INFECTION AND IMMUNITY, Mar. 1994, p. 897–903
0019-9567/94/$04.00+0

Vol. 62, No. 3

Characterization of *Pseudomonas aeruginosa* Mutants That Are Deficient in Exotoxin A Synthesis and Are Altered in Expression of *regA*, a Positive Regulator of Exotoxin A

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Received 18 October 1993/Returned for modification 2 December 1993/Accepted 3 January 1994

In *Pseudomonas aeruginosa*, production of exotoxin A, an ADP-ribosyltransferase, is a complex and highly regulated process. Two positively acting regulatory genes, *regA* and *regB*, have been cloned and characterized. To identify additional exotoxin A regulatory genes, we have characterized four *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-generated mutants of *P. aeruginosa* PA103 which are deficient in exotoxin A production. These mutants (PA103-8, PA103-15, PA103-16, and PA103-19) do not accumulate intracellular exotoxin A and are not complemented by the cloned *toxA* or *regAB* genes. This observation indicates that the lesion(s) in the mutants is probably in an exotoxin A regulatory gene(s) and is not in the genes for secretion of exotoxin A or in the *toxA* or *regAB* genes. To assess the effect of the putative regulatory mutations on the *toxA* and *regAB* genes, we compared the activity of the *toxA* and *regAB* promoters in the mutant and parental strains using plasmids containing the genes for β-galactosidase or chloramphenicol acetyltransferase under the control of either the *toxA* or the *regAB* promoter. The *toxA* promoter–β-galactosidase fusion plasmid could not be maintained in PA103-8. β-Galactosidase expression driven by the *toxA* promoter was absent in the mutant PA103-19 and occurred at a low level, which was not repressed by iron in mutants PA103-15 and PA103-16. The *regAB* genes are temporally controlled by two promoters, P1 and P2. In all four mutants, *regAB* P1 promoter activity was reduced; however, expression under the control of the *regAB* P2 promoter was normal. These observations suggest the existence of one or more regulatory genes which directly affect expression of both the *toxA* and the *regAB* P1 promoters.

Exotoxin A is one of the principal virulence factors produced by the opportunistic pathogen *Pseudomonas aeruginosa*. It inhibits eukaryotic protein synthesis by catalyzing ADP-ribosylation of elongation factor 2, thus causing cell death (21). Synthesis of exotoxin A is regulated in response to environmental stimuli, most notably the iron content of the culture medium (24). Iron concentrations greater than 5 μM significantly repress or even abolish exotoxin A production (3). The gene encoding exotoxin A, *toxA*, has been cloned and sequenced (15).

Accumulation of *toxA* mRNA occurs in a biphasic manner in the hypertoxigenic strain PA103 (11). The first phase occurs during early logarithmic growth with maximal mRNA accumulation at about 5 h in the growth cycle. This early accumulation is unaffected by the iron concentration of the culture medium and correlates with the presence of a small, but significant, amount of intracellular but not extracellular toxin. The second phase begins as the cells enter late logarithmic or early stationary phase and correlates with the appearance of extracellular toxin. During the second phase, accumulation of *toxA* mRNA is repressed by iron.

A positive regulator of *toxA*, the *regA* gene (*toxR*), has been cloned by complementation of the exotoxin A-deficient mutant PA103-29 (18). The *regA* gene encodes a 28,824-Da protein (19) which has been localized to the membrane fraction in *P. aeruginosa* PA103 (42). The transcription of *regA* shows the same biphasic pattern of mRNA accumulation and iron regulation as *toxA* transcription (12, 20). There are two different *regA* transcripts, T1 and T2, which are produced at different times during the bacterial growth cycle under the control of two different promoters, P1 and P2 (11, 36). The P1 promoter directs synthesis of the longer T1 transcript during early logarithmic phase and appears to be unaffected by iron. The P2 promoter is active after 5 h in the growth cycle and is repressed by iron. It is not known whether RegA regulates the transcription of *toxA* directly or through other intermediary factors or how *regA* itself is regulated.

Another regulatory gene *regB*, located 6 bp downstream of *regA*, may account for some variation in the amount of exotoxin A produced by different *P. aeruginosa* strains (41). For example, *regA* functions in the hypertoxigenic strain PA103 but not in strain PAO1, which produces less exotoxin A.

