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Phylogenetic Analysis of Phospholipase C Genes from *Clostridium perfringens* Types A to E and *Clostridium novyi*

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The phylogenetic interrelationships between strains of 5 toxin types (A to E) of *Clostridium perfringens* were examined by analysis of differences in the nucleotide sequences of phospholipase C genes (*plc* genes) among 10 strains, including 3 strains for which the *plc* gene sequences have been previously reported. A *plc* gene was also cloned from a *Clostridium novyi* type A strain and sequenced to analyze the interspecies diversity of *plc* genes. Phylogenetic trees constructed by the neighbor-joining method revealed that the phylogeny of *C. perfringens* strains is not related to toxin typing, in agreement with the results of a comparative genome mapping study by Canard et al. (B. Canard, B. Saint-Joanis, and S. T. Cole, Mol. Microbiol. 6:1421–1429, 1992). Various *C. perfringens* phospholipase C enzymes were purified from cultures of *Escherichia coli* cells into which the encoding *plc* genes had been cloned. All of the enzymes showed the same specific activity. On the other hand, the level of *plc* transcripts differed greatly (up to 40-fold) from one *C. perfringens* strain to another. No significant difference in the nucleotide sequence of the *plc* promoter region was observed for any of the *plc* genes. These results suggest that the variation in phospholipase C activity among different strains is not due to mutation in the *plc* coding region but to that in an extragenic region. The evolution of *C. perfringens* phospholipase C is discussed on the basis of similarities and differences between clostridial *plc* genes.

Clostridium perfringens belongs to the genus *Clostridium*, whose members are anaerobic gram-positive spore-forming bacilli, and is the most widely occurring pathogenic bacterium (14, 34). On the basis of the ability to produce major lethal toxins, the alpha-, beta-, epsilon-, and iota-toxins, *C. perfringens* strains are grouped into five toxin types (see Table 1) (25). Type A strains are widely distributed in nature and inhabit the human intestine, causing various diseases such as gas gangrene and food-borne diarrhea. Thus, they are a medically important pathogens. Strains of the other types, although rarely found in the environment, are inhabitants of the intestinal tracts of domestic animals and sometimes cause diseases of importance in veterinary medicine (14). Alpha-toxin (phospholipase C [PLC]) plays a key role in the pathogenicity of the organism (27), and its coding gene (*plc*) has already been cloned from three strains (two type A strains and one type C strain) and sequenced (20, 28, 31). We have undertaken to clone and sequence *plc* genes from other type A strains and also from strains of different types for several purposes.

The first aim is to determine the phylogenetic relationships between strains of different *C. perfringens* toxin types. Although the current classification based on toxin typing is convenient for clinical purposes, a comparative genome map study revealed that it does not reflect genetic relationships (7). Measurement of the evolutionary distances between strains of different types would facilitate the elucidation of their genetic relationships. Analysis of the rRNA nucleotide sequences has been performed to construct a phylogenetic tree for many clostridial species, including *C. perfringens* (6, 8, 30), as has been done for many bacteria (29, 30, 46). However, this analysis is not suitable for determining intraspecies relationships because of the high sequence conservation due to functional constraints and also because of the complexity arising from the microheterogeneity among multiple rRNA operons (4, 9). PLC is produced by strains of all five toxin types, unlike other major lethal toxins, and its encoding gene is located in a variable region close to the replication origin (7). In order to construct a phylogenetic tree for *plc* genes, we cloned and sequenced the *plc* genes from strains of all *C. perfringens* types.

The second aim of this work is to examine the interspecies diversity of *plc* genes. *Bacillus cereus* PLC (lecithinase), the crystal structure of which has been determined (17), is considered to be a prototype PLC (39). Recent studies involving protein engineering (41) and crystallography (3, 40) with *C. perfringens* PLC suggested that it consists of two domains: an N-terminal domain, which is functionally and structurally similar to that of *B. cereus* PLC, and a C-terminal one, which is postulated to confer sphingomyelinase activity on the enzyme (41). In the case of *B. cereus*, lecithinase and sphingomyelinase are encoded by two different genes, *cerA* and *cerB*, respectively (12), leading to the hypothesis that an ancestral gene of the present-day *C. perfringens plc* arose through the fusion of *cerA* and *cerB* homologs (12). However, the *cerB* product does not have any region exhibiting significant similarity to one in *C. perfringens* PLC, disproving this hypothesis. Comparison of the PLCs of more closely related species would provide insights into the molecular evolution of bacterial PLCs. For this purpose, we cloned and sequenced a *plc* gene from *Clostridium novyi* and compared it with two clostridial *plc* genes, from *C. perfringens* and *Clostridium bifermentans*, described previously (43).

