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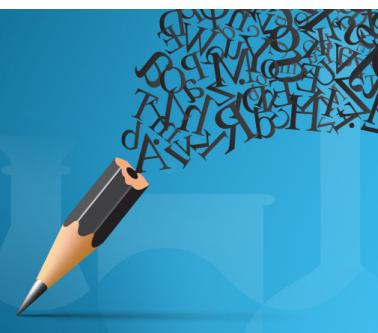


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Screening of *Bacillus thuringiensis* Natural Isolates to Find Promising Variants of Phosphatidylcholine-specific Phospholipase C

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Abstract. Phospholipase C (PLC) catalyzes the hydrolysis of phospholipids to diacylglycerol and phosphate monoesters. It has many applications in the enzymatic degumming of plant oils. Bacterial Phosphatidylcholine-specific PLC from *Bacillus spp.* is an optimal choice for industrial application in terms of its wide substrate spectrum, thermal stability, high activity, and approved safety. In this work, screening of *plc* gene sequences of *Bacillus thuringiensis* isolates, including bacteria collected from the geyser field on the Kamchatka Peninsula, was performed. Moreover, 96 *plc* gene variants were sequenced and analyzed. Finally, 12 coding sequences for different *plc* variants were identified. These coding sequences could be considered as promising candidates for the development of producer strains.

INTRODUCTION

Phospholipase C (PLC) is an enzyme that catalyzes the stereospecific hydrolysis of phospholipids into diacylglycerides and polar phosphate-containing groups [1]. PLCs carry out various functions and have been found in many living organisms, including plants, mammals, yeasts, bacteria, and fungi [2–5].

PLCs are usually divided to PLCs with specificity for phosphatidylinositol (PI-PLC) and phosphatidylcholine (PC-PLC). Bacterial PC-PLC from *B. cereus* and *B. anthracis* are widely used in industry due to its technological properties, including wide substrate spectrum, thermal stability, high activity and approved safety [6]. The protein structure of *Bacillus* PC-PLC has 24 a.a. N-terminal signal sequence to be directed into extracellular space. The active form of the enzyme is formed upon cleavage of the 14 amino acids from N-terminal prosequence by cellular proteases [7]. The mature enzyme is a small monomeric protein of 28 kDa consisting of 245 residues that binds three Zn²⁺ ions in its active site [8]. PC-PLC has broad substrate specificity and recognizes various phospholipid substrates that differ only in the structure of the head group: phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylglycerol, phosphatidylserine [9, 10].

PC-PLC is encoded by the *plc* gene (849 bp) and has a similar operon structure in the *B. cereus* group [11] [12]. The identification and phylogenetic relationships of bacteria within the *Bacillus cereus* group are still controversial. *B. thuringiensis* is genetically most similar to *B. cereus*, albeit phenotypically *B. thuringiensis* is distinguished by the production of crystal proteins [13, 14]. Although *B. cereus* and *B. thuringiensis* are classified as separate species, their taxonomy is still being discussed due to the genetic similarity of these bacteria. Not surprisingly, the phospholipase C of *B. thuringiensis* is highly homologous to the one of *B. cereus* and is a potential source of enzyme diversity.

The aim of this work was to study the diversity of PC-PLC amino acid sequences in natural isolates of *B. thuringiensis*.

MATERIALS AND METHODS

Bacterial Strains

Ninety-six isolates of *Bacillus thuringiensis* which were obtained from the collection of microorganisms of State Research Center of virology and biotechnology VECTOR (Koltsovo, Russia), including bacteria isolated from the geyser field on the Kamchatka Peninsula, were studied.

All isolates were characterized phenotypically and genetically by direct sequencing of 16S rRNA gene fragment as *B. thuringiensis* and then were tested for the phospholipolytic activity by the egg yolk agar plate method as described in [15].

