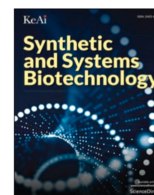
Synthetic and Systems Biotechnology 7 (2022) 989–1001

Contents lists available at ScienceDirect

Synthetic and Systems Biotechnology

journal homepage: www.keaipublishing.com/en/journals/synthetic-and-systems-biotechnology



Original Research Article

Deletion of COM donor and acceptor domains and the interaction between modules in bacillomycin D produced by *Bacillus amyloliquefaciens*

Ziyan Lv, Wenjie Ma, Ping Zhang, Zhaoxin Lu, Libang Zhou, Fanqiang Meng, Zuwei Wang, Xiaomei Bie *

Nanjing Agricultural University, College of Food Science and Technology, Nanjing, 210095, China

A R T I C L E I N F O

Keywords:
Bacillomycin D
COM domain
Interaction between modules
Lipopeptide analogs

A B S T R A C T

Bacillomycin D is a cyclic lipopeptide produced by *Bacillus amyloliquefaciens* fmbJ. At present, no relevant report has described the combinatorial biosynthesis of bacillomycin D. Due to the strong biosynthetic potential of the communication-mediating (COM) domains, its crosstalk between NRPS subunits has been studied to some extent, but the interaction of COM domain between modules is rarely reported. Therefore, in this study, we conducted the combinatorial biosynthesis of bacillomycin D through the deletion of the COM donor and acceptor domains between the modules and elucidated the interaction between the NRPS modules. The results showed that the deletion of the donor domain between modules 2 and 3 did not affect catalysis by upstream modules, but prevented downstream modules from catalysing the extension of the lipopeptide product, ultimately resulting in mutant complexes that could form linear dipeptides with the sequence β-NH_2FA-Asn-Tyr. However, the engineered hybrid bacillomycin D NRPSs lacking the donor domains between modules 3 and 4 and modules 6 and 7 could form multiple assembly lines that produced bacillomycin D and its analogs (linear tripeptides, cyclic hexapeptides and linear hexapeptides). In addition, all the acceptor domain deletion strains failed to produce bacillomycin D, only truncated peptides produced by module interruption (except for the acceptor domain deletion strains between modules 3 and 4, which also produced cyclic hexapeptides). In conclusion, deletion of the inter-module donor domains led to a more flexible hybrid biosynthetic system for the production of diverse peptide products; compared with the inter-subunit donor domain deletion strains that could only produce truncated peptides, the former had a greater biosynthetic capacity. Meanwhile, the acceptor domains between modules were an important part of module-module interactions and efficient communication within bacillomycin D synthetase.

1. Introduction

Bacillus amyloliquefaciens fmbJ belongs to the genus *Bacillus* and is a biocontrol microorganism with strong antagonistic effects on pathogenic fungi such as *Aspergillus flavus*, *Aspergillus ochraceus*, *Fusarium graminearum* and *Rhizopus stolonifer* [1–4]. Amphiphilic antimicrobial lipopeptides with β-hydroxy or β-amino fatty acid chains synthesized by nonribosomal pathways are widely present in *Bacillus* [5–7]. Due to their special amphiphilic structure, antimicrobial lipopeptides possess both antibacterial activity and biosurfactant properties [8], mainly consisting of three major families of surfactin, fengycin and iturin [9,10]. Bacillomycin D, belonging to the iturin family, is an antifungal compound with a cyclic lipopeptide structure composed of a β-amino aliphatic chain linked to a heptapeptide [11–13]. As shown in Fig. 1a, the synthase operon of bacillomycin D consists of four open reading frames, *bamD*, *bamA*, *bamB* and *bamC*, and bacillomycin D synthase is also a PKS/NRPS hybrid enzyme [14].

According to the molecular logic employed by NRPS, the biosynthesis of specific products relies on the selectivity of individual modules and their coordinated interactions [15,16]. Namely, in multienzyme complexes, the immobilized assembly line is required for interactions between adjacent modules while preventing interactions between nonadjacent modules. In general, the short terminal structure provides a pathway for the synthesis of intermediates along the correct assembly

Peer review under responsibility of KeAi Communications Co., Ltd.

* Corresponding author. Nanjing Agr Univ, Coll Food Sci & Technol, Nanjing, 210095, PR China.

E-mail addresses: 2020108005@stu.njau.edu.cn (Z. Lv), 2019108002@njau.edu.cn (W. Ma), 2019108003@njau.edu.cn (P. Zhang), fmb@njau.edu.cn (Z. Lu), zhoulb@njau.edu.cn (L. Zhou), 2016208025@njau.edu.cn (F. Meng), 2021108005@stu.njau.edu.cn (Z. Wang), bxm43@njau.edu.cn (X. Bie).

https://doi.org/10.1016/j.synbio.2022.05.007
Received 30 March 2022; Received in revised form 25 May 2022; Accepted 26 May 2022
Available online 10 June 2022
2405-805X/© 2022 The Authors. Publishing services by Elsevier B.V. on behalf of KeAi Communications Co. Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

line, similar to the docking domain in PKS synthase, known as the COM domain in NRPS [17]. As a linker, the COM domain is divided into a donor and an acceptor. The pairing of donor and acceptor domains promotes the correct positioning of the NRPS subunit in the multienzyme complex. The donor-acceptor structure of the COM domain usually consists of 15–30 amino acid residues [18], and sequence analysis predicts that both the donor and acceptor parts of the COM domain have an α-helical conformation [19,20], which is considered an important structure for the mutual selection of donors and acceptors. Furthermore, selective communication is presumed to be dominated by polar and electrostatic interactions [17]. Hahn [17,19] and colleagues mapped the position of the COM domain, revealing the highly conserved sequence T*PSD* within the E domain that is used as a fusion site between the E domain and the C-terminal donor domain (the exact fusion site is in italics). The conserved sequence L*(T/S) P*(M/L) QEG also exists at the N-terminus of the acceptor module and was identified as the fusion site between the C domain and the N-terminal COM acceptor domain. Therefore, three pairs of COM domains are present in bacillomycin D and located between module 2 and module 3 (COM^{D}_{BamB1}-COM^{A}_{BamB2}), module 3 and module 4 (COM^{D}_{BamB2}-COM^{A}_{BamB3}), and module 6 and module 7 (COM^{D}_{BamC1}-COM^{A}_{BamC2}) (see Fig. 1a and b). This differs from the positions of previously studied COM domains between subunits, such as the COM domains between TycA-TycB subunits and PpsA-PpsB subunits (see Fig. 1c and d). Therefore, throughout this paper, the comparison and discussion are carried out through the two expressions of inter-module COM domains and inter-subunit COM domains (the COM domains involved in this study are all located at the interface of the E-C domain, and the inter-modules here are located within subunits).

At present, the combinatorial biosynthesis of synthases such as surfactin [21], fengycin [22] and tyrocidine [23] has been widely reported, but the combinatorial biosynthesis strategy for bacillomycin D, a hybrid enzyme system, has not been studied. Previous researchers have successfully generated new analogs through the mutation, deletion and replacement of COM domains between NRPS subunits and suggested that the COM domain plays a decisive role in the interaction between NRPS subunits [18,22,24]. This makes it possible to uses COM domains to mediate module interactions and promote diverse polypeptide production. In this study, we analyzed the interaction between modules by deleting three pairs of COM domain donors and acceptors in bacillomycin D produced by *B. amyloliquefaciens* fmbJ. In addition, we determined whether the different positions of the donor and acceptor exerted different effects on the synthesis of bacillomycin D, whether the deletion of the COM domain affected the interaction between the multiple NRPS modules, and whether the compatibility of the COM domains between modules was consistent with the COM domains between NRPS subunits. Thus, our study complements the combinatorial biosynthesis mediated by the COM domain, and is expected to provide an additional reference for the versatility of the COM domain.

2. Materials and methods

2.1. Strains and culture conditions

B. amyloliquefaciens fmbJ, the cloned host strain *Escherichia coli* JM 109 and integration plasmid pKS2 (eliminated the *Bam*HI site) were all preserved by the Enzyme Engineering Laboratory of Nanjing Agricultural University. The demethylation host *Escherichia coli* JM110 was purchased from TransGen Biotech. Bacterial strains were routinely grown on Luria–Bertani (LB) agar plates or in LB broth at 37 °C. The seed medium contained 5.0 g L^{-1} beef extract, 5.0 g L^{-1} yeast extract, 10.0 g L^{-1} peptone and 5.0 g L^{-1} NaCl. The fermentation medium used for lipopeptide production was described previously [2,25].

2.2. Construction of deletion plasmids

The plasmids with donor and receptor domain deletions in bacillomycin D synthase are all derivatives of pKS2. The corresponding donor and acceptor domains were deleted using plasmids pKS2ΔCOM^{D}_{BamB1},

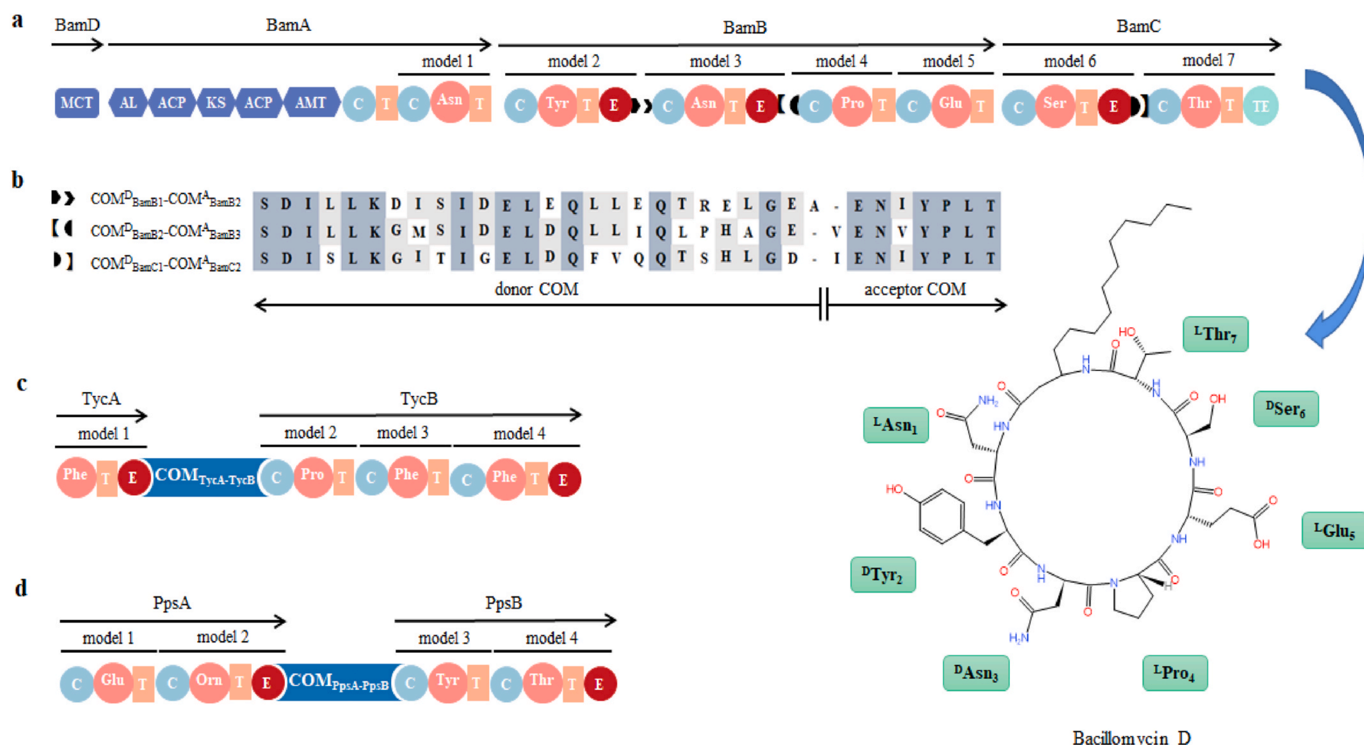


Fig. 1. Overview of bacillomycin D biosynthesis. (a) The bacillomycin D synthase operon and its chemical structure, in which the three pairs of black symbols represent the three pairs of COM domains in bacillomycin D, (b) complete amino acid sequence of the COM domain in bacillomycin D, (c) COM domain between TycA and TycB subunits in tyrocidine synthase, and (d) COM domain between PpsA and PpsB subunits in plipastatin synthase.

