Transcription and Expression of the Exotoxin A Gene of *Pseudomonas aeruginosa*

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The exotoxin A genes from *Pseudomonas aeruginosa* strains PA103 and PAO1 have been independently cloned in a pUC9-derived plasmid. In a non-toxigenic mutant of PAO1 as host, the cloned genes directed the synthesis of intact exotoxin A that expressed ADP-ribosyltransferase activity upon treatment with urea and dithiothreitol. Western-blot analysis of culture supernatants identified a polypeptide of 67 kDa, the molecular mass of intact exotoxin A. There was an approximately 15-fold increase in the toxin yield from *P. aeruginosa* cells carrying a cloned PA103 gene compared to PA103, and a 40-fold increase in the yield of toxin from cells carrying a cloned PAO1 gene compared to PAO1; cells carrying the cloned PA103 gene yielded about four times more toxin than those carrying the cloned PAO1 gene. Toxin expression was correlated with the presence of a transcript that was initiated 88 bp upstream from the translational start site. Little or no messenger RNA from either cloned gene could be detected in an *Escherichia coli* host, or in a *P. aeruginosa* host grown in the presence of 0.1 mM-Fe²⁺, a condition that inhibits toxin expression. The nucleotide sequences of two regions, each of approximately 500 bp, near the 5' and 3' termini of the structural gene were established. In these regions, the exotoxin A gene from PAO1 has ten base-pair differences compared to the PA103 gene, three in the non-coding region, and seven in the structural gene, four of which should lead to amino-acid differences. No apparent sequence similarities were found between the inferred promoter region of the exotoxin A gene and that of other *Pseudomonas* genes, nor with the consensus sequence of *E. coli* promoters.

INTRODUCTION

Pseudomonas aeruginosa exotoxin A, like diphtheria toxin, exerts its lethal effect on mammalian cells by inhibiting protein synthesis. This inhibition results from the toxin-catalysed transfer of the ADP-ribosyl moiety of NAD⁺ to elongation factor 2 (EF2), which blocks EF2 from further participation in protein synthesis (Iglewski & Kabat, 1975; Vasil *et al.*, 1977). While the enzymology of exotoxin A has been elucidated, it is not clear how the catalytic site of the toxin enters the cytosol to reach EF2, nor is the regulation of exotoxin A expression, which is controlled by iron, well understood. To better address questions of exotoxin A regulation and action, we (Mozola *et al.*, 1984), and others (Gray *et al.*, 1984) have been studying the cloned exotoxin A gene. We previously reported (Mozola *et al.*, 1984) the cloning of a segment of the exotoxin A gene of *P. aeruginosa* strain PA103. This segment was expressed both in *Escherichia coli* and in *P. aeruginosa*, as measured by transfer of the ADP-ribosyl moiety of NAD⁺ to EF2; but the polypeptide synthesized had a molecular mass of about 50 kDa, instead of the expected 67 kDa. We concluded that the carboxy-terminal region of the toxin had been cloned, and was fortuitously expressed from the tetracycline-resistance gene promoter of the vector pBR322.

Abbreviation: EF2, elongation factor 2.

Concurrently, Gray *et al.* (1984) were successful in cloning and sequencing the entire exotoxin A gene from PA103 together with 746 bp upstream of the gene. The gene was expressed in *E. coli* under the control of the *E. coli trp* promoter. Thus, in these initial experiments expression of the cloned exotoxin A gene from its natural promoter had not been achieved.

We report here the cloning and characterization of 2.76 kb *Pst*I-*Eco*RI chromosomal fragments that encode the intact exotoxin A genes from *P. aeruginosa* strain PA103, the classical exotoxin-producing strain (Liu, 1966), and from a derivative of PAO1, the strain used for most genetic studies of this species (Holloway *et al.*, 1979), including extensive studies related to exotoxin A (Bjorn *et al.*, 1977, 1978; Gray & Vasil, 1981; Hanne *et al.*, 1983; Sokol *et al.*, 1982). Both genes were expressed in a non-toxicogenic derivative of PAO1 from promoters located within the 2.76 kb cloned regions. A preliminary report of this work has appeared as S. T. Chen, E. Jordan, R. B. Wilson & R. C. Clowes (1986), ASM Annual General Meeting, Abstract no. B-124.

