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Molecular Studies of *Pseudomonas* Exotoxin A Gene

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A 2.7-kilobase DNA fragment carrying the entire exotoxin A (ETA) structural gene was divided into three nonoverlapping probes. Two probes covering the ETA structural gene were used in colony hybridization experiments to determine whether sequences homologous to the ETA gene could be detected in genera other than *Pseudomonas* or in *Pseudomonas* species other than *Pseudomonas aeruginosa*. The majority of strains examined other than the *P. aeruginosa* strains failed to react in the colony hybridization assays. Some *Pseudomonas* spp. other than *P. aeruginosa* and some *Bordetella* spp. did react in colony hybridization assays with the probes. However, additional studies in which we used Southern hybridization methods indicated that these reactions were apparently nonspecific and that the ETA gene is limited to *P. aeruginosa*. Studies in which we used all three ETA-related probes in Southern hybridization experiments to analyze the ETA gene and surrounding sequences in *P. aeruginosa* strains isolated from diverse sources revealed the following: (i) the incidence of the ETA gene in *P. aeruginosa* is ~95%; (ii) there are strains which have been isolated from human infections that do not carry the ETA structural gene; (iii) there is a maximum of one copy of the ETA gene per genome in any given strain; (iv) sequences within and 4 to 5 kilobases downstream of the ETA structural gene appear to be well conserved in different strains of *P. aeruginosa*; and (v) in contrast, sequences immediately upstream of the ETA structural gene are considerably rearranged from strain to strain. A multicopy plasmid carrying the entire cloned ETA gene was transferred to a *tox*$^-$ *P. aeruginosa* strain. This strain synthesized and secreted mature, full-length ETA, but the amount produced was small considering the multicopy nature of the plasmid. Synthesis of toxin in this strain was only minimally affected by iron. Our data suggest that the synthesis of ETA is positively regulated. Finally, we found that the presence of the ETA gene is independent of the ability of *P. aeruginosa* to produce several other recognized virulence factors, supporting the concept of the multifactorial nature of *P. aeruginosa* virulence.

Exotoxin A (ETA), a potentially important virulence factor of *Pseudomonas aeruginosa*, is a single polypeptide with a molecular weight of 66,600 and is secreted as a proenzyme. Upon proteolytic cleavage or denaturation and reduction it becomes an active ADP-ribosyl transferase (5, 15, 21, 22, 38). This enzyme has an intracellular mechanism of action identical to that of diphtheria toxin fragment A; that is, both of these molecules inhibit protein synthesis by covalently modifying elongation factor 2 in eucaryotic cells (15, 21). ETA, like diphtheria toxin, conforms to the A-B structure-function model of many bacterial toxins in that one portion of the molecule (fragment B) is necessary for interaction with a eucaryotic cell receptor, while the other (fragment A) is catalytic. However, *Pseudomonas* ETA and diphtheria toxin do not share any nucleotide or amino acid homology (9, 37).

While there is now a good deal of information regarding the biochemistry, mechanism of action, and structure-function relationships of ETA, little is known about the genetics of ETA regulatory and structural genes. It is known that the amount of ETA produced in vitro is strain dependent and that the amount produced is influenced by the concentration of iron and several environmental conditions (O_2, temperature, alanine concentration) (2, 4, 6). However, the mechanisms by which these factors influence expression of the ETA gene are not clear. It is possible that iron could regulate expression of ETA via the classical repressor-operator model, as has been proposed for regulation of diphtheria toxin by iron (20), or that a positive regulatory mechanism may be involved in regulating the expression of ETA, as has been recently described for cholera toxin (26, 27). It has also been suggested that the big differences in the absolute amounts of ETA produced in vitro by different strains could be the result of the duplication of the ETA gene in some strains. This mechanism for regulating the amount of toxin produced by a particular strain was recently shown to occur in *Vibrio cholerae* with the genes encoding enterotoxin (24).

In recent years some progress has been made in several laboratories toward understanding the genetics of ETA production. Several classes of interesting mutants have been isolated and partially characterized, including strains carrying regulatory or structural gene mutations, but the specific nature of these mutations and how they affect ETA production is not completely understood (10, 12). These studies have shown that regulation of ETA expression is a complex process, involving a number of genes or gene products because the mutations affecting ETA expression map distal to one another on the *P. aeruginosa* chromosome (85, 36, and 39 min) (10, 12).

We recently reported the cloning of the ETA structural gene and its entire nucleotide sequence (9). With the availability of this clone and information from the nucleotide sequence, we are now able to rationally address and hopefully answer many questions which were difficult or impossible to answer by using biochemical and classical microbial genetic approaches. In answer to some of these questions we report here (i) the use of specific ETA gene probes to determine whether other *Pseudomonas* species or other genera carry sequences homologous to the ETA gene; (ii) a

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TABLE 1. Bacterial strains and plasmids[a]

Strain or plasmid	Genotype or phenotype	Source	Reference(s)
Bacterial strain			
E. coli HB101	*leu thr pro lac gal* Str^r *recA* EndoI *hsdR hsdM*	A. L. Taylor	23
E. coli 294	*endA hsdR* Thi$^-$ Pro$^-$ Str^s	R. M. Berka	23
P. aeruginosa PA01[b]	Prototroph chl-3	B. W. Holloway	14
P. aeruginosa WR5	Prototroph Tox$^-$	P. A. Sokol	3, 31
Plasmid			
puc9BB	Ap^r *Bam*HI-*Bam*HI fragment of ETA		9, 37
ptoxETA	Tc^r Ap^s *Pst*I-*Eco*RI fragment carrying entire *tox* gene		9, 37
pGV-26	Ap^r Tc^s Hly$^+$ (PLC)[c]		36, 37
RSF1010	Sm^r Su^r	R. M. Berka	11
pRK2013	Km^r ColE1 with RK2 *tra*	D. Ohman	7
pCMtox	Hybrid of RSF1010 and ptoxETA		This study

[a] The genotype symbols have been described by Bachmann (1). Ap, Tc, Sm, and Su, Resistance to ampicillin, tetracycline, streptomycin, and sulfonamide, respectively; Tox, ETA.
[b] The remainder of the *P. aeruginosa* strains used in this study are listed in Table 3 along with their sources or references.
[c] pBR322 carrying the PLC gene of *P. aeruginosa*. For characteristics, see references 36 and 37.

molecular analysis of the copy number and structure of the ETA structural gene and surrounding sequences in *P. aeruginosa* strains isolated from a wide geographical distribution; (iii) the identification and characterization of naturally occurring *P. aeruginosa* strains that do not carry the ETA gene; (iv) the ability of a *P. aeruginosa* strain without a chromosomal copy of the ETA gene to produce ETA when the cloned *tox* gene is introduced on a multicopy plasmid; and (v) characterization of naturally occurring *P. aeruginosa* strains which lack the ETA gene.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Tables 1 and 2 along with relevant properties; if known, the source of each strain is also listed. The following investigators generously contributed strains used in this study: Barbara Iglewski, University of Oregon Medical School, Portland; Michael Janda, Mt. Sinai Hospital, New York, N.Y.; Olgerts Pavlovskis, Naval Medical Research Institute, Bethesda, Md.; Pamela Sokol and Donald Woods, University of Calgary, Calgary, Alberta, Canada; and Mary Jane Thomassen, Case Western Reserve University, Cleveland, Ohio.

