J. Antibiot. 60(11): 700–708, 2007

ORIGINAL ARTICLE

THE JOURNAL OF ANTIBIOTICS

Analysis of Modular-iterative Mixed Biosynthesis of Lankacidin by Heterologous Expression and Gene Fusion

Satoshi Tatsuno, Kenji Arakawa, Haruyasu Kinashi

Received: July 31, 2007 / Accepted: October 23, 2007

Abstract Lankacidin is a unique 17-membered macrocyclic antibiotic different from usual even-membered macrolides. Based on the gene organization of the lankacidin biosynthetic cluster coded on the linear plasmid pSLA2-L in *Streptomyces rochei*, we previously proposed a hypothesis of modular-iterative mixed polyketide biosynthesis for lankacidin. Two experimental evidences in this paper further strengthened this hypothesis. Heterologous expression of the lankacidin cluster (*lkcA-lkcO*) in *Streptomyces lividans* resulted in lankacidinol A production, indicating that the gene cluster is sufficient for the synthesis of the lankacidin skeleton. In addition, a gene fusant of *lkcF* and *lkcG* produced lankacidin at a similar level to the parent strain, suggesting that an iterative function of the LkcF protein is unlikely. These results are consistent with the hypothesis that LkcC is used four times and LkcA, LkcF and LkcG are used modularly to accomplish eight condensation reactions leading to the lankacidin skeleton.

Keywords antibiotic, polyketide synthase, *Streptomyces*, linear plasmid, biosynthesis, lankacidin

Introduction

Bacterial modular type-I polyketide synthases (PKSs) synthesize a variety of polyketide antibiotics such as macrolides, ansamycins, polyethers, and polyenes by sequential condensation reactions of short chain carboxylic acids [1]. Modular type-I PKSs are composed of modularly arranged sets of ketosynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) domains that are responsible for condensation reaction, the selection and transfer of extender units, and the retention of growing polyketide chains, respectively. In addition, modular PKSs contain modifying domains such as ketoreductase (KR), dehydratase (DH), and enoylreductase (ER), which give complex structural diversity to polyketide compounds. Thus, the domain organization in modular type-I PKSs has a strict colinear relationship with the order of biosynthetic steps.

However, metabolites without this relationship are sometimes produced by modular PKSs as minor components of fermentation. For example, 16-membered erythromycins were isolated from *Saccharopolyspora erythraea*, as a result of aberrant repeated use (termed PKS stuttering) of 6-deoxyerythronolide B synthases [2]. In contrast, ring-contracted epothilone derivatives were isolated as a result of PKS skipping in addition to ring-enlarged derivatives [3]. Different from such sporadic loss of colinear relationship, programmed iterative use of modular PKSs has been recently reported for stigmatellin in *Stigmatella aurantiaca* [4], borrelidin in *Streptomyces parvulus* [5], and aureothin in *Streptomyces thioluteus* [6]. In borrelidin biosynthesis, six modules catalyze eight rounds of chain elongation and modification, suggesting that module 5 (BorA5) is used three times [5].

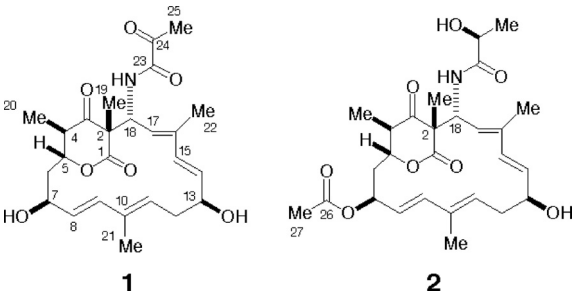
Lankacidin C, a unique 17-membered macrocyclic antibiotic (**1**, Fig. 1) produced by *Streptomyces rochei* 7434AN4, is another example of iterative use of modular PKSs. Strain 7434AN4 carries three linear plasmids, pSLA2-L, -M, and -S [7, 8], and the complete sequencing of pSLA2-L together with extensive gene disruption experiments revealed that the lankacidin synthase (*lkc*)

H. Kinashi (Corresponding author)**, S. Tatsuno, K. Arakawa:** Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, Higashi-Hiroshima 739-8530, Japan, E-mail: kinashi@hiroshima-u.ac.jp

gene cluster is located on pSLA2-L and spans 39 kb in size (Figs. 2 and 3A) [9, 10]. The *lkc* cluster contains one nonribosomal peptide synthetase (NRPS)-PKS hybrid gene (*lkcA*), three multidomain PKS genes (*lkcC*, *lkcF*, and *lkcG*), pyrroloquinoline quinone (PQQ) biosynthetic genes (*lkcK-lkcO*), and an amine oxidase gene (*lkcE*) [9]. In addition, the *lkc* cluster contains discrete AT (*lkcD*) and DH (*lkcB*) genes, both of which act *in trans* in lankacidin biosynthesis.

