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Expression and Secretion of the Cloned *Pseudomonas aeruginosa* Exotoxin A by *Escherichia coli*

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The exotoxin A gene from *Pseudomonas aeruginosa* PAK was expressed in *Escherichia coli* from recombinant plasmids when transcription was initiated from a promoter in the cloning vector. The exotoxin A polypeptide synthesized was found to have an electrophoretic mobility in sodium dodecyl sulfate-polyacrylamide gels of 66,000 daltons, identical in size to the mature exotoxin A made by *P. aeruginosa*. Analysis of the location of exotoxin A in various bacterial compartments by immunoblotting revealed that exotoxin A was exported by *E. coli* into its periplasmic space. Several functional assays, including analyses of disulfide bond formation, potentiation of ADP-ribosyltransferase activity, and HeLa cell cytotoxicity, were used to establish that the conformation of exotoxin A isolated from the *E. coli* periplasmic space is identical to that of exotoxin exported by *P. aeruginosa* to its extracellular space. Previous studies with recombinant plasmids expressing exotoxin A from *P. aeruginosa* PA103 (G. D. Gray, D. Smith, J. Baldridge, R. Markins, M. Vasil, E. Chen, and M. Heyneker, Proc. Natl. Acad. Sci. USA 81:2645–2649, 1984) showed a complete lack of processing and export of pre-exotoxin A in *E. coli*, differing from results reported here. These discrepancies may be explained by observed differences in the sequence of signal peptides encoded by the exotoxin A genes of PAK and PA103 strains of *P. aeruginosa*.

Extensive tissue damage during acute *Pseudomonas aeruginosa* infections is due to synthesis of a number of extracellular products by the invading microorganism. Exotoxin A, a 66,000-dalton polypeptide, is the most toxic component of these virulence factors (16). Exotoxin A has a well-characterized mode of action, which at the molecular level is identical to that of diphtheria toxin; both toxins kill susceptible cells by inhibiting elongation factor 2 via a catalytic ADP-ribosyltransferase reaction (11). Furthermore, synthesis of both of the toxins is repressed by high levels of iron in the medium, suggesting the presence of similar regulatory mechanisms (2).

Efficient excretion of toxins plays a key role in the ability of pathogenic microorganisms to cause disease, since these factors can act on targets that are distal from the site of colonization. Extracellular localization of proteins by gram-negative bacteria may involve a mechanism which could be fundamentally different from the normal secretion machinery, since excretion involves transfer of a polypeptide from the site of synthesis across the cytoplasmic as well as the outer membrane, the latter lacking a source of energy and proton motive force. While the outer membrane contains porins which allow free passage of hydrophilic solutes, the size of the pore is too small to allow free diffusion of large proteins from the periplasmic space. For these reasons, an alternative hypothesis for extracellular localization of proteins has been proposed (18) in which the excreted polypeptide bypasses the periplasm and is released from the cytoplasm through the sites of fusion between the inner and outer membranes (Bayer junctions).

In addition to excretion of exotoxin A and other proteins, *P. aeruginosa* contains the usual set of polypeptides in the cytoplasmic membrane, the periplasmic space, and the outer membrane. There must be an efficient sorting mechanism which allows a bacterium to distinguish and localize polypeptides into four different compartments. This sorting could be based on information within each polypeptide which is recognized and localized by a specific machinery in the bacterial cell. To identify the nature of the information and sorting machinery for extracellular localization of *P. aeruginosa* exotoxin A, we have sought to obtain expression of exotoxin A in *Escherichia coli*. We have chosen *E. coli* as a host for studies of expression and localization because this organism does not excrete many proteins and may allow identification of the information needed for extracellular localization of exotoxin A.

