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Regions of Toxin A Involved in Toxin A Excretion in *Pseudomonas aeruginosa*

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Toxin A is excreted by *Pseudomonas aeruginosa* as a mature 66,583-dalton protein. In this study, we used molecular cloning and deletion analysis to define specific regions of the toxin molecule involved in its excretion. Subclones that express either the amino terminus, the carboxy terminus, or toxin A molecules with internal deletions were constructed. The hypotoxigenic mutant PAO-T1 was used as a host for the expression of the toxin constructs. When overexpressed (by the presence of extra copies of the toxin A-positive regulatory gene, *regA*, in *trans*), toxin A-cross-reactive materials produced by most of these constructs were detected in the supernatant of PAO-T1. The supernatant of *P. aeruginosa* PAO-T1 contained proteolytic activity that degraded toxin A-derived products but not the intact toxin molecule. A single *SalI* intragenic deletion (coding for the leader peptide, the first 30 amino acids, and the last 305 amino acids of the toxin) resulted in a relatively stable product in the supernatant of PAO-T1. The product of the carboxy terminus construct (which codes for the last 305 amino acids of the toxin) was detected in the lysate of PAO-T1 only. The data suggest that the amino terminus region of toxin A (the leader peptide plus the first 30 amino acid of the mature protein) is sufficient for its excretion, and that a second region, amino acids 309 through 413, protects an internally truncated toxin A molecule from the proteolytic activity in the supernatant of *P. aeruginosa* PAO-T1.

Pseudomonas aeruginosa produces several extracellular factors that contribute to its virulence (22). The chromosomally encoded toxin A, an ADP-ribosyl transferase, is one of the most toxic of these factors (17). Toxin A is excreted from *P. aeruginosa* as a single polypeptide that is toxic but enzymatically inactive (40).

DNA sequence analysis of the cloned toxin A gene indicates that the toxin is synthesized as a 638-amino-acid precursor (13). The toxin is excreted as a 613-amino-acid mature protein upon the removal of its 25-amino-acid signal peptide. The proposed signal peptide shares characteristics of other procaryotic signal peptides, including a common peptidase site (13). X-ray crystallographic studies determined that the toxin A molecule is composed of three structural domains; an amino terminus domain (domain I), a middle domain (domain II), and a carboxy terminus domain (domain III) (1). Domain I contains the eucaryotic cell receptor site, domain III contains the enzymatic active site, and domain II is thought to facilitate transport across eucaryotic cell membranes (1, 16).

Lory et al. (24) suggested that toxin A is cotranslationally excreted in *P. aeruginosa*. A 71-kilodalton (kDa) precursor protein was detected on the outer membrane of *P. aeruginosa* in the presence of a membrane-perturbing agent (10% ethanol). They proposed a model for toxin A excretion in which the newly synthesized membrane-associated precursor passes through the inner-outer membrane junction (Bayer junction) to the outer surface of the outer membrane, where the leader peptide is cleaved, giving rise to the excreted mature protein.

In *Escherichia coli*, toxin A is not expressed from its own promoter (13). However, when it is expressed from an *E. coli* recognizable promoter, toxin A is efficiently processed and transported to the periplasm (11, 23). Recently, Chaudhary et al. (9) indicated that domain II of the toxin molecule is essential for the secretion of the toxin to the periplasm of *E. coli*.

In this study, we examined the specific regions of the toxin A molecule involved in its excretion in *P. aeruginosa*. Results indicate that the toxin A leader peptide and the first 30 amino acids of the mature toxin are required for the excretion of toxin A in *P. aeruginosa*.

MATERIALS AND METHODS

Strains and media. Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in LB medium (1% Bacto-Tryptone [Difco Laboratories, Detroit, Mich.], 0.5% yeast extract, and 1% sodium chloride). For toxin production, *P. aeruginosa* was grown in Trypticase (BBL Microbiology Systems, Cockeysville, Md.) soy broth dialysate to which 1% glycerol and 0.05 monosodium glutamate were added (31). To maximize toxin A production, *P. aeruginosa* was grown at 32°C for 18 h with shaking.

DNA manipulations. Plasmid pMS151 (which contains the toxin A gene) was the kind gift of Stephen Lory (University of Washington, Seattle) (11, 23). Restriction endonucleases, *E. coli* DNA polymerase I, T4 DNA polymerase, and T4 DNA ligase were purchased from New England Biolabs (Beverly, Mass.) and used according to their recommendations. The alkaline lysis procedure (5) was used for plasmid isolation. Plasmids were purified by CsCl density gradient centrifugation as described previously (27). Transformation of *P. aeruginosa* with plasmid constructs was done as described by Bagdasarian and Timmis (3).

Construction of the toxin A subclones. Based on previous information about other secreted proteins (12, 32), we constructed toxin A subclones that contain either the amino terminus region, the carboxy terminus region, or a deletion of certain internal regions, leaving both the amino and

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or phenotype[a]	Source or reference
Strains		
E. coli TB1	*endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*) *rpsL*	4
E. coli MM294	*endA hsdR thi pro*	2
P. aerugonisa PAO-T1	Toxin A hypotoxigenic mutant of PAO	30
Plasmids		
pUC18, pUC19	Amp^r ColE1	41
pKT230	Km^r Sm^r	3
pRK2013	ColE1, Km^r, Tra$^+$	10
pRO1614	Amp^r Tc^r	33
pFHK10[b]	Tc^r RegA$^+$	14
pMS 151-1[c]	Amp^r toxin A$^+$	Stephen Lory (23)

[a] Phenotype abbreviations indicate resistance (^{r}) to the following: Ap, ampicillin; Km, kanamycin; Sm, streptomycin; Tc, tetracycline. Tra, Transfer genes.

[b] pFHK10 is a pVK101 recombinant plasmid carrying a 3.0-kb *Xho*I fragment of the *P. aeruginosa* PA103 chromosomal DNA, which contains the toxin A-positive regulatory gene *regA* (14).

[c] pMS151 is a pUC18 recombinant plasmid carrying a 2.4-kb *Eco*RI-*Eco*RV fragment of the *P. aeruginosa* PAK chromosomal DNA, which contains the entire toxin A gene.

carboxy terminus regions intact. These constructs contain either one or more of the toxin A structural domains (Fig. 1).

