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Combinatorial Fine-Tuning of Phospholipase D Expression by *Bacillus subtilis* WB600 for the Production of Phosphatidylserine

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Phospholipase D has great commercial value due to its transphosphatidylation products that can be used in the food and medicine industries. In order to construct a strain for use in the production of PLD, we employed a series of combinatorial strategies to increase PLD expression in *Bacillus subtilis* WB600. These strategies included screening of signal peptides, selection of different plasmids, and optimization of the sequences of the ribosome-binding site (RBS) and the spacer region. We found that using the signal peptide amyE results in the highest extracellular PLD activity (11.3 U/ml) and in a PLD expression level 5.27-fold higher than when the endogenous signal peptide is used. Furthermore, the strain harboring the recombinant expression plasmid pMA0911-PLD-amyE-his produced PLD with activity enhanced by 69.03% (19.1 U/ml). We then used the online tool \RBS Calculator v2.0 to optimize the sequences of the RBS and the spacer. Using the optimized sequences resulted in an increase in the enzyme activity by about 26.7% (24.2 U/ml). In addition, we found through a transfer experiment that the retention rate of the recombinant plasmid after 5 generations was still 100%. The final product, phosphatidylserine (PS), was successfully detected, with transphosphatidylation selectivity at 74.6%. This is similar to the values for the original producer.

Keywords: Phospholipase D, phosphatidylserine, ribosome-binding site calculator, *Bacillus subtilis*, secretory expression

Introduction

As a member of the phospholipase family, phospholipase D (PLD, E.C. 3.1.4.4) undergoes two types of reactions: hydrolysis, which produces phosphatidic acid and a free alcohol by cleaving the terminal phosphodiester bond of glycerophospholipids, and transphosphatidylation, which forms new phospholipids by mediating exchange of the polar headgroup of PLs [1]. The transphosphatidylation reaction is useful for the synthesis of less abundant PLs such as phosphatidylglycerol (PG) [2, 3], phosphatidylethanolamine (PE) [4, 5], phosphatidylserine (PS) [6–8], and phosphatidylinositol (PI) [9, 10], indicating that transphosphatidylation plays an important role in phospholipid modification. From the perspective of application, these phosphatidyl derivatives are useful in the food and pharmaceutical industries, especially PS, which is a main component of nerves in the brain [11–14]. It activates enzymes for the repair of damaged cells in the brain. This unique role of PS is responsible for its ability to reduce the levels of stress hormones in mental workers and relieve stress, brain fatigue, and negative moods.

To date, the gene encoding PLD has been identified in different organisms, including mammals [15, 16], yeasts [17], bacteria [6, 18, 19], and plants [20, 21]. These wild-type species have low levels of PLD, which are far below the standard for industrial production [12]. Therefore, a number of researchers have committed to isolating strains capable of efficiently producing PLD [6] or to employing heterologous expression to improve the expression level of

PLD. PLDs from different sources have been successfully expressed in *E. coli* [22, 23], *Streptomyces lividans* [18, 24], *Pichiapastoris* [25], and *B. subtilis* [19]. Among these strains, the PLD produced by the recombinant *S. lividans* had the highest extracellular PLD activity (30 U/ml), which was about 90-fold of the enzyme activity of the wild type [24]. *S. lividans* is compatible with only a limited number of expression systems, and its growth process is complex. This results in a bottleneck in large-scale expression for industrial purposes.

In this study, we aimed to construct a food-grade strain with a mature expression system that can be used for the industrial production of PLD. To achieve this, we synthesized the PLD gene (Genbank: AB573232) to increase the expression of enzyme by combining strategies and expressed this modified version in *B. subtilis* WB600. Through signal peptide screening, plasmid selection, and optimization of the RBS and spacer sequences, we successfully produced PLD, which was detected in the culture medium, whose activity was increased from 1.8 U/ml to 24.2 U/ml. Moreover, the fermentation cycle was shortened to 36 h. In addition, we tested the stability of the recombinant strain and found that the retention rate of the recombinant plasmid after 5 generations was still 100%. The results indicate that the recombinant strain could potentially be used for the industrial production of PLD. Furthermore, this lays the foundation for the use of molecular modification to enhance PLD expression in *B. subtilis* WB600.

Materials and Methods

Bacterial Strains, Plasmids, and Materials

The gene sequence encoding PLD (Genbank: AB573232) from *Streptomyces racemochromogenes* strain 10-3 was codon-optimized for *B. subtilis* and then synthesized by Suzhou Hongxun