The gene organization of the *lkc* cluster raised two interesting questions. (i) How can five ketosyntase domains in the cluster accomplish eight condensation reactions necessary for lankacidin synthesis. (ii) How is formed the carbon-carbon linkage between C2 and C18, which generates a unique 17-membered macrocyclic skeleton. We have already answered the second question [10]; namely, the amine oxidase (LkcE) converts an acyclic amide (C18-N) intermediate (**3**, Fig. 2) to an imide (C18=N), which then receives a nucleophilic attack by C2 to form the C2~C18 linkage in lankacidinol A (**2**, Fig. 1). To the first question, we proposed a modular-iterative mixed biosynthesis hypothesis (Fig. 2), where the LkcC protein functions iteratively and the remaining three PKS proteins (LkcA, LkcF and LkcG) function modularly. This hypothesis exactly agrees with the chemical structure of lankacidin; namely, four times use of LkcC extends a polyketide chain from C14 to C7, and two KR domains in LkcF reduce the ketone groups at C7 and C5 positions. The repeated use of the MT domain in LkcC could introduce four methyl groups at C-2, 4, 10, 16 positions. However, the following alternative possibilities could not be ruled out; (i) additional PKSs coded on pSLA2-L or on the chromosome might be involved, and (ii) other PKS modules (LkcF and/or LkcG) might function iteratively.

To support the hypothesis of modular-iterative mixed polyketide biosynthesis for lankacidin, we carried out heterologous expression of the *lkc* cluster in *Streptomyces*



1 **2**

Fig. 1 Chemical structures of lankacidin C (**1**) and lankacidinol A (**2**).

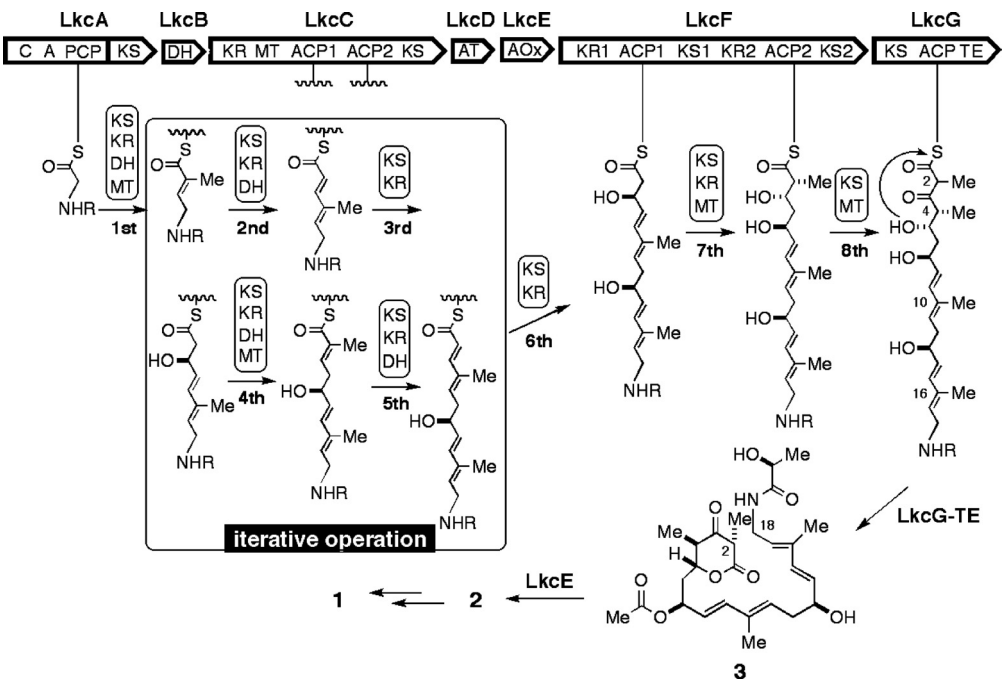


Fig. 2 Possible biosynthetic pathway of lankacidin.

