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Association between Transcript Levels of the *Pseudomonas aeruginosa regA*, *regB*, and *toxA* Genes in Sputa of Cystic Fibrosis Patients

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In this study, we examined the regulation of exotoxin A (ETA) production by *Pseudomonas aeruginosa* during chronic lung infections of cystic fibrosis (CF) patients. We used a recently developed technique termed population transcript accumulation in hybridization studies with RNA extracted from sputa. With this technique, we demonstrated that the structural gene for ETA, *toxA*, as well as two genes encoding positive regulators of ETA synthesis, *regA* and *regB*, were expressed in the lungs of CF patients infected with *P. aeruginosa*. These genes were always expressed together, never alone or in pairs, suggesting coincident expression and a possible regulatory role for *regA* and *regB* in this environment. Fluctuations in the levels of the three gene products were observed among samples, consistent with a regulatory phenomenon. The level of *regB* RNA detected never exceeded that of *regA*, although the ratio of *regA* RNA to *regB* RNA detected did change between samples. These observations are in agreement with in vitro observations which have shown that *regB* is located 3′ to *regA* in an operon which is expressed from two independently regulated promoters located upstream of *regA*. The presence of high levels of *toxA*, *regA*, and *regB* RNAs in some sputum samples prompted us to look for hyperproducing-toxin strains in the sputa of CF patients. In vitro, one such strain, 4384, had a transcript accumulation pattern for *toxA*, *regA*, and *regB* similar to that of a laboratory hyperproducer of ETA, strain PA103. These observations suggest that *regA* and *regB* are involved in the regulation of ETA production in strains of *P. aeruginosa* infecting the lungs of CF patients and that some of these strains may regulate ETA production in a manner similar to that of the hyperproducing-ETA strain PA103.

Cystic fibrosis (CF) is a genetic disease resulting from a defect in a chloride channel, CFTR (25, 44, 45), which causes abnormal water and electrolyte transport (2, 5, 9, 27, 55). This disease leads to pulmonary and pancreatic insufficiency (2, 5, 9, 55). In particular, constant, recurring lung infections are a hallmark of CF (15, 19, 52, 53, 56). These infections and the concomitant inflammatory response cause progressive lung deterioration and eventually result in death (11, 20, 37). The most frequent pathogen isolated from the sputa of CF patients is *Pseudomonas aeruginosa* (15, 19). This bacterium plays a key role in the destruction of the lungs of CF patients through its ability to persist after initial colonization (6, 37, 55, 56).

P. aeruginosa produces a number of virulence factors, both cell associated and secreted, which have been linked to initial colonization of and persistence in the CF lung (7, 8, 16, 22, 24, 26, 35, 39, 57). Among these are exotoxin A (ETA), elastase, exoenzyme S, alkaline proteinase, phospholipase C, and alginate (7, 8, 16, 22, 24, 26, 33, 35, 39, 57). ETA is one of the most cytotoxic of these virulence factors (23, 30, 32). The gene encoding ETA, *toxA*, is found in 90 to 95% of strains studied (3, 40). Infection by an ETA-producing strain is associated with a poor clinical outcome (4). In CF patients, ETA is found in association with immune complexes and may also function as a super antigen (28, 29, 34, 35). Further, high levels of antibody to ETA in serum correlate with exacerbations of lung infection (35). Fluctuating levels of anti-ETA antibody in the sera of CF patients chronically infected with *P. aeruginosa* suggest that ETA production may be regulated in this environment (22).

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To date, several genes which are involved in regulating the production of ETA have been identified. Among their products are PilD, a leader peptidase (51); LasR, a regulator of a number of virulence determinants produced by *P. aeruginosa* (14); AgmR, which regulates genes involved in glycerol metabolism (38); Anr, an oxygen-responsive regulator (41, 46); and Fur, which mediates gene expression in response to iron levels (41, 42). The *regAB* locus and its products appear to have the most direct effect on *toxA* expression. This locus consists of two genes expressed as an operon from two differentially regulated promoters (47, 48). The *regA* gene encodes a transcriptional activator of the *toxA* gene (17, 18), although the mechanism of action is poorly understood. The *regB* gene encodes a protein which enhances *toxA* expression by activating transcription from the P1 promoter of the *regAB* operon (48). Early expression of *regA* and *regB* from an upstream P1 promoter results in early, cell-associated production of ETA (13). Late expression of *regA* from the P2 promoter further downstream results in a later, secreted phase of ETA production (13). Transcription of the *toxA* locus closely follows expression of the *regAB* locus, and strain differences in the level of *regAB* expression have been shown to be responsible for differences in *toxA* expression (13, 48).

Although little is known about the actual mechanism of action of either RegA or RegB, the regulation of expression of the *regAB* operon is relatively well studied. Expression from P1 occurs early in the growth cycle in both low- and high-iron conditions while expression from P2 occurs late in the growth cycle only in low-iron conditions (12, 13, 47). Additionally, both *fur* and *anr* have been demonstrated to affect expression of the *regAB* locus, suggesting that the effects on *toxA* transcription are mediated through the *regAB* operon (39, 41).

Therefore, it appears that RegA and RegB are the major regulators of ETA production in *P. aeruginosa*.

The main objective of this study was to determine if the *regAB* operon was expressed in sputum populations of *P. aeruginosa* from CF patients and if that expression was coincident with *toxA* transcript accumulation. Such a correlation might indicate a regulatory role for *regA* and *regB* in the lung environment of CF patients. Utilizing population transcript analysis (50) on RNA extracted from the sputa of CF patients suffering from chronic lung infections with *P. aeruginosa*, we demonstrated that *regA*, *regB*, and *toxA* were expressed in this environment, their expression was not constitutive, and the level of expression varied among samples. In addition, we compared the relative levels of *regA* mRNA and *regB* mRNA in sputum samples and the ratios of *regA* to *regB* message detection to demonstrate that both the P1 and P2 promoters of the *regAB* operon were active in the CF lung. Finally, we demonstrated by enzyme-linked immunosorbent assay (ELISA) and Northern (RNA) blot analysis that high-ETA-producing strains of *P. aeruginosa* exist within the CF lung and that one of these isolates has *regAB* and *toxA* transcript accumulation patterns very similar to those of a hyperproducer of ETA, strain PA103. We conclude that the *regAB* locus is likely involved in the regulation of *toxA* expression in the environment of the chronically infected CF lung. Further, high-ETA-producing strains of *P. aeruginosa* involved in such lung infections may regulate ETA production by mechanisms similar to those of strains studied under in vitro conditions.

