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Original Research Article

Translocation of subunit PPSE in plipastatin synthase and synthesis of novel lipopeptides

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ABSTRACT

Nonribosomal peptide synthase (NRPS) is a unique molecular assembly mechanism with high hybridity. Its recombination is conducive to the development of novel lipopeptides. However, there are few reports on NRPS subunit recombination of plipastatin at present. In this paper, plipastatin synthase was modified by the forward movement of subunit PPSE and the replacement of the communication-mediating (COM) domain. The results showed that ppsABE, a new assembly line, could synthesize novel lipopeptides such as cycle pentapeptide ($C_{16\text{-}18}$β-OHFA-E-O-cyclo(Y-T-I), and its antimicrobial activity against *Rhizopus stolonifer* and *Staphylococcus aureus* was better than that of plipastatin. However, the reactivity of ppsABCE disappeared, but the substitution of $COM^{D}_{ppsC}/COM^{A}_{ppsD}$ or $COM^{D}_{ppsD}/COM^{A}_{ppsE}$ for $COM^{D}_{ppsC}/COM^{A}_{ppsE}$ could restore its activity and conduct the biosynthesis of linear hexapeptide ($C_{16\text{-}17}$β-OHFA-E-O-Y-T-E-A/V) and heptapeptide ($C_{17\text{-}18}$β-OHFA-E-O-Y-T-E-A-I). Collectively, these findings indicated that the COM donor domain at the C-terminus of PPSB could communicate with the COM acceptor domain at the N-terminus of PPSE and that the compatible COM domain is an important tool for communication between nonpartner subunits. Moreover, the integrity and selective compatibility of the COM acceptor domain of subunit PPSE are essential to promote the interaction between PPSE and other subunits. This work further complemented the rules of NRPS subunit recombination and provided a theoretical basis for the development of novel high-efficiency lipopeptides.

1. Introduction

Bacillus species are often applied as biocontrol strains owing to their ability to produce a variety of biocontrol agents [1–4]. Cyclic lipopeptides of *Bacillus*, such as surfactin, iturin, and plipastatin (fengycin), have well-known potential in biotechnology and biopharmaceutical applications due to their biosurfactant properties, antibacterial, antifungal, and antiviral activities [5–7]. Structurally, these lipopeptides share a typical cyclic structure comprised of β-amino or β-hydroxy fatty acid integrated into a peptide moiety. This particular structure also makes their chemical synthesis time-consuming and laborious. In contrast, some microorganisms could synthesize cyclic lipopeptides by the multicarrier thiotemplate mechanism of nonribosomal peptide synthase (NRPS). The NRPS has an interesting feature, its modular design, where the individual modules act as building blocks for incorporating single amino acid components into the final lipopeptide product [8]. Each module has a core C-A-T domain responsible for recognizing, activating, and loading the corresponding amino acid [9]. Some modules also have an Epimerization (E) domain, which can transform L-type amino acid to D-type amino acid to maintain the correct three-dimensional structure of the lipopetides [10,11]. In addition, there is a thioesterase (Te) domain at the end of the termination module, which is responsible for cyclizing and releasing the mature peptide products [12].

The modular structure endows NRPS systems with natural hybridity. In recent years, combinatorial biosynthesis mediated by the recombination of NRPS has become a potential way to synthesize novel lipopeptides. Jiang et al. [13] obtained high-efficient and low toxicity surfactin lipopeptide derivatives without Leu or Asp by knocking out a single module in surfactin synthase. In previous studies in our

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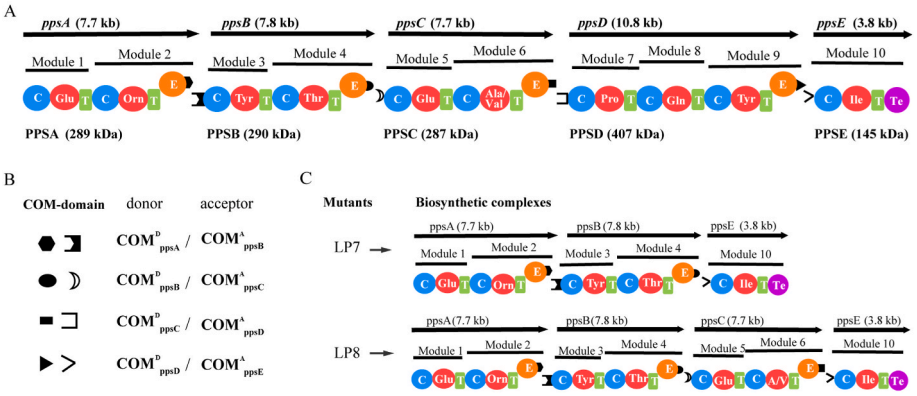

Fig. 1. Schematic representation of subunit PPSE translocation in plipastatin synthetases.(A) Plipastatin synthase operon; (B) COM domain between synthase subunits; (C) Strategy of subunit PPSE translocation.

Table 1
Plipastatin analogues produced by mutant strains.

Strains	Mass ions (m/z)	Peptide sequence	Products
pB2-L	1435.8, 1449.8, 1463.8, 1477.8, 1491.8, 1505.8, 1519.8, 1533.8	$C_{14\text{-}21}$ β-OHFA-E-O-Cyclo (Y-T-E-A/V–P-Q-Y-I/V)	Plipastatin
LP7	875.55, 889.57, 903.58	$C_{16\text{-}18}$ β-OHFA-E-O-cyclo (Y-T-I)	Cycle pentapeptide
LP8	–	–	–
LP9	980.5435, 994.5923, 1008.5950, 1022.6102	$C_{16\text{-}17}$ β-OHFA-E-O-Y-T-E-A/V	Linear hexapeptide
LP10	980.5435, 994.5923, 1008.5950, 1022.6102, 1107.6545, 1121.6705	$C_{16\text{-}17}$ β-OHFA-E-O-Y-T-E-A/V $C_{17\text{-}18}$ β-OHFA-E-O-Y-T-E-A-I	Linear hexapeptide Linear heptapeptide

laboratory, a new NRPS assembly line was constructed through the forward movement of the Te domain, and a series of linear lipopeptide products were synthesized [14].