METHODS

Bacterial strains and plasmids. *E. coli* K12 strain HB101 *hsdS20 recA13 ara14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44 λ⁻ F⁻* (Boyer & Roulland-Dussoix, 1969) was used for cloning and gene expression. The *P. aeruginosa* strains used are listed in Table 1. Strains PA103J and PAO286 were used as sources of the exotoxin A gene sequences studied. MAM2, a derivative of PAO286 that produces no exotoxin A, has a mutation that maps in or near the structural exotoxin A gene (*tox*A), and was described earlier (Mozola *et al.*, 1984). To produce a Rec⁻ derivative of this mutant, we first constructed a donor strain, PAO1818, which carried the fertility factor R68.45, by transferring this factor from PAO8(R68.45). We then crossed PAO1818(R68.45) with MAM2, selecting for Trp⁺ colonies, and screening for a Thr⁻ colony which we designated MAM3. MAM3 was then mated with PTO66(R68.45), selecting for Thr⁺ colonies, and screening cultures of recombinant colonies for a RecA⁻ phenotype by the ability to produce few or no prototrophic clones when transduced with a preparation of F116L bacteriophage that was previously grown on a *met-28*⁺ strain, and which produced more than 100 prototrophic transductant colonies on MAM2 (Fruh *et al.*, 1983). We designated one such Thr⁺ RecA⁻ colony, MAM4 (Table 1).

The plasmid pRO1614 was constructed by Olsen *et al.* (1982) by the insertion into the *Pst*I site of pBR322 of a 1.85 kb segment derived from a cryptic *Pseudomonas* plasmid. pRO1614 can replicate either in *E. coli* or *P. aeruginosa* (Olsen *et al.*, 1982) and has been used in previous studies (Mozola *et al.*, 1984; Wozniak *et al.*, 1987). pRO1614 and pUC9 (Vieira & Messing, 1982) were used for cloning and for the derivation of new vectors as described below.

Media. Nutrient agar contained (per litre) 25 g Oxoid Nutrient Broth no. 2, 5 g Difco yeast extract, and 12 g agar. Tryptic soy broth dialysate (TSBD) medium. (BBL: Becton Dickinson) deferrated with CaCl₂, and supplemented with 0.1 M monosodium glutamate and 1 % (v/v) glycerol was prepared as described by Iglewski & Sodoff (1979). It was also supplemented with 1 mM-MgSO₄, which was found to increase toxin yields approximately twofold (data not shown). TSBD medium was made inhibitory for exotoxin A production by the addition of 0.1

Table 1. *P. aeruginosa* strains used

Strain	Genotype	Derivation or source
PA103J	Prototrophic	G. Jacoby (Liu, 1966)
PA103L	Prototrophic	S. Leppla (Liu, 1966) (PA103-22)
PAO286*	<i>met-28 trp-6</i>	B. Holloway (Stanisich & Holloway, 1969)
PAO1818*	<i>leu-9001 pur-9013 thr-9001</i>	B. Holloway (Holloway <i>et al.</i> , 1979)
PAO8	<i>ilvD202 met-28 str-1</i>	T. Morgan (Isaac & Holloway, 1968) (source of R68.45) (Haas & Holloway, 1976; Morgan, 1982)
PTO66*	<i>his-4 ilv-1118 lys-12 proA82 trp-6 recA102</i>	D. Haas (Fruh <i>et al.</i> , 1983)
MAM2*	<i>met-28 trp-6 toxA</i>	Mutagenesis of PAO286 (Mozola <i>et al.</i> , 1984)
MAM3*	<i>met-28 thr-9001 toxA</i>	Cross PAO1818(R68.45) × MAM2†
MAM4*	<i>met-28 recA102 toxA</i>	Cross PTO66(R68.45) × MAM3†
MAM32	Prototrophic Tox ⁻	Mutagenesis of PA103J‡

* Derivative of PAO1 strain.

† This work.

‡ This work. Selection for a Tox⁻ strain by Elek test, which was complemented to Tox⁺ following transformation by pRC362. Methodology as previously described (Mozola *et al.*, 1984).

mm-FeSO₄. Solid TSBD was prepared by adding agarose (SeaKem) to 1.2% (w/v). Minimal glucose medium has been previously described (Holmans & Clowes, 1979); it was supplemented as necessary with amino acids, each to a concentration of 20 µg ml⁻¹. Where appropriate, disodium carbenicillin (Cb) or tetracycline (Tc) was added to concentrations of 300 µg ml⁻¹ and 50 µg ml⁻¹, respectively.