MATERIALS AND METHODS

Bacterial strains. RNA samples used as positive and negative controls were isolated from *P. aeruginosa* PA103 (pDF191.8-202), PAO, and PA103-29. RNA isolated from PA103(pDF191.8-202) served as a positive control for the detection of *regA*, *regB*, and *toxA* transcripts. PA103 is a hyperproducer of ETA and expresses *regA*, *regB*, and *toxA* (12). Plasmid pDF191.8-202 contains the entire *regAB* operon cloned into a pUC-based plasmid and results in an approximately 10-fold increase in *regA*, *regB*, and *toxA* transcription in PA103 (13). RNA isolated from PAO was used as a positive control for the detection of *regA* and *toxA* transcripts and as a negative control for the detection of *regB* transcripts. PAO is a prototypical producer of ETA and expresses *regA* and *toxA* but lacks *regB* (21, 43, 48). PA103-29 does not express *regA* or *regB* and produces barely detectable levels of the *toxA* transcript (36, 43). RNA isolated from PA103-29 was used as a negative control for the detection of *regA*, *regB*, and *toxA* transcripts. Strain 4384 is a clinical isolate of *P. aeruginosa* obtained from the sputum of a CF patient suffering from chronic lung infection.

Culture conditions. RNA was isolated from strains grown in Trypticase soy broth dialysate under conditions previously demonstrated to maximize ETA production (31). Primary cultures consisted of cells grown for 14 to 16 h in medium containing 10 μg of Fe^{2+} per ml, except for 4384, which was grown in low-iron medium (0.05 μg of Fe^{2+} per ml). These cultures were used to inoculate secondary cultures of low-iron medium (0.05 μg of Fe^{2+} per ml) or high-iron (10 μg of Fe^{2+} per ml) medium at a starting A_{540} of 0.02 per ml as measured with a Beckman DU50 spectrophotometer.

Sputum samples. RNA used to detect in vivo expression of *regA*, *regB*, and *toxA* was isolated from sputum samples of five adult CF patients attending the University of Calgary Medical Clinic and the Adult Cystic Fibrosis Clinic at Foothills Hospital, Calgary, Canada. All these patients were suffering from chronic lung infection with *P. aeruginosa*. RNA used as a negative control to ensure that *P. aeruginosa* RNA was being detected was isolated from the sputum of a patient with bronchiectasis who was uninfected with *P. aeruginosa*. All sputum samples were diluted 1:1 with Mucolyse (Pro-Lab, Inc.) to decrease the viscosity of each sample. Sputum samples treated with Mucolyse were stored at −70°C. RNA was isolated from a standardized volume of each sputum sample.

Bacterial quantitation. Numbers of *P. aeruginosa* were determined from serial saline dilutions of Mucolyse-treated sputum samples plated on MacConkey agar plates. Total viable bacterial cell counts and differential counts of colonial morphotypes were enumerated at 24 and 48 h postinoculation.

Extraction of total RNA. RNA was extracted both from sputum samples (50) and from bacterial cultures (12, 48) by methods previously described. RNA samples used as positive controls for the detection of *regA*, *regB*, and *toxA* transcripts were isolated as follows. Culture samples consisting of 2×10^{10} cells were removed from secondary cultures, and total RNA was isolated. RNA containing *regB* transcripts was isolated from culture samples of strain PA103(pDF191.8-202) removed at an optical density at 540 nm (OD_{540}) of 0.6. To detect *regA* and *toxA* transcripts, RNA was isolated from culture samples of strains PA103(pDF191.8-202) and PAO removed at an OD_{540} of 4.0. RNA samples used as negative controls for the detection of *regA*, *regB*, and *toxA* transcripts were isolated from culture samples of strain PA103-29 grown to an OD_{540} of 4.0 and from the sputum of a bronchiectasis patient uninfected with *P. aeruginosa*. RNA isolated from cultures of strains 4384 and PA103 was extracted at the indicated absorbances.

Slot blots of RNA. In experiments using RNA extracted from sputum samples and control bacterial cultures, 10 μg of RNA was blotted onto a Nytran membrane (Schleicher & Schuell) by a Minifold II slot blot system (Hoefer) as described previously (50). Fifty micrograms of RNA from strain 4384 was blotted in the same manner.

Northern blots. Northern blot analysis of 4384 RNA was carried out as previously described (12, 48). Ten micrograms of RNA was loaded in each well.

Hybridization, autoradiography, and measurement of sample intensity. Prehybridization and hybridization conditions were those recommended by the manufacturer of Nytran membranes. Each hybridization was standardized to contain the same amount of radioactively labelled DNA probe. Slot blots of RNA extracted from sputum samples were successively probed with radiolabelled DNA fragments complementary to the *regB*, *regA*, and *toxA* genes. This hybridization order was followed with all blots in order to prevent any carryover of radioactive signal between hybridizations as the *regB* probe consistently showed the weakest levels of hybridization, followed by those of the *regA* and *toxA* probes. All hybridized blots were exposed to X-ray film at −70°C for 72 h. Blots were washed to remove the radioactive probe between hybridizations under the conditions recommended by the manufacturer of Nytran membranes.

