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Nonribosomal Biosynthesis of Fusaricidins by *Paenibacillus polymyxa* PKB1 Involves Direct Activation of a D-Amino Acid

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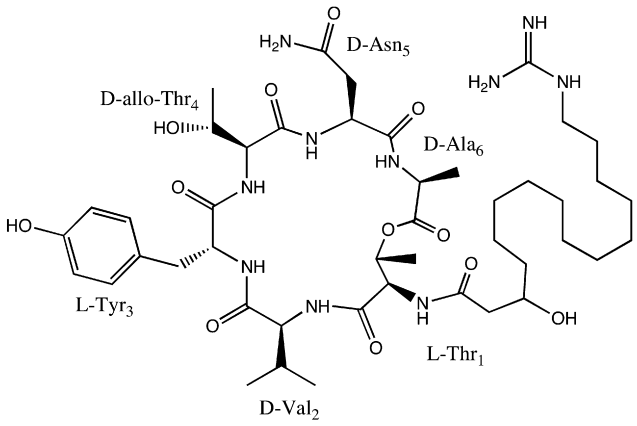
SUMMARY

Paenibacillus polymyxa **PKB1 produces fusaricidins, a family of lipopeptide antibiotics that strongly inhibits the growth of many plant pathogenic fungi. The fusaricidin biosynthetic gene cluster was cloned and sequenced, and it spans 32.4 kb, including an open reading frame (*fusA*) encoding a six-module nonribosomal peptide synthetase. The second, fourth, and fifth modules of fusaricidin synthetase each contain an epimerization domain, consistent with the structure of fusaricidins. However, no epimerization domain is found in the sixth module, corresponding to D-Ala. This sixth adenylation domain was produced at a high level in *Escherichia coli* and is shown to activate D-Ala specifically, providing evidence for direct activation of a D-amino acid by a prokaryotic peptide synthetase. The fusaricidin gene cluster also includes genes involved in the biosynthesis of the lipid moiety, but no genes for resistance, regulation, or transport functions were encountered.**

INTRODUCTION

Fusaricidins are a group of lipopeptide antibiotics produced by *Paenibacillus polymyxa* (formerly *Bacillus polymyxa*) and consist of a guanidinylated β-hydroxy fatty acid linked to a cyclic hexapeptide including four amino acid residues in the D-configuration (Figure 1) (Nakajima et al., 1972; Kurusu et al., 1987; Kajimura and Kaneda, 1996, 1997; Kuroda et al., 2000). The antifungal activity of the fusaricidins against *Leptosphaeria maculans*, a plant pathogenic fungus causing phoma stem canker (blackleg) disease in canola, makes *P. polymyxa* PKB1 of interest as a potential agent for biocontrol of blackleg disease (Kharbanda et al., 1997; Beatty and Jensen, 2002). Previously, we identified a fragment of a peptide synthetase gene (*fusA*) involved in the nonribosomal biosynthesis of fusaricidins (Li et al., 2007). Nonribosomal peptide synthetases (NRPSs) are large multienzyme complexes that are organized into modules (Marahiel et al., 1997). The number and order of modules are usually colinear with the amino acid sequence of the peptide product. Modules can be further divided into domains, each responsible for one catalytic step of peptide synthesis. The adenylation (A) domain is responsible for substrate recognition and activation as an aminoacyl adenylate. The activated amino acid is subsequently transferred to a 4′-phosphopantetheine (4′-PP) cofactor that is covalently tethered to the T domain, located downstream of the A domain. The condensation (C) domain, located between the T and A domains of consecutive modules, catalyzes peptide bond formation between two adjacent substrates. Finally, the fully assembled peptide chain is released from the enzyme template through cyclization or hydrolysis, which is typically carried out by a thioesterase (TE) domain located at the C-terminal end of the last module, although in some instances a reductase domain can also be responsible for release and cyclization (Kessler et al., 2004; Kopp et al., 2006).

Unlike ribosomally synthesized peptides and proteins, a common structural feature of nonribosomal peptides is the incorporation of nonproteinogenic amino acid residues, which dramatically increases the structural diversity and biological activity of peptides made by NRPSs. These nonproteinogenic residues include D-amino acids, and three different mechanisms of D-amino acid incorporation have been encountered to date. In most peptide synthetases, modules that incorporate D-configured residues contain an additional domain responsible for epimerization, found downstream of the T domain. An L-amino acid is activated, and the epimerization (E) domain then catalyzes L-to-D racemization of the thioester-bound amino acid (Stachelhaus and Walsh, 2000). A second mechanism for incorporating D-amino acids was observed in several NRPSs isolated from both actinomycete and *Pseudomonas* strains (Guenzi et al., 1998; McCafferty et al., 2002; Roongsawang et al., 2003; Scholz-Schroeder et al., 2003; Balibar et al., 2005; Yin and Zabriskie, 2006). In the lipopeptide arthrofactin, for example, there are no E domains detected in any of the 3 arthrofactin synthetases, although 7 of the 11 amino acids are in the D-configuration. Biochemical analyses demonstrated that A domains in modules corresponding to D-amino acids were specific for activation of L-isomers, and epimerase activity was provided by a new type of C domain with dual epimerization and condensation functions, located downstream of the T domain acylated with the amino acid undergoing epimerization. A third, very rare strategy for incorporation of D-amino acids involves the direct activation of D-isomers by the A domains. This mechanism was originally encountered in cyclosporin (Dittmann et al., 1994; Weber et al., 1994) and HC-toxin synthetases (Walton, 1987; Scott-Craig et al., 1992), both from fungal systems. Although these NRPSs purified from natural sources were shown convincingly to incorporate D-Ala, no biochemical studies with recombinant A domains have yet been conducted. Both gene clusters encode independent



Position	2	3	5
Amino acid substitutions	D-Val, D-*allo*-Ile, or D-Ile	L-Tyr, L-Phe, L-Val, L-*allo*-Ile, or L-Ile	D-Asn, or D-Gln

Figure 1. Structure of Fusaricidin C
Amino acid substitutions tolerated at three defined positions in fusaricidin variants are also presented. Residues are numbered according to the order of synthesis.

alanine racemases to generate the required D-Ala (Hoffmann et al., 1994; Cheng and Walton, 2000). Very recently, a third example of direct activation of a D-amino acid was reported in *Streptomyces atroolivaceus*, producer of the antitumor agent leinamycin. Leinamycin is a hybrid peptide/polyketide product in which a D-Ala residue serves as a starter unit for assembly of the molecule. The D-Ala residue is activated for this purpose by means of an isolated adenylation protein, and the specificity of this protein was shown to be for D- rather than L-Ala (Tang et al., 2007). Finally, the D-alanylation of lipoteichoic acids in *Bacillus subtilis* involves a D-alanyl-D-alanine carrier protein ligase that appears to activate D-alanine directly by a mechanism similar to that seen in the nonribosomal peptide synthatases (Perego et al., 1995).