Cloning procedures, Western-blot analysis, and assays of ADP-ribosyltransferase activity of exotoxin A. These procedures were as previously described (Mozola *et al.*, 1984), with several modifications. Horseradish-peroxidase-conjugated goat anti-rabbit IgG (Cappel) was used in place of ¹²⁵I-labelled Protein A (Hawkes *et al.*, 1982) to detect exotoxin-related polypeptides transferred to nitrocellulose. [³H]NAD⁺ (New England Nuclear), at a final concentration of 0.2 µCi ml⁻¹ (7.4 kBq ml⁻¹), was substituted for [¹⁴C]NAD⁺ in ADP-ribosyltransferase assays, and these assays were performed on supernatants from *P. aeruginosa* cultures grown at 32 °C (Liu, 1966).

DNA/DNA hybridization. To identify clones carrying exotoxin A gene sequences, transformed cells were transferred to nitrocellulose paper and screened by hybridization as described in Maniatis *et al.* (1982) with a DNA probe derived from the 520 bp *Bam*HI fragment of the exotoxin A gene previously cloned in pRC345 (Fig. 1; Mozola *et al.*, 1984). The purified fragment used as a probe was labelled with [α-³²P]dCTP by nick-translation with *E. coli* DNA polymerase I following the procedures recommended by Bethesda Research Laboratories, the suppliers of the nick-translation kit.

S1-nuclease mapping of RNA transcripts. The methodology of Berk & Sharp (1977) as described in detail by Chen & Clowes (1984) was employed, with several modifications when using RNA derived from *P. aeruginosa* strains. First, during the isolation of RNA from cells grown in TSBD, a heat pulse at 65 °C for 20 s was found to be adequate to promote lysis of *P. aeruginosa* cells, rather than the 100 °C pulse used to lyse *E. coli* (Dennis & Nomura, 1975). Second, to accommodate the higher GC content of *Pseudomonas* DNA, the temperature at which RNA/DNA hybrids were allowed to form was increased from 50 °C to 60 °C. RNA was isolated from cells after 19 h incubation in TSBD broth at 32 °C using the conditions noted in Fig. 2.

Nucleotide sequencing. DNA was sequenced by the standard method of Maxam & Gilbert (1980).

Construction of plasmid vectors. Several plasmid vectors capable of replication in both *E. coli* and *P. aeruginosa* were constructed from pUC9 by inserting the 1.85 kb *Pst*I fragment from pRO1614, which confers upon pBR322 the ability to replicate in a variety of hosts (Olsen *et al.*, 1982). Thus, pRC354 was constructed by ligating the 1.85 kb *Pst*I fragment of pRO1614 into *Pst*I-cleaved pUC9, and was shown to be able to replicate and confer carbenicillin resistance in *P. aeruginosa* hosts. pRC357 was derived from pRC354 by partial digestion with *Pst*I, followed by removal of the cohesive ends by treatment with S1 nuclease, blunt-end ligation with T4 DNA ligase and transformation into *P. aeruginosa*, selecting for carbenicillin-resistant (Cb^r) clones. The plasmid DNA of a number of transformants was first screened to detect those derivatives in which *Pst*I cleaved the DNA to linear monomers. Several such DNAs were further screened for the production, after *Pst*I-*Eco*RI digestion, of a linear DNA fragment of near monomer size of 4.51 kb. One such plasmid, which was concluded to have lost the *Pst*I site distal to the *Eco*RI site, was isolated, and was designated pRC357.

RESULTS

Cloning the intact exotoxin A gene in pUC9

From the data of Gray *et al.* (1984) it was predicted that the intact exotoxin A gene of *P. aeruginosa* would be carried on a 2.76 kb *Pst*I-*Eco*RI fragment. We digested chromosomal DNA from strain PAO286 with *Eco*RI and *Pst*I, separated the fragments by agarose-gel electrophoresis, transferred the DNA to nitrocellulose, and identified a 2.76 kb fragment that hybridized with the nick-translated 520 bp *Bam*HI fragment from pRC345 (Fig. 1). A 2.76 kb region from a similar gel was excised and the DNA was recovered by electroelution, purified, and ligated to a *Pst*I-*Eco*RI-cleaved preparation of pUC9, followed by transformation of *E. coli* HB101. (We used pUC9 and derivatives of pUC9 in these experiments because attempts to clone directly into pRO1614 resulted in the spontaneous loss of part of the 2.76 kb insert, together with part of the tetracycline-resistance gene of the vector.) Of 4500 Cb^r transformants screened, approximately 5% hybridized with the 520 bp probe, indicating the presence of exotoxin A gene sequences in these cell lines. Examination of plasmid DNA from ten of these colonies showed that each had acquired a plasmid of the size of pUC9 plus a 2.76 kb insert. To ensure that the 2.76 kb fragment was the correct one, the insert of one of these plasmid DNAs was digested with *Pst*I, *Eco*RI, *Bam*HI, *Sal*I or *Bgl*II. The fragments generated corresponded in size to those expected for the 2.76 kb *Pst*I-*Eco*RI exotoxin A gene fragment of PA103 DNA (Gray *et al.*, 1984) (Fig. 1). pUC9 carrying the 2.76 kb fragment of PAO286 was designated