Bacteriological characterization of *Pseudomonas* spp. strains. The following methods were used to verify the genera and species of the bacterial strains used in this study: (i) growth and pyocyanin production (blue-green pigment) on pseudomonas isolation agar (Difco Laboratories, Detroit, Mich.); (ii) growth at 26, 37, and 43°C; (iii) growth in oxidation-fermentation basal test media supplemented with glucose, sucrose, fructose, lactose, maltose, or mannitol; (iv) ornithine decarboxylase, lysine decarboxylase, and arginine dehydrolase activity tests; and (v) the oxidase reaction test (8).

Purification and characterization of DNA probes. Two plasmids which carry ETA sequences have been previously described (ptoxETA and pUCBB) (9). For the *Pst*I-*Nru*I (probe U) and *Bam*HI-*Eco*RI (probe A) *tox* gene probe ptoxETA was used as a source of DNA, and for the *Bam*HI-*Bam*HI *tox* gene probe (probe B) pUC9BB was used as a source of DNA (9) (Fig. 1). Plasmid pGV-26 was used as a source of the phospholipase C (PLC) gene probe (36). The desired probes were purified from vector sequences by digesting the hybrid plasmids with the required restriction endonucleases and electroeluting the appropriate DNA fragment from the gel following polyacrylamide or agarose gel electrophoresis. For each probe this procedure was done two or three times to ensure that the probes were free of vector sequences. The probes were initially checked by using the Southern hybridization methods described below; ^{32}P-labeled vector DNA was used as a probe to ensure that

TABLE 2. *Pseudomonas* spp. other than *P. aeruginosa* which failed to react with ETA DNA probes[a]

Species	No. of strains tested	Reactions with the following ETA probes:			Reaction with the PLC probe
		*Pst*I-*Nru*I	*Bam*HI-*Bam*HI	*Bam*HI-*Eco*RI	
P. cepacia	8	−	−	−	−
P. fluorescens	6	−	−	−	−
P. putida	6	−	−	−	−
P. malophilia	6	−	−	−	−
P. putrefaciens	3	−	−	−	−
P. schuylkilliensis	2	ND[b]	−	−	−
P. diminuta	2	ND	−	−	−
P. acidovorans	1	ND	−	−	−
P. alcaligenes	2	ND	−	−	−
P. pseudoalcaligenes	3	ND	−	−	−
P. stutzeri	2	ND	−	−	−
P. paucimobilis	1	ND	−	−	−

[a] All of the strains were tested by using the Southern hybridization method and high- and low-stringency conditions.
[b] ND, Not determined.

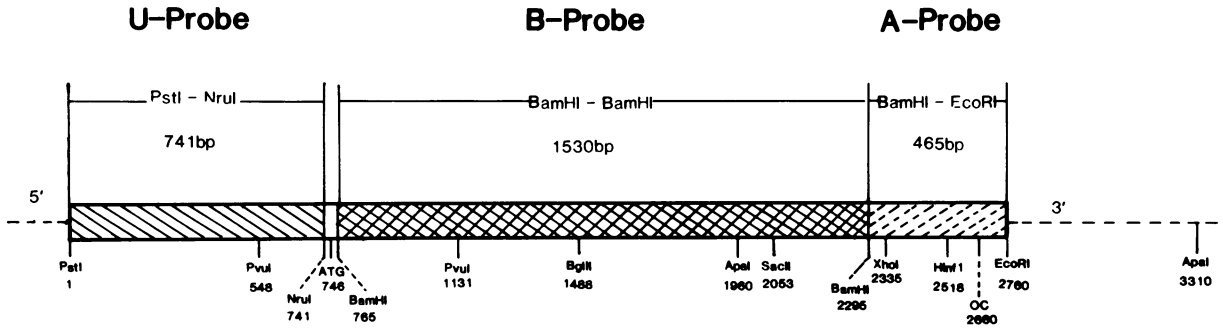

FIG. 1. Toxin probes.

the ETA and PLC DNA probes were free of vector sequences.

Colony hybridization. GeneScreen Plus colony/plaque screen hybridization transfer membrane disks (New England Nuclear Corp., Boston, Mass.) were used for colony hybridization studies. The disks were overlaid onto brain heart infusion (Difco) agar plates, and strains were patched onto the disks. The plates were incubated overnight to allow growth of the organisms. The colonies were then denatured and fixed to the disks as instructed by the manufacturers. Hybridization and washes were carried out as instructed by the manufacturer (New England Nuclear Corp.).

Southern hybridization. Bacterial chromosomal DNA was isolated by the Marmur method (18) after lysis of the bacteria by the Hirt method (13). The DNA (1 to 2 μg) was digested with various restriction endonucleases as instructed by the manufacturer. The digested DNA was electrophoresed on 0.7% agarose gels for approximately 18 h (30 V), after which the DNA was transferred to nitrocellulose or GeneScreen Plus (New England Nuclear Corp.) as described by Southern (35). Hybridization was carried out under conditions of high or low stringency. The high-stringency hybridization solution used contained 50% (vol/vol) formamide, 1 M sodium chloride, 10% dextran sulfate, 1% sodium dodecyl sulfate, and 250 μg of heat-denatured salmon testicle DNA per ml. The low-stringency hybridization solution was identical, except that 25% (vol/vol) formamide was used. Both high- and low-stringency hybridizations were carried out at 42°C for 18 h. The blots were washed as recommended by the manufacturer.

Production of ETA by *P. aeruginosa* strains and detection of ETA by Western blotting. *P. aeruginosa* strains were grown in iron-depleted medium for 18 to 24 h as previously described (4), and cell-free culture supernatants were electrophoresed on 10% polyacrylamide gels and blotted onto nitrocellulose as previously described (9). ETA was detected

