Analysis of Transcription of the Exotoxin A Gene of *Pseudomonas aeruginosa*

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Analysis of RNA isolated from *Pseudomonas aeruginosa* PA103 and PAKS grown under Fe^{2+}-limiting (0.08 μg/ml) and Fe^{2+}-sufficient (10 μg/ml) conditions demonstrated that exotoxin A (ETA) expression is regulated by Fe^{2+} at the level of transcription. S1 nuclease mapping revealed two 5' termini of the *tox* transcript, 89 base pairs (bp) (S1A) and 62 bp (S1B) 5' to the ETA initiation codon. There appeared to be no consensus promoter sequence for either *tox* transcript. An 8-bp direct repeat was found 5' to the start of transcript S1A. Transcript S1B mapped 8 bp upstream of a dodecamer sequence conserved between the ETA and phospholipase C genes of *P. aeruginosa*. Multicopy plasmids in which the expression of ETA is directed from (i) the *Escherichia coli* *trp* promoter (ptrpETA-RSF1010) or (ii) the *tox* promoter (pCMtox) were constructed and mobilized into a Tox^- *P. aeruginosa* strain, WR5. WR5 synthesized and secreted high levels of ETA when it was expressed from the *E. coli* *trp* promoter; however, the synthesis of ETA from its own promoter in this strain was very low. These and other data suggest that the expression of ETA is under a positive control mechanism. A fusion of the ETA promoter fragment to *lacZ* was constructed. Use of this fusion plasmid revealed that this DNA fragment directed the synthesis of β-galactosidase in *E. coli* at very low levels and that the synthesis of β-galactosidase from this fusion in *E. coli* was not regulated by Fe^{2+}.

It is now clear that many bacterial toxins are synthesized in a specifically regulated manner. Some of these toxins are regulated by ions required for growth of the bacterial cells. The production of *Shigella dysenteriae* type 1 toxin (7, 33) and *Corynebacterium diphtheriae* toxin (13, 22) is regulated by the amount of Fe^{2+} present in the culture medium. The amounts of exotoxin A (ETA), elastase, and alkaline protease produced by *Pseudomonas aeruginosa* are all reduced as the concentration of Fe^{2+} in the medium is increased (3, 4). More recently, other ions have been found to affect the production of certain bacterial toxins. For example, synthesis of the heat-labile hemolysin of *P. aeruginosa* (phospholipase C) was found to be regulated at the level of transcription by the P_i concentration of the medium (26). The synthesis of cholera toxin has also been reported to be influenced by the concentration of P_i (30).

Although it is evident from the above work that environmental conditions specifically affect the production of certain toxins, the actual regulatory mechanisms are just beginning to be understood. Recent studies by Mekalanos and colleagues have begun to shed some light on the molecular process involved in the regulation of toxin synthesis in *Vibro cholerae*. Mekalanos (16) has shown that production of cholera toxin may be regulated through amplification of genes encoding enterotoxin and that factors in the mammalian intestinal environment select for, or encourage, duplication of the genes encoding cholera toxin. In contrast, *P. aeruginosa* strains which produce various toxin levels all contain a single chromosomal copy of the ETA gene (34).

Another example of proposed mechanisms by which the expression of certain toxins is regulated is that maximal expression of the toxin requires a positive regulator protein which acts at the level of transcription. Miller and Mekalanos (18, 19) have recently presented evidence for such a regulatory protein which controls the expression of cholera toxin. Alternatively, Murphy and Bacha (20) have

postulated that the regulation of diphtheria toxin is achieved through a negative regulatory mechanism. They suggest that in the presence of Fe^{2+}, a negative regulator represses the synthesis of diphtheria toxin but that in the absence of Fe^{2+} the repressor is inactive.

The amount of ETA produced by *P. aeruginosa* is strain dependent, and the expression of ETA is regulated by Fe^{2+} and several environmental conditions (O_2, temperature, concentration of alanine) (3, 4, 6, 34). Toxin production usually decreases by 10-fold when clinical isolates of *P. aeruginosa* are grown in medium containing as little as 1 μg of Fe^{2+} per ml (3, 4). The cloning of a positive regulatory factor for ETA expression has been recently reported (11), and others have presented evidence that there are positive control mechanisms governing expression of ETA (34), but the genetics of Fe^{2+} regulation of ETA expression are not well understood at this time.

In this report we present data indicating that the regulation of ETA expression by Fe^{2+} is at the level of transcription and further evidence which supports a positive control mechanism governing the regulation of ETA expression, but at the present time the existence of negative, as well as positive, control elements cannot be overlooked.

MATERIALS AND METHODS

Bacterial strains. The *P. aeruginosa* and *Escherichia coli* strains that were used in this study are listed in Table 1.

Media. *E. coli* strains used in the β-galactosidase assays were grown in minimal medium 1XA (17) (60 mM K_2HPO_4, 33 mM KH_2PO_4, 7.5 mM $(NH_4)_2SO_4$, 1.7 mM sodium citrate) supplemented with 20 μg of B_1 per ml, 1 mM $MgSO_4$, and 0.4% glucose and containing 200 μg of carbenicillin per ml.

