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Effect of Iron on Accumulation of Exotoxin A-Specific mRNA in *Pseudomonas aeruginosa*

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A DNA probe from an internal fragment of the exotoxin A structural gene was used to study the effects of selected culture conditions on steady-state levels of exotoxin-specific mRNA in *Pseudomonas aeruginosa*. Cells grown under conditions of iron deprivation began to synthesize and excrete the exotoxin A polypeptide during the late exponential phase of growth and throughout the stationary phase of growth, concomitant with a sharp increase in exotoxin A mRNA pools in *P. aeruginosa* cells. The addition of iron to the medium resulted in the failure of these cells to synthesize exotoxin A mRNA, despite significantly enhanced growth. The inhibition of the production of exotoxin A and the accumulation of its mRNA by iron was dose dependent, with a half-maximal inhibitory concentration of $FeSO_4$ of 5 to 10 μM. A blockade of the initiation of transcription by rifampin resulted in the decay of exotoxin A mRNA, with a half-life of approximately 8 to 10 min, depending on the media used for growth. The addition of iron to cells actively engaged in exotoxin A synthesis also resulted in a gradual decrease in the amount of this mRNA in bacteria. However, the rate of decline of mRNA induced by iron was relatively slow (half-life, 90 min), with a considerable lag time between the iron addition and the first detectable effect on mRNA. While iron clearly appears to influence the production of exotoxin A at the transcriptional level, the molecular basis of this effect may involve several interacting factors affecting the initiation of transcription and perhaps mRNA turnover.

Most clinical strains of *Pseudomonas aeruginosa* produce a protein toxin called exotoxin A that appears to be the major virulence factor of this opportunistic pathogen (15). Exotoxin A has been purified and extensively characterized biochemically by several laboratories. It is excreted into the growth medium by *P. aeruginosa* as an M_r-66,000, single-chain polypeptide. This protein kills susceptible eucaryotic cells by ADP ribosylation of cytoplasmic elongation factor 2, inhibiting the translocation step of protein synthesis (10). The mechanism of action of exotoxin A is thus identical to that of the toxic polypeptide produced by *Corynebacterium diphtheriae* (23). Like diphtheria toxin, exotoxin A is found in the culture medium as an enzymically inactive but highly cytotoxic proenzyme. Full expression of the ADP-ribosyltransferase activity of exotoxin A requires the reduction of internal disulfide bonds and possibly the release of an active fragment (13, 16, 27).

The structural gene for exotoxin A, *toxA*, has been mapped to the 85-min location on the *P. aeruginosa* PAO chromosome (7). Recently, Gray and coworkers cloned the structural gene for exotoxin A in *Escherichia coli* and determined the complete nucleotide sequence corresponding to the coding region for the exotoxin A polypeptide (5). Remarkably, the primary amino acid sequence of exotoxin A bears no apparent homology to that of the functionally related diphtheria toxin. The recombinant plasmid containing the *toxA* gene lacked the necessary information to direct the synthesis of exotoxin A in *E. coli* unless the transcription of the cloned *toxA* gene was placed under the control of regulatory sequences derived from the tryptophan operon of *E. coli*. Mapping of various exotoxin A regulatory mutants to several different chromosomal locations provided additional suggestive evidence for a complex, multigenic mechanism controlling the expression of the *toxA* gene (6, 22).

The production of exotoxin A by *P. aeruginosa* is also regulated by environmental factors. Optimal synthesis of this toxin under laboratory conditions occurs during the stationary phase of growth, in a limited number of media, and under specific conditions of temperature and aeration (15). The most interesting aspect of the regulation of exotoxin A biosynthesis is the inhibitory influence of iron on the amounts of exotoxin A produced by *P. aeruginosa*. Media supplemented with 1 mM or higher concentrations of ferric or ferrous salts do not support the synthesis of exotoxin A. In contrast to the inhibitory effects of iron on exotoxin A formation, the addition of iron to media stimulates the growth of *P. aeruginosa* (1, 2). The production of other extracellular products, such as proteases, is similarly reduced when bacteria are grown in the presence of excess iron. The ability to detect conditions of iron limitation in the medium thus appears to be an important sensing mechanism, allowing the bacterium to induce a number of extracellular virulence factors when growth conditions are not optimal. This mechanism of iron-regulated expression of virulence factors is found in a number of pathogenic microorganisms. Elevated levels of iron in the growth medium block the formation of diphtheria toxin by interfering with the transcription of the *tox* gene in *C. diphtheriae* (11, 12).