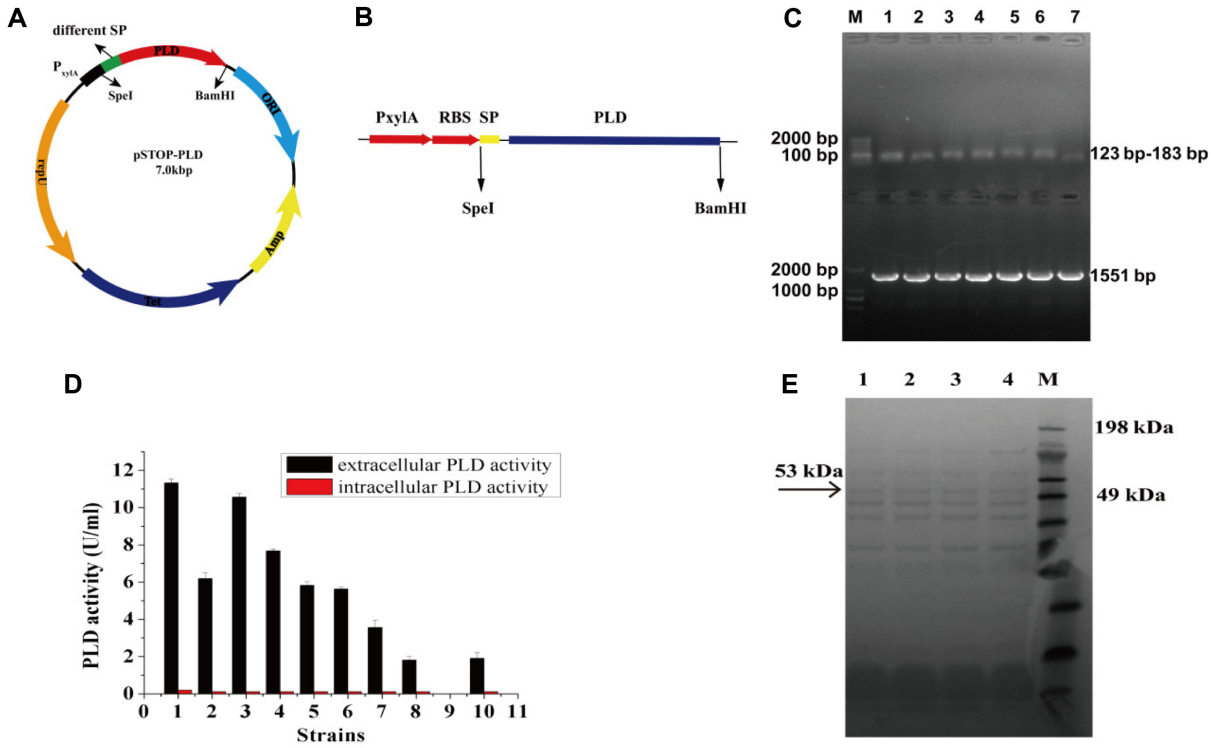

Fig. 1. The effect of different signal peptides (SP) on PLD activity.
(**A**) Construction of expression plasmid with different signal pepetides using vector pSTOP. (**B**) Schematic diagram of fusion fragment structure. (**C**) PCR analysis of seven signal peptides and PLD (The pattern in the top row, from 1 to 7 were signal peptides amyE, aprE, nprE, wapA, wprA, lipA, and ywbN, amplified from the genome of *B. subtilis* 168, respectively. The bottom row, from 1 to 7 represented PLD, which were fused with the corresponding signal peptides). (**D**) The extracellular (black) and intracellular (red) PLD activity using different signal peptides. 1-10: pSTOP-PLD-amyE, pSTOP-PLD-aprE, pSTOP-PLD-nprE, pSTOP-PLD-WapA, pSTOP-PLD-WprA, pSTOP-PLD-lipA, pSTOP-PLD-ywbN, pSTOP-PLD-Ori (the endogenous signal peptide), pSTOP (without out PLD gene), pSTOP-PLD-Zero (without signal peptide). (**E**) SDS-PAGE analysis of extracellular PLD of the recombinant strains. M: protein marker, from 1 to 4 represented extracellular protein secretion of strains 1, 3, 4, 5 (Fig. 1D) respectively.

Biotechnology Co., Ltd. (China). *E. coli* JM109 cells were used for plasmid construction, *B. subtilis* WB600 cells were used for PLD expression, and *B. subtilis* 168 (ATCC 23857) was used as the source of signal peptide sequences. The expression vectors used were pP43, pMA0911, and pSTOP. Silica gel was purchased from Qingdao Haiyang Chemical Co., Ltd. (China), and soybean lecithin (PC98) was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. (China). All other chemicals and reagents used were of analytical grade.

Construction of Phospholipase D Expression Plasmids with Different Signal Peptides

To study the effect of the signal peptide on the secretion of PLD, we separately fused seven different signal peptides to the N-terminus of mature PLD and expressed these fusion proteins in *B. subtilis* WB600 using the expression vector pSTOP (Table 2). We also compared the PLD secretion efficiency with its endogenous signal peptide and without any signal peptide respectively. The N-terminal sequence of the optimized PLD contained a sequence

Table 1. Primers used for vector cloning.