P. aeruginosa strains were grown in an Fe^{2+}-depleted medium (DTSB) prepared as described previously (4). Fe^{2+} was added from freshly prepared $FeSO_4$ stock. High Fe^{2+} medium contained 10 μg of Fe^{2+} per ml.

Subcloning of the *tox* promoter. The promoterless *lacZ* vector pMLB1034 (32) was used for subcloning the DNA

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TABLE 1. Bacterial strains and plasmids[a]

Strain or plasmid	Genotype or phenotype	Source	Reference(s)
Strain			
E. coli HB101	leu thr pro lac gal Str^r recA EndoI hsdR hsdM	A. L. Taylor	15
E. coli 294	endA hsdR Thi$^-$ Pro$^-$ Str^s	R. M. Berka	15
E. coli D1245	lac deletion of E. coli HB101	J. L. Betz	
P. aeruginosa WR5	Prototroph Tox$^-$ (lacks ETA structural gene [34])	P. A. Sokol	4, 24
P. aeruginosa PA103	Prototroph Tox$^+$		14, 25
P. aeruginosa PAKS-1	Prototroph Tox$^+$	B. Wretlind	25
Plasmid			
ptoxETA	Tc^r Ap^s PstI-EcoRI fragment carrying entire tox gene	G. Gray	9
RSF1010	Sm^r Su^r	R. M. Berka	10
pRK2013	Km^r ColE1 with RK2 tra	D. Ohman	8
pCMtox	Hybrid of RSF1010 and ptoxETA		34
pMLB1034	Tc^s Ap^r promoterless lacZ	R. M. Berka	32
ptrpETARSF1010	Hybrid of RSF1010 and ptrpETA (9)		This study
pCG39	pMLB1034 (32) carrying PvuII-BamHI tox promoter fragment		This study
pCG39It	pCG39 with transcription terminator inserted 5' to the ETA promoter fragment		This study

[a] The genotype symbols have been described by Bachman (1). Ap, Tc, Sm, and Su, Ampicillin, tetracycline, streptomycin, and sulfonamide, respectively; Tox, ETA.

fragment from ptoxETA (9) which carries the tox promoter (34). The procedure is outlined in Fig. 1. ptoxETA was digested with PvuII and BamHI (Boehringer Mannheim Biochemicals, Indianapolis, Ind., or Bethesda Research Laboratories, Inc., Gaithersburg, Md. [all enzymes used as directed by the supplier]), and the 760-base-pair (bp) fragment containing 24 bp of the structural ETA gene coding sequence and 736 bp 5' of the ETA initiation codon was isolated by electroelution from a 3.5% polyacrylamide gel. The 5' 4-bp cohesive terminus at the BamHI end was filled in with the Klenow fragment of T4 DNA polymerase (Boehringer Mannheim). pMLB1034 was digested with BamHI, and the 5' cohesive terminus was also filled in with the Klenow fragment and then treated with calf intestinal alkaline phosphatase (Boehringer Mannheim). The two DNA fragments were ligated overnight at 12°C in the presence of T4 DNA ligase (Boehringer Mannheim). This resulted in the tox initiation codon in frame with the lac reading frame. The ligation mix was transformed into E. coli D1245 as previously described (12). A transcription terminator, 5'-d(AGCCCGCCTAATGAGCGGGCTTTTTTTT)-3' (Pharmacia Diagnostics, Piscataway, N.J.) was inserted into pCG39 directly upstream of the ETA fragment as outlined in Fig. 1. Orientation of the tox fragment and insertion of the transcription terminator were verified by digestion of the plasmids with appropriate restriction endonucleases and verification of fragment sizes on ethidium bromide-stained agarose gels with molecular weight standards. Positive clones were cultured on minimal agar plates (17) containing 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside per ml and 200 μg of carbenicillin per ml for screening of β-galactosidase activity.

Construction of multiple-copy ETA plasmids. Construction of plasmid pCMtox, in which the synthesis of ETA is under the control of the tox promoter and which can replicate in P. aeruginosa, has been previously reported (34). A plasmid (ptrpETA-RSF1010) which carries the ETA structural gene under the control of the E. coli trp promoter and which can also replicate in P. aeruginosa was constructed as follows. EcoRI was used to digest ptrpETA, a plasmid previously constructed such that the expression of ETA is directed by the E. coli trp promoter (9), and RSF1010, a broad-host-range multicopy plasmid (40 to 50 copies per cell [10]), at their unique EcoRI sites. The two linearized plasmids were

ligated together overnight at 12°C in the presence of T4 DNA ligase (Boehringer Mannheim). The ligation mixture was transformed into E. coli 294 (12). This plasmid, as well as pCMtox, was mobilized into P. aeruginosa WR5 in a triparental mating as previously described (34).

β-Galactosidase assay. β-Galactosidase activity was assayed by the o-nitrophenyl-β-D-galactopyranoside assay as described by Miller (17).

Detection of ETA by Western blotting. Cell-free culture supernatants were electrophoresed on 10% polyacrylamide-sodium dodecyl sulfate gels and were electroblotted onto nitrocellulose as previously described (9). ETA was detected by using immunoaffinity-purified rabbit anti-ETA antibodies (60 ng/ml) and affinity-purified ^{125}I-labeled protein A (1 μCi/10-lane gel) (Amersham Corp., Arlington Heights, Ill.) as previously described (9).

