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Role of domain II of *Pseudomonas* exotoxin in the secretion of proteins into the periplasm and medium by *Escherichia coli*

(transforming growth factor α/signal sequence/alkaline phosphatase)

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ABSTRACT **_Pseudomonas_ exotoxin (PE) is composed of structural domains I, II, and III; when interacting with mammalian cells the function of domain I is cell recognition, the function of domain II is membrane translocation, and domain III functions in ADP ribosylation. PE is secreted by _Pseudomonas aeruginosa_ into its growth medium. The domain responsible for secretion has been examined by expressing modified PE genes in _Escherichia coli_ under the control of a T7 promoter. Without a signal sequence, PE accumulates within the cell, but PE is secreted into the periplasm when part or all of domain I is removed. PE appears in the periplasm and medium when domain I and part of domain II are removed. Domain II alone is secreted into the periplasm, whereas domain III alone remains within the cell. Addition of an OmpA signal sequence results in secretion of mature PE into the periplasm and secretion of domains II–III into the medium. A protein composed of transforming growth factor α fused to the amino terminus of domains II–III is secreted into the periplasm without a signal sequence and into the medium with a signal sequence. A protein composed of domain(s) II or II–III fused to the amino terminus of alkaline phosphatase is secreted into the periplasm and the medium with or without a signal sequence. We conclude that domain II contains important information for protein secretion.**

Proteins that are secreted into the periplasm of *Escherichia coli* or inserted into *E. coli* membranes have several specific features. Nearly all proteins destined to be exported have a signal sequence important for translocation (1–3). In addition, membrane or secreted proteins have structural features within the molecules that facilitate secretion or membrane insertion (4).

We have been constructing chimeric proteins in which portions of the *Pseudomonas* exotoxin (PE) gene are fused to growth factors, such as transforming growth factor type α (TGF-α) or interleukin 2, that direct the toxin to receptors on target cells (5, 6). The chimeric genes are expressed in *E. coli*, and the chimeric proteins are purified from the bacterial cells. X-ray crystallography of native PE showed that the molecule is composed of three distinct structural domains (7). To analyze domain function, various portions of the PE gene have been deleted and expressed in *E. coli* (8). Study of the expressed mutant proteins revealed that the amino-terminal domain I is responsible for receptor binding, middle domain II is responsible for translocation of the toxin across membranes, and the carboxyl-terminal domain III contains the ADP-ribosylating activity (Fig. 1). We now report that certain modified forms of PE are secreted into the periplasmic space and into the medium. Our data indicate that domain II of PE plays an essential role in secretion of toxin into the periplasm and medium and that domain II can also promote secretion of other molecules fused to it.

MATERIALS AND METHODS

Plasmid Construction (Fig. 1). (*i*) The pVC plasmids were created from the pJH series (8) by restricting them with enzymes *Sph* I and *Tth*111I. *pVC19:* pVC4 was cut with *Hin*dIII and *Tth*111I, treated with S1 nuclease, and the large fragment was ligated to itself. *pVC20:* pVC4 was cut with *Hin*dIII, treated with Klenow fragment of DNA polymerase I, and cut with *Pst* I to yield a 1.6-kilobase (kb) fragment. Separately, pVC4 was cut with *Bgl* II, treated with S1 nuclease, and then treated with *Pst* I. A 2.1-kb fragment was isolated and ligated to the 1.6-kb fragment to produce pVC20. *pVC85:* pVC8 (5) was treated sequentially with *Nde* I, S1 nuclease, and *Xba* I, and a 3.6-kb fragment was isolated. pINIII OmpA1 (11) was treated sequentially with *Eco*RI, Klenow fragment, and *Xba* I, and a 92-base pair (bp) fragment, carrying an OmpA Shine–Dalgarno region and an OmpA signal sequence, was isolated. The 3.6-kb fragment and the 92-bp fragment were ligated to produce pVC85. Other plasmids with OmpA signal sequences were created similarly.

(*ii*) The *phoA*-containing plasmids were constructed using a *Pst* I insert that contains the *phoA* gene (12). pVC229 was constructed by treating pVC8 with *Sst* II followed by T4 DNA polymerase to yield a 3.65-kb fragment that was dephosphorylated. pCH39 was treated with *Pst* I and T4 polymerase. A 3-kb fragment was isolated and ligated to the 3.65-kb fragment, and the recombinants were screened for proper orientation. pVC2295 was constructed from pVC85 similarly. pVC809 was constructed by treating pVC8 with *Ppu*MI followed by Klenow fragment to yield a 3.65-kb fragment. pCH2 was treated with *Pst* I and T4 polymerase to produce a 3.0-kb fragment. The two fragments were joined, and recombinants were checked for proper *phoA* orientation. pVC8095 was made from pVC85 similarly.

