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 toxA 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, 22, 24, 26, 35, 39, 56). 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).

To date, several genes which are involved in regulating the production of ETA have been identified. Among their products are PiID, 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 amp 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).

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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, that 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 diluents 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 Fe²⁺ per ml, except for 4384, which was grown in low-iron medium (0.05 μg of Fe²⁺ per ml). These cultures were used to inoculate secondary cultures of low-iron medium (0.05 μg of Fe²⁺ per ml) or high-iron (10 μg of Fe²⁺ per ml) medium at a starting A₅₄₀ 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 × 10⁶ 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 (OD₅₄₀) 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 OD₅₄₀ of 4.0. RNA samples used as negative controls for the detection of regB, the ratios of regA, regB, and toxA transcripts were isolated from culture samples of strain PA103-29 grown to an OD₅₄₀ 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.

#### 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 10⁷ *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 OD540 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 spumt 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 SalI internal DNA fragment. Two different probes were used to detect the regB-containing T1 transcript. One was a 491-bp BglI-PstI fragment which included the entire regB open reading frame. The other was a 449-bp AvaI 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 BamHI internal DNA fragment (12). All restriction enzymes were purchased from Bethesda Research Laboratories. DNA fragments were labelled with [32P]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>
<thead>
<tr>
<th>Patient</th>
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<th>toxA</th>
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<tbody>
<tr>
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<td>Negative</td>
<td>Positive</td>
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<td>9</td>
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<td>C</td>
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Table 1. Detection of regA, regB, and toxA messages in CF sputum samples.a

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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 32P-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
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 antiserum. 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

<table>
<thead>
<tr>
<th>Patient(s)*</th>
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<td>26</td>
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<tr>
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<tr>
<td>F</td>
<td>20</td>
<td>12</td>
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<tr>
<td>J</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>S</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>R</td>
<td>52</td>
<td>41</td>
</tr>
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</table>

Total 158 97 61

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

*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 ng 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 ng 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 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 32P-labelled *AvaI* 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.

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 OD260 of 0.3, 0.6, 1.0, 3.0, and 4.0. RNA extracted from low-iron cultures of strain PA103 at an OD260 of 0.3 was isolated as a control. Fifty micrograms of RNA was blotted onto a Nytran membrane and probed with a 32P-labelled *AvaI* 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.

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 OD260 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 OD260 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).
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 and high levels of regA 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 transcripts 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, ETA expression in the lungs 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 expression 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). 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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