To better understand the mechanism of toxigenesis in *P. aeruginosa*, I undertook a detailed analysis of the exotoxin A biosynthesis process from transcription of the exotoxin A mRNA to excretion of the active toxin into the growth medium. In this communication, I report the results of a study that quantitates exotoxin A mRNA induction and degradation kinetics under conditions of iron excess and iron limitation. My findings indicate that exotoxin A synthesis is directly related to the transcriptional activity of the *toxA* gene. Environmental factors, such as the availability of iron, exert their influence at the mRNA level by decreasing the

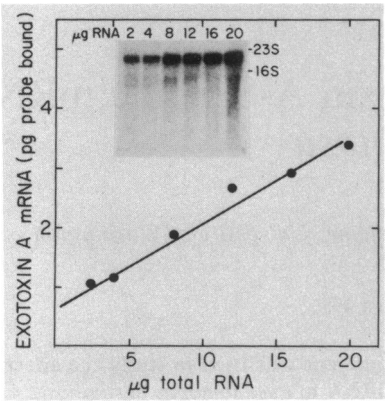

FIG. 1. Northern blot hybridization assay of total RNA from *P. aeruginosa*. RNA was extracted from *P. aeruginosa* PA103 grown for 20 h in dialyzed Trypticase soy broth supplemented with 1% glycerol and 50 mM glutamate. RNA was analyzed by formaldehyde-agarose gel electrophoresis and Northern blotting as described in Materials and Methods. The nitrocellulose blot was probed with the exotoxin A probe (labeled to a specific activity of 3.5×10^8 cpm/μg of DNA). Following autoradiographic visualization of the exotoxin A mRNA (inset), lanes were cut out and counted by liquid scintillation to determine the amount of probe bound.

rate of initiation of transcription or increasing the rate of mRNA degradation.

MATERIALS AND METHODS

Materials. All restriction enzymes, T4 DNA ligase, and the Klenow fragment of DNA polymerase were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md., and used in accordance with the specifications of the manufacturer. Trypticase soy broth was purchased from BBL Microbiological Systems, Cockeysville, Md., and Casamino Acids and yeast extract were purchased from Difco Laboratories, Detroit, Mich. Monosodium glutamate and glycerol were obtained from Sigma Chemical Co., St. Louis, Mo. [α-^{32}P]dATP and [^{14}C]adenine-labeled NAD were obtained from New England Nuclear Corp., Boston, Mass.

Bacterial strains and plasmids. *P. aeruginosa* PA103 was described by Liu (14, 15). *E. coli* HB101 was the host for plasmid pUC18 and its derivatives. *E. coli* JM101 (18) was used to propagate M13 phage.

Media and growth conditions. Dialyzed Trypticase soy broth (14) and Casamino Acids-yeast extract medium (19) were deferrated by calcium phosphate precipitation and additional treatment with Chelex-100 (Bio-Rad Laboratories, Richmond, Calif.) as described by Sokol et al. (25). Medium A (4) was not treated, but the iron supplement was omitted. Immediately before inoculation with bacteria, all three media were supplemented with 50 mM monosodium glutamate and 1% glycerol. When analyzed by the phenanthroline assay (24), iron was not detectable in any of the media. However, since the glassware was not acid washed, a trace amount of iron was presumably available for growth. All experiments reported in this study were carried out with a single lot of each medium. All incubations of cultures were carried out by vigorous aeration in a rotary shaker bath at 32°C.

Exotoxin A assay. Exotoxin A was assayed in culture fluids by the ADP-ribosyltransferase assay following activation of the protoxin with 4 M urea–5 mM dithiothreitol (3).

