Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 2645–2649, May 1984
Biochemistry

Cloning, nucleotide sequence, and expression in *Escherichia coli* of the exotoxin A structural gene of *Pseudomonas aeruginosa*

(secreted protein/ADP-ribosylation/active domain/diphtheria toxin)

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Communicated by David Botstein, December 28, 1983

ABSTRACT **A 2760-base pair DNA segment of the *Pseudomonas aeruginosa* strain PA103 chromosome encoding the exotoxin A (ETA) structural gene has been cloned in *Escherichia coli* and the nucleotide sequence has been determined. Analysis of the 5′- and 3′-flanking regions indicate that ETA is translated from a monocistronic message. Comparison of the deduced NH_2-terminal amino acid sequence with that determined by sequence analysis of the secreted protein indicates that ETA is made as a 638 amino acid precursor from which a highly hydrophobic leader peptide of 25 amino acids is removed during the secretion process. Data are presented that indicate a COOH-terminal location of the ADP-ribosylation activity of the molecule. Expression of the ETA coding sequence in *E. coli* under control of the *E. coli trp* promoter, but not the ETA promoter, results in the production of enzymatically and immunologically active protein. However, most of this material appears to be neither processed nor secreted. Comparison of the ETA amino acid and nucleotide sequences to those of diphtheria toxin revealed no significant homologies, indicating that these functionally similar toxins evolved from different genes.**

The exotoxin A (ETA; M_r, 66,000) of *Pseudomonas aeruginosa* belongs to a class of bacterial ADP-ribosyl transferases that includes diphtheria toxin (DT), cholera toxin, and *Escherichia coli* heat-labile enterotoxin (1). Like DT, ETA inhibits protein synthesis in eukaryotic cells by catalyzing the transfer of the ADP ribosyl moiety of oxidized NAD onto elongation factor 2 (EF-2). Biochemical studies have shown that ETA is similar to DT in other ways: (*i*) it is of similar size (2); (*ii*) it is secreted as a single polypeptide chain containing disulfide bridges and no free sulfhydryl groups (3); (*iii*) it is a proenzyme that must undergo alterations in covalent structure before ADP-ribosyltransferase activity is expressed (3, 4); (*iv*) it appears to modify the same site on EF-2 and possesses a similar binding constant for NAD^+(5); and (*v*) it is produced maximally in iron-deficient medium (6). Despite these similarities, the two toxins differ considerably: (*i*) they show little or no immunological cross-reactivity (1); (*ii*) they have different amino acid compositions (2); (*iii*) they differ in their mode of activation (3, 4); (*iv*) they bind to different cell receptors (7); and (*v*) intact DT binds ATP (8) and possesses NAD-glycohydrolase activity (9), whereas intact ETA lacks these properties (3, 8).

In contrast to that of DT, little is known about the *ETA* structural gene. A chromosomal locus has been demonstrated by linkage analysis of an *ETA* structural mutation (10). Attempts to isolate the gene in *E. coli* by using methods that depend on expression of ETA-related polypeptides have failed. We describe here the cloning of the *ETA* gene using oligonucleotide probes and also its preliminary characterization.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *E. coli* 294 (11) was used for transformations with pBR322-derived plasmids. For transformation with plasmids derived from pUC8 or pUC9 (12), *E. coli* strain JM83 (12) was used. pHGH207-1* is a plasmid that has been used for the expression of human growth hormone under *E. coli trp* promoter control (11). The M13 vectors mp8 and mp9 were used for all DNA sequence analyses as described (13). *P. aeruginosa* strain PA103 (2) was used for ETA production and genomic DNA preparation.