To identify additional genes that regulate exotoxin A synthesis and to extend our knowledge of *toxA* regulation, we describe here the characterization of the exotoxin A-deficient mutants, PA103-8, PA103-15, PA103-16, and PA103-19 (29). We have used fusions of the *toxA* and *regAB* promoters to reporter genes to examine the regulation of *toxA* and *regAB*. The results show that the mutants are defective in the functioning of the *toxA* promoters as well as the *regAB* P1 promoter.

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TABLE 1. Plasmids used in this study

Plasmid	Description	Reference
pSW201	pUC19 derivative containing the 1.8-kb *Pst*I fragment from pRO1614 (30)	This study
pMS151-1	*toxA* (including 412 bp of upstream sequence) from *P. aeruginosa* PAK and the 1.8-kb *Pst*I fragment from pRO1614 (30) inserted into pUC18; complements the exotoxin A-deficient phenotype of the *toxA* mutant PAO-T1 (16)	16
pMJ21	*toxA* (including 746 bp of upstream sequence) from *P. aeruginosa* PAO1 inserted into pUC18	17
pDF191.8-202	*regAB* from *P. aeruginosa* PA103 and the 1.8-kb *Pst*I fragment from pRO1614 (30) inserted into pUC19; complements the exotoxin A-deficient phenotype of the *regA* mutant PA103-29 (37)	12
pDF201	*regAB* from *P. aeruginosa* PA103 cloned into pCP13 (7)	20
pDF203	*toxA* from *P. aeruginosa* cloned into the *Eco*RI site of pLAFR (13)	20
pAH152	pAM21-1 (17) cloned into the *Eco*RI site of pKT230 (1); the promoterless *toxA* gene is under the control of the *lac* promoter	This study
pSW205	1.8-kb *Pst*I fragment from pRO1614 (30) inserted into pMLB1034 (33)	This study
pSW228	*P. aeruginosa* PAO *toxA* promoter region and the first 21 bp of the *toxA* coding region fused in frame to *lacZ* on pSW205	This study
pRL88	*regAB* P1 and P2 promoter region from *P. aeruginosa* PA103 fused to *lacZ* on pSW205	36
pQF26	Transcriptional fusion vector containing a promoterless CAT gene and stop codons in all three frames 5′ of the *cat* ribosome binding site to prevent translational readthrough	10
pP11	*regAB* P1 promoter region from *P. aeruginosa* PA103 inserted into pQF26	36
pP21	*regAB* P2 promoter region from *P. aeruginosa* PA103 inserted into pQF26	36

MATERIALS AND METHODS

Bacterial strains and plasmids. *P. aeruginosa* PA103 is an exotoxin A-hyperproducing strain which is deficient in the production of elastase and has been used extensively in studies characterizing exotoxin A and its regulation. Mutants PA103-8, PA103-15, PA103-16, and PA103-19 were generated by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine mutagenesis of PA103 and were selected for the failure to produce an extracellular immunologically cross-reactive exotoxin A molecule (29). Mutants PA103-15 and PA103-16 were isolated in the same mutagenesis experiment and, therefore, could be siblings. All strains were maintained on Luria-Bertani agar (27) at 37°C.

The plasmids used in this study are described in Table 1. The *lacZ* translational fusion vector pSW205 was constructed by insertion of the 1.8-kb *Pst*I fragment from pRO1614 (30) into the *Pst*I site within the ampicillin resistance gene on the *lacZ* fusion vector pMLB1034 (33). This fragment allows ColE1 replicons to be maintained in *P. aeruginosa*. pSW228 was constructed by insertion of a 760-bp *Pvu*II-*Hin*cII fragment from pMJ21 (17) into *Sma*I-digested pSW205. To fuse the first seven amino acids of *toxA* in frame with *lacZ*, this construction was digested with *Bam*HI, treated with the Klenow fragment of DNA polymerase I, and religated.

PA103 and the exotoxin A-deficient mutants were transformed with purified plasmid DNA as described by Bagdasarin and Timmis (2) or by electroporation as described by Smith and Iglewski (34). Transformants were selected on Luria-Bertani agar (27) containing 400 μg of carbenicillin per ml.

