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Identification and functional analysis of the fusaricidin biosynthetic gene of *Paenibacillus polymyxa* E681

Soo-Keun Choi [a], Soo-Young Park [a], Rumi Kim [b,1], Choong-Hwan Lee [b,2], Jihyun F. Kim [a], Seung-Hwan Park [a,*]

[a] *Systems Microbiology Research Center, KRIBB, 111 Gwahangno, Yuseong-gu, Daejeon 305-806, Republic of Korea*
[b] *Natural Medicines Research Center, KRIBB, Daejeon 305-806, Republic of Korea*

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Abstract

Fusaricidin, a peptide antibiotic consisting of six amino acids, has been identified as a potential antifungal agent from *Paenibacillus polymyxa*. Here, we report the complete sequence of the fusaricidin synthetase gene (*fusA*) identified from the genome sequence of a rhizobacterium, *P. polymyxa* E681. The gene encodes a polypeptide consisting of six modules in a single open-reading frame. Interestingly, module six of FusA does not contain an epimerization domain, which suggests that the sixth amino acids of the fusaricidin analogs produced by *P. polymyxa* E681 may exist as an L-form, although all reported fusaricidins contain D-form alanines in their sixth amino acid residues. Alternatively, the sixth adenylation domain of the FusA may directly recognize the D-form alanine. The inactivation of *fusA* led to the complete loss of antifungal activity against *Fusarium oxysporum*. LC/MS analysis confirmed the incapability of fusaricidin production in the *fusA* mutant strain, thus demonstrating that *fusA* is involved in fusaricidin biosynthesis. Our findings suggested that FusA can produce more than one kind of fusaricidin, as various forms of fusaricidins were identified from *P. polymyxa* E681.

Keywords: *Paenibacillus polymyxa*; Fusaricidin; Fusaricidin synthetase gene; NRPS

Fusaricidins are antibiotics that have been isolated from *Paenibacillus* sp., which have a ring structure composed of six amino acid residues in addition to 15-guanidino-3-hydroxypentadecanoic acid (GHPD) (Fig. 1A). Various analogs of fusaricidins were isolated and characterized from *Paenibacillus polymyxa*; these included LI-F03, LI-F04, LI-F05, LI-F06, LI-F07, and LI-F08 [1,2], as well as fusaricidins A–D (Fig. 1B) [3,4]. Fusaricidins have an excellent antifungal activity against plant pathogenic fungi such as *Fusarium oxysporum*, *Aspergillus niger*, *Aspergillus oryzae*, and *Penicillium thomii*, and fusaricidin B has particularly antagonistic activity against *Candida albicans* and *Saccharomyces cerevisiae*. Fusaricidins also have excellent germicidal activity to gram-positive bacteria such as *Staphylococcus aureus* [3,4]. In addition, they have antifungal activity against *Leptosphaeria maculans*, which causes black root rot of canola [5].

The amino acid chain of fusaricidin is not ribosomally synthesized by encoding, as are other general polypeptides, but instead is generated by a non-ribosomal peptide synthetase (NRPS) [6,7]. An NRPS combines each amino acid monomer in a stepwise manner to produce a peptide and, if necessary, modifies each amino acid to complete the synthesis of the entire amino acid chain or to form a ring structure. Each module of the NRPS is organized by at least three domains, which are referred to as A, C, and T domains. The A (adenylation) domain plays a role in the selection and activation of an amino acid monomer, the C (condensation) domain catalyzes peptide bond forma-

* Corresponding author. Fax: +82 42 860 4488.
E-mail address: shpark@kribb.re.kr (S.-H. Park).
[1] Present address: Institute of Hadong Green Tea, Hadong 667-805, Republic of Korea.
[2] Present address: Division of Bioscience and Biotechnology, Konkuk University, Seoul 143-701, Republic of Korea.