In this paper, we report the cloning and expression of *toxA*, the structural gene for exotoxin A from *P. aeruginosa* PAK, in *E. coli*. We also show that after cleavage of the signal sequence from pre-exotoxin A, the polypeptide is translocated into the periplasmic space of *E. coli*, where it assumes a folded conformation which is indistinguishable from that of the toxin which is synthesized and excreted by toxigenic *P. aeruginosa*. Finally, a comparison of signal peptides of pre-exotoxin A from two different *P. aeruginosa* strains revealed a significant variation in the sequences with no apparent effect on the efficiency of exotoxin A export from *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The source of chromosomal DNA for cloning was *P. aeruginosa* PAK, provided by D. Bradley, Memorial University of Newfoundland, St. John's, Newfoundland, Canada. *E. coli* HB101 (*hsd-20 recA13 ara-14 proA2 lac-41 galK2 mtl-1 xyl-5 supE44 rpsL2*) and *E. coli* DH5α [*endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*lacZYA-argF*)*U169* λ⁻ ϕ*80* d*lacZ* ΔM15] were used as hosts for construction of recombinant plasmids. *E. coli* K38 (HfrC) carrying the cloned T7 RNA polymerase on plasmid pGP1-2 was used with the T7 promoter vectors (25). Plasmids used in this study were pUC8, pUC9 (28), and pT7-6

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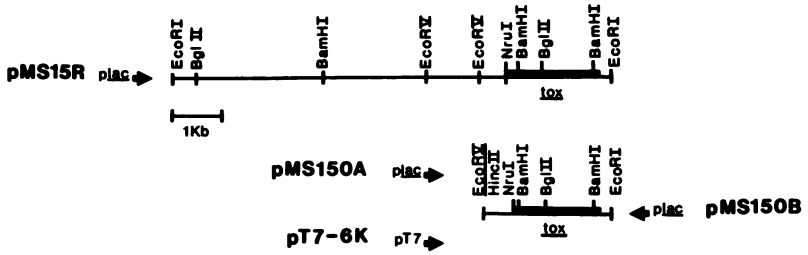

FIG. 1. Restriction map of *toxA*-containing recombinant plasmids. Arrows indicate the direction of transcription of the *lac* promoter in pUC8 and pUC9 in plasmids pMS15R, pMS150A, and pMS150B and the direction of the bacteriophage T7 promoter in pT7-6K. kb, Kilobase pairs.

(25). Recombinant plasmids generated in the course of this study are diagrammed in Fig. 1.

Enzyme assays. Determinations of ADP-ribosyltransferase activity were performed as described by Chung and Collier (3). β-Lactamase and β-galactosidase assays were performed as described by Jones et al. (13) and Miller (20), respectively.

Recombinant DNA techniques. All restriction enzymes, the Klenow fragment of T4 DNA polymerase, DNA ligase, and T4 polynucleotide kinase were purchased from Bethesda Research Laboratories (Gaithersburg, Md.) and were used according to the specifications of the supplier. Plasmid DNA was prepared by the alkaline procedure of Birnboim and Doly (1). Chromosomal DNA was extracted from *P. aeruginosa* as described previously (24).

Cloning of the *toxA* gene. *P. aeruginosa* chromosomal DNA was digested with *Eco*RI and ligated into the *Eco*RI site of plasmid pUC9. The recombinant plasmids were introduced into competent *E. coli* HB101 by transformation, and after selection on L agar containing 100 μg of ampicillin per ml, colonies were replicated onto nitrocellulose filters (BA-85; Schleicher & Schuell, Inc., Keene, N.H.). Colonies were lysed, and DNA was denatured by sodium hydroxide treatment before probing. The probe for *toxA* was an oligonucleotide (20-mer, synthesized by the Custom DNA Synthesis Laboratory, University of Washington) consisting of the antisense strand of the published sequence of *toxA* from *P. aeruginosa* PA103 (8) from position 841 to 852, which encodes the amino-terminal region of the mature exotoxin A polypeptide. The oligonucleotide was labeled with [γ^{32}P]ATP (New England Nuclear Corp., Boston, Mass.) with T4 polynucleotide kinase and was used to probe the filters as described by Maniatis et al. (19).

Cell fractionation. *E. coli* K38 (pGP1-2, pT7-6K) was grown to an optical density at 600 nm of 1 at 28°C in L broth containing 25 μg each of kanamycin and ampicillin per ml. The culture was shifted to 42°C for 20 min to induce the synthesis of T7 RNA polymerase and then returned to 30°C for an additional hour to allow transcription from the T7 promoter in pT7-6K and synthesis of exotoxin A. Cells were harvested by centrifugation, and periplasmic proteins were isolated by the modified cold osmotic-shock procedure of Hazelbauer and Harayama (9). Shocked cells were lysed by passage through a French pressure cell (6,000 lb/in^2), and membranes were separated from cytoplasmic proteins by centrifugation in a Beckman SW50.1 rotor at 100,000 × *g* for 1 h in a gradient consisting of equal volumes of 15% (wt/vol) sucrose over 60% (wt/vol) sucrose, in 50 mM Tris hydrochloride (pH 7.6). The membrane fraction was collected from the interface between the two sucrose solutions, diluted in 50 mM Tris hydrochloride (pH 7.6), and centrifuged as before. The exotoxin A content in each compartment was quantitated by the dot-immunobinding assay of Jahn et al. (12), with purified exotoxin A as a standard.