However, the success rate of the recombination of NRPS is relatively low. The main reason for this is that the docking between modules usually depends on specific protein–protein interactions [15,16]. For example, for a module where the C-terminus is an E domain, its communication with the downstream module depends on a short recognition region comprised of 15–25 amino acid residues known as the communication-mediating (COM) domain [17]. The COM domain consists of a donor domain COM^D and a receptor domain COM^A. COM^D is located at the C-terminus of the subunit that provides aminoacyl or peptide group, while COM^A is located at the N-terminal of the receiving subunit [18,19]. COM^D and COM^A from the two interacting subunits can form a pair of compatible COM domains responsible for the loading and transfer of nonribosomal peptide intermediates according to the established template sequence and prevent the communication and interaction with nonadaptive NRPS subunits [20].

Plipastatin, similar to fengycin in structure, is a cyclic lipopeptide antibiotic. It is assembled by five NRPS multienzymes, PPSA, PPSB, PPSC, PPSD, and PPSE [21,22]. To construct a novel lipopeptide assembly line with activity, combined with the protein–protein interactions specifically mediated by COM domain selection, this study carried out the forward movement of subunit PPSE in plipastatin synthase, constructed new lipopeptide producing strains, catalyzed the synthesis of shorter novel plipastatin derived lipopeptides [23], and discussed the effects of subunit PPSE forward movement and the selective interaction of COM domains on the new hybrid synthases. This is of great significance for deepening the understanding of the interaction between different NRPS subunits and enriching the natural lipopeptides library.

2. Materials and methods

2.1. Strains and culture media

The bacterial strains were grown in Luria Broth (LB) medium, supplemented when needed with erythromycin (5 g/mL for *B. subtilis*) and kanamycin (10 g/mL for *B. subtilis*, 50 g/mL for *E. coli*). Landy fermentation medium buffered with 0.1 M 3-(N-morpholino) propanesulfonic acid (MOPS) was used to produce lipopeptides [23].

2.2. Plasmid construction

All DNA fragments were PCR-amplified from the genomic DNA of *B. subtilis* 168 with pfu DNA polymerase (Fermentas, MA, USA). The primers (Table S1) were purchased from Sangon Biotech (Shanghai, China) and contain the desired restriction endonuclease sites for the subsequent cloning of PCR products. Standard procedures were applied for all DNA manipulations [24]. DNA sequencing confirmed the identity of all plasmids constructed.

A schematic representation of plasmid construction for deletion and substitution is shown in Fig. S1. The 1422 bp upstream fragment ppsB3' and the 945 bp downstream fragment ppsE5' were amplified with the primers ppsB-F/BEsoe-R and BEsoe-F/ppsE-R, respectively. Based on the 24 bp overlapping region in BEsoe-F and BEsoe-R, both fragments were used simultaneously as the template for the subsequent fusion PCR with the primers ppsB-F and ppsE-R. The resulting fusion fragment ppsB3′-ppsE5' (containing $COM^D_{ppsB}/COM^A_{ppsE}$) was cloned into the pMD19-T vector via TATA cloning. After digestion with SalI and KpnI, the fusion fragment was ligated into pKS2 to obtain the final deletion vector pKS-BE. Similarly, the fusion fragment ppsC3′-ppsE5' (containing $COM^D_{ppsC}/COM^A_{ppsE}$) was constructed by a splice overlap extension polymerase chain reaction (SOE-PCR) using the primers ppsC-F and CEsoe-R as well as CEsoe-F and ppsE-R to yield (after cloning) the deletion vector pKS-CE. The fusion fragment ppsC3′-ppsE5' (containing $COM^D_{ppsC}/COM^A_{ppsD}$) was amplified by SOE-PCR using the primers ppsCDU–F and ppsCDsoe-R as well as ppsCDsoe-F and ppsCDD-R to give pKS-CD. The fusion fragment ppsC3′-ppsE5' (containing $COM^D_{ppsD}/COM^A_{ppsE}$) was amplified by SOE-PCR using the primers ppsDEU-F and ppsDEsoe-R as well as ppsDEsoe-F and ppsDED-R to obtain pKS-DE.

2.3. B. subtilis strain construction

The translocation of subunit PPSE in plipastatin NRPS was achieved by a homologous recombination approach mediated with a two-step