The intensities of hybridization signals were measured with a soft laser scanning densitometer (BioMed Instruments). The relative intensities of sample hybridization were standardized to represent a population of 10^7 *P. aeruginosa* CFU as previously described (50). The hybridization signals for *regA* and *regB* were further standardized as follows. The *regB* gene is expressed with *regA* as part of an operon on a single transcript (48); therefore, differences in the hybridization of *regA* and *regB* probes to the T1 transcript might significantly alter the perceived relative level of *regB* expressed with respect to that

of *regA*. This would make direct comparisons of the levels of *regA* and *regB* expressed invalid. Therefore, in order to compare the relative levels of these gene products, differences in the hybridization of the two probes to the T1 transcript had to be taken into account. To correct for this, we grew strain PA103(pDF191.8-202) to an OD_{540} of 0.6. At this point in the growth curve, *regA* and *regB* should be transcribed at equal levels because only the T1 transcript encoding both genes is produced (47, 48, 54). Northern blot analysis with these two probes showed that the hybridization signals were not equal. Similar observations have been made in the past (reference 48 and unpublished data), indicating that this difference is not due to differences in the batches of RNA or the hybridization conditions. The most likely explanation for this observation is that the T1 transcript varies in stability along its length. It is possible that the internal *regA* probe detects a more stable portion of the T1 transcript than the 3′ *regB* probe does. We used the hybridization signals detected on these Northern blots to calculate a correction factor which makes the signals equivalent. This factor was then used to standardize the *regA* and *regB* hybridization signals detected in sputum samples. Such a correction was unnecessary for the *toxA* hybridization signals detected as we were only interested in comparing the relative levels of message, and since *toxA* is not expressed on the same transcript as *regA* and *regB*, differences in the hybridization of the probes did not greatly affect the trends seen.

Three points argue that the messages detected were specific for the genes in question. Firstly, no mRNA complementary to these genes was detected in RNA isolated from strain PA103-29 (36), a *regAB* null mutant, or from the sputum of a bronchiectasis patient uninfected with *P. aeruginosa*. Additionally, control RNA isolated from strains PAO and PA103 (pDF191.8-202) yielded detection patterns for *regA*, *regB*, and *toxA* mRNAs which exactly matched those previously reported (13, 43). Secondly, *regA*, *regB*, and *toxA* expressions were tightly linked in the sputum bacterial populations, as they are under laboratory conditions (12, 13, 48). Finally, sputum samples which were negative for *regA*, *regB*, and *toxA* expression tested positive for the presence of other *P. aeruginosa* transcripts (50), proving that the lack of detection was specific for *regA*, *regB*, and *toxA* and not due to an absence of mRNA.

DNA probes. The *regA* and *regB* probes were isolated from pDF191.8-202 (13) with a Gene Clean II kit (Bio 101, Inc.). The *regA* probe was a 400-bp *Sal*I internal DNA fragment. Two different probes were used to detect the *regB*-containing T1 transcript. One was a 491-bp *Bgl*I-*Pst*I fragment which included the entire *regB* open reading frame. The other was a 449-bp *Ava*I fragment which is complementary to the 5′ end of the *regAB* T1 transcript (12, 13). The *toxA* probe was isolated in a similar manner and is a 1,530-bp *Bam*HI internal DNA fragment (12). All restriction enzymes were purchased from Bethesda Research Laboratories. DNA fragments were labelled with [^{32}P]dCTP by a random primer DNA labelling system (Bethesda Research Laboratories).

Screening of isolates of *P. aeruginosa* for high-level production of ETA. Strains of *P. aeruginosa* isolated from the sputa of chronically infected CF patients were grown for 18 h under conditions previously shown to maximize ETA production (31). The amounts of ETA in the supernatants of cultures were measured by a competitive ELISA which employed polyclonal antisera to ETA. High-ETA-producing strains were defined as those producing >95 μg of ETA per ml.

TABLE 1. Detection of *regA*, *regB*, and *toxA* messages in CF sputum samples[a]

Patient	No. of sputum samples[b]					
	regB		*regA*		*toxA*	
	Positive	Negative	Positive	Negative	Positive	Negative
A	5	9	5	9	5	9
G	7	2	7	2	7	2
L	3	1	3	1	3	1
R	6	2	6	2	6	2
C	5	8	5	8	5	8

[a] Total RNA was extracted from sputa of CF patients chronically infected with *P. aeruginosa*. RNA was blotted onto a Nytran membrane and successively probed with ^{32}P-labelled DNA fragments complementary to *regB*, *regA*, and *toxA*. After autoradiography, positive samples were defined as those in which a hybridization signal was detected with a laser scanning densitometer.

[b] The total numbers of sputum samples positive and negative for detection of *regA*, *regB*, and *toxA* mRNA were 26 and 22, respectively.

RESULTS

Detection of *regA*, *regB*, and *toxA* transcripts in CF sputum samples. Transcripts encoding ETA have previously been detected in RNA isolated from sputa of CF patients suffering from chronic lung infections with *P. aeruginosa* (50). We wanted to determine if *regA* and *regB* transcripts could be detected in a similar fashion in order to analyze the regulation of ETA production in vivo.

We looked for *regA*, *regB*, and *toxA* messages in sputa from five individuals with CF who were chronically infected with *P. aeruginosa*. Fourteen sputum samples from patient A, four from patient L, eight from patient R, thirteen from patient C, and nine from patient G were used. Sputum samples were taken beginning in April and extending through December 1990. The interval between samples varied from 3 days to several months. In the 48 sputum samples analyzed, *toxA* transcripts were detected in 26 samples (Table 1). Messages corresponding to *regA* and *regB* were detected in the same 26 sputum samples (Table 1). This was a surprising finding, as *regA* and especially *regB* transcripts are difficult to detect in the laboratory even under optimal conditions. Strikingly, these three gene products were always detected together in a sputum sample, never alone or in pairs (Table 1). This result suggested that transcription of *toxA*, *regA*, and *regB* was tightly linked in the lungs of these patients.

Relative levels of *regA*, *regB*, and *toxA* transcripts detected in sputum samples. In *P. aeruginosa* grown under laboratory conditions, the levels of *regA*, *regB*, and *toxA* messages vary with respect to one another, depending on both the growth phase and various environmental signals (12, 13, 48). Comparison of the relative levels of *regA*, *regB*, and *toxA* transcript accumulation in sputum samples might yield further insight into the regulation of ETA production in the lungs of CF patients.