Polyacrylamide gel electrophoresis and Western immunoblot analysis. Polypeptides were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described by Laemmli (14). Samples were denatured by boiling in 2% SDS and 50 mM 2-mercaptoethanol, except where indicated. For immunoblotting, proteins were transferred to nitrocellulose sheets by the method of Towbin et al. (26). The nitrocellulose sheets were blocked for 1 h with 3% (wt/vol) nonfat dry milk in TBS (150 mM NaCl, 50 mM Tris hydrochloride, [pH 7.5]) and then reacted for 12 h with rabbit antitoxin serum. Preparation of antisera to purified exotoxin A was described previously (18). The filters were reacted with ^{125}I-protein A (New England Nuclear Corp., Boston, Mass.) for 1 h and then washed with TBS containing 0.5% Tween.

DNA sequencing. The template for Sanger et al. dideoxy sequencing (23) was M13mp19 bacteriophage containing the *Eco*RI-*Eco*RV insert from pMS15R. The reaction was primed with the same synthetic 20-mer oligonucleotide used as the probe for cloning the *toxA* gene.

RESULTS

Cloning and expression of the exotoxin A gene. We have isolated *toxA*, the structural gene for exotoxin A from *P. aeruginosa* PAK, as an 8-kilobase-pair DNA insert in plasmid pUC9. This recombinant plasmid, pMS15R, was used to prepare subclones of toxin, as shown in Fig. 1. The *Eco*RV-*Eco*RI fragment from pMS15R was ligated into pUC8, pUC9, and pT7-6. These subclones were constructed to determine whether the exotoxin A promoter is recognized by *E. coli* RNA polymerase or whether the transcription of the *toxA* gene in the cloning host is possible only when initiated from a vector promoter. Synthesis of exotoxin A in *E. coli* carrying *toxA* clones was determined by Western blot analysis of cell extracts, probed with anti-exotoxin A sera (Fig. 2).

E. coli containing pMS15R did not express detectable exotoxin A. This result suggests that the *toxA* gene is not expressed in *E. coli* because of either a transcriptional or a translational block. To determine whether the *toxA* promoter was inactive in *E. coli* or whether the failure to detect exotoxin A is due to the inability of *E. coli* to translate toxin mRNA, smaller subclones were constructed in vectors containing exogenous promoters. Plasmids pMS150A and pMS150B contain the same *Eco*RV-*Eco*RI fragment in vectors that differ only in the location of the *lac* promoter. In pMS150A, the direction of the transcription of the *toxA* gene is the same as transcription initiating at the *lac* promoter. In pMS150B, the *lac* promoter is in the opposite orientation with respect to *toxA* transcription.

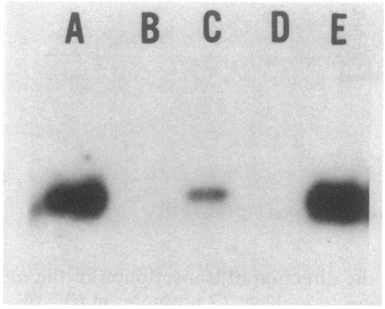

FIG. 2. Immunoblot analysis of *E. coli* carrying *toxA*-containing recombinant plasmids. Lanes: A, exotoxin A standard; B, *E. coli* DH5α (pMS15R); C, *E. coli* DH5α (pMS150A); D, *E. coli* DH5α (pMS150B), E, *E. coli* K38 (pGP1-2, pT7-6K).