Gene Sequencing

The gDNA of *B. thuringiensis* was isolated with a commercially available DNA isolation kit (Dia-m, Russia). The 16S rRNA gene fragment was amplified by standard PCR with known 27F (5'-AGAGTTTGATCMTGGCTCAG-3') sense primer and extended 1492RL (5'-CCCTACGGTTACCTTGTACGACTT-3') antisense primer to optimize melting temperature. The *plc* gene was amplified by a standard PCR protocol using one of the sense primers Bac_PLC_132F (3'-TAGTGTGGTCACGTTGACGACTG-5') or Bac_PLC_465F (3'-GAAAGGTGGATATTCTAGTCATAGGT-5') and antisense primer Bac_PLC_1505R (3'-CCTTTAGCAATTACCTTCACGT-5') that we designed based on the comparative analysis of known sequences.

Then, amplicons were purified with AMPureXP magnetic beads (Beckman Coulter Inc, USA) and sequenced using BigDye Terminator Ready Reaction Cycle Sequencing Kit 3.1 (Applied Biosystems, USA).

Gene Sequence and Encoded Amino Acid Structure Analysis

The identity of gene sequences and similarity were determined using RDP database (<http://rdp.cme.msu.edu>) for 16S rRNA and BLAST analysis for *plc*. Phylogenetic tree with *plc* gene was constructed using Maximum Likelihood algorithm with MEGA 10 software [16]. DNA fragment encoding PC-PLC was translated and aligned with MEGA 10 and MAFFT tools (<https://www.ebi.ac.uk/Tools/msa/mafft/>) [17].

RESULTS AND DISCUSSION

We have obtained DNA fragments overlapping *plc* gene sequences from 96 isolates of *B. thuringiensis*. Bac_PLC_465F and Bac_PLC_1505R primer pair led to the ~1000 bp PCR-product. However, we did not observe PCR amplification in several samples. Bac_PLC_132F and Bac_PLC_1505R primer pair led to ~1300, 2300, 2600, 4200 bp PCR products from some bacteria (Fig. 1). DNA sequencing and BLAST analysis revealed the presence of *plc* gene in all of the fragments along with some areas predicted as transposase, integrase, reporter protein and mobile element protein in long PCR-products (Fig. 2).

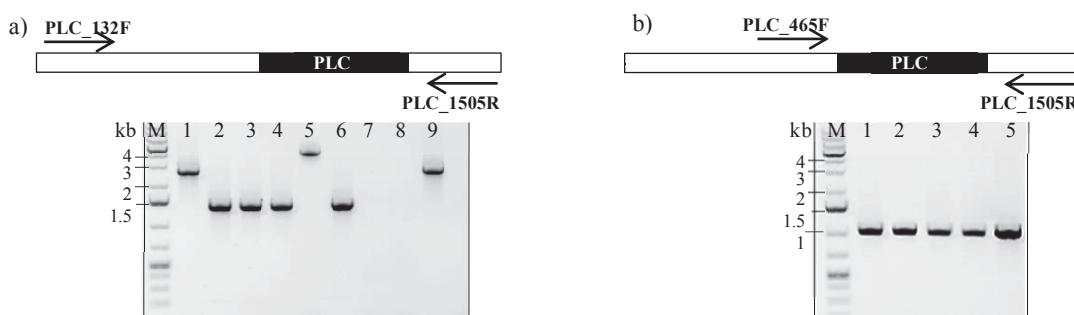


FIGURE 1. 1% agarose gel electrophoresis analysis of *plc* gene PCR products: a) line 1, Gi-466(7); line 2, Dg-1011(11); line 3, Gi-47; line 4, B-996; line 5, B-1001; line 6, B - 970; line 7, B-730; 8, B-914; line 9, B-915; b) line 1, Dg-1015; line 2, B-1000(11); line 3, B-932; line 4, B-959; line 5, B-962; M - 1Kb Plus DNA Ladder (Thermo Scientific, USA).

