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Nucleotide Sequence of the Gene for Perfringolysin O (Theta-Toxin) from *Clostridium perfringens*: Significant Homology with the Genes for Streptolysin O and Pneumolysin

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The nucleotide sequence was determined for the gene encoding the thiol-activated cytolysin, perfringolysin O (theta-toxin), from *Clostridium perfringens*. The nucleotide-sequence-derived primary structure of perfringolysin O is 499 residues long and exhibits a 27-amino-acid signal peptide. The calculated molecular weight of the secreted (mature) form of perfringolysin O is 52,469. The deduced amino-terminal sequence of perfringolysin O is identical to that determined for purified perfringolysin O. Hydropathy analysis indicated that, except for the signal peptide, no major stretches of hydrophobic residues are present. Extensive amino acid sequence homology (65%) was detected with the low-molecular-weight form of streptolysin O, and a lesser amount (42%) was detected with pneumolysin. The nucleotide sequence of the perfringolysin O gene (*pfo*) exhibits approximately 60% homology with the streptolysin O gene (*slo*) and 48% homology with the pneumolysin gene (*ply*). All three toxins contain an identical region of 12 amino acids, which includes the essential cysteine of all three toxins. The location of these 12 residues was conserved in all three toxins when the primary sequences were aligned for maximum homology.

Clostridium perfringens produces a variety of diseases in both humans and animals and is known to produce a plethora of toxins. One of the toxins is perfringolysin O (theta-toxin), a thiol-activated cytolysin which resembles other thiol-activated cytolysins from *Streptococcus*, *Bacillus*, and *Listeria* species (4). Related cytolysins have also been identified in seven other clostridial species (for a review, see reference 20). Collectively, these cytolysins are of interest from the standpoints of evolution, their structure/function relationships, and their roles in the diseases caused by the bacterial species which produce them. Features common to these thiol-activated cytolysins are their ability to lyse cholesterol-containing membranes, reversible inactivation by oxidation, and the ability to bind cholesterol. Since membrane binding and lysis depend on the presence of cholesterol, cholesterol is generally accepted as the membrane receptor for these cytolysins. Although a considerable amount of data has been published concerning the mechanism of action of these cytolysins, comparatively little is known about their structure/function relationships. Of particular interest is the role of the essential thiol group in the toxic mechanism, since it appears that several of these toxins contain a single cysteine. Recently, the genes for streptolysin O (SLO) (10, 11), pneumolysin (16, 21), and perfringolysin O (20a) have been cloned and the first two have been sequenced. Homology (42%) was detected between the DNA-deduced primary structures of SLO and pneumolysin. Less homology was observed at the DNA level for *slo* and *ply*, suggesting that both genes have undergone considerable divergence.

In this report, we present the complete nucleotide sequence of the gene encoding perfringolysin O from *C. perfringens* and the analysis of the nucleotide sequence and deduced primary structure of perfringolysin O.

MATERIALS AND METHODS

Bacterial strains, plasmids, enzymes, and bacteriophage. Plasmid pRT1B, which contains the perfringolysin O gene within a 3.2-kilobase fragment (20a), was used as the source of cloned DNA. Bacteriophage M13mp19 (24) and its DNA were used for all subcloning experiments and as a source of single-stranded DNA for sequencing. *Escherichia coli* JM109 (24) was the host strain for all subcloning experiments. Unless otherwise noted, all enzymes were obtained from Bethesda Research Laboratories, Gaithersburg, Md. All cloning experiments were done in accordance with National Institutes of Health guidelines that place cloning experiments with the oxygen-labile hemolysins, which are related to SLO, under EK1/BL1 containment.

DNA sequencing methods. Plasmid pRT1B was digested with *Sph*I, *Bgl*II or *Hin*dIII, and *Sst*I; *Bgl*II and *Hin*dIII cut within the toxin gene, and *Sph*I and *Sst*I cleave on either side of the cloned fragment. The DNA fragments were treated with S1 nuclease and DNA polymerase to provide blunt ends. The fragments were subcloned into the *Hin*cII site of coliphage M13mp19. The DNA was transformed into *E. coli* JM109, and transformants were isolated which contained the fragments in both orientations.