Primer	Sequence (5′ → 3′)	Restriction site
F1	GGACTAGTAAAGGAGGAAGGATCAATGTTTGCAAAACGATTCAAAACC	SpeI
R1	ATCCAGATGAGGTGTAGGTGATGCAGCACTCGCAGCCGCC	-
F2	GGACTAGTAAAGGAGGAAGGATCA ATGAGAAGCAAAAAATTGTGGATCA	SpeI
R2	ATCCAGATGAGGTGTAGGTGATGC AGCCTGCGCAGACATGTTG	-
F3	GGACTAGTAAAGGAGGAAGGATCAATGGGTTTAGGTAAGAAATTGTCTGTT	SpeI
R3	ATCCAGATGAGGTGTAGGTGATGC AGCCTGAACACCTGGCAGG	-
F4	GGACTAGTAAAGGAGGAAGGATCAATGAAAAAAAGAAAGAGGCGAAAC	SpeI
R4	ATCCAGATGAGGTGTAGGTGATGC TGCTAGTACATCGGCTGGCAC	-
F5	GGACTAGTAAAGGAGGAAGGATCA ATGAAACGCAGAAAATTCAGCTC	SpeI
R5	ATCCAGATGAGGTGTAGGTGATGC CGCTGCAGCTTTGGTTCC	-
F6	GGACTAGTAAAGGAGGAA GGATCAATGGCAAAGAAAGACGAACACC	SpeI
R6	ATCCAGATGAGGTGTAGGTGATGCCATGAGTTTTTTGAGCCCTGTATAGT	-
F7	GGACTAGTAAAGGAGGAAGGATCAATGAGCGATGAACAGAAAAAGCC	SpeI
R7	ATCCAGATGAGGTGTAGGTGATGCTGGCTTAGCCGCAGTCTGAAC	-
F11	GGCGGCTGCGAGTGCTGCATCACCTACACCTCATCTGGAT	-
R11	GCGGATCCTTACGCCTGGCAAAGGCC	BamHI
F22	CAACATGTCTGCGCAGGCTGCATCACCTACACCTCATCTGGAT	-
F33	CCTGCCAGGTGTTCAGGCTGCATCACCTACACCTCATCTGGAT	-
F44	GTGCCAGCCGATGTACTAGCAGCATCACCTACACCTCATCTGGAT	-
F55	GGAACCAAAGCTGCAGCGGCATCACCTACACCTCATCTGGAT	-
F66	ACTATACAGGGCTCAAAAAACTCATGGCATCACCTACACCTCATCTGGAT	-
F77	GTTCAGACTGCGGCTAAGCCAGCATCACCTACACCTCATCTGGAT	-
F9	GGACTAGTAAAGGAGGAAGGATCAATGGCATCACCTACACCTCATCTGGAT	SpeI
G1	GGAATTCATGTTTGCAAAACGATTCAAAACC	EcoRI
T1	CGGGATCC TTAGTGGTGGTGGTGGTGGTG CGCCTGGCAAAGGCCTC	BamHI
G2	GGGGTACCATGTTTGCAAAACGATTCAAAACC	KpnI
T2	TCCCCCGGG TTAGTGGTGGTGGTGGTGGTG CGCCTGGCAAAGGCCTC	SmaI
G3	GG ACTAGT AAAGGAGGAAGGATCAATGTTT	SpeI
T3	CGGGATCC TTAGTGGTGGTGGTGGTGGTGCGCCTGGCAAAGGCCTC	BamHI
P1	CCTCACATTTGTGCCACCTAACTTACTAAAGGAGGTTATTATATGTTTGCAAAACGATTCAA	-
S1	GTTTTGAATCGTTTTGCAAACATATAATAACCTCCTTTAGTAAG TTAGGTGGCACAAATGTGAG	-
P2	CCTCACATTTGTGCCACCTAACTTACTCCGAAGGAGGTTATTATATGTTTGCAAAACGATTCAA	-
S2	GTTTTGAATCGTTTTGCAAACATATAATAACCTCCTTCGGAGTAA GTTAGGTGGCACAAATGTGA	-
P3	CCTCACATTTGTGCCACCTAACTTACTCCGAAGGAGGTTATTTATGTTTGCAAAACGATTCAA	-
S3	GTTTTGAATCGTTTTGCAAACATAAATAACCTCCTTCGGAGTAA GTTAGGTGGCACAAATGTGA	-
P4	CCTCACATTTGTGCCACCTAACTTAGACCGAAGGAGGAAATTTATGTTTGCAAAACGATTCAA	-
S4	GTTTTGAATCGTTTTGCAAACATAAATTTCCTCCTTCGGTCTAA GTTAGGTGGCACAAATGTGA	-

The solid line portions refer to restriction sites, the dotted lines are synthetic RBS and spacers regions, and the wavy lines are His-tag sequence.

that was predicted by the SignalP 3.0 Server to encode a 26-amino acid endogenous signal peptide. From here on, we take the construction of the plasmid pSTOP-PLD-amyE as an example (Fig. 1A). Overlapping PCR was used (Fig. 1B) to construct the plasmids, and the sequences of all primers are listed in Table 1. The amyE signal peptide was amplified from the genome of *B. subtilis* 168 through PCR using the primers F1 and R1, the PLD gene was amplified from the plasmid pUC-PLD through PCR using the primers F11 and R11. After purifying the amplification products, the equal molar ratio of amplification products was mixed to serve as a template, and the full-length gene was cloned through PCR using the primers F1 and R11. A SpeI restriction site was introduced at the 5′ end of primer F1, while a BamHI restriction site was introduced at the 5′ end of primer R11. The fusion fragment was connected to the expression vector pSTOP through restriction enzyme digestion and ligation to the construct. Six other signal peptides, aprE, nprE, wapA, wprA, lipA, and ywbN, were similarly amplified from the genome of *B. subtilis* 168 using the primer pairs F2/R2, F3/R3, F4/R4, F5/R5, F6/R6, and F7/R7, respectively (Fig. 1C), and the corresponding gene constructs for the fusion proteins were amplified using the primer pairs F22/R11, F33/R11, F44/R11, F55/R11, F66/R11, and F77/R11 respectively. The selected signal peptides and their characteristics are shown in Table 2. The resulting recombinant plasmids were named pSTOP-PLD-amyE, pSTOP-PLD-aprE, pSTOP-PLD-nprE, pSTOP-PLD-WapA, pSTOP-PLD-wprA, pSTOP-PLD-lipA, pSTOP-PLD-ywbN, pSTOP-PLD-Ori, and pSTOP-PLD-Zero. pSTOP-PLD-Ori contains the putative endogenous signal peptide before the PLD sequence. The signal peptide-PLD fusion was inserted into the expression vector pSTOP through restriction enzyme digestion and ligation. On the other hand, the plasmid pSTOP-PLD-Zero did not contain any signal peptide before the PLD coding sequence. The inserted fragment was cloned through PCR using the primers F9 and R11.

Construction of Phospholipase D Expression Plasmids from Different Plasmid Backbones

The PLD gene fused to the amyE signal peptide was amplified from the plasmid pSTOP-PLD-amyE using the primers G1 and T1 and was inserted into the EcoRI-BamHI site of the vector pMA0911, which harbors an Hpa II promoter, to construct the expression vector (Fig. 2B). Similarly, using the primers G2 and T2, the PLD gene fused to the amyE signal peptide was amplified from the plasmid pSTOP-PLD-amyE and was inserted into the KpnI-SmaI site of the plasmid pP43, which encodes the P43 promoter (Fig. 2C). The primers G3 and T3 were used to introduce a His-tag into the vector pSTOP-PLD-amyE (Fig. 2A). The sequences of the constructed plasmids were confirmed. The plasmids were named pMA0911-PLD-amyE-his, pP43 -PLD-amyE-his, and pSTOP-PLD-amyE-his, and the strain *B. subtilis* WB600 containing the vectors were named ST2, ST3, and STT1, respectively. Enzyme activity and optical density (OD) were measured every 12 h.