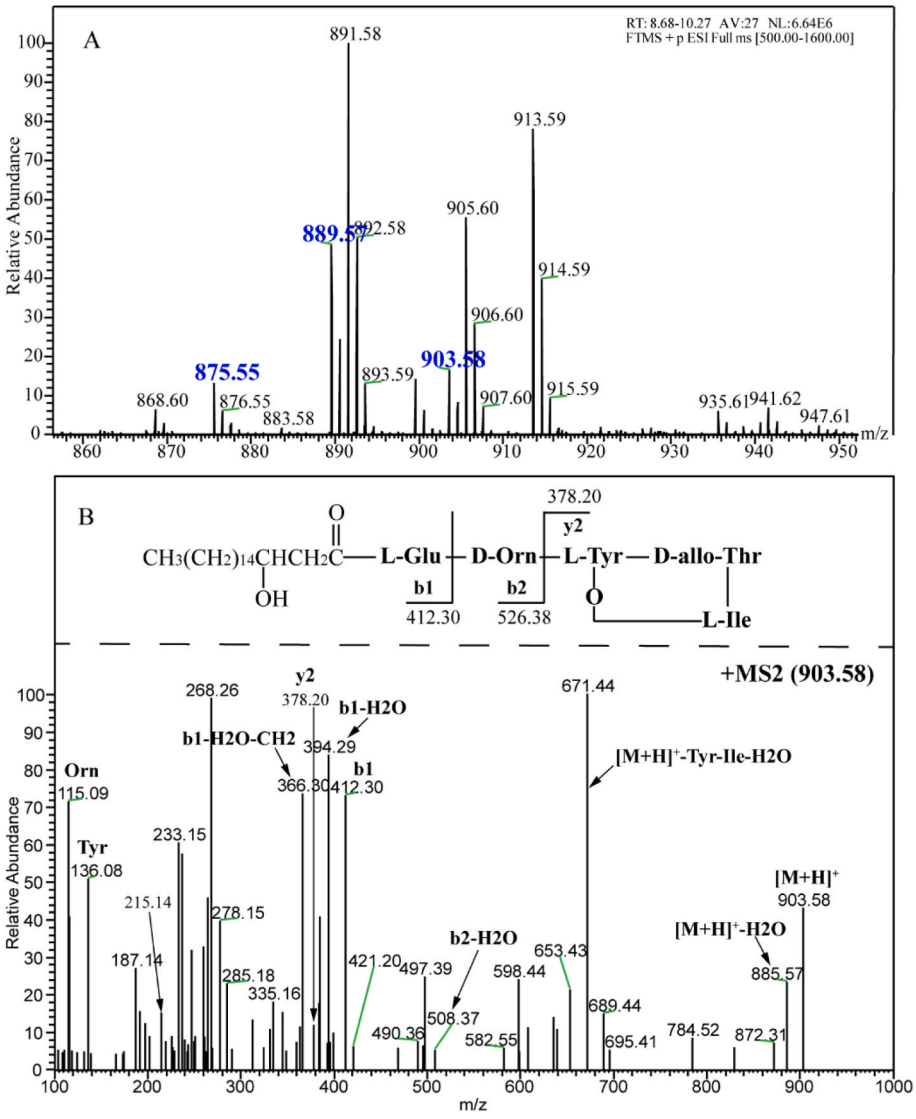

Fig. 2. ESI-MS/MS spectrum of plipastatin analogues isolated from LP7.(A) ESI-MS spectrum of plipastatin analogues isolated from mutant strain LP7; (B) ESI-MS/MS spectrum of $[M+H]^+$ ions at 903.58 *m/z*.

replacement recombination procedure [25]. Plasmids were transferred into *Bacillus subtilis* pB2-L by natural competence as previously described. After the transformation of the plasmid into the host, the entire plasmid was inserted into the chromosome via a single crossover between the target gene and a homologous sequence on the plasmid when grown in LB medium at 37 °C (a nonpermissive temperature for plasmid replication). Subsequent growth of the cointegrates in LB medium at the permissive temperature (30 °C) leads to a second recombination event, resulting in kanamycin- and erythromycin-sensitive clones with either the parental or mutant sequence. PCR analysis and sequencing were performed to confirm the mutant strain (the kanamycin and erythromycin resistance genes could not be amplified, but the upstream and downstream regions of the homologous sequence could be amplified). To this end, the deletion plasmid pKS-BE or pKS-CE was transformed into the wild-type strain *B. subtilis* pB2-L to give *B. subtilis* mutants LP7 (*ppsB*::COM^{D}_{ppsB}-COM^{A}_{ppsE}::*ppsE*) and LP8 (*ppsC*::COM^{D}_{ppsC}-COM^{A}_{ppsE}::*ppsE*). The mutant strain LP8 was transformed with plasmids pKS-CD or pKS-DE, generating *B. subtilis* mutants LP9 (*ppsC*::COM^{D}_{ppsC}-COM^{A}_{ppsD}::*ppsE*) and LP10 (*ppsC*::COM^{D}_{ppsD}-COM^{A}_{ppsE}::*ppsE*).

2.4. Product analysis

Production and purification of lipopeptides were performed as described previously. Extracts were analyzed by high-resolution liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) using a G2-XS Q-TOF mass spectrometer (Waters, USA). Samples were loaded onto a UPLC column (2.1 × 100 mm ACQUITY UPLC BEH C18 column containing 1.7 μm particles) and eluted with a solvent gradient of 5–95% buffer B for 22 min (buffer A, 0.1% [v/v] formic acid in H_2O; buffer B, 0.1% [v/v] formic acid in acetonitrile) at a flow rate of 0.4 mL min^{-1} and monitored at 205 nm.

Mass spectrometry was performed using an electrospray source in positive ion mode within a mass range of 50–1500 *m/z*. Ionization was performed with a capillary voltage of 2.5 kV, collision energy of 40 eV, source temperature of 120 °C, and desolvation gas temperature of 400 °C. Data acquisition and processing were performed using MassLynx 4.1 (Waters, USA).