Construction of pXY382 and pXY3825 will be described elsewhere (unpublished work). These plasmids contain a TGF-α cDNA fused to the 5′ end of DNA for domains II and III of PE. pXY3825 also carries an OmpA signal sequence. In all plasmids, genes are linked to a phage T7 late promoter.

(*iii*) Other plasmids and bacterial strains were previously described (5).

Expression and Localization of Recombinant Proteins. *E. coli* BL21(λDE3) cells carrying the appropriate plasmid were grown and induced with isopropyl β-D-thiogalactoside as described (5, 9). After induction, the culture (30 ml) was centrifuged at 3000 rpm for 10 min. To obtain periplasmic and spheroplast fractions (13), the cell pellet was suspended

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Abbreviations: PE, *Pseudomonas* exotoxin; PE_{40}, PE derivative containing domains II and III of PE; TGF-α, transforming growth factor type α; TGF-α–PE_{40}, a protein composed of TGF-α and domains II and III of PE; *phoA*, gene for alkaline phosphatase.

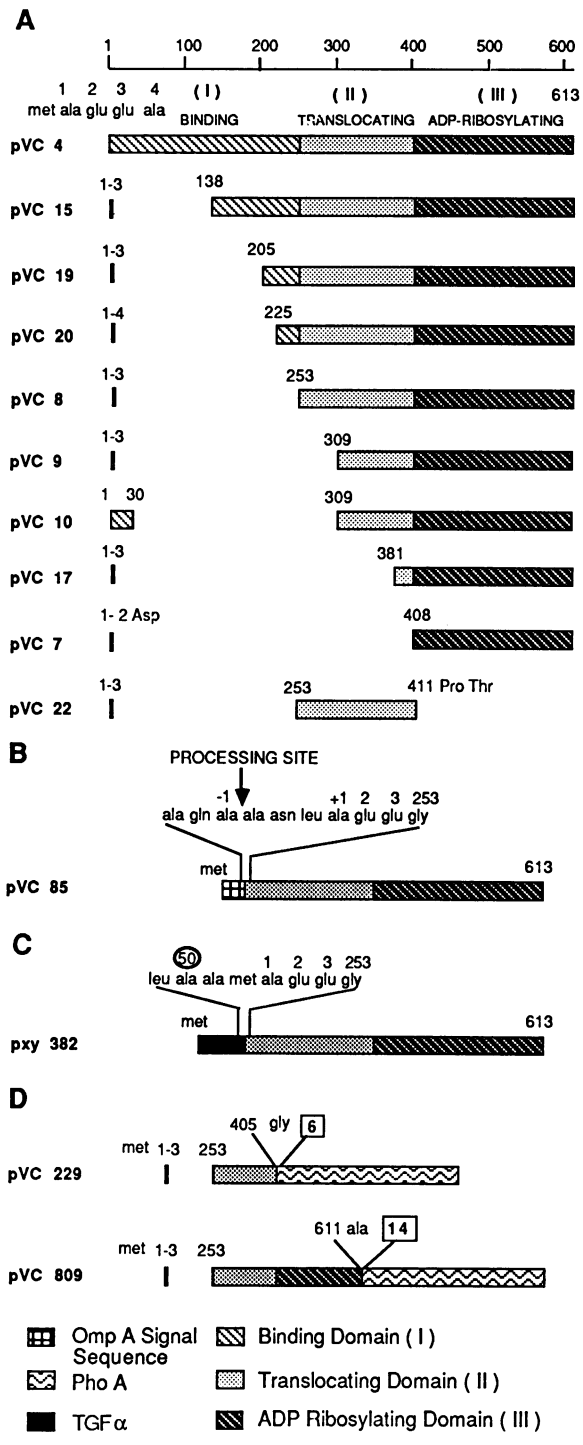

FIG. 1. Representation of various PE derivatives and fusion proteins. (*A*) Deletion mutants are shown, and numbers indicate amino acids present in native PE. A few extra amino acids were created during cloning. Genes of all PE derivatives are under control of a phage T7 late promoter and contain a T7 ribosome binding site (9). (*B*) Example of protein encoded by a plasmid that contains an OmpA Shine–Dalgarno region and a signal sequence. The signal sequence is processed away, leaving a tripeptide (Ala-Asn-Leu) in place of methionine. The number 5 at end of plasmid numbers indicates OmpA signal sequence. (*C*) Plasmid pXY382 contains TGF-α cDNA fused to the 5′ terminus of PE domains II and III. Fifty refers to the last amino acid of TGF-α. (*D*) pVC229 and 809 contain *phoA* genes fused to the 3′ terminus of the gene for domains II or II and III (PE_{40}). Unboxed numbers indicate PE amino acids; boxed numbers (6, 10) indicate *phoA*-encoded amino acids. Glycine and alanine were added during cloning. pVC2295 and pVC8095 are similar to pVC229 and pVC809, except for an OmpA signal sequence.

in 1.5 ml of sucrose solution (20% (wt/wt) sucrose/30 mM Tris, pH 7.4/1 mM EDTA), kept on ice for 10 min, and aliquots were removed for assay. The remainder was centrifuged at 6000 rpm, and the supernatant (sucrose wash) was discarded. The pellet was suspended in 1.4 ml of cold water, kept on ice for 10 min, and centrifuged at 8000 rpm. The supernatant is the periplasmic fraction. The pellet (spheroplasts) was suspended in 1.4 ml of TE buffer (50 mM Tris, pH 8.0/1 mM EDTA).