Analysis of the polypeptides in *E. coli*(pMS150A) showed the presence of a significant amount of exotoxin A antigen, whereas no exotoxin A was detected in *E. coli* pMS150B. These findings suggest that the expression of exotoxin A was dependent on the initiation of transcription from a vector promoter. To confirm these observations, the same *Eco*RV-*Eco*RI fragment was subcloned into T7-6, a plasmid containing the promoter from bacteriophage T7 (25). Exotoxin A was also synthesized in *E. coli* from this construction, provided that T7 RNA polymerase was made by the same cell (Fig. 2, lane E). These findings confirm the observations of Gray et al. (8), who also failed to obtain expression of exotoxin A from a *toxA* gene isolated from *P. aeruginosa* PA103 unless transcriptional initiation signals were engineered upstream from the *toxA* gene. Furthermore, a large quantity of the toxin polypeptide was made when transcription was directed from a strong promoter, such as that recognized by T7 RNA polymerase in pT7-6K; this excludes the possibility that lack of expression of the *toxA* gene in *E. coli* is due to failure of the translational machinery to recognize the *toxA* mRNA.

Localization of exotoxin A. A comparison of the size of authentic exotoxin A purified from culture fluids of *P. aeruginosa* to that of exotoxin A made by *E. coli* recombinants (Fig. 2) showed that they were identical. This observation suggested that *E. coli* is capable of correctly processing the exotoxin A precursor. To identify the cellular compartment that contains exotoxin A, *E. coli* expressing exotoxin A from plasmid pT7-6K was fractionated into cytoplasmic, periplasmic, and membrane fractions and analyzed for the presence of the exotoxin A antigen by Western blotting. Most of the exotoxin (ca. 80%) was found in the periplasmic compartment, while less than 10% was found in the cytoplasmic and membrane fractions (Fig. 3). No detectable toxin was excreted into the medium. More than 90% of β-lactamase activity and less than 5% of β-galactosidase activity was released by the osmotic shock procedure (data not shown), confirming that the periplasmic fraction is not contaminated with cytoplasmic proteins. A small amount of exotoxin A was found in the cytoplasmic fraction, which most likely represents contamination with periplasmic proteins since approximately 5 to 8% of β-lactamase activity was also detectable in the cytoplasm. Similarly, exotoxin A was nearly completely periplasmically localized in *E. coli* harboring pMS150A. These findings suggest that the export information on the exotoxin A polypeptide is recognized by *E. coli* and that the polypeptide is secreted into the periplasmic space. Our findings differ from those of Gray et al. (8), who studied localization of recombinant *P. aeruginosa*

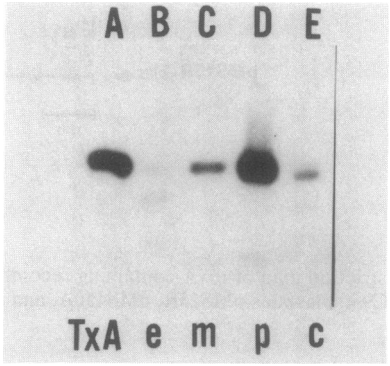

FIG. 3. Cellular location of exotoxin A in *E. coli* K38 (pGP1-2, pT7-6K). Lanes show purified exotoxin A (A), extracellular (B), membrane (C), periplasmic (D), and cytoplasmic (E) fractions.

PA103 exotoxin A in *E. coli* and found that the *E. coli* host accumulated the exotoxin A polypeptide in a precursor form in the cytoplasm.

Conformation of *E. coli*-synthesized exotoxin A. To determine whether exotoxin A isolated from the *E. coli* periplasmic space is folded in a different conformation than that of the native toxin from *P. aeruginosa*, several assays were performed. Electrophoretic mobility through polyacrylamide gels is an indication of relative compactness of a polypeptide chain, and this property depends on, among other factors, the extent of intrachain disulfide cross-linking (4). To determine whether exotoxin A secreted into the *E. coli* periplasm contains cysteines in either sulfhydryl- or disulfide-linked forms, we have compared the relative electrophoretic mobilities of the denatured and reduced recombinant exotoxin A to similarly treated *P. aeruginosa*-made exotoxin A. Figure 4, lanes A and B, demonstrates that even in the presence of SDS, exotoxin A with all four disulfide bonds intact migrates faster than the completely reduced, more extended polypeptide. Exotoxin A extracted from the *E. coli* periplasmic space (Fig. 4, lanes C and D) shows a similar alteration in mobility upon reduction. We therefore conclude that secretion of the exotoxin A into the *E. coli*