In this study, we cloned and characterized the complete fusaricidin biosynthetic gene cluster from *P. polymyxa* PKB1, including *fusA*, which encodes a single peptide synthetase enzyme involved in fusaricidin biosynthesis. Substrate specificity of the A domain from the sixth module was investigated, and D-Ala was shown to be activated directly, providing clear evidence of direct selection and activation of a D-amino acid in a typical prokaryotic NRPS system.

RESULTS AND DISCUSSION

Cloning and Sequencing the Fusaricidin Biosynthetic Gene Cluster, *fus*

Previously, we constructed a SuperCos-1 genomic library of *P. polymyxa* PKB1, and isolated a cosmid, Col-19, carrying a partial ORF (designated *fusA*) that encodes two modules typical of NRPSs (Li et al., 2007). Disruption of *fusA* completely abolished the antifungal activity of strain PKB1, indicating that it is part of the fusaricidin biosynthetic gene cluster (*fus*). However, additional sequence analysis of Col-19 indicated that rearrangement of the insert DNA or incorporation of non-contiguous genomic DNA fragments had occurred, preventing complete analysis of the *fus* gene cluster. A second SuperCos-1 genomic library of PKB1 showed similar defects in all *fusA*-bearing clones, despite careful attention to size selection of insert DNA fragments during library construction. To circumvent these apparent instability problems, a third genomic library of PKB1 was constructed by using pSMART-FOS, a single-copy fosmid vector designed for the stable maintenance of DNA inserts. The fosmid library was screened by using a probe derived from the 3′ end of the previously sequenced *fusA* gene fragment, and two overlapping fosmids, 4G9 and 6D11, were selected from a group of positive clones for further study. Southern analyses showed no evidence of rearrangement of the fosmid insert DNA, and sequence analysis yielded~48 kb of contiguous DNA sequence information (Figure 2A). Subsequent analyses suggest that this DNA sequence covers the entire fusaricidin biosynthetic gene cluster as well as flanking regions, although definitive proof would require heterologous expression of the entire gene cluster.

Overall Organization of the *fus* Gene Cluster

The *fus* gene cluster itself covers 32.4 kb and includes 8 ORFs. The organization and assigned functions of these ORFs are shown in Figure 2A and Table 1, respectively. The boundaries of the *fus* cluster are predicted based on the nature of the gene products encoded and on gene-disruption analyses. Genes identified in the biosynthetic gene cluster include those necessary for assembly of the peptide backbone as well as synthesis and incorporation of the lipid moiety, but genes for regulation, export, and resistance were not detected in the region.

Modular Organization of the Fusaricidin Synthetase

The *fus* gene cluster includes a large ORF of about 23.7 kb, encoding a protein of 7,908 amino acids with a molecular mass of 888,101 Da. The predicted gene product shows the highest similarity to bacitracin synthetase (BacC) from *Bacillus licheniformis*. Sequence analysis revealed that the partial ORF cloned previously (Li et al., 2007) corresponds to the 5′ end of this NRPS gene. Therefore, we have retained the designation for the complete ORF as the fusaricidin synthetase gene, *fusA*. The translation of *fusA* appears to start with an ATG codon at nt 17,682 of the deposited sequence, 7 bp downstream of a putative ribosome-binding site (5′-AGGAGT-3′). By comparison with other peptide synthetases, six functional modules were identified within the deduced amino acid sequence of FusA (Figure 2B). Each module contains the three catalytic domains common to NRPSs, an A, a T, and a C domain.

FusA contains five typical C domains that presumably catalyze peptide bond formation between the adjacent amino acid residues of fusaricidin. An additional C domain was detected at the N terminus of the first module of FusA. C domains are not normally present in the initial modules of NRPSs, except for in lipopeptides and chromodepsipeptides, subgroups of nonribosomal peptides with acyl groups and chromophores, respectively,