RNA extraction. P. aeruginosa strains to be used for RNA extraction were grown for 12 and 15 h in DTSB at 32°C with vigorous aeration with or without the addition of 10 μg of Fe^{2+} per ml. A_{590} of cultures with no added Fe^{2+} was: 12 h, 4.2; 15 h, 4.7. A_{590} of cultures with 10 μg of Fe^{2+} added per ml was: 12 h, 7.1; 15 h, 9.5. The production of ETA was assayed by Western blot analysis of cell-free culture supernatants from which the cells were used for RNA extraction.

RNA was extracted from 500-μl samples of P. aeruginosa cultures essentially as described by Buck and Groman (5). The cells were sedimented by centrifugation, the supernatant was collected for Western blot analysis, and the cell pellets were suspended in diethyl pyrocarbonate-treated 0.02 M sodium acetate (pH 5.5)–4% sodium dodecyl sulfate–1 mM EDTA (pH 8.0)–1 mg of yeast tRNA per ml. The samples were incubated at 100°C for 90 s followed by the addition of an equal volume of chloroform-isoamyl alcohol, (24:1, vol/vol). The suspension was then vortexed for 90 s. Phenol (500 μl) saturated with diethyl pyrocarbonate-treated 0.1 M sodium acetate–1 mM EDTA was added, and the sample was incubated with mixing at 65°C for 30 min. The mixture was then centrifuged, and the aqueous phase was collected. An additional phenol extraction (65°C, 15 min) was followed by two chloroform-isoamyl alcohol extractions. The RNA was precipitated with ethanol and stored at −20°C until used.