R, CH_3–CH(OH)C(=O)–; C, condensation domain; A, adenylation domain; PCP, peptidyl carrier protein; KS, *β*-ketoacyl-ACP synthase; ACP, acyl carrier protein; KR, *β*-ketoacyl-ACP reductase; MT, *C*-methyltransferase; TE, thioesterase; AT, acyltransferase; DH, *β*-hydroxy-acyl-ACP dehydratase; AOx, amine oxidase.

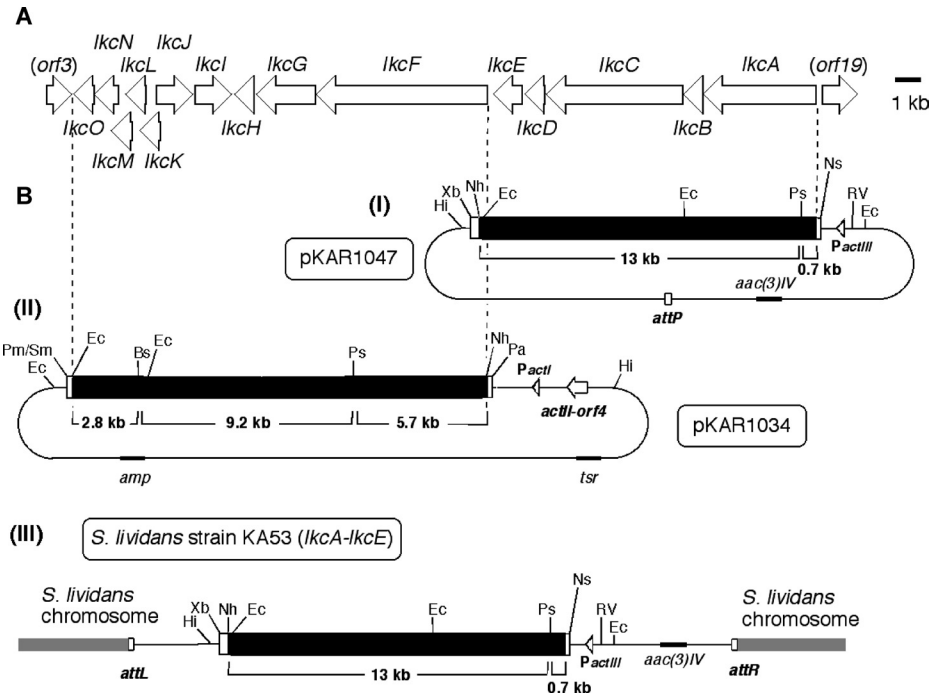


Fig. 3 Heterologous expression of the *lkc* cluster.

(A) Gene organization of the *lkc* cluster. (B) Cloning and integration of the *lkc* subcluster. (I) pKAR1047 carrying *lkcA-lkcE*. (II) pKAR1034 carrying *lkcF-lkcO*. (III) *S. lividans* KA53 in which the *lkcA-lkcE* region was integrated into the chromosome at the *attP* site. *actII-orf4*, transcriptional activator gene; P*actI*, *actI* promoter; P*actIII*, *actIII* promoter; *attP*, phage attachment site; *aac(3)IV*, apramycin resistance gene; *amp*, ampicillin resistance gene; *tsr*, thiostrepton resistance gene; Nh, *Nhe*I; Ec, *Eco*RI; Xb, *Xba*I; Hi, *Hin*dIII; 47, *Eco*47III; Ps, *Pst*I; Ns, *Nsi*I; Nd, *Nde*I; RV, *Eco*RV; Bs, *Bsp*EI; Bg, *Bgl*II; Pm, *Pme*I; Sm, *Sma*I.

lividans and gene fusion of *lkcF* and *lkcG*, the results of which are described in this paper.