Analyses. To measure ADP ribosylation, samples were diluted in 4 volumes of extraction buffer (7 M guanidine hydrochloride/100 mM Tris, pH 7.0/5 mM EDTA), kept on ice for 1 hr with intermittent mixing, and centrifuged. An aliquot was diluted in TE buffer containing 0.1% bovine serum albumin and used for ADP-ribosylation assays. Samples were incubated at 23°C for 15 min with wheat germ extracts and 2.4 μM [^{14}C]NAD. Some samples were treated with urea and dithiothreitol (8). β-Galactosidase and alkaline phosphatase were measured as described (10, 14). Gel electrophoresis (PAGE) and immunoblotting were previously described (5, 15). Enzymes and chemicals were obtained from standard sources.

RESULTS

Localization of PE Molecules with Various Portions Deleted (Table 1). pVC4 encodes the entire PE protein with a methionine placed before the alanine at position 1. When PE gene is expressed in *E. coli*, the toxin has been found to accumulate within the cell (8). Table 1 shows that >98% of this molecule is found associated with spheroplasts. Proteins with deletions of progressive amounts of domain I encoded by pVC15, pVC19, and pVC20 were found mostly associated with spheroplasts, but significant amounts appeared in the periplasm (Table 1). When all domain I was deleted (pVC8), 98% of the synthesized PE was secreted into the periplasm (Table 1, Fig. 2*A*). The protein produced by pVC8 has a M_r of 40,000 and hence is referred to as PE_{40}. Deletion of increasing portions of domain II, as in pVC9, pVC10, and pVC17, caused about 9% of the protein to appear in the medium—with the rest appearing mostly in the periplasm. When all domain II was deleted (pVC7), the resulting protein, which consists of only domain III, remained within the cell (Table 1). Because pVC17 contains only 27 amino acids not present in pVC7, these amino acids must be important for secretion. When domain II was expressed alone (pVC22), the protein was found in the periplasm (Table 1, Fig. 2*D*). These results show that sequences present in domain I apparently inhibit the secretion promoted by domain II. Removal of all domain I allows large amounts of the toxin to be secreted into the periplasm, and removal of domain I and of portions of domain II causes some secretion into the medium.

Effect of OmpA Protein Signal Sequence on Secretion (Table 2). Because signal sequences present in proteins of Gram-negative bacteria have been found to promote secretion of proteins into the periplasmic space or insertion of proteins into cell membranes, we examined the effect of an OmpA signal sequence on cellular distribution of various molecules derived from PE. Table 2 shows that the OmpA signal sequence in pVC45 promoted the secretion of full-length PE into the periplasm, but not into the medium. A similar result was recently reported by Douglas *et al.* (16). The OmpA signal sequence apparently overcomes the inhibitory action of domain I.