Fig. 1. Exotoxin A gene. (a) The segment cloned in pRO1614 to produce pRC345. Part of the cloned exotoxin A gene sequences is shown by the black bar; pRC1614 sequences are shown by the wiggly line. *Eco*, *Bam*, *Apa*, *Xho* and *Hin* are restriction-enzyme sites mapped for *Eco*RI, *Bam*HI, *Apa*I, *Xho*I and *Hin*I, respectively. The arrows spanning the '520' and '450' fragments represent those segments used as probes. P2 is the *tet* promoter in pBR322 and ATG is the start codon of the tetracycline-resistance gene. (b) The entire exotoxin A gene sequence as cloned on a 2.76 kb *Pst*I-*Eco*RI fragment (Gray *et al.*, 1984). The numbers are nucleotides numbered from the *Pst*I site. (*Pst*, *Pvu*, *Sal*, *Bgl* and *Sau* represent cleavage sites for *Pst*I, *Pvu*I, *Sal*I, *Bgl*I and *Sau*3AI, respectively.) The hatched double line represents the coding region of exotoxin A, ATG the initiation codon and P(ex), the initiation site of exotoxin transcription. (c) I, II, III represent segments of the gene used as probes in S1-nuclease experiments. (d) The arrows represent the regions over which the nucleotide sequences were determined from the asterisked sites.

pRC350. pRC351 was constructed by ligating the 1.85 kb *Pst*I fragment of pRO1614 into *Pst*I-cleaved pRC350. pRC352 was isolated from pRC351 after deletion of the *Pst*I site distal to the *Eco*RI site, as described for the construction of pRC357 from pRC354 (see Methods).

The exotoxin A gene from PA103 was cloned on a 10 kb *Eco*RI fragment of PA103J chromosomal DNA, which had been shown to hybridize with the 520 bp fragment of pRC345. An *Eco*RI fragment was selected, since we found that chromosomal DNA isolated from two sources of PA103 (PA103J and PA103L) was not cleaved by *Pst*I. The 10 kb *Eco*RI fragment was ligated with an *Eco*RI-cleaved, alkaline-phosphatase-digested preparation of pUC9. After transformation of competent *E. coli* HB101 cells, 3000 Cb' clones were obtained, approximately 1% of which hybridized with the 520 bp probe. Plasmid DNA was isolated from eight clones and each was found to consist of a 10 kb *Eco*RI fragment inserted into pUC9. One of these plasmids, designated pRC361, produced a 2.76 kb fragment when digested with *Eco*RI plus *Pst*I. This fragment was isolated, purified, and cloned into *Pst*I-*Eco*RI-cleaved pRC357 to produce pRC362, and into similarly cleaved pUC9 to produce pRC360. When digested with *Bam*HI, *Kpn*I, *Sal*I, *Ava*I, *Bgl*II or *Dde*I, both pRC360 and pRC362 produced fragments with sizes consistent with the sequence information provided for the 2.76 kb *Pst*I-*Eco*RI fragment cloned by Gray *et al.* (1984). Similar cleavage-site correlations were found in the clones pRC350 and pRC352 derived from PAO286, except that one *Rsa*I site (at nucleotide 1127) was missing, suggesting that the nucleotide sequences of the two genes from different sources were not identical; this was substantiated by nucleotide-sequencing studies (see below). Plasmid DNA from either pRC352 or pRC362 isolated from a MAM4 host could be readily cleaved with *Pst*I. However, when a *Tox*⁻ derivative of PA103, MAM32, was transformed with pRC352 or pRC362, and the plasmid DNA was reisolated, it could not be cleaved with *Pst*I. We conclude that our sources of PA103, as distinct from that used by Gray *et al.* (1984), express a system for *Pst*I site modification.