2.5. Analysis of biological activity

Using *Rhizopus stolonifer*, *Fusarium oxysporum*, *Aspergillus ochraceus*, and *Staphylococcus aureus* as indicator microorganisms, the antibacterial

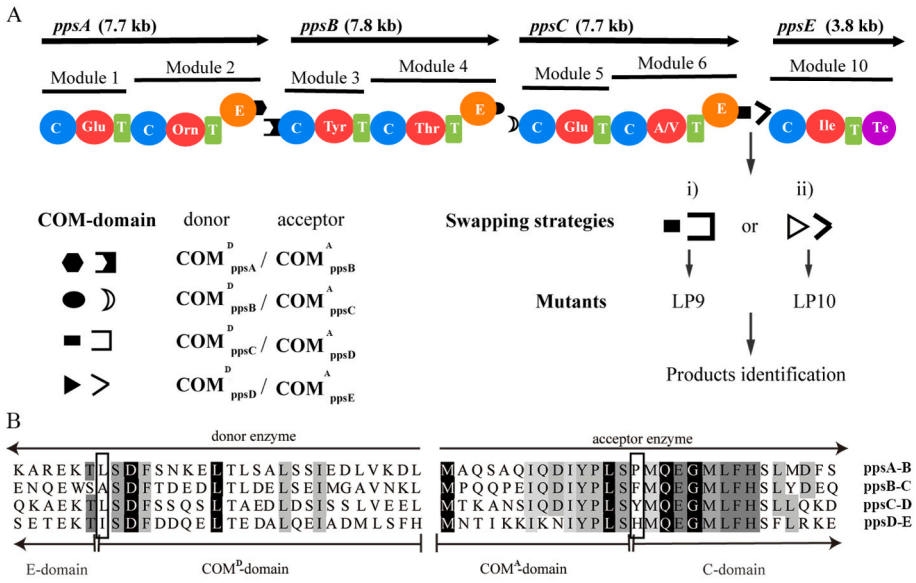

Fig. 3. Substitution strategy and sequence alignment of COM domain.(A) Schematic diagram of swapping the COM domain between subunit PPSC and PPSE in novel hybrid synthetases; (B) Sequence alignment of donor and acceptor domain derived from the plipastatin biosynthetic system. The box indicates the site of COM domain replacement.

activity of the samples was detected by the Oxford cup plate diffusion method (double-layer nutrient agar, 10 mL in the upper layer and 5 mL in the lower layer). The methanol extract of the fermentation product of mutant strains LP7, LP8, and LP9 was filtered and sterilized with a 0.45 μm PVDF membrane. Then, 100 μL of filtrate was added to the Oxford cup and cultured at 37 °C for 24 h, and the bacteriostatic effect was observed and recorded.

3. Results

3.1. Translocation of the PPSE subunit in plipastatin synthetases

To obtain plipastatin derivatives with a shorter peptide sequence, the PPSE subunit, comprising a module for incorporating Ile and the terminal Te domain, was moved forward into the end of the PPSB or PPSC subunit in plipastatin synthase. The knockout plasmids (pKS-BE and pKS-CE) containing the appropriate constructs were transferred into *B. subtilis* pB2-L, and then translocations of the subunit PPSE were generated by completely deleting PPSC and PPSD to give the corresponding *B. subtilis* mutants LP7 and LP8, as shown in Fig. 1C. The native donor COM^{D}_{ppsB} domain at the C-terminus of subunit PPSB and native acceptor COM^{A}_{ppsE} domain at the N-terminus of subunit PPSE were maintained to facilitate the interaction between the nonpartner subunit PPSB and PPSE in mutant strain LP7. Similarly, the constructed pair $COM^{D}_{ppsC}/COM^{A}_{ppsE}$ was used to facilitate contact between the nonpartner subunits PPSC and PPSE in mutant strain LP8.

After verifying the correct genotype, the fermentation products of the recombinant strains *B. subtilis* LP7 and LP8 were analyzed by high-resolution ESI-MS. A series of molecular ions with m/z values at 875.5488, 889.5644, and 903.5801 (Table 1 and Fig. 2A) were detected from the crude extract of LP7, which were consistent with the mass ions of the predicted shortened pentapeptide $C_{16\text{-}18}$β-OHFA-Glu-Orn-cyclo (Tyr-Thr-Ile). Further support for this result was obtained from an analysis of the ESI-MS/MS spectrum of $[M+H]^+$ ions at 903.5801 m/z, as shown in Fig. 2B. The cyclic molecule fragments yielded characteristic products -y_3 (378.20) and -b_2 broken at the Orn-Tyr bonds, corresponding to the macrocyclic moiety and fatty acid chain along with Glu-Orn residues. Other fragment ions at m/z 115.09 $(Orn)^+$, 136.3 $(Tyr)^+$, and internal fragmentation ions at m/z 215.14 $(Tyr + Ile)^+$, m/z 689.44 $(M + H\text{-}Tyr\text{-}Ile)^+$, m/z 671.44 $(M + H\text{-}Tyr\text{-}Ile\text{-}H_2O)^+$, m/z 653.43 $((M + H\text{-}Tyr\text{-}Ile\text{-}2H_2O)^+$, and m/z 885.57 $(M + H\text{–}H_2O)^+$ were also detected, which confirmed the sequence of the cyclic pentapeptide as C_{18}β-OHFA-Glu-Orn-cyclo(Tyr-Thr-Ile). The precursor ions m/z 875.5488 and 889.5644 differed from m/z 903.5801 with multiples of 14 Da in mass, indicating that the cyclic pentapeptides with identical peptide sequences had variable chain lengths of the β-OH fatty acid (C_{16} and C_{17}).

In contrast, we failed to detect any lipopeptide related to plipastatin in crude extracts of *B. subtilis* LP8, which suggested that the hybrid enzyme ppsABCE was inactivated. Previous studies have shown that the interaction between subunits of the NRPS complex relies on the interplay of compatible sets of donor and acceptor COM domains [26]. Thus, we speculated that the COM pair $COM^{D}_{ppsC}/COM^{A}_{ppsE}$ was incompatible, prevented the crosstalk between enzymes PPSC and PPSE, resulting in the abrogation of this hybrid biosynthetic system in LP8.