Results and Discussion

Heterologous Expression of the *lkc* Cluster (*lkcA-lkcO*) in *S. lividans*

Although extensive gene disruption experiments have delimited the lankacidin biosynthetic (*lkc*) cluster in the range from *lkcA* to *lkcO* (Fig. 3A) [9, 10], we could not completely rule out the possibility that additional genes located on pSLA2-L or the chromosome might be involved. To exclude this possibility, heterologous expression of the *lkc* cluster was carried out in *S. lividans*. For this purpose, the *lkc* cluster was divided into two regions, *lkcA-lkcE* and *lkcF-lkcO*, because whole cluster is too large to be incorporated in one vector. Consequently, the *lkcA-lkcE* region was integrated into the chromosome of *S. lividans*, while the *lkcF-lkcO* region was inserted into the *E. coli-Streptomyces* shuttle vector pKAR1026-4, a pHGF7604-based vector [11]. To express the divided clusters in *S. lividans*, we used the ActII-Orf4/P*actI*-P*actIII* expression system. ActII-Orf4 is a transcriptional activator that binds to the pathway-specific *actI* and *actIII* promoters (P*actI* and P*actIII*) and initiates actinorhodin synthesis in *Streptomyces coelicolor* [12]. This system has been used for the expression of various antibiotic biosynthetic genes due to its strong promoter activity [11, 13].

To construct the *lkcA-lkcE* region, a 0.67-kb DNA fragment containing the 5′ terminus of *lkcA* (nt 35,881～35,217 of pSLA2-L) and multiple cloning sites was amplified by polymerase chain reaction (PCR) (Fig. 3B-I). Then, the *actII-orf4*/P*actIII* region was amplified by PCR and introduced into this fragment, and finally the 13-kb *Nhe*I-*Pst*I (nt 22,293～35,217) fragment carrying the rest part of *lkcA* and the *lkcB-lkcE* genes was inserted to give pKAR1047 on the integrative shuttle vector pSET152 [14]. pKAR1047 was transformed into *S. lividans* TK64 and finally gave strain KA53, in which the *lkcA-lkcE* region was integrated into the chromosome at the ϕC31 *attP* site (Fig. 3B-III).

To construct the *lkcF-lkcO* cassette, a 0.48-kb PCR fragment containing the 5′ terminus of *lkcF* and multiple cloning sites was obtained (Fig. 3B-II). Then, the 5.7-kb *Pst*I-*Nhe*I (nt 16,616～22,293) fragment, the 2.8-kb *Eco*RI-

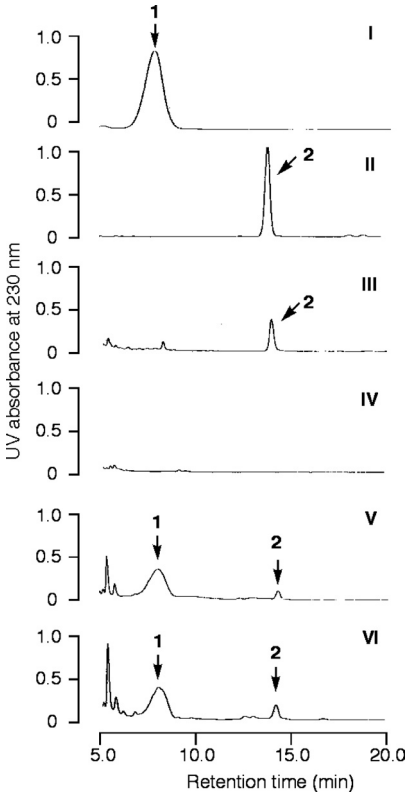


Fig. 4 Analysis of lankacidin production by reverse-phase HPLC.

(I) Lankacidin C (**1**), (II) lankacidinol A (**2**), (III) *S. lividans* KA53 (*lkcA-lkcE*) carrying pKAR1034 (*lkcF-lkcO*), (IV) KA53 (*lkcA-lkcE*) carrying pKAR1026-4 (control), (V) *S. rochei* 51252 (parent), and (VI) *S. rochei* KA49 (*lkcFG* fusant).

*Bsp*EI (nt 4,659～7,450) fragment, and the 9.2-kb *Bsp*EI-*Pst*I (nt 7,450～16,616) fragment were successively introduced into this fragment. The obtained 18.2-kb fragment containing whole *lkcF-lkcO* region was cloned into pKAR1026-4 carrying *actII-orf4* and P*actI* to give pKAR1034. This plasmid was transformed into *S. lividans* KA53 containing integrated *lkcA-lkcE* genes.