All toxin A subclones were generated from plasmid pMS151. This plasmid was constructed by cloning a 2.4-kilobase (kb) *EcoRI-EcoRV* chromosomal fragment, containing the entire toxin A gene, from *P. aeruginosa* PAK into the *Sma*I-*EcoR*I sites of vector pUC18 (23; Stephen Lory, personal communication). Plasmids pAH100 and pAHN104 were constructed by inserting a transcriptional terminator and translational stop codons at the unique *Bgl*II and *Xho*I sites of pMS151, respectively. We used the 2.0-kb Sp^r Sm^r *Sma*I omega fragment (Amersham Corp., Arlington Heights, Ill.), which contains the T4 transcriptional terminator and translational stop codon in both orientations (35). Plasmid pAH100 was constructed by digesting the *Sma*I omega fragment with *Bam*HI and cloning it into the *Bgl*II site of pMS151 (Fig. 1). Plasmid pAHN104 was constructed by digesting pMS151 with *Xho*I, filling in the 5′ ends with the Klenow fragment of the *E. coli* DNA polymerase I (27), and religating it with the *Sma*I omega fragment (Fig. 1). Plasmid pAH104 was constructed by cloning the 1,950-base-pair (bp) *Hin*dIII-*Xho*I fragment from pMS151 into the *Hin*dIII-*Sal*I sites of pUC18 (Fig. 1). Plasmid pAH130 was constructed by cloning the 1,186-bp *Hin*dIII-*Ava*I fragment from pMS151 into the *Hin*dIII-*Sal*I sites of pUC18 (Fig. 1). In both plasmids pAH104 and pAH130, the toxin promoter directs the transcription of the toxin gene in the opposite direction of the Lac promoter. To obtain a subclone that only codes for the carboxy terminus of the toxin molecule (pAH103), we cloned the 957-bp *Sal*I-*Eco*I fragment from pMS151 into the *Sal*I-*Eco*RI sites of pUC19 (Fig. 1). The toxin gene in this subclone is expressed under the constitutive Lac promoter. The internal deletion subclone pAH129 was constructed by *Ava*I-*Xho*I digestion of pMS151 and religation (Fig. 1). The deletion of the 764-bp *Ava*I-*Xho*I fragment in pAH129 caused a shift in the reading frame of the toxin gene at amino acid 506. Plasmids pAH132, pAH134, pAH136, and pAH146 are in-frame internal deletions in the toxin structural gene. Plasmid pAH132 (in which the internal 847-bp *Bgl*II-*Xho*I fragment of the toxin gene was deleted) was constructed by digesting plasmid pMS151 with *Bgl*II and *Xho*I, filling in the protruding 5′ ends with the Klenow fragment, and religating (Fig. 1). The construction of plasmid pAH134 was done by doubly digesting pMS151 with *Kpn*I and *Sac*II, converting the protruding 3′ ends to blunt ends with the T4 DNA polymerase (27), and religating (Fig. 1). Thus, a 923-bp *Kpn*I-*Sac*II internal fragment of the toxin gene is deleted in plasmid pAH134. To construct plasmid pAH136, plasmid pMS151 was doubly digested with *Bgl*II-*Sac*II enzymes, and the 3′ and 5′ ends were changed to blunt ends with T4 DNA polymerase and religated. The resulting plasmid, pAH136, has a 565-bp *Bgl*II-*Sac*II internal deletion in the toxin gene (Fig. 1). Plasmid pAH145 contains a deletion of the 834-bp internal *Sal*I fragment of the toxin gene (Fig. 1). It was constructed by double digesting plasmid pMS151 with *Hin*dIII and *Xba*I (toxin A gene has no restriction site for either enzyme) to remove the pUC18 *Sal*I site. The 5′ ends were then filled by using the T4 DNA polymerase (27) and religated to generate plasmid pAH144 (Fig. 2). Plasmid pAH144 was digested with *Sal*I to remove the toxin A gene internal *Sal*I fragment, and the resulting plasmid was pAH145 (Fig. 2).

Toxin A subclones were introduced into PAO-T1 by either mobilization or transformation (3, 10). Plasmids pAH104, pAH130, pAH102, pAH128, pAH132, pAH134, pAH136, and pAH145 were subcloned into the *EcoR*I site of pKT230, generating recombinant plasmids pAH105, pAH131, pAH103, pAH129, pAH133, pAH135, pAH137, and pAH146, respectively (Fig. 1). An example of such constructions is shown in Fig. 2. These recombinant plasmids were mobilized to PAO-T1 by using plasmid pRK2013 as a helper (10).

A 1.8-kb *Pst*I fragment obtained from plasmid pRO1614 (33) was cloned into the unique *Pst*I site of plasmids pMS151, pAH100, pAHN104, and pAH145, generating plasmids pMS151-1, pAH101, pAHN105, and pAH162, respectively (Fig. 1). This 1.8-kb *Pst*I fragment carries the origin of replication gene of plasmid RP1, which allows ColE1 plasmids to replicate stably in *P. aeruginosa* (33). Plasmids pMS151-1, pAH101, pAHN105, and pAH162 were introduced into PAO-T1 by transformation (3).

The expression of the toxin A subclones was examined by using the *P. aeruginosa* toxin A hypotoxigenic mutant PAO-T1 (30). The failure of PAO-T1 to produce any identifiable toxin A protein or cross-reactive materials (CRM), combined with its ability to be complemented with plasmid pMS151-1 (which carries the whole toxin A gene under its own promoter), suggests that the mutation in PAO-T1 is either in the upstream region or in the very beginning of the toxin A structural gene (data not shown). This makes PAO-T1 a suitable host for the examination of the expression of toxin A subclones. We also explored using the toxin A-negative strain WR5 (34) as a host; unfortunately, we detected only a low level of toxin A expression in this strain when it was transformed with plasmid pMS151-1 (A. N. Hamood and B. H. Iglewski, unpublished results).