pKS2ΔCOM^{D}_{BamB2}, pKS2ΔCOM^{D}_{BamC1}, pKS2ΔCOM^{A}_{BamB2}, pKS2ΔCOM^{A}_{BamB3} and pKS2ΔCOM^{A}_{BamC2}. Primers were designed according to the genome sequence of *B. amyloliquefaciens* fmbJ [25], and the primer sequences are shown in Table 1. For pKS2ΔCOM^{D}_{BamB1}, the upstream (548 bp) and downstream (527 bp) DNA fragments of the donor COM^{D}_{BamB1} were amplified by PCR with the primer pairs COM^{D}_{BamB1}-UF/R and COM^{D}_{BamB1}-DF/R, respectively. These two fragments were used as templates for splice overlap extension polymerase chain reaction (SOE-PCR) with primers COM^{D}_{BamB1}-UF and COM^{D}_{BamB1}-DR. The upstream and downstream fusion fragments of COM^{D}_{BamB1} were obtained and cloned into the *Sal*I-*Kpn*I sites of pKS2 using a one-step recombinase to obtain pKS2ΔCOM^{D}_{BamB1}. The obtained plasmid was sequenced and verified by GenScript Bio. The other deletion plasmids, pKS2ΔCOM^{D}_{BamB2}, pKS2ΔCOM^{D}_{BamC1}, pKS2ΔCOM^{A}_{BamB2}, pKS2ΔCOM^{A}_{BamB3} and pKS2ΔCOM^{A}_{BamC2}, were constructed in the same manner as pKS2ΔCOM^{D}_{BamB1}.

2.3. Construction of Bacillus amyloliquefaciens deletion mutants

Each deletion plasmid was electroporated into *B.amyloliquefaciens* fmbJ [26]. Using the kanamycin and erythromycin markers present in the pKS2 plasmid, the positive transformants were screened with 20 μg mL^{-1} kanamycin and 5 μg mL^{-1} erythromycin at 30 °C. The deletion mutants were selected using a two-step replacement recombination procedure described previously [5,27]. The strain was grown at 37 °C (a temperature not allowed for plasmid replication) in the presence of kanamycin and erythromycin to select clones in which the plasmid was integrated into the chromosome between the target gene and a homologous sequence on the plasmid through a single crossover [5]. Afterward, a separate clone of the integrant was cultured in LB medium at 30 °C for 5 generations (12 h per generation) to induce a second crossover event and excise the plasmid. The obtained kanamycin- and erythromycin-sensitive strains with original or deleted sequences were verified by PCR and sequencing (see Table 2 for the primers used in this experiment). The mutant strains were named fmbJD1, fmbJD2, fmbJD3, fmbJA1, fmbJA2 and fmbJA3 (see Table 3).

Table 2
Primer sequences used to verify donor and acceptor deletion strains.

Name	Sequence(5'∼3')
ΔCOM^{D}_{BamB1}-F	CGCCTAATTCACGCGTCTG
ΔCOM^{A}_{BamB2}-F	GGCGTTAATGGATAAATATTTTCCGCTTC
ΔCOM^{D}_{BamB2}-F	CCAATTCGTCAATGGACATGCCT
ΔCOM^{A}_{BamB3}-F	AGTGAGCGGATACACGTTTTCAACC
ΔCOM^{D}_{BamC1}-F	TGATCCAATTCACCGATCGTTATACC
ΔCOM^{A}_{BamC2}-F	CGGGGTTAATGGGTATATATTTTCAATGTC

The upstream primer sequences all contain the corresponding donor and acceptor sequences. If no bands appear after gel electrophoresis, the donor or acceptor domains have been deleted; the downstream primer sequences are the corresponding DR primers listed in Table 1 above.

2.4. Extraction and identification of lipopeptide antibacterial substances

Single colonies of the wild-type fmbJ and mutant strains obtained after activation were inoculated into the seed medium, cultured at 37 °C and 180 rpm to the logarithmic growth phase, inoculated into the fermentation medium at an inoculum concentration of 5% and cultured at 30 °C for 120 h with shaking at 180 r·min^{-1}. After centrifugation, the fermentation broth supernatant was collected, and its pH was adjusted to 2.0 with 6 M HCl. Then, the solution was stored at 4 °C until further treatment. Subsequently, the precipitate was collected after centrifugation, and the supernatant was discarded. Methanol was added to dissolve the pellet, and then the pH was adjusted to 7.0 with NaOH and the sample was centrifuged at 10,000 g for 10 min to obtain the crude lipopeptide extract [1,25]. After the crude extract was filtered through a 0.22 μm membrane, a Waters Xevo G2-XS Q-TOF mass spectrometer was used for high-resolution liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). HPLC and mass spectrometry conditions were described in the studies by Gong and Qian et al. [1,2].

3. Results

3.1. Donor and acceptor knockout strain acquisition and HPLC detection

As shown in Table 3, we deleted three pairs of COM domain donors

Table 1
Primer sequences used to construct donor and acceptor deletion strains.

Name	Sequence (5'∼3')	Restriction site
pKS2-F	GgtcgacGGTATCGATAAGCTT	*Sal*I
pKS2-R	ggtaccCAATTCGCCCTATAGTG	*Kpn*I
COM^{D}_{BamB1}-UF	TATAGGGCGAATTGggtaccCGGCCGAGCCATTCAATATATTG	*Kpn*I
COM^{D}_{BamB1}-UR	AGCAGACTTCTCTCACACCAGAAAATATTTATCCATTAACGCCGATGC	
COM^{D}_{BamB1}-DF	TGGTGTGAGAGAAGTCTGCTTCCGGCTTACGCAATGTG	
COM^{D}_{BamB1}-DR	GCTTATCGATACCgtcgacCTCGTGAATTTGGAAGGCCAC	*Sal*I
COM^{A}_{BamB2}-UF	TATAGGGCGAATTGggtaccCAATAAGCCGTGGCCTCTTTC	*Kpn*I
COM^{A}_{BamB2}-UR	CGCGTGAATTAGGCGAAGCGCCGATGCAGAAGGGCATG	
COM^{A}_{BamB2}-DF	CGCTTCGCCTAATTCACGCGTCTGCTCCAAAAGCTGTTCC	
COM^{A}_{BamB2}-DR	GCTTATCGATACCgtcgacCCACAAGCCAGTACCCTGTC	*Sal*I
COM^{D}_{BamB2}-UF	TATAGGGCGAATTGggtaccGCTGCCGATGACGGTTCTG	*Kpn*I
COM^{D}_{BamB2}-UR	AACAATCGGAACTGACGCCAGTTGAAAACGTGTATCCGCTCAC	
COM^{D}_{BamB2}-DF	TGGCGTCAGTTCCGATTGTTCTTTATGAACGCAATGCGTAATGATGG	
COM^{D}_{BamB2}-DR	GCTTATCGATACCgtcgacCGCATTGGTCAGGACTGGAAC	*Sal*I
COM^{A}_{BamB3}-UF	TATAGGGCGAATTGggtaccGGCGGTCAAGCCACTCGATATAG	*Kpn*I
COM^{A}_{BamB3}-UR	AACTGCCGCATGCAGGTGAGCCGATGCAGAAAGGAATGCTC	
COM^{A}_{BamB3}-DF	CTCACCTGCATGCGGCAGTTGAATGAGGAGCTGATCCAATTCGTC	
COM^{A}_{BamB3}-DR	GCTTATCGATACCgtcgacCGTATCCGGTTGTGCTGCACG	*Sal*I
COM^{D}_{BamC1}-UF	TAGGGCGAATTGggtaccGGGGCTGTATGGAGTGACGGC	*Kpn*I
COM^{D}_{BamC1}-UR	ATCAAATTCACCTGACGCCAATTGAAAATATATACCCATTAACCCCGATGC	
COM^{D}_{BamC1}-DF	TGGCGTCAGGTGAATTTGATCTTGAGCATCACAATGTGCG	
COM^{D}_{BamC1}-DR	TATCGATACCgtcgacCTCGGCTATTCATCGTTGGACCGG	*Sal*I
COM^{A}_{BamC2}-UF	TAGGGCGAATTGgttaccGGCCAATAGGCTGACGCCTG	*Kpn*I
COM^{A}_{BamC2}-UR	AGACGAGTCATCTCGGTGACCCGATGCAGAAAGGAATGTTGTTC	
COM^{A}_{BamC2}-DF	GTCACCGAGATGACTCGTCTGCTGCACAAATTGATCCAATTCAC	
COM^{A}_{BamC2}-DR	TATCGATACCgtcgacCTCGGTGGGATGGTTCACAAGCCT	*Sal*I

Lowercase bases represent the restriction site, and underlined bases represent the homologous sequence.

Table 3
The strains used in this study and their lipopeptide products.