It is well known that the level of PLC activity produced in the culture medium greatly differs from one strain to another. It is not clear whether this is due to a difference in the *plc* coding region, to a difference in other regions, or both. We previously reported that type A and C strains differed in the transcriptional activity of the *plc* gene, probably because of a

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TABLE 1. Bacterial strains used and *plc* genes cloned and sequenced in this study

Species	Toxin type	Toxin production				Strain	Accession number of *plc* gene	Source or reference
		Alpha	Beta	Epsilon	Iota			
C. pefringens	A	+	−	−	−	NCTC8237	M24904	28
						8-6	X17300	31
						PB6K	D32123	This study
						13	D32127	This study
						KZ211	D32124	This study
	B	+	+	+	−	NCIB10691	D32128	This study
	C	+	+	−	−	NCIB10662	D10248	20
	D	+	−	+	−	L9	D49968	This study
						NCIB10663	D49969	This study
	E	+	−	−	+	NCIB10748	D32126	This study
C. novyi						CL49	D32125	This study
C. bifermentans						ATTC 638		43

difference in the extragenic regulation of *plc* gene expression (20). To assess the possibility that the difference between the two strains could be generalized, we determined the specific activities of PLCs and the levels of *plc* transcripts produced by strains of various types. This paper describes the phylogenetic relationships of *C. perfringens plc* genes at the intraspecies and interspecies levels and differences in *plc* transcriptional activity among *C. perfringens* strains. It also describes the evolution of a *plc* gene.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *C. perfringens* strains used in this study included four type A strains (NCTC8237, 13 [21], PB6K [35], and KZ211 [18]), a type B strain (NCIB10691), a type C strain (NCIB10662), two type D strains (L9 [47] and NCIB10663), and a type E strain (NCIB10748). All of the strains other than strains 13 and NCTC8237 were collected by and stored at the Department of Bacteriology, School of Medicine, Kanazawa University, Kanazawa, Japan. *C. novyi* type A strain CL49 was obtained from the Institute for Veterinary Hygiene, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Japan. All strains were cultured in GAM broth (Nissui Co., Tokyo, Japan), as described previously (20). *Escherichia coli* JM109 (48) was used as the host strain for plasmid pUC19 (48), pBluescript II KS+ (2), and their derivatives. It was grown in Luria-Bertani broth (Gibco BRL, Paisley, United Kingdom) containing 50 μg of ampicillin per ml with shaking at 37°C.

Preparation of chromosomal DNA. Chromosomal DNA of *C. perfringens* was prepared as follows. Cultures were grown in 600 ml of GAM broth to an optical density at 600 nm (OD) of approximately 1.2 and then harvested by centrifugation at 4,000 × *g* for 10 min. Each cell pellet was resuspended in 18 ml of 25 mM Tris-HCl–10 mM EDTA (pH 8) containing 50 mM glucose and 10 mg of lysozyme per ml and then incubated for 30 min at 37°C. Two milliliters of 10% sodium dodecyl sulfate (SDS) and 100 μl of a proteinase K solution (20 mg/ml; Sigma Chemical Co., St. Louis, Mo.) were added to the suspension. After incubation at 37°C for 1 h, 3.6 ml of 5 M NaCl and 3 ml of a hexadecyltrimethylammonium bromide (CTAB)-NaCl solution (45) were added to the mixture. Subsequent treatment was performed as described elsewhere (45). Cultures of *C. novyi* were grown in 600 ml of GAM broth to an OD of 0.3. After 12 ml of 0.5 M EDTA and 600 mg of penicillin G had been added, the cultures were further incubated for 1 h. Cells were collected by centrifugation and washed twice with 20 ml of 25 mM Tris-HCl–50 mM EDTA (pH 8) containing 50 mM glucose. Subsequent procedures for chromosome preparation were as described above.

Southern hybridization. DNA was digested with *Eco*RI and/or *Hin*dIII, and then Southern hybridization was performed as described previously (23). A 531-bp *Sau*3AI fragment within the coding region of the *plc* gene and a 2,031-bp *plc* gene-containing fragment from *C. perfringens* NCTC8237 (20) were used as probe DNAs.

Northern (RNA) hybridization. *C. perfringens* strains were grown in GAM broth at 37°C. When cultures reached an OD of 0.8, total RNA was prepared by the SDS-phenol method (1). The DNA probe used was a 1.4-kb *Ssp*I-*Hin*dIII fragment, which was prepared from the NCTC8237 *plc* gene and labeled with digoxigenin-dUTP. Northern hybridization was performed as described previously (23). The levels of *plc* transcripts were determined by semiquantitative Northern analysis with various amounts (0.025 to 5.0 μg) of RNA. In order to determine band intensities, a luminogram image was digitized on a flat-bed scanner (model JX-330M; Sharp, Tokyo, Japan) and then analyzed with a Macintosh Quadra 650 computer by using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov). The relative amounts of *plc* mRNAs were determined with a calibration curve, which was obtained with RNA prepared from the NCTC8237 strain, which contained the highest level of *plc* mRNA.

