

## Cloning of a Gene Involved in Regulation of Exotoxin A Expression in *Pseudomonas aeruginosa*

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We have cloned a gene from *Pseudomonas aeruginosa* that stimulates the expression of exotoxin A. A recombinant library of genomic DNA from strain PA103 constructed with a broad-host-range plasmid vector containing chromosomal insert fragments generated by *Sau3A* was used to transform the hypotoxigenic mutant strain PA103-29. A recombinant plasmid, pFHK6, was isolated from a PA103-29 transformant which displayed increased toxin production. From pFHK6, which contained a 20-kilobase-pair chromosomal insert, a 3-kilobase-pair *XhoI* fragment was isolated and subcloned into the plasmid cloning vector pVK101 to give pFHK10. In toxigenic *P. aeruginosa* strains containing pFHK10, toxin expression was increased 10-fold and high levels of iron in the culture medium only partially inhibited the overproduction. Expression studies suggested that pFHK10 did not contain the toxin structural gene. In addition, Southern analysis with the 3-kilobase-pair *XhoI* fragment suggested that the putative toxin regulatory gene is common among different strains of *P. aeruginosa* including previously reported nontoxigenic strains.

The opportunistic bacterial pathogen *Pseudomonas aeruginosa* secretes several toxic proteins that are thought to act as virulence factors in the susceptible host. Of these, exotoxin A is the most toxic (30) and has been the most intensively studied. Identical in mode of action to diphtheria toxin, exotoxin A catalyzes the transfer of the ADP-ribosyl moiety of NAD onto elongation factor 2, thereby inhibiting protein synthesis in eucaryotic cells (15, 16). Exotoxin A is chromosomally encoded (10) and is produced by most (>90%) *P. aeruginosa* strains (4).

Little is known about the regulation of exotoxin A gene expression. Gray and Vasil (9) have mapped a genetic locus, *tox-2*, near 35 min on the genetically well-characterized *P. aeruginosa* strain PAO1 (32) chromosome, that appears to be involved in the regulation of exotoxin A gene expression. The *tox-2* mutant produces 100-fold less toxin yet still maintains parental levels of protease and total extracellular protein. Since this *tox-2* locus is well separated from the toxin structural gene of PAO1 (*toxA-1*) at 85 min, as determined by Hanne et al. (10), it has been suggested that the expression of the toxin-encoding gene is positively regulated by the gene product of the *tox-2* locus. Gray et al. (8) recently cloned the exotoxin A structural gene into *Escherichia coli* but expression of toxin could not be detected without placing the gene under the control of *E. coli* transcriptional/translational signals. While inefficient recognition of the toxin gene promoter in *E. coli* may have been the reason for the inability to detect expression, it is also possible that *E. coli* lacks one or more *P. aeruginosa* positive regulatory factors necessary for efficient expression of exotoxin A.

Of additional interest is the fact that excess iron in the culture medium inhibits toxin gene expression (2, 3), and this

inhibition is alleviated by a mutation in the *toxC* gene of the PAO1 strain.

In a previous report we described the cloning of a 20-kilobase-pair (kbp) fragment of *P. aeruginosa* DNA which resulted in an increase in exotoxin A expression in several strains of *P. aeruginosa* (R. C. Hedstrom, C. R. Funk, O. R. Pavlovskis, and D. R. Galloway, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, B151, p. 42). Here we further report the subcloning of a segment of *P. aeruginosa* DNA that stimulates the expression of exotoxin A in toxigenic strains after transformation of such strains with recombinant plasmids containing the cloned segment. The cloned DNA segment does not contain the toxin structural gene, as determined by expression studies, and DNA hybridization analysis shows that the cloned DNA is present in the genomes of both toxigenic and nontoxigenic *P. aeruginosa* strains.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** The *P. aeruginosa* strains used and their reported exotoxin A phenotypes are listed in Table 1. *P. putida* was strain 12633 and *E. coli* was strain HB101. The plasmids used for cloning were pJBK68 (J. B. Kaper, unpublished data) and pVK101 (20), each a derivative of the broad-host-range cloning vector pRK290 (6). In addition to the tetracycline resistance gene of pRK290, both pJBK68 and pVK101 contain a kanamycin resistance gene used for detection of insert DNA by marker inactivation and are low-copy-number plasmids. The helper plasmid pRK2013 (6) was used for mobilization of plasmids into *P. aeruginosa* strains during triparental matings. Bacteria were cultured at 37°C in LB medium for DNA isolation and plasmid selection procedures. For exotoxin A expression studies and for toxin purification, bacteria were cultured in tryptic soy broth dialysate at 32°C with vigorous aeration (17). Antibiotic concentrations for the selection of plasmid-bearing *P. aeruginosa* were 100 µg of tetracycline per ml and 1 mg of kanamycin per ml, whereas plasmid-bearing *E. coli* organisms were selected with 15 µg of tetracycline per ml and 100 µg of kanamycin per ml.