Deletion strategies		Strains	Lipopeptide products	Molecular formula	Mass(m/z)	Peptide sequence	Yield (%)
Wild type		fmbJ	bacillomycin D	$C_{48}H_{74}N_{19}O_{15}$ $C_{49}H_{76}N_{19}O_{15}$ $C_{50}H_{78}N_{19}O_{15}$ $C_{51}H_{80}N_{19}O_{15}$	1031.5413 1045.5570 1059.5726 1073.5883	$C_{14\text{-}17}$β-NH_2FA-Asn-Tyr-Asn-Pro-Glu-Ser-Thr	100
Donor deletion	the donor between modules 2 and 3 (COM^{D}_{BamB1})	fmbJD1	linear dipeptide	$C_{27}H_{44}N_4O_6$ $C_{28}H_{46}N_4O_6$ $C_{29}H_{48}N_4O_6$	521.3327 535.3483 549.3625	$C_{14\text{-}16}$β-NH_2FA-Asn-Tyr	100
	the donor between modules 3 and 4 (COM^{D}_{BamB2})	fmbJD2	bacillomycin D	$C_{48}H_{74}N_{19}O_{15}$ $C_{49}H_{76}N_{19}O_{15}$ $C_{50}H_{78}N_{19}O_{15}$ $C_{51}H_{80}N_{19}O_{15}$	1031.5404 1045.5569 1059.5721 1073.5881	$C_{14\text{-}17}$β-NH_2FA-Asn-Tyr-Asn-Pro-Glu-Ser-Thr	89.05
			linear tripeptide	$C_{31}H_{50}N_6O_8$ $C_{32}H_{52}N_6O_8$ $C_{33}H_{54}N_6O_8$	635.3770 649.3930 663.4085	$C_{14\text{-}16}$β-NH_2FA-Asn-Tyr-Asn	9.75
			cyclic hexapeptide	$C_{43}H_{67}N_9O_{14}$ $C_{44}H_{69}N_9O_{14}$ $C_{45}H_{71}N_9O_{14}$ $C_{45}H_{73}N_9O_{14}$ $C_{46}H_{75}N_9O_{14}$	934.4902 948.5037 962.5222 976.5385 990.5536	$C_{14\text{-}18}$β-NH_2FA-Asn-Tyr-Asn-Glu-Ser-Thr	1.20
	the donor between modules 6 and 7 (COM^{D}_{BamC1})	fmbJD3	bacillomycin D	$C_{48}H_{74}N_{19}O_{15}$ $C_{49}H_{76}N_{19}O_{15}$ $C_{50}H_{78}N_{19}O_{15}$ $C_{51}H_{80}N_{19}O_{15}$	1031.5404 1045.5571 1059.5723 1073.5874	$C_{14\text{-}17}$β-NH_2FA-Asn-Tyr-Asn-Pro-Glu-Ser-Thr	91.88
			linear hexapeptide	$C_{44}H_{69}N_9O_{14}$ $C_{45}H_{71}N_9O_{14}$ $C_{46}H_{73}N_9O_{14}$	948.5020 962.5234 976.5341	$C_{14\text{-}16}$β-NH_2FA-Asn-Tyr-Asn-Pro-Glu-Ser	8.12
Acceptor deletion	the acceptor between modules 2 and 3 (COM^{A}_{BamB2})	fmbJA1	linear dipeptide	$C_{27}H_{44}N_4O_6$ $C_{28}H_{46}N_4O_6$ $C_{29}H_{48}N_4O_6$	521.3337 535.3492 549.3650	$C_{14\text{-}16}$β-NH_2FA-Asn-Tyr	100
	the acceptor between modules 3 and 4 (COM^{A}_{BamB3})	fmbJA2	linear tripeptide	$C_{31}H_{50}N_6O_8$ $C_{32}H_{52}N_6O_8$ $C_{33}H_{54}N_6O_8$	635.3776 649.3937 663.4080	$C_{14\text{-}16}$β-NH_2FA-Asn-Tyr-Asn	57.85
			cyclic hexapeptide	$C_{43}H_{67}N_9O_{14}$ $C_{44}H_{69}N_9O_{14}$ $C_{45}H_{71}N_9O_{14}$	934.4950 948.5126 962.5281	$C_{14\text{-}16}$β-NH_2FA-Asn-Tyr-Asn-Glu-Ser-Thr	42.15
	the acceptor between modules 6 and 7 (COM^{A}_{BamC2})	fmbJA3	linear hexapeptide	$C_{44}H_{69}N_9O_{14}$ $C_{45}H_{71}N_9O_{14}$	948.5079 962.5276	$C_{14\text{-}15}$β-NH_2FA-Asn-Tyr-Asn-Pro-Glu-Ser	100

The yield here is only the relative proportion of different lipopeptides produced by the same strain, which is obtained by integrating the peak area, not the real yield of lipopeptides.

and acceptors in bacillomycin D to study the interaction between modules. First, the constructed deletion plasmids were electroporated into the target strain *B. amyloliquefaciens* fmbJ, and then the mutant strains fmbJD1, fmbJD2, fmbJD3, fmbJA1, fmbJA2 and fmbJA3 were obtained through markerless deletion using temperature-induced homologous recombination double crossovers. The kanamycin- and erythromycin-sensitive strains were verified by PCR, and the strains that verified correct were sent to GenScript Bio Company for sequencing.

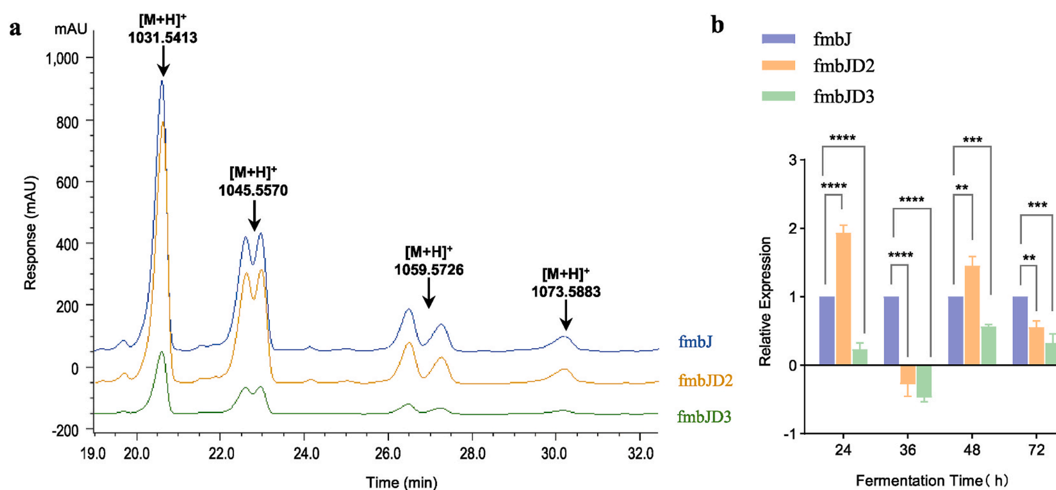


Fig. 2. Deletion of the donor domain COM^{D}_{BamB2} does not affect the production of bacillomycin D. (a) HPLC analysis of fermentation crude extracts. The production of bacillomycin D (four homologs of C14-17) in the fermentation crude extracts of strains fmbJ, fmbJD2 and fmbJD3 were 331.51 ± 5.94, 335.69 ± 10.31 and 49.73 ± 11.93 mg L^{-1}, respectively. (Standard curve: y = 7.6396x-2.3576; x, content of bacillomycin D, mg·L^{-1}; y, peak area, mAU·h), and (b) determination of relative expression of TE domain.

According to the sequencing results, the donor and acceptor domain deletion strains have been obtained (supplementary materials: Fig. S1).

The crude antibacterial lipopeptide extracts obtained by the fermentation of the 6 mutant strains described above were analyzed using HPLC. It was found that the presence of bacillomycin D was only detected in the fermentation crude extracts of the donor deletion strains fmbJD2 and fmbJD3. The bacillomycin D production level of fmbJD2 was comparable to that of wild-type fmbJ, while the bacillomycin D production of fmbJD3 was significantly decreased (see Fig. 2a). The expression level of TE domain in fmbJD3 was significantly decreased at all times during the fermentation process, especially at 36 h of fermentation; while the donor deletion strain fmbJD2 did not show a decrease in the expression of the TE domain throughout the fermentation process (see Fig. 2b). This was due to the fact that the donor COM^{D}_{BamC1} was located between modules 6 and 7, and the TE domain that mediated product release was located in module 7. And the deletion of the donor COM^{D}_{BamC1} affected the interaction with the downstream module (module 7) to a certain extent, resulting in a significant decrease in the production of bacillomycin D.

3.2. *Identification of lipopeptide analogs in fermentation products of donor deletion strains*

Pure HPLC analysis is unable to determine whether the donor deletion strains fmbJD1, fmbJD2 and fmbJD3 synthesize bacillomycin D and its analogs; therefore, we further detected and analyzed the crude lipopeptide extracts using high-resolution ESI-LC-MS. Both fmbJD2 and fmbJD3 synthesized bacillomycin D, but fmbJD1 did not synthesize bacillomycin D. In addition, the fermentation product of the mutant strain fmbJD1 also exhibited a signal with an ionic molecular weight of *m/z* 521.3327, as shown in Fig. 3a. This signal was identical to the preliminarily predicted molecular weight of a novel linear dipeptide (C_{14}β-NH_2FA-Asn-Tyr) whose predicted molecular formula was $C_{27}H_{44}N_4O_6$. Signals were also observed at *m/z* 535.3483 and *m/z* 549.3625, which were the molecular weights of C_{15} and C_{16}, respectively. The precursor ion at *m/z* 521.3327 was selected for MS/MS analysis and the dipeptide sequence was determined to be C_{14}β-NH_2FA-Asn-Tyr. As shown in Fig. 3c, the C-terminal y-ion fragments *m/z* 296.1230 and *m/z* 182.0805 in the MS/MS spectrum were consistent with the predicted fragmentation values of linear dipeptide molecules, and fragment ion peaks at *m/z* 521.3318 and *m/z* 115.0501 were detected; thus, we determined that the assembly line of the lipopeptide analog was C_{14}β-NH_2FA-Asn-Tyr. These results indicated that the deletion of the donor COM^{D}_{BamB1} did not affect catalysis by upstream modules, but prevented the downstream modules from catalysing the extension of the lipopeptide product, ultimately resulting in mutant complexes that could form linear dipeptides with the sequence β-NH_2FA-Asn-Tyr.

The fermentation product of the donor deletion strain fmbJD2 exhibited a signal with an ionic molecular weight of *m/z* 934.4902 in addition to a signal with an ionic molecular weight of *m/z* 635.3770, as shown in Fig. 4a and b. The aforementioned signal was the same as the