Cloning and sequencing of *plc* genes. Chromosomal DNA from each *C. perfringens* strain was digested with *Eco*RI and *Hin*dIII and then separated on a 0.8% agarose gel. DNA fragments (approximately 2 kb) electroeluted from a gel slice were ligated into the *Eco*RI and *Hin*dIII sites of pUC19 and then introduced into *E. coli* JM109. Transformants were screened for hemolysis on overlaid blood agar (28) and also for PLC activity on egg yolk agar (28). Plasmid DNAs were purified and DNA was sequenced as described previously (23, 49). The *plc* gene from *C. novyi* CL49 was cloned and sequenced in the same manner as described above, except that pBluescript II KS+ was used as the cloning vector and the clones to be sequenced were generated by nested deletion with exonuclease III. A plasmid, pTOX5, containing the *plc* gene from *C. perfringens* 8-6 was obtained from Stewart T. Cole (Laboratoire de Génétique Moléculaire Bactérienne, Institut Pasteur, Paris, France). A 2.0-kb *Eco*RI-*Hin*dIII fragment containing the *plc* gene was subcloned from pTOX5 into pUC19. *E. coli* JM109 transformed with the resultant plasmid was used to purify strain 8-6 PLC.

Phylogenetic analysis. Sequences used in the phylogenetic analysis of *plc* genes included those from 10 strains of *C. perfringens*, *C. novyi* CL49, and *C. bifermentans* ATTC 638, which are listed in Table 1. A total of 1,194 nucleotide positions for the entire segment of the *plc* coding region were used in this analysis. A *cerA* gene encoding *B. cereus* phosphatidylcholine-specific PLC was used as an outgroup (12). Phylogenetic tree construction was performed by using the programs contained in the Clustal V package described by Higgins et al. (15). Nucleotide sequence alignment was carried out with the multiple alignment algorithm described by Higgins and Sharp (16) and standard parameters. The phylogenetic tree was constructed by the neighbor-joining method of Saitou and Nei (32). The reliability of tree nodes was analyzed by generating 1,000 bootstrap trees (11).

Purification of PLC. *E. coli* JM109 cells carrying pUC19 derivatives with a cloned 2-kb *Eco*RI-*Hin*dIII *plc* gene were grown in 2 liters of LB broth containing ampicillin (50 μg/ml) to an OD of 2.5. The cultures were harvested by centrifugation, and then the cell pellets were resuspended in 40 ml of 50 mM Tris-HCl (pH 7.5) containing polymyxin B sulfate (10,000 U/ml; Taito-Pfizer Co., Tokyo, Japan). The cell suspension was incubated for 30 min at 37°C and then centrifuged at 20,000 × *g* for 20 min at 4°C. Ammonium sulfate was then added to the supernatant to give 80% saturation. The precipitate was collected by centrifugation at 13,000 × *g* for 20 min at 4°C. The pellet was dissolved in 10 ml of 50 mM Tris-HCl (pH 7.5) and then dialyzed against 1 liter of the same buffer. The material was then subjected to affinity chromatography on a Sepharose 4B-linked egg yolk lipoprotein column as described by Takahashi et al. (38), except for the column size (2.0 by 3.2 cm). The pooled PLC fractions were concentrated to approximately 100 μl by ultrafiltration with a Centricon 10 (Amicon, Danvers, Mass.). The sample was then subjected to size exclusion fast protein liquid chromatography (Superdex 75 HR 10/30; Pharmacia LKB Biotechnology, Uppsala, Sweden). The column was eluted with 50 mM Tris-HCl (pH 7.5)–0.15 M NaCl–0.1 mM $ZnCl_2$. The pooled PLC fractions were concentrated, rechromatographed as described above, and stored at −80°C until used.

Assays for PLC. PLC activity was assayed by the turbidimetric method (19) with egg lecithin (Wako Pure Chemicals, Osaka, Japan). Protein concentrations were determined with the Pierce bicinchoninic acid protein assay reagent (33), with bovine serum albumin as a standard.

Nucleotide sequence accession numbers. The nucleotide sequence accession numbers for the *plc* genes sequenced in this study are given in Table 1.