**DNA preparation and analysis.** Plasmids were isolated

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TABLE 1. *P. aeruginosa* strains

Strain	Exotoxin A phenotype	Reference
PA103	+	Liu (23)
PA103-29	-	Ohman et al. (28)
PAO1	+	Holloway et al. (13)
PAO-PRI	CRM <sup>a</sup>	Cryz et al. (5)
PAO-T1	-	Ohman et al. (27)
PA 220	+	Pavlovskis et al. (29)
388	-	Iglewski et al. (18)
WR5	-	Bjorn et al. (4)

<sup>a</sup> Nontoxic, immunologically cross-reactive exotoxin A.

according to Birnboim and Doly (1) and further purified by centrifugation to equilibrium in ethidium bromide-CsCl gradients. Chromosomal DNA was prepared as described by Hull et al. (14). Southern blot analysis was performed under high-stringency conditions, as described by Mekalanos (24). The 3-kbp probe used in the Southern analysis was isolated from a 1% agarose gel after electrophoresis of an *Xho*I digestion of pFHK10 DNA.

**Transformation.** *P. aeruginosa* PAO1 cells were cultured at 42°C as described by Holloway (12). Otherwise, all strains were grown at 37°C. Transformation was carried out according to a modification of a previously published procedure (31). Briefly, exponentially growing cells (20 ml) were washed in 10 ml of ice-cold 100 mM MgCl<sub>2</sub>, resuspended in 10 ml of buffer containing 75 mM CaCl<sub>2</sub>-10 mM MOPS, (morpholinepropanesulfonic acid; pH 6.5)-0.5% (wt/vol) glucose, and placed on ice for 20 min. These cells were then pelleted by centrifugation and resuspended in 1.0 ml of the same buffer. Cells of *E. coli* HB101 were treated in the same manner except that they were not washed in MgCl<sub>2</sub> and the CaCl<sub>2</sub> concentration was increased to 100 mM. Cells were used for transformation by plasmid DNA in the usual manner.

**Enzymes.** Restriction endonucleases, T4 DNA ligase, and bacterial alkaline phosphatase were obtained from Bethesda Research Laboratories (Rockville, Md.) and used as recommended by the supplier.

**Quantitative dot-immunoblotting assay for exotoxin A.** Exotoxin A antigen in culture supernatants was quantitated based on a method described by Jahn et al. (19). Briefly, twofold dilutions of culture supernatant samples in immunoblot buffer (50 mM Tris hydrochloride, pH 7.5, 150 mM NaCl, 0.15% [wt/vol] sodium azide) were made, and 20 µl of each was added to wells of a dot-blot apparatus (Minifold; Schleicher & Schuell, Inc., Keene, N.H.) containing a sheet of nitrocellulose paper. The sheet had been prewetted with immunoblot buffer and each well was overlaid with 100 µl of the same buffer. After blotting of the samples by suction, the sheet was removed and incubated in blocking buffer (3% [wt/vol] bovine serum albumin in immunoblot buffer) for 3 h. The entire blotting procedure was performed on a rotary shaker at ambient temperature. The nitrocellulose sheet was incubated overnight with polyclonal rabbit antitoxin (7) diluted 1:1,000 in blocking buffer. Excess antibody solution was removed, and the sheet was washed for 1 h in wash buffer (0.2% [wt/vol] gelatin) followed by 1 h in 0.05% (wt/vol) Nonidet P-40 in immunoblot buffer. To the washed and drained sheet was added 10<sup>6</sup> cpm of <sup>125</sup>I-labeled staphylococcal protein A (New England Nuclear, Boston, Mass.) per ml in blocking buffer. After a 3-h incubation, excess solution was removed and the sheet was washed as before. Finally, individual wells were cut out and the amount of

bound radioactivity was measured in a gamma counter. Standard curves were prepared for each sheet by blotting dilutions of purified exotoxin A. With this method, values for the standards were linear from 4 to 125 µg of exotoxin A, and from this linear portion of the standard curve the determination of the amount of toxin in the unknown samples was made.