S1 nuclease mapping. S1 nuclease protection experiments were performed by the method of Berk and Sharp (2) as
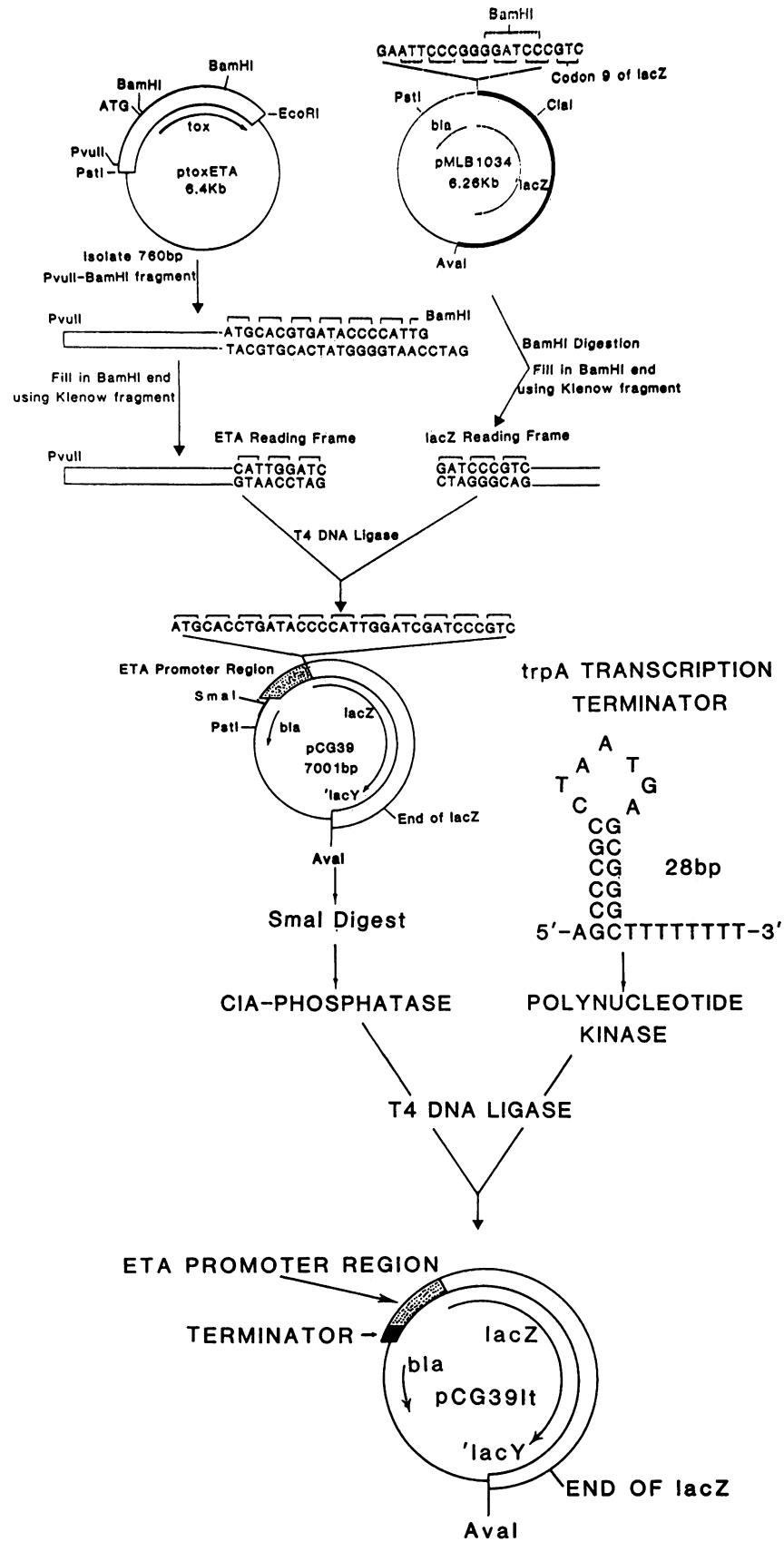
FIG. 1. Construction of ETA promoter–β-galactosidase fusion plasmids pCG39 and pCG39It. Kb, Kilobases.
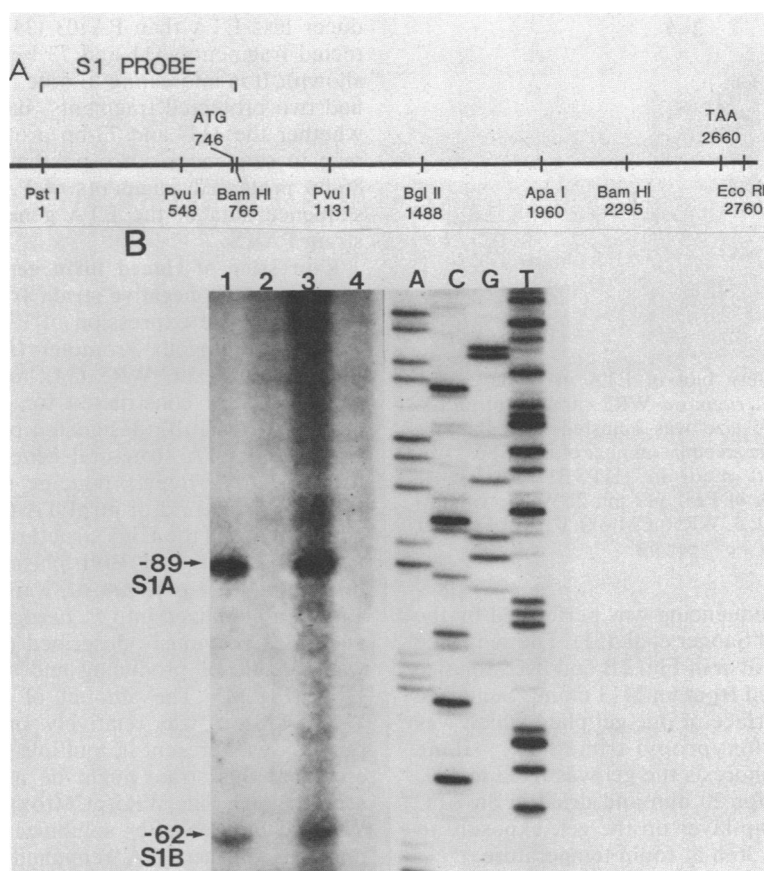
FIG. 2. S1 nuclease mapping of the 5' end of the ETA transcript from *P. aeruginosa* PA103. (A) Partial restriction endonuclease map of the cloned ETA fragment. *PstI-BamHI* DNA fragment (uniquely end labeled at the *BamHI* site) used as the S1 probe is overlined. (B) Autoradiograph of a sequencing gel of S1 nuclease analysis of the *tox* transcript in *P. aeruginosa* PA103. Fe^{2+} was added to deferrated basal medium (DTSB). Lanes 1, 2, 3, and 4, S1 nuclease digestion products obtained with RNA from lanes: 1, 12 h of growth, 0 μg of Fe^{2+} per ml; 2, 12 h of growth, 10 μg of Fe^{2+} per ml; 3, 15 h of growth, 0 μg of Fe^{2+} per ml; 4, 15 h of growth, 10 μg of Fe^{2+} per ml. Lanes A, C, G, and T, Sequence ladder determined by the method of Sanger et al. (31). Arrows, Major S1 nuclease digestion products. Numbers indicate the corresponding site in the sequence upstream of the *tox* initiation codon (see Fig. 5).

modified by Weaver and Weissmann (36). The RNA concentration of each preparation was determined by UV absorption at 260 nm. A 50-μg portion of each RNA sample was added to the S1 reaction. The *P. aeruginosa* ETA gene probe was a 765-bp *PstI-BamHI* DNA fragment of ptoxETA which contains 24 bp of ETA-coding sequence and 741 bp 5' of the ETA initiation codon (Fig. 2A). The DNA fragment was uniquely end labeled at the *BamHI* end (bp 765) with [γ-^{32}P]ATP (New England Nuclear Corp., Boston, Mass.) and T4 polynucleotide kinase (New England Bio-Labs, Inc., Beverly, Mass.) (15). The DNA probe (150,000 cpm) was hybridized with total cell RNA at 55°C (80% formamide, 40 mM PIPES [piperazine-*N*,*N*'-bis(ethanesulfonic acid)] [pH 6.7], 0.4 M NaCl, 1 mM EDTA) for 15 h and then incubated with 100 U of S1 nuclease (Boehringer Mannheim) for 45 min at 37°C (8% formamide, 4 mM PIPES [pH 6.7], 290 mM NaCl, 1 mM EDTA, 30 mM sodium acetate [pH 4.6], 1 mM $ZnSO_4$). The samples were then precipitated with ethanol, and the pellets were suspended in 3 μl of formamide dye mix (53% [vol/vol] deionized formamide, 33 mM Tris borate [pH 8.3], 0.67 mM EDTA, 0.067% [wt/vol] xylene cyanol, 0.067% bromophenol blue), incubated at 100°C for 2 min, and electrophoresed through an 8 M urea–6% polyacrylamide sequencing gel (40 cm by 0.3 mm) for 4 h at 30 mA (bromophenol blue run 40 cm).
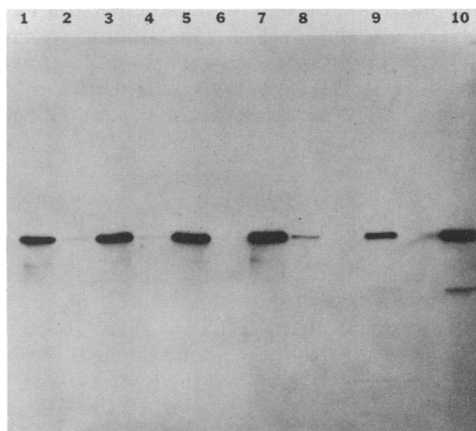