Ordered deletions were prepared for DNA sequencing by the method of Henikoff (5). Briefly, the DNA of either subclone was digested first with *Sma*I and then with *Sst*I; these enzymes left a blunt end proximal to the perfringolysin O gene fragments and a 3′ overhang next to the sequencing primer site. Approximately 5 to 10 μg (total volume, 200 μl) of either DNA was digested with approximately 50 U of exonuclease III at 37°C. Samples (10 μl) were removed from the reaction mixture every 45 s, and the exonuclease digestion was stopped by the addition of EDTA to 10 mM. Each sample was then extracted with phenol and passed through a spun column of Sephadex G-50F (Pharmacia, Inc., Piscat-

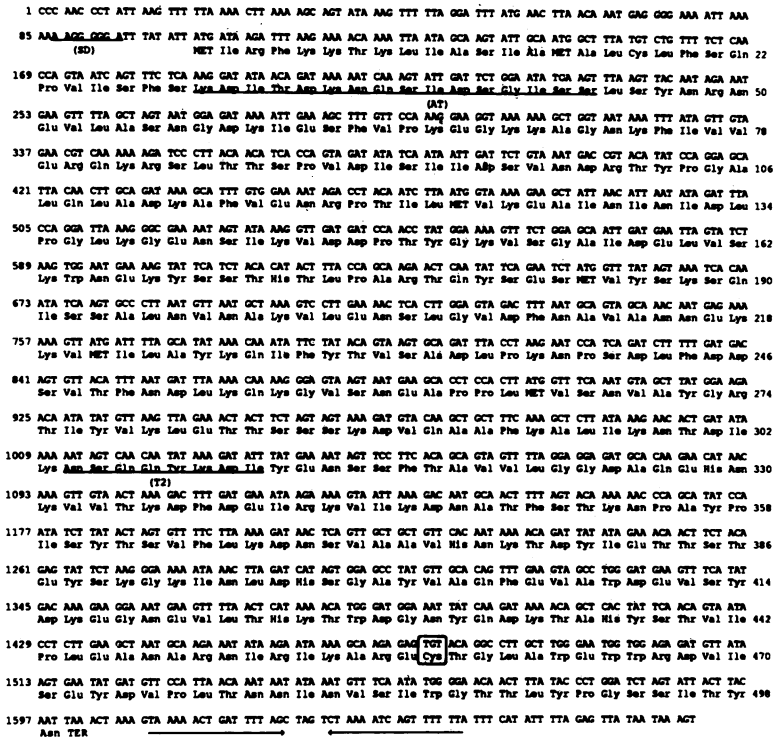

FIG. 1. Nucleotide sequence of the perfringolysin O gene (*pfo*). A putative ribosome-binding site (SD) is shown 9 bp upstream of the putative initiation codon at nucleotide 104. The amino-terminal sequence (AT) determined from the purified perfringolysin O is underlined. Homology between the deduced primary structure of perfringolysin O and a partial sequence of a tryptic peptide deduced by Ohno-Iwashita et al. (15) is shown (T2). Also shown are the position of the single essential cysteine (box) and a region of dyad symmetry (arrows).

away, N.J.) to remove buffer salts and residual phenol. The samples were treated with S1 nuclease (67 Vogt units per ml) to remove the long regions of 3′ overhang left by exonuclease III. The samples were treated with phenol and passed over spun columns of Sephadex G-50F. Each sample was treated with DNA polymerase (large fragment) and then ligated with DNA ligase for 3 to 6 h. Approximately one-fifth of the total reaction volume (50 μl) from each sample was transformed into competent cells of *E. coli* JM109, which yielded 1,000 to 2,000 transformants per transformation. From each transformation 5 to 10 plaques were picked and analyzed for insert size. The results indicate that exonuclease III removed about 200 nucleotides per minute. Clones were picked which contained fragments separated by about 150 to 200 base pairs (bp).