TABLE 3. Summary of Southern hybridization data

Strain[a]	Reference(s) or source[b]	Sizes of fragments (kb)										
		*Pst*I-*Nru*I ETA probe				*Bam*HI-*Bam*HI ETA probe			*Bam*HI-*Eco*RI ETA probe			*Stu*I-*Pst*I PLC probe *Bam*HI fragments
		*Xho*I fragments	*Sal*I fragments	*Bgl*II fragments	*Hin*fI fragments	*Xho*I fragments	*Bam*HI fragments	*Bgl*II-*Xho*I fragments	*Apa*I fragments	*Bgl*II fragments	*Sac*II fragments	
PA103	2, 9	5.4	4.7	9.7	2.9	5.4	1.53	0.85, 4.6	1.35	5.9	2.3	6.1
PAO1	14	13.5	11.6	9.7	2.8	13.5	1.53	0.85, 8.8	1.35	5.9	2.3	6.1
ATCC 15692	14	13.5	11.6	9.7	2.8	13.5	1.53	0.85, 8.8	1.35	5.9	2.3	6.1
PAKS-1	31	6.3	5.1	7.7	2.1	6.3	1.53	0.85, 5.5	1.35	5.9	2.3	6.1
Ps388	16	20.0	10.8	4.8	3.0	20.0	1.53	0.85, 4.0	1.35	5.9	5.9	6.1
M2	31	2.8	11.8	8.5	3.0	2.8	1.53	0.85, 2.0	1.35	5.9	2.3	6.1
WR35	3	15.5	12.7	5.2	2.1	15.5	1.53	0.85, 4.3	1.35	5.9	2.3	6.1
PsUA	CI	7.4	11.0	ND[c]	ND	7.4	1.53	ND	1.35	5.9	ND	6.1
WR715	3	19.0	10.0	9.5	2.4	19.0	1.53	0.85, 8.6	1.35	5.9	2.3	6.1
Damon classic	CI	19.0	ND	ND	2.3	19.0	1.53	0.85, 9.5	1.35	5.9	2.3	6.1
Damon mucoid	CI	9.5	ND	ND	3.1	9.1	1.53	0.85, 4.3	1.35	5.9	2.3	6.1
Booth rough	CI	19.0	ND	ND	2.3	19.0	1.53	0.85, 9.5	1.35	5.9	2.3	6.1
Booth mucoid	CI	19.0	ND	ND	2.3	19.0	12.0	0.85, 9.5	1.35	5.9	2.3	6.1
CL-60	CI	7.6	ND	ND	2.1	7.6	1.53	0.85, 7.7	1.35	5.9	2.3	6.1
1137732	CI	2.7	ND	ND	3.1	2.7	1.53	0.85, 1.8	1.35	5.9	2.3	6.1
FRD2 Alg$^-$	28	2.2	ND	ND	2.2	2.2	1.53	0.85, 1.4	1.35	ND	ND	6.1
FRD2 Alg$^+$	28	2.2	ND	ND	ND	2.2	1.53	0.85, 1.4	1.35	ND	ND	6.1
PCF-1 Alg$^-$	28	7.0	ND	ND	ND	7.0	1.53	0.85, 6.1	1.35	ND	ND	6.1
PCF-1 Alg$^+$	28	7.0	ND	ND	ND	7.0	1.53	0.85, 6.1	1.35	ND	ND	6.1
V209 Alg$^-$	28							ND		ND	ND	6.1
V209 Alg$^+$	28				ND			ND		ND	ND	6.1
WR5	3, 31	13.6	3.8	3.7	ND						ND	6.1

[a] Classic, mucoid, and rough refer to colony type.
[b] CI, Clinical isolate from Mary Thomassen, Case Western Reserve University, Cleveland, Ohio, or from Swedish Medical Center, Denver, Colo.
[c] ND, Not determined.

by using immunoaffinity-purified rabbit anti-ETA antibodies (60 ng/ml) and affinity-purified ^{125}I-labeled protein A (1 μCi per 10-lane gel) as previously described (9).

RESULTS

Characteristics of DNA probes. The DNA probes used for the ETA structural gene and the 5′ regulatory elements were isolated and purified as described in Materials and Methods and are shown diagrammatically in Fig. 1. Based on the known nucleotide sequence of the ETA gene from strain PA103 (9), we selected three different nonoverlapping probes to examine the sequences upstream, downstream, and within the ETA structural gene of *P. aeruginosa* strains from diverse sources. The *Pst*I-*Nru*I 741-base-pair (bp) fragment designated probe U does not have any ETA structural gene sequences but starts 5 bp upstream of the ATG initiation codon and carries the promoter for the ETA gene (Vasil and Grant, unpublished data). The *Bam*HI-*Bam*HI 1,530-bp fragment designated probe B consists only of sequences within the structural gene. It does not carry any sequences encoding the enzymically active portion (ADP-ribosyl transferase) of the toxin because the *Bam*HI-*Eco*RI fragment (see below) is sufficient to encode the enzymically active portion of the toxin and these two fragments do not overlap (9, 37). The *Bam*HI-*Eco*RI 465-bp fragment designated probe A is slightly ambiguous in terms of the ETA structural gene because it carries 100 bp in addition to the sequences encoding C-terminal fragment A (ADP-ribosyl transferase) of ETA. However, probe A is unambiguous in that it carries the entire catalytic site of ETA and does not overlap the other probes. The probe used for the PLC studies (see below) (Tables 2 through 4) is a 1,420-bp *Stu*I-*Pst*I fragment carrying sequences which only encode the PLC structural gene (A. Pritchard and M. L. Vasil, submitted for publication).

Colony hybridization studies with ETA-specific probes. In order to rapidly determine whether genera other than *Pseudomonas* and species other than *P. aeruginosa* carry sequences homologous to the ETA gene, we first used a DNA colony hybridization method and probe B (Fig. 1) because this probe carries only ETA-specific sequences. Using this approach, we tested a variety of organisms, including *Corynebacterium diphtheriae* (*tox*$^+$), recent *Escherichia coli* clinical isolates, *Vibrio cholerae*, *Campylobacter fetus*, *Campylobacter jejuni*, *Plesiomonas shigelloides*, *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Salmonella enteritidis*, *Bordetella pertussis*, and *Bordetella parapertussis*. None of these organisms except the *Bordetella* species reacted with probe B. Because this probe does not carry sequences encoding the catalytic site (ADP-ribosyl transferase site) of ETA and some of the organisms tested produce toxins with a similar or identical enzymic activity, we repeated the experiments with probe A. The results were essentially identical to the results obtained when probe B was used. We then did the same kinds of experiments with several different *Pseudomonas* species. Typical results are shown in Fig. 2. Depending on the species, we noted variable results in this set of experiments. Some *Pseudomonas* species gave negative or very weak reactions, while others gave apparently significant reactions (Fig. 2). None of the reactions with *Bordetella* spp. or non-*P. aeruginosa* pseudomonads were as strong as the reactions with Tox$^+$ *P. aeruginosa* strains. It is unlikely that the positive results with the non-*P. aeruginosa* strains were due to nonspecific sticking of the probes to the bacterial debris on the filters because we reproducibly obtained the same kind of results when we used highly

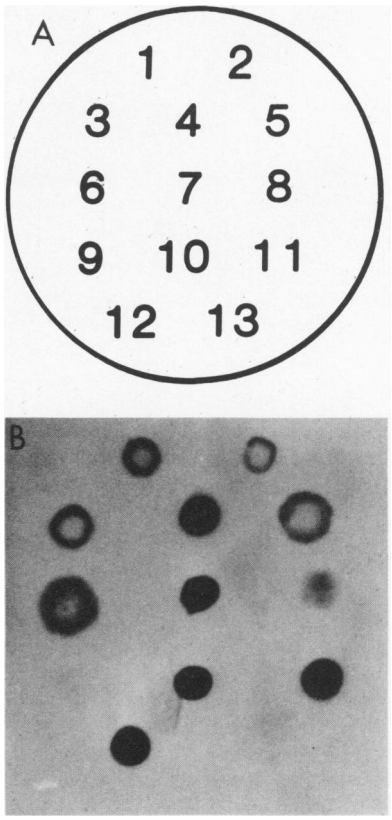

FIG. 2. Colony hybridization assay for sequences homologous to the ETA gene, using probe B. Lanes 1 through 3, *Pseudomonas maltophilia* isolates; lane 4, *P. aeruginosa*; lanes 5 and 6, *P. fluorescens* isolates; lanes 7 and 8, *P. putida* isolates; lane 9, *P. cepacia*; lanes 10 through 12, *P. aeruginosa*; lane 13, *P. putrefaciens*.