**Antitoxin preparation.** The preparation of rabbit antitoxin and purified immune monoclonal antitoxin antibodies has previously been described in detail (7).

**Other methods.** Electrophoretic transfer of proteins, separated by electrophoresis through sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) (21) onto nitrocellulose sheets, was performed as described previously (11). Immunoblotting of these sheets was carried out as described above. Exotoxin A was purified according to the procedure described by Leppla (22) with previously noted modifications (7).

## RESULTS

**Genomic library construction and selection of pFHK10.** The chromosomal DNA of *P. aeruginosa* strain PA103 was partially digested with *Sau*3A and fractionated by size through a 5 to 20% NaCl gradient (35,000 rpm, 4.5 h, SW41 rotor, 40 µg of DNA per gradient). Fractions containing DNA of 15 to 25 kbp in size were pooled, and the DNA in these fractions was ligated to pJBK68 vector DNA that had been digested with *Bgl*III and treated with bacterial alkaline phosphatase. The ligation mixture was used to transform *P. aeruginosa* strain PA103-29 cells, a mutant hypotoxigenic strain. Samples of the transformation mixture were spread on tetracycline plates. Colonies from these plates were screened for kanamycin resistance. Kanamycin-sensitive clones were assayed for toxin production, using the Chinese hamster ovary cell cytotoxicity assay according to a previously published method (5, 17). Seven clones positive in this assay (thus, positive toxin phenotype) were further tested for toxin production with the ADP-ribosyl transferase assay (17). The plasmid DNA from one such clone, designated pFHK6, was isolated and found to contain a 20-kbp genomic insert that stimulated expression of toxin in PA103-29(pFHK6) cells (data not shown). Plasmid FHK6 DNA was digested with *Xho*I, producing three separate fragments which were ligated to *Xho*I digests of pVK101 DNA. After transformation and antibiotic selection of transformants, several clones were found that demonstrated increased toxin expression. From one of these, a recombinant plasmid, designated pFHK10, was isolated and shown to contain a 3-kbp *Xho*I insert.

**Effect of pFHK10 on exotoxin A expression in toxigenic and nontoxigenic *P. aeruginosa*.** Plasmid pFHK10 contained an insert of *P. aeruginosa* genomic DNA that stimulated toxin expression in each of the toxigenic strains tested (Table 2; Fig. 1). This was determined by mobilizing pFHK10 from *E. coli* HB101, using a triparental mating system (6), into each of the *P. aeruginosa* strains and calculating toxin levels by radioimmunoassay and enzymatic activity in the culture supernatant fraction of the resultant transconjugants. The results shown in Table 2 indicate that toxin production was stimulated at least 10-fold. These results are supported by the immunoblotting data shown in Fig. 1A. Here it can be seen that culture supernatants from pFHK10-transformed strains contain significantly increased quantities of toxin A as compared with the recombinant control strains containing only the plasmid vector. In addition to culture supernatant levels, significant amounts of toxin remained within the cell

of toxigenic strains containing the cloned DNA segment (Fig. 1B). We were unable to detect exotoxin A production in strain 388, previously reported to be a nonproducer of exotoxin A (18, 26); however, pFHK10-containing cells of strain 388 produced a significant amount of exotoxin A (Table 2). This result was verified by immunoblotting data, using specific antitoxin A antibody (Fig. 2).

It was important to determine whether the increased toxin production resulted from the presence of the toxin structural gene in the 3-kbp insert of pFHK10. Consequently, pFHK10 was used to transform a toxin structural gene mutant of strain PAO1, known as PAO-PR1, which produces a nontoxic yet immunologically cross-reactive analog of toxin A (CRM toxin). The results shown in Fig. 1 (lanes 6 and 7), when compared with the PAO-PR1 data in Table 2, demonstrate that PAO-PR1(pFHK10) produces an increased amount of CRM toxin, but no native toxin A (no ADP-ribosyl activity). To demonstrate the complete absence of native toxin A expression in PAO-PR1(pFHK10), toxins were purified from strains PA103, PAO1(pFHK10), and PAO-PR1(pFHK10) and compared by immunoblot analysis, using a monoclonal antitoxin specific for native exotoxin A, but previously shown to be nonreactive with the CRM toxin (7). The results are shown in Fig. 3. The ability of the monoclonal antitoxin to distinguish between CRM and native toxin A demonstrates that the cloned genomic insert of pFHK10 does not contain the toxin structural gene since native toxin A is not produced or expressed by the recombinant PAO-PR1(pFHK10).