Culture conditions. TSBD broth, an iron-depleted medium that supports optimal production of exotoxin A, was prepared as described by Ohman et al. (29). Briefly, Trypticase soy broth (Difco Laboratories, Detroit, Mich.) was treated with Chelex-100 (−400 mesh; Bio-Rad Laboratories, Hercules, Calif.) to remove iron and then was dialyzed. The dialysate was supplemented with 0.05 M monosodium glutamate and 1% glycerol. For iron-replete conditions, TSBD broth was supplemented with 37 μM $FeCl_3$-$6H_2O$.

For measurement of exotoxin A production, TSBD broth (10 ml) was inoculated with 0.1 ml of an overnight TSBD culture of *P. aeruginosa* PA103 or one of the exotoxin A-deficient mutants and was incubated at 32°C with shaking for 18 h. The cultures were centrifuged for 10 min at 13,000 × *g*. The cell pellets were resuspended in 1/10 volume of the original culture medium with phosphate-buffered saline. Both the resuspended cell pellets and the supernatant fractions were stored at −80°C until assayed for exotoxin A activity as described below.

For measurement of reporter gene activity under the control of the *toxA* or *regAB* promoter, TSBD broth (10 ml) was inoculated to an optical density at 600 nm of 0.02 with an overnight iron-replete TSBD culture from *P. aeruginosa* PA103 or one of the exotoxin A-deficient mutants containing the appropriate reporter plasmid. The cultures were incubated at 32°C with shaking for 14 to 18 h. At the indicated times, samples were taken, and the optical densities at 540 and 600 nm were determined. The samples were centrifuged at 13,000 × *g* for 2 min, the supernatant was removed, and the cell pellets were stored at −80°C for further analysis of reporter gene activity.

Assay for exotoxin A. For determination of extracellular exotoxin A levels, culture supernatants (10 μl) were activated by treatment with urea and dithiothreitol and were assayed for ADP-ribosyltransferase activity as described by Ohman et al. (29) and Chung and Collier (6). Partially purified elongation factor 2 from wheat germ was the substrate (6). Exotoxin A activity is expressed as counts per minute per optical density unit of culture supernatant.

For determination of intracellular exotoxin A levels, the resuspended cell pellets were passaged twice through a French pressure cell (SLM Instruments, Inc., Urbana, Ill.) at 10,000 lb/in^2. Cellular debris was removed by centrifugation at 13,000 × *g* for 10 min at 4°C. Exotoxin A activity was assayed as described above.

Assays of β-galactosidase and CAT activities. β-Galactosidase activity was measured as described by Miller (27). Chloramphenicol acetyltransferase (CAT) assays were performed as described by Neumann et al. (28) with ^{14}C-acetyl coenzyme A. Samples were routinely incubated at 37°C for 50, 100, and 150 min. Units of CAT activity were calculated from standard curves generated with purified *Escherichia coli* CAT (Pharmacia LKB Biotechnology, Piscataway, N.J.).

TABLE 2. Exotoxin A production by exotoxin A-deficient mutants *P. aeruginosa* PA103

Strain	ADP-ribosyltransferase activity[a] (cpm/10 μl [%])	
	Culture supernatant	Cell lysate
PA103	13,487 [100]	4,813 [100]
PA103-8	166 [1]	376 [9]
PA103-15	169 [1]	35 [1]
PA103-16	130 [1]	320 [8]
PA103-19	113 [1]	49 [1]

[a] Determined as described by Ohman et al. (29) and Chung and Collier (6).

TABLE 3. Effects of the cloned *toxA* and *regAB* genes on exotoxin A production by *P. aeruginosa* PA103 mutants

Strain	ADP-ribosyltransferase activity[a] (cpm/10 μl [%])		
	Vector[b]	*toxA*[c]	*regAB*[d]
PA103	4,968 [100]	6,925 [100]	8,082 [100]
PA103-8	54 [1]	232 [3]	144 [2]
PA103-15	43 [1]	21 [0]	72 [1]
PA103-16	39 [1]	149 [2]	202 [3]
PA103-19	37 [1]	184 [3]	51 [1]

[a] Of culture supernatants, determined as described by Ohman et al. (29) and Chung and Collier (6).
[b] pSW201.
[c] pMS151-1.
[d] pDF191.8-202.