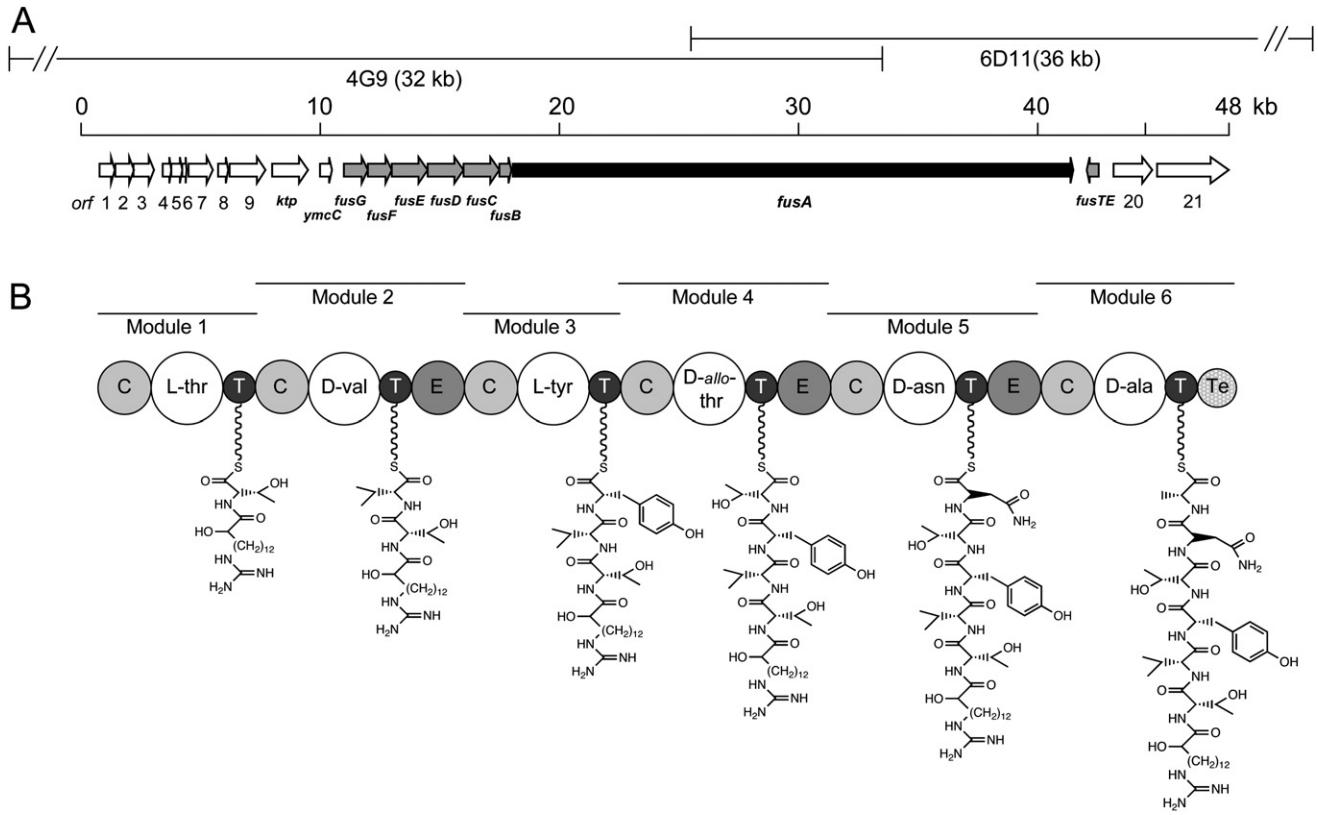


Figure 2. Diagrammatic Representation of the Fusaricidin Biosynthetic Gene Cluster

(A) ORF arrangement of the *fus* gene cluster and flanking regions. The black arrow represents the peptide synthetase gene *fusA*, whereas other fusaricidin biosynthetic genes are shown in gray; genes flanking the cluster are in white. The overlapping fosmid inserts cloned in this study are also shown.

(B) Module and domain organization of the fusaricidin synthetase encoded by *fusA*.

attached at the N terminus (Schmoock et al., 2005). The presence of N-terminal C domains (C_N) has been reported for a number of lipopeptide synthetases, including those from actinomycete, *Bacillus*, and *Pseudomonas* species (Guenzi et al., 1998; Roongsawang et al., 2003; Cosmina et al., 1993; Duitman et al., 1999; Konz et al., 1999; Lin et al., 1999; Tsuge et al., 2001; Miao et al., 2005, 2006) and is suggested to catalyze acylation of the first amino acid with a fatty acid as an early step of lipopeptide biosynthesis. Since they catalyze the coupling of fatty acids rather than amino acids to the first amino acid of the peptide chain, these initial C domains may share structural features not found in regular C domains, which could explain the greater similarity seen among C domains from within this group than to other typical C domains (Figure 3). The remaining five C domains in FusA can be subdivided into two groups according to their locations: they are the C_D domains (in FusA-C3, FusA-C5, and FusA-C6), which are preceded by an E domain and accept an upstream D-amino acid at the donor site, and C_L domains (in FusA-C2 and FusA-C4), which are located immediately downstream of a T domain and accept an upstream L-amino acid at the donor site. Distinct differences between the C_D and C_L domains and similarities within the same domain type were seen in the amino acid sequences of the conserved motifs. This may reflect the stereoselectivity of C domains toward the C-terminal amino acid of the growing peptidyl chain in the course of the condensation reaction.

Modifying E domains were detected at the C-terminal ends of the second, fourth, and fifth modules, consistent with the incorporation of D-amino acids in these positions of fusaricidin peptides. However, the sixth module, FusA-M6, corresponding to D-Ala, lacks such an E domain, and instead terminates with a C-terminal TE domain responsible for the cyclization and release of the peptide product.

The T domains upstream of the E domains in FusA-M2, FusA-M4, and FusA-M5 all contain a core sequence of LGG**D**SIK. The aspartate residue (in bold) in front of the conserved serine residue is essential for proper interaction between the T domain and the downstream E domain, and subsequent racemization of the thioester bound L-amino acid to its D-isomer (Linne et al., 2001). In contrast, the core sequence of the T domain found in FusA-M6 (LGG**H**SL) matches that of T domains not associated with E domains (xGG**H**SL), such as those found in the first and third modules. This finding is consistent with the absence of an E domain in the final module of FusA. Furthermore, since the amino acid activated by FusA-M6 occupies the C-terminal position of the fusaricidin peptide, this excludes the possibility that epimerization of an L-Ala residue is catalyzed by a downstream dual C/E domain, as seen in some pseudomonads and

Table 1. Summary of ORFs from the Fusaricidin Biosynthetic Gene Cluster and Flanking Regions