3.2. Swapping of the $COM^{D}_{ppsC}/COM^{A}_{ppsE}$ domain

To validate our speculation and restore the productivity of the hybrid synthase ppsABCE, we substituted the incompatible $COM^{D}_{ppsC}/COM^{A}_{ppsE}$ domain with the native compatible COM pair $COM^{D}_{ppsC}/COM^{A}_{ppsD}$ and $COM^{D}_{ppsD}/COM^{A}_{ppsE}$ (Fig. 3A). This cloning step was performed in a way that ensured the maintenance of the primary sequences of the conserved core motifs "TLSD" and "MQEGMLFH", which were used as fusion sites (Fig. 3B).

The corresponding COM domain swaps were generated, and the integrity of the resulting *B. subtilis* strains LP9 ($COM^{D}_{ppsC}/COM^{A}_{ppsD}$) and LP10 ($COM^{D}_{ppsD}/COM^{A}_{ppsE}$) was verified as described above. Subsequently, lipopeptide production was analyzed. As shown in Fig. 4A, the high-resolution ESI-MS revealed a series of molecular ions with m/z values of 980.5435, 994.5923, 1008.5950, and 1022.6102, which were consistent with the mass ions of the truncated hexapeptide $C_{16\text{-}17}$β-OHFA-Glu-Orn-Tyr-Thr-Glu-Ala/Val. The structures of the derivatives were further confirmed using ESI-MS/MS analysis. For example, in the ESI-MS/MS spectrum of the $[M+H]^+$ ions at 1022.6102 m/z (Fig. 4B), the observed b- and y-fragment ions permitted coverage of the entire sequence. This confirmed that the amino acid composition of the putative linear hexapeptide was Glu-Orn-Tyr-Thr-Glu-Val, where the β-OH fatty acid containing 17 carbons was linked to the N-terminus of the peptide. Other molecular ions were derived from this peptide moiety, and the 14 Da differences again indicated variations of the sixth amino

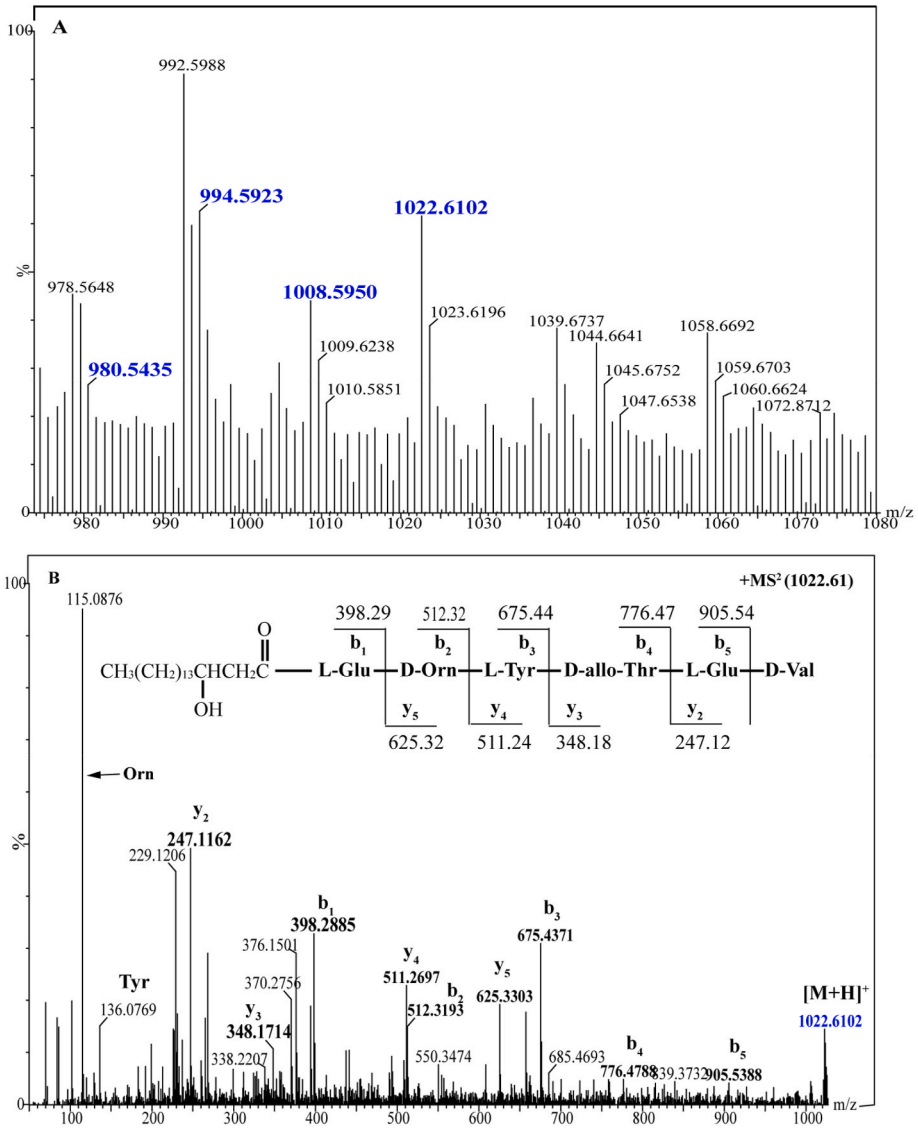

Fig. 4. High-resolution ESI-MS/MS spectrum of plipastatin derivatives isolated from LP9.(A) High-resolution ESI-MS spectrum of plipastatin derivatives isolated from mutant strain LP9; (B) MS/MS spectrum of $[M+H]^+$ ions at 1022.61 m/z.