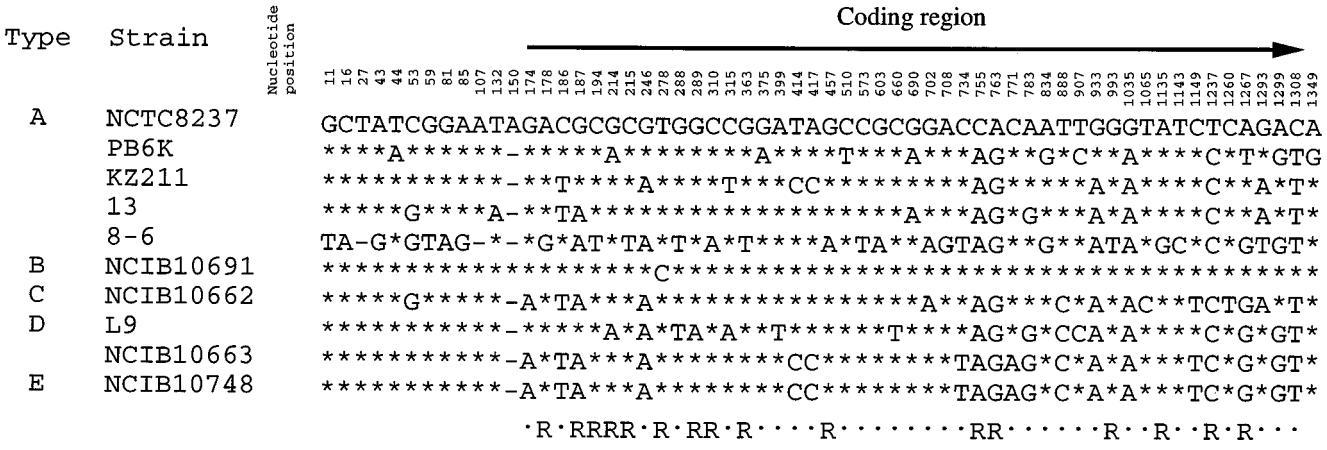

FIG. 1. Nucleotide differences among the *plc* genes from 10 strains of *C. perfringens*. Invariant sites are not shown. The nucleotides are numbered (listed vertically), with the first adenine (A) of 5′-AATTTGAATTG-3′, which is located immediately upstream of a direct repeat within the promoter upstream region, numbered 1 (20). The coding region is denoted by the arrow. R indicates nucleotide substitutions that result in an amino acid replacement.

RESULTS

Southern analysis and cloning of *plc* genes. In our previous study, the *plc* genes from both *C. perfringens* type A NCTC8237 and type C NCIB10662 were mapped within a 2-kb *Eco*RI-*Hin*dIII fragment (20, 28). To examine whether *plc* genes and their flanking regions are conserved in other *C. perfringens* strains and *C. novyi*, and also to determine an appropriate restriction fragment for the cloning of each *plc* gene, their chromosomal DNAs were digested with *Eco*RI and/or *Hin*dIII and then subjected to Southern analysis with DNA probes derived from the NCTC8237 *plc* gene. For *Eco*RI, *Hin*dIII, and *Eco*RI-*Hin*dIII digests of the DNAs from all of the *C. perfringens* strains, the hybridized bands were 2, 3.1, and 4.4 kb long, respectively. In contrast, all three digests of *C. novyi* DNA showed different profiles of hybridized bands, which were slightly larger than the corresponding bands of the *C. perfringens* strains (data not shown). Therefore, the flanking regions of the *plc* gene seem to be homogenous at the intraspecies level but heterogeneous at the interspecies level. For all of the digests, only one band was detected, indicating that PLC is encoded by a single *plc* gene in all of the strains. The chromosomal DNAs from seven strains of *C. perfringens* and *C. novyi* CL45 were digested with *Eco*RI and *Hin*dIII, and the fragments corresponding to the hybridized bands were ligated into the *Eco*RI and *Hin*dIII sites of the plasmid vectors. Then *plc* genes were cloned and sequenced as described in Materials and Methods.

Comparison of *C. perfringens plc* genes. The nucleotide sequences of the *plc* genes from 10 *C. perfringens* strains, including those from the 3 strains reported previously, were compared over nucleotide positions 1 to 1350. For convenience, the NCTC8237 *plc* gene was tentatively used as the reference, and only nucleotides different from those of the reference sequence are aligned in Fig. 1. In the 1,350 bp sequenced there were 60 polymorphic sites. Although a one-base deletion exists in the upstream noncoding region, all nucleotide differences in the coding region are base substitutions. The sequences of pairs of strains differed, on average, in 1.4% of the nucleotide sites. In the 1,194-bp coding region, the sequences of pairs of strains differed, on average, in 1.3% of the nucleotide sites and 1.2% of the amino acid positions. Surprisingly, NCIB10691 (type B) differs in only one nucleotide from NCTC8237 (type A), while the latter differs in 37 nucleotides from strain 8-6 (type A). It should also be noted that NCIB10663 (type D) coincides in all nucleotides with NCIB10748 (type E) but differs in 15 nucleotides from L9 (type D). Adenine at nucleotide 150, which is located between the Shine-Dalgarno sequence and the first Met codon, is deleted in most of the *plc* genes. This does not seem to affect the translational efficiency very much, since this adenine is present in the *plc* genes from both a strain producing high levels of PLC (NCTC8237) and one producing low levels of PLC (NCIB10691) but not in another one producing high levels of PLC (PB6K).