Lankacidin production was analyzed by reverse-phase HPLC. *S. lividans* KA53 harboring pKAR1034 showed a peak of lankacidinol A (**2**) (Fig. 4-III), while strain KA53 harboring the control plasmid pKAR1026-4 did not (Fig. 4-IV). The identity of the metabolite with **2** was confirmed by mass spectrometry [$(M+Na)^+=526.1$]. The production level of **2** was relatively low (0.40 mg/liter) compared with the parent strain 51252 (8.0 mg/liter of lankacidin C (**1**) and 0.25 mg/liter of **2**). However, this result clearly indicated that the gene cluster (*lkcA-lkcO*) contains all genes necessary for the synthesis of the lankacidin skeleton, and ruled out a possibility that additional genes located on pSLA2-L or the chromosome are involved in this process.

Interestingly, *S. lividans* KA53/pKAR1034 did not produce **1** but produced only **2**, although the former is a main product in strain 51252. **2** may be converted to **1** by subsequent oxidation of C-24 and hydrolysis of an acetoxy group on C-7 [10]. Therefore, additional enzymes necessary for these modifications may be coded on the *Streptomyces rochei* chromosome, but not on the *S. lividans* chromosome.

lkcF-lkcG Fusant Produced Lankacidin at a Similar Level to the Parent Strain

Heterologous expression of the *lkc* cluster in *S. lividans* confirmed that eight condensation reactions for the lankacidin skeleton are accomplished by five KS domains in the cluster; one KS domain in LkcA, one in LkcC, two in LkcF, and one in LkcG. This means that one (or some) of the four PKS proteins functions iteratively. LkcA is an NRPS-PKS fusion protein, recognizing a glycine starter unit and condensing it with a malonate unit. Therefore, LkcA could be eliminated from the candidate enzymes performing iterative condensation. Similarly, LkcG could be eliminated, because it contains a thioesterase (TE) domain and terminates chain elongation reaction. Thus, LkcC and LkcF remained as candidates by elimination.

Similar iterative use of modular PKSs was proposed for stigmatellin in *Stigmatella aurantiaca* [4], borrelidin in *Streptomyces parvulus* Tü4055 [5], and aureothin in *Streptomyces thioluteus* [6]. Based on the gene organization, it was suggested that either StiI or StiJ may be used twice for stigmatellin synthesis, BorA5 three times for borrelidin, and AurA twice for aureothin. However, any causative sequence differences between modular and iterative PKSs have not been recognized. Fusion of the iterative BorA5 protein with the neighbor PKSs (BorA4 or BorA6) did not abolish borrelidin production, but the production yield was greatly decreased (about 20% of the parent strain) [5]. Based on the former fact (positive production), Olano *et al.* [5] concluded that three copies of Bor5 were not used sequentially in borrelidin synthesis but one copy was used repeatedly. However, we rather focused on the latter fact (low production yield), because fusion of the typical modular PKSs, 6-deoxyerythronolide B synthases, did not affect the production yield of erythromycin [15]. Thus, it was suggested that fusion of iterative and modular PKSs decreases a production yield, which we utilized to distinguish an iterative PKS from modular PKSs in the following experiments.

To test an iterative function of LkcF, we constructed a gene fusant of *lkcF* and *lkcG*. The 3′ terminus of *lkcF* and the 5′ terminus of *lkcG* were combined to make a fused *lkcFG* gene, which codes a fused protein with the amino