Isolation of mRNA and Northern hybridization. Total RNA was isolated from *P. aeruginosa* PA103 by the phenol-sodium dodecyl sulfate method of von Gabain et al. (28) and stored at −20°C as an ethanol precipitate. The concentration of RNA was determined spectrophotometrically (1 A_{260} unit corresponds to 40 μg of RNA per ml).

RNA was analyzed by formaldehyde gel electrophoresis following denaturation in 50% formamide–2.2 M formaldehyde–20 mM morpholinepropanesulfonic acid (MOPS)–50 mM sodium acetate–1 mM EDTA and heating at 60°C for 10 min. A total of 10 to 15 μg of RNA was electrophoresed in 1.2% agarose gels. The electrophoresis buffer consisted of 20 mM MOPS, 50 mM sodium acetate, and 2.2 M formaldehyde. Size-fractionated RNA was transferred onto nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H.) and probed with a ^{32}P-labeled probe as described by Thomas (26). Unlabeled M13 mp18 DNA (10 μg/ml) was added to hybridization solutions to minimize nonspecific binding of the probe to nitrocellulose. Following extensive washing of the nitrocellulose sheets, bands were visualized by autoradiography.

The amount of RNA hybridizing with the probe was quantitated by excising the portion of the nitrocellulose corresponding to the exotoxin A mRNA band and determining bound radioactivity by liquid scintillation counting. For each blot, background radioactivity was subtracted by counting a similar region of the nitrocellulose containing blotted total *E. coli* RNA prepared by the same procedure as *P. aeruginosa* RNA. The amount of probe hybridized was calculated from specific activities of the probe.

Preparation of DNA probes. Radiolabeled probes were derived from the recombinant plasmid pMS151, a construction in vector pUC18 containing a 2.4-kilobase *Eco*RV-*Eco*RI fragment that includes the exotoxin A gene from *P. aeruginosa* PAK, which was cloned in this laboratory. A 1.5-kilobase *Bam*HI fragment consisting only of the exotoxin A-coding sequence was inserted into the *Bam*HI site of the replicative form of M13 mp18, and single-stranded phage were isolated from infected *E. coli* as described by Messing (18). The hybridization probe was prepared by labeling this M13 mp18 DNA template, which contained the 1.5-kilobase *Bam*HI insert in the negative-strand orientation, with the large fragment of T4 DNA polymerase, by using the hybridization probe primer (Bethesda Research Laboratories) in the presence of [^{32}P]dATP as described by Hu and Messing (9). The specific activity of the probe was usually between 1×10^8 and 3×10^8 cpm/μg of DNA.

RESULTS

Northern blot analysis of exotoxin A mRNA. Total RNA was isolated from *P. aeruginosa* PA103 grown in deferrated Trypticase soy broth medium and fractionated by formaldehyde-agarose gel electrophoresis. Exotoxin A-specific mRNA was identified by hybridization to a ^{32}P-labeled probe consisting in its entirety of the exotoxin A-coding sequence (Fig. 1). This probe hybridized to a single mRNA band that migrated between the 23S (2,900 bases) and 16S (1,500 bases) rRNA standards. The size of the exotoxin A mRNA is therefore in good agreement with the size predicted from the coding sequence of the exotoxin A polypeptide (1,914 base pairs), with additional short regulatory regions.

The amount of probe hybridizing to the toxin mRNA was linear with respect to the concentration of total RNA applied to the gels, indicating that the probe was in excess of complementary sequences contained within at least 20 μg of

total RNA electrophoresed in each lane of the gel. In all subsequent experiments, 10 to 15 μg of total RNA was analyzed in each lane of the gel to assure accurate quantitation of mRNA hybridizing to the probe.

Induction of exotoxin A mRNA synthesis. The synthesis of exotoxin A by *P. aeruginosa* is regulated by various environmental conditions, including concentrations of iron in the growth medium. Figure 2 shows a comparison of the kinetics of accumulation of exotoxin A and its mRNA in a culture of *P. aeruginosa* PA103 propagated in Trypticase soy broth in the presence or absence of iron. Exotoxin A, measured by the appearance of ADP-ribosyltransferase activity in the culture supernatant, was found only in media lacking the iron supplement despite a significantly higher cell mass in the iron-enriched culture. In the iron-limited culture, the formation of exotoxin A was first detected during the second half of the exponential phase of growth and continued at the maximal rate during the early portion of the stationary phase of growth.