Polyacrylamide gel electrophoresis and Western blot (immunoblot) analysis. *P. aeruginosa* PAO-T1 containing different subclones was grown at 32°C for 18 h to an optical density at 540 nm of about 3.5 to 4.0. The serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) was added (except where noted) to the cultures just before centrifugation to avoid protein degradation during processing of the cultures. Cells were centrifuged, suspended in 1/10 volume

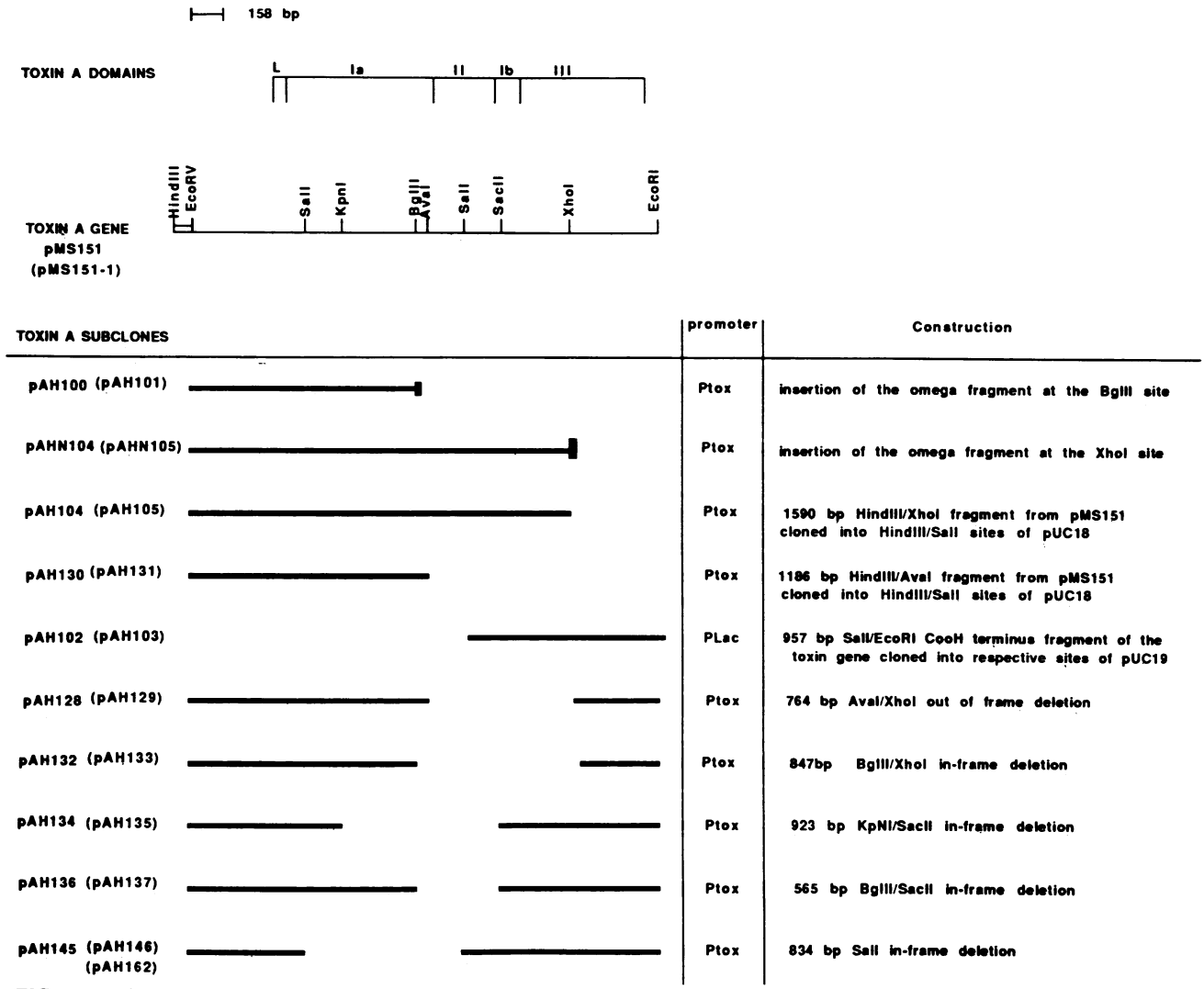

FIG. 1. Toxin A subclones. The position of the transcriptional terminator and translational stop codon, the omega fragment (see text) is indicated (■). The small open bar (□) in the map of the toxin A gene indicates the multiple cloning region of pUC18 (not drawn to scale). Plasmids between brackets are the recombinant plasmids that were introduced into *P. aeruginosa* PAO-T1. This was done either by subcloning the original plasmids in to the *P. aeruginosa* vector pKT230 or by cloning the 1.8-kb *Pst*I fragment (stability fragment) into them (see Materials and Methods). L, Toxin A leader peptide.

of the original culture medium with phosphate-buffered saline, and lysed with a French pressure cell (SLM Instruments, Inc., American Instruments Co., Urbana, Ill.) at 10,000 lb/in^2. Culture supernatants were concentrated 20× by using minicon concentrators (Amicon, Division of W. R. Grace & Co., Danvers, Mass.). It is important to note that the supernatants were concentrated at 4°C and that both supernatant and lysate samples were aliquoted and stored at −20°C before examination. The amount of protein in the lysates and supernatants was determined by the method of Lowry et al. (25). Proteins from supernatants and lysates were separated on 10 to 20% polyacrylamide gradient gels (36). After separation, proteins were electrophoretically transferred to nitrocellulose filters by the method of Towbin et al. (39). The filters were probed with rabbit anti-toxin A serum and ^{125}I-labeled protein A (New England Nuclear Corp., Boston, Mass.) and exposed to X-ray films (X-Omat AR; Eastman Kodak Co., Rochester, N.Y.). Polyclonal toxin A-specific antiserum was used as a source of anti-toxin immunoglobulin G for detecting toxin A-related polypeptides. Specific toxin A antiserum was produced in rabbits, and the immunoglobulin G fraction was isolated by specific precipitation of the crude antiserum with ammonium sulfate followed by DE52 filtration as previously described (18).

Enzyme assays. The ADP-ribosyl transferase activity of the supernatants and lysates of PAO-T1 containing toxin A subclones was determined as previously described (30), except that the reaction was allowed to proceed at 25°C for 10 min instead of 60 min. The assay for glucose-6-phosphate dehydrogenase was done as previously described by Malamy and Horecker (26).