RESULTS

Ohman et al. (29) found that the exotoxin A-deficient mutants PA103-8, PA103-15, PA103-16, and PA103-19 produced extracellular exotoxin A at 0.3% or less of the parental strain levels. We have obtained similar results (Table 2). Ohman et al. (29) proposed that these mutants contain lesions in loci involved in the regulation, synthesis, and/or secretion of exotoxin A. To determine whether these four mutants are deficient in secretion of exotoxin A and therefore accumulate it intracellularly, we assayed cell lysates for ADP-ribosyltransferase activity. Intracellular exotoxin A produced by the mutants was between 1 and 9% of that made by the parental strain, suggesting that the lesions do not affect the secretory apparatus (Table 2). To confirm that the mutants were capable of secreting enzymatically active toxin, we introduced pAH152 into each mutant and assayed for production of extracellular exotoxin A. pAH52 contains the *toxA* gene under the control of the *E. coli lac* promoter, and thus, exotoxin A is produced independently of any *P. aeruginosa* exotoxin A regulatory mechanism. ADP-ribosyltransferase activity was detected in the supernatants of all four mutants and ranged from 2,000 to 15,000 cpm/μl. On the basis of these results, we concluded that these four mutants did not contain mutations in a gene required for secretion of exotoxin A, but rather in the *toxA* gene or in an exotoxin A regulatory gene.

Complementation analysis with the cloned *toxA* and *regAB* genes. Failure to produce exotoxin A could be due to a lesion in the *toxA* gene itself or in the *regAB* genes which regulate *toxA*. To examine this possibility, we introduced the cloned *toxA* gene and *regAB* operon under the control of their own promoters into the mutant and parental strains and assayed for extracellular ADP-ribosyltransferase activity to determine whether these genes would restore exotoxin A production in the mutants. This analysis would also distinguish between *regA* mutants and mutants containing lesions in other exotoxin A regulatory genes. Neither the cloned *toxA* gene nor the *regAB* operon when present on the vector pSW201 restored exotoxin A production in any of the mutants (Table 3). Exotoxin A production increased when the same plasmids were introduced into the parental strain. Similar results were obtained when the *toxA* and *regA* genes were present on the low-copy-number plasmids pDF201 and pDF203, respectively (data not shown).

***toxA* and *regAB* promoter activities in the mutant strains.** If the defect in these mutants is in a gene(s) that affects the activity of the *regA* promoter, then both the *regA* and the *toxA* promoters should be inactive. Alternatively, if the defect is in a gene(s) which acts in concert with *regA* to activate *toxA* transcription, then *regA* promoter activity should be normal and *toxA* promoter activity should be absent. To differentiate between these two hypotheses, we compared the expression of a reporter gene under the control of either the *toxA* or the *regAB* promoters in the mutants with expression in the parental strain.

For assessment of *toxA* promoter activity, each mutant and the parental strain PA103 were transformed with pSW228, which contains the *lacZ* gene under the control of the *toxA* promoter, or with pSW205, the same plasmid without the *toxA* promoter. β-Galactosidase activity was measured at intervals throughout the growth curve. Results are shown in Fig. 1. In the parental strain PA103, there was an early phase of β-galactosidase expression in iron-replete medium; this expression was maximal at about 6 h and decreased as the culture entered stationary phase. In iron-deficient medium, the expression of high levels of β-galactosidase activity driven by the *toxA* promoter in strain PA103 began in late log phase and continued throughout stationary phase. This pattern of *toxA* promoter activity is similar to that reported by Vasil et al. (40). In mutants PA103-15 and PA103-16, expression was approximately 3 to 10% of parental levels throughout all phases of growth and was unaffected by iron (Fig. 1). In mutant PA103-19, expression of β-galactosidase under the control of the *toxA* promoter in iron-deficient and -replete media was less than 1%