Evolutionary tree. A neighbor-joining evolutionary tree for the entire segment of the coding region of the *plc* gene is shown in Fig. 2. The root of the tree was placed by use of the sequence of a *cerA* gene encoding *B. cereus* PLC as the outgroup, and the reliability of the branching order was determined by bootstrap analysis of 1,000 computer-generated trees. The sequences of three clostridial species formed a monophyletic group. The sequences of *C. perfringens* strains are closely related to one another, forming a monophyletic group. They were more closely phylogenetically linked to the sequence of *C. novyi* than to that of *C. bifermentans*, coinciding with the results of phylogenetic analysis of rRNA (8). Among the *C. perfringens* sequences, that of strain 8-6 is the most divergent. The branching order among sequences of the other strains is uncertain, as indicated by the low bootstrap value. However, it is evident that the sequences of two strains, NCTC8237 (type A) and NCIB10691 (type B), are tightly linked to each other, forming a cluster distinct from others. One type D strain, NCIB10663, is tightly linked to a type E strain, NCIB10748, forming a cluster distinct from the other cluster comprising one type A and another type D strain. These results clearly indicate that the toxin type does not agree with the genotype.

Comparison of three clostridial PLCs. To examine the interspecies diversity of clostridial PLCs, the nucleotide sequence and deduced amino acid sequence of the *plc* gene from *C. perfringens* NCTC8237 were compared with those of *C. novyi* and *C. bifermentans*. The nucleotide sequences of the *plc* coding regions of *C. perfringens* and *C. novyi* show 69% identity, while those of *C. perfringens* and *C. bifermentans* show 64% identity. An alignment of the deduced amino acid sequences of the three PLCs is shown in Fig. 3. All precursor forms of the PLCs consist of 398 amino acid residues. Since the N-terminal