Sequencing of the ordered deletions was carried out by the method of Sanger et al. (18), as modified by Johnston-Dow et al. (8), using adenosine-5′-[α-^{35}S]thiotriphosphate (3,000 Ci/mmol; Dupont/New England Nuclear, Wilmington, Del.).

Determination of amino-terminal sequence of perfringolysin O. The first 17 amino acids of purified perfringolysin O were determined by sequence analysis on an Applied Biosystems (Foster City, Calif.) model 470A gas phase amino acid analyzer equipped with an on-line model 120A phenylthiohydantoin analyzer, in accordance with the instructions of the manufacturer.

RESULTS AND DISCUSSION

Nucleotide sequence of the perfringolysin O gene. The percent A+T of the perfringolysin O gene (*pfo*) sequence (Fig. 1) is 70%, which is consistent with that for clostridia in general (19). This is also reflected in the codon usage in the gene, which is heavily biased toward the use of A, and to a lesser extent T, in position 3 of the codons. Similar observations were reported by Garnier and Cole (3) for the *bcn* gene of *C. perfringens*. Located 8 bp upstream of the start site of the perfringolysin O coding region is a putative ribosome-binding site. This region is identical to the putative ribosome-binding site of the *celD* gene of *Clostridium thermocellum* (9). No clear consensus promoter sequences were detected in the 5′ region of the sequence preceding the coding region. A region of dyad symmetry is located 8 bp downstream of the termination codon for the perfringolysin O gene that can form a stem-and-loop structure with 15 bp in the stem and 5 bp in the loop. This corresponds to a rho-independent transcriptional terminator in *E. coli* (6) which is also present in the *bcn* gene of *C. perfringens* (3).

A comparison of the sequences of the *pfo*, *slo*, and *ply* genes (Fig. 2) showed that there is greater homology between *pfo* and *slo* than between *pfo* and *ply*. There is approximately 60% homology between *pfo* and *slo*; about 48% homology was found between *pfo* and *ply*. These results explain why Kehoe and Timmis (10) were unable to detect sequence homology at 80% stringency between *slo* and *pfo* when they used a *slo*-derived probe. The majority of the homology between *pfo* and *slo* is in the coding region for the secreted form of perfringolysin O and that for the low-molecular-weight form of SLO (1, 11). Interestingly, the beginning of the nucleotide homology between *pfo* and *slo* corresponds to the initiation codon of *pfo* and an out-of-phase ATG codon of *slo* at position 398 (11). A potential coding region for about 32 amino acids (not including the ATG codon) is present when the sequence from nucleotide