The inset in Figure 2 shows an analysis of exotoxin A mRNA levels during growth of a *P. aeruginosa* culture in iron-depleted Trypticase soy broth medium. The first visible mRNA band was detectable in samples taken at 8 h, with a steady increase in the intracellular mRNA pool over the next 3 to 4 h. This increase was followed by a period of constant exotoxin A mRNA levels. No exotoxin A mRNA was

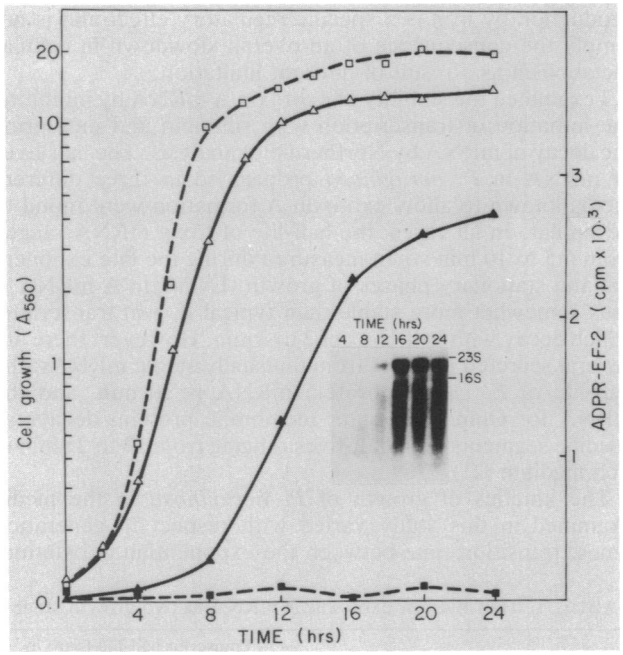

FIG. 2. Kinetics of synthesis of exotoxin A and its mRNA. Two cultures of *P. aeruginosa* were grown in a medium containing dialyzed Trypticase soy broth, 1% glycerol, and 50 mM monosodium glutamate, with or without a 100 μM $FeSO_4$ supplement. Growth was monitored by reading the optical density at 560 nm. Exotoxin A production was assayed by sampling 0.5 ml from each culture and measuring the ADP-ribosyltransferase activity (ADPR-EF-2) in the growth media following removal of the bacteria by brief centrifugation. EF-2, Cytoplasmic elongation factor 2. The pelleted bacteria were used to extract RNA for Northern blot analysis (inset). Symbols: △ and □, A_{560} of iron-depleted and iron-supplemented cultures respectively; ■ and ▲, ADP-ribosyltransferase activity in culture filtrates of *P. aeruginosa* grown in the presence (■) or absence (▲) of iron. Inset: Northern blot of mRNA from an iron-depleted culture.

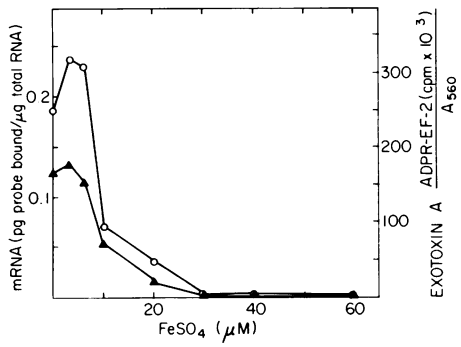

FIG. 3. Dose response of the effect of iron in the growth medium on exotoxin A production and mRNA accumulation in *P. aeruginosa*. Dialyzed Trypticase soy broth containing 1% glycerol and 50 mM monosodium glutamate and supplemented with various concentrations of $FeSO_4$ was inoculated with *P. aeruginosa* PA103 to an A_{560} of 0.1 and incubated for 20 h at 32°C. The exotoxin A content of the growth medium and exotoxin A-specific mRNA levels in the bacteria were determined as described in Materials and Methods. Symbols: ▲, ADP-ribosyltransferase activity (ADPR-EF-2); ○, mRNA. EF-2, Cytoplasmic elongation factor 2.

detectable at any stage of growth in cultures supplemented with 100 μM ferrous sulfate. Thus, the appearance of exotoxin A correlates well with the increased level of toxin-specific mRNA in bacteria during the period of maximal exotoxin A production. Furthermore, the inhibitory effect of iron on exotoxin A yields was a transcriptional effect, due to the inability of cells to accumulate mRNA for translation into the exotoxin A polypeptide.