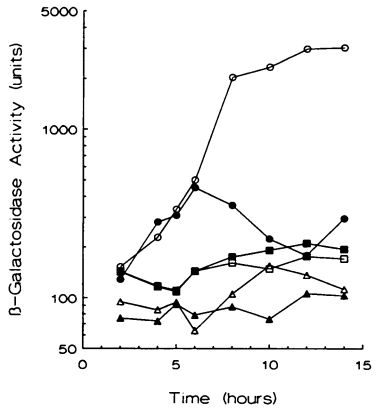

FIG. 1. Expression of β-galactosidase directed by the *toxA* promoter in PA103, PA103-15, and PA103-16. The parental and mutant strains containing pSW228 were grown in TSBD broth (29) without added iron (open symbols) and with 37 μM $FeCl_3$-$6H_2O$ (closed symbols) at 32°C with aeration. There were no significant differences in the rate of growth between the parent and the mutant strains. β-Galactosidase activity was determined at appropriate intervals throughout the growth cycle as described by Miller (27). Circles, PA103; squares, PA103-15; triangles, PA103-16. β-Galactosidase activities in PA103 and the mutants containing the control plasmid pSW205 were negligible (0.1 to 0.5 U).

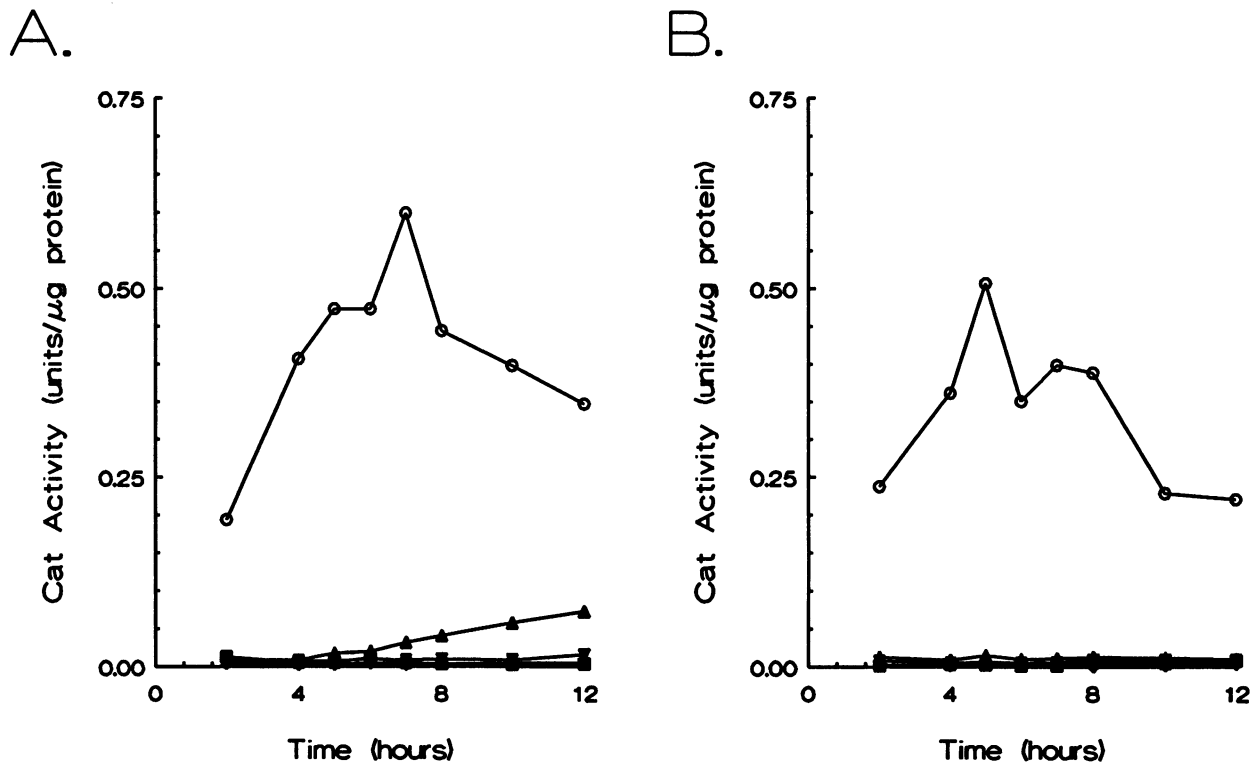

FIG. 2. Expression of CAT directed by the *regAB* P1 promoter in PA103, PA103-8, PA103-15, PA103-16, and PA103-19. The parental and mutant strains containing pP11 were grown in TSBD broth (29) containing 400 μg of carbenicillin per ml without added iron (A) and with 37 μM $FeCl_3$-$6H_2O$ (B) with aeration. There were no significant differences in the rate of growth between the parent and the mutant strains. CAT activity was determined at the indicated intervals throughout the growth cycle as described by Neumann et al. (28). ○, PA103; ▼, PA103-8; ■, PA103-15; ▲, PA103-16; ◆, PA103-19. Approximately 0.005 U of CAT activity per μg of protein was detected for both the parent and the mutants when they contained only the vector pQF26.