To compare the levels of mRNAs within a sputum sample, we measured the amount of radioactive signal detected after hybridization with *regA*, *regB*, and *toxA* probes with a laser scanning densitometer. The relative levels of *regA*, *regB*, and *toxA* transcripts detected varied among sputum samples (Fig. 1). Several sputum samples (A12, C3, L2, and L3) contained levels of *regA*, *regB*, and *toxA* messages greater than or similar to those of positive control RNA (Fig. 1). In the remaining sputum samples, we detected either moderate (A4, R3, R5, R8, G4, and G6) or low levels of these gene products relative to those of positive control RNA (Fig. 1). Variations in the

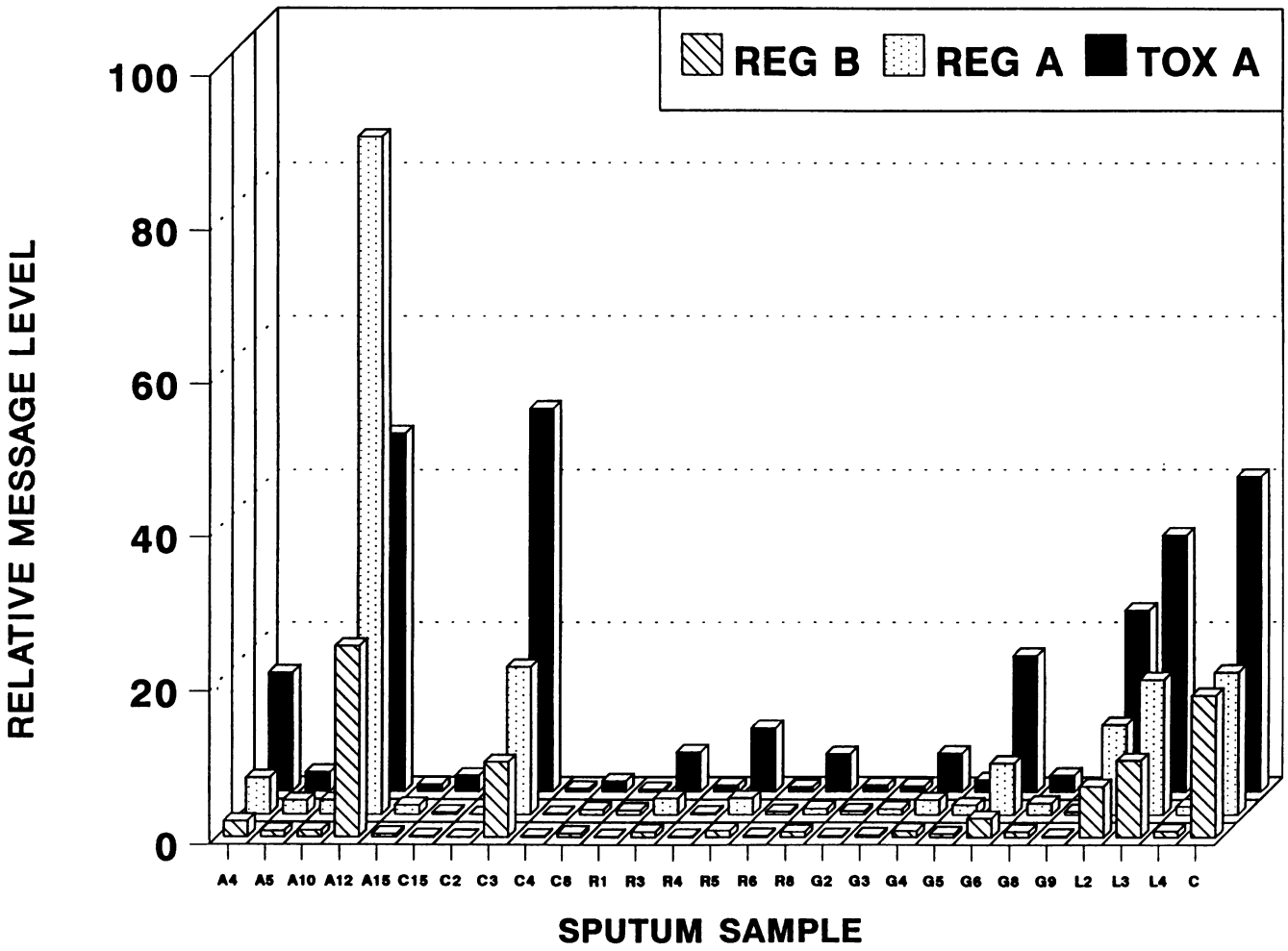

FIG. 1. Measurement of *regA*, *regB*, and *toxA* population transcript accumulation. Sputum samples from five CF patients chronically infected with *P. aeruginosa* were centrifuged, and total RNA was extracted from the pelleted bacteria. Ten micrograms of RNA from each sputum sample was blotted onto a Nytran membrane and hybridized with the *regB* probe. Autoradiographs were exposed for 72 h, after which blots were washed for probe removal. Following hybridization with the *regA* probe, autoradiograph exposure, and being washed, the blots were hybridized with the *toxA* probe and autoradiographed a final time. The relative intensities of the signals were measured with a soft laser scanning densitometer. The results were normalized so that each sample represented 10^7 CFU of *P. aeruginosa*. Samples from the same patient begin with the same letter. C, RNA extracted from strain PA103(pDF191.8-202) at an OD_{540} of 0.6.

levels of transcript accumulation were seen among sputum samples from single patients in five different cases (Fig. 1). This suggested that ETA production may vary in chronically infected CF patients. Interestingly, when high levels of *toxA* message were detected, those of *regA* and *regB* were also high (Fig. 1, A12, C3, L2, and L3). Similarly, when *toxA* transcript detection was low, so were the levels of *regA* and *regB* messages (Fig. 1, A15, C4, R6, and G9), suggesting not only coincident expression but a tight association in the levels of expression as well. The same pattern is seen under the laboratory conditions used to study ETA regulation (12, 13). Therefore, our data suggested that *regA* and *regB* were involved in regulating ETA production in the chronically infected lungs of CF patients.