OmpA signal sequence had little effect on secretion of molecules in which various portions of domain I were deleted and which were already being secreted into the periplasm—e.g., pVC155 and pVC205 with deletion of amino acids 4–137 or 5–224, respectively. But the OmpA

Table 1. Cellular localization of PE molecules with deletion of portions of domains I and II

Plasmid	Amino acid sequence	Domain	Medium, %	Periplasm, %	Spheroplast, %
pVC4	Met, 1–613	I II III	<1	1 (0.6)	99 (6.7)
pVC15	Met, 1–3, 138–613	I* II III	<1	14 (3.2)	86 (20.0)
pVC19	Met, 1–3, 205–613	I* II III	<1	8 (2.0)	92 (24.0)
pVC20	Met, 1–4, 225–613	I* II III	<1	28 (2.8)	72 (23.0)
pVC8	Met, 1–3, 253–613	II III	<1	98 (5.0)	2 (0.6)
pVC9	Met, 1–3, 309–613	II* III	9 (3.3)‡	79 (28.0)	12 (4.0)
pVC10	Met, 1–30, 309–613	II* III	9 (3.3)	78 (30.0)	13 (5.2)
pVC17	Met, 1–3, 381–613	II* III	9 (3.0)	90 (31.6)	1 (0.6)
pVC7	Met, 1–3, Asp, 408–613	III	<1	<5†	>95†
pVC22	Met, 1–3, 253–411, Pro, Thr	II	<1	>95†	<5†

*A portion of domain is deleted.
†Based on immunologic blot analysis.
‡Values in parentheses are mg of toxin per liter of culture based on measurements of ADP-ribosylation activity.

signal sequence did cause secretion into the medium of a molecule containing only domains II and III (Table 2, Fig. 2*B*). Addition of OmpA signal sequence also resulted in the secretion of domain II by itself into the medium (Fig. 2*E*). Thus, when an OmpA signal sequence precedes a protein otherwise composed of only domain II or of domains II and III, that protein is secreted into the medium. The PE molecules in the periplasm and in the medium were slightly smaller than the molecules in the spheroplasts, indicating removal of the signal sequence (Fig. 2 *B* and *E*). We have purified PE_{40} from the medium of cells producing it and found the amino-terminal sequence to be Ala-Asn-Leu-Ala, indicating correct processing of the signal sequence (23). We also saw that the protein produced by pVC105, which lacks all domain I and some domain II, was also secreted into the medium when the OmpA signal sequence was present (Table 2). To control for cell lysis, β-galactosidase was measured in all experiments, and <1% of its total activity was found in the medium, indicating that lysis had not occurred.

Secretion of Chimeric Protein TGF-α–PE_{40} (Table 3). We previously reported on a chimeric protein made by fusing a cDNA encoding TGF-α to the 3′ terminus of a PE gene encoding domains II and III (5). The resulting protein, PE_{40}–TGF-α, accumulated within *E. coli* and could be purified from *E. coli* extracts. We now have placed a cDNA encoding TGF-α at the 5′ terminus of a gene encoding domains II and III to produce a TGF-α–PE_{40} fusion protein. This molecule is ≈10-fold more active in killing cells bearing epidermal growth factor receptors than was PE_{40}–TGF-α (unpublished work). Without OmpA signal sequence, TGF-α–PE_{40} was found mainly within the cell, but ≈25% was found in the periplasm (Table 3), and a small but significant amount was found in the medium. Addition of OmpA signal sequence resulted in large amounts of TGF-α–PE_{40} appearing in the medium, as well as in the periplasm. TGF-α–PE_{40} molecules in the medium and periplasm are slightly smaller than TGF-α–PE_{40} molecules found within the cell, indicating that processing has occurred (Fig. 2*G*). Thus, sequences found within domain II of PE promote secretion of TGF-α–PE_{40} into the periplasm, with some protein appearing in the medium, and the further addition of a signal sequence, such as that of OmpA, results in increased secretion of TGF-α–PE_{40} into the medium.

***phoA* Fusions Are Secreted (Table 4).** Because a molecule containing only domain III was not secreted (Table 1), important information for secretion seemed to be located within domain II of PE. Therefore, DNA sequences encoding domain II alone or domains II and III were fused to the *phoA* structural gene encoding alkaline phosphatase in *E. coli*. Alkaline phosphatase was chosen because it is secreted into the periplasm only when attached to a signal sequence (17). OmpA signal sequences were included in some constructions (Table 4). Appearance of fusion proteins in the periplasm and medium was measured in several ways. Aliquots were subjected to PAGE and transferred to nitrocellulose, and the proteins were located by immunoblotting using an antibody to PE. All fusion proteins had the expected size (for example, Fig. 2 *H* and *I*). Protein amounts were estimated from the intensity of the antibody reaction and were confirmed by measurements of ADP-ribosylating or alkaline phosphatase activity. Alkaline phosphatase activity is only exhibited by those proteins secreted out of the cellular interior (17). *E. coli* BL21 contains a normal alkaline phosphatase gene that is not expressed because the cells are grown in LB broth containing high phosphate levels.