Optimization of Ribosomal Binding Sites and Spacer Sequence

The plasmid pMA0911-PLD-amyE, which comprises RBS and spacers (AAAGGAGGAAGGATCA), was used. In order to measure translation efficiency, the 30-bp DNA sequence starting from the +1 position at the Hpa II promoter to the amyE signal peptide sequence was inputted as the protein coding sequence for analysis using the online tool RBS Calculator v2.0 [26, 27]. The sequence is shown in Fig. 3A. Then, according to the software prediction results, the RBS sequences with translation intensities 3, 6, 9, or 12 times higher than that of the original RBS sequence were selected (Table 3). The original RBS sequence of the vector was then replaced through overlap PCR. Using the plasmid pMA0911-PLD-amyE as a backbone, a series of recombinant plasmids containing RBS and spacers with different translation efficiencies were constructed. The plasmids pMA0911-PLD-amyE-3, pMA0911-PLD-amyE-6, pMA0911-PLD-amyE-9, and pMA0911-PLD-amyE-12 containing RBS and spacers 1, 2, 3, and 4, were constructed using the primer pairs P1/S1, P2/S2, P3/S3, and P4/F4, respectively. The resulting mixture after amplification was treated with DpnI for 2 h to remove the template. The resulting recombinant plasmids were named pMA0911-PLD-amyE-3, pMA0911-PLD-amyE-6, pMA0911-PLD-amyE-9, and pMA0911-

Table 2. The signal peptides used for PLD secretion in *B. subtilis* WB600.

Name	Amino acid sequence	The net charge at N domain[a]	Hydrophobicity [%][b]	D-scor[c]
amyE	MFAKRFKTSLLPLFAGFLLLFHLVLAGPAAASA	3	78.79	0.904
aprE	MRSKKLWISLLFALTLIFTMAFSNMSVQA	3	74.19	0.349
nprE	MGLGKKLSVAVAASFMSLSISLPGVQA	2	66.7	0.450
wapA	MKKRKRRNFKRFIAAFLVLALMISLVPADVLA	8	65.63	0.918
wprA	MKRRKFSSVVAAVLIFALIFSLFSPGTKAAA	4	67.74	0.450
lipA	MKFVKRRIIALVTILMLSVTSLFALQPSAKA	4	64.52	0.874
ywbN	MSDEQKKPEQIHRRDILKWGAMAGAAVAIGASGLGGLAPLVQTAAKP	0	62.79	0.735

[a]The net charge at the N domain was calculated with aspartate and glutamate defined as −1, arginine and lysine defined as +1, and any other amino acid was defined as 0.
[b]The percentage of hydrophobic residues in each signal sequence was calculated with amino acids G, A, V, L, I, M, F, W and P, which were defined as hydrophobic, and the other residues were characterized as hydrophilic. [c]SignalP 3.0 was used to calculate (http://www.cbs.dtu.dk/services/SignalP/)