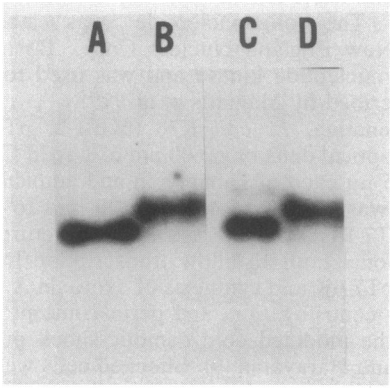

FIG. 4. Effect of reduction of disulfide bonds on mobility of exotoxin A in SDS-polyacrylamide gels. Exotoxin A purified from culture supernatant of *P. aeruginosa* (lanes A and B) and extracted from *E. coli* K38 (pGP1-2, pT7-6K) (lanes B and C) was denatured at room temperature in 1% SDS, with (lanes B and D) or without (lanes A and C) 20 mM dithiothreitol. Samples were treated for 20 min with 50 mM iodoacetamide to block reformation of disulfide bonds and then electrophoresed on 7.5% SDS-polyacrylamide gels, followed by Western transfer and immunoblotting with antitoxin.

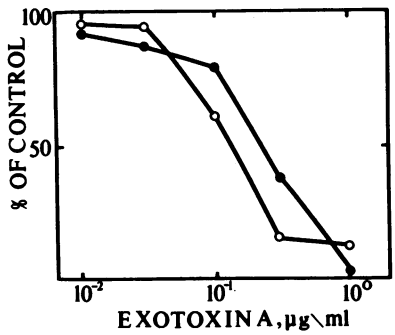

FIG. 5. Inhibition of protein synthesis of HeLa cells by exotoxin A. Monolayers of HeLa cells, in 48-well microtiter plates, were exposed to various concentrations of exotoxin A for 24 h. Inhibition of protein synthesis was determined after 30-min labeling with [^{35}S]methionine. Cells were lysed with 0.1% SDS, and proteins were precipitated with 10% trichloroacetic acid and collected on glass fiber filters. The radioactivity incorporated into acid-insoluble material was determined by counting the filters by liquid scintillation. ●, *P. aeruginosa*-made exotoxin A; ○, exotoxin A made by *E. coli* K38 (pGP1-2, pT7-6K).

periplasmic space is followed by formation of most, if not all, of the disulfide bonds found in the native exotoxin.

For exotoxin A to exert its full toxic effect on animals and cultured cells, it is absolutely necessary that all eight cysteines form specific disulfide linkages (15, 27). A comparison between exotoxin A made by *P. aeruginosa* and that made by recombinant *E. coli* in their ability to inhibit protein synthesis in HeLa cells is shown in Fig. 5. Both toxins exert a 50% inhibitory effect at a similar concentration range, suggesting that they do not differ dramatically in conformation.

We have previously shown that controlled reduction of approximately one-half of the four disulfide bonds in exotoxin A results in expression of maximal ADP-ribosyltransferase activity in in vitro assays (17). We have used the potentiation of the ADP-ribosyltransferase activity as an additional criterion for comparison of the conformation of exotoxin A made by recombinant *E. coli* and *P. aeruginosa*.

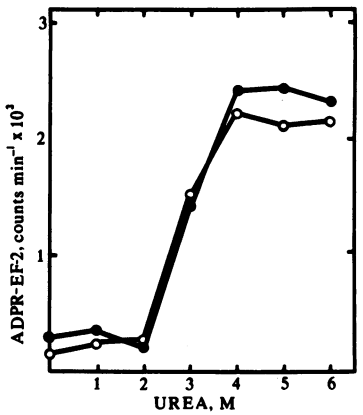

FIG. 6. Activation of the ADP-ribosyltransferase activity of exotoxin A in response to urea-dithiothreitol treatments. Samples (1 μg/ml) of exotoxin A was treated for 15 min with various concentrations of urea and 20 mM dithiothreitol. The reaction was terminated by 20-fold dilution with 50 mM Tris hydrochloride (pH 7.8), and 10-μl aliquots were assayed for ADP-ribosyltransferase activity. ●, Exotoxin A purified from *P. aeruginosa* culture supernatant; ○, exotoxin A isolated from the periplasmic space of *E. coli* K38 (pGP1-2, pT7-6K).

