg of iron per ml decreased the yields of toxin A, proteases, and HA by at least 80%, as compared to the yields of these products in medium containing only 0.05 μg of iron per ml. The kinetics of release of these extracellular products were also similar at a given iron concentration (Fig. 3). Toxin A, proteases, and HA were first detectable at about 10 h in the low-iron medium and followed approximately the same kinetics of release through 18 h. In contrast, when WR-9 was grown in the high-iron medium, the kinetics of release of these products were markedly decreased (Fig. 3). These results suggest that in strain WR-9 iron regulates the release of toxin A, proteases, and HA either by some common mechanism or through equally sensitive independent mechanisms.

Yields of diphtheria, *S. dysenteriae* type 1, and *Pseudomonas* A toxins have been shown to decrease as the iron concentration of the growth medium increases (1, 5, 16, 24, 26, 29). This study extends this effect of iron to include additional extracellular products of *P. aeruginosa*. The mechanism(s) by which iron exerts this control is unknown.

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