Isolation of ETA Clones. Purified ETA was subjected to NH_2-terminal amino acid sequencing by the Edman method (14). The sequence revealed was Ala-Glu-Glu-Ala-Phe-Asp-Leu-Trp-Asn-Glu-Cys-Ala-Lys-Ala-Cys-Val-Leu-Asp-Leu-Lys-Asp-Gly-Val-Arg-Ser-Ser-Arg-Met. Oligonucleotide probes d(G-C-$^{G}_{A}$-C-A-$^{T}_{C}$-T-C-$^{G}_{A}$-T-T-C-C-A) potentially complementary to the region of lowest genetic degeneracy (positions 8–12) were synthesized (15) and ^{32}P-labeled at the 5′ end with T4 polynucleotide kinase and [γ-^{32}P]ATP. The probe pool was hybridized to a recombinant pUC9-PA103 library of *Bam*HI-cleaved genomic DNA constructed essentially as described (16). Hybridization and washing of filters were carried out at low stringency as described by Pennica *et al.* (17). Plasmid DNA was isolated from several positive clones, digested with *Bam*HI, and found always to contain a 1.5-kilobase insert fragment. One such fragment, from clone pUC9BB, was isolated by electroelution after polyacrylamide gel electrophoresis (18), and then the sequence was determined by the dideoxy chain-termination method (19). An 84-base pair (bp) region (Fig. 1*b*, bp 821–904), potentially coding for the stretch of 28 NH_2-terminal amino acids described above, was found 55 bp from one of the *Bam*HI termini and was used to orient the fragment. The 5′ and 3′ overlapping ETA clones, pUC9PS and pBR322RS (Figs. 1*a* and 2*a*), were then isolated by standard methods (20–22) using pUC9BB-derived ^{32}P-labeled restriction fragments as hybridization probes. Their inserts were then analyzed.

Expression of the *ETA* Gene and its COOH-Terminal Fragments in *E. coli*. The *ETA* gene was reassembled into plasmid vectors to allow its expression in *E. coli* under the control of its own (postulated) promoter or the *E. coli trp* promoter as outlined in Fig. 2*a*. *E. coli* 294 cells transformed with these vectors were grown overnight at 37°C in LB medium supplemented with ampicillin at 20 μg/ml. After harvesting by centrifugation, cells were resuspended in 10 mM Tris·HCl (pH

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Abbreviations: bp, base pair(s); EF-2, elongation factor 2; DT, diphtheria toxin; ETA, exotoxin A.