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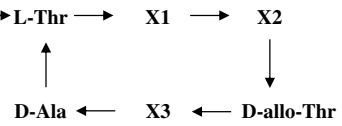
A

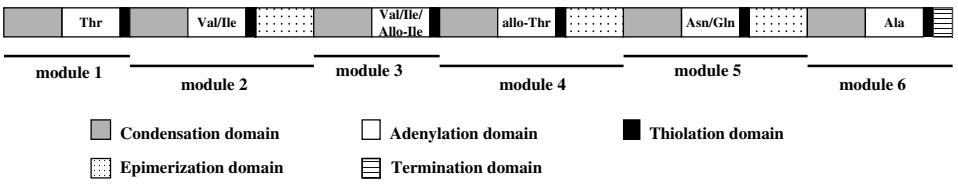


B

fusaricidin	Amino acid positions			$(M+H)^+$
	X1	X2	X3	
LI-F03a (C)	D-Val	L-Tyr	D-Asn	947
LI-F03b (D)	D-Val	L-Tyr	D-Gln	961
LI-F04a (A)	D-Val	L-Val	D-Asn	883
LI-F04b (B)	D-Val	L-Val	D-Gln	897
LI-F05a	D-Val	L-Ile	D-Asn	897
LI-F05b	D-Val	L-Ile	D-Gln	911
LI-F06a	D-allo-Ile	L-Val	D-Asn	897
LI-F06b	D-allo-Ile	L-Val	D-Gln	911
LI-F07a	D-Val	L-Phe	D-Asn	931
LI-F07b	D-Val	L-Phe	D-Gln	945
LI-F08a	D-Ile	L-allo-Ile	D-Asn	911
LI-F08b	D-Ile	L-allo-Ile	D-Gln	925

Fig. 1. (A) Primary structure of the fusaricidin-type antibiotics. GHPD indicates 15-guanidino-3-hydroxypentadecanoic acid. *X*1, *X*2, and *X*3 indicate three variable positions in fusaricidins. (B) Amino acid substitutions at three variable positions in previously reported fusaricidins and the molecular weights of the fusaricidins.

A



B

A domain	Amino acid residues involved in substrate recognition										Predicted substrate
	235	236	239	278	299	301	322	330	331	517	
FusA A1	D	F	W	N	I	G	M	V	H	K	Thr
FusA A2	D	A	F	T	L	G	C	T	F	K	Val/Ile
FusA A3	D	A	S	T	L	A	G	V	C	K	Val/Ile/allo-Ile
FusA A4	D	F	W	N	I	G	M	V	H	K	allo-Thr
FusA A5	D	L	T	K	I	G	E	V	C	K	Asn/Gln
FusA A6	D	F	P	N	F	C	I	V	Y	K	Ala

Fig. 2. (A) Domain organization of the FusA enzyme. (B) Amino acid residues of FusA A domains involved in substrate recognition and predicted substrate specificity of A domains of the fusaricidin synthetase.

tion, and the T (thiolation, also called PCP) domain is involved in the rotating phosphopantheteine group to incorporate an amino acid monomer into the growing peptide chain. In addition to these major domains, there are the E (epimerization) domain, which plays a role in the conversion of an L-amino acid into a D-amino acid, and the TE (termination) domain, which is characterized by a specific amino acid motif.

In this study, we identified and analyzed the complete sequence of the fusaricidin synthetase gene, *fusA*, and confirmed its role in fusaricidin biosynthesis by targeted mutagenesis, mass spectrometry, and antifungal assays.

Materials and methods

Strains and culture condition. *P. polymyxa* E681, which was isolated from the roots of winter barely in the Republic of Korea [8], was cultured in Katznelson and Lochhead medium (KL medium) [9] for the analysis of fusaricidin and BHIS (BHI containing 10% sucrose) broth for transformation.

Antifungal assays. *P. polymyxa* E681 was cultured in the KL medium under aerobic conditions at 30 °C for 24 h. After centrifugation of the culture, the cell pellet was extracted by methanol. The antifungal activities of the cell pellet extract and supernatant were measured against *F. oxysporum* using the disk diffusion method.

LC/MS analysis. The methanol extract of the cell pellet was analyzed by LC/MS (Thermo electron Co., USA) using a mixed solvent of water and acetonitrile containing 0.1% formic acid at a rate of 0.2 ml/min. To confirm LI-F series structures, ring-opened peptides were prepared by hydrolysis of the LI-F complex with MeOH—H_2O–28% aqueous NH_3 (4:1:1, pH 9.0) for 24 h according to the method of Kuroda et al. [1].