Additional evidence in support of this conclusion was obtained by the pFHK10 transformation of *P. aeruginosa* strain WR5, which has been shown to lack the toxin A structural gene (M. Vasil, personal communication), and *P. putida*, also a non-toxin-producing pseudomonad. The results in Table 2 and Fig. 1 indicate that pFHK10 does not result in the expression of toxin A in these strains. The inability of pFHK10 to complement the toxin structural gene mutation of PAO-PR1 and the lack of toxin production in pFHK10-containing cells of the nontoxigenic strains WR5 (4) and *P. putida* indicate that the cloned DNA segment does not contain the toxin structural gene. Thus, the overproduction of toxin in toxigenic strains containing pFHK10 does

TABLE 2. Exotoxin A levels in culture supernatant from toxigenic and nontoxigenic *P. aeruginosa* strains containing the vector plasmid pVK101 or the recombinant plasmid pFHK10 and effect of excess (5.0  $\mu\text{g}/\text{ml}$ ) iron added during cell growth

Strain	Enzymatic activity <sup>a</sup>		Exotoxin A <sup>b</sup>
	Low iron	High iron	
PA103(pVK101)	598	13	35
PA103(pFHK10)	6,224	2,510	243
PA103-29(pVK101)	12	0	1
PA103-29(pFHK10)	6,121	2,532	239
PAO1(pVK101)	325	4	15
PAO1(pFHK10)	4,374	906	153
PAO-PR1(pVK101)	0	0	5
PAO-PR1(pFHK10)	0	0	49
388(pVK101)	0	0	0
388(pFHK10)	101	0	3
WR5(pVK101)	0	0	0
WR5(pFHK10)	0	0	0

<sup>a</sup> ADP-ribosyl transferase activity expressed as counts per minute per microgram of culture supernatant protein.

<sup>b</sup> Solid-phase radioimmunoassay expressed as nanograms of toxin per microgram of culture supernatant protein in low-iron cultures.