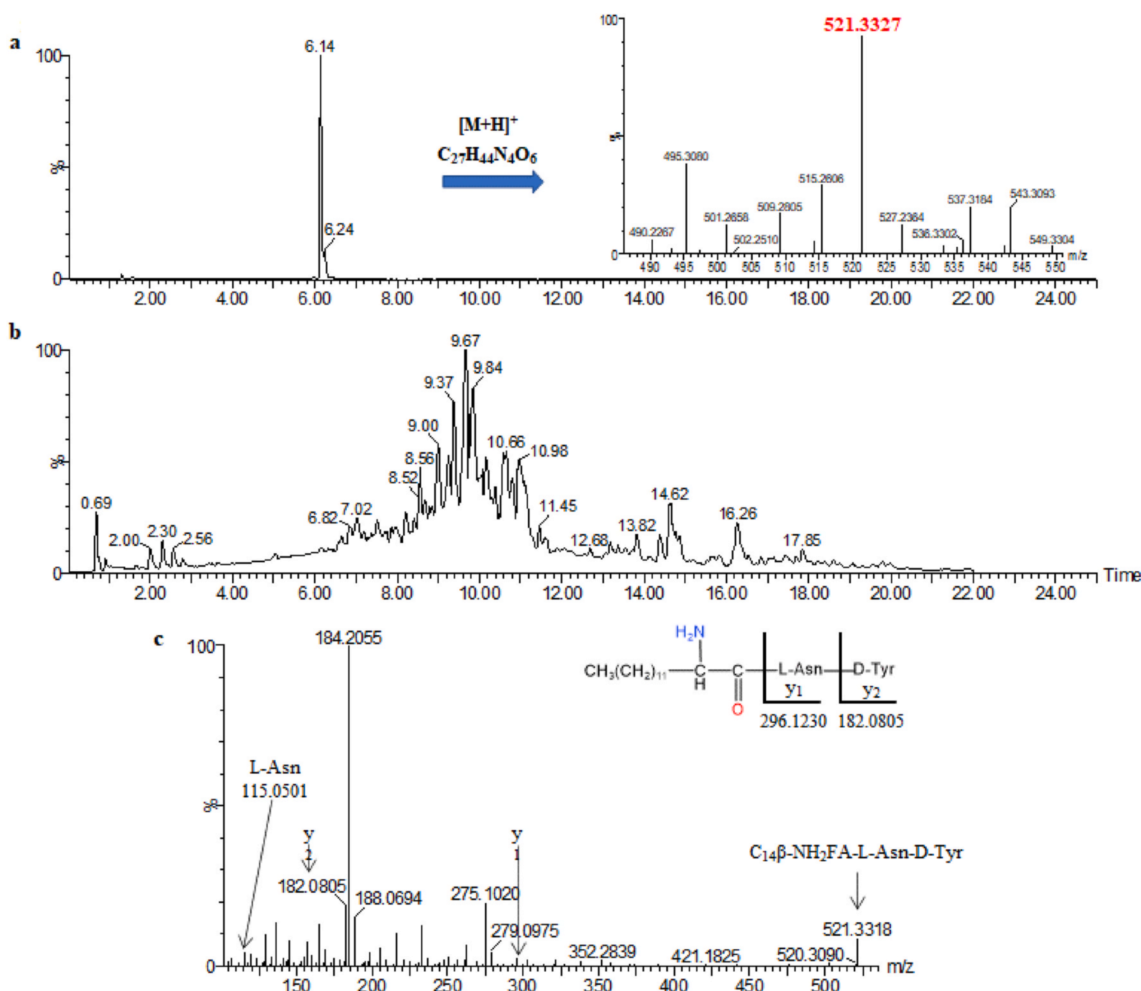


Fig. 3. LC-MS/MS analysis of the crude extract from the mutant strain fmbJD1. (a) Chromatogram corresponding to *m/z* 521.3327, (b) ESI-LC-MS total chromatogram of the crude extract from the mutant strain fmbJD1, and (c) ESI-MS/MS fragment ion analysis at *m/z* 521.3327.

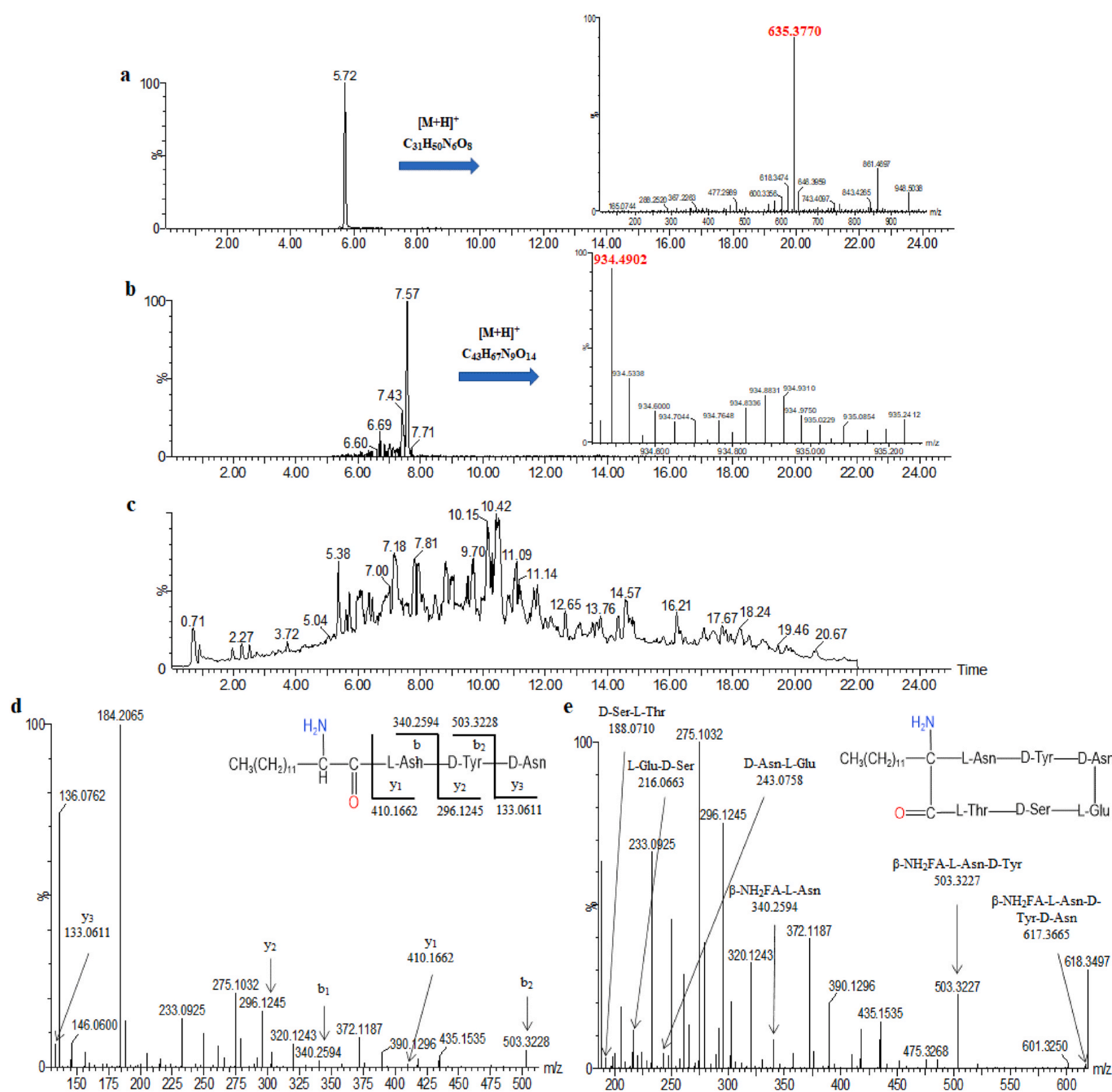


Fig. 4. LC-MS/MS analysis of the crude extract from the mutant strain fmbJD2. (a) Chromatogram corresponding to *m/z* 635.3770, (b) chromatogram corresponding to *m/z* 934.4902, (c) ESI-LC-MS total chromatogram of the crude extract from the mutant strain fmbJD2, (d) ESI-MS/MS fragment ion analysis at *m/z* 635.3770, and (e) ESI-MS/MS fragment ion analysis at *m/z* 934.4902.

preliminarily predicted molecular weight of a novel linear tripeptide (C_{14}β-NH_2FA-Asn-Tyr-Asn) and a novel cyclic hexapeptide (C_{14}β-NH_2FA-Asn-Tyr-Asn-Glu-Ser-Thr). The molecular formulas were $C_{31}H_{50}N_6O_8$ and $C_{43}H_{67}N_9O_{14}$, respectively. In addition, signals at *m/z* 649.3930 and *m/z* 663.4085 were detected as linear tripeptides with molecular weights of C15 and C16 and at *m/z* 948.5037, *m/z* 962.5222, *m/z* 976.5385 and *m/z* 990.5536 as cyclic hexapeptides with molecular weights of C15, C16, C17 and C18. We speculated that the deletion of COM^{D}_{BamB2} may cause the assembly line of bacillomycin D to undergo module skipping, namely, to skip module 4, to generate a new cyclic hexapeptide assembly line.

The precursor ions *m/z* 635.3770 and *m/z* 934.4902 were selected for MS/MS analysis. As shown in Fig. 4d, the N-terminal b-ion fragments *m/z* 503.3228 and *m/z* 340.2594 and the C-terminal y-ion fragments *m/z* 410.1662, *m/z* 296.1245 and *m/z* 133.0611 were consistent with the predicted fragment values of linear tripeptide molecules. The assembly line of the lipopeptide analogs was determined to be C_{14}β-NH_2FA-Asn-Tyr-Asn. The MS/MS analysis of the ion molecular weight of *m/z* 934.4902 is shown in Fig. 4e. Fragment ion peaks *m/z* 617.3665, *m/z* 503.3227 and *m/z* 340.2594 were N-terminal b-ion fragments, in addition to the fragment ion peaks *m/z* 243.0758, *m/z* 216.0663 and *m/z* 188.0710, which were consistent with the predicted fragment values of cyclic hexalipopeptide molecules. From this information, we deduced that the compound structure of *m/z* 934.4902 was β-NH_2FA-Asn-Tyr-Asn-Glu-Ser-Thr. After the deletion of the donor domain COM^{D}_{BamB2}, new assembly lines, namely, the linear tripeptide assembly line and cyclic hexapeptide assembly line, were generated due to module interruption and module skipping in the presence of bacillomycin D. This suggested that in this complex biosynthetic system, a series of modules upstream of module 4 retained the ability to assemble the precursor tripeptide chain. However, due to the different strengths of the interactions between the modules, competition for connection resulted in different assembly lines for the production of different lipopeptides. Among them, the yield of bacillomycin D could account for 89.05%, and the yield of linear tripeptides and cyclic hexapeptides accounted for 9.75% and 1.2% (see Table 3); that is, the interaction between modules 3 and 4 was stronger

than the hydrolysis of the TE domain and the interaction between modules 3 and 5.

Similarly, the fermentation product of the mutant strain fmbJD3 also had the predicted molecular weight of *m/z* 948.5020 due to module interruption, as shown in Fig. 5a. We preliminarily speculated that the assembly line was disconnected from module 6 and module 7, resulting in the assembly line of the linear hexapeptide β-NH_2FA-Asn-Tyr-Asn-Pro-Glu-Ser, in addition to the existence of linear hexapeptide homologs at C_{15} and C_{16}. The MS/MS analysis of the ion molecular weight of *m/z* 948.5020 is shown in Fig. 5c. A series of ion fragment peaks (609.2510 → 446.1871 → 332.1452 and 617.3663 → 503.3229 → 340.2590) was consistent with the fragment values for the novel linear hexapeptide C_{14}β-NH_2FA-Asn-Tyr-Asn-Pro-Glu-Ser. Thus, after the deletion of the donor COM^{D}_{BamC1} in bacillomycin D, a series of modules located upstream of module 7 retained the ability to assemble the precursor hexapeptide chain. However, since the interaction between modules 6 and 7 was stronger than the hydrolysis of the TE domain, two assembly lines were generated to direct the production of bacillomycin D (91.88%) and linear hexapeptide (8.12%) (see Table 3).