PCR-targeted mutagenesis. PCR primers for targeted mutagenesis are listed in Table 1, and the strategies for *fusA* disruption are presented in Fig. 3. In brief, the fosmid DNA, pFusA, which contained the *fusA* gene in a 36.8 kb chromosomal DNA fragment cloned into pCC1fos (EPICENTRE Biotechnologies), was introduced into *Escherichia coli* BW25113 carrying Red recombinase by pKD46 [10]. The chloramphenicol acetyl transferase (*cat*) gene of pFusA was replaced with a tetracycline-resistance gene (Tc) using a λ Red recombination system to construct fosmid pFusA-Tc. The Tc gene was amplified from pBC16 with the Foscm-TCF and Foscm-TCR primers bearing 70-bp side arms that bind to the flanking regions of the *cat* gene of pCC1fos (EPICENTRE Biotechnologies). For the inactivation of the *fusA* gene, a chloramphenicol resistance gene–kanamycin resistance gene (*cat–kan*) cassette was introduced into the *fusA* structural gene of pFusA-Tc using a λ RED recombination system. The *cat–kan* cassette was constructed as follows. The *cat* gene was amplified by PCR with primers Cm1 and Cm2 from pDG1661 [11], and was then introduced into pGem7zf(+) (Invitrogen Inc.) with an EcoRI and BamHI cleavage site. The resulting plasmid was digested with the NarI restriction enzyme and ligated with the PCR product containing the kanamycin resistance gene that was amplified from pKD4 [10] using the Kd4kanF and Kd4kanR primer set. The constructed *cat–kan* cassette was amplified with primers FusckF and FusckR, giving 60-bp homologous arms of the target site to each ends. The amplified *cat–kan* cassette was inserted into pFusA-Tc to construct the fosmid pDfusA. To remove the pKD46 plasmid completely, kanamycin-resistant transformants were transferred onto a fresh agar medium containing kanamycin and incubated at 37 °C. The disruption of *fusA* with the *cat–kan* cassette was confirmed by PCR with FusdelF and FusdelR primers, which bind to the outer regions of the

Table 1
Primers used in this study

Primers	Oligonucleotide sequences[a]
Foscm-TCF	5′-TATCGAGATTTTCAGGAGCTAAGGAAGCTAAAATGGAGAAAAAAATCACTGGATATACCACCGTTGATAGATACAAGAGAGGTCTCTCG-3′
Foscm-TCR	5′-GGCACCAATAACTGCCTTAAAAAAATTACGCCCCGCCCTGCCACTCATCGCAGTACTGTTGTAATTCATAACAAACGGGCCATATTGTTG-3′
CatF	5′-AAA*GGATCC*TCATGTTTGACAGCTTATCATCG-3′
CatR	5′-AAA*GAATTC*CCACGCCGAAACAAGCGCTC-3′
Kd4kanF	5′-CC*ATCGAT*GTGTAGGCTGGAGCTGCTTC-3′
Kd4kanR	5′-CC*ATCGAT*ATGGGAATTA GCCATGGTCC-3′
FusckF	5′-TACTATTGTTCGACATGCATCATATTGTCTCAGATGGGGTTTCTATGAATATTCTCATAGTCATGTTTGACAGCTTATCATCG-3′
FusckR	5′-AACAGCGCATGGATCGTCTTGTCACGAGGATAATCTGCCTTGGCTTCGTCCGGCGCTTTCCCACGCCGAAACAAGCGCTC-3′
FusdelF	5′-AGCTCCATTGCTGCGGGTCG-3′
FusdelR	5′-ATCTTCACATACGACTGCCAC-3′

[a] The underlined sequences indicate the targeted region for Red recombinase and italicized forms mean the synthetic restriction sites.