ORF	Gene	Nucleotide Position	Highest BLAST Hit (%Identity/%Similarity)
1	*gat*	853–1428	Glutamine amidotransferase of anthranilate synthase (*Thiobacillus denitrificans*) YP_315982 (72/87)
2	*adl*	1432–2328	4-amino-4-deoxychorismate lyase (*Bacillus sp.*) ZP_01173359 (51/61)
3	*dps*	2361–3191	Dihydropteroate synthase (*Geobacillus kaustophilus*) YP_145922 (64/76)
4	*dna*	3390–3752	Dihydroneopterin aldolase (*Bacillus coagulans*) ZP_01697879 (54/75)
5	*hpk*	3762–4310	2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase (*Bacillus cereus*) ZP_00240852 (50/67)
6	*xre*	4271–4462	Probable transcriptional regulator (*Bacillus sp.*) ZP_01173355 (50/71)
7	*dus*	4508–5536	tRNA-dihydrouridine synthase (*Bacillus clausii*) YP_173620 (74/88)
8	*greA*	5730–6227	Transcription elongation factor (*Pelotomaculum thermopropionicum*) YP_001210812 (71/87)
9	*lysRS*	6338–7858	Lysyl-tRNA synthetase (*Geobacillus kaustophilus*) YP_145927 (74/86)
10	*ktp*	7949–9286	K^+ uptake transporter (Trk family) (*Geobacillus kaustophilus*) YP_146065 (48/71)
11	*ymcC*	9579–10112	YmcC (unknown function) (*Rubrobacter xylanophilus*) YP_645537 (49/68)
12	*fusG*	10739–11473	Enoyl-(acyl carrier protein) reductase (*Bacillus cereus*) NP_977660 (62/79)
13	*fusF*	11534–12946	Acyl CoA ligase (*Streptomyces aizunensis*) AAX98201 (36/56)
14	*fusE*	12991–14214	Hypothetical protein (aldehyde dehydrogenase) (*Saccharopolyspora erythraea*) YP_001102874 (40/65)
15	*fusD*	14211–15911	Acetolactate synthase large subunit (*Saccharopolyspora erythraea*)YP_001102875 (42/62)
16	*fusC*	16118–17176	3-oxoacyl-(acyl carrier protein) synthase (*Bacillus licheniformis*)YP_078422 (54/74)
17	*fusB*	17181–17588	(3*R*)-hydroxymyristoyl-[acyl carrier protein] dehydratase (*Bacillus cereus*) NP_832645 (48/75)
18	*fusA*	17682–41408	Bacitracin synthetase 3; BacC (*Bacillus licheniformis*) O68008 (42/61)
19	*fusTE*	41926–42549	Hypothetical protein (α/β hydrolase) (*Gloeobacter violaceus*) NP_924256 (47/66)
20	*rrnA-16 s*	43187–44711	16S ribosomal RNA gene (*Paenibacillus polymyxa*) EF532687 (99%)
21	*rrnA-23 s*	45016–47943	23S ribosomal RNA gene (*Bacillus licheniformis*) CP000002 (86%)

actinomycetes (Balibar et al., 2005; Yin and Zabriskie, 2006). Taken together, these findings suggest that either an external racemase is involved in the transformation of thioester-bound L-Ala to D-Ala, or that free D-Ala is directly selected and activated by the A domain of FusA-M6, as was shown for the isolated adenylation protein from leinamycin synthetase (Tang et al., 2007) and strongly suggested for cyclosporin and HC-toxin biosynthesis (Dittmann et al., 1994; Weber et al., 1994; Walton, 1987; Scott-Craig et al., 1992).

Substrate Specificities of Adenylation Domains

By comparison with the amino acids lining the substrate-binding pocket in the Phe-activating domain of gramicidin S synthetase GrsA, a "nonribosomal code" specifying important residues involved in substrate recognition by A domains has been defined by two independent groups (Stachelhaus et al., 1999; Challis et al., 2000). The corresponding amino acid residues were examined in the six A domains of FusA, and the substrate specificity of each A domain was predicted (Table 2). The A domain in the first module (FusA-A1) incorporating L-Thr and in the fourth module (FusA-A4) incorporating D-*allo*-Thr both share the same signature sequence as the Thr-activating domain from fengycin synthetase (FenD, GenBank accession number CAA09819). The substrate-recognition sequence of FusA-A2 is most similar to the Val-activating A domain from surfactin synthetase (SrfA-B, GenBank accession number BAA08983), which also activates Ile to a lesser extent (Elsner et al., 1997). The incorporation of D-Val, D-Ile, or D-*allo*-Ile as substrates at this position in fusaricidins indicates an even broader substrate specificity, possibly due to substitutions of Ile299→Leu and Gly322→Cys in FusA-A2 compared to SrfA-B. FusA-A3 contains a signature sequence most closely resembling the 3-hydroxy-L-Tyr-activating domain from chloroeremomycin synthetase (CepB, GenBank accession number CAA11795). However, considerable similarity to the Phe-activating domains from TycA and GrsA synthetases (GenBank accession numbers AAC45928 and CAA33603, respectively) was also noted, which may explain the overall relaxed specificity toward the aromatic and hydrophobic amino acid residues seen at this position in the fusaricidin variants. The specificity code of FusA-A5 matches that of the Asn-activating A domain from tyrocidine synthetase (TycC, GenBank accession number AAC45930). Replacement of D-Asn by D-Gln at the fifth position in several fusaricidin variants indicates that this conservative substitution is tolerated by the corresponding A domain. The amino acid substrate for FusA-A6 cannot be predicted because its signature sequence shows no similarity to A domains with assigned specificities, including those activating L- and D-Ala (Figure 4). With the exception of FusA-M6, the predicted substrates for the A domains of each module of fusaricidin synthetase are consistent with the residues actually found at the respective positions of the fusaricidin peptides.

In order to determine the substrate specificity of FusA-A6, a DNA fragment corresponding to the A domain of FusA-M6 was amplified from fosmid 6D11 and was cloned into pET-19b. Based on previously described A domain borders (Mootz and Marahiel, 1997), the N-terminal end of the FusA-A6 domain was set at 101 aa upstream from the core motif A2 (LKAGGA), and the C-terminal end was set at 17 aa upstream from the core motif T (LGGHS). After expression in *E. coli*, a soluble protein with a molecular mass of 64 kDa was obtained and purified

	C1	C2	C3	C4	C5	C6	C7
consensus	SxAQxR[LW/MY]xL	RHExLRTxF	MHHxISDG[W/S/V]S	YxD[F/Y]AVW	[I/V]GxFVNT[QC/LA]xR	H[Y/QD/N]PFE	RDxSRNPL
C_N domains							
FusA-C1	TNAQKRIWYT	QYDAFRIRI	MHHIISDGIS	YIQYIAD	IGMFVSTAAAR	HQKYPYN	KDI-QRLFG
SrfA-A-C1	TDAQKRIWYT	RNDAMRLRL	VHHVISDGIS	FIDHVLS	LGMFVSTVPLR	HQKYPYN	SSL-TKLFT
DptA1-C1	TAAQQSVWLA	ETEALRTRF	YHHIALDGYG	LAGVLTE	PCMLANDVPLR	HQRFRGE	AGL-ARVTV
ArfA-C1	TAAQLDIWLD	RHDALRTIL	AHHLIVDGWG	YIDFIEA	LGLFAQVSAVR	HQRFPVS	RSQ-LFEVT
C_L domains							
FusA-C2	SSAQKRLYVL	RHESLRTGF	MHHIISDGVS	YKDYAVW	IGMFVNTLALR	HQDYPFE	RDV-SRNPL
FusA-C4	SSAQKRLFIL	RHGSLRTRF	MHHIVSDGVS	YTDYAVW	IGMFVGTVALR	NQDYPFE	RDL-SRNPL
C_D domains							
FusA-C3	TPMQKGMLFH	RHAILRTNF	FHHIVMDGWC	YSRYIEW	IGLFINTIPVR	YDTYPLF	QDLISHIMV
FusA-C5	TPMQKGMLFH	RHAILRTGF	FHHIVMDGWC	YSRYIEW	VGLFINTVPIR	YDTYPLY	QDLISHIMI
FusA-C6	TPMQKGMLFH	RHAILRTNF	SHHIILDGWC	YSQYIQW	IGLFINTVPVR	YDSYPLY	QDLISHIMV