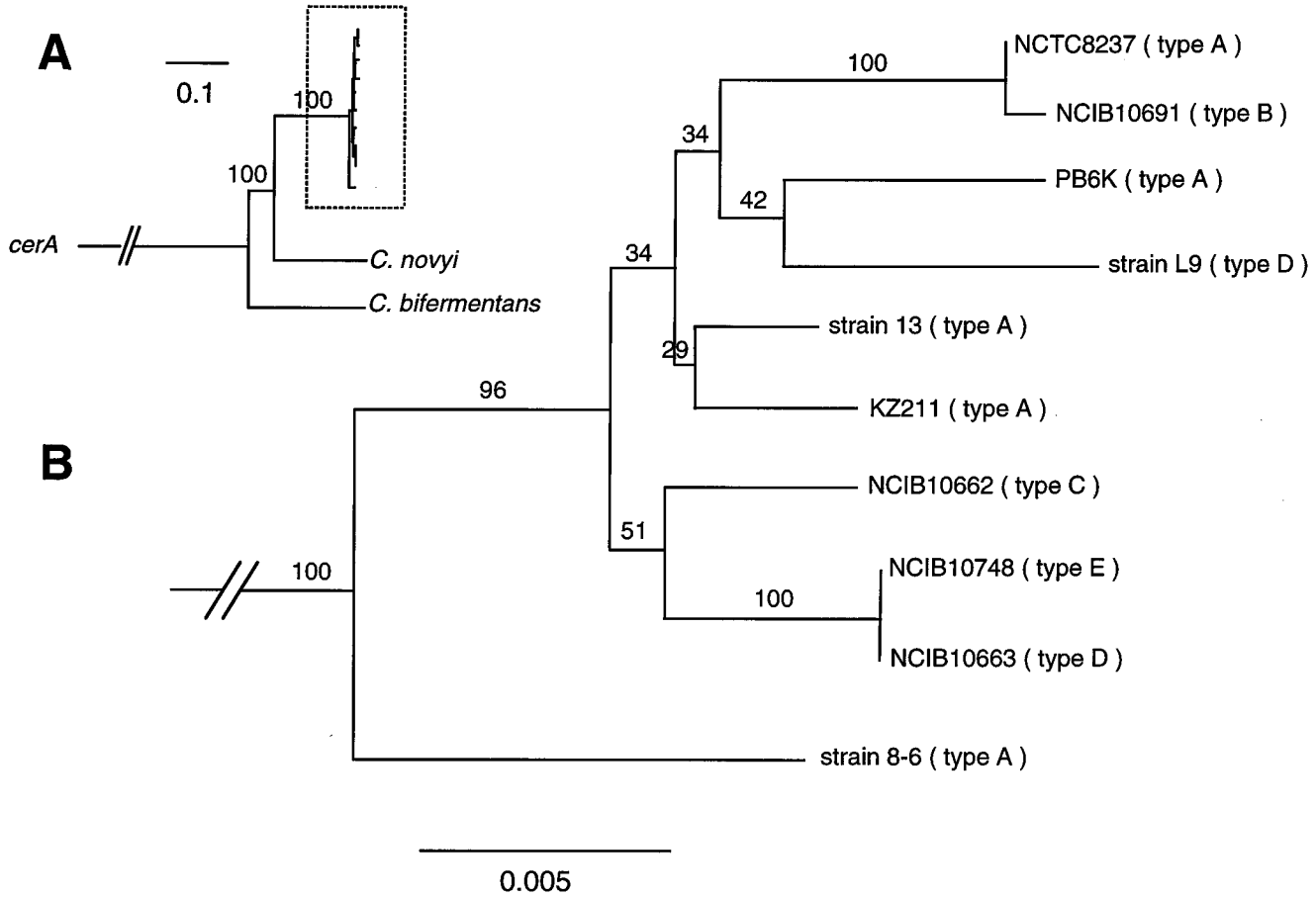

FIG. 2. (A) Phylogenetic tree for the *plc* gene sequences of 10 strains of *C. perfringens*, *C. novyi* type A strain CL49, and *C. bifermentans* ATCC 638 (43), constructed by the neighbor-joining method. The rooted tree was constructed by using *cerA*, encoding *B. cereus* PLC, as the outgroup. (B) Enlargement of the enclosed area in panel A. The bootstrap (confidence) values shown on the branches were calculated for 1,000 trees. The marker bars in panels A and B represent 0.1 and 0.005 expected substitutions per nucleotide position, respectively.

amino acid of the *C. perfringens* PLC has been shown to be Trp-1, the mature enzyme should consist of 370 amino acid residues (28). If premature PLCs are processed at the same site in *C. novyi* and *C. bifermentans* as in *C. perfringens*, the mature PLCs would consist of 370 and 372 amino acid residues, respectively. The overall amino acid identities between *C. perfringens* and *C. novyi* PLCs and between *C. perfringens* and *C. bifermentans* PLCs are 58 and 51%, respectively. Nine amino acid residues of *B. cereus* PLC (histidine, glutamic acid, tryptophan, and aspartic acid) which have been shown to coordinately bind zinc ions (17, 44), are located at similar positions in all three PLCs. There are two variable regions; one is a signal peptide, and the other is the region from Ser-250 to Lys-256. The region from the N-terminal portion up to the latter variable region is highly similar in all three PLCs, supporting the hypothesis that this region is a prototype of bacterial PLCs (39).

Comparison of PLC activity and level of *plc* transcripts among various type strains. It is well known that the level of PLC activity produced in the culture medium greatly differs from one *C. perfringens* strain to another. This is also the case for the strains used in this study (data not shown). We previously reported that the difference in PLC activity between type A and C strains is mainly due to a difference in the transcriptional activity of the *plc* gene (20). However, this may not be the case for strains of the other types, and it is possible that even minor differences in the amino acid residues may affect the specific activity of PLC. No strains, other than two strains producing high levels of PLC, NCTC8237 and PB6K, produce PLCs in amounts sufficient that their PLCs can be easily purified. Therefore, PLCs were purified from cultures of various recombinant *E. coli* cells by means of a rather simple method, which consisted of extraction from the periplasmic space and purification by affinity chromatography and gel filtration. Analysis by SDS-polyacrylamide gel electrophoresis showed that no polypeptide other than a 43-kDa one corresponding to PLC was detected (data not shown). The specific activity of NCTC8237 PLC obtained from recombinant *E. coli* cultures was approximately 80% of that for *C. perfringens* NCTC8237 cultures and was similar to that reported by Tso and Siebel (43) for PLC from recombinant *E. coli*. However, the samples from recombinant *E. coli* were pure enough to compare the specific activities of various PLCs with each other. There was no marked difference in specific activity between any of the PLCs purified from representative recombinant *E. coli* cells (Table 2). It seems very likely that all base substitutions found in the *plc* coding region are neutral, since strain 8-6 PLC showed nearly the same specific activity as the other PLCs, despite being the most divergent. This indicates that the difference in PLC activity between different strains is due to a difference in the level of production of the enzyme and suggests that it is probably due to a difference at the transcriptional level, as was