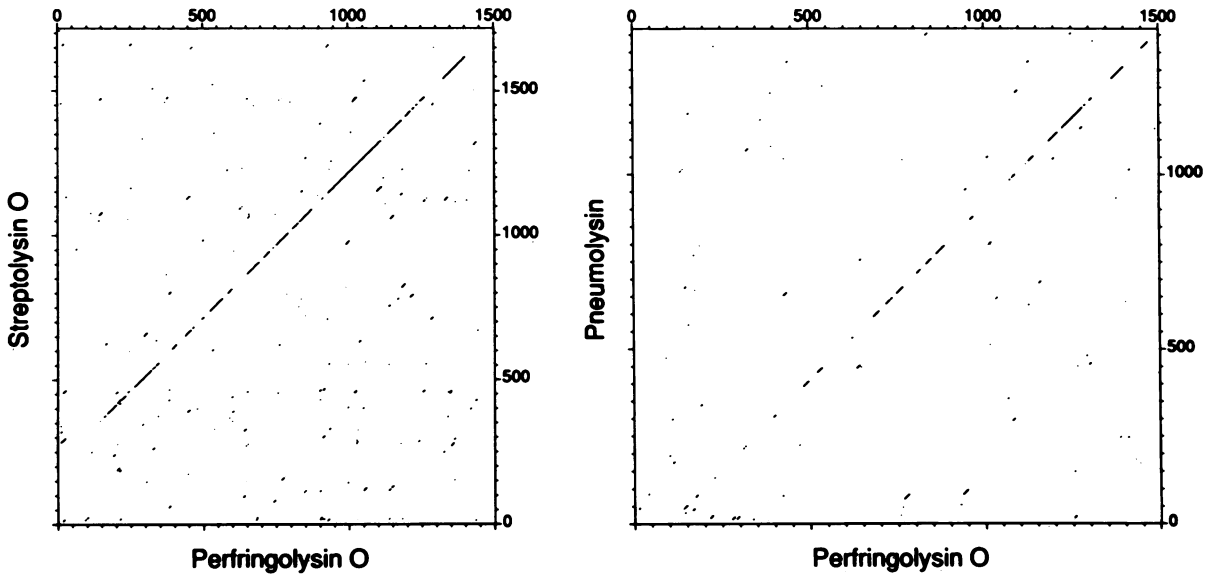

FIG. 2. Dot matrix homology analysis of the genes for perfringolysin O, SLO, and pneumolysin. Each dot represents 14 matched bases out of a total of 21 bp. Only the coding regions for each protein were used for the comparisons. The program described by Maizel and Lenk (13) was used for the comparisons.

398 to the start of the coding region for the low-molecular-weight form of SLO (nucleotide position 498) is considered. This corresponds to the coding region for the signal peptide of perfringolysin O and may be the remnants of the coding region for a signal peptide to a progenitor of the *slo* gene, if *slo* originally resembled *pfo*. We examined this out-of-frame sequence of *slo* for the characteristic features of a signal peptide but have not found them. However, it is probable that this region has undergone various mutations that would obscure the original sequence. Little homology was found with the remaining coding region for the hydrophilic 67 amino-terminal residues of SLO, a fragment which is easily lost after secretion of SLO (1, 11). It is interesting that the perfringolysin O nucleotide and amino acid sequences exhibit significantly higher levels of homology with SLO and its gene (Fig. 2 and 3) than does pneumolysin with SLO. A higher degree of homology would have been expected in the genes derived from the same genus. This and the general similarity of the cytolysin primary structures suggest that intergenic transfer rather than divergence of a single primordial gene may have been responsible for the dissemination of the thiol-activated hemolysin genes among the various genera.

Features of the deduced perfringolysin O amino acid sequence. The sequence of the perfringolysin O gene was predicted to start from the second in-frame methionine codon (Fig. 1) at nucleotide position 103. The start of the coding region for the mature protein was determined to be at nucleotide position 187, based on the complete homology of the DNA-deduced primary structure with the first 17 amino acids of purified perfringolysin O. This sequence differs by at least two residues from that determined for perfringolysin O by Ohno-Iwashita et al. (15). They determined that the amino-terminal sequence is Lys-Lys-Ile-Thr-X-Ile-Asn-Gln-X-Ile-Asp-X-Gly-Ile-X-X-Leu, whereas the amino-terminal sequence presented here, which is based on both our amino-terminal sequence analysis of the perfringolysin O protein and the DNA sequence, is Lys-Asp-Ile-Thr-Asp-Lys-Asn-Gln-Ser-Ile-Asp-Ser-Gly-Ile-Ser-Ser-Leu. The first difference is at position 2 (Asp→Lys), and the second is at