FIG. 3. Detection by Western blot of ETA produced by *P. aeruginosa* PA103 in crude culture supernatant. Sample size was adjusted such that at a given time point the supernatant was from an equal number of cells. Fe^{2+} was added to deferrated basal medium (DTSB). Lanes: 1, 12 h, 0 μg of Fe^{2+} per ml; 2, 12 h, 10 μg of Fe^{2+} per ml; 3, 15 h, 0 μg of Fe^{2+} per ml; 4, 15 h, 10 μg of Fe^{2+} per ml; 5, 18 h, 0 μg of Fe^{2+} per ml; 6, 18 h, 10 μg of Fe^{2+} per ml; 7, 21 h, 0 μg of Fe^{2+} per ml; 8, 21 h, 10 μg of Fe^{2+} per ml; 9, ETA standard, 0.1 μg; 10, ETA standard, 0.5 μg.
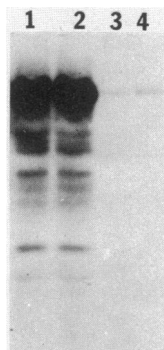
FIG. 4. Detection by Western blot of ETA in crude culture supernatant produced by *P. aeruginosa* WR5 carrying ptrpETA-RSF1010 or pCMtox. Sample size was adjusted such that the amount of supernatant was from an equal number of cells. Fe^{2+} was added to deferrated basal medium (DTSB). Lanes: 1, WR5(ptrpETA-RSF1010), 0 μg of Fe^{2+} per ml; 2, WR5(ptrpETA-RSF1010), 10 μg of Fe^{2+} per ml; 3, WR5(pCMtox), 0 μg of Fe^{2+} per ml; 4, WR5(pCMtox), 10 μg of Fe^{2+} per ml.

DNA sequencing. DNA sequencing was performed by the chain termination method of Sanger et al. (31). The sequence ladder (not of the probe) shown in Fig. 2B and used for size determinations was prepared from an M13 clone. Sequence gels were bonded to the surface of one gel plate which was coated with α-methacryloxypropyl-trimethoxy silane (Pharmacia). After electrophoresis the gel was fixed in 10% methanol–10% acetic acid for 20 min and dried in an 80°C oven. Without a Saran Wrap layer on the gel, exposure to Kodak XAR-5 film was for 36 h at room temperature.

RESULTS

Iron regulation of ETA transcription. The level of ETA expression in *P. aeruginosa* PA103 grown in Fe^{2+}-limiting and Fe^{2+}-sufficient conditions as assayed by Western blotting is shown in Fig. 3. The only difference between these cultures is the Fe^{2+} content of the medium. Therefore the synthesis of ETA is being regulated by the concentration of Fe^{2+} in the medium. To determine whether the Fe^{2+} regulation of ETA is at the level of transcription, we compared ETA mRNA levels from *P. aeruginosa* cells grown under various conditions. ETA mRNA was detected in Northern blot analysis of RNA extracted from Fe^{2+}-limited cultures of *P. aeruginosa*. In contrast, RNA extracted from cells grown in the presence of excess Fe^{2+} did not detectably hybridize to the ETA gene probe, suggesting that the amount of Fe^{2+} in the culture influenced the level of ETA mRNA (data not shown). Since S1 nuclease mapping is a more sensitive means of transcript detection (13), we extracted RNA from strain PA103 grown with or without 10 μg of Fe^{2+} per ml added to the basal medium (DTSB). These RNA preparations were annealed to the ^{32}P-end-labeled 765-bp *PstI*-*BamHI* ETA probe (Fig. 2A), and the hybrids were digested with S1 nuclease as described above. Figure 2B shows an autoradiograph of an S1 gel. DNA fragments of 113 and 86 bp corresponding to positions −89 and −62 bp, respectively, upstream of the ETA initiation codon were protected when the probe was annealed to RNA extracted from PA103 cells grown under Fe^{2+}-limiting conditions (Fig. 2B, lanes 1 and 3). RNA extracted from cells grown under Fe^{2+}-sufficient conditions failed to protect the ^{32}P-labeled DNA probe (Fig. 2B, lanes 2 and 4). In a similar experiment with RNA extracted from *P. aeruginosa* PAKS, a strain which pro-

duces less ETA than PA103 (34), two Fe^{2+} regulated protected fragments (111 and 73 bp) were also seen (data not shown). It is interesting to note that both PA103 and PAKS had two protected fragments, but at this time it is unclear whether the 111- and 73-bp protected fragments of PAKS map to sequences corresponding to those of the 113- and 86-bp protected fragments of PA103 owing to the lack of sequence data of the ETA gene and regulatory region of strain PAKS.