ADP-ribosyltransferase activity from cloned DNA

The ADP-ribosyltransferase activity produced by *P. aeruginosa* strains PA103, PAO286, the *Tox*⁻ derivative of PAO286, MAM4, carrying pRC352 or pRC362, and *E. coli* HB101 carrying pRC352 or pRC362 was measured in culture supernatants by assaying transfer of [³H]ADP-

Fig. 2

Fig. 3

Fig. 2. ADP-ribosyltransferase activities of various exotoxin-A-producing cultures as a function of growth. Overnight cultures in TSBD broth were diluted 1:200, subcultured in TSBD and incubated at 32 °C with aeration. At various times, samples were removed, diluted where appropriate, and assayed for culture density (OD_{600}) and for the ADP-ribosyltransferase activities of the supernatants (assessed as μg exotoxin ml^{-1}). ●, ▲, OD_{600} values for cultures of PA103 and MAM4(pRC362) respectively [growth of MAM4(pRC352) and PAO286 was similar to MAM4(pRC362)]. The open symbols represent ADP-ribosyltransferase activities expressed in terms of μg exotoxin A per OD_{600} unit, measured as previously described (Iglewski & Sadoff, 1979), in supernatants of MAM4(pRC362) (△), MAM4(pRC352) (▽), PA103 (○) and PAO286 (□). The amounts of exotoxin A produced by PAO286 cultures were too small to be visible on this graph, but were well above the detectable level of $0.05 \mu\text{g ml}^{-1}$ – see Table 2.

Fig. 3. Western-blot analysis of exotoxin A in culture supernatants. Procedures were as described by Mozola *et al.* (1984). Lane 1, $1 \mu\text{g}$ purified exotoxin A. Lanes 2 to 5, $30 \mu\text{l}$ supernatant from 150 ml cultures grown as described and for which ADP-ribosyltransferase activities are shown in Table 2: lane 2, PA103; 3, PAO286; 4, MAM4(pRC362); 5, MAM4(pRC352).

Table 2. ADP-ribosyltransferase activity in *P. aeruginosa* cultures

The methodology was as described by Iglewski & Sadoff (1979). Activity was measured in $10 \mu\text{l}$ (diluted 1:10 or 1:100 as appropriate) of the supernatant of a 150 ml culture derived from a 1:200 subculture of an overnight culture into TSBD and grown at 32 °C for a further 20 h, when exotoxin A activity reached a maximum (see Fig. 2).

Culture	OD_{600}	ADP-ribosyltransferase activity* ($\mu\text{g ml}^{-1}$)	($\mu\text{g per } OD_{600} \text{ unit}$)
PA103	14.0	20.1	1.44
PAO286	8.4	1.22	0.145
MAM4(pRC362)	7.4	162	21.90
MAM4(pRC352)	7.5	43.7	5.83

* The amount of exotoxin A per sample was calculated using a standard curve of known concentrations of exotoxin A; the results shown are the means of triplicate samples.

ribose from $[^3\text{H}]\text{NAD}^+$ to wheat-germ EF2 (Table 2, Fig. 2). As previously observed in PA103 cultures (Bjorn *et al.*, 1978), exotoxin A synthesis was not detected until the early stationary phase of growth (Fig. 2) and no toxin ($<0.05 \mu\text{g ml}^{-1}$) could be detected in any of the supernatants until the cell density exceeded $2 \times 10^9 \text{ cells ml}^{-1}$. In culture supernatants of PA103, there was an approximately tenfold greater concentration of exotoxin A (expressed as activity per cell mass) than in PAO286 cultures after 20 h of growth (Table 2). When the cloned genes were expressed in strain MAM4, there was a 15-fold increase in exotoxin A activity in

cultures of cells carrying the cloned PA103 gene and a 40-fold increase in those of cells carrying the cloned PAO286 gene. When iron was added to 0.1 mM, toxin concentrations never exceeded, in any culture, more than 1% of those attained in cultures without added iron. No exotoxin A activity ($<0.05 \mu\text{g ml}^{-1}$) was detected in cultures of *E. coli* HB101 carrying either of the cloned genes (data not shown).

Western-blot analysis

Supernatants derived from the exotoxin-A-producing cultures (Table 2) were subjected to SDS-PAGE and transferred to nitrocellulose. A single immunologically active band, which comigrated with purified exotoxin A, was found in preparations derived from PA103, PAO286, MAM4(pRC352) or MAM4(pRC362) (Fig. 3). The intensity of each band (Fig. 3) was roughly proportional to the ADP-ribosyltransferase activity of the samples (Table 2). No bands were visible when supernatant fluids of cultures of *E. coli* HB101(pRC362), or of *P. aeruginosa* strains that carried an active exotoxin A gene but which were grown in 0.1 mM-FeSO₄, were tested (data not shown).