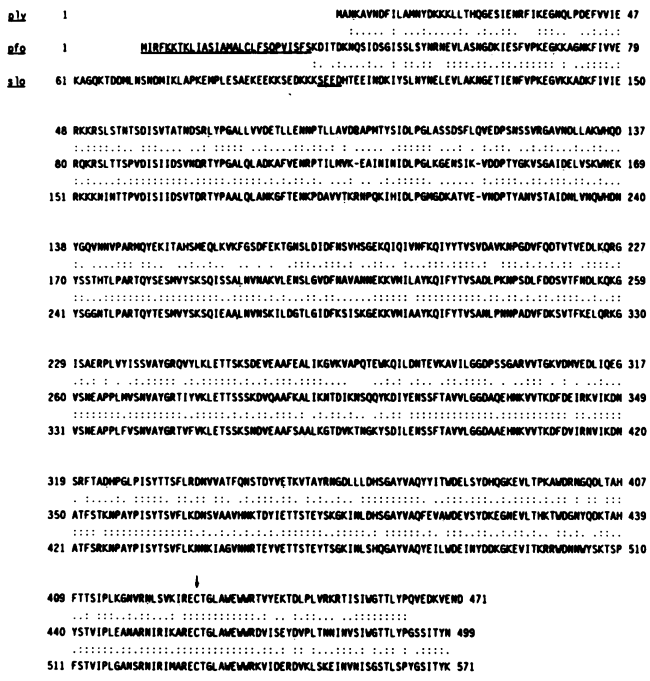

FIG. 3. Alignment of the amino acids of the primary structures of perfringolysin O, SLO, and pneumolysin. The primary structures were aligned by using the program FASTP of Wilbur and Lipman (22). The double dots represent perfect matches, and the single dots represent conservative amino acid substitutions. Both the pneumolysin and perfringolysin O sequences start at amino acid 1 of the deduced primary structure. The SLO sequence starts at residue 61; no significant homology with either perfringolysin O or pneumolysin was detected in the primary sequence of SLO prior to residue 100. The signal peptide of perfringolysin O is underlined. The position of the essential cysteine in all three proteins is indicated by the arrow.

TABLE 1. Amino acid composition of perfringolysin O

Amino acid	No. of residues[a]			
	+SP	−SP	Yamakawa et al. (23)	Mitsui et al. (14)
Ala	36	33	33	62–75
Val	41	40	38	44–53
Leu	31	28	26	44–47
Ile	37	33	33	26–35
Pro	17	16	22	15–30
Met	6	4	5	*
Phe	16	13	13	19–30
Trp	7	7	*	*
Gly	21	21	25	57–70
Ser	51	47	43	33–64
Thr	34	33	33	34–37
Cys	2	1	1	0
Tyr	23	23	22	10–24
Asn	38	38	]–72	]–47–67
Asp	34	34		
Gln	14	13	]–38	]–22–60
Glu	27	27		
Lys	45	42	42	28–39
Arg	13	12	11	15–25
His	6	6	6	9–12

[a] The DNA-sequence-derived amino acid composition is given for perfringolysin O with (+SP) and without (−SP) the signal peptide. The amino acid compositions of perfringolysin O derived by Yamakawa et al. (23) and Mitsui et al. (14) are also shown. The data of Mitsui et al. (14) are given as the high and low values presented in their report on two different forms of perfringolysin O. Total glutamate plus glutamine and total aspartate plus asparagine were determined by Mitsui et al. (14) and Yamakawa et al. (23). *, Number of residues not determined.

position 6 (Lys→Ile). There may be additional differences (X's in their sequence); however, most of the undetermined residues in their sequence correspond in our sequence to serine, an amino acid that has a low yield during sequence analysis and can be difficult to identify. Assuming that both sequences are correct, it appears that the primary sequence of perfringolysin O may vary between strains of *C. perfringens*. In addition, Ohno-Iwashita et al. (15) determined an amino-terminal sequence for a trypsin fragment, designated T2, of perfringolysin O (Asn-X-Gln-Gln-Tyr-X-Asp-Ile) which corresponds to amino acids 304 to 311 of the DNA-derived sequence (Fig. 1). The deduced amino acid composition (Table 1) of perfringolysin O agrees with that determined by Yamakawa et al. (23) but not with the amino acid analysis reported by Mitsui et al. (14). The reasons for the discrepancy are unclear; however, the preparation of Mitsui et al. (14) may have contained contaminants. Both Yamakawa et al. (23) and Mitsui et al. (14) purified perfringolysin O from *C. perfringens* PB6K, whereas in this study the perfringolysin O protein and its gene were derived from *C. perfringens* ATCC 13124.