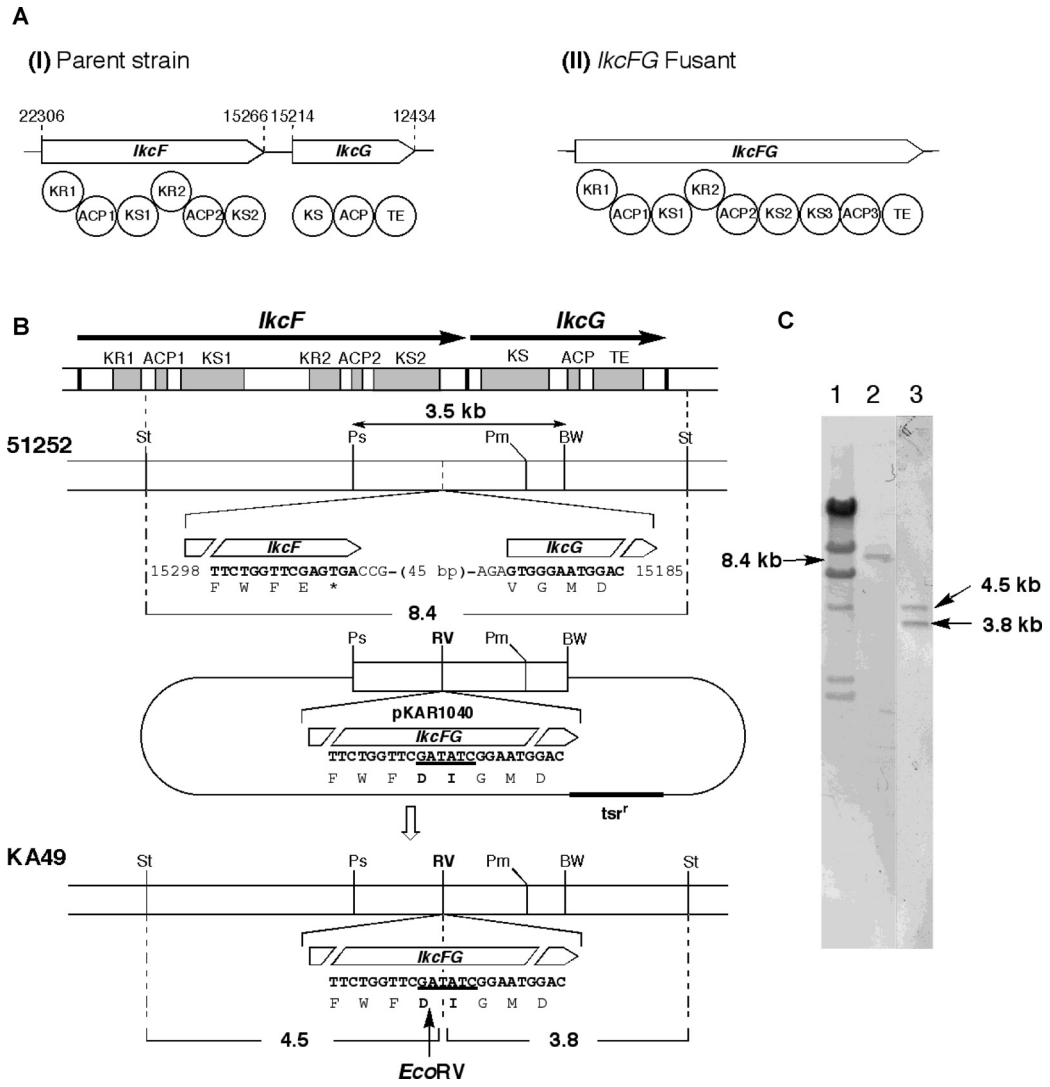


Fig. 5 Gene fusion of *lkcF* and *lkcG*.

Domain organization of LkcF and LkcG in strain 51252 (I) and fusant KA49 (II). Numbers indicate the nucleotide positions in pSLA2-L. (B) Construction scheme of fusant KA49. The *N*- and *C*-terminal amino acid sequences in 51252 and the fused sequence in KA49 are shown. An EcoRV site was generated in KA49. Ps, *Pst*I; St, *Stu*I; BW, *Bsi*WI; Pm, *Pme*I; RV, *Eco*RV. (C) Southern blot analysis. Lane 1, λ/*Hin*dIII; lane 2, 51252/*Stu*I-*Eco*RV; lane 3, KA49/*Stu*I-*Eco*RV. Probe, 3.5-kb *Pst*I-*Bsi*WI fragment in (B).

acid sequence FWFDIGMD in place of the *C*-terminal FWFE of LkcF and the *N*-terminal VGMD of LkcG (Figs. 5A and B). Plasmid pKAR1040 containing the fused *lkcFG* gene on the shuttle vector pRES18 [16] was transformed into *Streptomyces rochei* strain 51252, which finally gave mutant KA49 by gene replacement (Figs. 5B and C). As shown in Fig. 4-VI, fusant KA49 produced **1** and **2** at a similar level with the parent strain (Fig. 4-V). This result suggested that LkcF and LkcG are modular PKSs, leaving only LkcC as an iterative PKS by elimination. Although gene fusants of *lkcA-lkcC* and *lkcC-lkcF* are under construction, we have not yet obtained them due to the presence of intervening genes, *lkcB* between *lkcA* and *lkcC*, and *lkcD* and *lkcE* between *lkcC* and *lkcF*.