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          -25                                                  -12
PA103  Met-His-Leu-Ile-Pro-His-Trp-Ile-Pro-Leu-Val-Ala-Ser-Leu-
PAK    Met-His-Leu-Thr-Pro-His-Trp-Ile-Pro-Leu-Val-Ala-Ser-Leu-

          -11                          -3      -1  +1  +2
PA103  Gly-Leu-Leu-Ala-Gly-Gly-Ser-Ser-Ala-Ser-Ala-Ala-Glu-
PAK    Gly-Leu-Leu-Ala-Gly-Gly-Leu-Ser-Arg-Ser-Ala-Ala-Glu-
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FIG. 7. Comparison of leader peptides of exotoxin A made by *P. aeruginosa* PA103 (8) and *P. aeruginosa* PAK. Position +1 is the first amino acid of the mature exotoxin A from PA103. Underlined are amino acids that differ between the two sequences.

Exotoxin A obtained from the periplasmic fraction of *E. coli* (pT7-6K) was treated with dithiothreitol in the presence of increasing concentrations of urea (Fig. 6). Progressive denaturation of exotoxin A, with simultaneous reduction of disulfide bonds, led to an increase in ADP-ribosyltransferase activity, starting at background levels of activity at urea concentrations of up to 2 M, to maximal activity at concentrations of urea of 4 M or greater. Both natural and recombinant exotoxin A showed nearly identical transition profiles between native enzymatically inactive and denatured and reduced fully active forms.

Amino acid sequence of the exotoxin A signal peptide. In a previous publication, Gray et al. (8) reported expression and cytoplasmic localization of the exotoxin A precursor in *E. coli*. Their results, obtained with the cloned *toxA* gene from *P. aeruginosa* PA103, differ from those reported in this communication. Since we have used a different strain (PAK) for our studies, we sought to determine whether the two toxins differ in their signal peptides, which might account for the lack of export in *E. coli* of exotoxin A cloned from PA103 and periplasmic localization of the same protein from PAK. Figure 7 shows a comparison of the deduced amino acid sequences for the two proteins. If one assumes that the site of cleavage of the signal peptidase is between two alanines, indicated as −1 and +1 in Fig. 7, then the two sequences are highly homologous but not identical. Differences are found at position −22 (where Ile in PA103 is replaced with Thr in PAK), at position −5 (Ser replaced with Leu), and at position −3 (Ala replaced with Arg).

DISCUSSION

The *toxA* gene of *P. aeruginosa* encodes a 66,000-dalton polypeptide, termed exotoxin A, which is one of the major virulence factors of this organism. When the site of initiation of transcription was mapped, no homology to any known bacterial promoters was observed (7). Positively regulated genes very often lack a characteristic −10, −35 sequence recognized by the major form of procaryotic RNA polymerase (21). Indeed, Hedstrom et al. (10) identified a gene, *toxR*, which appears to be the transcriptional activator of *toxA*. It is, therefore, not surprising that expression of the cloned *toxA* gene in *E. coli* is dependent on transcription initiating from an exogenous promoter (Fig. 2). Furthermore, pMS15R, the largest fragment containing the *toxA* gene, fails to direct synthesis of exotoxin A in *E. coli*, suggesting that the genes for accessory regulatory elements are not found within the 6-kilobase-pair region flanking the *toxA* gene. Expression of exotoxin A in vectors containing the *lac* or T7 phage promoters suggests that the toxin mRNA is efficiently translated into the exotoxin A polypeptide and that the failure of *E. coli* RNA polymerase to initiate transcription at the *toxA* promoter is the sole limiting factor in preventing synthesis of this polypeptide in *E. coli*.

The synthesis of exotoxin A directed by various expression vectors resulted in a polypeptide which comigrated with

exotoxin A purified from the growth medium of *P. aeruginosa* on SDS-polyacrylamide gels. It thus appears that pre-exotoxin A can serve as a substrate for one of the *E. coli* signal (leader) peptidases. The major signal peptidase, encoded by the *lep* gene, has a broad substrate specificity (5) and is likely to be the enzyme which cleaves the signal sequence on the exotoxin A precursor. We cannot exclude the possibility, however, that an as yet uncharacterized signal peptidase of *E. coli* is involved in processing of pre-exotoxin A.