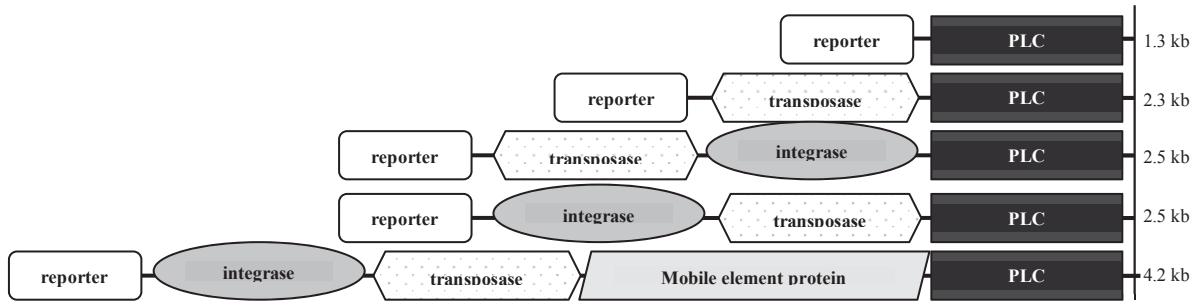


FIGURE 2. Distribution of genes in fragments obtained by PCR using PLC_132F and PLC_1505R primer pair.