Based on the amino-terminal sequence data and the putative start codon, a 27-residue signal peptide is present on perfringolysin O. The putative signal peptide has the typical structure of a signal peptide, including a basic amino-terminal region, a hydrophobic center, and a Ser-Phe-Ser sequence prior to the signal cleavage site (17). Comparison of the amino acid sequence of perfringolysin O with the deduced primary sequences of SLO and pneumolysin showed that significant homology is present among all three proteins (Fig. 3). Surprisingly, perfringolysin O (secreted form) exhibits more homology (65%) with SLO (low-molecular-weight form) than pneumolysin exhibits with either SLO or perfringolysin O (42%). If conservative amino acid substitutions are taken into consideration, perfringolysin O exhibits 96% homology with the low-molecular-weight form of SLO. The largest stretch of completely homologous primary sequence among all three proteins surrounds the essential cysteine. This is the only cysteine present in the deduced primary sequence for the secreted form of perfringolysin O, although another cysteine is present in the signal peptide. The position of the cysteine was also conserved within all three proteins when they were aligned for maximum homology (Fig. 3). There have been no reports of perfringolysin O, or any of the other thiol-activated cytolysins, forming a disulfide with other toxin molecules or membrane proteins on the target membranes. In addition, we have not detected any larger forms of perfringolysin O resulting from the formation of intermolecular disulfides with itself or membrane proteins of erythrocytes (R. K. Tweten, unpublished data). Therefore, the role of the essential thiol may not be to form a disulfide, but rather it may have some other role, such as the formation of a hydrogen bond or hydrophobic interaction. Interestingly, the pH activity curve determined by Mitsui et al. (14) for perfringolysin O shows an optimum activity at approximately pH 7.0, whereas at pH 8.5 the activity is only about 50%. One explanation for this is that ionization of the proton of the cysteine results in a loss of activity. The pK_a of the cysteine proton is 8.33; no other amino acid R group has a pK_a close to this value.

This region is also unusual in that it contains three of the six tryptophan residues found in perfringolysin O. Tryptophan has the largest aromatic side chain of the amino acids and therefore may impart a local hydrophobic character to the environment surrounding the cysteine. Iwamoto et al. (7) have found that chemical modification of the cysteine results in a 100-fold decrease in membrane binding by perfringolysin O, presumably as a result of decreased affinity for cholesterol. If the cysteine and surrounding residues are involved in cholesterol binding, then the tryptophan residues may stabilize binding by the formation of hydrophobic interactions with the cholesterol molecule.

All three toxins exhibit a general similarity in their primary structures (Fig. 3) and hydropathic profiles (Fig. 4). The hydropathy plots of the perfringolysin O and low-molecular-weight form of SLO are nearly superimposable, whereas the hydropathy plot of pneumolysin exhibited some minor differences. The largest difference resides in residues 188 to 208 of perfringolysin O (260 to 280 of SLO and 158 to 178 of pneumolysin). Both perfringolysin O and SLO are relatively hydrophobic in this region, whereas the same region in pneumolysin is hydrophilic. No significant regions of hydrophobic residues are present in perfringolysin O; this was also observed by Kehoe et al. (11) for SLO and pneumolysin. This suggests that membrane-bound perfringolysin O may not span the lipid bilayer. This is consistent with the observation of Cowell et al. (2), who found that the thiol-activated cytolysin cereolysin did not appear to span the membrane bilayer when they examined membrane-bound cereolysin by electron microscopy. Nevertheless, it is possible that perfringolysin O, or any of the related cytolysins, may fold to juxtapose appropriate sequences to form a membrane-spanning region.