Expression of cloned toxin gene fragment in a naturally occurring ETA-negative strain from the *tox* and *E. coli trp* promoters. The expression of ETA from the *tox* promoter and a heterologous promoter (*E. coli trp*) in a Tox⁻ *P. aeruginosa* strain, WR5 (34), was analyzed. Two hybrid plasmids were constructed for this purpose. The first is ptoxETA-RSF1010 (designated pCMtox) (34). This plasmid carries the ETA structural gene and the 5′ 746-bp region from the toxin-hyperproducing strain PA103. The second plasmid is a hybrid of ptrpETA (9) and RSF1010 (10). This plasmid carries the ETA structural gene under control of the *E. coli trp* promoter. Both plasmids, which are capable of replicating in *P. aeruginosa*, were transferred to *E. coli* 294 and then mobilized into *P. aeruginosa* WR5 in a triparental mating as previously described (34). Strain WR5(pCMtox) was capable of producing and secreting full-sized mature ETA (Fig. 4). The amount of toxin produced by strain WR5(pCMtox) was relatively small, even though plasmid pCMtox was present in multiple copies (34). It was considered that this strain might be making more toxin but not secreting it. The WR5(pCMtox) cells were examined by Western blotting after solubilization of the cells, and less than 5% of the ETA remained with the cells (data not shown). In contrast to wild-type cells, ETA produced from WR5(pCMtox) is not regulated by Fe^{2+} (Fig. 3, lanes 1 to 8; Fig. 4, lanes 3 and 4). It was possible that strain WR5 was, for some unknown reason, incapable of producing and secreting high levels of ETA. For this reason we assayed ETA levels produced by WR5(ptrpETA-RSF1010) in which ETA synthesis is under the control of the *E. coli trp* promoter. The synthesis of ETA from the *trp* promoter was substantially higher than that produced from the *tox* promoter (Fig. 4, lanes 1 and 2), indicating that strain WR5 is capable of synthesizing and secreting high levels of ETA. The levels of ETA did not decrease when this strain was grown in an Fe^{2+}-sufficient medium (Fig. 4, lanes 1 and 2) nor was the synthesis of ETA from the *trp* promoter regu-

TABLE 2. β-Galactosidase activity of *E. coli* strains carrying the plasmid of the fusion of the ETA promoter to *lacZ*

E. coli strain	Fe^{2+} added (μg/ml)	β-Galactosidase activity (U)[a]
HB101	0	1.533
	10	1.588
D1245	0	0.123
	10	0.124
D1245(pMLB1034)	0	0.098
	10	0.043
D1245(pCG39)[b]	0	0.200
	10	0.205
D1245(pCG39It)[c]	0	0.200
	10	0.174

[a] As defined by Miller (17).

[b] pCG39 carries the ETA promoter in pMLB1034 in the same orientation with respect to *tox*.

[c] pCG39It is pCG39 with a *trpA* transcription terminator inserted directly upstream of the ETA promoter fragment.

A

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562
a                                                          b    8bp  DIRECT  REPEAT
CCCGTTCGCACATTCACCACTCTGCAATCCAGTTCATAAATCCCATAAAAGCCCTCTTCCGCTCCCCGCCAGCCTCCCCG
                                                   -35?
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642          S1a                          S1b    DODECAMER
CATCCCGCACCCTAGACGCCCCGCCGCTCTCCGCCGGCTCGCCCGACAAGAAAAACCAACCGCTCGATCAGCCTCATCCT
-10?         -89                          -62    c
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722
                        d
TCACCCATCACAGGAGCCATCGCG  MET HIS LEU ILE PRO HIS TRP ILE PRO LEU VAL ALA SER LEU
      S D                 ATG CAC CTG ATA CCC CAT TGG ATC CCC CTG GTC GCC AGC CTC
                                                       Bam H1
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B

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        -25              -13
    AAAAACCACCCG          PLC