acid (Ala or Val) and the length of the β-OH FA chain. Remarkably, no expected shortened heptapeptide could be observed in the crude lipopeptide extracts of *B. subtilis* LP9. According to these results, the $COM^{D}_{ppsC}/COM^{A}_{ppsD}$ domain swap restored the productivity of ppsABCE, leading to a truncated assembly line with PPSA, PPSB, PPSC, and Te domains that produces hexapeptides with the sequence $C_{16\text{-}17}$β-OHFA-Glu-Orn-Tyr-Thr-Glu-Ala/Val but does not allow for a productive interaction between the natural nonpartner subunits PPSC and PPSE.

In contrast, ESI-MS and ESI-MS/MS detection results for *B. subtilis* LP10 ($COM^{D}_{ppsD}/COM^{A}_{ppsE}$) revealed the formation of the expected shortened heptapeptide product. As shown in Fig. 5, the predicted linear heptapeptides C_{17}β-OHFA-Glu-Orn-Tyr-Thr-Glu-Ala-Ile (m/z calcd for $C_{54}H_{90}N_8O_{16}$ $[M+H]^+$, found 1107.6545, RT: 11.59 min) and C_{18}β-OHFA-Glu-Orn-Tyr-Thr-Glu-Ala-Ile (m/z calcd for $C_{55}H_{92}N_8O_{16}$ $[M+H]^+$, found 1121.6705, RT: 12.22 min) were detected. Using MS/MS spectra of the precursor ions [M+H]+ at m/z 1121.6705, b- and y-fragment ions were assigned, which confirmed the sequence of the heptapeptide as C_{18}β-OHFA-Glu-Orn-Tyr-Thr-Glu-Ala-Ile (Fig. 6). These results indicated that the compatible $COM^{D}_{ppsD}/COM^{A}_{ppsE}$ domain swap restored the productivity of the hybrid NRPS system ppsABCE, enforced communication between the natural nonpartner enzymes PPSC and PPSE, and eventually catalyzed the synthesis of shortened heptapeptides.

3.3. Biological activity

Based on the above results, the antimicrobial activity of lipopeptide analogues from LP7, LP9, and LP10 was analyzed. Previous studies have shown that the biological activity of plipastatin is mainly reflected in its antifungal activity [27,28]. As shown in Fig. 7, the cyclic pentapeptide produced by LP7 maintained good antifungal activity, especially against *Rhizopus stolonifer*, but the antifungal activity of linear hexapeptide and linear heptapeptide were decreased significantly.

In addition, plipastatin also has antimicrobial activity against *Staphylococcus aureus* [29]. It can reduce the intestinal colonization ability of *Staphylococcus aureus* by inhibiting its quorum sensing [30] and thus has good application prospects in the treatment of infections caused by *Staphylococcus aureus*. As shown in Fig. 7, the methanol extracts of pB2-L and LP7 had a clear inhibition zone on *Staphylococcus aureus*, and the diameter of LP7 was significantly larger than that of pB2-L. In contrast, LP9 and LP10 had no antibacterial activity against *Staphylococcus aureus*. This result showed that the change in structure

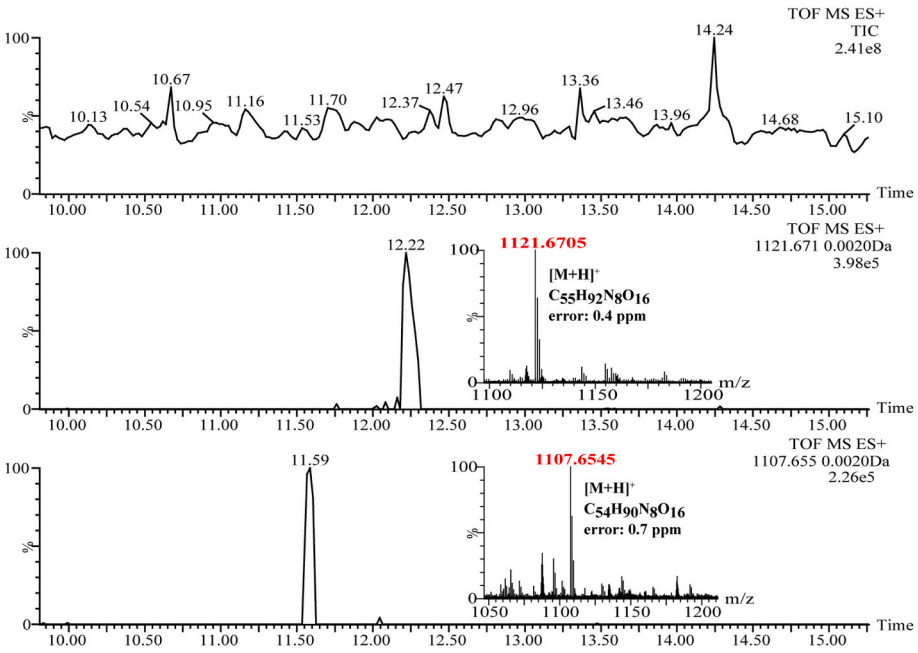

Fig. 5. LC-ESI-MS chromatogram of crude extract lipopeptides isolated from mutant strain LP10.