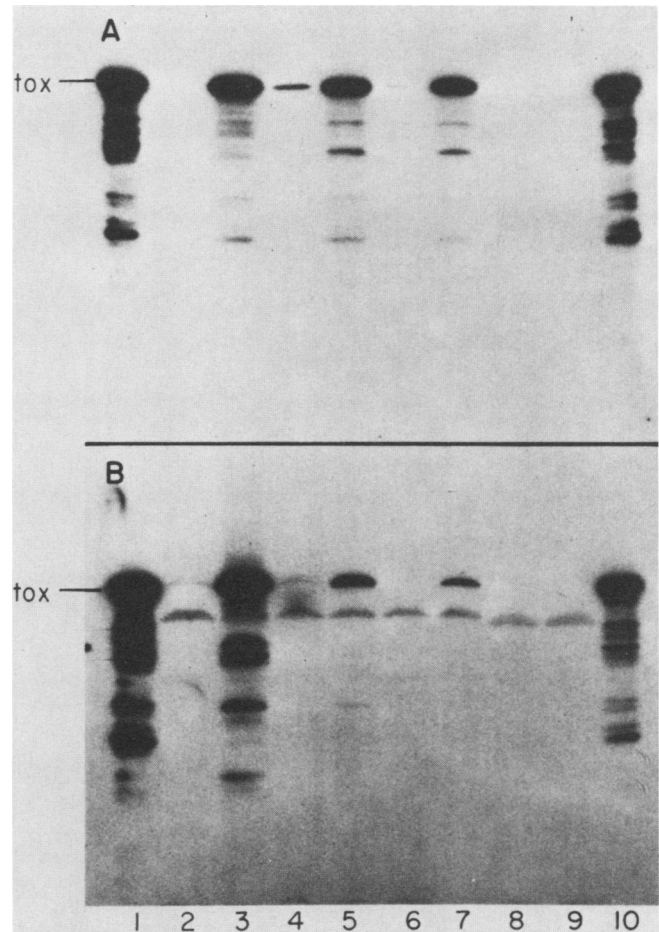


FIG. 1. Immunoblot analysis of 10% SDS-PAGE profiles of culture supernatants (A) and the corresponding cell pellets (B) with rabbit polyclonal antitoxin. (Note: Lower-molecular-weight bands below the toxin band in lanes 1 and 10 probably represent fragments of the purified toxin.) Lanes contain the following: 1, purified toxin from *P. aeruginosa* PA103; 2, *P. aeruginosa* PA103-29(pVK101); 3, *P. aeruginosa* PA103-29(pFHK10); 4, *P. aeruginosa* PAO1(pVK101); 5, *P. aeruginosa* PAO1(pFHK10); 6, *P. aeruginosa* PAO-PR1(pVK101); 7, *P. aeruginosa* PAO-PR1(pFHK10); 8, *P. putida* pVK101; 9, *P. putida* pFHK10; 10, purified toxin from *P. aeruginosa* PAO1(pFHK10).

not appear to be due to the coincidental cloning of the toxin structural gene.

Since excess iron is known to have an inhibitory effect on toxin expression, we compared its effect on toxin expression in strains containing pFHK10 and in strains containing the vector alone (Table 2; Fig. 2). As expected in the presence of iron, toxin production was substantially reduced in strains containing only the vector plasmid; however, excess iron only partially reduced toxin expression in toxigenic strains containing pFHK10. Thus, the stimulating effect on toxin gene expression by pFHK10 largely overcame the inhibitory effect of the chromosomally encoded iron regulatory element.

To explore the possibility that the pFHK10 effect on toxin expression was part of a more general phenomenon affecting additional exoproducts, experiments designed to measure elastase and alkaline protease activity have been conducted. Specifically, these exoproducts have been examined in both vector- and pFHK10-transformed strains, using immuno-

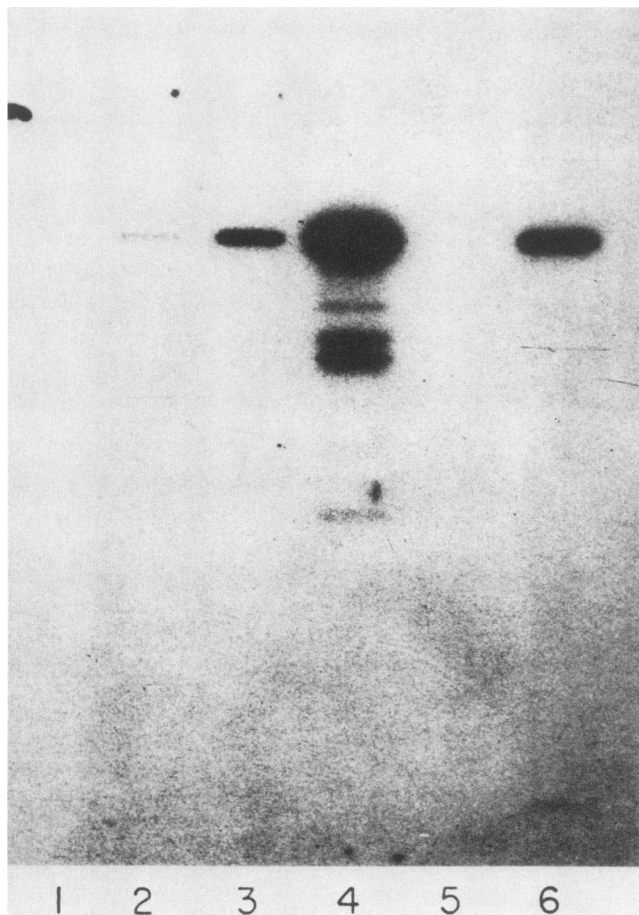


FIG. 2. Immunoblot analysis of 10% SDS-PAGE profiles of culture supernatants with rabbit polyclonal antitoxin. Cultures were grown in tryptic soy broth dialysate under low-iron (lanes 1 to 4) or high-iron (5  $\mu\text{g}/\text{ml}$ ; lanes 5 to 6) conditions. Lanes contain the following: 1, *P. aeruginosa* 388(pVK101); 2, *P. aeruginosa* 388(pFHK10); 3, *P. aeruginosa* PAO1(pVK101), low iron; 4, *P. aeruginosa* PAO1(pFHK10), low iron; 5, *P. aeruginosa* PAO1(pVK101), high iron; 6, *P. aeruginosa* PAO1(pFHK10), high iron.

blotting and protease assays. The results of these experiments show that pFHK10 does not cause any increase in the expression of these exoproducts, either extracellularly or intracellularly (data not shown).