3.3. Identification of lipopeptide analogs in fermentation products of acceptor deletion strains

No bacillomycin D signal was detected in the fermentation product of the acceptor deletion strain fmbJA1, but it also showed the ionic molecular weight of the linear dipeptide *m/z* 521.3337, as shown in Fig. 6a, and the predicted molecular formula was $C_{27}H_{44}N_4O_6$. Signals were also detected at *m/z* 535.3492 and *m/z* 549.3650, which were the molecular weights of C_{15} and C_{16}, respectively. The precursor ion at *m/z* 521.3337 was selected for MS/MS analysis, and the results are shown in Fig. 6c. The C-terminal y-ion fragments *m/z* 296.1232 and *m/z* 182.0805 present in the MS/MS spectrum were consistent with the predicted fragment values of linear dipeptide molecules. In addition to the fragment ion peaks *m/z* 521.3335 and *m/z* 115.0501, we determined that the assembly line of the lipopeptide analog is C_{14}β-NH_2FA-Asn-Tyr. The aforementioned results showed that the deletion of the acceptor

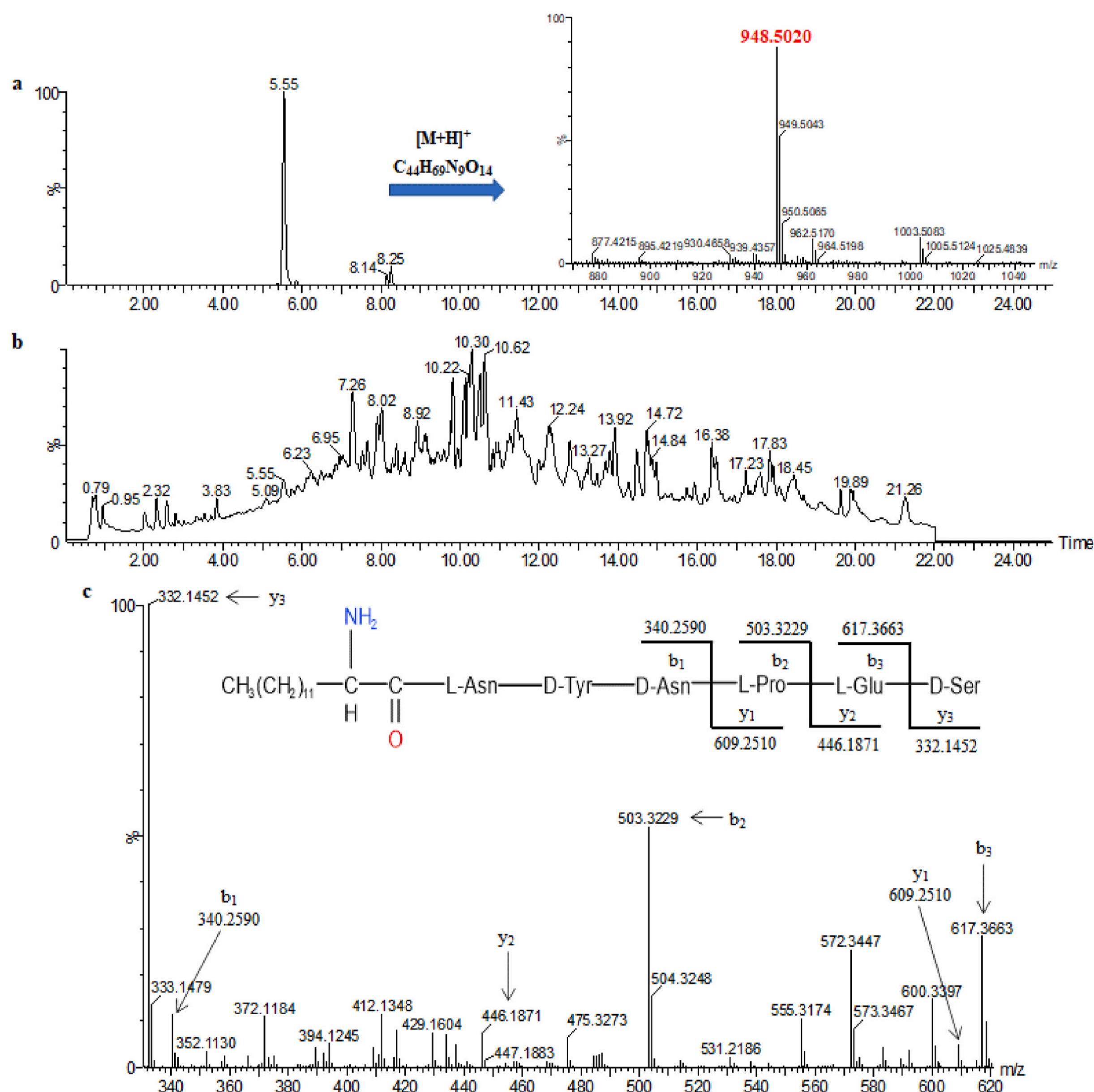


Fig. 5. LC-MS/MS analysis of the crude extract from the mutant strain fmbJD3. (a) Chromatogram corresponding to *m/z* 948.5020, (b) ESI-LC-MS total chromatogram of the crude extract from the mutant strain fmbJD3, and (c) ESI-MS/MS fragment ion analysis at *m/z* 948.5020.

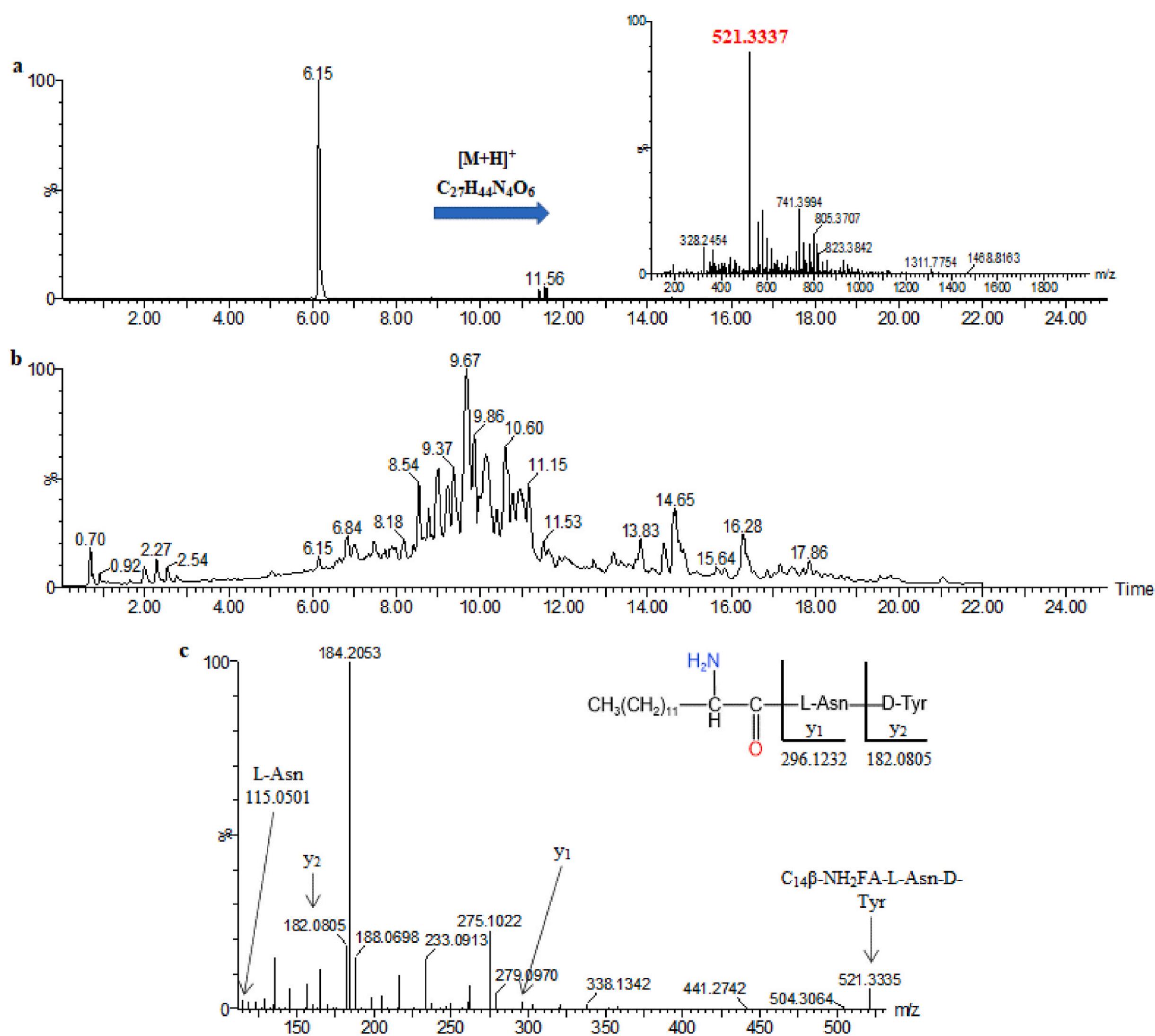


Fig. 6. LC-MS/MS analysis of the crude extract from the mutant strain fmbJA1. (a) Chromatogram corresponding to *m/z* 521.3337, (b) ESI-LC-MS total chromatogram of the crude extract from the mutant strain fmbJA1, and (c) ESI-MS/MS fragment ion analysis at *m/z* 521.3337.

COM^{A}_{BamB2} also prevented the downstream modules from catalysing the extension of the lipopeptide product, which ultimately guided the synthesis of linear dipeptides by the assembly line β-NH_2FA-Asn-Tyr.

The fermentation product of the acceptor deletion strain fmbJA2 was further detected and analyzed using high-resolution ESI-LC-MS, and bacillomycin D was also absent. However, the product exhibited the same ion molecular weight signals of *m/z* 635.3776 and *m/z* 934.4950 as the donor deletion strain fmbJD2 (see Fig. 7a and b). We speculated that the deletion of the acceptor COM^{A}_{BamB3} not only caused module interruption but also skipped module 4 to generate the assembly line of the cyclic hexapeptide. The predicted molecular formulas were $C_{31}H_{50}N_6O_8$ and $C_{43}H_{67}N_9O_{14}$. In addition, signals were detected at *m/z* 649.3937 and *m/z* 663.4080 as the molecular weights of the linear tripeptides at C15 and C16, respectively, and signals at *m/z* 948.5126 and *m/z* 962.5281 as the molecular weights of the cyclic hexapeptides at C15 and C16, respectively.

The precursor ions *m/z* 635.3776 and *m/z* 934.4950 were selected for MS/MS analysis. As shown in Fig. 7d, the predicted linear tripeptide b- and y-fragment ion peaks at *m/z* 503.3226, *m/z* 340.2572 and *m/z* 410.1655, *m/z* 296.1237 were present in the MS/MS spectrum. Thus, the assembly line of the lipopeptide analog was determined to be C_{14}β-NH_2FA-Asn-Tyr-Asn. The MS/MS analysis of the ion with molecular weight *m/z* 934.4950 is shown in Fig. 7e. Fragment ion peaks at *m/z* 746.4059, *m/z* 619.3723, *m/z* 503.3210 and *m/z* 340.2600 were N-terminal b-ion fragments, and the fragment ion peak at *m/z* 656.3706 was consistent with the predicted fragment value for the cyclic hexalipeptide molecule. Based on this information, the structure of the compound at *m/z* 934.4950 was determined to be C_{14}β-NH_2FA-Asn-Tyr-Asn-Glu-Ser-Thr. The acceptor domain COM^{A}_{BamB3} deletion strain generated a hybrid enzyme complex that produced two types of bacillomycin D analogs. Among them, the hydrolysis of the TE domain was slightly stronger than the interaction of modules 3 and 5, resulting in a slightly higher yield of linear tripeptides (57.85%) than that of cyclic hexapeptides (42.15%) (see Table 3).