TABLE 4. Characteristics of selected strains used in this study[a]

Strain	PLC[b]		Exotoxin S[c]		ETA[d]	
	Act. U	Probe	Act.	Ab.	Ab.[b]	Probe[a]
PA103	ND	+	ND[e]	ND	+	+
PAO1	235	+	ND	ND	+	+
PAKS-1	181	+	ND	ND	+	+
M2	305	+	ND	ND	+	+
PsUA	ND	+	−	±	ND	+
WR35	152	+	ND	ND	ND	+
WR26	644	+	ND	ND	ND	+
Ps388	385	+	+	+	+	+
WR5	80	+	−	+	−	−
V209 Alg$^-$	295	+	+	+	−	−
V209 Alg$^+$	41	+	ND	ND	−	−

[a] All strains were positive for the following biotype characteristics: oxidase, growth at 43°C, protease production (measured on casein plates by using the method of Sokol et al. [34]), pyocyanin production (measured as previously described [17, 37]), and hemolysis on sheep blood agar.

[b] Act., Heat-labile hemolysin (PLC), measured as previously described (36). Probe indicates whether the genomic DNA reacted with cloned fragment encoding PLC in Southern hybridization tests. See Table 2 and references 36 and 37.

[c] Production of exoenzyme S was kindly tested by Donald Woods by assaying for enzymic activity (Act.) and by immunological methods (Ab.) (16).

[d] Ab., Assayed by Western blotting (see Fig. 5); Probe, determined by Southern hybridization (see Table 3).

[e] ND, Not determined.

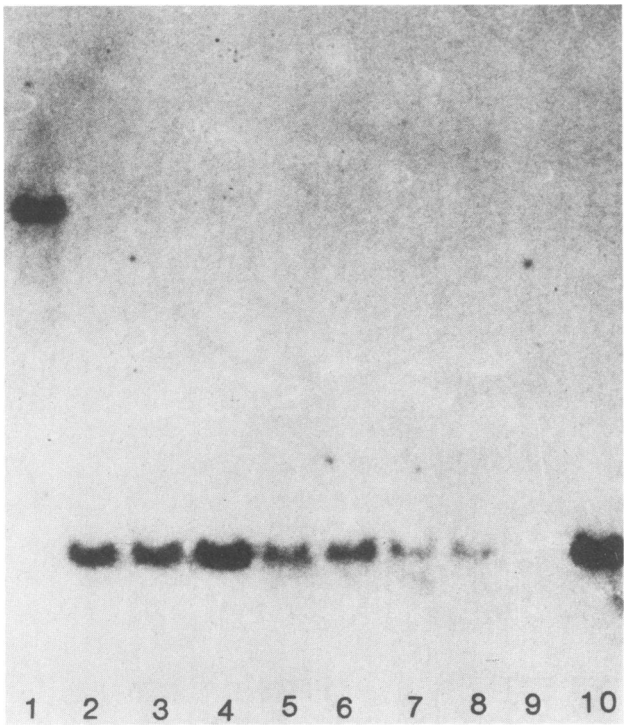

FIG. 3. Southern hybridization of selected *P. aeruginosa* strains. Genomic DNAs were digested with *Bam*HI and probed with probe B. Lane 1, Booth mucoid strain; lane 2, Booth rough strain; lane 3, strain ATCC 15692 (=PAO1); lane 4, strain 1137732; lane 5, strain PsUA; lane 6, strain PAKS-1; lane 7, strain WR715; lane 8, strain WR35; lane 9, strain WR5; lane 10, strain PA103.

purified genomic DNA and the reactions could be abrogated by pretreating the DNA with DNase and then inactivating the DNase before the sample was spotted onto the filters. If the results were reproducibly negative under high- and low-stringency conditions, then that was taken to mean that a strain did not carry sequences homologous to the ETA gene of *P. aeruginosa*. If the reaction in the colony hybridization test was positive (weak to strong), then the genomic DNA of the strain was purified and analyzed by Southern hybridization methods.

Analysis of genomic DNA by Southern hybridization with ETA probes. Because of the ambiguities encountered when we used colony hybridization methods, all further experiments with the ETA probes were done with restriction endonuclease-digested genomic DNAs and Southern hybridization methods. Using this approach, we examined more than 60 *P. aeruginosa* strains and more than 40 non-*P. aeruginosa* pseudomonads, including fresh clinical isolates from different kinds of infections, well-characterized laboratory strains, and strains isolated from diverse geographic sources (Tables 2 and 3). We also examined *P. aeruginosa* strains which have been reported previously to be Tox^-, as determined by immunological or enzymic assays (3, 16, 31). Furthermore, because the *Bordetella* spp. gave positive reactions in the colony hybridization tests, we examined fresh clinical isolates of this genus. As shown in Table 2, all of the non-*P. aeruginosa* strains which we tested with the three distinct probes (Fig. 1) failed to react under high- or low-stringency conditions in Southern hybridization tests. The same was true for the *Bordetella* spp. strains (data not shown). Therefore, we concluded from these studies that neither non-*P. aeruginosa* pseudomonads nor the *Bordetella* spp. carry sequences specifically homologous to the ETA gene. It is not clear why the colony hybridization assays were positive with some of the non-*P. aeruginosa* strains and the *Bordetella* spp. However, a phenomenon similar to these apparently nonspecific reactions was reported by Koomey and Falkow (19) in their studies with the immunoglobulin A protease gene of *Neisseria* spp. These authors reported that some commensal *Neisseria* species react in a colony hybridization assay even though they do not have the gene encoding immunoglobulin A protease.