The principal difference between perfringolysin O and pneumolysin is the lack of a signal sequence on the pneumolysin. This is consistent with the fact that pneumolysin remains intracellular in *Streptococcus pneumoniae* and is released only by cellular lysis. In contrast, SLO exhibits an additional 67-residue fragment between its signal peptide and

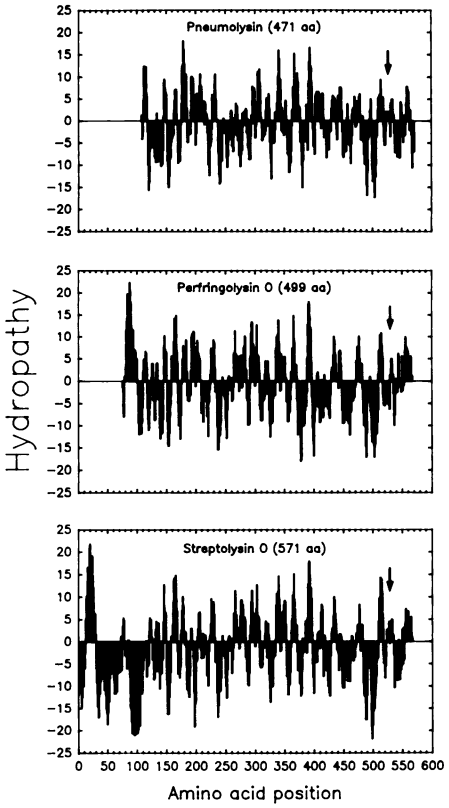

FIG. 4. Hydropathy analysis of perfringolysin O, SLO, and pneumolysin. The hydropathy analysis was based on the method of Kyte and Doolittle (12). Positive values indicate hydrophobic regions, and negative values indicate hydrophilicity. Numbering is based on the SLO sequence; however, the hydropathy plots are aligned for maximum homology as determined by the alignment presented in Fig. 3. The relative position of the essential cysteine is shown by the arrow. aa, Amino acids.

the beginning of the low-molecular-weight form (1, 11). This region is extremely hydrophilic (Fig. 4) and is apparently not necessary for the activity of SLO. The complete lack of similarity of this region of SLO with the primary structures of either perfringolysin O or pneumolysin suggests that this region was formed or added after *Streptococcus pyogenes* acquired the *slo* gene. Any function this additional sequence has in the activity of SLO is unclear since it is rapidly lost after secretion (1) and does not appear to affect the in vitro lytic activity of SLO. It will be of interest whether this feature is conserved in SLO molecules produced by other strains of *S. pyogenes* or whether it is present in the primary sequences of cytolysin molecules produced by strains other than those of the streptococci.

The sequence of the perfringolysin O gene coupled with the elevated expression of perfringolysin O in *E. coli* (20a) will help us to effectively use this system for the study of the structure/function relationships in perfringolysin O. Several questions are of interest with respect to the structure/function relationships in the thiol-activated cytolysin; for instance, how does the essential thiol function in the mechanism of cell lysis and where is the cholesterol molecule bound by these proteins? In addition, comparatively little is known of the contribution of perfringolysin O to the various diseases caused by *C. perfringens*; therefore, the cloned gene will be a useful tool to examine the role of perfringolysin O in disease.