**Southern blot analysis.** The 3-kbp *Xho*I insert fragment from pFHK10 was isolated and used to probe genomic DNA digests in Southern blot analysis (Fig. 4). These results indicate that sequences homologous to the putative regulatory gene are present in a variety of toxigenic strains as well as the nontoxigenic strain WR5. Genomic digests generated with *Hind*III (Fig. 4), as well as ones generated with *Pst*I and *Eco*RI (data not shown), suggest that the gene is present in *P. aeruginosa* as a single copy. The restriction fragment length polymorphism associated with this probe and genomic fragments from independently isolated strains indicate that this gene resides in a genomic environment that is not especially well conserved.

#### DISCUSSION

We have cloned a segment of DNA from *P. aeruginosa* that contains a gene involved in the regulation of exotoxin A

expression. The putative regulatory gene was cloned by screening a genomic library for plasmids capable of complementing the mutation in the nontoxigenic mutant PA103-29. Complementation of this mutation underlies the importance of this gene in virulence since PA103-29 has been shown to be less virulent than its parental strain (27, 30). The cloned gene was isolated on a 3-kbp *Xho*I fragment and a recombinant plasmid containing this fragment, pFHK10, was shown to stimulate the expression of toxin 10-fold in the culture supernatant of all toxigenic *P. aeruginosa* strains tested. It seemed unlikely that a product regulating increased secretion of toxin was encoded by the 3-kbp fragment because a significant amount of toxin remained associated with the cell pellet fraction of pFHK10-containing cell cultures. The expression of elastase, another exoproduct, was not increased in pFHK10-transformed strains, which suggests that the 3-kbp *Xho*I fragment does not contain a gene involved in general exoproduct processing or transport. The specificity of the cloned gene(s) for regulation of exotoxin A expression is supported by the fact that pFHK10 causes the increased expression of an altered form of exotoxin A when used to transform the structural gene mutant PAO-PR1. In this case, the 3-kbp *Xho*I fragment specifically increases the expression of a CRM toxin from the mutant toxin structural gene without producing any native exotoxin A. This also supports our contention that the cloned insert does not contain the exotoxin A structural gene. Additional evidence is provided by the fact that strain WR5, known to lack the exotoxin A structural gene, could not be complemented by pFHK10.

An interesting observation revealed in these studies is the fact that *P. aeruginosa* strain 388, previously reported to be a toxin A-negative strain (18, 26), actually does produce exotoxin A when transformed with pFHK10 and contains a chromosomal sequence homologous to this putative regula-

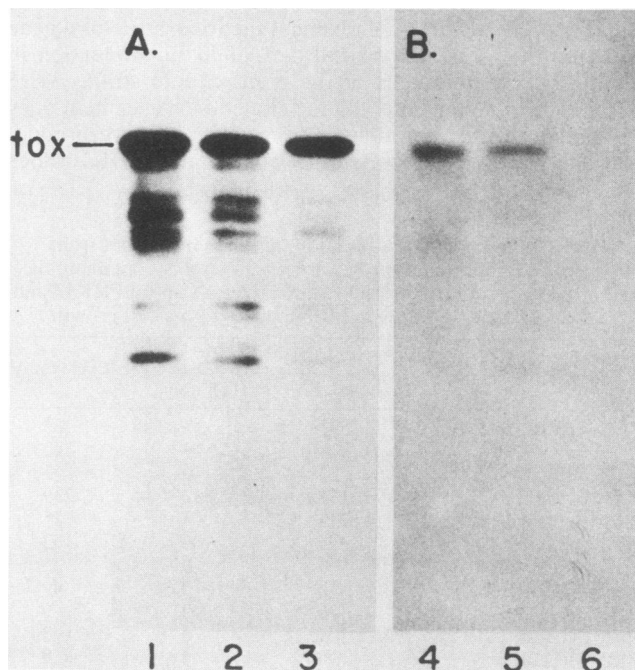


FIG. 3. Immunoblot analysis of 10% SDS-PAGE profiles of purified toxin with rabbit polyclonal (A) and mouse monoclonal (B) antitoxin. Toxin was purified from the following strains: lanes 1 and 4, *P. aeruginosa* PA103; lanes 2 and 5, *P. aeruginosa* PAO1(pFHK10); lanes 3 and 6, *P. aeruginosa* PAO-PR1(pFHK10).