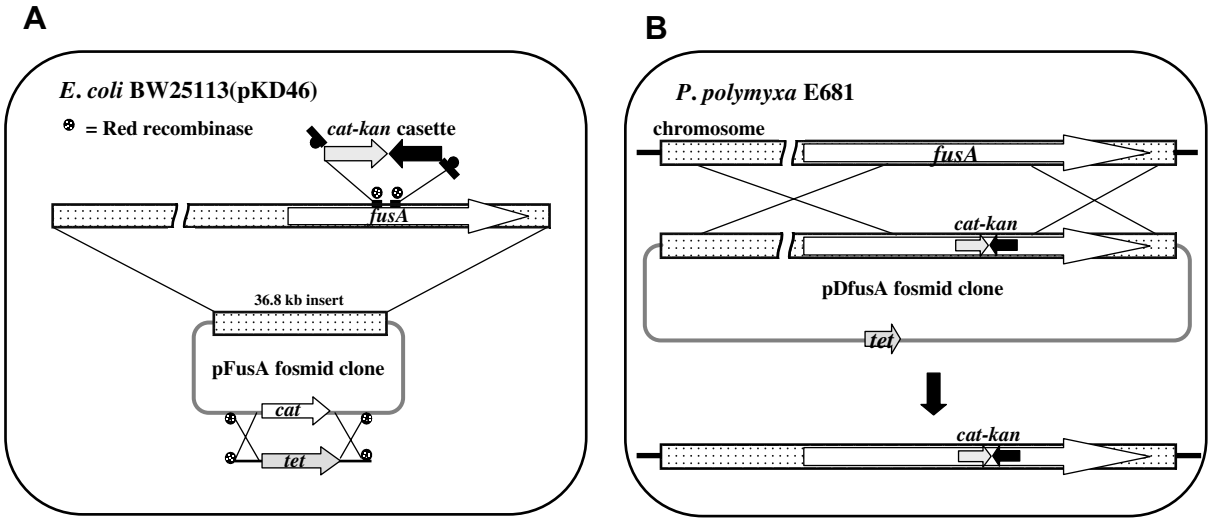


Fig. 3. Scheme for the construction of a fosmid containing the *fusA* disruption in *E. coli* by using the λ Red recombination system (A) and a *fusA*-disrupted *P. polymyxa* strain (B).

homologous arm. The fosmid pDfusA was introduced into *P. polymyxa* E681 to generate a fusaricidin-defective mutant.

Transformation of P. polymyxa. Transformation of *P. polymyxa* was performed by electroporation. Competent cells were prepared using the following procedure. A single colony of strain E681 that had been grown on a TSA plate was cultured in BHIS broth for 20 h at 30 °C and 200 rpm. Two milliliters of pre-culture were inoculated in 200 mL of BHIS and cultured at 30 °C and 200 rpm. When the cells reached an OD_{600} of 0.5, the culture was put in ice for 10 min and bacterial cells were centrifuged at 5000*g* for 10 min at 4 °C. After washing the cells twice with cold SM buffer (10% sucrose and 1 mM $MgCl_2$), the competent cells were resuspended in SM buffer at a concentration of 10^{10} cells/ml. Electroporation was performed using a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.). Competent cells that had been kept frozen were thawed on ice, mixed with DNA, transferred to 2 mm cuvettes, and placed on ice for at least 5 min. The sample was pulsed with a voltage of 6.25 kV cm^{-1}, a capacitance of 25 μF, and a resistance of 200 Ω. One milliliter of pre-warmed BHIS was added and incubated at 30 °C for 3 h. After incubation, the cells were cultured on TSA plates containing chloramphenicol. The *fusA* disruption was confirmed by PCR with FusdelF and FusdelR primers.

Nucleotide sequence accession number. The GenBank Accession Number for the fusaricidin synthetase gene (*fusA*) is EU184010.

Results and discussion

Identification of fusaricidins from P. polymyxa E681

Paenibacillus polymyxa E681 showed antifungal activity against *Fusarium oxysporum* (Fig. 4A). To identify the causal antibiotic, *P. polymyxa* E681 cultures were separated into supernatant and cell pellet by centrifugation, and the cell pellet was extracted using methanol. The result of the antifungal assay with the cell pellet extract and supernatant showed that the cell extract had a high antifungal activity, but the supernatant fraction had a weak activity (Fig. 4B). This implies that the antifungal material is tightly associated with bacterial cells or cell wall-bound polymer. The methanol extract of the cell pellet was analyzed by LC/MS (Thermo electron Co., USA). $(M+H)^+$ ion peaks were 883 and 897 at a retention time of 13.81 (Fig. 4E), 897 and 911 at a retention time of 14.46 (Fig. 4F), and 911 and 925 at a retention time of 15.13 (Fig. 4G). Analysis of ring-opened LI-F complex based on the previous report [1] showed the many fragment ions cleaved at each peptide linkage (cleavage at CO—NH of peptide bond) as weak peaks (Table 2). Comparison of these results with the report of Kuroda et al. [1] revealed that *P. polymyxa* E681 produced LI-F04a (fusaricidin A), LI-F04b (fusaricidin B), LI-F05a, LI-F05b, LI-F08a, and LI-F08b. These results indicate that *P. polymyxa* E681 produced the various fusaricidins under the culture conditions used in this study.