Figure 3. Comparison of the Conserved Core Motifs within Condensation Domains of FusA

Alternative amino acids at a particular position are indicated. The N-terminal C domains (C_Ns) of surfactin synthetase SrfA-A (*Bacillus subtilis*, GenBank accession number BAA02522), daptomycin synthetase DptA (*Streptomyces roseosporus*, accession number AAX31557), and arthrofactin synthetase ArfA (*Pseudomonas sp*. MIS38, accession number BAC67534) are shown in comparison to the C_N of FusA. C_L and C_D domains of FusA represent condensation domains following modules activating L- and D-amino acid residues, respectively. Shading indicates identity with the consensus sequence. The C domains in each peptide synthetase are numbered according to their order in the protein.

by Ni^{2+}-affinity chromatography (Figure 5A). Enzymatic activity of the purified recombinant FusA-A6 domain was determined by an ATP-PPi exchange assay with various amino acids as substrate. From the results obtained (Figure 5B), it was evident that FusA-A6 activates D-Ala, with very low tolerance for L-Ala (less than 1% of D-Ala), or any of a variety of amino acids, including both L- and D-isomers (1%–4% of D-Ala). In previous studies of cyclosporin and HC-toxin synthetases (Dittmann et al., 1994; Weber et al., 1994; Walton, 1987; Scott-Craig et al., 1992), the specificity for D-Ala activation by respective A domains was first predicted based on the colinearity rule of NRPSs and then confirmed by showing direct incorporation of D-Ala by using native NRPS proteins isolated from the producer fungi (Walton, 1987; Cheng and Walton, 2000; Zocher et al., 1986). In contrast, direct activation of D-Ala was demonstrated by using a recombinant adenylation protein for leinamycin biosynthesis in *S. altooolivaceus* (Tang et al., 2007); however, this is an unusual system in which the D-Ala residue is incorporated into a hybrid peptide/polyketide by an isolated adenylation protein rather than an NRPS module.

The data from the present study show that the recombinant A domain from module six of fusaricidin synthetase recognizes and activates D-Ala as its substrate. To our knowledge, this represents the first example of a typical NRPS of prokaryotic origin in which direct activation of a D-amino acid was observed. Ala-specific A domains, whether for L- or D-isomers, show relatively weak similarity. However, since the side chain of D-Ala might be expected to extend toward the opposite face of the amino acid-binding pocket compared to L-Ala, it is not clear that the signature amino acids that define the nonribosomal code in A domains have any relevance for D-isomers. Furthermore, the small size of the Ala side chain may allow greater variability in

Table 2. Amino Acid Residues Lining the Substrate-Binding Pockets of Adenylation Domains in Fusaricidin Synthetase

Adenylation Domain	Residue Positions Involved in Substrate Recognition[a]									Predicted Substrate	Corresponding Residue in Fusaricidin
	235	236	239	278	299	301	322	330	331		
FusA-A1	D	F	W	N	I	G	M	V	H	Thr	L-Thr
FusA-A2	D	A	F	W	L	G	C	T	F	Val	D-Val, D-*allo*-Ile, or D-Ile
FusA-A3	D	A	S	T	L	A	G	V	C	3H-Tyr[b]	L-Tyr, L-Phe, L-Val, L-Ile, or L-*allo*-Ile
FusA-A4	D	F	W	N	I	G	M	V	H	Thr	D-*allo*-Thr
FusA-A5	D	L	T	K	I	G	E	V	G	Asn	D-Asn or D-Gln
FusA-A6	D	F	P	N	F	C	I	V	Y	*[c]	D-Ala

[a] Lys517 was not included because it is conserved in all six A domains.
[b] 3H-Tyr represents 3-hydroxy-L-tyrosine.
[c] * represents no match.