of the parental levels (data not shown). We were unable to transform pSW228 into PA103-8; therefore, we did not evaluate *toxA* promoter activity in this mutant. β-Galactosidase activities in PA103 and the mutants containing the control plasmid pSW205 were negligible throughout the growth cycle (0.1 to 0.5 U).

For assessment of *regAB* promoter activity, we transformed each mutant and the parent PA103 with the recombinant plasmid pRL88. pRL88 is a translational fusion of the *regAB* promoter region to the *E. coli* β-galactosidase gene and contains both the P1 and the P2 promoters of *regAB*. In PA103, the pattern of expression directed by the *regAB* promoters resembles that of the *toxA* promoter, except that in iron-replete medium *regAB* promoter activity peaks slightly earlier (12). However, in the four mutants the peak of activity associated with expression from the P1 promoter was absent, suggesting that *regAB* transcription from the P1 promoter was absent (data not shown). To confirm that the activity from the *regAB* P1 promoter is impaired in the mutants, the P1 and P2 promoters were examined separately. Each mutant and parental PA103 were transformed with plasmids containing the *regAB* P1 (pP11) or P2 (pP21) promoter joined in transcriptional fusions to the CAT gene (36). In the parent strain PA103, CAT expression driven by the *regAB* P1 promoter occurred early in the growth cycle, was maximal between 5 and 7 h, and was partially repressed by iron during stationary phase (Fig. 2). In contrast, little or no P1 promoter activity was observed for PA103-8, PA103-15, and PA103-19 (Fig. 2). In PA103-16, some expression of CAT driven by the P1 promoter was seen, but it was markedly reduced in iron-deficient medium compared with the parental strain carrying the same plasmid and was negligible in iron-replete medium (Fig. 2). In low-iron medium, CAT expression from the P2 promoter in all four mutants was similar to that of the parent (Fig. 3). In iron-replete medium, P2 promoter activity was repressed to the same extent in both the parent and the four mutants (data not shown). These observations confirm that the *regAB* P2 promoter, but not the *regAB* P1 promoter, functions in these mutants and suggests that the mutation is in a gene which affects, in a positive manner, transcription from the *regAB* P1 promoter.

Comparison of the *toxA* and *regAB* promoters. The absence of expression from the *toxA* and *regAB* P1 promoters suggested that these promoters might bind the same factors and thus contain similar nucleotide sequences. Therefore, we compared the nucleotide sequences of the *toxA* and *regAB* promoter regions (Table 4). Two transcriptional start sites have been mapped for each gene (5, 12, 14). The two *toxA* transcriptional starts have been designated S1a and S1b. When either of the *toxA* start sites is aligned with the start site of the *regAB* P1 promoter region, a high degree of similarity is detected in the region 1 to 42 nucleotides 5′ of the start sites (Table 4). The region of highest similarity lies between 16 and 41 bp 5′ of the transcription initiation site. Within this region, the similarity between the *regAB* P1 promoter and either *toxA* promoter is 56 to 60%, while the two *toxA* promoter regions are 68% similar. The *toxA* and *regAB* P1 promoters are exceedingly rich in guanosine and cytosine residues. The GC content of the *toxA*

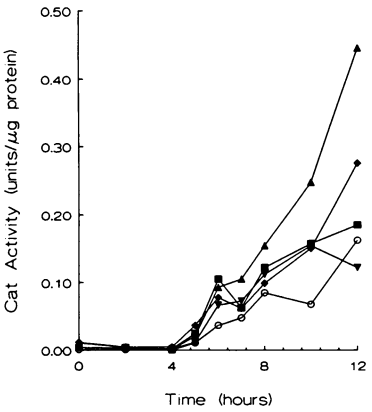

FIG. 3. Expression of CAT directed by the *regAB* P2 promoter in PA103 and the four exotoxin A-deficient mutants grown in low-iron medium. The parental and mutant strains containing pP21 were grown and CAT activity was determined as described in the legend to Fig. 2. There were no significant differences in the rate of growth between the parent and the mutant strains. ○, PA103; ▼, PA103-8; ■, PA103-15; ▲, PA103-16; ◆, PA103-19.