Domain analysis of a fusaricidin synthetase gene

During the whole genome sequencing of *P. polymyxa* E681 that has been completed recently in our laboratory (unpublished), an NRPS gene was identified as a potential fusaricidin biosynthetic gene. Domain analysis indicated that FusA, a polypeptide composed of 7908 amino acids, contains six modules. Each module is composed of three or four domains, such as C–A–T, C–A–T–E, C–A–T, C–A–T–E, C–A–T–E, and C–A–T–TE (Fig. 2A). The A domains were analyzed according to the method of Ansari et al. [12], and the active site residues for amino acid recognition are shown in Fig. 2B. The first, fourth, and sixth amino acids are well conserved in all previously reported fusaricidins (Fig. 1A), which suggests that the A1, A4, and A6 domains of FusA can recognize threonine, allo-threonine, and alanine, respectively. However, module six of FusA does not contain an epimerization domain, thus implying that the sixth amino acids of fusaricidin analogs produced by *P. polymyxa* E681 may exist as an L-form, although all reported fusaricidins contain D-form alanines in their sixth amino acid residues. However, we do not exclude the possibility that the sixth A domain of FusA can directly recognize the D-form alanine. The active site residues of the second A domain of FusA match the A domains of TycC A4 [13], LicB A1 [14], GrsB A2 [15], and SrfAB A1 [16], which suggests that the amino acid activated by FusA A2 is a valine. However, the detection of LI-F08a and LI-F08b from E681 (Table 2) suggests that A2 can also recognize isoleucine. The amino acid sequence of the FusA A3 was 53% identical to that of TycB A3 [13], which activates phenylalanine. The active site residues of FusA A3 also match well to those of TycB A3. However, analysis of the fusaricidins of E681 suggested that FusA A3 activates valine, isoleucine, and allo-isoleucine. The active site residues of FusA A5 perfectly match those of TycC A1 [13], ItuA A2, ItuB A2, ItuC A1 [17], MycA A2, MycB A2, MycC A2 [18], and BacC A5 [19], all of which activate asparagine. While the fifth amino acids of LI-F04a, LI-F05a, and LI-F08a are asparagines, those of LI-F04b, LI-F05b, and LI-F08b are glutamines. Thus, FusA A5 may activate both asparagine and glutamine. These results indicate that the fusaricidin biosynthesis gene isolated from the *P. polymyxa* E681 strain can produce more than one type of fusaricidin.

Construction and analysis of a fusA mutant strain

Li and colleagues recently reported a partial sequence of *fusA* including the first two modules [20]. They confirmed that *fusA* is essential for the biosynthesis of fusaricidin by PCR-targeted mutagenesis. The deduced amino acid sequence of the FusA homolog of our E681 strain exhibited 98.3% identity to that of the first two modules of the FusA of the PKB1 strain [20]. This finding strongly suggests that the FusA protein that was identified in this study is involved in fusaricidin biosynthesis. To confirm this, we constructed and characterized a *fusA* mutant strain. The antifungal activity of the *fusA* mutant of *P. polymyxa* E681 was completely abolished in a bioassay against *F. oxysporum* (Fig. 4A). The same result was obtained from the subsequent antifungal assay with the methanol extract of the mutant (Fig. 4B). LC/MS data supported the results by showing that the peak corresponding to fusaricidin could not be detected in the *fusA* mutant (Fig. 4D). Taken