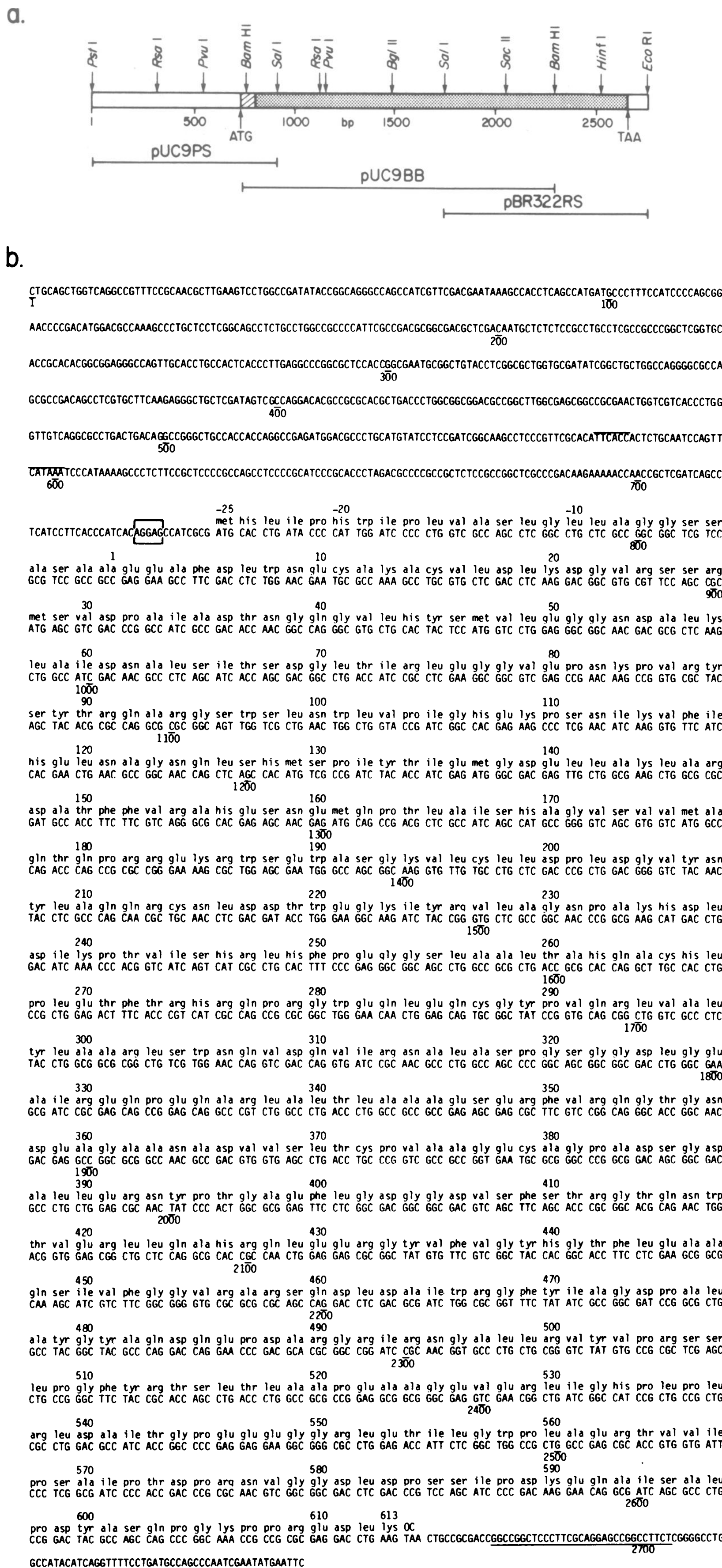

FIG. 1. (*a*) Restriction endonuclease map of the 2760-bp ETA DNA. The numbers and sizes of fragments were estimated by electrophoresis on 6% polyacrylamide gels. Positions of sites were confirmed by DNA sequence analysis. The shaded region represents the coding region for the postulated mature ETA protein, the leader peptide is indicated by a cross-hatched region, and the open regions show the 3′- and 5′-noncoding sequences. The lines below the map indicate the three partial ETA clones. (*b*) The nucleotide sequence of the ETA DNA. The putative leader peptide is represented by amino acid residues −25 through −1. The boxed sequence is the postulated Shine–Dalgarno region, the overlined sequences represent possible −10 and −35 sequences, and a potential transcription terminator sequence is underlined. Numbers above each line refer to amino acid position, and numbers below each line refer to nucleotide position.

7.0), lysed by sonication (10 bursts of 2 sec), and analyzed for ETA-related polypeptides. Immunoblot analysis was carried out exactly as described (11) except that the antiserum used was immunoaffinity-purified rabbit anti-ETA (23). ADP-ribosyl:EF-2 transferase assays were carried out on activated samples as described (4).