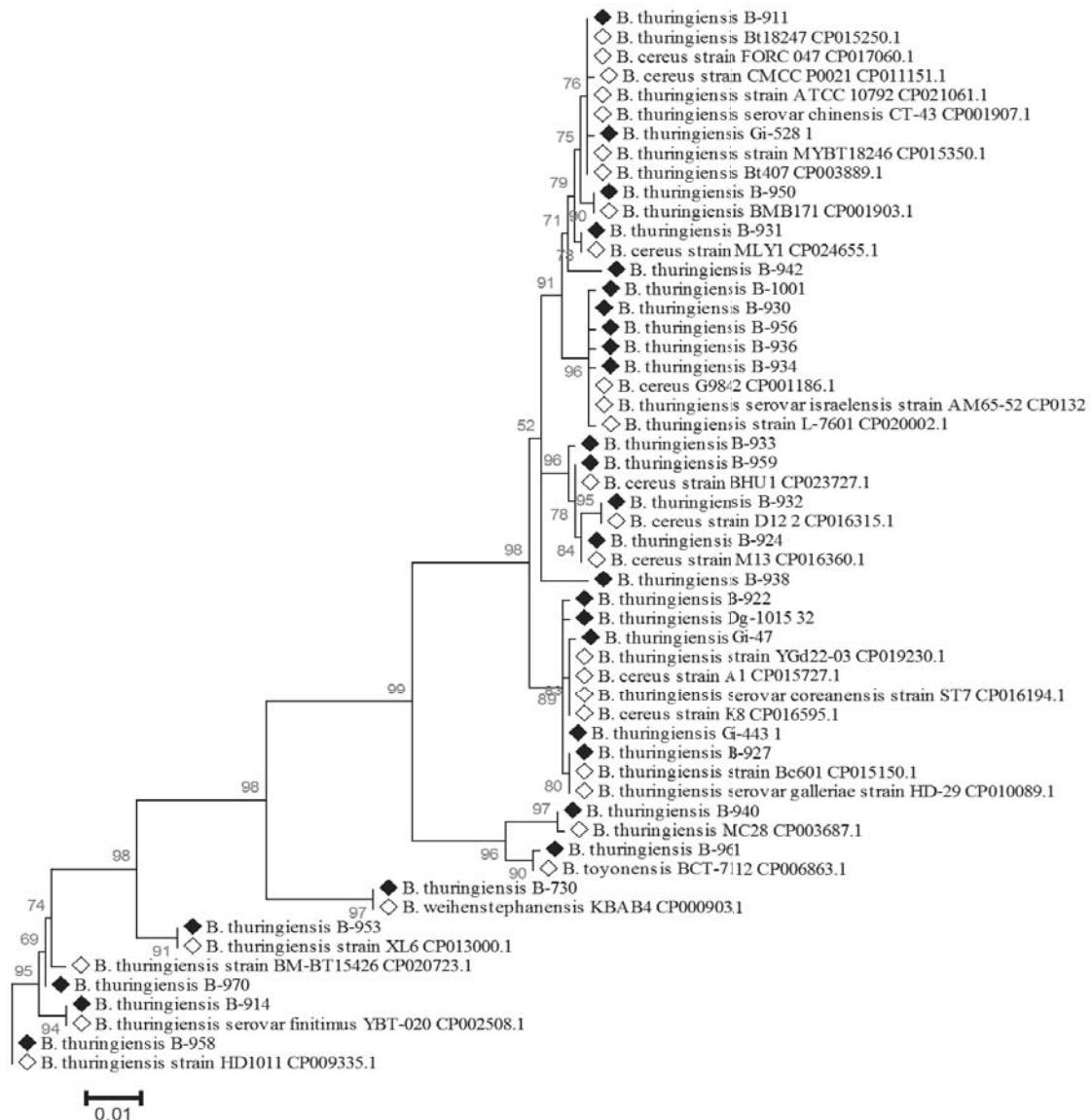


FIGURE 3. Phylogenetic tree deduced from the *plc* gene sequences of *B. thuringiensis* (filled square mark) and reference sequences (empty square mark) by maximum likelihood algorithm. Branch points supported with bootstrap values $\geq 50\%$ are indicated. The scale below shows substitution per site.

All the obtained PCR fragments encoding PC-PLC revealed high level (80–100%) of sequence similarity with *B. cereus* group genes. No unique sequences were found in bacteria isolated from Kamchatka geysers. A nonsense mutation was detected in the *plc* gene of B-934 isolate resulting in the formation of a stop codon and truncated protein production.

Phylogenetic tree constructed with *plc* gene sequences using maximum likelihood algorithm showed that the isolates are clustered closely with the members of the *B. cereus* group (Fig. 3). Interestingly, the data obtained in the egg yolk agar phospholipase activity test do not correlate with above mentioned *plc* tree: some of bacterial isolates from the same branches differed markedly in their enzymatic activity. This may depend on the properties of gene expression as well as on the activity of the other phospholipases.

As expected, amino acid sequences predicted from all ORFs revealed that primary structure of PC-PLC protein is similar to *B. cereus* enzyme, however, some amino acid substitutions were found. Phylogenetic tree constructed with PC-PLC amino acid sequences showed 12 divisions (Fig. 4, a).