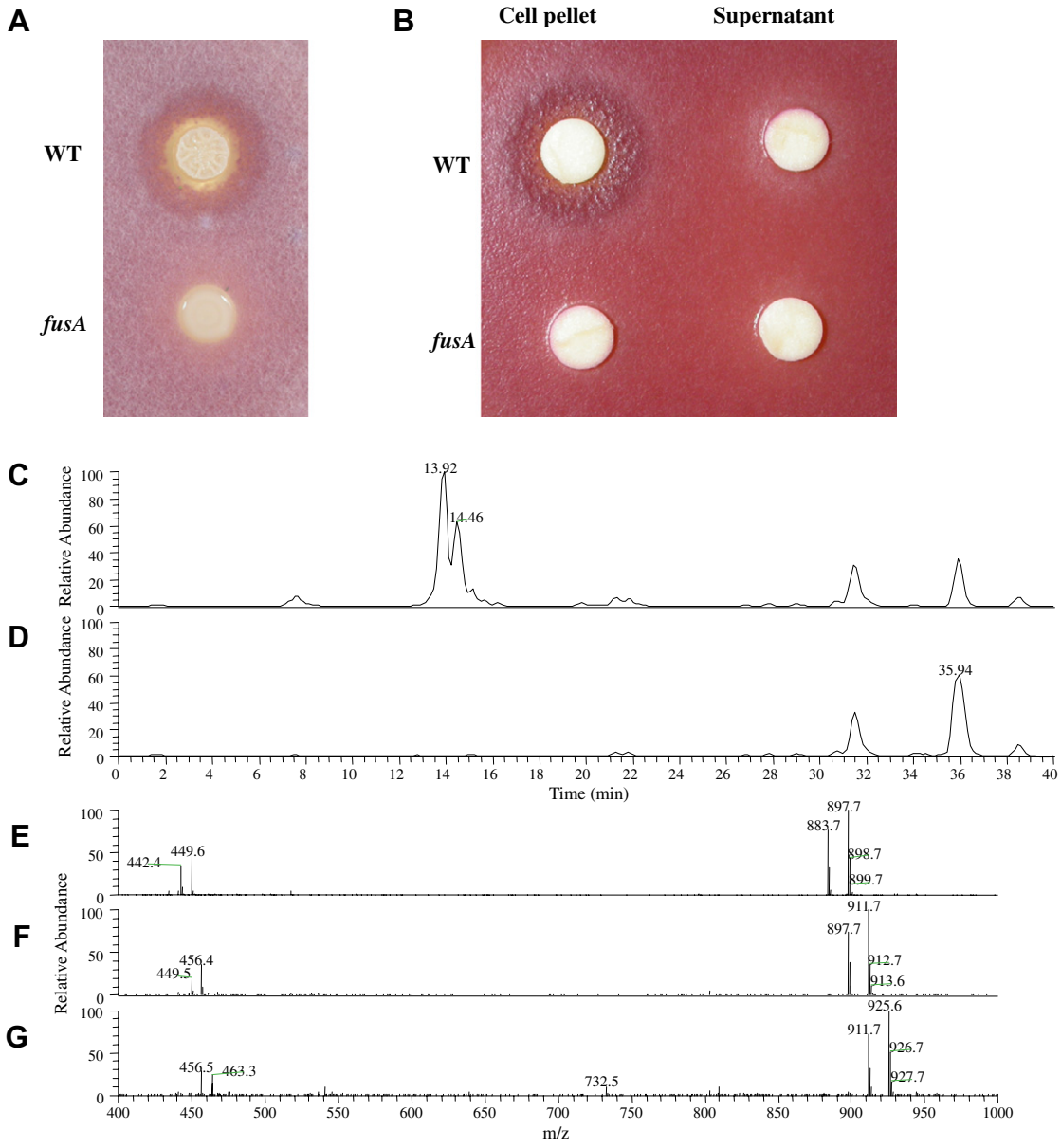


Fig. 4. (A) Antifungal activities of the wild-type E681 and the *fusA* mutant strain co-cultured with *F. oxysporum* on a PDA plate. (B) Antifungal activities of the methanol-extracted cell pellet, the culture supernatant of E681, and the *fusA* mutant against *F. oxysporum*. LC/MS total ion chromatogram (positive ion mode) of cell pellet methanol extracts of E681 (C) and *fusA* mutant (D) with MS data at retention times of 13.81 (E), 14.46 (F), and 15.13 min (G), respectively.

together, these results demonstrated that the *fusA* gene is involved in fusaricidin biosynthesis.