Determination of the site of transcriptional initiation

Gray *et al.* (1984) determined that the initiating codon of exotoxin A began at base-pair 746 of the *Pst*I-*Eco*RI fragment, with the first base-pair of this fragment designated as base-pair 1 (Fig. 1). The probe we initially used to determine whether transcription occurs in the region upstream of the initiating codon was a 1488 bp *Pst*I-*Bgl*II fragment (Fig. 1) isolated from pRC350 and 5'-end-labelled at the *Bgl*II site only. S1-digestion experiments of the probe hybridized with RNA isolated from *P. aeruginosa* MAM4(pRC352) after 20 h subculture produced a protected sequence of 845 ± 30 nucleotides, indicating that transcription was initiated from nucleotide 645 ± 30 (data not shown). A smaller probe, consisting of the 357 bp *Pvu*I-*Sal*I fragment from pRC350, 5'-end-labelled at the *Sal*I site only, produced a band of 253 ± 10 nucleotides under similar conditions with MAM4(pRC352) RNA (Fig. 4, lane 3), indicating that transcription had been initiated at nucleotide 655 ± 10 . Bands of a similar size, but less intense, were also seen when RNA from PA103 or PAO286 cultures was used to protect this probe (Fig. 4, lanes 4 and 5). No bands of 253 ± 10 were seen when the RNA was derived from strain MAM4 carrying the vector pRC357, or from *E. coli* HB101(pRC352) (Fig. 4, lanes 7 and 8). Finally, a 214 bp *Pvu*I-*Bam*HI fragment from pRC350 was used as the probe DNA with RNA from MAM4(pRC362) and this reaction was run in parallel with a nucleotide-sequence determination of that segment. From this experiment it was concluded that initiation of transcription takes place at nucleotide 658. An autoradiograph of this experiment is shown in Fig. 5. In parallel experiments, using RNA derived from toxigenic cultures which were grown in the presence of added 0.1 mM-Fe²⁺ and in which no toxin was produced, no protection of the DNA probe was seen. The result of such an S1 experiment using RNA derived from MAM4(pRC362) is also shown in Fig. 5.

Nucleotide-sequence determinations

pRC350 and pRC360 were each cleaved with *Bam*HI to produce two fragments, one of 1530 bp (765 to 2295), and the other of 3890 bp (including the vector plasmid) (Fig. 1). The fragments were end-labelled at the 5'-termini of the *Bam*HI sites using T4-polynucleotide kinase and [γ -³²P]ATP. After cleavage with *Pvu*I, the four single, end-labelled fragments were separated and sequenced by the technique of Maxam & Gilbert (1980).

The nucleotide sequence from 2020 to 2560 of pRC362 (from PA103J) was identical to that published for the PA103 strain used by Gray *et al.* (1984). The sequence in the same region of pRC352 (from PAO1) is almost identical, varying at only four positions: at 2287 there is a GC for AT substitution, and at 2341 a TA for CG substitution, neither of which should result in an amino-acid substitution; at 2039 there is an AT substitution for a GC which should substitute Ile in PAO286 for Val in PA103, and at 2363 there is substitution of GC for AT which should substitute Gly in the PAO286 sequence for Ser in the PA103 sequence. The 409 bp nucleotide sequence from nucleotides 553 to 961 of the PA103J strain used in this study was also identical to that determined by Gray *et al.* (1984). DNA from the analogous region of PAO286 differed in six

Fig. 4

Fig. 5

Fig. 4. S1-nuclease assay of exotoxin A probe. Autoradiograph of urea polyacrylamide gel following separation of a 357 bp end-labelled *PvuII*-*SalI* fragment (probe II in Fig. 1c) after hybridization with RNA and digestion with S1 nuclease. Lanes 1 and 6, pBR322 *HpaII* standards with sizes (bp) shown at side. Lane 2, probe II (0.15 μ l); lanes 3, 4, 5, 7 and 8 were loaded with 7.5 μ l of probe II following its hybridization with RNA and S1-digestion. RNAs isolated after 19 h subculture were 60 μ g MAM4(pRC352) in lane 3; 175 μ g PA103 in lane 4; 100 μ g PAO286 in lane 5; 84 μ g MAM4(pRC357) in lane 7; 100 μ g *E. coli* HB101(pRC352) in lane 8. Bands in lanes 3, 4, 5, 7, 8 at 360 bp are due to undigested probe. The identity of the band at about 220 bp in lane 8 is unknown.