Testing of clinical isolates of *P. aeruginosa* for high-level production of ETA. Several sputum samples (A12, C3, L2, and L3) contained *regA*, *regB*, and *toxA* message levels similar to or higher than those of positive-control RNA isolated from a hyperproducing-ETA strain of *P. aeruginosa*, PA103 (Fig. 1). Additionally, *regB* RNA, which has been demonstrated to be responsible for the high level of ETA produced by PA103 (48, 54), was detected in all the sputum samples that were positive for *regA* and *toxA* transcript detection (Table 1) (Fig. 1). These data suggested that strains of *P. aeruginosa* which produce high levels of ETA may be present in the chronically infected CF lung.

We screened 158 *P. aeruginosa* isolates from the sputa of eight CF patients suffering from chronic infection for high-level production of ETA (Table 2). We measured ETA production by an ELISA using anti-ETA antisera. Each isolate was grown for 18 h in Trypticase soy broth dialysate medium prior to analysis. Under these conditions, the laboratory hyperproducer of ETA, strain PA103, produced approximately 95 μg of ETA per ml (data not shown). Sixty-one percent of the clinical isolates of *P. aeruginosa* produced approximately the same level of ETA (Table 2). The percentage of isolates which produced >95 μg of ETA per ml in any one patient varied from 0% in patient S to 79% in patient R (Table 2). These isolates produced amounts of ETA which varied from

TABLE 2. Screening of clinical isolates of *P. aeruginosa* from chronically infected CF patients for high-level production of ETA

Patient(s)[a]	No. of isolates[b]		% Positive
	Tested	Positive	
T	46	26	57
M	5	3	60
H	6	4	67
G, S	12	8	67
F	20	12	60
J	9	3	33
S	8	0	0
R	52	41	79
Total	158	97	61

[a] Each patient is represented by a single letter. G, S represents isolates from two patients.

[b] Isolates were grown for 18 h in low-iron Trypticase soy broth dialysate. Bacterial cells were removed by centrifugation, and competitive ELISAs using polyclonal antisera to ETA were done on spent culture medium. Positive isolates were defined as those strains of *P. aeruginosa* producing >95 μg of ETA per ml. This level was the amount of ETA produced by the hyperproducing strain PA103 grown under the same conditions.

150 to >300 μg of ETA per ml (data not shown). In the majority of patients tested, approximately 60% of the isolates from sputum produced levels of ETA comparable to those of PA103 (Table 2). These findings demonstrated that strains of *P. aeruginosa* in the lungs of chronically infected CF patients were able to produce high levels of ETA.

Transcription of the *regAB* and *toxA* loci in clinical isolate 4384. To determine whether clinical isolates of *P. aeruginosa* which produced high levels of ETA had *regAB* and *toxA* transcription patterns similar to those of strain PA103, we examined the accumulation of these messages in a CF isolate. This isolate, one of the strains from patient R (Table 2) and designated 4384, was chosen because it produced high levels of ETA.

Maximal *toxA* transcription occurred late in the growth curve under low-iron conditions with isolate 4384 (Fig. 2A), as

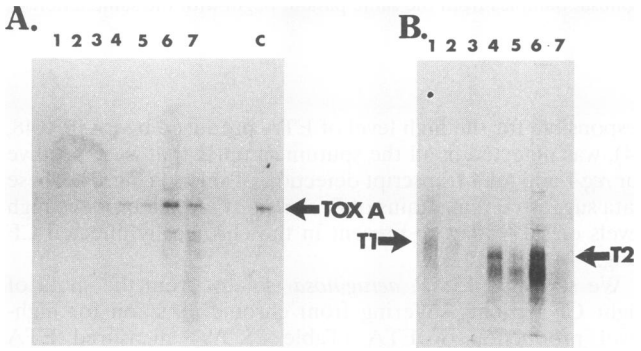

FIG. 2. Northern blot analysis of *regA* and *toxA* transcript accumulation in clinical isolate 4384. Total RNA was extracted from low-iron Trypticase soy broth dialysate cultures at OD_{540}s of 0.3, 0.6, 1.0, 2.0, 3.0, 4.0, and 5.0 (lanes 1 to 7, respectively). Lane C, RNA extracted from a low-iron Trypticase soy broth dialysate culture of strain PA103 at an OD_{540} of 4.0. Twenty micrograms of RNA was treated with glyoxal and separated on a 1.2% agarose gel. RNA was blotted onto a Nytran membrane and probed with a *regA* probe (B). Autoradiographs were exposed for 72 h, and the blots were washed for probe removal. Blots were then hybridized with a *toxA* probe and exposed for 72 h (A).

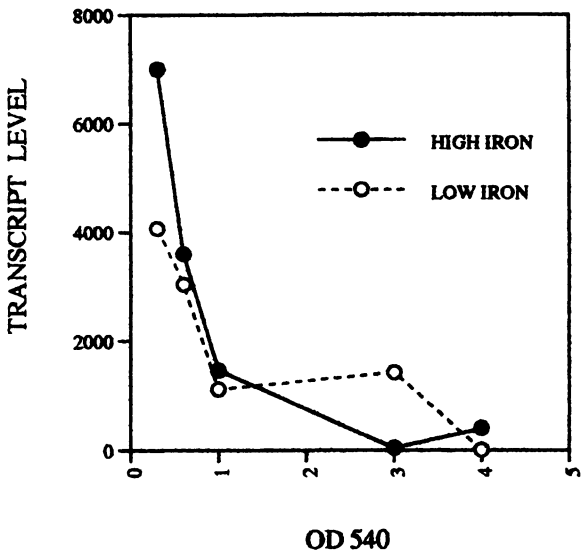

FIG. 3. Slot blot analysis of *regAB* T1 expression in clinical isolate 4384. Total RNA was extracted from low- and high-iron Trypticase soy broth dialysate cultures at OD_{540}s of 0.3, 0.6, 1.0, 3.0, and 4.0. RNA extracted from low-iron cultures of strain PA103 at an OD_{540} of 0.3 was isolated as a control. Fifty micrograms of RNA was blotted onto a Nytran membrane and probed with a ^{32}P-labelled *Ava*I fragment complementary to the upstream region of the *regAB* T1 transcript. Blots were washed and exposed to X-ray film at −70°C. Results were measured with a soft laser scanning densitometer.