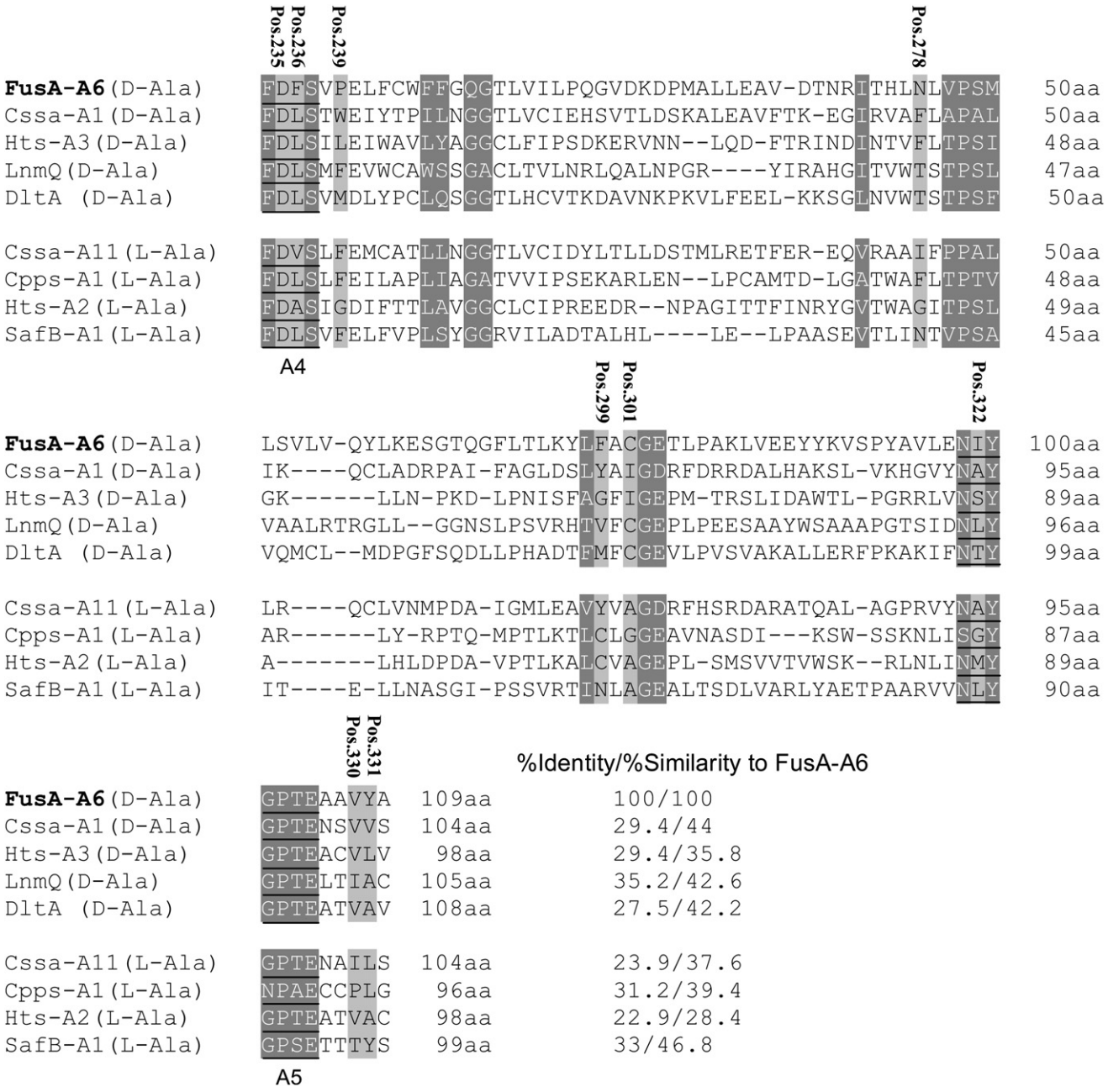


Figure 4. Alignment of the Amino Acid Sequences in Core Motifs A4 and A5 of FusA-A6 with Those of Other A Domains Known to Activate L- or D-Ala

Deduced specificity-conferring residues are shaded light gray. Conserved motif sequences A4 and A5 are underlined. Structural anchor residues are shaded dark gray. Abbreviations for proteins are as follows: Cssa, cyclosporin synthetase (Tolypocladium inflatum, accession number Z28383); Hts, HC-toxin synthetase (C. carbonum, accession number M98024); LnmQ, leinamycin synthetase (Streptomyces atroolivaceus, accession number AF484556); DltA, d-alanine-d-alanyl carrier protein ligase (Bacillus subtilis, accession number NP_391729.1); Cpps, d-lysergyl peptide synthetase (Claviceps purpurea, accession number CAB39315); SafB, saframycin Mx1 synthetase B (Myxococcus xanthus, accession number YP_632693). The A domains in each peptide synthetase are numbered according to their module order in the protein.

the residues lining an Ala-binding pocket to be tolerated (Challis et al., 2000). Interestingly, in each case in which direct activation of a D-amino acid has been shown, the residue in question is D-Ala. This allowable variability apparently can extend to accommodate the two chiral forms of Ala, whereas accommodating different chiral forms of larger amino acids may require so great a variation in sequence that A domain function would be compromised. Whereas the cyclosporin and HC-toxin biosynthetic gene clusters each encode a distinct alanine racemase catalyzing conversion of L- to D-Ala (Hoffmann et al., 1994; Cheng and Walton, 2000), an alanine racemase specific for the *fus* cluster was not encountered, nor was one reported for the leinamycin

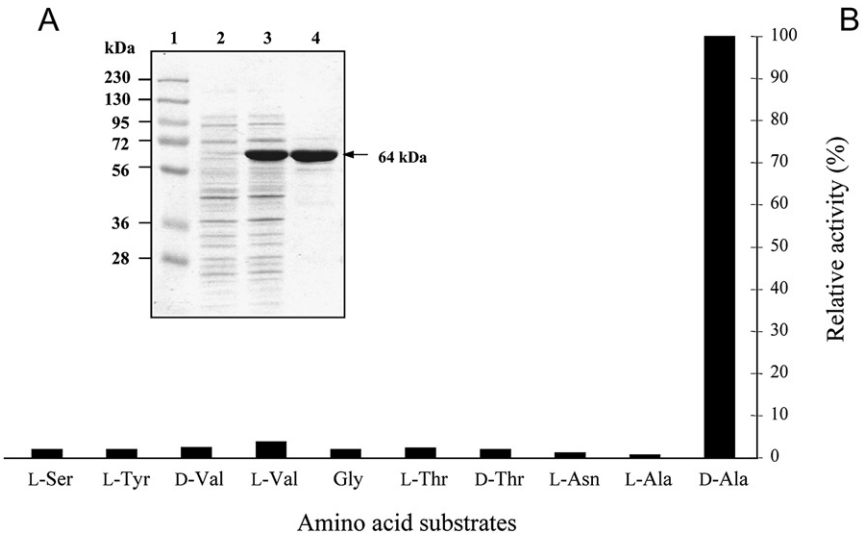


Figure 5. Analysis of His-Tagged Recombinant FusA-A6 Protein

(A) SDS-PAGE analysis of recombinant FusA-A6 protein. Lane 1, prestained protein ladder; lane 2, whole-cell extract obtained before IPTG induction; lane 3, whole-cell extract obtained after a 4 hr induction with IPTG; lane 4, protein purified by Ni^{2+}-affinity chromatography.

(B) Relative activities of purified FusA-A6 protein for various amino acid substrates determined by using the ATP-PPi exchange assay. The control reaction without amino acids was subtracted from all reactions with amino acid substrates.

system (Tang et al., 2007). However, alanine racemases are widely present in bacteria, where they provide D-Ala, essential for cell wall synthesis.