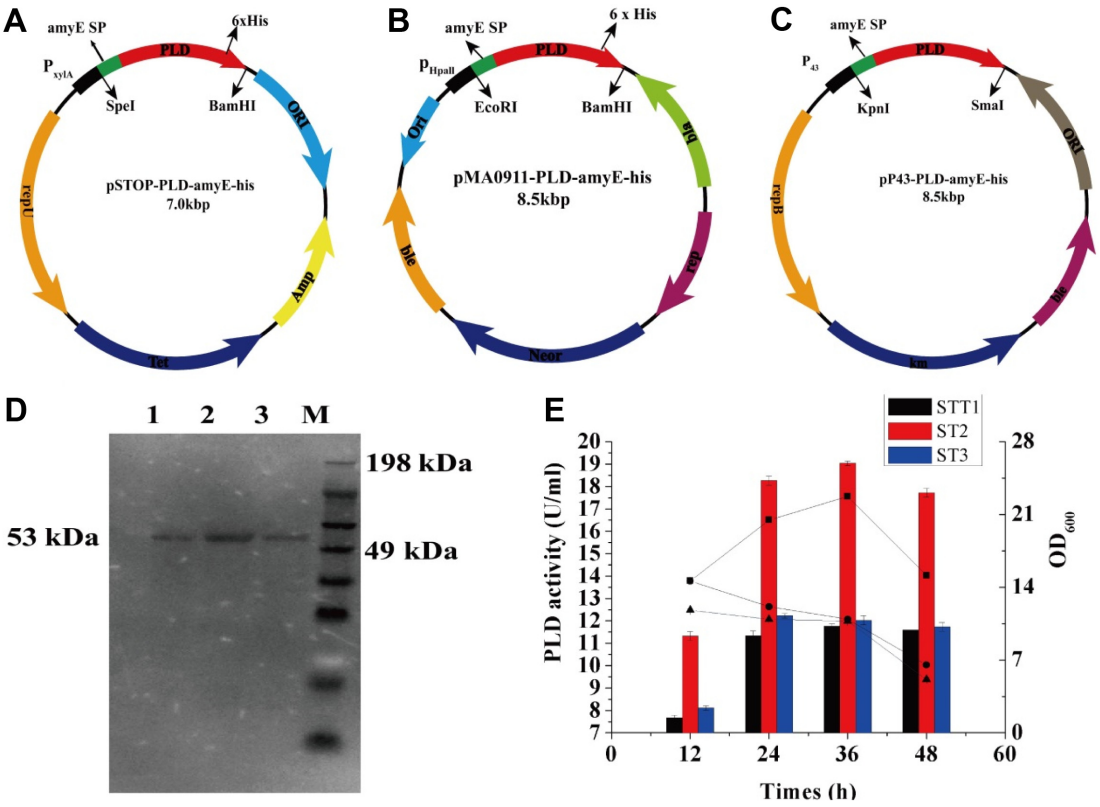

Fig. 2. The effect of different vectors on the production of PLD by the recombinant *B. subtilis* WB600.
(**A**) Expression plasmid pSTOP-PLD-amyE-his, containing the Pxyl promoter. (**B**) Expression plasmid pMA0911-PLD-amyE-his, containing the Hpa IIpromoter. **(C)** Expression plasmid pP43-PLD-amyE-his, containing the P43 promoter. All of the three recombinant plasmids were labeled with His-tag downstream of the PLD gene. (**D**) Western blot analysis of extracellular PLD of the recombinant strains. 1: STT1 (expressing PLD by strain STT1 harboring plasmid pSTOP-PLD-amyE-his), 2: ST2 (expressing PLD by strain ST2 harboring plasmid pMA0911-PLD-amyE-his), 3: ST3 (expressing PLD by strain ST3 containing plasmid pP43-PLD-amyE-his), M: protein marker. (**E**) The time profile of PLD production and OD of strains STT1 (squares), ST2 (circles), ST3 (triangles).

PLD-amyE-12 and were transformed into *B. subtilis* WB600. The resulting strains were named RS1, RS2, RS3, and RS4, respectively. The sequences of the primers used to mutate the original RBS and spacer sequences are shown in Table 1.

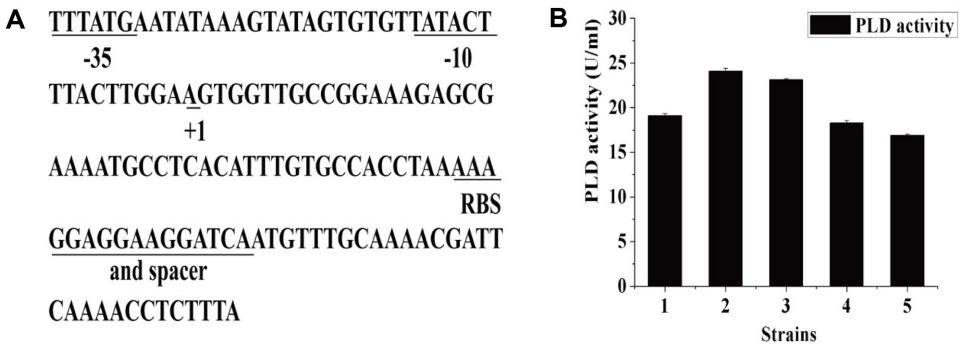

Fig. 3. Optimizing RBS and spacers sequence of vector pMA0911-PLD-amyE-his.
(**A**) Sequence to detect translation efficiency of RBS and spacers region using "RBS Calculator v2.0". (**B**) Enzyme activities with varied strength of RBS and spacers. 1: strain ST2 containing original RBS and spacers region. 2-5 the RBS sequence of translation intensity was increased by 3, 6, 9, 12 times compared to the original RBS sequence respectively.

Table 3. Sequences and parameters of RBS and spacers region with different strength evaluated by "RBS Calculator v 2.0".

Number	Strain	Multiple	Translation Initiation Rate (au)	RBS and spacers sequence
1	ST2	1	2661.39	AAAGGAGGAAGGATCA
2	RS1	3	7959.34	CTAAAGGAGGTTATTAT
3	RS2	6	16885.61	CTCCGAAGGAGGTTATTAT
4	RS3	9	23013.77	CTCCGAAGGAGGTTATTT
5	RS4	12	31535.83	GACCGAAGGAGGAAATTT

The strength of initial RBS and spacers was defined as 1 and the RBS and spacers sequences of translation intensity were increased by 3, 6, 9, and 12 times compared to the original RBS sequences which were defined as 3, 6, 9, 12, respectively.

Expression of Phospholipase D

Each constructed PLD expression plasmid was transformed into *B. subtilis* WB600. A single colony of the transformants harboring each of the constructed plasmids was inoculated into 20 ml of LB medium containing 50 μg/ml of kanamycin and was incubated at 37°C up to the logarithmic stage. Then, the seed culture was inoculated into 20 ml of fermentation medium (1.2% tryptone, 2.4% yeast extract, 0.4% glycerol, 17 mM KH_2PO_4, 72 mM K_2HPO_4) containing the same amount of kanamycin in a 250-ml flask and was incubated at 37°C with shaking at 220 rpm for 36 h. Cell growth was monitored by measuring the optical density of the culture broth at 600 nm (OD_{600}). To analyze PLD activity, 1 ml of the culture was centrifuged (8,000 ×*g* for 5 min at 4°C), and the supernatant, corresponding to the extracellular fraction, was obtained.

Genetic Stability of Recombinant Strains

A fresh single colony of the recombinant strain was transferred into 20 ml of liquid LB medium with kanamycin and incubated at 37°C with shaking at 220 rpm for 12 h. Every 12 h, aliquots of this culture were transferred into 20 ml of liquid LB medium without any antibiotics to a 3% concentration. The genetic stability of the recombinant bacteria in liquid LB medium with kanamycin was defined as 100%. The time points at which transfers were performed were recorded as the times of generations, resulting in a total of 5 generations acquired. After the bacterial solutions were diluted by a factor of about 10^3, they were then spread-plated for cultivation. Then, 100 single colonies were selected from corresponding positions in the non-resistant plate and resistant plate. Once the 100 colonies on the non-resistant plate had grown, the colonies formed on the resistant plate (with kanamycin) were counted. The number of colonies with the recombinant plasmid was then used to determine the genetic stability of the recombinant plasmid. The genetic stability of the recombinant plasmid was defined as: b/a, where "a" is the number of colonies on the non-resistant plate and "b" is the number of colonies on the resistant plate.