In contrast to the non-*P. aeruginosa* strains, almost all of the *P. aeruginosa* strains reacted with the toxin-specific probes (probes A and B). As Fig. 3 and Table 3 show, when the genomic DNAs of *P. aeruginosa* strains were digested with *Bam*HI and analyzed by using a Southern hybridization and the 1.53-kilobase (kb) probe B, the majority of strains displayed a single band at 1.53 kb. The only exceptions among the more than 60 *P. aeruginosa* strains which we examined were a strain which displayed a 12.0-kb band with

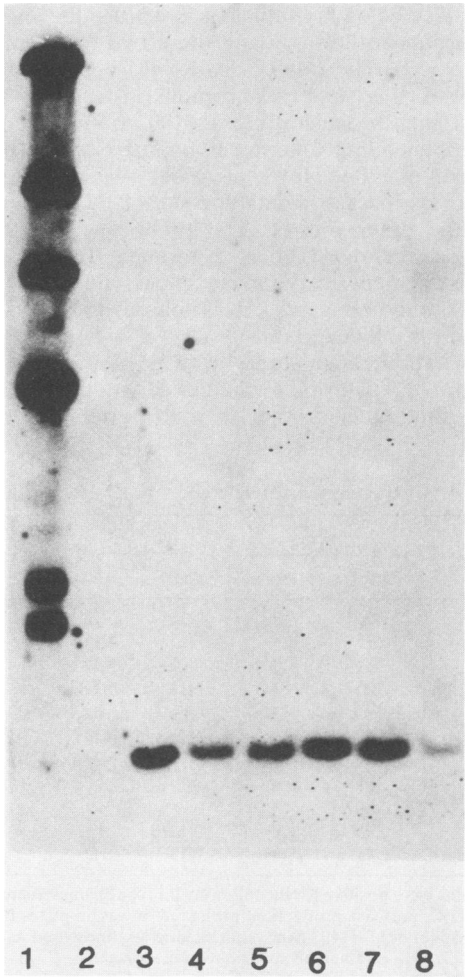

FIG. 4. Southern hybridization of selected *P. aeruginosa* strains. Genomic DNAs were digested with *Apa*I and probed with probe A. Lane 1 contained the following *Hin*dIII molecular weight markers (from top to bottom): 23, 9.4, 6.6, 4.4, 2.3, and 2.1 kb. A 0.6-kb marker is not shown. Lane 2, Blank; lane 3, strain PA103; lane 4, strain PA01; lane 5, strain WR35; lane 6, strain PAM2; lane 7, strain CL-60; lane 8, strain Ps388.

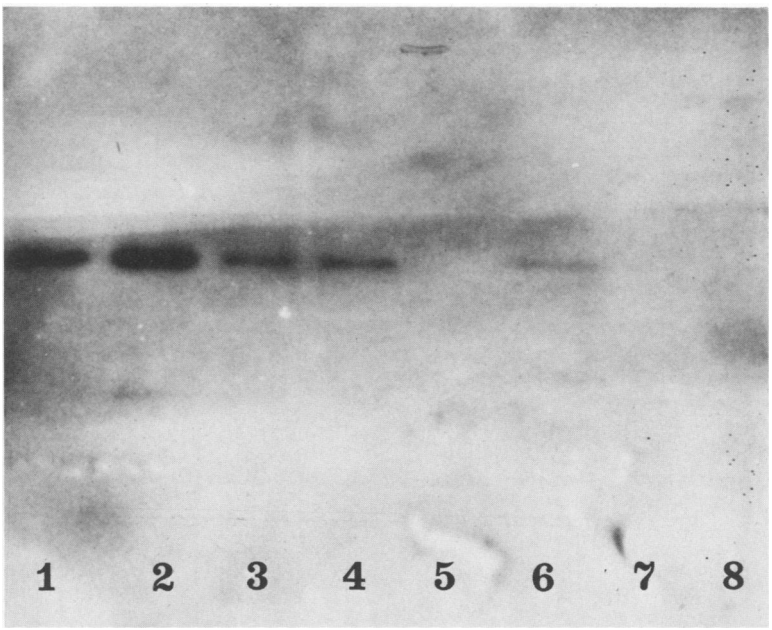

FIG. 5. Detection by Western blotting of ETA in crude culture supernatants of selected *P. aeruginosa* strains. Lane 1, Strain PAO1 (25 μl); lane 2, strain PAKS-1 (25 μl); lane 3, strain PAM2 (25 μl); lane 4, strain ATCC 15692 (25 μl); lane 5, strain WR5 (100 μl); lane 6, strain Ps388 (50 μl); lane 7, strain V209 Alg^- (100 μl); lane 8, strain V209 Alg^+ (100 μl).

probe B and four strains which failed to react with this probe (see below).

Other restriction endonuclease sites within the structural gene of ETA from various strains were then examined to determine whether the ETA sequences were relatively conserved from strain to strain or whether there were any additional restriction site polymorphisms in the ETA gene like the polymorphism observed with the Booth mucoid strain. It was also possible that a strain could carry portion B of the toxin gene and not portion A. Table 3 and Fig. 4 show that all of the strains that were reactive with probe B were also reactive with probe A. In all of the cases which we examined except one (the Booth mucoid strain), the sizes of the restriction fragments within the ETA gene were identical (Table 3). These data indicate that there is very little restriction site variability within the ETA structural gene and that the sequences within this gene may be relatively well conserved from strain to strain.

The restriction endonuclease enzymes used in the study described above did not cut within the probes. Therefore, because each strain displayed only one band after Southern hybridization (Fig. 3 and 4 and Table 3), it is evident that all of the tox^+ *P. aeruginosa* strains examined had only one copy of the ETA structural gene per genome. This was as true for strains which produce relatively high amounts of ETA, such as strain PA103, as it was for strains which produce only very low amounts of toxin, such as strain Ps388 (Fig. 5).

Strains WR5 and V209 and two other strains which failed to react with probe B also failed to react with probe A. Strain WR5 was previously reported to be Tox^-, which was verified in our laboratory by Western blotting (Fig. 5 and 6), and we found that strain V209 is also Tox^- by using the same procedure (Fig. 5). The two other strains which failed to react with probes A and B (data not shown) were recently isolated from cystic fibrosis patients. It is evident from our data that there are some naturally occurring strains of *P.*

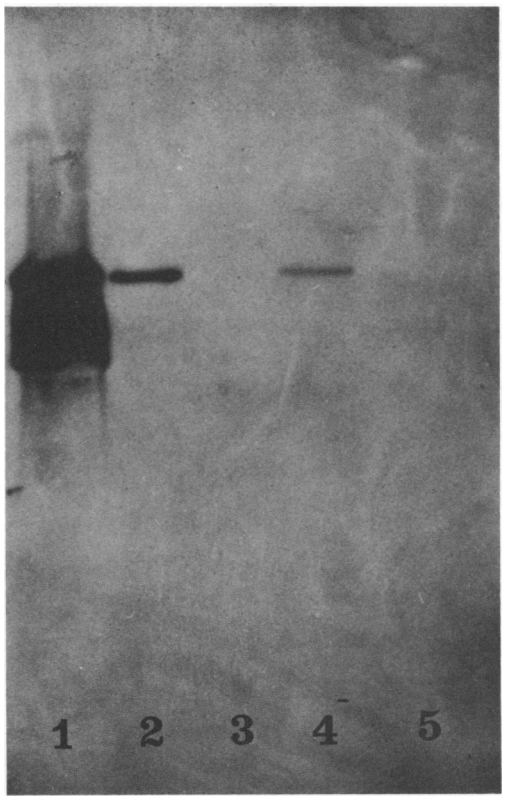

FIG. 6. Detection in crude culture supernatants by the Western blot technique of ETA produced by wild-type *P. aeruginosa* strains and strain WR5 carrying pCMtox. Lane 1, Strain PA103 (50 μl); lane 2, strain PAO1 (50 μl); lane 3, strain WR5(pBR322::RSF1010) (50 μl); lane 4, strain WR5(pCMtox) (50 μl); lane 5, strain WR5 (100 μl).