N-Terminal Lipidation of Fusaricidins

Fusaricidins belong to a group of nonribosomal peptides that are modified at the N terminus with a fatty acid. The lipid side chains of lipopeptide antibiotics are believed to play an important role in their interactions with their cellular target, i.e., cell membranes. The predicted gene products of six ORFs (*fusB*, *fusC*, *fusD*, *fusE*, *fusF*, and *fusG*) upstream of *fusA* show homology to enzymes involved in fatty acid or amino acid synthesis (Table 1) and, accordingly, are likely involved in the synthesis and/or modification and activation of a fatty acid precursor for attachment to the amino group of the N-terminal L-Thr residue of fusaricidin. The acyl-CoA ligase encoded by *fusF* may begin the process of lipid side chain synthesis by coupling coenzyme A (CoA) to an acyl group, perhaps arising from primary metabolism or generated by the action of the FusD gene product. The resulting CoA derivative would then be transferred to an acyl carrier protein (ACP) for elongation by the *fusB*, *fusC*, and *fusG* gene products, although no gene encoding an ACP-like protein was found in the *fus* cluster. Possibly, an ACP from fatty acid biosynthesis or from some other NRP or polyketide gene cluster fills this need in fusaricidin biosynthesis. Similarly, there is no obvious candidate gene in the *fus* cluster to provide a ketoreductase function during fatty acid biosynthesis, unless the aldehyde dehydrogenase encoded by *fusE* fulfills this role. Rather than starting the process of side chain synthesis, it is also possible that FusF ends the process by activating the fully formed lipid side chain to a CoA derivative before transfer to the initial C domain of FusA.

During lipid side chain synthesis, a β-OH group must be preserved or introduced into the growing fatty acid chain, and, ultimately, the activated fatty acid must be further modified by addition of a guanidino group. Alternatively, perhaps an arginine residue is deaminated to 5-guanidino pentanoic acid, then activated and transferred to ACP for elongation by a process analogous to fatty acid biosynthesis. Acylation of the N-terminal amino acid with the modified fatty acid is presumably catalyzed by the initial C domain in the first module of FusA. Additional studies are needed to clarify the catalytic roles of the fatty acid and amino acid biosynthetic genes upstream of *fusA* in fusaricidin biosynthesis.

Boundaries of the *fus* Cluster

Downstream of *fusA* and oriented in the opposite orientation, a 624 bp ORF encoding a putative protein of 207 amino acids was detected (Figure 2A). This gene product shows the greatest similarity to proteins of the α/β hydrolase fold family (~45% identity) and has one conserved GxSxG motif typical of thioesterases. Therefore, we designated this ORF *fusTE*, although it shows no similarity to genes encoding type II thioesterases from other NRPS gene clusters. It also shows no significant similarity to the C-terminal TE domain of FusA, but it may catalyze hydrolysis of acyl or aminoacyl groups inappropriately attached to the 4′-PP cofactors of fusaricidin synthetase, as has been demonstrated for other type II thioesterases (Schwarzer et al., 2002). Alternatively, FusTE may function as an acyltransferase for transfer of the fatty acid of fusaricidin onto the first condensation module of FusA, as has been demonstrated for SrfD, the type II thioesterase of surfactin biosynthesis (Steller et al., 2004). DNA sequence further upstream of *fusTE* showed 99% identity to a *P. polymyxa* 16S rRNA gene, and beyond that a 23S rRNA gene is apparent (Table 1). Therefore, *fusTE* was assigned as the 3′ boundary of the *fusA* gene cluster even though no gene-disruption studies were conducted to confirm its involvement.

About 10 kb of DNA sequence upstream of *fusG* was also examined in order to identify additional genes involved in fusaricidin production (Figure 2A). The next ORF (*ymcC*) upstream of *fusG* encodes a protein product of 177 aa with similarity to YmcC (a protein with unassigned function) from various species. The counterpart of *ymcC* in the *B. subtilis* 168 genome precedes a cluster of putative polyketide synthase genes. In order to determine whether *ymcC* plays a role in fusaricidin biosynthesis, a *ymcC* mutant was created by using a previously described PCR-targeting mutagenesis method (Li et al., 2007). The resulting *ymcC* mutant was assessed for fusaricidin production by bioassay of culture extracts against the indicator fungus *L. maculans*, but fusaricidin production was unchanged compared to the wild-type (data not shown). Another ORF oriented in the

same transcriptional direction, as *ymcC* is located further upstream. The predicted gene product of 445 aa shares sequence similarity with potassium-uptake transporter proteins and so was designated *ktp*. However, the PKB1 mutant in which *ktp* was replaced by an antibiotic-resistance cassette also produced wild-type levels of fusaricidins, thus indicating that the gene product of *ktp* is not required for export of, or resistance to, fusaricidins. In the sequenced region further upstream from *ktp*, a variety of ORFs was encountered, but none encoded functions obviously related to fusaricidin biosynthesis (Table 1). Therefore, the 5′ boundary of the *fus* cluster is likely defined by *fusG*.

SIGNIFICANCE

The gene cluster for production of fusaricidin-type antifungal antibiotics has been cloned and characterized from *Paenibacillus polymyxa* PKB1, a potential biocontrol agent for blackleg disease of canola. Other than *fusA*, encoding the peptide synthetase, and genes associated with the synthesis and attachment of the N-terminal guanidino-lipid, no genes for regulation, resistance, or transport were found in the cluster. Genes encoding regulators and ABC-type transporters are frequently found in gene clusters for lipopeptides, although their involvement in peptide production has not always been established (Roongsawang et al., 2003; Miao et al., 2005; Mootz and Marahiel, 1997). In fusaricidin production, these functions must be provided by genes from elsewhere on the chromosome. D-amino acid residues are important elements of nonribosomally produced peptides. Examination of the fusaricidin gene cluster revealed that two strategies for D-amino acid incorporation are employed by fusaricidin synthetase. Incorporation of D-amino acids in the second, fourth, and fifth positions of fusaricidins is mediated by E domains in the corresponding modules, whereas no E domain is found in the sixth module corresponding to D-Ala. The sixth A domain of FusA exhibits stereospecificity for D-Ala, a characteristic only reported previously in two eukaryotic NRPSs and one prokaryotic hybrid peptide/polyketide synthetase. Cloning of the fusaricidin NRPS gene makes it possible to consider genetic manipulation to increase the antifungal activity of the PKB1 strain. Fusaricidin is a mixture of at least 12 depsipeptides, resulting from the relaxed substrate specificity of fusaricidin synthetase. Alterations of A domain selectivity by mutation of specificity-conferring residues (Eppelmann et al., 2002; Uguru et al., 2004) may make it possible to narrow the substrate specificities of the relevant FusA modules, thereby increasing yields of fusaricidin A and B, variants associated with the greatest antifungal activity (Beatty and Jensen, 2002).