Measurement of Phospholipase D Activity

The hydrolytic activity of PLD was measured using the method described by Ogino [28]. The reaction mixture (total volume: 200 μl) consisted of 0.5% (w/v) egg yolk lecithin, 0.1% (v/v) Triton X-100, 40 mM Tris-HCl (pH 7.4), and 100 μl of an enzyme sample. After incubation at 37°C for 10 min, the reaction was terminated by the addition of 50 μl of a solution containing 50 mM EDTA and 200 mM Tris-HCl (pH 7.4) and heating at 95°C for 10 min. After cooling down, the reaction mixture was added to 500 μl of 20 mM potassium phosphate buffer (pH 7.6) containing 21 mM phenol, 0.59 mM 4-aminoantipyrine, 5 μl of 10 U/ml choline oxidase, and 3 μl of 10 U/ml horseradish peroxidase. After incubation at 37°C for 1 h, the absorbance of the reaction mixture was measured at 505 nm. A calibration curve was obtained by replacing the enzyme solution with a standard solution of choline chloride. One unit (U) of hydrolysis activity of PLD was defined as the amount of enzyme that produced 1 μmol of choline per minute.

Phosphatidylserine (PS) Synthesis in an Aqueous–Solid System

Soybean lecithin and L- serine were used as substrates, and purified PLD was used as the catalyst to synthesize PS through transphosphatidylation. The supernatant from cultures of the recombinant bacteria RS1, which harbors pMA0911-PLD-amyE-3, was concentrated using an ultrafiltration tube (Millipore, 10-kDa cut) and was purified using Magextractor-His Tag (TOYOBO). The purified PLD was dialyzed against 40 mM Tris-HCl (pH 7.4), and its presence was confirmed through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A single band corresponding to a protein size of about 53 kDa was observed on the gel.

Herein an environment-friendly aqueous-solid system was carried out to produce PS [29] instead of a biphasic system [30–35] or an expensive ionic liquids system [36–39] to avoid the generation of hydrolysis byproduct (PA). First, 50 mg of PC was dissolved in 5 ml of ethyl acetate and 100 mg silica gel. Then, 5 ml of a mixture consisting of ethyl acetate and acetone (1:1 v/v) was added. The resulting mixture was incubated at 37°C with shaking at 200 rpm for 3 h. The carrier-adsorbed PC was collected by centrifugation (3,500 ×*g*, 20 min, 15°C) and then washed with distilled water thrice. The precipitates were resuspended in 10 ml of 0.11 Macetic acid–sodium acetate buffer (pH 5.5) containing 1.31 M L-serine, and 100 μl of the PLD solution (15 U/ml) was added. The reaction mixture was incubated at 37°C with shaking

at 220 rpm for 24 h. After incubation, the mixture was separated by centrifugation. The precipitates were washed three times with distilled water to remove PLD and L-serine, and phospholipids adsorbed on carriers were eluted with eluting solvent (chloroform/methanol, 2:1 v/v, 3 ml × 5) [30].

Following this, 20-µl samples were taken from the elution buffer, and PC, PS, and PA were detected through HPLC-ELSD. The detection method was performed according to the *Chinese pharmacopoeia*, 2015 edition. The HPLC system used was the Dionex UltiMate U-3000, and the column used was a Sepax HP-Silica (250 mm × 4.6 mm × 5 µm). Mobile phase A was composed of methyl alcohol/water/acetic acid (85:15:0.5, v/v/v) with 0.05% triethylamine (TEA), and mobile phase B was composed of n-hexane/2-propanol/mobile A (20:48:32, v/v/v). Mobile phase B was loaded at 100% at 0 min–5 min, and was decreased gradually to 78% from 5 min to 15 min. The gradient continued to be decreased from 78% to 10% in 12 min, then 10% B was increased from 10% to 100% in 1 min. Then, 100% B was maintained for 4 min to precondition the column for the next injection. The column temperature was maintained at 40°C and flow rate at 1 ml/min. The different classes of phospholipids were detected through ELSD with the following settings: evaporation temperature, 60°C; sensitivity gain, 6; flow rate of N_2 gas, 1 l/min. Nebulizer temperature was set at 30°C. The transphosphatidylation conversion rate (%) was defined as: [PS] × 100/[PS]+[PC]+[PA], and selectivity (%) was defined as: [PS] × 100/[PS]+[PA].

Results and Discussion

Screening of Signal Peptides for Enhanced Secretion of PLD

To study the effect of the signal peptide on the secretion of PLD, seven signal peptides (AmyE, AprE, NprE, WapA, WprA, LipA, and YwbN) were separately fused to mature PLD upstream of the coding sequence. The resulting fusion protein was then expressed in *B. subtilis* WB600 using the vector pSTOP. According to our analysis of enzyme activity (Fig. 1D), PLD was successfully expressed in *B. subtilis* WB600, with the fusion protein containing the signal peptide amyE having the highest extracellular PLD activity (11.3 U/ml) and the fusion protein containing ywbN having the lowest extracellular activity (3.2 U/ml). The strain harboring pSTOP-PLD-amyE was named ST1. Fusion proteins containing aprE, wapA, wprA, or lipA had higher extracellular PLD activities than that containing ywbN. We also found that the presence of the endogenous signal peptide and the absence of any signal peptide resulted in low extracellular enzyme activities (1.8 U/ml). All cells with the PLD gene exhibited low levels of intracellular PLD activity, and cells without the gene did not show any intracellular or extracellular enzyme activity. As indicated in Fig. 1E, with relatively high extracellular enzyme activity by SDS-PAGE analysis of the extracellular protein secretion of strain 1, 3, 4, 5 (Fig. 1D), the band corresponding to the ~53-kDa target protein was observed faintly in the culture supernatants.