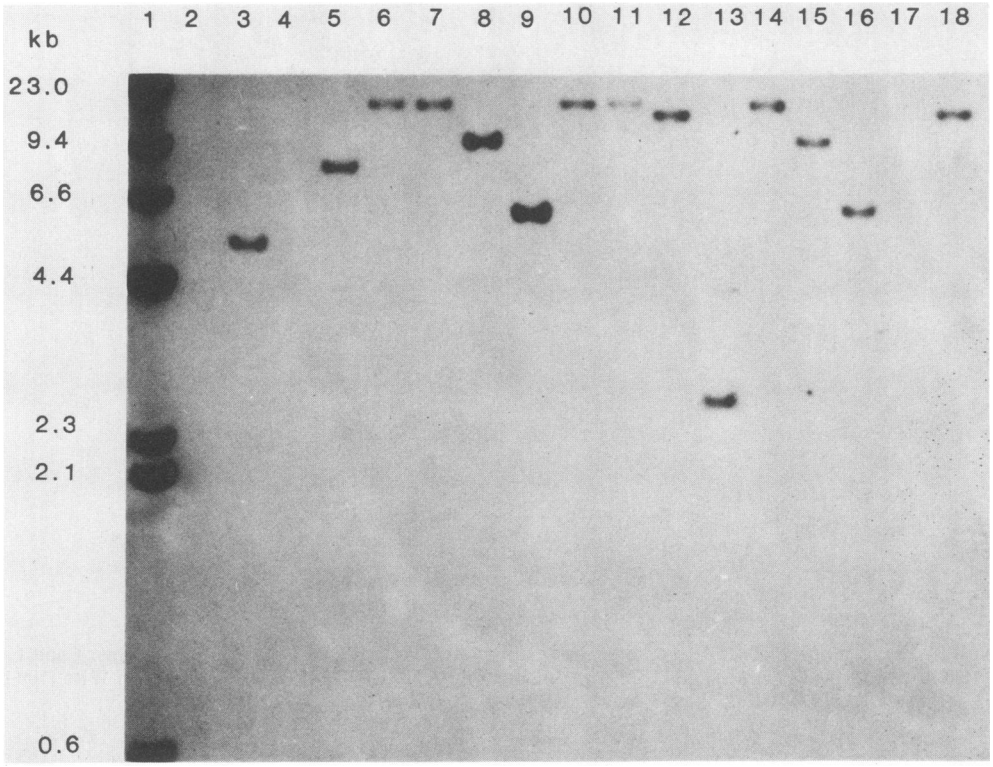

FIG. 7. Southern hybridization of selected *Pseudomonas* spp. strains (unless indicated otherwise, all strains were *P. aeruginosa* strains). Genomic DNAs were digested with *Xho*I and probed with probe B. Lane 1, *Hin*dIII molecular weight markers; lane 2, blank; lane 3, strain PA103; lane 4, *P. maltophilia*; lane 5, strain CL-60; lane 6, strain 3217 NMRI; lane 7, Damon classic strain; lane 8, Damon mucoid strain; lane 9, strain PAKS-1; lane 10, Booth rough strain; lane 11, Booth mucoid strain; lane 12, strain ATCC 15692; lane 13, strain 1137732; lane 14, strain WR715; lane 15, strain 7632 NMRI; lane 16, strain WRPG29; lane 17, strain WR5; lane 18, strain WR35. Whenever fragments sizes were out of the range of the molecular weight standards (e.g., strains ATCC 15692 and 7632 NMRI), the gels were redone by using higher or lower percentages of agarose as necessary and additional molecular weights standards in order to obtain more accurate molecular weights of the fragments.

aeruginosa which lack the ETA gene (Table 3 and Fig. 5). There is no doubt that these *tox*$^-$ strains are in fact *P. aeruginosa* strains and not members of some other *Pseudomonas* sp. because they clearly have properties which are distinct to *P. aeruginosa* (8, 17).

We next used probe U or in some cases probe B to verify that there was only one copy of the ETA gene in the strains which we examined and to compare the locations of restriction sites among different strains in the region located just upstream of the ETA gene. When the genomic DNAs of various *P. aeruginosa* strains were digested with *Xho*I and hybridized to either probe U or probe B, we found only one copy of the ETA gene per genome (Fig. 7 and 8 andTable 3). However, in sharp contrast to the homogeneity of fragment sizes within the ETA gene and downstream of the ETA gene among the strains, we found that there was a considerable range of sizes which reacted with these two probes, reflecting heterogeneity in the region just upstream of the ETA structural gene (Fig. 7 and 8 and Table 3). In fact, it was relatively uncommon to observe probe-reactive *Xho*I fragments that were the same size from different strains. This could have been due to: (i) simple polymorphisms at the *Xho*I site upstream of the ETA gene, (ii) a chemical modification of the DNA just upstream of the ETA gene which affected cutting of the genomic DNAs by *Xho*I, or (iii) DNA rearrangements in the different strains close to the ETA structural gene. Evidence that the *Xho*I sites within the ETA gene were conserved among the different strains is presented in Table 3. As predicted from Fig. 1, a 850-bp *Bgl*II-*Xho*I fragment within the ETA structural gene was found in all of the strains. Because the probe-reactive fragments were also different when we used other restriction enzymes (*Sal*I, *Bgl*II, and *Hin*fI) (Table 3), the second interpretation described above (that there are DNA rearrangements) appears to be correct. In fact, we examined more than 60 *P. aeruginosa* strains from different sources or patients and did not find two strains which had identical restriction patterns when the sites upstream of the ETA gene were examined (data not shown). By comparison, in the same strains we found only one restriction site variation in one strain within or 5 kb downstream of the ETA gene (Fig. 3 and Table 3). We can usually distinguish ~90% of the strains by using only two restriction enzymes (J. W. Ogle and M. L. Vasil, manuscript in preparation).

From the data shown in Table 3 and Fig. 8 it appears that the DNA rearrangements described above do not occur readily in vitro. Strain PAO1 was obtained directly from Bruce Holloway (Monash University, Clayton, Victoria, Australia) several years ago. Strain ATCC 15692 is the same strain but was deposited in the American Type Culture Collection by B. Holloway and was obtained directly from that organization. These isolates of strain PAO1, despite the fact that they were stored under different conditions, had identical restriction patterns no matter what probe we used or what restriction enzyme we used to digest the genomic DNA. Also, alginate (mucoid)-producing strains (Alg$^+$) and

in vitro-derived Alg$^-$ isogenic variants obtained from Dennis Ohman (University of California, Berkeley) (28) both had identical restriction patterns for all of the enzymes which we examined (Fig. 8 and Table 3).