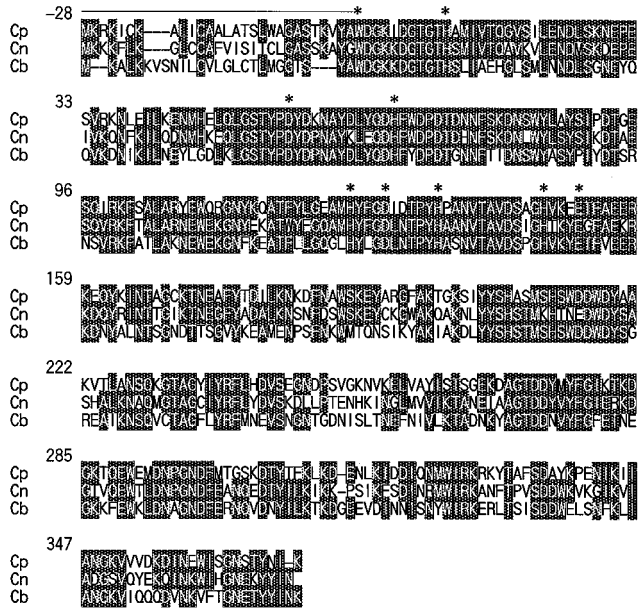

FIG. 3. Amino acid sequence alignment of the PLCs from *C. perfringens* type A strain NCTC8237 (Cp) (28), *C. novyi* (Cn), and *C. bifermentans* (Cb) (43). Residues conserved in at least two sequences are indicated by a black background. The numbering is according to the amino acid sequence of the mature *C. perfringens* PLC protein. The signal peptide determined for *C. perfringens* NCTC8237 PLC is overlined. The amino acid residues of *C. perfringens* PLC suggested to be involved in binding three zinc ions (44) are indicated by asterisks.

previously demonstrated for the type A and C strains (20). To confirm this, RNA was prepared from cultures of various *C. perfringens* strains at the mid-log growth phase and then subjected to Northern hybridization analysis. The level of *plc* mRNA differed markedly from one strain to another. The relative amounts of *plc* mRNA were determined by semiquantitative Northern analysis (Table 3). A 40-fold difference was observed between a strain producing high levels of PLC (NCTC8237) and two strains producing low levels of PLC (NCIB10691 and NCIB10663). Type A strains vary considerably in the *plc* mRNA level, which is significantly higher than those in strains of the other types. Therefore, the variation in the PLC activity produced in the culture medium by different strains is mainly, if not wholly, due to variation in the level of *plc* transcripts.

TABLE 2. Specific activities of PLCs purified from *E. coli* cultures expressing *plc* genes of various *C. perfringens* strains[a]

Toxin type	Strain	Sp act (U/mg of protein)[b]
A	NCTC8237	9,730 ± 170
	8-6	7,630 ± 170
B	NCIB10691	8,530 ± 80
C	NCIB10662	7,970 ± 230
E	NCIB10748	8,750 ± 80

[a] *E. coli* JM109 cells carrying pUC19 with a 2-kb *Eco*RI-*Hin*dIII fragment of a *plc* gene cloned from the *C. perfringens* strains listed were grown in 2 liters of Luria-Bertani broth containing 50 μg of ampicillin per ml. The periplasmic fraction was prepared by treatment with polymyxin B. PLC was purified as described in Materials and Methods. The PLC activity was determined with egg lecithin as a substrate.

[b] Values are means ± standard deviations for triplicate determinations.

TABLE 3. Levels of *plc* mRNA in various strains of *C. perfringens*[a]

Toxin type	Strain	Relative *plc* mRNA level
A	NCTC8237	42.9
	PB6K	23.9
	KZ211	4.9
	13	5.6
B	NCIB10691	1.1
C	NCIB10662	1.8
D	L9	4.3
	NCIB10663	1.0
E	NCIB10748	3.0

[a] RNA was extracted from cultures when they reached an OD of 0.8. Various amounts of RNA (0.025 to 5 μg) were subjected to Northern hybridization with a 1.4-kb *Ssp*I-*Hin*dIII fragment prepared from the NCTC8237 *plc* gene. The relative amounts of *plc* mRNA were determined as described in Materials and Methods.