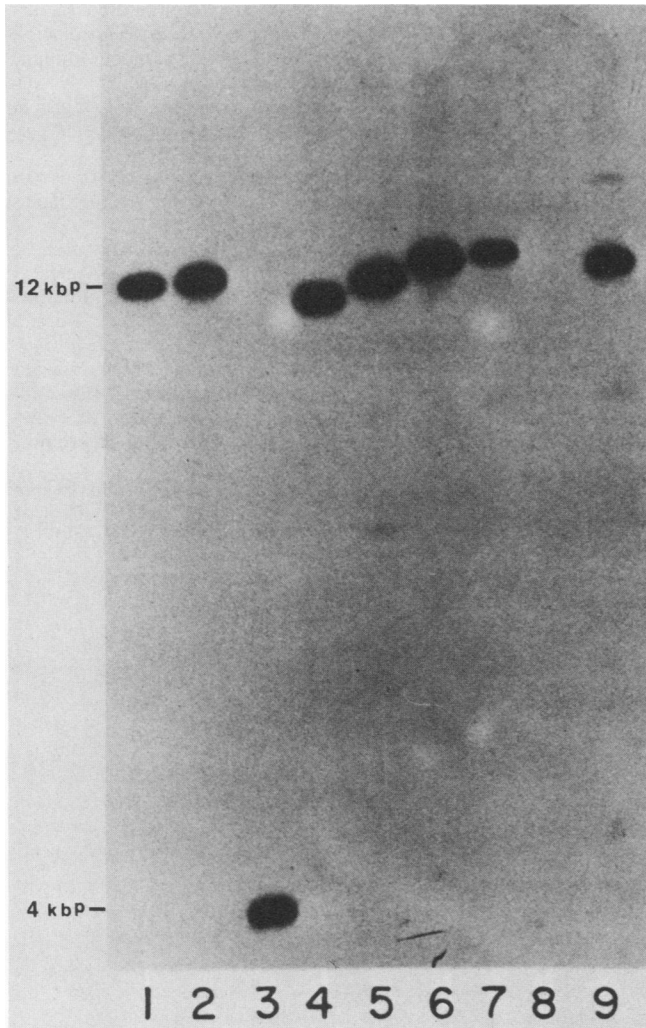


FIG. 4. Southern blot hybridization analysis of the 3-kbp *Xho*I insert of pFHK10 to restriction fragments of chromosomal DNA under conditions of high stringency. Purified chromosomal DNA from toxigenic and nontoxigenic *P. aeruginosa* strains and *P. putida* was digested with *Hind*III, fractionated by electrophoresis in agarose gel, blotted onto nitrocellulose, hybridized with the <sup>32</sup>P-labeled insert, and autoradiographed. The lanes contain DNA from the following: 1, *P. aeruginosa* PAO1; 2, *P. aeruginosa* PAO-PR1; 3, *P. aeruginosa* WR5; 4, *P. aeruginosa* 388; 5, *P. aeruginosa* T1; 6, *P. aeruginosa* PA103; 7, *P. aeruginosa* PA220; 8, *P. putida*; 9, *P. aeruginosa* PA103-29.

tory gene. Since pFHK10 does not appear to contain the exotoxin structural gene, this suggests the possibility that strain 388 carries a mutation which is complemented by pFHK10. In all strains the addition of excess iron to the culture medium reduced but did not completely suppress the stimulatory effect on toxin expression. However, these results do not rule out the possibility that iron is involved with this regulatory gene.

Our results could be explained by a positive regulatory element for toxin expression analogous to the *toxR* gene of *Vibrio cholerae* recently described by Miller and Mekalanos (25), in which *toxR* encodes a positive regulatory element for cholera toxin transcription. As with the *toxR* gene of *V. cholerae*, sequences homologous to this putative regulatory gene of *P. aeruginosa* are found in a nontoxigenic strain and

these sequences appear to be present in the genome as a single copy. Unlike *toxR*, this *P. aeruginosa* gene appears to be associated with a region of the genome that displays restriction fragment length polymorphism. Whether this gene encodes a positive regulatory element of toxin transcription must await further analysis with a toxin probe that could be used to detect exotoxin and specific transcription products. Such experiments are currently in progress.

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