has been previously demonstrated with strain PA103 (12). The level of *toxA* mRNA detected in isolate 4384 at the peak of expression was comparable to that in strain PA103 (Fig. 2A, compare lanes 6 and C), as would be predicted from the amount of ETA detected with our ETA ELISA (43). Northern blot analysis of RNA from isolate 4384 with a ^{32}P-labelled DNA fragment of *regA* revealed the expression of a *regA*-encoding transcript of the same size as the T1 message produced by PA103 early in the growth cycle (Fig. 2B). The T1 transcript has previously been shown to encode both *regA* and *regB* (48). In isolate 4384, the T1 transcript was followed by expression of a smaller T2 transcript late in the growth cycle (Fig. 2B). The T2 transcript has been shown to encode only *regA* (12, 13, 48). This pattern of *regA* and *regB* transcription was very similar to that previously observed in strain PA103 (12, 13, 48).

To confirm that the transcripts detected with the *regA* probe represented two different messages corresponding to T1 and T2, we did slot blots of RNA isolated from isolate 4384 under both low- and high-iron conditions and hybridized with a T1 upstream probe previously demonstrated to define T1 expression in strain PA103 (13). Slot blots were scanned with a soft laser scanning densitometer to measure the level of transcript detected with the *Ava*I T1 upstream probe. Message complementary to this fragment was detected in RNA isolated from both low- and high-iron cultures only at early time points in the growth curve (Fig. 3). This is the same as the pattern of T1 expression in PA103 previously identified (13) and correlates well with the expression of the T1-sized transcript detected with a *regA* probe on Northern blots (Fig. 2B). Additionally, the levels of message detected with the *Ava*I T1 upstream probe at early time points in the growth curve were similar to those detected in control RNA isolated from PA103 (data not shown).

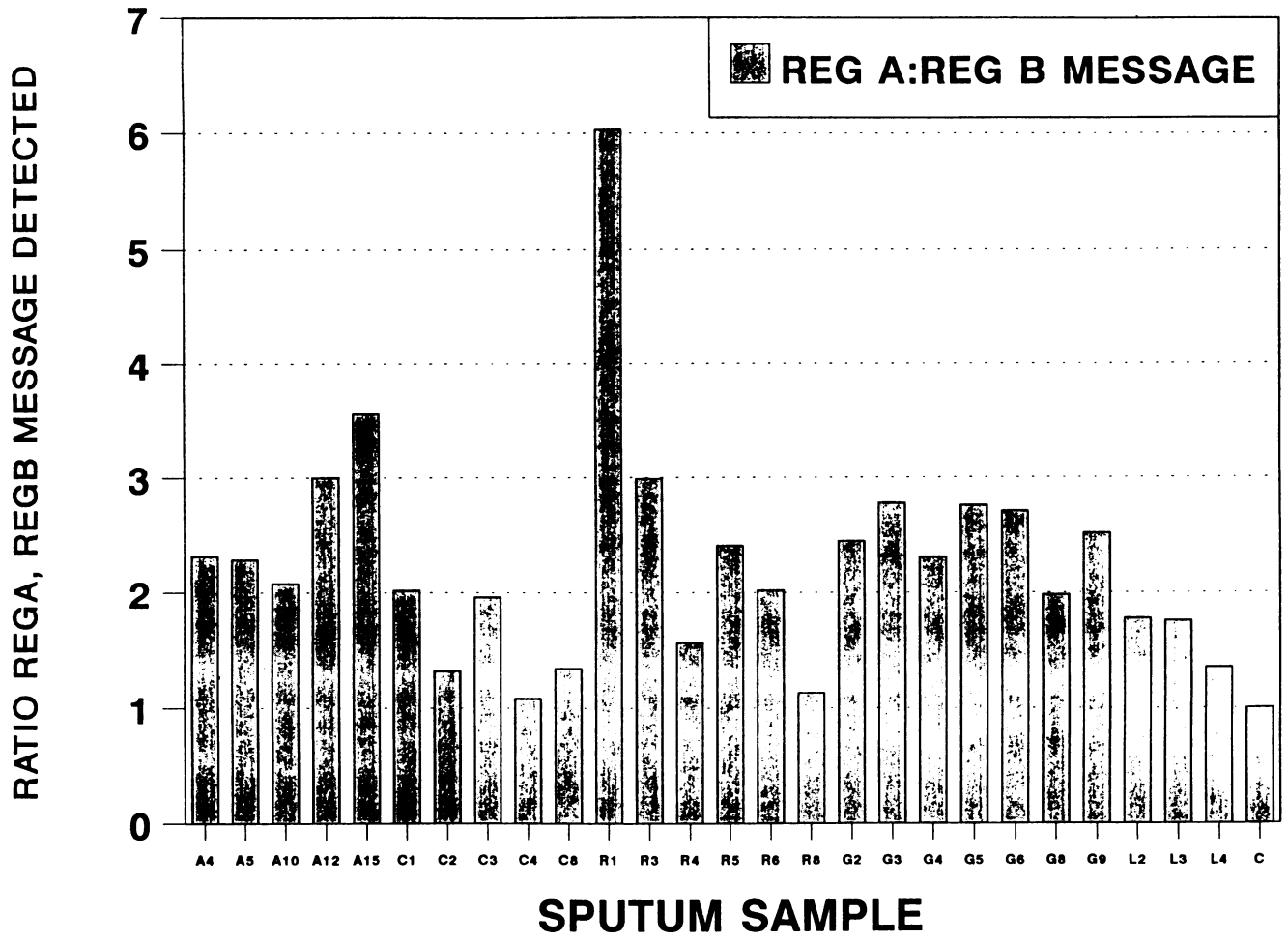

FIG. 4. Ratios of *regA* to *regB* transcript levels in 26 sputum samples positive for detection of *regA*, *regB*, and *toxA* messages. Ratios were derived from the relative levels of *regA* and *regB* transcripts detected in Fig. 1. *regB* message levels were corrected such that the ratio of *regA* to *regB* transcript was equal to 1 at an OD_{540} of 0.6 in control RNA isolated from strain PA103 (C).