I examined the effect of different concentrations of iron on the production of exotoxin A and the accumulation of its mRNA (Fig. 3). The yields of exotoxin A as well as the amount of probe hybridizing to mRNA showed a dose dependent response to the amount of iron added to the *P. aeruginosa* culture medium. The addition of trace amounts (1 to 2 μM) of ferrous sulfate resulted in an increase in the level of exotoxin A mRNA per cell, with a concomitant increase in the amount of exotoxin A in the culture supernatant. At higher concentrations of iron (up to 30 μM), the amount of exotoxin A found in the culture supernatant declined at the same rate as mRNA, after which neither exotoxin A nor hybridizable mRNA was detected. Because of the apparent stoichiometric relationship between the levels of mRNA and of exotoxin A, it is likely that iron regulates exotoxin A synthesis at the level of mRNA accumulation by altering rates of synthesis or degradation.

Since the presence of iron in the growth medium prevented the accumulation of exotoxin A mRNA, I investigated the effect of the addition of iron on cells fully engaged in the synthesis of exotoxin A. These measurements were carried out on *P. aeruginosa* cultures following 14 h of incubation at the onset of the stationary phase of growth, when the level of exotoxin A mRNA was at its highest. The addition of 50 μM ferrous sulfate resulted in a brief 30-min lag before the accumulation of exotoxin A began to slow down (Fig. 4). After 2 h, exotoxin A mRNA began to decline at a constant rate, with a half-life of approximately 1.5 h. Identical kinetics of mRNA accumulation were observed when the spent medium was replaced with fresh broth at the time of the addition of iron, suggesting that the culture growth medium did not contain any factors that would interfere with the uptake of iron.

Stability of exotoxin A mRNA. The accumulation of signif-

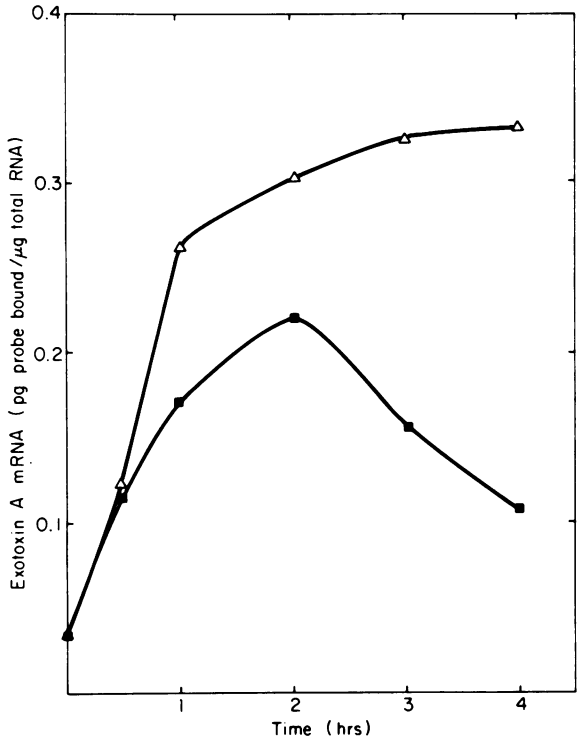

FIG. 4. Effect of iron on exotoxin A mRNA accumulation. To a culture of *P. aeruginosa* grown for 14 h in dialyzed Trypticase soy broth at 32°C was added iron to a final concentration of 50 mM. At hourly intervals, bacteria were collected by centrifugation, and total RNA was extracted for Northern blot analysis. The exotoxin A mRNA content in iron-treated cells (■) was compared with that in control cells not treated with iron (△).

icant amounts of exotoxin A in *P. aeruginosa* during the stationary phase of growth, when other metabolic activities were slowing down, raised the possibility that the mRNA is unusually stable. I investigated the decay of mRNA following the inhibition of new mRNA synthesis with rifampin. The MIC of rifampin for most *P. aeruginosa* strains is between 50 and 75 μg/ml. At a concentration of 200 μg/ml, the incorporation of [^{3}H]uridine into RNA was reduced by >98% (data not shown), so this concentration was subsequently used for all mRNA decay experiments.