Signal peptide screening has been shown to be an effective method to improve the extracellular production of a target protein in *B. subtilis* [40]. Due to the specificity of the signal peptide, the secretion pathways of different kinds of proteins in *B. subtilis* are different [41, 42]. Some studies have shown that the net charge at the N domain and the hydrophobicity at the H domain of a signal peptide markedly impacts the secretion of protein [43, 44]. Based on previous research, we opted to fuse the signal peptide of PLD and seven other signal peptides with different charges at the N domain and degrees of hydrophobicity at the H domain to the PLD gene upstream of the coding sequence. We found that the signal with the highest degree of hydrophobicity and a low charge, amyE, resulted in the highest degree of extracellular protein secretion, which was 69.03% higher than that of the wild-type signal peptide (Fig. 1D). According to previous research [45, 46], one possible explanation for this effect is that hydrophobicity at the H domain of signal peptides plays an important role in maintaining an alpha-helical conformation, which allows the protein to insert into the cytoplasmic membrane. Signal peptides with higher degrees of hydrophobicity at their H domain are more efficient than those with low hydrophobicity at transporting proteins through the cytoplasmic membrane.

PLD Expression with Different Plasmids

To further explore other possible strategies to improve PLD expression, we chose the other two plasmids that are commonly used in the laboratory: pMA0911 and pP43. Fusion fragments of amyE and PLD were inserted into these two vectors. As shown in Fig. 2E, all three recombinant strains reached the highest PLD activity after 36 h of cultivation, and the intracellular residual enzyme activities of the three strains were all lower than 1 U/ml at 36 h fermentation (data not shown). The extracellular enzyme activities of ST2 and ST3 were higher than that of STT1 (compared with strain ST1, STT1 was introduced His-tag on the N end of the PLD gene, according to experimental analysis, there was no difference in the enzyme activity of STI and STT1), with the enzyme activity of ST3, which was about 69.03% higher than that of STT1, being the highest. An evident single band 53 kDa in size was observed on the western blot (Fig. 2D). This is

consistent with the size of the target protein observed in the recombinant strain. In addition, we found that the band on the blot corresponding to the protein from the strain ST2, which harbors pMA0911-PLD-amyE-his, was markedly brighter than those from the other two recombinant bacteria (Fig. 2D), which was in accordance with the observed enzyme activity. Although plasmid pP43-PLD-amyE-his has the strongest promoter among those used (p43), the activity of the enzyme from the strain harboring this plasmid was not the highest. One possible reason for this is that a strong promoter may cause the transcription and translation speeds of PLD to be too fast for it to fold correctly, thus preventing the secretion of PLD.

Meanwhile, an interesting phenomenon was observed when OD was measured at different stages of the strain cultures. After 12 h of fermentation, as fermentation time was extended, the OD value of strain STT1 became much higher than that of ST2 and ST3. We analyzed possible reasons for this phenomenon: One possible reason is that STT1 contains the vector pSTOP-PLD-amyE-his, harboring the inducible promoter Pxyl, which requires the addition of 5 g/l xylose to induce PLD expression. In contrast, ST2 and ST3 contain plasmids with constitutive promoters. Thus, the STT1 medium may have contained an additional carbon source in the form of xylose. The carbon source in the culture medium was adequate at the start of the 12 h incubation; thus, the three recombinant bacteria grew at similar rates. However, upon extension of the incubation time, the carbon sources (0.4% v/v glycerol) in the culture media of ST2 and ST3 were gradually depleted, whereas extra xylose was present in the STT1 culture medium as an additional carbon source. Thus, its growth was not affected seriously. On the one hand, these results indicate that the carbon source in the medium that was used was relatively scarce and therefore could not fully meet bacterial growth requirements. Optimization of the initial amount of carbon source, or the addition of a carbon source through flow plus can be adopted to satisfy this growth requirement. On the other hand, we found that the OD of the STT1 culture was the highest, but the activity of the enzyme from this culture was not the highest after 36 h of incubation. This indicated that bacterial growth is not positively correlated with enzyme expression.

Optimizing Translation Initiation Sequence Using the RBS Calculator v2.0

To calculate the impact of the RBS and the spacer region on translation efficiency and to screen for RBSs and spacers with the optimal strength to further improve the PLD expression at the translational level, four RBSs with different strengths were screened using the online tool RBS Calculator v2.0 [26, 27]. These were tested in the context of *B. subtilis* WB600. As shown in Fig. 3B, the activities of enzymes from strains RS1, RS2, and RS3 were higher than those of enzymes from ST2, which did not have optimized RBS and spacer sequences. In particular, the activity of the RS1 enzyme was further improved by about 26.7%, reaching 24.2 U/ml, more than 14-fold of the enzyme activity of the PLD gene that was cloned from *E. coli* K_{12} and expressed in *B. subtilis* DB 104 [19]. On the contrary, in the strain RS4, which had the strongest RBS translation rate, enzyme activity decreased by 11.8%. We found, with increasing RBS strength, PLD activity first increased and then decreased. This may suggest that an RBS that is not relatively stronger for secreting PLD is required. This phenomenon was consistent with previous findings that an optimal RBS strength could balance target gene transcription and translation [47]. In addition to analyzing the impact of RBS strength, we also explored the role of the bases in the vicinity of the RBS and the spacers. When we changed certain bases adjacent to RBS upstream, namely those from the +1 position at the promoter Hpa II to RBS, we found that the translation rate predicted by the software was significantly affected.

The Stability of Recombinant Plasmids

Since PLD is mainly used in food and medicines, substances that are toxic and harmful to humans, such as antibiotics, should not be used in the production process. Therefore, we determined the stability of plasmids without antibiotics. The genetic stability of recombinant bacteria is determined by the holding rate of the recombinant plasmid. As shown in Table 4, the retention rate of the recombinant plasmids after 5 generations was still 100%, indicating that the recombinant strain has high genetic stability. Five generations are equivalent to the expansion of a bacterial culture by 10^6. In industrial production, secondary seeds are cultivated in 100 ml flasks and inoculated into 100-ton fermentation tanks, thus, the fermentation culture medium expands in multiples of 10^5–10^6. Therefore, the genetic stability of the recombinant

Table 4. Genetic stability of recombinant strains pMA0911-PLD-amyE-his in *B. subtilis* WB600.