RESULTS

Expression of the toxin A constructs. The CRM products expressed from the toxin A constructs were analyzed in the culture supernatant and the cell lysate of PAO-T1 by Western blotting experiments with rabbit antitoxin A immunoglobulin G. Earlier experiments with the immunoprecipitation technique showed that, with the exception of the

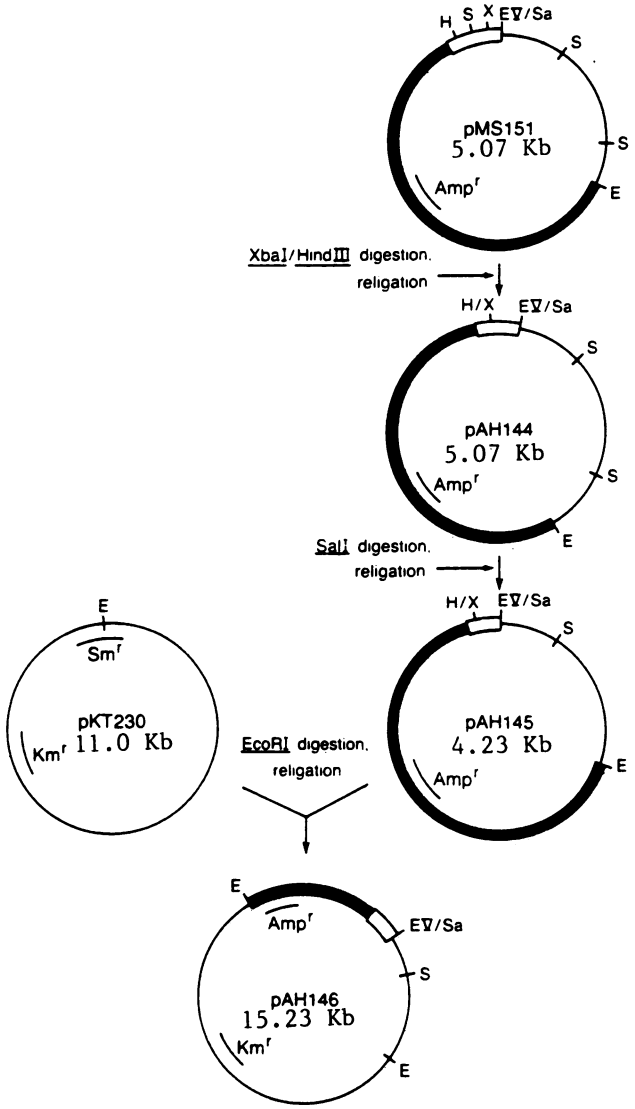

FIG. 2. Construction of plasmid pAH146. Restriction sites: E, *Eco*RI; S, *Sal*I; EV, *Eco*RV; Sa, *Sma*I; H, *Hin*dIII; X, *Xba*I. In plasmids pMS151, pAH144, and pAH145, thick lines represent vector (pUC18) DNA and thin lines represent toxin A DNA.

products of pMS151-1, no toxin A-CRM was seen in the culture supernatant of PAO-T1 containing toxin A constructs (data not shown). The 66-kDa mature toxin encoded by pMS151-1 was detected in both the supernatant and lysate (data not shown).

Previous studies have shown that multiple copies of the *P. aeruginosa* toxin A-positive regulatory gene (*regA*) significantly increases the amount of toxin A produced by different strains of *P. aeruginosa* (14, 15). Since the expression of all toxin A constructs, with the exception of pAH103, is under control of the toxin A promoter (Fig. 1), additional copies of the *regA* gene were introduced in *trans* to overexpress toxin A-CRM products. Plasmid pFHK10, carrying *regA* gene on a 3.0-kb *Xho*I fragment (14), was mobilized into PAO-T1 containing different toxin A constructs. The resulting PAO-T1 exconjugants contained at least two extra copies of the *regA* gene in addition to the original chromosomal copy (plasmid pFHK10 was derived from the P1 incompatibility low-copy-number plasmid RK2) (7, 21).

Toxin A-CRM products of all constructs, with the exception of the pAH103, were detected in both cell lysates and culture supernatants of PAO-T1(pFHK10) (Fig. 3A and B). Constructs pAHN105 and pAH105 produced no detected products and were not examined further. The CRM product of pAH103 was detected only in the cell lysate of PAO-T1(pFHK10) (Fig. 3A, lane 6; Fig. 3B, lane 6). About 85 to 90% of the glucose-6-phosphate dehydrogenase (cytoplasmic marker) activity was localized to the whole-cell portion of PAO-T1 containing toxin A constructs, whereas 10 to 15% of the activity was detected in the culture supernatant (data not shown). This indicates that cell lysis was not responsible for the appearance of the toxin A-CRM products in the culture supernatant of PAO-T1. Besides the higher-molecular-weight (MW) bands (which correlate approximately with the calculated MWs of the CRM product (Table 2), other smaller bands were seen (Fig. 3A). These smaller products may be caused by a limited proteolytic degradation of the toxin molecule and the CRM products. These results indicate that CRM product of the carboxy terminus construct pAH103 is not excreted in PAO-T1, whereas the CRM products of all other constructs are excreted.

The observed MW of the CRM products of constructs pAH129, pAH133, pAH135, and pAH137 correlated closely with their expected MWs based on the nucleotide sequencing; the MWs of the products expressed by pAH101 and pAH131 were higher than their calculated MWs (Fig. 3A and B, lanes 4 and 5, Table 2). Although construct pAH131 lacks a translational stop codon (Fig. 1), the direction of the transcription of its toxin A gene is opposite to the direction of the transcription of the *lac* gene. Thus, it is not likely that the discrepancy in MW is due to a read through the *lac* gene. Construct pAH103 produced a 40-kDa product, which was larger than the predicted 32-kDa polypeptide product (Fig. 3A, lane 6; Table 2). The same size product was detected when the lysate of the *E. coli* MM294 containing this construct was examined (data not shown). pAH103 still retains the translational stop codon of toxin A (Fig. 1). Another construct which produced a product with a higher MW than its expected MW was pAH146 (Fig. 3A and B, lane 11; Table 2). This construct, expressed from the toxin A promoter, was an in-frame deletion in the toxin gene (Fig. 1). We do not know the reason for these variations between the calculated and detected MWs of the CRM products. Plasmids carrying different toxin A constructs replicated stably in PAO-T1 with either pRO1614 or pKT230 replicons. Intrinsic differences between replicons and their copy numbers may cause variations in the amount of the CRM products expressed by the toxin A constructs. However, toxin A constructs carried on either plasmid produced detectable products (Fig. 3).