When alkaline phosphatase gene was placed at the carboxyl terminus of domains II and III (PE_{40}) or of domain II alone (Fig. 1*D*), ≈8% of the fusion protein appeared in the medium (Table 4). Because domain II alone is not secreted, we conclude that the alkaline phosphatase sequences are not inert, but promote secretion of the fusion protein. Addition of OmpA signal sequence at the amino terminus of the

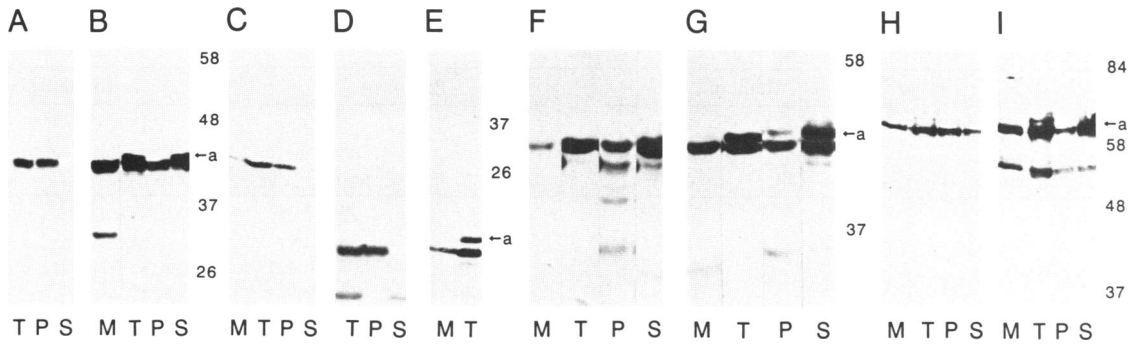

FIG. 2. Cellular localization of PE derivatives by immunoblotting. An aliquot of total cell (T), periplasm (P), spheroplast (S), and medium (M) was electrophoresed on 10% (*A*, *B*, *F–I*) or 12.5% (*C–E*) gels and immunoblotted with antibodies to PE. (*A*) pVC8, (*B*) pVC85, (*C*) pVC17, (*D*) pVC22, (*E*), pVC225, (*F*) pXY382, (*G*) pXY3825, (*H*) pVC229, (*I*) pVC2295 (for structures, see Fig. 1). a, Unprocessed protein.

Table 2. Effects of OmpA signal sequence on secretion of PE molecules into periplasm and medium

Plasmid	Amino acid sequence	Domain	Medium, %	Periplasm, %	Spheroplast, %
pVC45	L*-1-613	I II III	2 (0.1)†	84 (3.0)	14 (0.5)
pVC155	L-1-3, 138-613	I† II III	3 (0.6)	12 (1.5)	85 (11.0)
pVC205	L-1-4, 225-613	I† II III	2 (0.7)	61 (23.0)	37 (14.0)
pVC85	L-1-3, 253-613	II III	13 (7.1)	45 (25.0)	42 (23.0)
pVC105	L-1-30, 308-613	I† II‡ III	22 (10.0)	64 (30.0)	14 (6.5)

*L represents the OmpA signal sequence that is removed upon processing and leaves an amino-terminal sequence of Ala-Asn-Leu (see also Fig. 1*B*).
†Values in parentheses are in mg per liter of culture determined by measuring ADP-ribosylation activity.
‡Only a portion of that domain is present.

protein did not result in any further increase in secretion into the medium of a fusion protein with only domain II, but did increase somewhat the secretion of molecules containing both domains II and III.