S1a and S1b promoter regions is 73 and 80%, respectively, while that of the *regAB* P1 promoter is 70%. The *toxA* promoter region contains multiple direct repeats but no regions of dyad symmetry (5, 14, 39). In contrast, the *regAB* P1 promoter does contain a region of imperfect dyad symmetry (12). No sequence similarities either between the *toxA* promoter and the *regAB* P2 promoter or between the *regAB* P1 promoter and the P2 promoter were detected (Table 4). Additionally, the *toxA* and *regAB* P1 promoters are distinct from other *P. aeruginosa* promoters (9, 32).

DISCUSSION

To increase our understanding of the mechanism which regulates exotoxin A production, we have characterized four putative exotoxin A regulatory mutants, PA103-8, PA103-15, PA103-16, and PA103-19, described by Ohman et al. (29). We hypothesized that the lesion(s) in these four mutants was either in the *toxA* structural gene, in a gene required for secretion of *toxA*, in *regAB*, or in another regulatory gene affecting expression of *toxA*. We eliminated the possibility that the mutants contained a lesion in either the *toxA* or the *regAB* genes themselves, since neither the *toxA* nor the *regAB* genes complemented any of the mutants. Also, these mutants do not contain mutations in genes controlling secretion, since they secreted enzymatically active exotoxin A when the *toxA* gene was placed under the control of the *E. coli lac* promoter.

toxA and *regAB* P1 promoter activities were absent or significantly reduced in all four mutants. Two patterns of expression controlled by the *toxA* promoter were observed. *toxA* expression was absent in mutant PA103-19 and occurred at a consistent but low level, which was not repressed by iron in mutants PA103-15 and PA103-16. *toxA* promoter activity could not be evaluated for PA103-8, since the reporter plasmid (pSW228) could not be maintained in this mutant. The patterns of *regAB* promoter activity were similar in all four mutants. That is, the P2 promoter functioned normally, whereas the P1 promoter failed to direct expression of the reporter gene. These observations suggest that the mutants contain an altered regulatory gene which affects both the *toxA* and the *regAB* P1 promoters.

The lack of CAT expression from the *regAB* P1 promoter on the transcriptional fusion vector pP11 indicates that the defect in all four mutants is in a positive activator of transcription from the *regAB* P1 promoter. We believe that *toxA* expression in these four mutants is also blocked at the transcriptional level; however, we cannot conclude this, because the *toxA* fusion was a translational fusion rather than a transcriptional fusion.

The significance of the differential regulation of *regAB* by two promoters is not clear at this time. However, expression of *regA* does not appear to be sufficient for *toxA* expression, since in all four mutants *regA* is expressed from the P2 promoter with no concurrent induction of *toxA* expression.

Alternative sigma factors are required for transcription of genes encoding several *P. aeruginosa* virulence factors, such as pilin, flagellin, and *algD* (22, 25, 35, 38). A potential role for this putative exotoxin A regulatory gene(s) could be as an alternative sigma factor. The *toxA* promoter contains no significant homology compared with other known *P. aeruginosa* promoters or with the consensus sequences for *E. coli* promoters (8, 32). Other *P. aeruginosa* promoters, such as the pilin, elastase, and porin F promoters, have homology with either the *E. coli* sigma^{70} or sigma^{54} consensus sequences (8, 32). Additionally, the *toxA* promoter apparently is not recognized by the *P. aeruginosa* homolog of the *E. coli* sigma^{54} gene (*rpoN*), as mutations in *P. aeruginosa rpoN* have no effect on exotoxin A production (38). The lack of similarity to other promoters and the unusual structure of the *toxA* and *regAB* P1 promoter regions, that is, the presence of multiple direct repeats and the exceedingly high G+C content, suggest that an alternative sigma factor may be required for recognition of these promoters by RNA polymerase holoenzyme.