Effect of the PAO-T1 supernatant on the detectability of the CRM products of toxin A subclones. It is possible that the conformation of the CRM products encoded by the toxin A subclones differs from the conformation of the intact toxin A molecule. This change in conformation could make the CRM products a target for the proteolytic activity in the supernatant of PAO-T1. This possibility was examined in vitro by comparing the detectability of the CRM products with that of the intact toxin A molecule in the supernatant and lysate of PAO-T1 after preincubation at two different conditions: 22° and 37°C. We selected construct pAH146 because its product was more stable in the supernatant of PAO-T1 than the CRM products of other constructs. The cell lysates and culture supernatants of PAO-T1(pMS151-1)(pFHK10) and PAO-T1(pAH146)(pFHK10) were incubated at 22 and 37°C

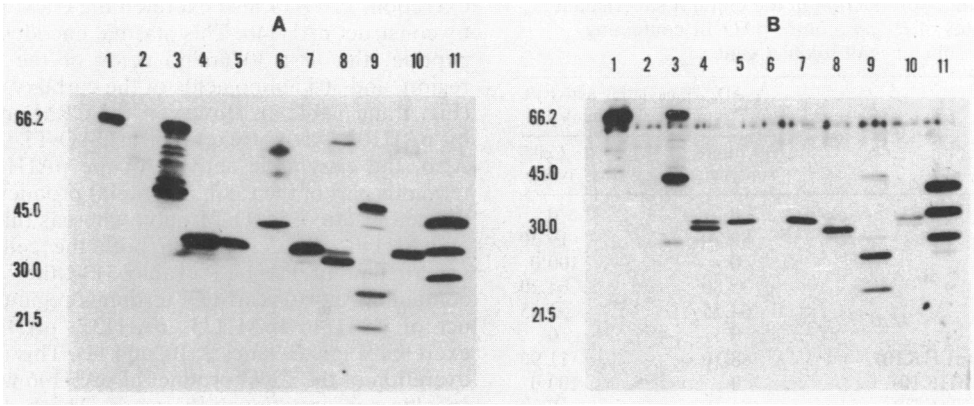

FIG. 3. Detecting the products of toxin A subclones produced in PAO-T1 containing extra copies of the toxin A-positive regulatory gene (*regA*) by immunoblot analysis. (A) Lysate fractions (lanes): 1, toxin A (positive control); 2, PAO-T1; 3, PAO-T1(pMS151-1); 4, PAO-T1(pAH101); 5, PAO-T1(pAH131); 6, PAO-T1(pAH103); 7, PAO-T1(pAH129); 8, PAO-T1(pAH133); 9, PAO-T1(pAH137); 10, PAO-T1(pAH135); 11, PAO-T1(pAH146). (B) Supernatant fractions of samples in A. The sizes of the molecular size standards (in kilodaltons) are shown to the left. The amount of protein loaded in each lane was 40 to 50 μg.

for 1 h before loading on acrylamide gels. There was no change in the amount of the intact toxin detected after preincubation at either temperature (Fig. 4, lanes 2 through 5). No CRM product was detected in the supernatant of PAO-T1(pAH146)(pFHK10) preincubated at 37°C (Fig. 4, lanes 6 and 8). Furthermore, after preincubation at 22°C, the product of pAH146 in the supernatant of PAO-T1 was reduced from 40 to about 35 kDa (Fig. 4, lane 6). A partial intracellular degradation was observed with the toxin A CRM product from the cell lysate of PAO-T1(pAH146)(pFHK10) upon preincubation at 22 and 37°C (Fig. 4, lanes 7 and 9). However, preincubation of the cell lysate of PAO-T1(pAH146)(pFHK10) with the culture supernatant of PAO-T1 at 37°C for 1 h resulted in the total loss of the CRM product (Fig. 4, lane 10). These data indicate that CRM product degradation is most likely due to proteolytic activity in the supernatant of PAO-T1. The addition of the protease inhibitor PMSF to the supernatant of PAO-T1(pAH146)(pFHK10) at 37°C partially abolished this degradation effect (Fig. 4, lane 11). The excreted CRM products of other constructs were not detected in the supernatant of PAO-T1 after only 10 min of preincubation at 22°C in the absence of PMSF (data not shown).

Enzymatic activity of the CRM products of the toxin A constructs. The intact toxin A molecule produced by pMS151-1 and the CRM products of subclones pAH103, pAH146, and pAH162 had ADP-ribosyl transferase activity (Table 3). The products of other constructs were enzymatically inactive (data not shown). These results are consistent with previously published observations concerning portions of the toxin A molecule involved in its enzymatic activity (13, 16). The ADP-ribosyl transferase activity was detectable in both the supernatant and lysate of PAO-T1(pMS151-1) and PAO-T1(pAH146) but only in the lysate of PAO-T1(pAH103) (Table 3). This confirms that the CRM product

TABLE 2. Calculated and detected sizes of the CRM products of toxin A constructs

Plasmid	Amino acid sequence[a]	Size of the toxin A CRM products (kDa)	
		Calculated[b]	Detected
pMS151-1	−25 to +613	65.74	66
pAH101	−25 to +224	24.02	35
pAHN105	−25 to +506	54.26	60[c]
pAH105	−25 to +506	54.26	60[c]
pAH131	−25 to +250	26.90	35
pAH103	+308 to +613	32.71	40
pAH129	−25 to +250 and +506 to +613	38.37	35
pAH133	−25 to +224 and +506 to +613	35.49	33
pAH135	−25 to +102 and +413 to +613	32.38	35
pAH137	−25 to +224 and +413 to +613	45.47	48
pAH146	−25 to +30 and +308 to +613	35.92	45

[a] Amino acids −25 to +1 correspond to the toxin A leader peptide, and amino acids +1 to +613 correspond to the mature toxin A protein (based on nucleotide sequence analysis).

[b] These values represent the mature toxin and the CRM products, from which the 25-amino-acid leader peptide is removed. No toxin A precursor protein was detected in the lysate of PAO-T1 (see text).

[c] The CRM products of this construct were detectable in the lysate of PAO-T1 by immunoprecipitation experiments only.