    AAAAACCAACCG          ETA
    -54              -43
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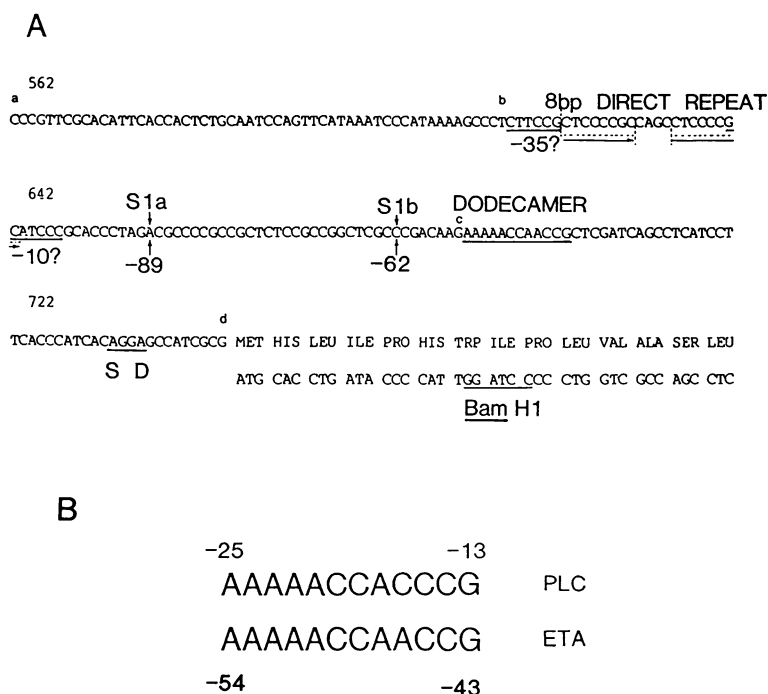
FIG. 5. Features of the regulatory region of the ETA gene. (A) Nucleotide sequence of the 5' upstream region and the 5' coding region of the ETA cloned fragment from *P. aeruginosa* PA103 adapted from sequence data presented in reference 9. The 5' termini of the *tox* mRNA are designated S1A and S1B and initiate at positions −89 and −62 bp, respectively, from the translational start signal. SD, Putative ribosome-binding site. Dotted lines indicate the direct repeat in the *tox* regulatory region. (B) Conserved dodecamer sequence of the 5' untranslated region of the ETA and phospholipase C (26) transcripts. See text for description of noted features.

lated by tryptophan in *P. aeruginosa* (M. L. Vasil, unpublished data).

Expression of β-galactosidase from the *tox* promoter in *E. coli*. A 760-bp *Pvu*II-*Bam*HI fragment from the cloned ETA fragment which contains the first 24 bp of the ETA structural gene as well as 736 bp directly 5' of the initiation codon, which had been previously shown to carry the ETA promoter (34), was used in the construction of a fusion of the ETA promoter to a promoterless *lacZ* gene (Fig. 1). To ensure that the synthesis of β-galactosidase was directed from the ETA promoter and not a vector promoter or a promoterlike sequence of the vector (32), a transcriptional terminator was inserted directly upstream of the ETA promoter fragment (Fig. 1). The two plasmids, pCG39 and pCG39It, were transformed into *E. coli* D1245, a *lac* deletion derivative of *E. coli* HB101. D1245(pCG39) and D1245(pCG39It) gave pale blue colonies on 5-bromo-4-chloro-3-indoyl-β-galactoside plates compared with the control D1245(pMLB1034), indicating low but detectable synthesis of β-galactosidase from the ETA promoter fragment. To obtain more quantitative results, we assayed β-galactosidase activity with the chromogenic substrate *o*-nitrophenyl-β-D-galactopyranoside (17). These results (Table 2) indicate that there is synthesis of β-galactosidase in *E. coli* at low but detectable levels. Moreover, this synthesis was not regulated by Fe^{2+} in *E. coli*.

DISCUSSION

It had been previously shown that the synthesis of ETA by *P. aeruginosa* was repressed when the cells were grown in an Fe^{2+}-supplemented medium (3). We used S1 nuclease analysis of RNA extracted from cells grown under Fe^{2+}-limiting (0.08 μg of Fe^{2+} per ml) and Fe^{2+}-sufficient (10 μg of

Fe^{2+} per ml) conditions to show that the regulation of ETA by Fe^{2+} is at the level of transcription. These S1 nuclease experiments revealed the presence of two protected fragments. The 5' termini of the *tox* transcripts were found to be at 89 and 62 bp upstream of the ETA initiation codon in strain PA103. The 5' terminus of the longer transcript (S1A) is within 1 bp of that recently reported by Chen et al. (S.-T. Chen, E. Jordan, R. B. Wilson, and R. C. Clowes, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, B124, p. 45); however, they do not report observing the second protected fragment (S1B). At this time it is unclear whether the two protected fragments represent two unique transcripts or whether the shorter one is a degradation product of the larger transcript. It is interesting to note that the 5' terminus of the smaller transcript (S1B) is located only 8 bp upstream of a conserved dodecamer also found in the transcript of the phosphate-regulated phospholipase C gene of *P. aeruginosa*. The 5' terminus of the phospholipase C transcript is located 2 bp upstream of the conserved dodecamer sequence (26). It is possible that this is a transcriptional regulatory sequence; however, it is not possible to make any significant conclusions regarding this sequence with the present data. Analysis of the DNA sequence of this 5' region of the ETA cloned fragment (9) is shown in Fig. 5. At the spatial positions of the −10 and −35 regions of the longer transcript (S1A) and the corresponding positions for the shorter transcript (not shown) there appears to be no homology to the consensus −10 (TATAAT) and −35 (TTGACA) regions of procaryotic promoters. It is of interest to note that when there is a great variation from the consensus −10 and −35 regions, then an activator protein is usually required to help the polymerase bind and efficiently begin transcription in *E. coli* (27).