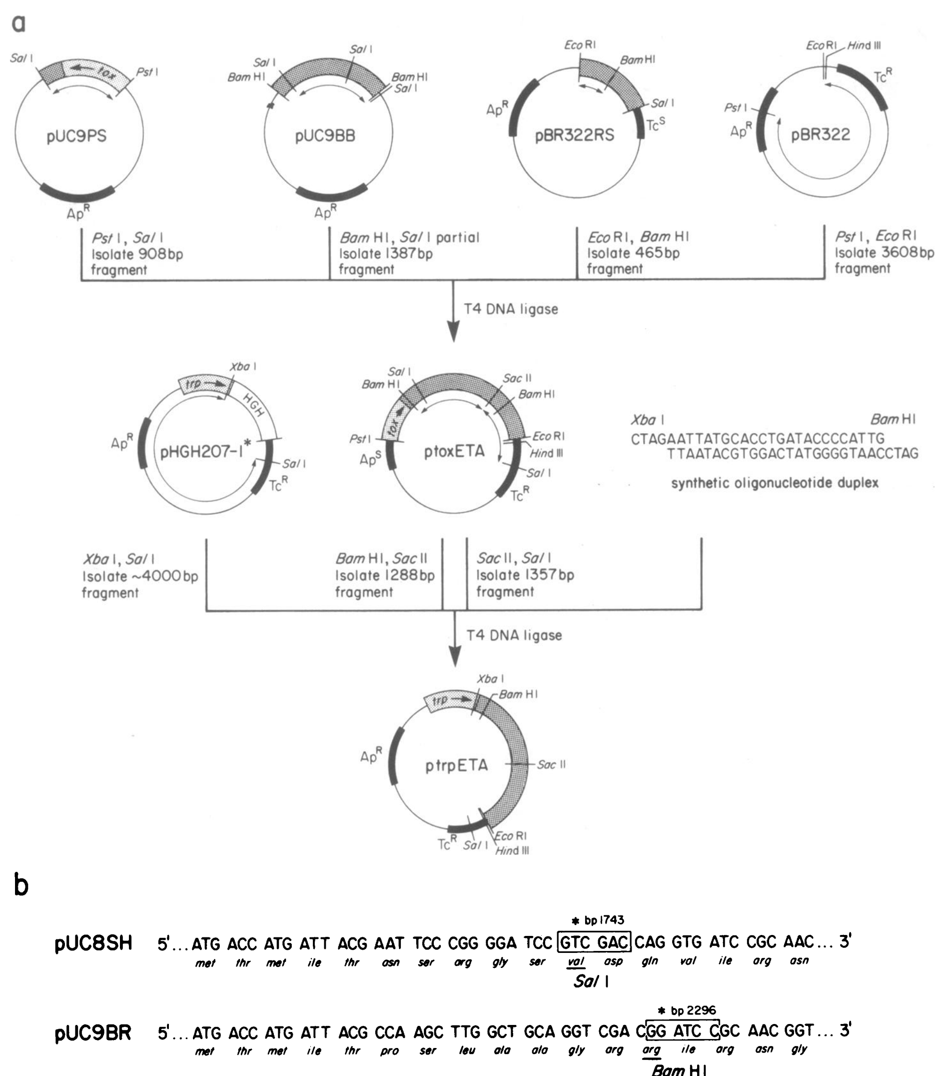

FIG. 2. Construction of ETA expression vectors. (*a*) Vectors for expression of the complete *ETA* gene. ETA coding sequences are represented by solid boxes; open box shows the human growth hormone (*HGH*) gene; promoters and the direction of transcription are shown in stippled boxes. Double arrows indicate fragments isolated. The oligonucleotides (synthesized by the phosphotriester method; ref. 15) were annealed by heating at 95°C for 5 min and then by cooling at room temperature. They were not 5′-phosphorylated. (*b*) DNA and NH_2-terminal amino acid sequences of *lacZ* gene fusions in vectors for expression of COOH-terminal ETA polypeptides. Asterisks indicate the beginning of ETA-derived DNA. The amino acid beginning the ETA region of each polypeptide is underlined. Boxes indicate DNA sequences of restriction endonuclease cleavage sites at which *lacZ* DNA and ETA DNA were fused.

For expression of COOH-terminal regions of ETA, two vectors were constructed: (*i*) A *Sal*I to *Hin*dIII fragment (containing ETA bp 1743–2760) from ptoxETA (Fig. 2*a*) was inserted into *Sal*I, *Hin*dIII-cleaved pUC8 to give pUC8SH. (*ii*) a *Bam*HI to *Eco*RI (containing ETA bp 2296–2760) fragment from pBR322RS (Figs. 1*a* and 2*a*) was inserted into *Eco*RI, *Bam*HI-cleaved pUC9 to give pUC9BR. The nucleotide and NH_2-terminal amino acid sequences of the resultant *LacZ–ETA* gene fusions are shown in Fig. 2*b*. *E. coli* JM83 cells transformed with these vectors were cultured and prepared for ETA-related polypeptide analysis by the ADP-ribosyl:EF-2 transferase assay as described above.