What Does Determine Modular or Iterative Condensation?

The Lkc-PKS proteins, especially LkcC, have several interesting features, which distinguish them from usual type-I PKS proteins. They do not contain AT or DH domains, both of which are present as discrete proteins, LkcD or LkcB. The domain order (KR-ACP-KS) of LkcC and LkcF is different from those (KS-AT-KR-ACP) of usual type-I PKSs such as DEBS1 for

erythromycin synthesis; namely, the KS domain is located at the *C*-terminus of protein in place of the *N*-terminal position. In addition, LkcC contains two tandemly aligned ACP domains. Are these properties related to iterative condensation reaction?

Different from the Lkc proteins, the PKS proteins for stigmatellin, borrelidin and aureothin biosynthesis contain AT and DH domains in normal domain arrangement. Thus, the unique domain order and the discrete AT domain of the Lkc proteins may not be related to iterative function. However, they gave us an interesting question on the evolution of PKS genes. It was suggested that monofunctional type-II PKS genes have been fused into a multifunctional type-I PKS gene in the evolutional history [17]. Therefore, in the generation process of the *lkc*-PKS genes, the KS gene was fused with other genes in a unique order, while the AT and DH genes were not fused and remained as separate genes. The tandem alignment of two ACP domains was also found in the albicidin PKS [18] and several fungal type-I PKSs, for example, WA for naphtopyrone [19, 20] and StcA for sterigmatocystin [21]. Huang *et al.* [18] proposed that the second ACP domain in the albicidin PKS serves as a waiting position for growing chains to facilitate iterative condensation, although no experimental evidence was provided. Fujii *et al.* [21] disrupted each of the two tandem ACP domains in WA and showed that either ACP alone was enough for naphtopyrone synthesis. It is noteworthy that tandemly aligned doublet and triplet ACP domains were found even in the modular PKSs for mupirocin in *Pseudomonas fluorescens* [22]. All of these results suggest no relationship between tandem ACPs and iterative condensation. However, disruption of tamdem ACPs in LkcC is in progress to get a final answer on their function in lankacidin synthesis.

Despite of accumulated data, little information has been obtained on the question how the PKS enzymes for lankacidin, stigmatellin, borrelidin, and aureothin could distinguish and perform modular and iterative condensations. Although we extensively introduced amino acid replacement into the active sites of three KR domains in the *lkcC* and *lkcF* genes, we have not isolated any metabolites to confirm our hypothesis (unpublished results). Such metabolites have neither been isolated for stigmatellin, borrelidin, or aureothin. A new strategy with a totally different aspect may be necessary to obtain conclusive evidence.

In conclusion, heterologous expression of the lankacidin cluster and gene fusion of *lkcF* and *lkcG* in this study suggested a plausible hypothesis that LkcC is used four times and LkcA, LkcF and LkcG are used modularly to accomplish eight condensation reactions leading to the lankacidin skeleton.

Experimental

Bacterial Strains and Culture Conditions

Streptomyces rochei strain 51252 [7] carrying only pSLA2-L was used as the parent strain of lankacidin producer. All of the strains, plasmids, and PCR primers used in this study are listed in Table 1. DNA manipulations for *Escherichia coli* [23] and *Streptomyces* [24] were performed according to the standard procedures. *Streptomyces* strains were grown in YEME medium [24], treated with lysozyme to make protoplasts, regenerated on R1M medium [25], and overlaid with soft nutrient agar containing thiostrepton (final concentration; 10 μg/ml) or apramycin (50 μg/ml) to select transformants. YM medium (yeast extract 0.4%, malt extract 1.0%, and glucose 0.4%, pH 7.3) was used for antibiotic production. *Escherichia coli* XL1-blue was grown in Luria Bertani medium containing either ampicillin (100 μg/ml) or apramycin (50 μg/ml).