There are some other interesting features of this region of the *tox* promoters. (i) The G+C content of the ETA struc-

tural gene is 68.5%, and the overall G+C content of *P. aeruginosa* is 67%. In contrast, the sequence upstream of the promoter region (a to b, Fig. 5A) is 48% G+C, whereas the region of the promoter and transcript start sites (b to c, Fig. 5A) is 76% G+C; the sequence from the end of the promoter region to the start of the gene (c to d, Fig. 5A) then decreases to 56% G+C content. (ii) There is an 8-bp direct repeat present between the spatially determined −35 and −10 sites of S1A. The possible role these regions play in the transcription and regulation of ETA expression, if any, is unclear at this time. Isolation of mutants with changes in these regions will further the understanding of the role these sequences play in the regulation and expression of ETA.

There are several possible reasons for the lack of Fe^{2+} regulation of ETA in *P. aeruginosa* when the gene is in high copy number with its own promoter and also when the ETA structural gene is present in *E. coli*. It should be noted that the diphtheria toxin gene, while under Fe^{2+} regulation in *C. diphtheriae*, is also not regulated by Fe^{2+} in *E. coli* (13). As mentioned above, this could be due to the lack of recognition of transcriptional or translational signals or both by *E. coli*, or *E. coli* lacks a factor required for transcription or regulation or both. Leong and Murphy (13) have demonstrated that the diphtheria toxin promoter is efficiently recognized by the *E. coli* RNA polymerase and that transcription of the diphtheria toxin gene begins at the same place in both *C. diphtheriae* and *E. coli*. It appears that the lack of Fe^{2+} regulation of diphtheria toxin may be due to the absence of a negative Fe^{2+} regulatory element. The proposed model for Fe^{2+} regulation of diphtheria toxin is one in which the inhibitory effect of Fe^{2+} is mediated through an aporepressor which acts as a negative controlling factor (20). The lack of repression of diphtheria toxin in *E. coli* could be due to the absence of such a repressor.

It could be that Fe^{2+} regulates ETA in a similar manner, through a negative regulator. However, we are not aware of any data available which support a negative control element. In contrast, there are data which are consistent with the notion that a positive activator is necessary for ETA expression. The ETA promoter is not well recognized in *E. coli*, in contrast to the recognition of the diphtheria toxin promoter by *E. coli* RNA polymerase (13). Also, the *recA* gene (21) and the pilin gene (23) of *P. aeruginosa* have been cloned into *E. coli* and have been expressed from their own promoters. These data suggest that the expression of ETA from the *tox* promoter requires an activator, a factor essential for high levels of expression from the *tox* promoter, a factor present in *P. aeruginosa* but not in *E. coli*. In support of this hypothesis, data are presented which demonstrate that the amount of ETA produced by WR5(pCMtox) is small considering the potential for ETA production from the 40 copies of the ETA gene present. In contrast, strain WR5(ptrpETA-RSF1010), in which toxin production is controlled by the *E. coli trp* promoter, produces significantly more ETA than WR5(pCMtox), in which the expression of ETA is under the control of its own promoter. These data suggest that the *tox* gene is under positive transcriptional control rather than a negative control system. If there was a repressor one would expect to see derepression of ETA synthesis in WR5 (pCMtox) because of the multiple copies of the hypothetical *tox* operator, which would titrate out the repressor (28). Further evidence that ETA is in fact positively regulated is a recent report of the cloning of a gene from hypertoxin-producing strain PA103 encoding a *trans*-acting product which regulates the expression of ETA in a positive manner (11). This regulator acts to increase the expression of ETA

10-fold in toxigenic *P. aeruginosa* strains tested, and high levels of Fe^{2+} in the culture medium only partially inhibited the overproduction (11). It is worthwhile to note that sequences homologous to this cloned activator are present in strain WR5 (11).

It should be stressed that the presence of a positive regulator does not rule out the possibility of a negative regulatory mechanism. Current evidence supports the presence of an activator for ETA expression but does not address the notion of a negative regulatory mechanism as well. One of the best-understood regulation systems, the lytic-lysogenic phase maintenance of bacteriophage lambda, involves the regulation of the expression of the repressor *cI*. *cI* acts as both a positive and negative regulator of its own synthesis. A second regulator, *cro*, also acts as a negative regulator of *cI* expression (27). The *E. coli phoB* gene of the *pho* regulon is also regulated by both positive (*phoR, phoB*) and negative (*phoR*) regulatory factors (35), as is the regulation of expression of the *lac* (29) and *ara* (37) operons of *E. coli*. Therefore, the presence in a cell of both a positive and negative regulator for one gene is not mutually exclusive.

It is clear that the regulation of ETA synthesis is complex, in which there are multiple genes and multiple environmental controls (3, 4, 6, 34). This study has taken a step toward the discovery of the regulatory mechanism of this gene with identification of the level of transcription as the site of Fe^{2+} regulation. Also, data presented are consistent with the presence of a positive regulator for ETA expression; however, additional studies will be necessary to elucidate the mechanism of this gene control. Toward these goals we also described in this report the construction of a fusion of the ETA promoter to the β-galactosidase gene. This hybrid plasmid was integrated into the chromosome of strain PA103, which now expresses β-galactosidase activity (unpublished data). Further studies with this strain will be useful in the reconstruction of the mechanisms which govern the synthesis of ETA.

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