Time of generation	1	2	3	4	5
Non-resistant plate	100	100	100	100	100
Resistant plate with Kanamycin	100	100	100	100	100

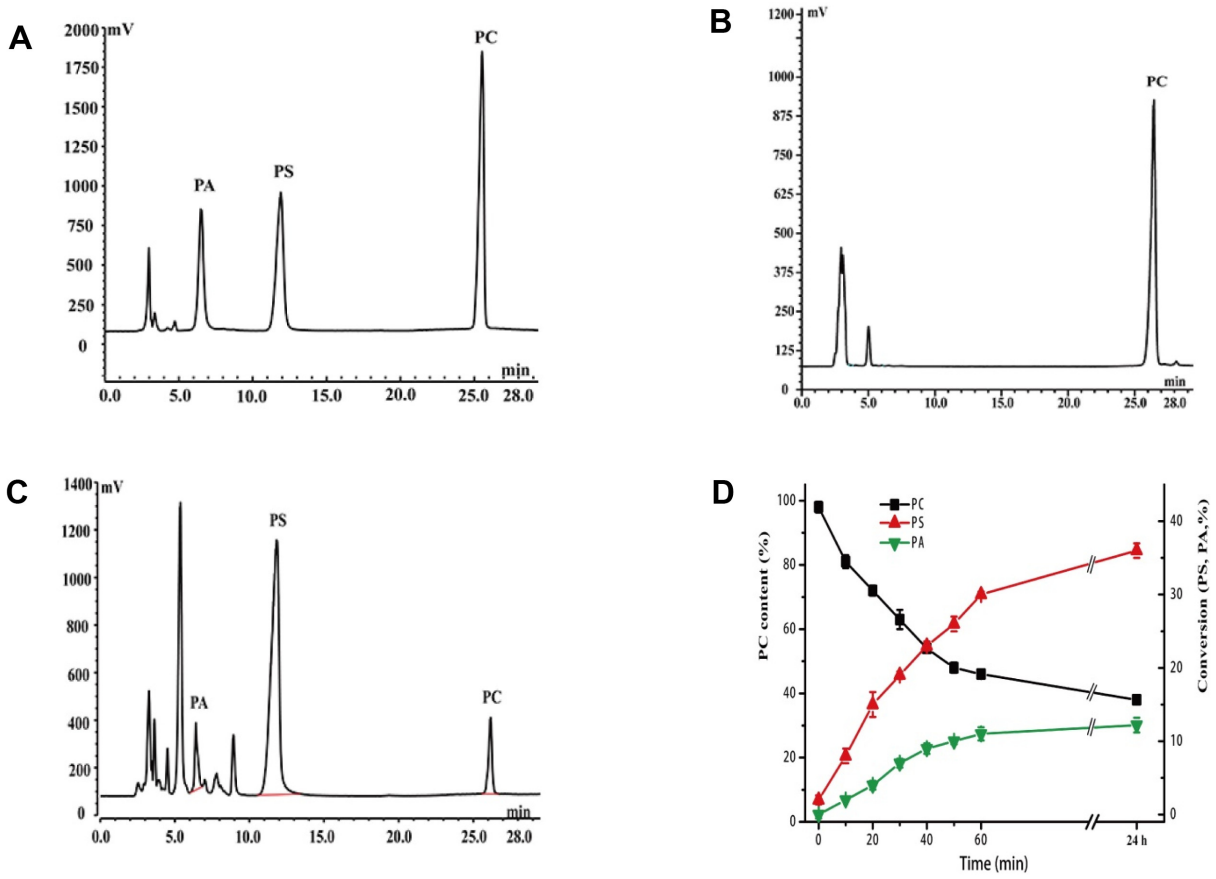

Fig. 4. The HPLC-ELSD chromatograms of PLD reaction products.

(**A**) The chromatographic peak of PS, PA, PC standard solution. (**B**) Chromatogram of PLD reaction products at 0 h. (**C**) Synthesis of PS and PA from PC by rPLD. (**D**) Time course of transphosphatidylation reaction between phosphatidylcholine and L-serine. ■, phosphatidylcholine (PC); ▲, phosphatidylserine (PS); ▼, phosphatidic acid (PA)

strains meets the needs of industrial fermentation and during production, antibiotics may not be added without the risk of a large loss of plasmids, which could cause a significant decline in production.

PS-Producing Transphosphatidylation by PLD

In order to test the transphosphatidylation capability of the PLD secreted by *B. subtilis* WB600, we used an aqueous-solid system to produce PS [29]. Detection of PS through HPLC-ELSD (Figs. 4B and 4C) suggested that lecithin and L-serine can be successfully converted into PS through the enzymatic action of recombinant PLD. The PC loading of 79.4%, and the molar ratio of PC to serine was about 1:20, and the time course of transphosphatidylation reaction indicated that the rate of PC degradation and the rate of PS formation were found to be almost identical. We detected 1.59 mmol of PS and a small amount of by-product PA (0.54 mmol) in the reaction fluid after 24 h. Moreover, the transphosphatidylation conversion rate was 36%, and selectivity was 74.6% (Fig. 4D), with the latter having a similar value to the wild type. The following experiments will systematically study the optimization of the PS generation process, such as the amount of L-serine and PLD, the selection of adsorbent for PC, the molar ratio of substrates, adsorption time and so on, to obtain the optimum reaction conditions for maximum PS conversion rate.

In summary, the PLD gene from *Streptomyces racemochromogenes* strain 10-3 was successfully expressed in *B. subtilis* WB600 and was improved through a series of combinatorial strategies, including signal peptide screening, the use of different plasmids, and optimization of the RBS and the spacer region. PLD activity increased from 1.8 U/ml to 24.2 U/ml, and fermentation duration was shortened to 36 h. Upon analysis of the transphosphatidylation reaction

catalyzed by the enzyme and of the genetic stability of the recombinant strain, we have demonstrated that the strains generated are viable, and we have succeeded in laying the foundation for a strain that can be used for the industrial production of PLD. Further work, such as pilot amplification on fermentation tanks, needs to be performed to explore industrial application.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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