Localization of ETA-Related Polypeptides in *E. coli* 294/ptrpETA Cells. *E. coli* 294/ptrpETA cells grown overnight in LB medium (20 μg of ampicillin per ml and 1 mM isopropylthiogalactoside) were pelleted by centrifugation. Supernatants were saved and cells were fractionated by the osmotic-shock procedure (24). Fractions were analyzed enzymatically for β-galactosidase (25) and β-lactamase (11) and both enzymatically and by immunoblot analysis for ETA. In a separate experiment, cells were sonically disrupted (10 bursts of 2 sec) and separated into membrane and nonmembrane fractions by high-speed centrifugation as described (26). These fractions were similarly analyzed except that immunoblot analysis was omitted.

RESULTS

The Nucleotide Sequence of ETA. The DNA sequence of the cloned *ETA* gene and the deduced primary structure of the protein encoded by this gene are shown in Fig. 1*b*. The first 28 amino acids of the deduced mature coding sequence (positions +1 through +28) correspond completely to the NH_2-terminal sequence as determined by amino acid sequence analysis of the purified secreted protein. The ATG, which is inferred to be the initiation codon, is located 75 bp upstream of the mature coding sequence and begins the code for a larger precursor containing a highly hydrophobic sequence of 25 amino acids (positions −25 through −1), presumed to be the ETA signal peptide. In common with other prepeptides in prokaryotes (27), this signal peptide has basic residues close to its NH_2-terminus: histidines at positions −24 and −20.

In agreement with previous studies, the deduced mature primary structure contains eight cysteines, all of which are

known to participate in intrachain disulfide bonds (3). Complete chemical cleavage of ETA at cysteines or methionines results in the generation of enzymatically active fragments of M_r ≈30,000 and 50,000, respectively (3). The former fragment probably occurs by cleavage at the most COOH-terminal cysteine at position 379, thus generating a COOH-terminal fragment of 234 amino acids (M_r, 24,880), whereas the latter fragment must have been generated by scission at the most COOH-terminal methionine at position 176, thus generating a COOH-terminal fragment of 437 amino acids (M_r, 47,574). These results imply that the ADP-ribosyltransferase catalytic center of ETA resides in a COOH-terminal region of the molecule, in contrast to an NH_2-terminal placement in the functionally similar DT.

Expression of Full Length *ETA* in *E. coli*. To further confirm the identity of this DNA sequence as the *ETA* gene, the DNA was engineered for expression in *E. coli*. Cells containing plasmid ptoxETA (Fig. 2*a*), designed to express *ETA* under the control of its own (postulated) promoter, produced no ETA as determined by its enzyme activity (Table 1) or immunoblot analysis (data not shown). In *P. aeruginosa*, iron limitation is required for ETA production. However, the use of low iron medium (6) did not stimulate ETA synthesis in the *E. coli*/ptoxETA cells. Thus we constructed ptrpETA (Fig. 2*a*), designed to express *ETA* under control of the *E. coli trp* promoter. Cells transformed with this plasmid produced high levels of cell-associated ETA enzyme activity (Table 1) with essentially no detectable activity in the culture supernatant. A single ETA-related band with an apparent molecular weight slightly greater than that of ETA purified from *P. aeruginosa* PA103 supernatants was detected (Fig. 3, lane 2). This band probably represents unprocessed ETA precursor. The cellular localization of the ETA was probed by analysis of fractions obtained by the osmotic-shock method. The presence of the majority of this protein in the shocked cell fraction suggests that most of it resides primarily, if not exclusively, in a nonperiplasmic location (Table 2; Fig. 3, lanes 7 and 10). The segregation of most of the β-lactamase into the osmotic-shock fluid, whereas the β-galactosidase remained in shocked cells, indicates the effectiveness of the osmotic-shock procedure. Sonically disrupted cells separated into membrane and nonmembrane fractions were found to contain most of the ETA enzyme activity in the nonmembrane fraction. Together these data indicate a cytoplasmic location for most of the ETA protein produced in 294/ptrpETA cells, although they do not rule out a loose membrane attachment.