Fig. 5. Initiation site for exotoxin A mRNA transcription. RNA was isolated after 20 h incubation at 32 °C from a subculture of MAM4(pRC362) grown in TSBD, or in TSBD with 0.1 mM-Fe²⁺, and the degree of protection of a ³²P-labelled 214 bp *PvuII*-*BamHI* fragment (probe III in Fig. 1c) from pRC360 afforded after hybridization with this RNA was determined. A 50% urea/8% polyacrylamide gel in TBE buffer was run under constant power (45 W) for 4 h. After electrophoresis, the gel was exposed to Kodak XAR-2 film at -70 °C overnight. Lane 1, RNA from MAM4(pRC362) grown in TSBD; lane 2, RNA from MAM4(pRC362) grown in TSBD + 0.1 mM-Fe²⁺; lanes 3, 4, 5, and 6, Maxam and Gilbert nucleotide sequence reactions for G, G + A, T + C, C, respectively; lane 7, probe without RNA. The letters and numbers to the left of the photograph indicate the nucleotide sequence complementary to part of that shown in Fig. 6.

Fig. 6. Nucleotide sequence of part of the exotoxin A gene upstream of the initiating codon. The 5' to 3' sequence from nucleotide 596 to 666 shown is that for PA103 published by Gray *et al.* (1984) and confirmed in this study for PA103J. The numbers below the sequence indicate distances from the first base of the initiating codon at 746. The sequence of PAO286 is identical in this region, except at nucleotides 614 and 644, where the bases shown above the line in parentheses were found. The arrow above nucleotide 658 indicates the site inferred for the initiation of transcription of exotoxin A. The underlining indicates an 8 bp direct repeat.

positions from the sequence found for PA103J. Three of these differences were upstream of the coding region: TA instead of CG at position 614, AT for TA at 644, and AT for CG at 732. The three differences in the coding region were CG for TA at 756 (which should result in the substitution of Ile by Thr), TA for CG at 810 (substituting Phe for Ser), and GC for AT at 859 (which should not result in a change of amino acid).

DISCUSSION

The exotoxin A genes from *P. aeruginosa* strains PA103 and PAO286 (a derivative of PAO1) have each been cloned on a 2.76 kb *Pst*I-*Eco*RI fragment. The cleavage patterns of the genes of PAO286 (in pRC352) and of PA103 (in pRC362) for a number of restriction enzymes correspond to those of the PA103 gene reported by Gray *et al.* (1984) (see Fig. 1). The conclusion that the intact genes had been cloned was confirmed by measuring ADP-ribosylating activity (Table 2, Fig. 2) and the production of polypeptides which comigrated with purified exotoxin A (molecular mass 66 583 Da, as predicted from the sequence of Gray *et al.*, 1984), and interacted specifically with antibody to exotoxin A (Fig. 3). Moreover, these exotoxin-A-related polypeptides were synthesized in *P. aeruginosa* hosts, but no similar product was seen when the plasmids were introduced into an *E. coli* host. This finding is consistent with the presence on the 2.76 kb *Pst*I-*Eco*RI cloned fragments of promoters for transcription of the exotoxin A genes which are recognized in *P. aeruginosa*, but not in *E. coli*.

The ADP-ribosyltransferase assays showed an approximately tenfold difference in the amounts of exotoxin A produced by PA103 compared to PAO286 (Table 2). When the exotoxin A genes of PA103 or PAO286 were cloned, exotoxin A synthesis increased approximately 15- and 40-fold, respectively. This finding is consistent with a gene dosage effect due to the multicopy state of plasmids such as pRC357 and pRO1614 (Olsen *et al.*, 1982), especially in cells in early-stationary-phase cultures, where increases in copy number occur (Lin-Chao & Bremer, 1986). These results differ from those recently reported by Vasil *et al.* (1986), in which the PA103 gene was inserted into a pBR322/RSF1010 hybrid, multicopy plasmid, and the recombinant plasmid was introduced into the wild-type *P. aeruginosa* strain WR5, which appears to be deleted for exotoxin A gene sequences. This construct produced less exotoxin A than the parental PA103 strain, and the synthesis of the toxin was not reduced in media containing $10 \mu\text{g Fe}^{2+} \text{ ml}^{-1}$. The reason for these discrepancies is unknown, but may be due to differences in the host strains used.