Similarly, no bacillomycin D signal was detected in the fermentation product of the acceptor deletion strain fmbJA3, and only the predicted linear hexapeptide ion with a molecular weight *m/z* 948.5079 produced by the interruption of the module was present, as shown in Fig. 8a. A signal was detected at *m/z* 962.5276 for the linear hexapeptide homolog of C_{15}. The ion with a molecular weight of *m/z* 948.5079 was selected as the precursor ion for MS/MS analysis, as shown in Fig. 8c. A series of ion fragment peaks (843.4588 → 714.4067 → 617.3638 → 503.3219 → 340.2557 and 609.2522 → 446.1873) were consistent with the fragment values for the novel linear hexapeptide C_{14}β-NH_2FA-Asn-Tyr-Asn-Pro-Glu-Ser. These results showed that after the deletion of the acceptor COM^{A}_{BamC2} in bacillomycin D, the assembly line was unable to pass the

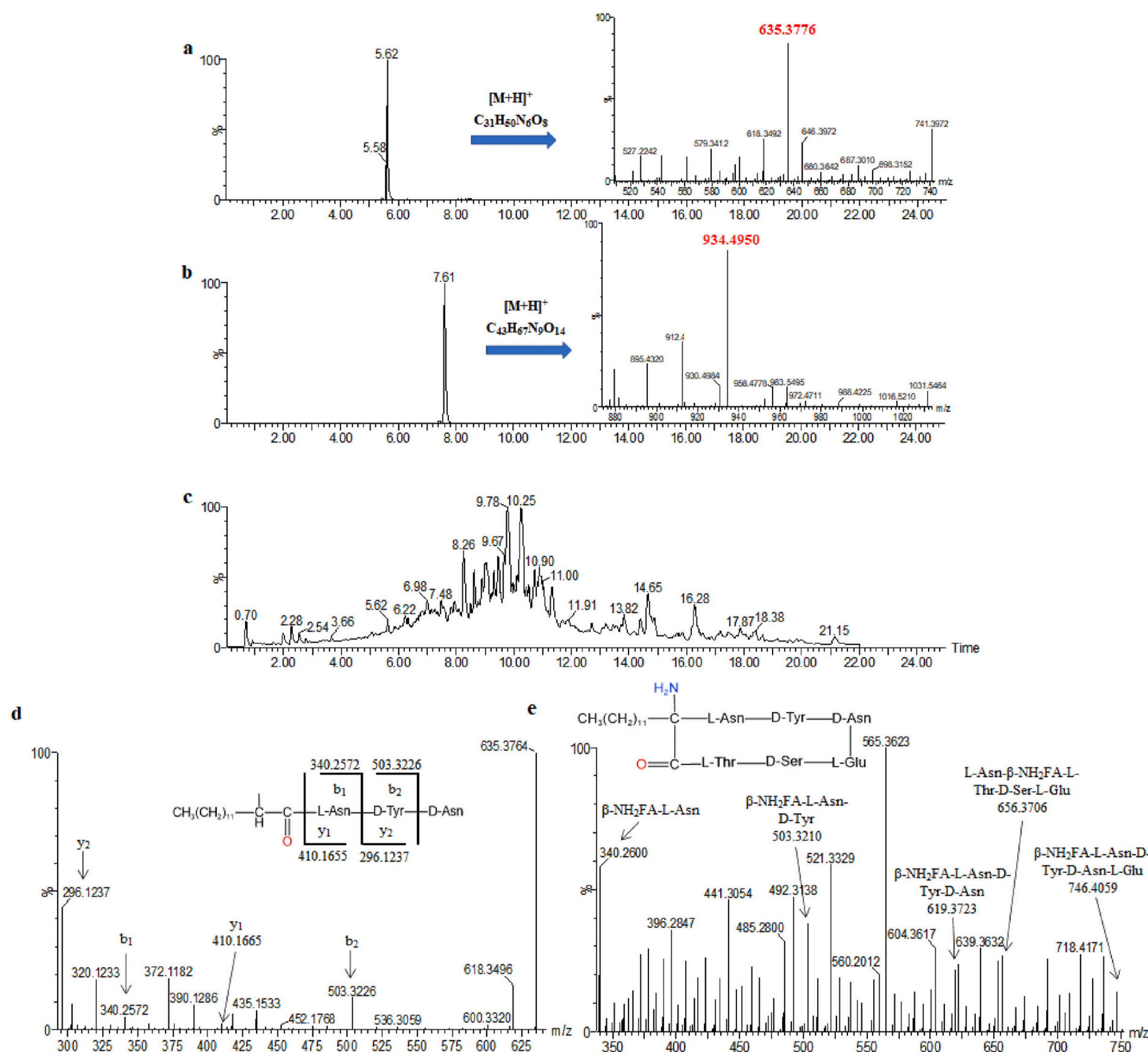


Fig. 7. LC-MS/MS analysis of the crude extract from the mutant strain fmbJA2. (a) Chromatogram corresponding to *m/z* 635.3776, (b) chromatogram corresponding to *m/z* 934.4950, (c) ESI-LC-MS total chromatogram of the crude extract from the mutant strain fmbJA2, (d) ESI-MS/MS fragment ion analysis at *m/z* 635.3776, and (e) ESI-MS/MS fragment ion analysis at *m/z* 934.4950.

substrate from module 6 to module 7, and could only produce linear hexapeptides ($C_{14-15}\beta$-NH_2FA-Asn-Tyr-Asn-Pro-Glu-Ser).

4. Discussion

With the advent of synthetic biology, knowledge of the modularity of the NRPS system, and a better understanding of the structure of the assembly line of NRPSs [28], combinatorial biosynthesis has emerged as a powerful strategy for the reprogramming of NRPSs [29,30]. In the past few decades, various reprogramming strategies have been developed for NRPSs. The first was gatekeeper domain exchange (deletion), which mainly focused on the replacement of the gatekeeper domain (A-PCP or A domain) [5,31,32]. The purpose was to load different monomers into the assembly line to obtain new analogs. The second strategy was extended to different domains, such as deletion and advancement of the TE domain in the module [33,34]. The third involved the exchange (deletion) of single or multiple complete modules within the assembly line [5,35]. Fourth, some researchers recently proposed the concept of XUs [36], and by exchanging XU units, they were able to create novel peptides at high yields. However, one limitation of this approach is the amino acid specificity of the downstream C domain. Therefore, authors identified a new fusion point within the C domain to overcome this problem, proposing another concept, XUCs [37]. This strategy was considered to have the potential to generate peptide libraries. While all of the aforementioned methods might lead to the synthesis of the desired NRP product, a significant reduction in product yield is often observed. Meanwhile, the current knowledge of the NRPS systems was mostly based on domain, dual domain and single NRPS module information [38,39]. However, little was known about the interactions between modules in multimodule NRPSs [40].

Bioinformatics analyses unveiled the prevalence of both inter- and intra-subunit COM interfaces throughout biosynthetic assembly lines, including two types of PCP domain-bridging interfaces not previously characterized [41]. Fage and co-workers validated the importance of the PCP-COM^D domain in inter-subunit communication, demonstrating the versatility of COM regions in promoting functional domain-domain interactions beyond those of the canonical E and C domain pair [41]. Through the exchange of E-C COM domains between modules 4 and 5 and modules 5 and 6, Kaniusaite and co-workers demonstrated that the E-C COM domain between modules was similar to the E-C COM domain between subunits in that a successful domain exchange required compatible linkers to connect the upstream and downstream modules of

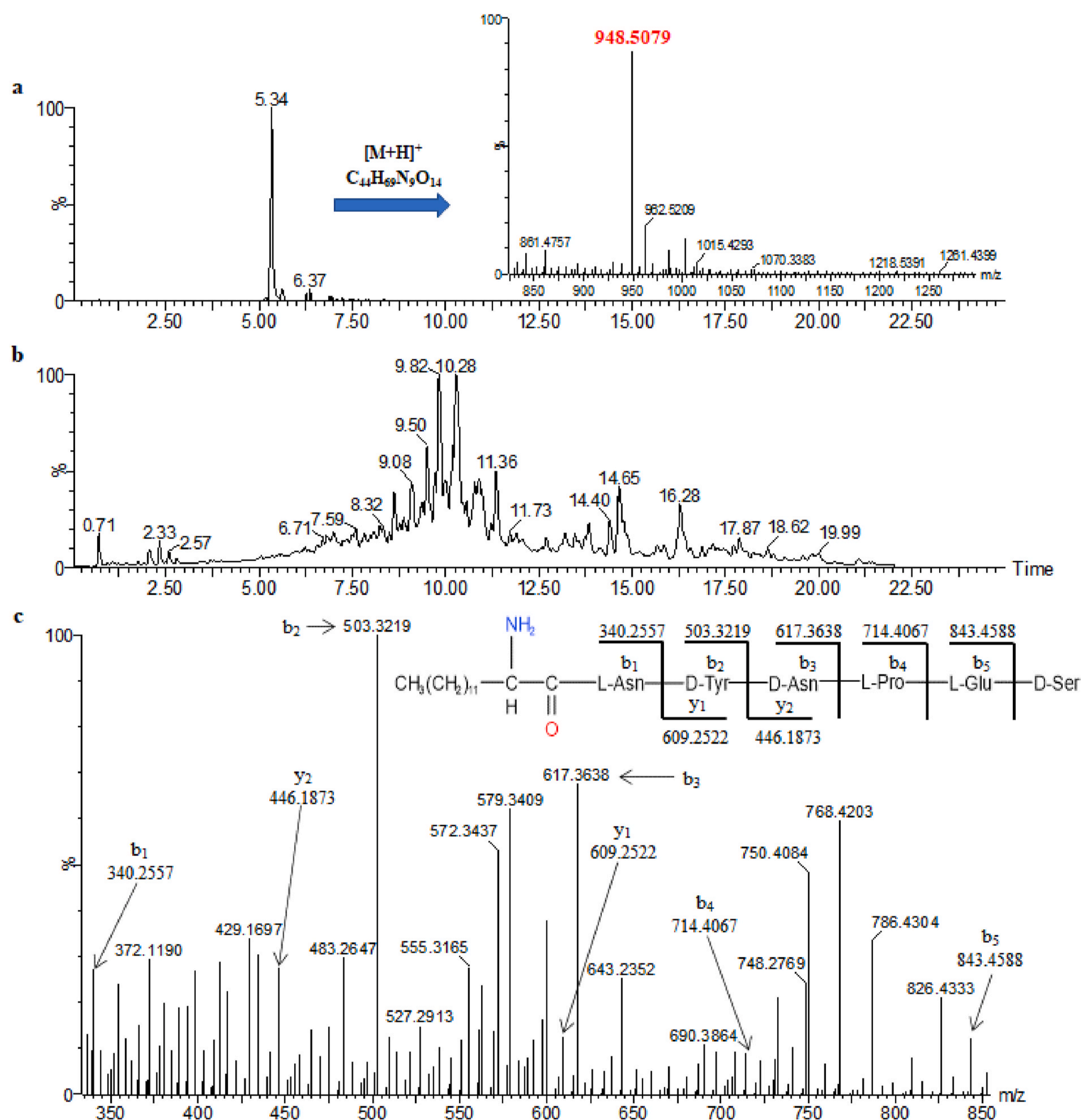


Fig. 8. LC-MS/MS analysis of the crude extract from the mutant strain fmbJA3. (a) Chromatogram corresponding to *m/z* 948.5079, (b) ESI-LC-MS total chromatogram of the crude extract from the mutant strain fmbJA3, and (c) ESI-MS/MS fragment ion analysis at *m/z* 948.5079.

interest [42]. In this study, we first located the specific position and sequence of the COM domain in bacillomycin D through the conserved sequences of the donor and acceptor domains at the E-C domain interface determined by Hahn [17,19] et al. (see Fig. 1). The position of the COM domain in bacillomycin D was located between modules, similar to the E-C COM domains between modules 4 and 5 and between modules 5 and 6 in teicoplanin non-ribosomal peptide synthetase [42], all of which belong to the intra-subunit COM interfaces reported by Fage [41]. To further clarify whether the compatibility of COM domain donors and acceptors between modules was consistent with the compatibility between subunits. And whether the different positions of the donor and acceptor in the assembly line exerted different effects on the synthesis of bacillomycin D. We explored these by deleting the donor and acceptor domains of three pairs of COM domains at different positions in bacillomycin D.