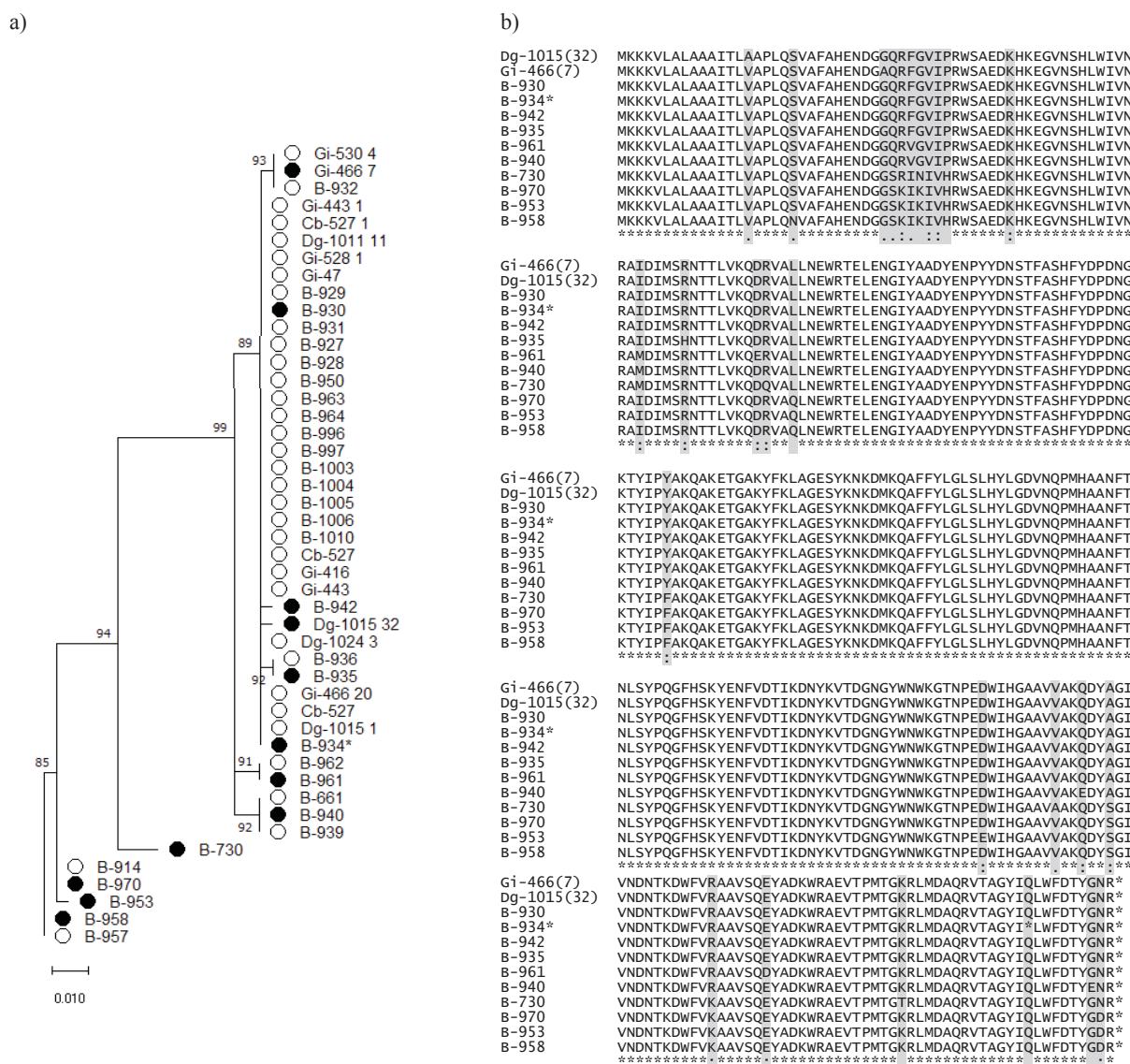


FIGURE 4. Diversity of amino acid sequences of PC-PLC from *B. thuringiensis*: a) phylogenetic tree deduced from the PC-PLC amino acid sequences by maximum likelihood algorithm: filled circle, variants of PC-PLC selected for alignment; *, truncated protein. Branch points supported with bootstrap values $\geq 50\%$ are indicated. The scale below shows substitution per site; b) multiple alignment of amino acid prosequences of selected PC-PLCs (CLUSTAL W).

One representative was selected from each division and aligned with each other (Fig. 4, b). The largest number of amino acid substitutions are localized in the prosequence regions, the remaining substitutions are diffusely scattered in whole sequences. These data revealed that some amino acid substitutions are localized close to amino acid residues that form the active site of the enzyme or bind Zn²⁺ ions. Such substitutions could significantly alter the final properties of the enzyme. Thus, the obtained PC-PLC variants may be of interest for studying the enzyme and development of recombinant enzymes with improved properties.

CONCLUSIONS

We screened 96 *Bacillus thuringiensis* isolates, including bacteria from the geyser field on the Kamchatka Peninsula, by PCR amplification and DNA Sanger sequencing to find unique variants of the *plc*. Using comparative and phylogenetic analysis of gene structures we identified 12 different PC-PLC variants from *B. thuringiensis* collection. All 12 *plc* gene variants encoding phosphatidylcholine-specific phospholipase C were cloned into the expression vector to be used as a promising enzyme source to create industrial producers.

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