DISCUSSION

Genetic relationships between *C. perfringens* strains of various types inferred from comparative genome mapping do not reflect the current classification based on toxigenicity (7). We have compared the nucleotide sequences of *plc* genes from *C. perfringens* strains of all toxin type with each other to examine their evolutionary relationships. The phylogenetic tree constructed by the neighbor-joining method revealed that toxin typing of *C. perfringens* strains is unrelated to their evolutionary relationships. Therefore, major lethal toxins other than PLC seem to be encoded by extrachromosomal genes, the loss or acquisition of which should cause toxin type variations (7). Such complexity arising from alteration of toxigenicity is also encountered in other clostridial species. Two organisms have been reported to produce botulinal toxins but would otherwise have been identified as *Clostridium butyricum* and *Clostridium barati* (13, 24). *Clostridium botulinum* includes some strains which are more closely related to *C. novyi* than to *C. botulinum* (26). This was attributed to conversion mediated by infecting phages (10). Thus, the ability to produce a toxin, which is the basis for the identification of pathogenic clostridia, is unreliable in a phylogenetic scheme.

This paper also describes the cloning and sequencing of the *plc* gene from *C. novyi. C. novyi* produces two different PLCs: PLC, which is specific to phosphatidylcholine and sphingomyelin, and PI-PLC, which is specific to phosphatidylinositol. *E. coli* JM109 containing the cloned *C. novyi plc* gene showed weak but positive PLC activity on egg yolk agar. On blood agar, it showed hot-cold hemolysis, which is characteristic of a sphingomyelin-degrading PLC (39). Therefore, the cloned gene should encode PLC but not PI-PLC. Taguchi and Ikezawa purified PLC from *C. novyi* type A strain IID140, which was later shown to contain PI-PLC (36, 37). While the latter has been purified and well characterized, the former has not been completely purified yet. We attempted to purify PLC from *C. novyi* and recombinant *E. coli* cultures. However, the purification was unsuccessful because they produced only trace amounts of the enzyme. While the *plc* gene from *C. perfringens* possesses a strong promoter, which is well expressed not only in *C. perfringens* but also in *E. coli*, the *C. novyi plc* gene lacks the promoter consensus sequence or its homolog. The difference in PLC production between the two species seems to be at least partly due to a difference in the transcriptional activity of the *plc* genes. It also seems possible that the activity of *C. novyi* PLC is as low as that of *C. bifermentans* PLC, which is 50-fold less active than *C. perfringens* PLC (43).

B. cereus PLC, the three-dimensional structure of which has

been determined (17), can hydrolyze lecithin but not sphingomyelin, unlike *C. perfringens* PLC. *B. cereus* PLC, consisting of 245 amino acid residues, has been shown to be homologous to the N-terminal 248 amino acid residues of *C. perfringens* PLC (39, 41). The three-dimensional structures of the homologous regions can be superimposed almost completely (40). Thus, the additional C-terminal portion present in *C. perfringens* PLC may be associated with sphingomyelinase activity and various toxicities characteristic of *C. perfringens* PLC (39, 43). When the N- and C-terminal portions of the *C. perfringens* PLC were compared separately with the corresponding ones of the other clostridial PLCs, 169 of 248 residues (68.1%) were found to be identical to the N-terminal domain of the *C. novyi* PLC, and 60 of 122 (49.1%) were identical to its C-terminal domain; 142 of 248 residues (57.2%) were identical to the N-terminal domain of *C. bifermentans* PLC, and 54 of 124 (43.5%) were identical to its C-terminal domain. Such differences may imply that the two portions evolved under different constraints. Tyrosine, a hydrophobic residue, has been implicated in the interaction of the enzyme with membrane phospholipids (39). Of seven tyrosine residues present in the C-terminal portion of *C. perfringens* PLC, only four and three matched those in *C. novyi* PLC and *C. bifermentans* PLC, respectively (Fig. 3). This may be responsible for the difference in enzyme activity. Further analysis of the three PLCs by gene manipulation, for example, by construction of a truncated or chimeric form, should provide insights into the structure-function relationship of PLC.

In contrast to diversification of PLCs at the interspecies level, all of the *C. perfringens plc* genes are highly conserved. None of the mutations seen in the different strains affects the enzyme activity significantly. The promoter of the *C. perfringens plc* gene is one of the strongest promoters (42). It is unique in that it possesses a TG motif at position −12 and an AT block at −43, both of which compensate for a −35 region with poor resemblance to the consensus sequence (5, 31). Furthermore, the activity of this promoter is increased by an intrinsically curved DNA located immediately upstream (22). These elements increasing the promoter activity are all conserved in all of the *C. perfringens* strains examined in this study. Thus, *C. perfringens* seems to have an upgraded *plc* gene in terms of both the activity and the amount of the PLC. It may be possible that a mutation occurred in a regulatory gene of type A strains producing high levels of PLC, with the *plc* gene thereby being transcribed to the maximal level.

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