Alternatively, the putative exotoxin A regulatory gene or genes could encode a transcriptional activator required for initiation of transcription from the *toxA* and the *regAB* P1 promoters. The *toxA* promoter contains a series of pyrimidine-rich direct repeats which share the sequence CCGC. The presence of multiple direct repeats is characteristic of a group of bacterial promoters, which includes the promoters for *araB*,

TABLE 4. *toxA* promoter region and sequence comparison with the *regAB* promoters

Promoter	Sequence[a]	% G+C
regAB P2	CGACGAAAGACCTTGATTCGTGGGAGGTAGGGTCG	58
regAB P1	AAAAG CCGCCACCAA CCAGGCCTGG CGGCGACACC GGAAGTACCCTC	70
toxA S1a	CCCTCTT CCGCTCCCCG CCA·GCCTCC CCGCATCCCG CACCCTAGA	73
toxA S1b	CCGCATC CCGCACCCTA G·ACGCCCCG CCGCTCTCCG CCGGCTCGCC	80
Consensus	ccnnntn CCGCncCCna ccAnGCCtcg CcGCnnccCg cnnncTagcc	

[a] The 3′ nucleotide represents the transcription start site. The start sites for *toxA* transcription have been mapped at 89 bp (S1a) and 62 bp (S1b) 5′ to the *toxA* initiation codon (5, 14). The starts of transcription from the *regAB* P1 and P2 promoters are located at 164 and 75 bp 5′ of the *regA* initiation codon, respectively (12).

malP, *malE*, *malK*, *phoA*, and *ompC* in *E. coli*, in which transcription initiation involves the weak binding of activating proteins (31). Thus, the putative *toxA* regulatory gene could be similar to the positive activators which enhance transcription of these genes.

Recently, it has been demonstrated that transcription of the genes encoding many virulence factors is regulated by signal-transducing regulatory pairs which recognize specific environmental signals (26). Such a mechanism may exist for transcription initiation from the *toxA* gene. In the case of these mutants, the environmental signal recognized by a signal transduction pair does not appear to be the iron content of the growth medium, since expression from the *regAB* P2 promoter was subject to the same iron repression as in the parent. However, numerous other environmental stresses have been shown to affect *toxA* expression. These stimuli include carbon source, temperature, aeration, calcium content of the medium, and perhaps, other unidentified factors (4, 24).

Histone-like proteins have been shown to be involved in the regulation of bacterial virulence factors. For example, *algR3* (*algP*), which has nucleotide sequence homology with sea urchin histone H1 is required for transcription of *algD*, an alginate biosynthetic enzyme (8, 23). It has been postulated that *algR3* (*algP*) may control the local DNA conformation or topology and thus affect protein intermediates at specific promoters. It is conceivable that a histone-like protein could be involved in stabilization of local DNA structures involved in expression of *toxA* and *regAB* from the P1 promoter.

The phenotype of these mutants suggests that the mutations are mutant alleles of one or more genes whose product(s) affects expression from both the *toxA* and the *regAB* P1 promoters. Alternatively, the mutations could be in one or more genes that have similar functions or that are part of a regulatory scheme or cascade. In addition to being deficient in exotoxin A production, the mutants examined in this study produce reduced levels of protease and several other extracellular secreted proteins (29). Thus, the gene(s) affected in these mutants may also regulate synthesis of protease and other secreted proteins; that is, it may be part of a regulon involved in virulence expression. Alternatively, the deficiency in protease production could be the result of a mutation in another gene, since *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis is known to produce multiple mutations. Detailed mapping studies and/or cloning the gene or genes which complement the mutations in these strains will be necessary to distinguish between these possibilities and to establish the role of the putative exotoxin A regulatory gene(s). Studies are under way to clone and characterize the regulatory gene(s) in these mutants.

ACKNOWLEDGMENTS

This study was supported by a Cystic Fibrosis Foundation research fellowship (FO649C-2) (S.E.H.W.), by the University of Wisconsin—Madison Graduate School (S.E.H.W.), and by PHS grant AI33713 (B.H.I.).

Plasmids pMS151, pDF191.8-202, pDF201, pDF203, pRL88, pRLX5, and pMJ21 were kind gifts of Steve Lory, Dara Frank, Doug Storey, and Mary Jo Wick. We acknowledge Jim Cook and Ellen B. Cook for excellent technical assistance.

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