Characteristics of ETA-negative strains and selected ETA-positive strains. Because we identified naturally occurring strains which do not carry the ETA gene (ETA negative), we wanted to characterize these strains further, particularly for their ability to produce other virulence factors. We particularly wanted to determine whether ETA-negative strains were capable of producing exotoxin S, which is also an ADP-ribosyl transferase toxin of *P. aeruginosa* but has a different substrate specificity than ETA (16). Donald Woods (University of Calgary) kindly assayed several ETA-negative and ETA-positive strains for the production of exotoxin S by using both enzymic (16) and immunoblot assays and monoclonal antibodies against exotoxin S. As Table 4 shows, it is clear that there is no correlation between the production of exotoxin S and whether a strain is ETA positive or ETA negative. The same was found to be true for PLC. All of the *P. aeruginosa* strains which we tested, including the four ETA-negative strains, had the PLC gene and produced active PLC. Overall, these ETA-negative strains did not appear to be significantly different than other *P. aeruginosa* strains in their ability to produce the other major virulence factors, including protease, exotoxin S, hemolysin, and PLC (Table 4). Thus far we have assayed for both the production of ETA and the presence of the intact ETA gene in about 25 strains but have not encountered any strain which has the ETA gene and is incapable of producing and secreting full-size mature ETA (data not shown). Only those *P. aeruginosa* strains which did not have the ETA gene failed to produce toxin, as determined by Western blotting.

Expression of the cloned toxin gene in naturally occurring ETA-negative strains. Figure 9 shows the construction of a plasmid which carries the ETA structural gene and the 5′ 746-bp region from hyperproducing strain PA103. This RSF1010-ptoxETA hybrid (designated pCMtox) was transferred into *E. coli* 294 by transformation, and then this hybrid plasmid, which could replicate in *P. aeruginosa*, was conjugated into strain WR5 in a triparental mating involving strain WR5, *E. coli* 294(pCMtox), and *E. coli* HB101(pRK2013). Plasmid pRK2013 is an RK2 derivative which has *tra* functions and can operate in *trans* (7). Additionally, we transferred a hybrid of pBR322 and RSF1010 to strain WR5 to be sure that there was nothing produced by the vector sequences which cross-reacted with ETA. As Fig. 6 shows, strain WR5(pCMtox) was capable of producing and secreting full-size mature ETA. The control, strain WR5 with only the vector sequences, did not show any reaction on the Western blot (Fig. 6). The amount produced by strain WR5(pCMtox) was relatively small, even though plasmid pCMtox was present in multiple copies (~40). It was possible that this strain was making considerable amounts of toxin but was not secreting it. The strain WR5(pCMtox) cells were examined by Western blotting after they were gently lysed, and we found that only a very small amount (less than 5%) of the ETA remained attached to the cells (data not shown). We also examined the production of ETA by strain WR5 (pCMtox) in media containing high and low iron concentrations. There was only a modest decrease (less than 50%) in the amount of toxin produced in media containing 10 μg of iron per ml compared with cells grown in iron-deficient media (0.01 μg of iron per ml). These results are not similar to the results obtained when *tox*$^+$ strains are grown in high- or low-iron media. Usually the amount of toxin produced by clinical isolates is decreased by 90% when cells are grown in media containing as little as 1 μg of iron per ml (2, 4). This lack of iron regulation of the ETA gene in strain WR5(pCMtox) may have been due to the increased number of *tox* regulatory elements because of the multicopy nature of plasmid pCMtox (Grant and Vasil, unpublished data).

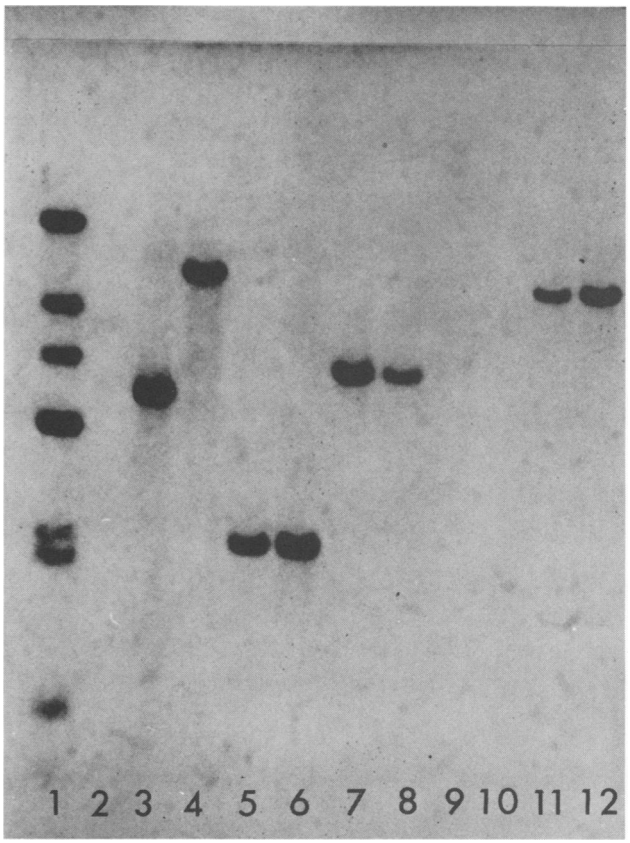

FIG. 8. Southern hybridization of selected *P. aeruginosa* strains. Genomic DNAs were digested with *Xho*I and probed with probe U. Lane 1, *Hin*dIII molecular weight standards; lane 2, blank; lane 3, strain PA103; lane 4, strain PAO1; lane 5, strain FRD2 Alg$^+$; lane 6, strain FRD2 Alg$^-$; lane 7, strain PCF-1 Alg$^+$; lane 8, strain PCF-1 Alg$^-$; lane 9, strain V209 Alg$^+$; lane 10, strain V209 Alg$^-$; lane 11, strain PA107-2 Alg$^+$; lane 12, strain PA107-2 Alg$^-$.

DISCUSSION

In this study we obtained data which we used to unambiguously answer specific questions about the genetics of ETA production in *Pseudomonas* spp. We concluded (i) that the production of ETA and the presence of the ETA gene are probably limited to *P. aeruginosa* and are not found in other *Pseudomonas* spp. or in a number of other genera; (ii) that duplication of the ETA gene is not a mechanism by which production of ETA is regulated and that among the strains which carry the ETA gene there is only one copy per genome; (iii) that there are naturally occurring ETA-negative *P. aeruginosa* strains which can be isolated from human infections (strain WR5 from a wound, strain V209, and two other strains from cystic fibrosis patients) and that the frequency of these strains lacking the ETA gene does not significantly differ from the previously reported frequency of Tox$^-$ strains, as determined by detection of the translated product (3); and (iv) that the sequences within the ETA