Expression of COOH-Terminal Toxin Fragments. As described above, comparison of the deduced ETA primary structure with ETA chemical-cleavage results allowed a tentative assignment of the catalytic center to the COOH-terminal half of the molecule. To verify this, we constructed pUC8 or pUC9 expression vectors in which 3′ fragments of the ETA-coding DNA sequence were fused with the DNA encoding the NH_2-terminus of the *lacZ* gene contained in the pUC plasmids (12) (Fig. 2*b*). JM83/pUC8SH cells containing a gene fusion involving the 306 COOH-terminal ETA amino acids (M_r, 32,714) produced high levels of enzymatically active ETA-related protein (Table 1). JM83/pUC9BR cells containing a gene fusion involving a smaller 122 amino acid COOH-terminal fragment (M_r, 13,075) also produced an enzymatically active but highly unstable ETA-related protein.

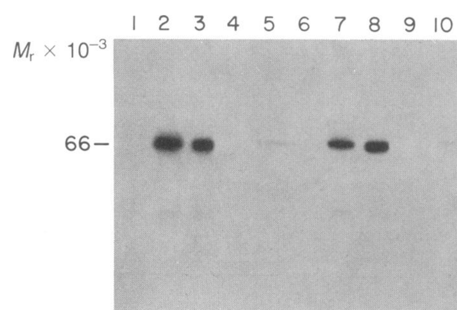

FIG. 3. Immunological detection and size determination of ETA-related polypeptides produced by *E. coli* 294. Culture supernatants, whole-cell extracts, and cell fractions obtained by the osmotic-shock method were analyzed. Proteins were separated on a $NaDodSO_4$/10% polyacrylamide gel and then transferred to a nitrocellulose sheet. The sheet was treated with purified rabbit antibodies to ETA. Antigen–antibody complexes were detected with ^{125}I-labeled protein A and autoradiography. Each lane was loaded with a protein sample prepared from 0.3 ml of culture ($A_{550\ nm}$ = 1.0). Lanes: 1, 294/pBR322 cell extract; 2, 294/ptrpETA cell extract; 3, 0.5 μg of ETA standard; 4, 294/pBR322 supernatant; 5, 294/ptrpETA supernatant; 6, 294/pBR322 shocked-cell extract; 7, 294/ptrpETA shocked-cell supernatant; 8, 0.5 μg of ETA standard; 9, 294/pBR322 osmotic-shock fluid; 10, 294/ptrpETA osmotic-shock fluid.

DISCUSSION

The deduced molecular size of the mature portion of ETA (M_r, 66,583) agrees closely with published values, which range from 66,000 to 70,500. The agreement of the deduced amino acid composition with that determined biochemically (2) (see Table 3 for comparison) strongly supports the identity of the DNA segment as the *ETA* gene. Furthermore, expression of the gene in *E. coli* using the *trp* promoter results in the production of a protein that cross-reacts with purified antibodies to ETA and has high levels of ADP-ribosyl: EF-2 transferase activity.

Table 1. ADPR:EF2 transferase activity of ETA-related polypeptides in *E. coli* 294, *E. coli* JM83, and *P. aeruginosa* PA103 cell extracts and culture supernatants

	Activity, cpm*	
Strain/plasmid	Cell extract	Culture supernatant
294/pBR322	10	10
294/ptoxETA	20	10
294/ptrpETA	1930	30
JM83/pUC9	68	ND
JM83/pUC8SH	3610	ND
JM83/pUC9BR	1843	ND
PA103†	512	4400

ND, not determined.
*Results corrected to reflect the ETA production in either fraction by a constant number of cells.
†PA103 cells grown in DTSB medium (6).

Table 2. Localization of ETA-related polypeptides in *E. coli* 294/trpETA cells

Fraction	% β-galactosidase	% β-lactamase	% ETA
	Experiment 1		
Culture supernatant	4.1	9.8	0.8
Cell wash	0.0	3.1	1.6
Osmotic-shock fluid	1.3	87.0	21.6
Shocked cells	94.6	0.0	76.1
	Experiment 2		
Cytoplasm	83.3	26.7	76.5
Total membranes	16.7	73.3	23.5

In experiment 1, stationary-phase cells were fractionated by the osmotic-shock method (24). In experiment 2, cells were disrupted by sonication and fractionated by high-speed centrifugation (26). Each enzyme activity is expressed as a percentage of the total in the culture.