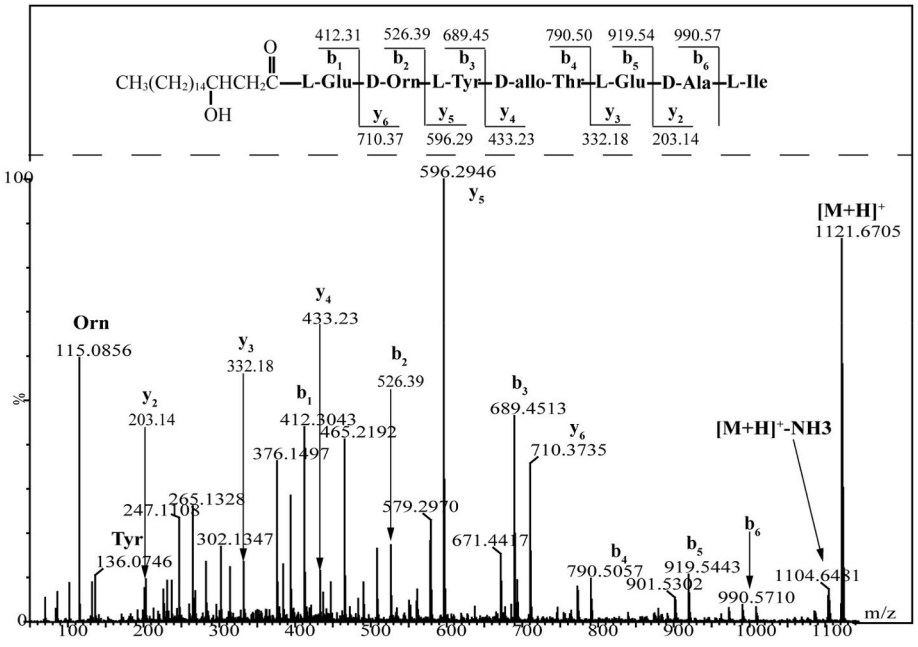

Fig. 6. MS/MS spectrum of $[M+H]^+$ ions at 1121.6705 m/z isolated from mutant strain LP10.

affected the biological activity of plipastatin. The new cyclic pentapeptide exerted an excellent inhibitory effect on *Staphylococcus aureus* and fungi, which provided a new and efficient potential drug for the treatment of *Staphylococcus aureus*.

4. Discussion

In NRPS, in addition to the selective specificity of the amino acid fusion module, the synthesis of specific lipopeptides also depends on proper communication between subunits [31–33]. The COM domain plays a vital role in promoting proper communication between cooperative enzyme subunits, preventing nonselective interactions between noncooperative enzyme subunits, and finally ensuring the synthesis of peptides according to specific assembly lines. With molecular dynamics simulation, Fage et al. [20] proposed that through T7D mutation, the selection specificity of COM^{D}_{ppsB} could be converted to COM^{A}_{ppsE}, and its recognition activity to the homologous receptor COM^{A}_{ppsB} could be weakened. This result showed that the COM domain is easy to transpose.

In this study, a new biosynthetic assembly line, ppsABE, was successfully constructed by moving the subunit PPSE forward to the C-terminus of PPSB. It could catalyze the synthesis of a new cyclic pentapeptide (shown in Table 1). These results indicated that COM^{D}_{ppsB} and COM^{A}_{ppsE} have strong compatibility and could promote the protein–protein interaction between subunits PPSB and PPSE and condense the peptidyl substrate Glu-Orn-Tyr-Thr-S-ppan with the substrate Ile. However, it should be noted that this compatibility is not universal. For

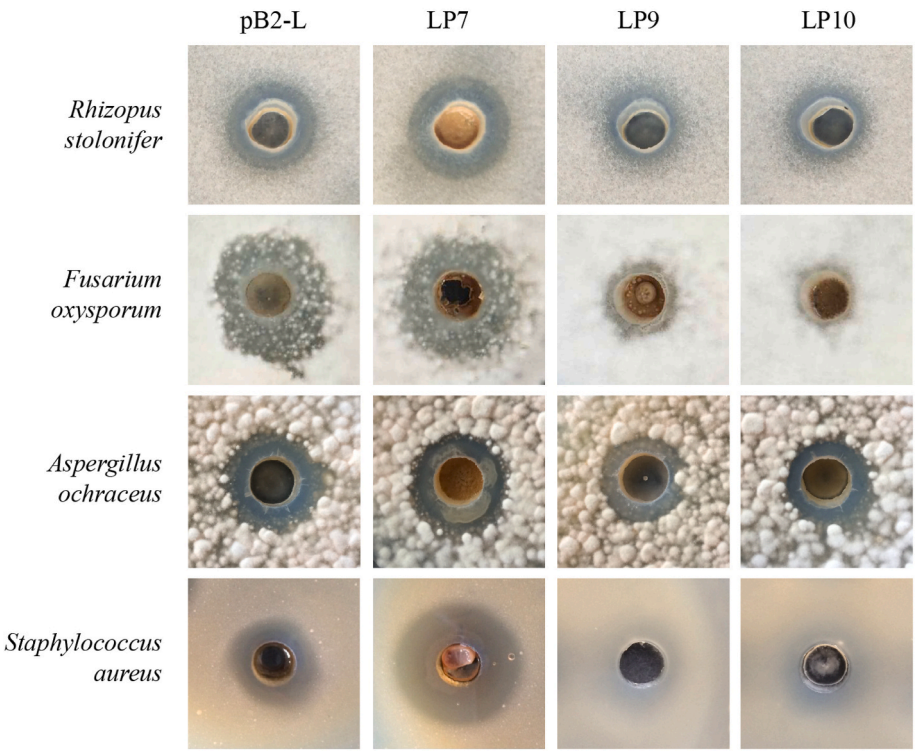

Fig. 7. Antimicrobial activity of fermentation products of mutant strains.

example, knockout of PPSD in this study caused a loss of the ability to synthesize lipopeptides in the hybrid synthetase ppsABCE in the mutant strain LP8, indicating that COM^{D}_{ppsC} and COM^{A}_{ppsE} are mismatched and incompatible with each other, just as COM^{D}_{TycA} cannot interact with COM^{A}_{TycC1} [17].