The rate of decay of exotoxin mRNA was measured in *P. aeruginosa* cultures at two stages of growth in Trypticase soy broth, in Casamino Acids-yeast extract medium, and in medium A, all of which had reduced iron contents. While the growth kinetics of *P. aeruginosa* in Trypticase soy broth and Casamino Acids-yeast extract medium were similar, strain PA103 grew considerably more slowly in medium A. However, exotoxin A yields per cell mass were comparable to each other under all three culture conditions.

To determine the half-life of exotoxin A mRNA, I inhibited the initiation of RNA synthesis with rifampin in the late exponential phase of growth, defined as the time during which the growth curve deviated from the linear part of the exponential phase for 1 h. Similarly, the rate of RNA decay was measured in the stationary phase, which followed 6 h later. Table 1 summarizes the half-life measurements of exotoxin A transcripts. While the growth kinetics and exotoxin A yields were different in each medium, the mRNA decay kinetics were similar. In the two rich media tested, the half-life of exotoxin A mRNA was 8 to 10 min. In medium A, the half-life was slightly shorter (6.5 min in the late exponential phase and 7.4 min in the stationary phase). The half-life of exotoxin A mRNA was slightly longer than those reported for most procaryotic mRNAs, about 2 to 3 min (21). Since no data are available on the other mRNAs of *P. aeruginosa*, I cannot compare the stability of exotoxin A mRNA to that of any other mRNAs of the same organism.

DISCUSSION

In this report, I have shown that the appearance of exotoxin A in cultures of *P. aeruginosa* during the late exponential and early stationary phases of growth is the result of the accumulation of exotoxin A mRNA in these cells. These findings further demonstrate that the synthesis of exotoxin A is regulated, in part, at the level of transcription. The Northern blot analysis used in this study only measured the pools of specific mRNAs; therefore, I cannot determine whether this regulation is at the level of initiation of transcription or mRNA degradation.

No detectable mRNA was found in cells cultured under conditions of iron excess, suggesting that iron concentration in the growth medium plays a key role in preventing the transcription of the *toxA* gene. This conclusion is further supported by observations (Fig. 2) that bacteria grown in the presence of an iron supplement reached the stationary phase later and at significantly higher cell yields but did not synthesize exotoxin A. Thus, the inhibition of exotoxin A production by iron is a specific regulatory effect and is not simply the consequence of an overall slowdown in cellular metabolism as a result of nutrient limitation.

I examined the stability of exotoxin A mRNA by inhibiting the initiation of transcription with rifampin and measuring the decay of mRNA by Northern blot analysis. The half-lives of mRNA in *P. aeruginosa* propagated in three different media known to allow exotoxin A formation were found to be similar. In all cases, the half-life of toxin mRNA ranged from 6.5 to 10 min when measured during the late exponential and stationary phases of growth. Exotoxin A mRNA is thus somewhat more stable than typical *E. coli* transcripts, which decay with half-lives of 2 to 3 min. However, there are several secreted proteins from unusually stable mRNAs: the half-life of *E. coli* lipoprotein mRNA is 20 min, and the mRNA for OmpA, an outer membrane protein, decays as specific segments with half-lives ranging from 13 to 25 min in rich medium (21).