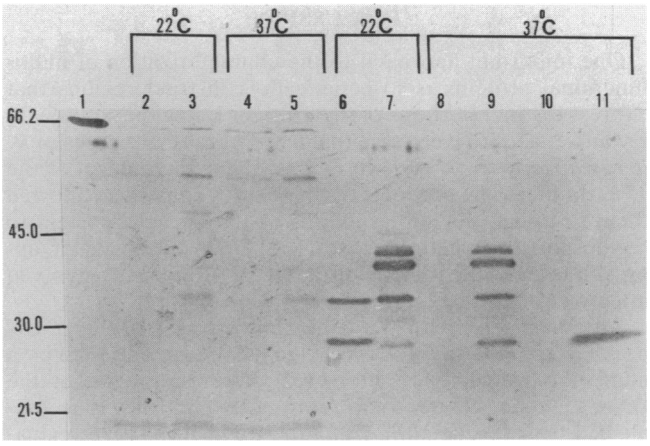

FIG. 4. In vitro degradation of the toxin A-CRM product of subclone pAH146. The supernatant and lysate of PAO-T1, containing either pMS151-1 or pAH146 plasmids, were incubated at the indicated conditions for 1 h and analyzed by immunoblotting. Lanes: 1, toxin A (positive control); 2 and 4, PAO-T1(pMS151-1) supernatant; 3 and 5, PAO-T1(pMS151-1) lysate; 6 and 8, PAO-T1(pAH146) supernatant; 7 and 9 PAO-T1(pAH146) lysate; 10, PAO-T1(pAH146) lysate plus PAO-T1 supernatant; 11, PAO-T1(pAH146) supernatant plus PMSF (protease inhibitor). The sizes of the molecular size standards (in kilodaltons) are shown to the left.

TABLE 3. Toxin A production in the cultural supernatant and cell lysates of *P. aeruginosa* pAO-T1 containing subclones of toxin A gene

Strain	% ADP-ribosylating activity[a] (cpm/μg of protein)	
	Culture supernatant	Cell lysate
pAO-T1[b]	0	0
pAO-T1(pMS151-1)	80.70	19.30
pAO-T1(pAH103)	0	100.0
pAO-T1(pAH146)	35.80	64.20
pAO-T1(pAH162)	64.35	35.65
pAO-T1(pFHK10)[c]	0	0
pAO-T1(pMS151-1)(pFHK10)	88.10	11.90
pAO-T1(pAH103)(pFHK10)	0	100.0
pAO-T1(pAH146)(pFHK10)	84.46	15.54
pAO-T1(pAH162)(pFHK10)	88.32	11.68

[a] ADP-ribosyl transferase activity was determined in the cell free cultural supernatant and cell lysate.

[b] For toxin A production, cells were grown in Trypticase soy dialysate treated with Chelex-100 at 32°C with maximum aeration. Cells were grown for 18 h to a cell density (optical density at 546 nm) of about 4.0.

[c] pFHK10 is a pVK101 recombinant plasmid carrying a 3.0-kb *Xho*I fragment of the *P. aeruginosa* pA103 chromosomal DNA, which contains the toxin A-positive regulatory gene (*regA*) (14).

of pAH146 is excreted. The higher enzymatic activity observed with the product of pAH103 is presumably due to its higher expression under the constitutive Lac promoter. Plasmid pAH162, which codes for the same CRM product as pAH146 and was constructed by insertion of the 1.8-kb *Pst*I (stability fragment), gave comparable results (Table 3). This excluded the effect of the replicon difference between plasmids pMS151-1 and pAH146 and allowed us to compare the enzymatic activity of their products in the lysate and supernatant of PAO-T1. In the presence of extra copies of the *regA* gene, the enzymatic activity was significantly increased in the lysate and supernatant of both PAO-T1(pMS151-1) and PAO-T1(pAH146) (Table 3).

DISCUSSION

One important approach to the characterization of multifunctional proteins is to genetically construct regions that express distinct portions of the protein. Using this approach, Hwang et al. (16) were able to express different regions of *P. aeruginosa* toxin A gene in *E. coli* under the control of the T7 promoter. The products of the toxin A constructs allowed them to assign distinct functions of the toxin A protein to regions of its structural gene (16). We used a similar approach to examine regions of the toxin A molecule involved in its excretion in *P. aeruginosa*.

All constructs, with the exception of pAH103, were expressed from the toxin A promoter, which is controlled by a number of regulatory factors (22). Overexpression of the DNA sequences under toxin A promoter control was accomplished by introducing in *trans* the toxin A-positive regulatory gene *regA* (14, 15). The CRM products of these overexpressed genes were detected in both the culture supernatant and the cell lysate of *P. aeruginosa* PAO-T1 (Fig. 3). These toxin A derivatives should be useful for further structure-function studies of toxin A in *P. aeruginosa*.

The ability to detect a CRM product encoded by construct pAH101 in the supernatant of PAO-T1 indicates that this subclone contains all the information required for toxin A excretion. PAO-T1 also excreted the CRM product encoded by construct pAH146. This plasmid encodes the toxin leader peptide, the first 30 amino acids of the amino terminus region, and 305 amino acids of the carboxy terminus region. (Fig. 1 and Table 2). However, the CRM product expressed by pAH103 was not excreted in pAO-T1 (Fig. 3, lanes 6). Also, the enzymatic activity of the pAH103 (the carboxy terminus part of the toxin A protein) product was detected in the cell lysate of PAO-T1 only, whereas that of the product of pAH146 was detected in both the cell lysate and the supernatant of PAO-T1 (Table 3). Other CRM products, containing shorter carboxy terminus regions than the product of pAH146 (pAH133, pAH135, pAH137), were also excreted (Fig. 3B, lanes 9, 10, and 11). This indicates that the excretion of the CRM product of pAH146 was not due to its specific conformational character. These data suggest that the toxin A leader peptide plus the first 30 amino acids of the mature toxin may be sufficient for its excretion in *P. aeruginosa*. To examine this possibility further, we constructed plasmid pAH121, which codes for the leader peptide and the first 30 amino acids of the mature toxin. However, no product was detected in the cell lysate or the culture supernatant of PAO-T1 containing this plasmid (data not shown). This construct might be producing an unstable product, or its product might not be recognized by our antiserum. Recently, Storm and Lory (37) fused the *P. aeruginosa* pilin gene to the alkaline phosphatase (*phoA*) gene and showed that the first 51 amino acids of the pilin protein are sufficient for its secretion in both *E. coli* and *P. aeruginosa*. A fusion protein encoded by the *P. aeruginosa* pilin leader sequence fused to the *phoA* gene was not secreted in either *E. coli* or *P. aeruginosa* (37). Currently, we are exploring the use of Tn-phoA vectors (28, 38) to produce fusion proteins with the NH_2 region of the toxin molecule to determine the minimum number of NH_2 amino acids of the mature toxin A protein required for its excretion in *P. aeruginosa*.