The results of COM domain substitution showed that after replacing the receptor domain COM^{A}_{ppsE} of subunit PPSE in the hybrid synthetase ppsABCE with COM^{A}_{ppsD} to form a matched and compatible COM^{D}_{ppsC}/COM^{A}_{ppsD}, the mutant strain LP9 could produce linear hexapeptide. This result revealed that COM^{D}_{ppsC}/COM^{A}_{ppsD} restored the ability of ppsABCE to synthesize lipopeptides to a certain extent. However, it cannot guide the interaction between subunit PPSC and PPSE and promote the condensation of the peptidyl substrate Glu-Orn-Tyr-Thr-Glu-Ala/Val-S-ppan with substrate Ile. In contrast, after COM^{D}_{ppsC} in ppsABCE was replaced with COM^{D}_{ppsD}, the matched and compatible COM^{D}_{ppsD}/COM^{A}_{ppsE} might promote the communication and interactions between subunit PPSC and PPSE and encourage the condensation of the peptidyl substrate Glu-Orn-Tyr-Thr-Glu-Ala-S-ppan with Ile to form a short heptapeptide intermediate, which was finally hydrolyzed and released by thioesterase to form a new linear heptapeptide $C_{17\sim18}$β-OHFA-Glu-Orn-Tyr-Thr-Glu-Ala-Ile. Similarly, Siewers [34] reported that COM^{D}_{TycA} and COM^{A}_{TycB} could match each other to realize the protein–protein interaction between the TycA and SrfAC modules and then synthesize dipeptide intermediates. These results suggest that the appropriate and compatible COM domain plays an indispensable role in protein–protein interactions and directing peptide product synthesis.

In the plipastatin synthase system, COM^{D}_{ppsB}/COM^{A}_{ppsC} are a pair of compatible and matched COM domains. However, this study demonstrated that COM^{D}_{ppsB} and COM^{A}_{ppsE} were also compatible in the hybrid synthetase ppsABE. Hahn et al. [35] proposed that the replacement of COM domains with high homology could maintain the ability of selective interactions between subunits. However, the results of the homology comparison showed that the amino acid sequence homology between receptor COM^{A}_{ppsC} and COM^{A}_{ppsE} is only 30%. It could be speculated that homology is not the main reason for the compatibility. In addition, Chiocchini et al. [18] indicated that polar and electrostatic interactions might influence the selective communication of COM domains. Previous studies in our laboratory showed that the mutation of Thr in the amino acid sequence of COM^{D}_{ppsB} to Asp changed the interaction selectivity of subunit PPSB from the original subunit PPSC to PPSD [36]. Therefore, we speculated that the interaction between COM^{D}_{ppsB} and COM^{A}_{ppsE} might also be related to the polarity and electrostatic action of some amino acids.

Changes in biological activity often accompany the new products obtained by modifying NRPS synthase. For example, the antibacterial activity of the cyclic pentapeptide against *Rhizopus stolonifer* and *Staphylococcus aureus* is significantly better than that of plipastatin. It has a broader application space in medical treatment and biological prevention and treatment. The difference is likely due to the changes in the amino acid composition and structure of the peptide chain. The antifungal activity of plipastatin is mainly realized through its damage to the cell membrane, so the change in antifungal activity is closely related to the binding ability between plipastatin and the cell membrane. Sreyoshi et al. [37] pointed out that the hydrophobic interaction between Tyr and Ile and the favorable electrostatic interaction between Glu and Orn and the lipid head group play an important role in the aggregation of plipastatin. The forward movement of PPSE directly led to changes in Tyr, Glu, and other related amino acids, which may affect the activity of lipopeptides. In addition, the ring structure also seems to have a significant impact on the antimicrobial activity of plipastatin [30]. This also explained the significant decrease in the antibacterial activity of the linear hexapeptide and linear heptadeptide obtained in our study. These results provide a direction for the development of new high-efficiency lipopeptides.

5. Conclusion

In our previous research, we found that the 10IleT-Te linker plays an important role in the forward transformation of the Te domain [14]. In contrast, this study demonstrated that the interaction and compatibility between COM domains are of great significance for maintaining the activity of NRPS synthase and found that COM^{D}_{ppsB} could selectively

interact with COM^{A}_{ppsE} in addition to COM^{A}_{ppsC}. With the interaction between COM domains, a new assembly line ppsABE and ppsABCE was constructed, and ppsABE could synthesize a cyclic pentapeptide, which has better antibacterial activity against *Rhizopus stolonifer* and *Staphylococcus aureus* than plipastatin. It would have good application prospects in the agriculture and medical industries. This study further improved and perfected the NRPS subunit recombination rules in plipastatin, offered a theoretical basis for the recombination of NRPS, and provides a method to develop new efficient lipopeptides through the recombinant NRPS assembly line.

CRediT authorship contribution statement

Ling Gao: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft. **Wenjie Ma:** Conceptualization, Formal analysis, Investigation, Data analysis, Writing – original draft. **Zhaoxin Lu:** Methodology, Resources. **Jinzhi Han:** Investigation, Validation. **Zhi Ma:** Formal analysis. **Hongxia Liu:** Validation. **Xiaomei Bie:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2022.09.001.

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