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions

Media for growth of *P. polymyxa* PKB1 and *Escherichia coli* have been previously described (Li et al., 2007). *E. coli* DH5α and XL1-Blue were used for preparation of recombinant plasmids. *E. coli* strain *E. cloni* Replicator (Lucigen, Middleton, WI) was used as the host for the fosmid genomic library and preparation of fosmid DNA. High-level production of recombinant proteins was performed in *E. coli* BL21(DE3).

DNA Manipulations

Chromosomal DNA from *P. polymyxa* was isolated as previously described (Li et al., 2007). The *P. polymyxa ymcC* and *ktp* mutants were constructed by using the previously described PCR-targeted mutagenesis technique (Li et al., 2007). The PCR primers used to amplify the Apra^r Cm^r *oriT* disruption cassette are as follows: *ymcC* mutant, JRL42-RD (5′-AAATACCAATTTCTAATTTGAAAGGAATCATCTATTATGATTCCGGGGATCCGTCGACC-3′) and JRL43-RD (5′-ACCAGCCAGATATCATCTAAGTGTAAGTCTTAACCTTTATGTAGGCTGGAGCTGCTTC-3′); *ktp* mutant, JRL31-RD (5′-CTCTTTTTCATAAGAACGGATGGAGAGAATACTCTAATGATTCCGGGGATCCGTCGACC-3′) and JRL32-RD (5′-GCTAATCAGCACGGGTACATCCTTTTTATAGATACATTATGTAGGCTGGAGCTGCTTC-3′). The λ Red recombination plasmid pKD46 and fosmid 4G9 were used to prepare the disruption constructs. All other DNA manipulations were carried out according to standard techniques (Sambrook et al., 1989).

Cloning and Sequencing of the *fus* Gene Cluster

A genomic library of *P. polymyxa* PKB1 was constructed with the fosmid vector pSMART-FOS (Lucigen, Middleton, WI), by following the manufacturer's instruction. Chromosomal DNA from the PKB1 strain was partially digested with Sau3AI and was size fractioned on a 0.8% low-melting-point agarose gel. The region containing DNA fragments of 35–45 kb was excised and recovered from the gel by agarase digestion. After precipitation with isopropanol, the purified genomic DNA fragments were ligated with BamHI-digested, dephosphorylated pSMART-FOS vector at 16°C for 16 hr. The resulting ligation mixture was packaged in vitro with Gigapack III XL (Stratagene) and then introduced into the *E. cloni* Replicator cells by transfection. A total of 550 clones were transferred onto a Hybond-N nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) and screened according to the manufacturer's protocols. The nucleotide sequences of positive fosmid inserts were determined in part by SeqWright DNA Technology Services (Houston, TX). Gaps were closed by direct sequencing of fosmid DNA, carried out by the Molecular Biology Facility at University of Alberta. GeneTools 2.0 and PepTool 1.0 (BioTools, Inc., Edmonton, Canada) were used for analysis of the DNA sequence and the amino acid sequence, respectively. Nucleotide and amino acid sequence similarity searches were performed by using the online program BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

Overproduction and Purification of the His-Tagged Adenylation Domain Corresponding to D-Ala

A DNA fragment encoding the sixth A domain (FusA-A6) was amplified from fosmid 6D11 by using the Expand High-Fidelity PCR system (Roche, Mannheim, Germany) with primers JRL37 (5′-TAAGGATCCCCAAATCTGCGCTAGTTCTAC-3′) and JRL38 (5′-CTGCATATGCGTATTGATGAGCTGGAGTTGA-3′) (restriction sites are underlined). PCR products were first cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA), and then excised as *Nde*I-BamHI fragments and ligated into a similarly digested pET-19b (Novagen, Darmstadt, Germany). *E. coli* BL21(DE3) cells were then transformed with the recombinant expression plasmid, and cultures yielded an N-terminally His-tagged protein when grown in Luria-Bertani medium to an OD_{600} of 0.6, followed by induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and then 4 hr of growth at 22°C. Purification of soluble His-tagged FusA-A6 protein was performed by Ni^{2+}-affinity chromatography (QIAGEN) according to the manufacture's protocol. Purified protein was then desalted into assay buffer (50 mM sodium phosphate [pH 8.0], 10 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM EDTA) by using a NAP™-5 column (Amersham Pharmacia Biotech, Uppsala, Sweden). The purity of the recombinant protein was checked by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis with Coomassie Brilliant blue staining. Protein concentration was determined by using a calculated ε_{280} for purified FusA-A6 of 65,375 $M^{-1}cm^{-1}$.

ATP-PPi Exchange Assay

The amino acid specificity of purified recombinant FusA-A6 protein was determined by using an ATP-PPi exchange assay, performed as previously described (Mootz and Marahiel, 1997), with minor modifications. Reaction mixtures contained 50 mM sodium phosphate (pH 8.0), 2 mM $MgCl_2$, 2 mM dithiothreitol, 2 mM ATP, 1 mM amino acid substrate, 0.2 μCi tetrasodium [^{32}P]-pyrophosphate (Perkin Elmer, Boston, MA) and 0.1 mM tetrasodium pyrophosphate. Reactions were initiated by the addition of recombinant FusA-A6

protein to a final concentration of 250 nM in a total volume of 100 μl. After incubation at 37°C for 15 min, reactions were stopped by adding 0.5 ml termination mixture (1.2% [w/v] activated charcoal, 0.1 M tetrasodium pyrophosphate, and 0.5 M $HClO_4$). The charcoal was sedimented by centrifugation and washed once with 1 ml H_2O. The radioactivity bound to the charcoal was determined by liquid scintillation counting.

ACCESSION NUMBERS

The nucleotide sequence of the fusaricidin biosynthetic gene cluster and flanking regions from *P. polymyxa* PKB1 has been deposited in GenBank by appending it to accession number EF451155.

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Note Added in Proof

An article has appeared recently describing the sequence of a similar fusaricidin synthetase-encoding gene from another strain of *Paenibacillus polymyxa*. See: Choi, S.K., Park, S.Y., Kim, R., Lee, C.H., Kim, J.F. and Park, S.H. (2008). Identification and functional analysis of the fusaricidin biosynthetic gene of *Paenibacillus polymyxa* E681. Biochem. Biophys. Res. Commun. *365*, 89–95.