Conclusions and perspectives

The adenylation domain (A domain) specifically recognizes and activates one amino acid. Based on the crystal structure of the phenylalanine activating A domain of the NRPS gramicidin synthetase A (GrsA), Conti et al. determined 10 residue positions that are crucial to substrate binding and catalysis [21]. Comparison of these 10 residues with those of other A domains can extract a specificity-conferring code [12,22]. In the case of FusA, the codes of A domains could be determined by analyzing fusaricidin analogs from the *P. polymyxa* E681 strain because the active site residues of A domains were not exactly matched with those of previously reported A domains, except A1 and A4. Moreover, the E681 strain produced various fusaricidin analogs, which suggested that the substrate-binding pockets of A2, A3, and A5 of FusA may have flexible structures. The production of various fusaricidin analogs may enhance the antagonistic ability of the E681 strain against plant pathogenic fungi.

According to the recent reports on the excellent germicidal activity of fusaricidin against pathogenic gram-positive bacteria and plant pathogenic fungi [3–5], fusaricidin

Table 2
Fragment ions (*m*/*z*) of ring-opened peptides (b*n*, B′*n*, β*n*, y*n*, and Y*n* ions)[a]

Ion	LI-F0-					
	4c	4d	5c	5d	8c	8d
$[M+H]^+$	901 vs	915 vs	915 vs	929 vs	929 m	943w
$[MH-NH_3]^+$	884 s	898 vs	898 s	912 m	912 s	926 m
$[MH-H_2O]^+$	883 b	897 v	897 s	911 s	911 s	925 vs
$[MH-CO_2]^+$	857 vs	871 s	871 vs	885 vs	885 vs	899 s
S	830 vs	844 m	844 vs	858 vs	858 vs	872 vs
$[S-H_2O]$	812 m	826 m	826 s	840 vs	840 s	854 s
b1	399 vw	399 vw	399 vw	399 vs	399 vw	399 vw
b2	498 vw	498 w	498 vw	498 vw	512 vw	512 w
b3	597 w	597 w	611 vw	611 vw	625 vw	625 w
b4	698 m	698 m	712 m	712 m	726 m	726 m
b5	812 m	826 m	826 s	840 vs	840 s	854 s
β1	346 vw	346 w	346 w	346 w	346 w	346 w
β2	445 m	445 s	445 vw	445 s	459 s	459 s
β3	544 vw	544 vw	558 m	558 vw	572 w	572 vw
β4	645 vw	645 w	659 w	659 vw	673 vw	673 w
y3	305 vw	319 w	305 vw	319 vw	—	319 w
y4	404 w	418 m	418 m	432 vw	418 w	432 w
y5	503 m	517 m	517 m	531 m	531 m	545 m
y6	604 vw	618 w	618 vw	632 vw	632 vw	646 vw
y′7	859 m	873 m	873 m	887 m	887 m	901 m
Y3	288 w	302 w	—	—	—	—
Y4	387 vw	104 w	401 w	415 vw	401 vw	—
Y5	486 vw	500 w	500 w	—	514 vw	—
Y6	—	—	601 w	—	(615)	—

[a] Relative abundances of the ions for the base peak are listed with the following abbreviations: vs means >50%, s means 20–50%, m means <20% and >5%, w means 2–5% and vw means <2%.

seems to have great potential for industrial use. As part of efforts to increase the productivity of an antibiotic, an antibiotic biosynthesis gene was inserted into a host strain to industrially mass produce the antibiotic [23,24]. Attempts have also been made to substitute a promoter of the antibiotic biosynthesis gene with a stronger promoter in order to increase productivity [17]. There have been attempts to develop novel antibiotics by re-constructing modules or domains of antibiotic biosynthesis genes [25–27] or replacing a certain amino acid in a domain [28]; such attempts were expected to contribute to the development of a novel antibiotic that would have excellent activity. Therefore, the fusaricidin synthetase gene can be effectively used for the development of fusaricidin-based novel antibiotics and the improvement of fusaricidin productivity.

Acknowledgments

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