These findings demonstrate that 4384, a CF isolate of *P. aeruginosa*, had *regAB* and *toxA* transcript accumulation patterns similar to those of another strain which produces high levels of ETA, PA103.

Ratios of *regA* to *regB* transcripts detected in sputum samples. In vitro, *regA* and *regB* are expressed on a T1 transcript from the P1 promoter of the *regAB* operon (48) while a T2 mRNA encoding only *regA* is expressed from the P2 promoter (12, 13, 47, 48). Therefore, the presence of *regB* messages and the consistently greater amount of *regA* transcript detected in sputum samples (Fig. 1) suggested that expression from both of these promoters occurs in the chronically infected CF lung.

To further investigate the regulation of *toxA* expression in the CF lung, we analyzed the ratios of *regA* to *regB* transcripts detected in sputum samples by employing the relative levels of transcripts measured in Fig. 1 (Fig. 4). The ratio of *regA* to *regB* message detected never fell below 1 (Fig. 4), consistent with the previous finding that *regB* is expressed on a transcript with *regA* from the P1 promoter of the *regAB* operon (48). The *regA* transcript was never observed alone (Fig. 1 and 4) (Table 1), suggesting that expression from P1 occurred consistently at some level when *toxA* was being expressed in the CF lung. The ratios of *regA* to *regB* transcript detected varied, ranging from just above 1 (Fig. 4, C4 and R8) to 6 (Fig. 4, R1). Previous studies have shown that *regA* can be expressed on a transcript without *regB* from the P2 promoter downstream of P1 (12, 13, 48). Therefore, the variations in the ratio of *regA* to *regB* transcript detected among samples most likely reflect changing levels of transcription from the P2 promoter relative to those from the P1 promoter of the *regAB* operon. These changes might indicate fluctuations in the levels of P1 and P2 expression within the major population(s) of *P. aeruginosa* over time in response to environmental alterations in the CF lung. Alternatively, they may reflect a variety of *P. aeruginosa* populations at different stages of growth throughout the lung.

Approximately two-thirds of the sputum samples contained a ratio of *regA* to *regB* transcript of ≥ 2, suggesting that expression from the P2 promoter was equal to or greater than that from the P1 promoter in the CF lung. Indeed, in some samples (e.g., A12, A15, R1, and R3 in Fig. 4), P2 expression appeared to be predominant. These data suggested that both the P1 and P2 promoters of the *regAB* operon were active in the CF lung.

Different ratios of *regA* to *regB* mRNA were seen in association with different levels of *toxA* message. In sputum samples containing the highest levels of *toxA* message, C3, G8, L2, and L3 (Fig. 1), the ratio of *regA* to *regB* transcript averaged around

2 (Fig. 1 and 4). An exception to this observation was sample A12, in which a ratio of 6 was seen with a high level of *toxA* message (Fig. 1 and 4). This might be explained by the observation that sample A12 was the only one in which higher levels of *regA* message than of *toxA* were detected (Fig. 1). What conditions might lead to this situation in the chronically infected lung are unknown; perhaps another regulator of *toxA* expression plays a role. A correlation between low levels of *toxA* message and average ratios of *regA* to *regB* transcript was not observed (Fig. 1 and 4). While these observations say little with respect to situations in which low levels of *toxA* are being expressed, they might indicate that when *toxA* is transcribed at a high level, there are equal numbers of T1 and T2 transcripts in the CF lung, suggesting that both *regA* and *regB* play an important role in regulating ETA production in this setting.

DISCUSSION

The results of this study suggested that the *regAB* locus plays a role in regulating the production of ETA in strains of *P. aeruginosa* involved in chronic lung infections in CF patients. This conclusion is supported by analysis of the levels of *regA*, *regB*, and *toxA* mRNAs derived from the lungs of patients chronically infected with *P. aeruginosa* as well as by transcript accumulation studies of a CF isolate. These results were consistent with previous works which showed that the *regAB* locus encodes two transcriptional activators which mediate regulation of the level of expression of the *toxA* locus (12, 13, 17, 18, 48, 54).

The *regA*, *regB*, and *toxA* loci were expressed by populations of *P. aeruginosa* infecting the lungs of CF patients (Table 2) (Fig. 1). Demonstration that the levels of anti-ETA antibodies in the blood of chronically infected CF patients fluctuate (22) might lead one to expect this result. However, other studies suggest that the detection of these gene products is somewhat surprising. In particular, mucoid strains of *P. aeruginosa*, which are isolated frequently from the lungs of CF patients (6), have been demonstrated to produce lower levels of virulence factors, including ETA, in vitro (57). Since conversion to the mucoid phenotype is thought to occur soon after infection, it might be expected that virulence factor production would be low in an adult CF patient colonized for many years. These data and a previous study (50) show that populations of *P. aeruginosa* involved in chronic lung infections are capable of expressing high levels of virulence factors, particularly ETA, over the course of infection.

The detection of *regB*-specific mRNA in sputum samples was surprising. *regB* has been demonstrated to be transcribed in only two strains to date, PA103 and PAO Fe18 (1, 48). Additionally, many clinical isolates of *P. aeruginosa* produce PAO levels of ETA when cultured in vitro (57), suggesting that these clinical isolates may be more similar to strain PAO than the high-level producer of ETA, strain PA103 (36). In contrast, the data presented here indicate that both the transcription of *regB* and high-level production of ETA may be common occurrences. It is possible that the environment of the CF lung provides some signal which results in a higher level of ETA production. Alternatively, perhaps strains of *P. aeruginosa* which have the capability of producing high levels of ETA have a selective advantage in the environment of the CF lung and are therefore present in greater numbers. It is not known whether the isolates of *P. aeruginosa* from a single patient screened for high-level production of ETA represent one or several strains. Therefore, two possibilities exist. Firstly, the isolates which produced high levels of ETA may be different strains from those which produced low levels of ETA. These strains might exist as specific subpopulations within the CF lung which upregulate ETA production in response to specific cues. Alternatively, as isolates from a single patient were taken at different times over the course of infection, it is possible that they represent a single strain which varies its production of ETA over time in response to varying conditions in the CF lung.