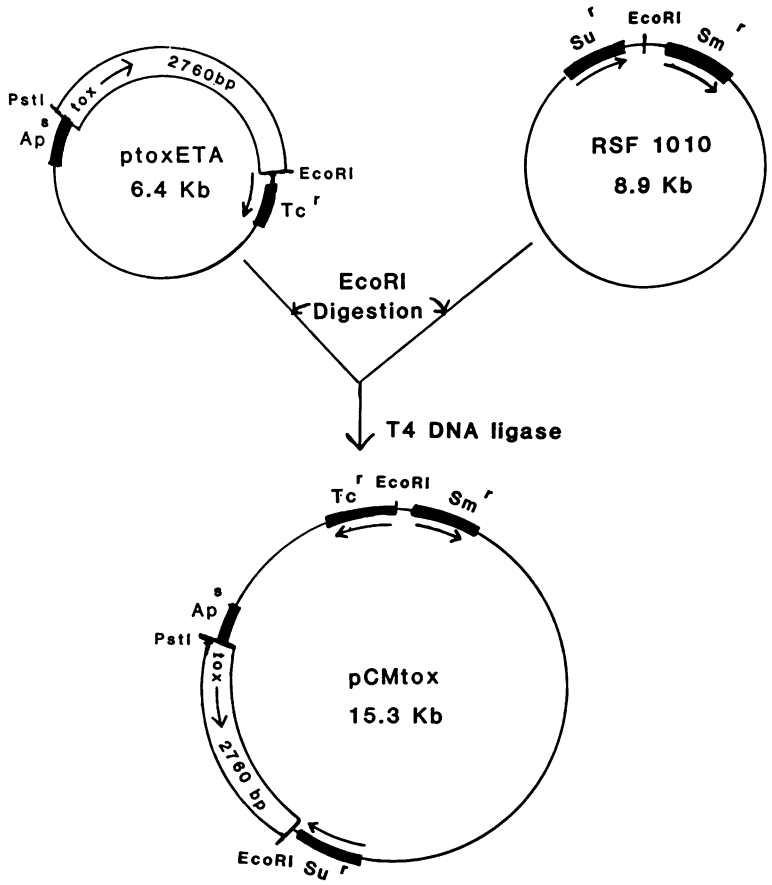

FIG. 9. Construction of hybrid plasmid pCMtox.

structural gene appear to be well-conserved from strain to strain.

The reasons for the apparently nonspecific reactions with the toxin probes which we observed with the non-*P. aeruginosa* pseudomonads and the *Bordetella* spp. strains in the colony hybridization studies remain unexplained, but these reactions were probably not due to the presence of ETA sequences in these bacteria. We thought that it was possible that these reactions might be due to the relatively high guanine-plus-cytosine contents of the *Pseudomonas* spp. (usually more than 60 mol%) and *Bordetella* spp. (67 mol%). This does not appear to be the case because the *Aeromonas* spp. strains which we tested were uniformly negative in the colony hybridization reactions and had guanine-plus-cytosine contents of 63 mol%. Whatever the nature of these reactions, it is evident from our data and the data of other investigators (19) that colony hybridization tests can yield a significant number of false-positive results even under the best conditions.

The unexpected observation of DNA rearrangements in the various *P. aeruginosa* strains just upstream of the ETA structural gene compared with the structural gene itself and downstream of the structural gene raises interesting new questions. For example, is it possible that the differences in the arrangement of DNA in the various strains influence the expression of ETA? There are essentially the following two kinds of regulation of ETA expression: (i) wide differences in the absolute amount of ETA which each strain produces (Fig. 5 and 6) and (ii) apparent specific effects which environmental conditions like iron concentration have on the expression of ETA (2, 6). It is possible that the DNA rearrangements play a role in one or both of these kinds of regulation. At this point, it is also possible that the rearrangements upstream of *tox* have nothing to do with expression of *tox*, but they could play a role in the expression of some gene unrelated but adjacent to *tox*. The rearrangements might be involved in expression of pili in *P. aeruginosa*, like DNA rearrangements which are involved in expression of pili in *Neisseria gonorrhoeae* (25). The pili of *P. aeruginosa* are immunologically variable, as they are in *N. gonorrhoeae* (39; D. E. Woods, personal communication). Thus, the recent cloning of the genes encoding pili from *P. aeruginosa* should permit testing of this hypothesis (30).

Another possibility which has been considered and could explain this restriction site variability is that the *tox* gene is located on a movable genetic element and that the restriction site variability is not due to a localized rearrangement but is a reflection of different locations of the *tox* gene in different strains. The fact that there was no observed variability in the downstream region may have been due to the limitations of the probes available to accurately examine sites more than ~5.0 kb downstream. Thus far the structural gene for *tox* has been mapped in only one strain (strain PAO1). This gene maps to the region at 85 min on the chromosome (12). Mapping of the ETA structural gene in other *P. aeruginosa* strains might provide insight as to whether the ETA structural gene is located in different regions of the chromosome in different strains and might support the hypothesis that *tox* is located on a movable genetic element.

The availability of the ETA structural gene and the 5′ regulatory elements (promoter and others?) should now allow us to address questions pertaining to the mechanism of regulation of the ETA gene (specifically, the mechanism of regulation by iron). Toward these goals we have presented preliminary data which indicate that strains which lack the chromosomal ETA gene but carry the cloned ETA gene are capable of synthesizing and secreting mature ETA. This information and these strains (strains WR5 and V209) could also be of value in future studies on the structure-activity relationships of ETA, in which cross-reacting toxins could be produced and secreted in sufficient quantities to permit relatively easy purification. This is desirable because the amount of ETA produced in *E. coli* appears to be limited and because ETA is not secreted from *E. coli* (9) as it is from *P. aeruginosa*. However, the amounts of toxin produced by constructions like strain WR5(pCMtox) are small considering the potential for production given the number of gene copies present. To overcome this problem, we recently observed that if *tox* is controlled by an *E. coli trp* promoter, we can obtain a 50- to 100-fold increase in the synthesis of a truncated cross-reacting toxin by strain WR5 compared with when it is under the control of its own promoter on a multicopy plasmid (M. L. Vasil, A. I. Vasil, and R. E. Gill, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, D89, p. 80). These data suggest that the *tox* gene is under a positive transcriptional control mechanism rather than a negative control system, such as one involving a repressor. If there were a repressor, we would expect to see derepression of *tox* synthesis in strain WR5(pCMtox) because of the multiple copies of the hypothetical *tox* operator, which would titrate out the relatively small amount of repressor (32). More direct evidence that *tox* is in fact positively regulated has been presented recently by Hedstrom et al. (R. C. Hedstrom, C. R. Funk, D. R. Galloway, and O. R. Pavlovskis, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, D91, p. 69); these authors reported the cloning of a gene from hypertoxigenic strain PA103 which increased the synthesis of toxin severalfold when it was introduced into low-toxin-producing strains, such as strains PAO1 and Ps388.

Finally, we have found that ETA production is independent of the production of any other virulence factor of *P. aeruginosa*. This observation supports the concept that the pathogenesis of *P. aeruginosa* infections is multifactorial and that the virulence of this opportunistic pathogen is not solely dependent on a single aggressin or toxin, but that optimal virulence may depend upon the contribution and interaction of many bacterial factors, as well as host factors. Further studies in which researchers use the cloned ETA gene and other recently available cloned genes which encode specific virulence factors (pili [30], PLC [36, 37], elastase [P. A. Schad, T. I. Nicas, L. F. Hanne, and B. H. Iglewski, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, B83, p. 32], and alginate production [29]) will undoubtedly make significant contributions to understanding the complex pathogenicity and basic biology of *P. aeruginosa*.

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