The presence of a promoter sequence on the cloned DNA was confirmed, and its position located, by the S1-nuclease experiments. The results of experiments in which RNA from toxigenic cultures was used to protect three defined probes are consistent with the initiation of transcription being within the cloned DNA at nucleotide 658, which is 88 bp upstream of the translational initiation site (Figs 1, 4 and 6). It may also be concluded that initiation takes place at the same site on each of the cloned genes, and on the chromosomal genes of both PA103 and PAO286. However, we cannot exclude the possibility that the initiation site may be upstream of nucleotide 658, and that a longer transcript is synthesized and processed. [After this paper was submitted, Grant & Vasil (1986) reported that exotoxin A mRNA transcription from the chromosomal gene of PA103 was initiated at 89 bp and at 62 bp upstream of the initiating codon.]

The extent of exotoxin A synthesis in different cultures, measured either as enzyme activity (Table 2) or as the amount of immunologically cross-reacting protein (Fig. 3), is roughly paralleled by the level of transcription that may be inferred from S1-nuclease studies (Fig. 4). It seems likely, therefore, that the fourfold differences observed in the levels of enzyme activity in cells carrying the cloned genes from PA103 or PAO286 (Fig. 2) can be correlated with similar differences in the total exotoxin A protein synthesis (Fig. 3), which reflect the levels of mRNA transcription (Fig. 4). These differences may therefore result from quantitative differences in the regulation of exotoxin A synthesis, perhaps due to differences in the nucleotide sequences upstream of the coding regions of the genes.

The S1-nuclease experiments reported here show that in the presence of added iron, when exotoxin A production in *P. aeruginosa* is repressed, exotoxin A mRNA was reduced or absent in strains PA103 and PAO286, and also in a non-toxicogenic host carrying a cloned exotoxin gene from either of these strains. This reduction in the level of mRNA may be due to inhibition of initiation of exotoxin transcription, or to enhanced degradation of the transcripts.

The sequence immediately upstream of the inferred site of transcriptional initiation at position 658 (Fig. 6) shows no obvious similarities to the consensus sequence for promoters recognized by the σ^{70} RNA polymerase holoenzyme of *E. coli* (Hawley & McClure, 1983; Rosenberg & Court, 1979), and the sequence is particularly GC rich, in contrast to the AT-richness of the *E. coli* consensus promoter sequence. However, it may be noteworthy that this region carries a direct repeat of the sequence CTCCCCGC, and that within a region that would correspond approximately to the -10 box of the *E. coli* promoter consensus, there is a T (at nucleotide 644) in the PA103 sequence, but an A in the PAO286 sequence. Also, 44 nucleotides upstream of the initiation site, at nucleotide 614, the PAO286 sequence has a T in place of the C in the PA103 sequence. It is possible that these sequence differences may affect RNA polymerase binding, which together with other differences in the regulatory region may influence the level of expression and contribute to the fourfold difference found between the expression of the two genes cloned from PA103 and PAO286 in the same host strain, MAM4.

Comparisons of sequences within this assumed promoter region of the exotoxin A gene also show no obvious similarities with other regions recently tentatively identified as promoters in *Pseudomonas*. These include promoters of the TOL plasmid [the *xylABC* operon (Inouye *et al.*, 1984a), the *xylDEFG* operon (Inouye *et al.*, 1984b; Mermod *et al.*, 1984) and the *xylR* regulatory gene (Inouye *et al.*, 1985)]. However, sequence similarities between the promoter region of the exotoxin A gene and that of the alginate (*algD*) gene of *P. aeruginosa* have recently been identified (Deretic *et al.*, 1987). All these genes are only inefficiently expressed in *E. coli*, and regions resembling the *E. coli* σ^{70} -promoter consensus sequence have not been found, either in the region appropriately located to the site of transcriptional initiation, or in the region extending 100 bp upstream of this.

There appear to be at least two types of sequences that can be recognized as promoters in *P. aeruginosa*. One type, characterized by the β -lactamase promoter on RP1, has close similarities with the consensus sequence of *E. coli* and is also recognized by *E. coli* RNA polymerase, probably by the holoenzyme with a σ^{70} factor (Chen & Clowes, 1987). The other type, as found in the exotoxin A gene, is clearly different and is not recognized by *E. coli* RNA polymerase. Since exotoxin A is not synthesized until early stationary phase, it is possible that its synthesis first requires the synthesis of a specific RNA polymerase, or of a σ -factor able to react with the core enzyme. Such a σ -factor would function as a positive regulator to increase exotoxin synthesis, like that identified as the product of the recently cloned *toxR* gene (Hedstrom *et al.*, 1986; Wozniak *et al.*, 1987).

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