Regarding the deletion of the donor domain, deletion of COM^{D}_{BamB1} (in module 2), COM^{D}_{BamB2} (in module 3), and COM^{D}_{BamC1} (in module 6) all resulted in module disruption, resulting in truncated peptides, namely, linear dipeptides ($C_{14\text{-}16}\beta$-NH_2FA-Asn-Tyr), linear tripeptides ($C_{14\text{-}16}\beta$-NH_2FA-Asn-Tyr-Asn) and linear hexapeptides ($C_{14\text{-}16}\beta$-NH_2FA-Asn-Tyr-Asn-Pro-Glu-Ser) (the synthetic mechanisms are shown in Fig. 9a, b and 9d). These results were consistent with previous reports that the selectivity of the ppsC acceptor was blocked and led to a novel assembly line for the synthesis of hexalipopeptides (ppsA/ppsB/ppsC) when the ppsC donor was deleted [22]. However, in our study, we found that in addition to COM^{D}_{BamB1}-deleted strains, COM^{D}_{BamB2}- and COM^{D}_{BamC1}-deleted strains had the original bacillomycin D assembly line. In particular, the production of bacillomycin D was not significantly reduced after the deletion of COM^{D}_{BamB2}. Therefore, the position of the donor domain exerted different effects on the entire assembly line, and the COM domain at the front end played a more important role in the synthesis of bacillomycin D. Similar results have also been reported in previous studies of the deletion of the T domain at various positions in plipastatin. Deletion of the T domain of module 6 completely inactivated the plipastatin complex,

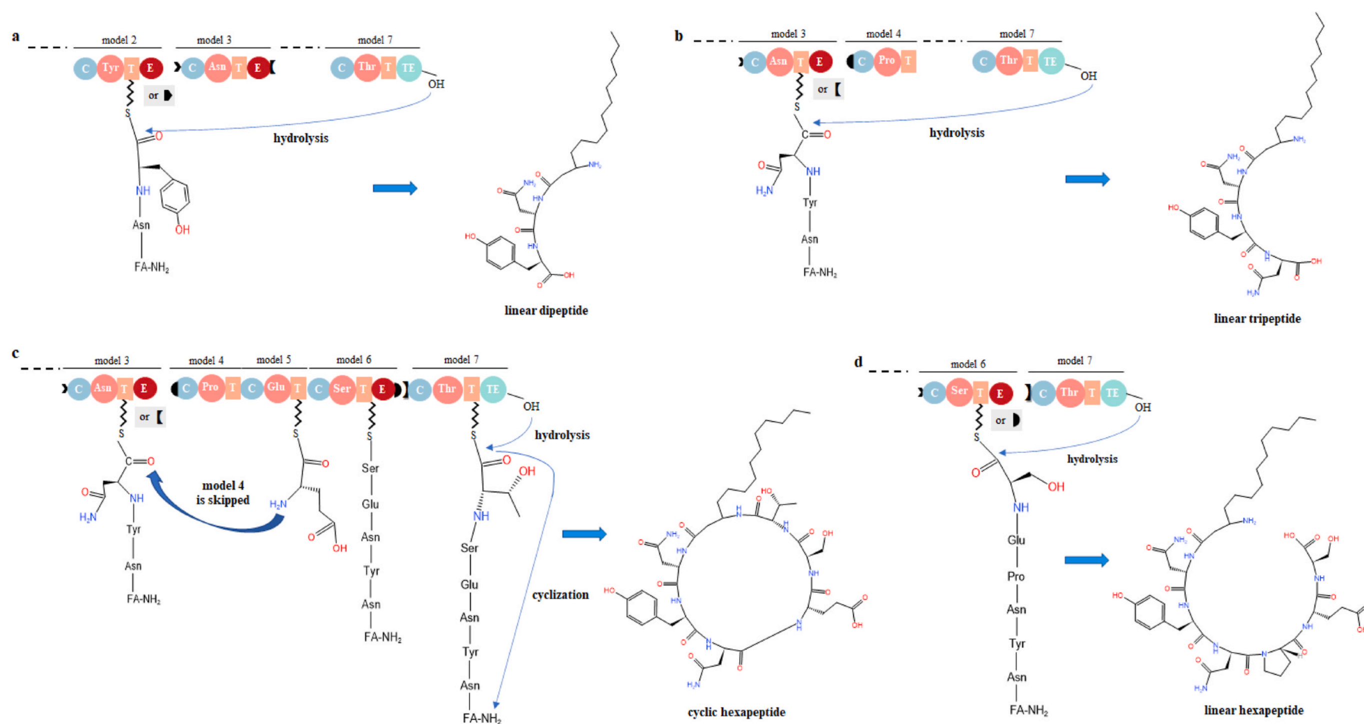


Fig. 9. Generation mechanism of four novel lipopeptides. (a) Formation of linear dipeptide due to premature hydrolysis of the thioesterase (TE) domain, (b)formation of linear tripeptide due to premature hydrolysis of the TE domain, (c) formation of cyclic hexapeptide by complete module 4 skipping, and (d) formation of linear hexapeptide due to premature hydrolysis of the TE domain.

whereas deletion of the T domain of module 7 resulted in the presence of a linear hexapeptide [5]. Thus, the T domain of module 6 played an important role in the overall structural conformation of the plipastatin NRPS complex. Based on these results, the deletion of the donor domain between modules or between NRPS subunits altered the interaction of upstream and downstream modules to varying degrees. However, the donor domains between NRPS subunits appear to play a more important role in the overall structural conformation of the NRPS complex than the donor domains between modules [22]. Interestingly, we also discovered a new assembly line for the synthesis of cyclic hexapeptides ($C_{14\text{-}18}$β-NH_2FA-Asn-Tyr-Asn-Glu-Ser-Thr) in the fermentation product of the COM^{D}_{BamB2}-deleted strain (see Fig. 9c). This product has never been obtained from previous donor and acceptor deletions between NRPS subunits. The existence of a more flexible hybrid biosynthetic system suggests that the inter-module donor domain deletion strains have greater biosynthetic capacity than the inter-subunit donor domain deletion strains.

In contrast, deletion of the acceptor domains between modules showed lower compatibility. After deleting COM^{A}_{BamB2} (in module 3), COM^{A}_{BamB3} (in module 4) and COM^{A}_{BamC2} (in module 7), we found that all strains were incapable of producing bacillomycin D and all strains generated truncated peptides due to module interruption: linear dipeptides ($C_{14\text{-}16}$β-NH_2FA-Asn-Tyr), linear tripeptides ($C_{14\text{-}16}$β-NH_2FA-Asn-Tyr-Asn) and linear hexapeptides ($C_{14\text{-}15}$β-NH_2FA-Asn-Tyr-Asn-Pro-Glu-Ser). These results were similar to those obtained through donor domain deletions. Thus, the deletion of acceptors did not affect the catalysis of the upstream module, but prevented the downstream module from catalysing the elongation of the lipopeptide product, ultimately leading to assembly line disruption. By comparing donor and acceptor substitutions, we found that donor substitution and donor deletion only generated truncated peptides. However, acceptor replacement only resulted in a reduction in yield and did not affect the original assembly line [18,22]. Therefore, we postulated that the acceptor domain between modules was more important for the overall structural conformation of the NRPS complex enzyme than the acceptor domain between NRPSs, indicating that the acceptor domain was an important part of module-to-module interactions and efficient communication within bacillomycin D synthetase.

In addition, we also found the $C_{14\text{-}16}$β-NH_2FA-Asn-Tyr-Asn-Glu-Ser-Thr assembly line for the synthesis of cyclic hexapeptide in the COM^{A}_{BamB3}-deleted strain. This result indicated that the deletion of either the donor domain or the acceptor domain in modules 3 and 4 would lead to the skipping of module 4, resulting in the connection of module 3 and module 5. This suggested a strong interaction between module 3 and module 5 in bacillomycin D. The previous correct connection was attributed to the correct pairing of the COM domain donor and acceptor. When either the donor or acceptor was absent, the interaction between module 3 and module 4 was affected, resulting in this unusual assembly line (cyclic hexapeptides, Fig. 9c). Unusual biosynthetic pathways have also been shown in studies of teicoplanin synthase reprogramming, resulting in the presence of pentapeptides (M5-6a + M3+M4+M3) due to the strong interaction mediated by the M3/M4 interface [42].

5. Conclusion

In this study, the COM domains between the bacillomycin D synthetase modules were reported for the first time, and the interaction between NRPS modules was elucidated by the deletion of the donor and acceptor domains. In conclusion, deletion of the intermodular COM donor and acceptor domains might be used to reprogram bacillomycin D synthase in *B. amyloliquefaciens*, resulting in novel lipopeptides, namely, linear dipeptides, linear tripeptides, linear hexapeptides, and cyclic hexapeptides. Among them, cyclic hexapeptides were generated by module skipping. The presence of multiple assembly lines suggested that the combinatorial biosynthetic potential of the COM domains was greater than previously reported. In particular, the donor domain between modules did not affect the original bacillomycin D yield at all, which had never been reported before. However, the intermodule acceptor domain was an important part of module-module interactions and efficient communication in bacillomycin D synthetase. This study

further complemented the current lack of studies on the interaction between NRPS modules and provided a theoretical basis for the generation of novel lipopeptides mediated by the intermodule COM domains.

CRediT authorship contribution statement

Ziyan Lv: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. **Wenjie Ma:** Methodology. **Ping Zhang:** Formal analysis. **Zhaoxin Lu:** Resources, Supervision. **Libang Zhou:** Validation. **Fanqiang Meng:** Validation. **Zuwei Wang:** Investigation. **Xiaomei Bie:** Conceptualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (grant no. 31972174).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2022.05.007.

References

[1] Gong Q, Zhang C, Lu F, Zhao H, Bie X, Lu Z. Identification of bacillomycin D from *Bacillus subtilis* fmbJ and its inhibition effects against *Aspergillus flavus*. Food Control 2014;36:8–14. https://10.1016/j.foodcont.2013.07.034.