The kinetics of growth of *P. aeruginosa* in the media examined in this study varied with respect to generation times, transition time between the exponential and station-

TABLE 1. Half-lives of exotoxin A mRNA at two growth stages[a]

Growth medium	Transcript half-life (min) in indicated growth phase:	
	Late exponential	Stationary
Dialyzed Trypticase soy broth	8.2	7.8
Casamino Acids-yeast extract medium	9.3	10.1
Medium A	6.5	7.4

[a] Cultures of *P. aeruginosa* PA103 were grown to the late exponential and stationary phases of growth, at which time the transcription of RNA was blocked by the addition of rifampin. Total cellular RNA was isolated at 2, 4, 8 and 15 min after the addition of rifampin, and exotoxin A-specific transcripts were quantitated by Northern blotting. Values shown represent the average half-lives of transcripts from two independent determinations.

ary phases, and growth yields. However, the mRNA pools were nearly identical at the late exponential and early stationary phases when corrected for total cell number. Thus, while the availability of nutrients determines the extent of bacterial growth and, with it, the total amount of exotoxin A, the rate of transcription and the amount of accumulated mRNA are constant. These results suggest the exotoxin A synthesis is regulated by specific factors in the medium and not by the overall availability of metabolizable nutrients.

I examined the effect of added iron on mRNA pools when *P. aeruginosa* was cultivated under conditions of maximal toxin synthesis. When iron was added to the cultures at the late exponential phase, the effect was rapid. Within 10 min there was no increase in mRNA accumulation, followed by a rapid decline. The addition of iron to the cultures at the early stationary phase resulted in a lowering of exotoxin A mRNA levels, but this decrease in mRNA levels was much slower (half-life, about 1.5 h) than that observed when transcription was blocked by rifampin. Furthermore, a lag of approximately 1 h preceeded the first detectable change in the exotoxin A mRNA levels. This delay in the effect of iron on mRNA may represent the time needed to synthesize the necessary components of a sensing mechanism by which the change in the iron composition of the medium is detected by the cell and relayed to the transcriptional apparatus. Such an iron-sensing mechanism almost certainly includes iron-binding siderophores, their membrane receptors, and additional factors regulating the expression of the *toxA* gene.

The evidence presented here suggests that iron affects the production of exotoxin A by altering the concentration of mRNA available for translation. Since these studies were geared to measure the levels of total exotoxin A mRNA, one cannot assume that the effect is at the level of initiation of transcription, as is the case in classical, negatively regulated operons. Indeed, the decrease in mRNA levels may be due to increased degradation. Degradation may be influenced by the saturation of the mRNA with ribosomes, and thus translational regulation cannot be excluded. Finally, since exotoxin A is secreted via a cotranslational mechanism (17), interference with exotoxin A export could result in a decreased rate of exotoxin A polypeptide synthesis, thus increasing the rate of *toxA* mRNA degradation.

Work from several laboratories has recently demonstrated that diphtheria toxin synthesis is also regulated at the transcriptional level by iron (11, 12). Furthermore, localization of the site of initiation of transcription within a palindromic sequence suggested that this sequence may be recognized by an iron-sensitive repressor, as originally suggested by Murphy and Bacha (20). The transcription of exotoxin A in *P. aeruginosa*, on the other hand, appears to be regulated by both positive and negative elements. Hedstrom et al. (8) described the cloning of a gene encoding the putative positive regulator of the *toxA* gene by complementation of a hypotoxigenic mutant of *P. aeruginosa* PA103 (22). Additional mutants of *P. aeruginosa* that are resistant to the inhibitory effect of iron have been described (25). Thus, iron regulation of mRNA levels may involve several mechanisms. One such mechanism postulates the binding of an iron-induced or iron-sensitive repressor to the site of initiation of transcription. Alternatively, a polypeptide synthesized in response to the presence of elevated levels of iron in the medium could bind to and inactivate a transcriptional activator. Finally, the production of exotoxin A by *P. aeruginosa* could be quantitatively influenced by iron at the posttranscriptional level by an alteration of the rates of translation of mRNA, protein export, or mRNA turnover.

While this study examined some aspects of the regulation of exotoxin A synthesis in well-characterized laboratory media, it is likely that similar factors influence the biosynthesis of exotoxin A in natural environments, including infected human hosts. Thus, studies aimed at the elucidation of molecular details of the regulation of this virulence factor could lead to a better understanding of *P. aeruginosa* pathogenesis as well.

ACKNOWLEDGMENTS

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