As we demonstrated in this study, and as has been shown previously for other bacterial proteins (6, 8, 19, 21), one major problem in this approach is the instability of the hybrid or truncated products. The CRM product of construct pAH146 was more resistant to the degradation effect in the supernatant of PAO-T1 at 22°C (Fig. 4, lane 6), whereas the excreted CRM products of all other constructs were rapidly degraded. Other internal deletion constructs share considerable common regions with pAH146 (Fig. 1). For example, the CRM products of pAH137 and pAH133 overlap with the NH_2 region of the CRM product of pAH146 but lack 196 and 104 amino acids from its carboxy terminus region, respectively (Fig. 1 and Table 2). Taken together, these results suggest that the 105-amino-acid region (amino acids 305 through 413 in the toxin molecule) (Table 2) contributes to the stability of the toxin molecule in the supernatant of PAO-T1. One possible function of this region is to protect the toxin molecule, through its position in the folded mature protein, against any degradation. The absence of this protection, as in the CRM products of other constructs, leads to their degradation in the supernatant of *P. aeruginosa*.

The product of pAH146 was subjected to more than one type of degradation: (i) an intracellular degradation which was shared by the toxin molecule and other toxin A CRM products (Fig. 3A; Fig. 4, lane 7); (ii) a partial extracellular degradation that occurred at 22°C (Fig. 4, lane 6); (iii) a complete extracellular degradation that occurred at 37°C (Fig. 4, lanes 8 and 11); and (iv) a partial, extracellular, PMSF-resistant degradation that occurred at 37°C (Fig. 4,

lane 11). The excreted products of other constructs in the supernatant of PAO-T1 were completely degraded when preincubated at 22°C for 10 min (data not shown). The fact that the supernatant of PAO-T1 was able to degrade the product of the toxin A constructs while having no effect on the intact toxin molecule indicates that changing the conformation of the toxin A molecule resulted in its increased susceptibility to degradation by *P. aeruginosa* extracellular proteases. Part of this degradation is likely caused by *Pseudomonas* alkaline protease, which is more active at 37°C than at 32 or 22°C (30) (Fig. 4, lanes 4, 8, and 10). This conclusion is strengthened by the fact that the degradation was inhibited by the addition of the serine protease inhibitor PMSF, which inhibits alkaline protease but not elastase (29) (Fig. 4, lane 11).

A recent study by Chaudhary et al. (9) indicated that domain II of the toxin A molecule plays an essential role in the secretion of toxin A to the periplasm of *E. coli*. Alkaline phosphatase, fused to the carboxy terminus part of domain II, was also excreted to the periplasm of *E. coli* (9). Domain II of the toxin molecule contains amino acids 253 through 364 (16). The products of pAH133, pAH135, and pAH137 do not contain domain II, whereas the product of pAH146 contains only 59 amino acids of domain II (Table 2). However, all these deletion products were excreted by PAO-T1 to the medium (Fig. 3, lanes 9 through 12). These results indicate that the mechanism of toxin A excretion in *P. aeruginosa* is different from that of toxin A secretion in *E. coli*. Unlike the case in previous studies with *E. coli* (9), we were not able to detect toxin A protein or toxin A CRM product in the periplasm of PAO-T1 containing pMS151-1 or pAH146 (data not shown). Furthermore, the lysate of PAO-T1/pMS151-1 contained the 66-kDa mature toxin A but not the 71-kDa precursor (Fig. 4A, lane 3). Our results support the previous model of Lory et al. (24) for toxin A excretion in *P. aeruginosa*. Based on that model, toxin A in *P. aeruginosa* is synthesized on membrane-associated polysomes and excreted through membrane fusion regions (Bayer junctions) (24). Accordingly, toxin A does not pass through the periplasmic space of *P. aeruginosa* during the process of its excretion to the medium. However, the detection of the mature toxin and the excreted CRM products in the lysate of PAO-T1 suggest that the excretion of toxin A in *P. aeruginosa* might be posttranslational rather than cotranslational. However, further studies are required to confirm this.

In conclusion, we have shown that the leader peptide and the first 30 amino acids of the mature toxin A protein are sufficient for its excretion in *P. aeruginosa*. The data also suggest that the internal region of the toxin A molecule (amino acids 309 through 413) protects the toxin molecule from the proteolytic activity in the PAO-T1 supernatant.