[2] Qian S, Lu H, Sun J, Zhang C, Zhao H, Lu F, et al. Antifungal activity mode of *Aspergillus ochraceus* by bacillomycin D and its inhibition of ochratoxin A (OTA) production in food samples. Food Control 2016;60:281–8. https://10.1016/j.foodcont.2015.08.006.

[3] Sun J, Li W, Liu Y, Lin F, Huang Z, Lu F, et al. Growth inhibition of *Fusarium graminearum* and reduction of deoxynivalenol production in wheat grain by bacillomycin D. J Stored Prod Res 2018;75:21–8. https://10.1016/j.jspr.2017.11.002.

[4] Tang Q, Bie X, Lu Z, Lv F, Tao Y, Qu X. Effects of fengycin from *Bacillus subtilis* fmbJ on apoptosis and necrosis in *Rhizopus stolonifer*. J Microbiol 2014;52:675–80. https://doi.org/10.1007/s12275-014-3605-3.

[5] Gao L, Guo J, Fan Y, Ma Z, Lu Z, Zhang C, et al. Module and individual domain deletions of NRPS to produce plipastatin derivatives in *Bacillus subtilis*. Microb Cell Factories 2018;17. https://doi.org/10.1186/s12934-018-0929-4.

[6] Cochrane SA, Vederas JC. Lipopeptides from *Bacillus* and *Paenibacillus spp.*: a gold mine of antibiotic candidates. Med Res Rev 2016;36:4–31. https://10.1002/med.21321.

[7] Caulier S, Nannan C, Gillis A, Licciardi F, Bragard C, Mahillon J. Overview of the antimicrobial compounds produced by members of the *Bacillus subtilis* group. Front Microbiol 2019;10. https://10.3389/fmicb.2019.00302.

[8] Yang R, Lei S, Xu X, Jin H, Sun H, Zhao X, et al. Key elements and regulation strategies of NRPSs for biosynthesis of lipopeptides by *Bacillus*. Appl Microbiol Biotechnol 2020;104:8077–87. https://10.1007/s00253-020-10801-x.

[9] Zhao H, Shao D, Jiang C, Shi J, Li Q, Huang Q, et al. Biological activity of lipopeptides from *Bacillus*. Appl Microbiol Biotechnol 2017;101:5951–60. https://doi.org/10.1007/s00253-017-8396-0.

[10] Yaseen Y, Gancel F, Drider D, Béchet M, Jacques P. Influence of promoters on the production of fengycin in *Bacillus spp*. Res Microbiol 2016;167:272–81. https://doi.org/10.1016/j.resmic.2016.01.008.

[11] Peypoux F, Besson F, Michel G, Lenzen C, Dierickx L, Delcambe L. Characterization of a new antibiotic of iturin group: bacillomycin D. J Antibiot 1980;33:1146–9. https://10.7164/antibiotics.33.1146.

[12] Peypoux F, Besson F, Michel G, Delcambe L. Structure of bacillomycin D, a new antibiotic of the iturin group. Eur J Biochem 1981;118:323–7. https://10.1111/j.1432-1033.1981.tb06405.x.

[13] Peypoux F, Pommier MT, Das BC, Besson F, Delcambe L, Michel G. Structures of bacillomycin D and bacillomycin L peptidolipid antibiotics from *Bacillus subtilis*. J Antibiot 1984;37:1600–4. https://10.7164/antibiotics.37.1600.

[14] Moyne AL, Cleveland TE, Tuzun S. Molecular characterization and analysis of the operon encoding the antifungal lipopeptide bacillomycin D. FEMS Microbiol Lett 2004;234:43–9. https://doi.org/10.1016/j.femsle.2004.03.011.

[15] Beck C, Garzon JFG, Weber T. Recent advances in Re-engineering modular PKS and NRPS assembly lines. Biotechnol Bioproc E 2020;25:886–94. https://doi.org/10.1007/s12257-020-0265-5.

[16] Siewers V, San-Bento R, Nielsen J. Implementation of communication-mediating domains for non-ribosomal peptide production in *Saccharomyces cerevisiae*. Biotechnol Bioeng 2010;106:841–4. https://10.1002/bit.22739.

[17] Hahn M, Stachelhaus T. Selective interaction between nonribosomal peptide syntheltases is facilitated by short commuinication-mediating domains. P Natl Acad Sci Usa 2004;101:15585–90. https://10.1073/pnas.0404932101.

[18] Chiocchini C, Linne U, Stachelhaus T. In vivo biocombinatorial synthesis of lipopeptides by COM domain-mediated reprogramming of the surfactin biosynthetic complex. Chem Biol 2006;13:899–908. https://10.1016/j.chembiol.2006.06.015.

[19] Hahn M, Stachelhaus T. Harnessing the potential of communication-mediating domains for the biocombinatorial synthesis of nonribosomal peptides. P Natl Acad Sci Usa 2006;103:275–80. https://10.1073/pnas.0508409103.

[20] Dehling E, Volkmann G, Matern JCJ, Dörner W, Alfermann J, Diecker J, Mootz HD. Mapping of the communication-mediating interface in nonribosomal peptide synthetases using a genetically encoded photocrosslinker supports an upside-down helix-hand motif. J Mol Biol 2016;428:4345–60. https://doi.org/10.1016/j.jmb.2016.09.007.

[21] Hu F, Liu Y, Li S. Rational strain improvement for surfactin production: enhancing the yield and generating novel structures. Microb Cell Factories 2019;18. https://doi.org/10.1186/s12934-019-1089-x.

[22] Liu H, Gao L, Han J, Ma Z, Lu Z, Dai C, et al. Biocombinatorial synthesis of novel lipopeptides by COM domain-mediated reprogramming of the plipastatin NRPS complex. Front Microbiol 2016;7. https://10.3389/fmicb.2016.01801.

[23] Hur GH, Meier JL, Baskin J, Codelli JA, Bertozzi CR, Marahiel MA, Burkart MD. Crosslinking studies of protein-protein interactions in nonribosomal peptide biosynthesis. Chem Biol 2009;16:372–81. https://10.1016/j.chembiol.2009.02.009.

[24] Kegler C, Bode HB. Artificial splitting of a non-ribosomal peptide synthetase by inserting natural docking domains. Angew Chem, Int Ed 2020;59:13463–7. https://10.1002/anie. 201915989.

[25] Sun J, Qian SQ, Lu J, Liu Y, Lu F, Bie X, Lu Z. Knockout of rapC improves the bacillomycin D yield based on de novo genome sequencing of *Bacillus amyloliquefaciens* fmbJ. J Agric Food Chem 2018;66:4422–30. https://doi.org/10.1021/acs.jafc.8b00418.

[26] Zakataeva NP, Nikitina OV, Gronskiy SV, Romanenkov DV, Livshits VA. A simple method to introduce marker-free genetic modifications into the chromosome of naturally nontransformable *Bacillus amyloliquefaciens* strains. Appl Microbiol Biotechnol 2010;85:1201–9. https://doi.org/10.1007/s00253-009-2276-1.

[27] Chong L. Molecular cloning - a laboratory manual. Science 2001;292:446. https://10.1126/science.1060677. third ed.

[28] Dehling E, Ruschenbaum J, Diecker J, Dorner W, Mootz HD. Photo-crosslink analysis in nonribosomal peptide synthetases reveals aberrant gel migration of branched crosslink isomers and spatial proximity between non-neighboring domains. Chem Sci 2020;11:8945–54. https://10.1039/d0sc01969k.

[29] Baltz RH. Synthetic biology, genome mining, and combinatorial biosynthesis of NRPS-derived antibiotics: a perspective. J Ind Microbiol Biotechnol 2018;45:635–49. https://10.1007/s10295-017-1999-8.

[30] Watzel J, Duchardt-Ferner E, Sarawi S, Bode HB, Wohnert J. Cooperation between a T domain and a minimal C-terminal docking domain to enable specific assembly in a Multiprotein NRPS. Angew Chem, Int Ed 2021;60:14171–8. https://10.1002/anie.202103498.

[31] Calcott MJ, Owen JG, Lamont IL, Ackerley DF. Biosynthesis of novel pyoverdines by domain substitution in a nonribosomal peptide synthetase of *Pseudomonas aeruginosa*. Appl Environ Microbiol 2014;80:5723–31. https://10.1128/AEM.01453-14.

[32] Stachelhaus T, Schneider A, Marahiel MA. Rational design of peptide antibiotics by targeted replacement of bacterial and fungal domains. Science 1995;269:69–72. https://doi.org/10.1126/science.7604280.

[33] Gao L, Liu H, Ma Z, Han J, Lu Z, Dai C, et al. Translocation of the thioesterase domain for the redesign of plipastatin synthetase. Sci Rep-Uk 2016;6. https://10.1038/srep38467.

[34] Trauger JW, Kohli RM, Mootz HD, Marahiel MA, Walsh CT. Peptide cyclization catalysed by the thioesterase domain of tyrocidine synthetase. Nature 2000;407:215–8. https://doi.org/10.1038/35025116.

[35] Nguyen KT, Ritz D, Gu J, Alexander D, Chu M, Miao V, et al. Combinatorial biosynthesis of novel antibiotics related to daptomycin. P Natl Acad Sci Usa 2006;103:17462–7. https://doi.org/10.1073/pnas.0608589103.

[36] Bozhueyuek KAJ, Fleischhacker F, Linck A, Wesche F, Tietze A, Niesert C, et al. De novo design and engineering of non-ribosomal peptide synthetases. Nat Chem 2018;10:275–81. https://10.1038/NCHEM.2890.

[37] Bozhuyuk KAJ, Micklefield J, Wilkinson B. Engineering enzymatic assembly lines to produce new antibiotics. Curr Opin Microbiol 2019;51:88–96. https://doi.org/10.1016/j.mib.2019.10.007.

[38] Samel SA, Schoenafinger G, Knappe TA, Marahiel MA, Essen L. Structural and functional insights into a peptide bond-forming bidomain from a nonribosomal peptide synthetase. Structure 2007;15:781–92. https://10.1016/j.str.2007.05.008.

[39] Corpuz JC, Sanlley JO, Burkart MD. Protein-protein interface analysis of the non-ribosomal peptide synthetase peptidyl carrier protein and enzymatic domains. Synthetic and Systems Biotechnology 2022;7(2):677–88. https://10.1016/j.synbio.2022.02.006.

[40] Tarry MJ, Haque AS, Bui KH, Schmeing TM. X-Ray crystallography and electron microscopy of cross- and multi-module nonribosomal peptide synthetase proteins

reveal a flexible architecture. Structure 2017;25:783. https://10.1016/j.str. 2017.03.014.

[41] Fage CD, Kosol S, Jenner M, Oster C, Gallo A, Kaniusaite M, et al. Communication breakdown: dissecting the COM interfaces between the subunits of nonribosomal peptide synthetases. ACS Catal 2021;11(17):10802–13. http://10.1021/acscatal.1c02113.

[42] Kaniusaite M, Goode RJA, Tailhades J, Schittenhelm RB, Cryle MJ. Exploring modular reengineering strategies to redesign the teicoplanin non-ribosomal peptide synthetase. Chem Sci 2020;11(35):9443–58. http://10.1039/d0sc03483e.