Most of the exotoxin A made by *E. coli* was secreted into the periplasmic space. In contrast, *P. aeruginosa* excretes exotoxin A immediately after completion of its synthesis, and no detectable pools are ever found in any of the subcellular compartments, including the periplasmic space (18). Exotoxin A made by *E. coli* was indistinguishable from that made by *P. aeruginosa* and purified from culture filtrates in a number of functional assays, suggesting that in the periplasmic space, the recombinant toxin assumed the native conformation. Thus, it appears that the necessary information on the pretoxin is recognized by the *E. coli* export apparatus and exotoxin A enters the periplasmic export pathway.

We have previously proposed a model for extracellular localization of exotoxin A by *P. aeruginosa* in which the polypeptide bypasses the periplasmic space (18). According to this model, the first stage of the excretion pathway is interaction with the inner membrane, followed by lateral fusion to the outer membrane through the regions of fusion between the two membranes (Bayer junctions). As soon as the toxin enters the outer membrane portion of the Bayer junction, it undergoes a conformational change and is released into the extracellular space. The findings presented in this report are not inconsistent with this model. In *P. aeruginosa*, the maintenance of association with the inner membrane is essential for allowing the lateral movement to the Bayer junction along or within the inner membrane. Complete translocation across the inner membrane would result in a "trapping" of exotoxin A, since by definition periplasmic space proteins are not exported across the outer membrane.

The differential localization of exotoxin A by *E. coli* and *P. aeruginosa* further suggests that *P. aeruginosa* may contain additional components in its export machinery which interact with the signal sequence or a sequence within the mature polypeptide chain to prevent translocation of the polypeptide chain across the inner membrane. Absence of this machinery in *E. coli* results in the export of exotoxin A into the periplasmic space, where it folds and assumes a conformation identical in all physical and biological assays to that of exotoxin A excreted by *P. aeruginosa*. The periplasmic form of the exotoxin, while correctly folded with all eight cysteines forming the correct disulfide bonds, is in a compartment from which it cannot be further translocated. The periplasmic localization of exotoxin A therefore represents an aberrant pathway of export.

Recent work in several laboratories has provided evidence that folded proteins cannot be translocated (6, 22). The preservation of the unfolded state may be the function of specific components of the export apparatus. Excretion via the Bayer junction may require exotoxin A to be in a partially or completely unfolded conformation. Individual cysteines in exotoxin A could play an important role in maintenance of the unfolded state by forming disulfide-bonded intermediates during export. Breaking of such bonds and formation of the correct set of disulfide bridges during passage through the Bayer junction would stabilize a native, correctly folded conformation. Such conformation may be incompatible with the outer membrane environment, resulting in the rapid release of exotoxin A.

Our observation, that the recombinant exotoxin A is secreted into the periplasmic space by *E. coli*, differs from the findings of Gray et al. (8). These authors observed that the expression in *E. coli* of the *toxA* gene from *P. aeruginosa* PA103 resulted in the synthesis of the precursor exotoxin A, which was found entirely in the cytoplasm. One possible explanation for these differences in localization could be the presence of mutations in the regions of the polypeptide that specify export. We compared the amino acid sequence of the signal peptide of exotoxin A made by strain PAK and used in this study with the signal peptide of exotoxin A form PA103 (Fig. 7). Although the two signal peptides are identical in size, three amino acid differences were found in positions −3, −5, and −22 from the putative signal peptidase cleavage site. At position −3, the PAK signal contains an arginine residue, whereas an alanine occupies the same position in the PA103 signal peptide. Amino acids with large, charged side groups at this position are not found in any exported polypeptide (29), raising the possibility that exotoxin A expressed by PAK is not processed at the same amino acid position as PA103. Several additional potential cleavage sites can be found in the sequence of the exotoxin A precursor. Cleavage of the signal sequence between positions +1 and +2 (Ala-Glu) or −2 and −1 (Ser-Ala) could also occur, yielding a mature exotoxin A from PAK which would be similar in size to that synthesized by PA103. To account for the differences in export of the two polypeptides in *E. coli*, one has to assume that the difference of three amino acids in the PA103 exotoxin A signal peptide results in a conformation of this sequence which is not recognized by the secretion apparatus of *E. coli*, whereas the signal peptide of PAK has a structure which allows it to enter the export pathway of periplasmic proteins.

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