The fluctuating levels of anti-ETA antibodies in the sera of chronically infected CF patients suggest that ETA production is not constitutive in the environment of the CF lung (16, 22). Similarly, our data showed that *toxA* transcript levels vary over a period of time (days to months) (Fig. 1), demonstrating that ETA production within the CF lung is likely regulated. These events are unlikely to be caused by shifts in the strains colonizing the lungs, as most CF patients remain colonized with one or several clonal types of *P. aeruginosa* once infected (10). Additionally, we found that none of the patients in this study became colonized with new *P. aeruginosa* morphotypes but they consistently had the same morphotypes in their sputa. Although this observation is not proof that the strains remained the same, it suggests that the *P. aeruginosa* populations in each patient did not undergo rapid changes during this study. Thus, it seems likely that ETA production is regulated in the chronically infected CF lung.

Although numerous genes are involved in the regulation of ETA production (*fur*, *lasR*, and *anr*) (14, 41, 42, 46, 51), the *regAB* locus appears to have the most direct effect on expression of *toxA* (12, 13, 17, 18, 48). The strongest evidence for this is the tight linkage of *regAB* and *toxA* transcription observed under laboratory conditions in which increased levels of *regAB* transcription are followed by increased levels of *toxA* expression (12, 13). In addition, strain differences in the levels of ETA production have been demonstrated to be due to increases or decreases in expression of the *regAB* locus (48, 54). Similarly, the tight linkage of expression of these loci in the CF lung suggests that *regA* and *regB* may play an equally important role in the regulation of ETA production in the chronic lung infections of CF individuals. *regA* and *regB* were expressed concurrently with *toxA* in the lung; none of these genes were ever expressed alone or in pairs. Statistical analyses show that the correlations between detection of these three messages are highly significant ($P < 0.01$) (43). Indeed, the correlation in expression of *regA*, *regB*, and *toxA* was higher than that seen for any two loci analyzed in other population transcript studies (43), including the correlation between *toxA* and *lasR*, another modulator of *toxA* transcription (14, 49). Further, the relative levels of *regA* and *regB* messages paralleled those of *toxA*, suggesting a direct link in the CF lung between the levels of expression of these two loci. Although our results do not preclude the possibility that other regulators modulate the expression of *toxA*, it seems probable that ETA production is regulated in the lungs of CF patients by the *regAB* locus in a manner similar to that seen in the laboratory strains studied to date.

This hypothesis is further supported by transcriptional studies of strain 4384, a CF isolate of *P. aeruginosa*. Strain 4384 expresses the *toxA* and *regAB* genes in a manner very similar to the hyperproducer of ETA, strain PA103. We have shown that other strains of *P. aeruginosa* which produce high levels of ETA are involved in CF lung infections (Table 2). It is possible that ETA production is regulated in a similar manner in these strains as well. Additionally, the observation that isolate 4384 demonstrates relationships between *regAB* and *toxA* message levels similar to those seen in populations of *P. aeruginosa* within the lungs (i.e., the relative levels of *regAB* and *toxA* expression increased or decreased together) suggests that

regAB and therefore *toxA* expression may be controlled by the same environmental cues in the CF lung which control expression in vitro. Alternatively, different, unidentified cues may act through the *regAB* locus in a similar fashion to regulate *toxA* transcription.

Two pieces of evidence suggest that both the P1 and P2 promoters of the *regAB* operon are active in the CF lung. Firstly, a *regB* probe detected mRNA in sputum samples (Fig. 1). Since in vitro work demonstrates that *regB* is expressed only from the P1 promoter of the *regAB* operon, this suggests that transcription from the P1 promoter is occurring in the chronically infected CF lung. Secondly, detection of higher levels of *regA* in some samples suggests that there are more transcripts encoding *regA* than *regB* present (Fig. 1). Again, this is consistent with in vitro observations that show that a T2 transcript lacking *regB* is expressed from the P2 promoter of the *regAB* operon. Therefore, it seems possible that transcription of the *regAB* operon in strains of *P. aeruginosa* infecting the CF lung occurs from two promoters, as it does in strain PA103.

Comparison of the ratios of *regA* to *regB* message detected in sputum samples suggests that the relative levels of expression from these two promoters vary in the CF lung. As these promoters are regulated by different environmental and temporal cues under laboratory conditions, these observations indicate that more than one cue is involved in triggering *regAB* and therefore *toxA* expression in the CF lung. The fact that P1 activity was always present to some extent suggests that the autoinducing activity of *regB* may play an important role in this environment. A low iron level in the medium is the only environmental cue to date that has been demonstrated to result in activity from both promoters of the *regAB* operon (although at different points in the growth cycle) (12, 13). Therefore, since *regA* and *regB* transcripts were consistently detected together with *toxA* transcripts in the chronically infected CF lung, the level of iron may be an important signal, triggering ETA production in this environment. Alternatively, the cues which result in *regAB* expression and therefore *toxA* expression in the CF lung may as yet be unidentified. Potential candidates include osmoregulation, the growth phase, and oxygen availability. It is also uncertain whether altered levels of P1 and P2 expression reflect responses by the entire *P. aeruginosa* population within the lung or specific adaptations by subsets of the population in response to local environmental changes. The latter seems likely, as one would expect local differences within the lung with respect to such factors as drug concentration, oxygen availability, and biofilm formation, etc. The constant flux in P1 and P2 activities suggests that the strains of *P. aeruginosa* involved in chronic lung infections are constantly regulating the expression of *toxA* in response to different environmental cues.

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