Table 3. Comparison of the amino acid composition deduced from the putative mature ETA codons with that measured by analysis of an ETA hydrolysate (2)

Residue	Measured	Deduced	Residue	Measured	Deduced
Asp/Asn	57	57	Met	5	6
Ser	39	37	Ile	24	26
Glu/Gln	68	70	Leu	67	67
Pro	38	38	Tyr	18	18
Gly	56	56	Phe	14	14
Ala	67	67	Lys	15	15
Cys	7	8	His	14	15
Val	33	37	Arg	45	44
Thr	28	27	Trp	11	11

Prokaryotic signal peptides always contain one or more basic residues near their NH_2-termini, including at least one arginine or lysine (27). The presence of only histidines in the ETA leader peptide is therefore unprecedented and leads us to speculate that this weakly basic amino acid may be an important feature of signal peptides of proteins destined for extracellular transport in *Pseudomonas*.

ETA expression in *E. coli* contrasts strongly with that in the natural host: most of the material remains unprocessed and cell-associated, apparently residing in the cytoplasm. These results differ somewhat with the findings of Lory and Tai (28), who studied the expression in *E. coli* of the gene for phospholipase C, another extracellular enzyme of *P. aeruginosa*. They found that this enzyme was outer-membrane associated but did not report whether it was processed from its precursor. We note that the observed presence of ETA in the nonmembrane fraction of *E. coli* cells does not rule out a loose attachment to the outer membrane that was disrupted by the sonication step of our fractionation method. The failure of *E. coli* to secrete *P. aeruginosa* extracellular proteins into the medium indicates that all the components of the required mechanism may not be present in this species.

The absence of any extended open reading frame in the 745 bp 5′-untranslated sequence and the presence of a sequence resembling a *rho*-independent terminator (29) just downstream of the termination codon (underlined in Fig. 1*b*) indicate that *ETA*, like many other genes of *P. aeruginosa*, is translated from a monocistronic message. Although the 5′-untranslated sequence contains a typical Shine–Dalgarno region, A-G-G-A-G (29), just upstream of the initiation codon, the best fitting −10 and −35 promoter sequences (overlined in Fig. 1*b*) correspond rather poorly to those of *E. coli*. This may partially explain why *E. coli* does not produce ETA under the control of the ETA promoter. As expected, codon usage in the *ETA* gene is strongly reflective of the high G+C content of the DNA (68.5%). Except for glutamic acid and histidine codons, adenine or thymine occur rarely in the degenerate position (5.4%).

The observation that urea-solubilized ETA is maximally activated after reduction of at least two of its four disulfide bonds led Lory and Collier (3) to propose that these bonds were involved in shielding the catalytic center in the proenzyme from its substrates. The activity of the putative ETA precursor expressed in 294/ptrpETA cells is stimulated by denaturation but not by reduction (data not shown). This result may reflect its reduced state in the highly reducing environment of the bacterial cytoplasm (30).

The COOH-terminal occurrence in ETA of the ADP-ribosyl:EF-2 transferase center contrasts with an NH_2-terminal location in DT. This result indicates that amino acid sequence homologies between ETA and DT, if any, are likely to occur between misaligned regions. Thus we searched for such homologies by comparing consecutive 20 amino acid residue stretches of the ETA sequences with all of the DT sequences, using a computer program derived from a published algorithm (ref. 31). No obvious homologies were found, even between the COOH-terminal region of ETA and the NH_2-terminal region of DT, which contain the catalytic centers. Similarly, comparison of consecutive 60-nucleotide stretches of the ETA coding region with all of the DT coding sequences revealed no significant homologies. Furthermore, ETA DNA does not hybridize to DNA derived from the corynebacteriophage β even under conditions of low stringency (unpublished observations). These results imply that the functional similarities between DT and ETA are not due to the evolution of any of their DNA sequences from the same ancestral gene.

We thank Kim Dinkelspiel and Peter Ng for preparation of synthetic oligonucleotides, and Jeanne Arch and Alane Gray for assistance in manuscript preparation. This study was supported in part by Public Health Service Grant AI15940 from the National Institute of Allergy and Infectious Diseases to M.L.V.

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