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LITERATURE CITED

1. **Allured, V. S., R. J. Collier, S. F. Carroll, and D. B. McKay.** 1986. Structure of exotoxin A of *Pseudomonas aeruginosa* at 3.0 Angstrom resolution. Proc. Natl. Acad. Sci. USA **83:** 1320–1324.
2. **Backman, K., M. Ptashne, and W. Gilbert.** 1976. Construction of plasmids carrying the cI gene of bacteriophage Lambda. Proc. Natl. Acad. Sci. USA **73:**4174–4178.
3. **Bagdasarian, M., and K. Timmis.** 1982. Host: vector systems for gene cloning in *Pseudomonas*. Curr. Top. Microbiol. Immunol. **96:**47–67.
4. **Bethesda Research Laboratories, Inc.** 1984. *Escherichia coli* TB1 host for pUC plasmids. Focus **6**(4):7.
5. **Birnboim, H. C., and J. Doly.** 1979. A rapid extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1525.
6. **Bremer, E., S. T. Cole, I. Hindennach, E. Beck, C. Kurz, and H. Schaller.** 1982. Export of a protein into the outer membrane of *Escherichia coli* K12. Stable incorporation of the ompA protein requires less than 193 amino-terminal amino-acid residues. Eur. J. Biochem. **122:**223–231.
7. **Bukhari, A. I., J. A. Shapiro, and S. L. Adhya.** 1977. DNA insertion elements, plasmids, and episomes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
8. **Celis, R. T. F.** 1981. Chain-terminating mutants affecting periplasmic binding protein involved in the active transport of arginine and ornithine in *Escherichia coli*. J. Biol. Chem. **256:**773–779.
9. **Chaudhary, V. K., Y. H. Xu, D. Fitzgerald, S. Adhya, and I. Pastan.** 1988. Role of domain II of *Pseudomonas* exotoxin in the secretion of the protein into the periplasm and medium by *Escherichia coli*. Proc. Natl. Acad. Sci. USA **85:**2939–2943.
10. **Ditta, G., S. Starfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for gram-negative bacteria. Construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77:**7347–7351.
11. **Douglas, C. M., C. Guidi-Rontani, and R. J. Collier.** 1987. Exotoxin A of *Pseudomonas aeruginosa*: active, cloned toxin is secreted into the periplasmic space of *Escherichia coli*. J. Bacteriol. **169:**4962–4966.
12. **Ghrayeb, J., and M. Inouye.** 1984. Nine amino acid residues at the NH2-terminal of lipoprotein are sufficient for its modification, processing, and localization in the outer membrane of *Escherichia coli*. J. Biol. Chem. **259:**463–467.
13. **Gray, G. L., D. H. Smith, J. S. Baldridge, R. N. Harkins, M. L. Vasil, E. Y. Chen, and H. L. Heyneker.** 1984. Cloning, nucleotide sequence, and expression in *Escherichia coli* of the exotoxin A structural gene of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **81:**2645–2649.
14. **Hedstrom, R. C., C. R. Funk, J. B. Kaper, O. R. Pavloviskis, and D. R. Galloway.** 1986. Cloning of a gene involved in regulation of exotoxin A expression in *Pseudomonas aeruginosa*. Infect. Immun. **51:**37–42.
15. **Hindahl, M. S., D. W. Frank, and B. H. Iglewski.** 1987. Molecular studies of a positive regulator of toxin A synthesis in *Pseudomonas aeruginosa*. Antibiot. Chemother. **39:**279–289.
16. **Hwang, J., D. J. Fitzgerald, S. Adhya, and I. Pastan.** 1987. Functional domains of Pseudomonas exotoxin identified by deletion analysis of the gene expressed in *E. coli*. Cell **48:** 129–136.
17. **Iglewski, B. H., and D. Kabat.** 1975. NAD-dependent inhibition of protein synthesis by *Pseudomonas aeruginosa* toxin. Proc. Natl. Acad. Sci. USA **72:**2284–2288.
18. **Iglewski, B. H., and J. C. Sadoff.** 1979. Toxin inhibition of protein synthesis: production, purification, and assay of *Pseudomonas aeruginosa* toxin A. Methods Enzymol. **60:**780–793.
19. **Ito, K., and J. Beckwith.** 1981. Role of the mature protein sequence of maltose-binding protein in its secretion across the *E. coli* cytoplasmic membrane. Cell **25:**143–150.
20. **Knauf, V. C., and E. W. Nester.** 1982. Wide host range cloning vectors: a cosmid clone bank of an *Agrobacterium* Ti plasmid. Plasmid **8:**45–54.
21. **Koshland, D., and D. Botstein.** 1980. Secretion of the β-lactamase requires the carboxy end of the protein. Cell **20:**749–760.
22. **Liu, P. V.** 1974. Extracellular toxins of *Pseudomonas aeruginosa*. J. Infect. Dis. **130**(Suppl.):94–99.
23. **Lory, S., M. S. Storm, and K. Johnson.** 1988. Expression and secretion of the cloned *Pseudomonas aeruginosa* exotoxin A by *Escherichia coli*. J. Bacteriol. **170:**714–719.

24. **Lory, S., P. C. Tai, and B. Davis.** 1983. Mechanism of protein excretion by gram-negative bacteria: *Pseudomonas aeruginosa* exotoxin A. J. Bacteriol. **156**:695–702.
25. **Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
26. **Malamy, H. M., and B. L. Horecker.** 1964. Release of alkaline phosphatase from cells of *Escherichia coli* upon lysozyme spheroplast formation. Biochemistry **3**:1889–1893.
27. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Manoil, C., and J. Beckwith.** 1985. TnphoA: a transposon probe for protein export signals. Proc. Natl. Acad. Sci. USA **82**: 8129–8133.
29. **Morihara, K., and J. Y. Homma.** 1985. Pseudomonas proteases, p. 41–79. *In* I. A. Holder (ed.), Bacterial enzymes and virulence. CRC Press, Inc., Boca Baton, Fla.
30. **Ohman, D. E., R. B. Burns, and B. H. Iglewski.** 1980. Corneal infections in mice with toxin A and elastase mutants of *Pseudomonas aeruginosa*. J. Infect. Dis. **142**:547–555.
31. **Ohman, D. E., J. C. Sadoff, and B. H. Iglewski.** 1980. Toxin A deficient mutants of *Pseudomonas aeruginosa* PA103: isolation and characterization. Infect. Immun. **28**:899–908.
32. **Oliver, D.** 1985. Protein secretion in *Escherichia coli*. Annu. Rev. Microbiol. **39**:615–648.
33. **Olson, R. H., G. Debusscher, and W. R. McCombie.** 1982. Development of broad host range vectors and gene banks: self cloning of the *Pseudomonas aeruginosa* PAO chromosome. J. Bacteriol. **150**:60–69.
34. **Pavloviskis, O. R., B. H. Iglewski, and M. Pollack.** 1978. Mechanism of action of *Pseudomonas aeruginosa* exotoxin A in experimental mouse infections: adenosine diphosphate ribosylation of elongation factor 2. Infect. Immun. **19**:29–33.
35. **Prentki, P., and H. M. Krisch.** 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene **29**:303–313.
36. **Richardson, K., and C. D. Parker.** 1985. Identification and characterization of *Vibrio cholerae* surface proteins by radioiodination. Infect. Immun. **48**:87–93.
37. **Storm, M., and S. Lory.** 1987. Mapping of export signals of *Pseudomonas aeruginosa* pilin with alkaline phosphatase fusions. J. Bacteriol. **169**:3181–3188.
38. **Taylor, R. K., V. L. Miller, D. B. Furlong, and J. J. Mekalanos.** 1987. Use of *phoA* gene fusions to identify a pilus colonization factor cooridinately regulated with cholera toxin. Proc. Natl. Acad. Sci. USA **84**:2833–2837.
39. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
40. **Vasil, M. L., D. Kabat., and B. H. Iglewski.** 1977. Structure-activity relationships of an exotoxin of *Pseudomonas aeruginosa*. Infect. Immun. **16**:353–361.
41. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.