Effect of OmpA Protein Signal Sequence on Level of Expression. Beginning with the T7 expression plasmid (9), an ATG codon was placed in front of various PE molecules (Fig. 1 and Table 1). When the plasmids containing OmpA signal sequence were constructed, we used a DNA fragment containing both the OmpA Shine–Dalgarno region and signal sequence and fused it to various PE molecules. Some of these plasmids consistently made more recombinant protein than those that did not contain these additions (Tables 1–3), and possibly the production level affects the distribution of molecules found in different cellular compartments. Several reasons might explain enhanced expression of these plasmids: (*i*) OmpA has a more efficient Shine–Dalgarno sequence. (*ii*) Translation initiation codons in the OmpA signal sequence are enriched in adenine and thymine, and the newly formed RNA can more readily dissociate from DNA template to associate with ribosomes. That replacement of basic amino acids of the signal sequence of the outer membrane lipoprotein with acidic residues at the amino terminus caused a 2- to 5-fold reduction in lipoprotein synthesis (18, 19) is interesting in this context. In PE an acidic amino terminus was changed to a basic terminus by the addition of a signal sequence (Fig. 1).

Table 3. Cellular localization of TGF-α–PE_{40} with and without OmpA protein signal sequence

Plasmid	Domain*	Location, %		
		Medium	Periplasm	Spheroplast
pXY382	TGF-α–II–III	4	25	70
pXY3825	L–TGF-α–II–III	33	27	40

Distribution was determined by ADP-ribosylation activity. L, OmpA protein signal sequence.
*The TGF-α gene has been fused at the 5′ terminus of domains II and III (see also Fig. 1*C*).

DISCUSSION

We have shown that molecules containing domain II of PE are secreted into the periplasm of *E. coli* and, under certain conditions, are also secreted into the medium. We initially studied a molecule termed PE_{40} that contains both domains II and III of PE, and we found it to be secreted into the periplasm when lacking an OmpA signal sequence and to be secreted into the medium when the signal sequence was added; this result suggested that sequences within domain II in concert with a signal sequence promoted secretion into the medium. However, this hypothesis was proven incorrect when deletion of all domain I of PE and part of domain II also generated molecules that were secreted into the medium and periplasm without a signal sequence. In addition, placement of alkaline phosphatase at the carboxyl terminus of a toxin molecule containing domain II alone or domains II and III also led to secretion into the medium.

Domain II contains many hydrophobic amino acids. The amino terminus of each recombinant PE protein made without an OmpA sequence was constructed to consist of Met-Ala-Glu-Glu. Thus, the amino terminus of our constructs differs from a conventional leader sequence, which must possess positively charged amino acids in the amino-terminal region (20, 21). Substitution of some random sequences for a signal sequence has been reported to lead to the secretion of invertase in yeast (22). Our results suggest that sequences within domain II of PE are important for secretion of molecules across *E. coli* membranes. When domain II is combined with a signal sequence, such as that found in OmpA, or with a protein that itself can be transported across a membrane (alkaline phosphatase), secretion into the periplasm and into the medium results. Furthermore, a protein such as TGF-α can be efficiently transported into the medium or periplasm when fused to domains II and III of PE.

The mechanism by which sequences present in domain II of PE promote secretion remains to be elucidated. Domain II is composed of a series of α-helices, and stretches of hydrophobic amino acids are present in these helices. Do-

Table 4. Cellular location of PE–alkaline phosphatase fusion proteins

Plasmid	Sequence	Medium		Periplasm		Spheroplast	
		ADP-ribosylating activity*, %	Alkaline phosphate activity†, %	ADP-ribosylating activity*, %	Alkaline phosphate activity†, %	ADP-ribosylating activity*, %	Alkaline phosphate activity†, %
pVC229	II-*phoA*	ND	8 (0.37)	ND	79 (3.3)	ND	13 (0.51)
pVC2295	L–II-*phoA*	ND	8 (3.13)	ND	33 (12.4)	ND	59 (22.0)
pVC809	II–III-*phoA*	9 (0.3)	8 (0.06)	57 (1.8)	64 (0.42)	34 (1.03)	28 (0.18)
pVC8095	L–II–III-*phoA*	14 (2.1)	15 (2.4)	21 (3.0)	55 (9.0)	65 (9.4)	30 (5.0)
pVC8	II–III	<1	ND	88 (4.2)	ND	12 (0.6)	ND
pVC85	L–II–III	20 (5.0)	ND	39 (9.4)	ND	41 (10.0)	ND

L, OmpA protein signal sequence. ND, not detectable.
*Values in parentheses are mg/liter of culture measured by ADP ribosylation.
†Values in parentheses are OD units at 420 nm/ml of culture.

main II is also required for efficient translocation of PE into the cytoplasm of animal cells during endocytosis. Discovering how domain II promotes secretion may clarify that biologically important process.

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