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LITERATURE CITED

1. **Bhakdi, S., M. Roth, A. Sziegoliet, and J. Tranum-Jensen.** 1984. Isolation and identification of two hemolytic forms of streptolysin O. Infect. Immun. **46**:394–400.
2. **Cowell, J. L., K. Kim, and A. Bernheimer.** 1978. Alteration by cereolysin of the structure of cholesterol-containing membranes. Biochim. Biophys. Acta **507**:230–241.
3. **Garnier, T., and S. T. Cole.** 1986. Characterization of a bacteriocinogenic plasmid from *Clostridium perfringens* and molecular genetic analysis of the bacteriocin-encoding gene. J. Bacteriol. **168**:1189–1196.
4. **Geoffrey, C., and J. E. Alouf.** 1984. Antigenic relationships between sulfhydryl-activated toxins, p. 241–243. *In* J. E. Alouf, J. Fehrenbach, J. H. Freer, and J. Jeljaszewicz (ed.), Bacterial toxins. Academic Press, Inc. (London), Ltd., London.
5. **Henikoff, S.** 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28**:351–359.
6. **Holmes, W. M., T. Platt, and M. Rosenberg.** 1983. Termination of transcription in *E. coli*. Cell **32**:1029–1032.
7. **Iwamoto, M., Y. Ohno-Iwashita, and S. Ando.** 1987. Role of the essential thiol group in the thiol-activated cytolysin from *Clostridium perfringens*. Eur. J. Biochem. **167**:425–430.
8. **Johnston-Dow, L., E. Mardis, C. Heiner, and B. Roe.** 1987. Optimized methods for fluorescent and radio labeled DNA sequencing. Biotechniques **5**:754–765.
9. **Joliff, G., P. Beguin, and J. P. Aubert.** 1986. Nucleotide sequence of the cellulase gene *celD* encoding endoglucanase D of *Clostridium thermocellum*. Nucleic Acids Res. **11**:8605–8613.
10. **Kehoe, M., and K. N. Timmis.** 1984. Cloning and expression in *Escherichia coli* of the streptolysin O determinant from *Streptococcus pyogenes*: characterization of the cloned streptolysin O determinant and demonstration of the absence of substantial homology with determinants of the other thiol-activated toxins. Infect. Immun. **43**:804–810.
11. **Kehoe, M. A., L. Miller, J. A. Walker, and G. J. Boulnois.** 1987. Nucleotide sequence of the streptolysin O (SLO) gene: structural homologies between SLO and other membrane-damaging, thiol-activated toxins. Infect. Immun. **55**:3228–3232.
12. **Kyte, I., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157**:105–132.
13. **Maizel, J. V., and R. P. Lenk.** 1981. Enhanced graphic matrix analysis of nucleic acid and protein sequences. Proc. Natl. Acad. Sci. USA **78**:7665–7669.
14. **Mitsui, K., N. Mitsui, and J. Hase.** 1973. *Clostridium perfringens* exotoxin. II. Purification and some properties. Jpn. J. Exp. Med. **43**:377–391.
15. **Ohno-Iwashita, Y., M. Iwamoto, K. Mitsui, H. Kawasaki, and S. Ando.** 1986. Cold-labile hemolysin produced by limited proteolysis of theta toxin from *Clostridium perfringens*. Biochemistry **25**:6048–6053.
16. **Paton, J. C., A. M. Berry, R. A. Lock, D. Hansman, and P. A. Manning.** 1986. Cloning and expression in *Escherichia coli* of the *Streptococcus pneumoniae* gene encoding pneumolysin. Infect. Immun. **54**:50–55.
17. **Perlman, D., and H. O. Halvorson.** 1983. A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptides. J. Mol. Biol. **167**:391–409.
18. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
19. **Smith, L. D. S., and G. Hobbs.** 1974. Genus III. *Clostridium* Prazmowski 1880, 23, p. 551–572. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.

20. **Smyth, C. J., and J. L. Duncan.** 1978. Thiol-activated (oxygen-labile) cytolysins, p. 129–183. *In* J. Jeljaszewicz and T. Wadstrom (ed.), Bacterial toxins and cell membranes. Academic Press, Inc. (London), Ltd., London.

20a. **Tweten, R. K.** 1988. Cloning and expression in *Escherichia coli* of the perfringolysin O (theta-toxin) gene from *Clostridium perfringens* and characterization of the gene product. Infect. Immun. **56**:3228–3234.

21. **Walker, J. A., R. L. Allen, P. Falmagne, M. K. Johnson, and G. J. Boulnois.** 1987. Molecular cloning, characterization, and complete nucleotide sequence of the gene for pneumolysin, the sulfhydryl-activated toxin of *Streptococcus pneumoniae*. Infect. Immun. **55**:1184–1189.

22. **Wilbur, W. J., and D. J. Lipman.** 1983. Rapid similarity searches of nucleic acid and protein data banks. Proc. Natl. Acad. Sci. USA **80**:726–730.

23. **Yamakawa, Y., A. Ito, and H. Sato.** 1977. Theta toxin of *Clostridium perfringens*. I. Purification and some properties. Biochim. Biophys. Acta **494**:301–313.

24. **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.