Heterologous Expression of the *lkc* Cluster (*lkcA-lkcO*)

Introduction of *lkcA-lkcE* Genes into the Chromosome of *S. lividans* TK64

Using cosmid B10 of pSLA2-L [9] as a template and primers KAR-1801 and KAR-1802 (Table 1), a 0.67-kb DNA fragment containing the *N*-terminus of *lkcA* (nt 35,881～35,217 of pSLA2-L) and multiple cloning sites was amplified. After treatment with *Nsi*I-*Xba*I, this fragment was cloned into pKAR1024 to give pKAR1027. To introduce the P*actIII* promoter and an *Eco*RV site, another PCR product was prepared using pKAR1027 as a template and primers KAR-1802 and KAR-1803. The resulting DNA cut with *Pac*I-*Xba*I was cloned into pNEB193 (New England Biolabs) to afford pKAR1044. Its vector fragment digested with *Sma*I-*Xba*I was replaced by pBluescript SK-plus (Stratagene) digested with *Eco*RV-*Xba*I to give pKAR1045. A 13-kb *Nhe*I-*Pst*I fragment (nt 22,293～35,217) was introduced into the corresponding sites of pKAR1045 to give pKAR1046, the vector part of which was replaced by pSET152 [14] digested with *Eco*RV-*Xba*I to afford an integrative *lkcA-lkcE* expression plasmid, pKAR1047. This plasmid was transformed into *S. lividans* TK64 and transformants were cultured in the presence of apramycin (20 μg/ml) to give strain KA53, in which the *lkcA-lkcE* genes were integrated into the chromosome.

Construction of *lkcF-lkcO* Expression Plasmid

Using cosmid B10 as a template and primers KAR-1301 and KAR-1303, a 0.48-kb fragment was amplified. After

Table 1 Bacterial strains, plasmids, and primers used in this study

Strains/plasmids/primers	Properties/oligonucleotide sequences	Source/ref.
Strains		
S. rochei 7434AN4	Wild type (pSLA2-L, M, S)	Ref. 7
S. rochei 51252	Strain from 7434AN4 (pSLA2-L)	Ref. 7
S. rochei KA49	in-frame gene fusion of *lkcF* and *lkcG*	This study
S. lividans KA53	Strain TK64 with integration of pKAR1047, *aac3(IV)*	This study
Plasmids		
pHGF7505	pNEB193-based plasmid carrying *actII-orf4* and P*actIII-actI*	Ref. 11
pHGF7604	SCP2*-derived vector, bla, tsr	Ref. 11
cosmid B10	45.4-kb pSLA2-L DNA (nt 3,341-48,756) cloned into SuperCos-1 at *Bam*HI site	Ref. 9
pKAR1024	1.2-kb *Swa*I-*Pme*I fragment eliminated from pHGF7505	This study
pKAR1025	*Eco*RI site eliminated from pKAR1024	This study
pKAR1026	1.5-kb *Eco*RI-*Hin*dIII fragment carrying *actII-orf4* and P*actIII-actI* cloned into pHGF7604	This study
pKAR1026-4	1.5-kb *Eco*RI-*Bgl*II PCR product cloned into pKAR1026	This study
pKAR1027	0.67-bp *Nsi*I-*Xba*I PCR product cloned into pNEB193	This study
pKAR1029	0.48-bp *Pac*I-*Sma*I PCR product cloned into pKAR1025	This study
pKAR1030	5.2-kb *Nhe*I-*Pst*I fragment (nt 22,293∼16,616) cloned into pKAR1029	This study
pKAR1032	2.8-kb *Bsp*EI-*Eco*RI fragment (nt 7,450∼4,659) cloned into pKAR1030	This study
pKAR1033	9.2-kb *Pst*I-*Bsp*EI fragment (nt 16,616∼7,450) cloned into pHGF1032	This study
pKAR1034	18-kb *Pac*I-*Sma*I fragment cloned into pKAR1026-4 at *Pac*I-*Pme*I site	This study
pKAR1044	0.67-bp *Pac*I-*Xba*I PCR product cloned into pNEB193	This study
pKAR1045	0.67-bp *Sma*I-*Xba*I fragment from pKAR1044 cloned into pBluescript SK+ at *Eco*RV-*Xba*I site	This study
pKAR1046	13-kb *Pst*I-*Nhe*I fragment (nt 35,217∼22,293) cloned into pKAR1045	This study
pKAR1047	14-kb *Eco*RV-*Xba*I fragment from pKAR1046 cloned into pSET152	This study
pKAR1038	3.5-kb *Bsi*WI-*Pst*I fragment cloned into *Acc*65I-*Pst*I site of Litmus28i	This study
pKAR1039	1.3-kb *Age*I-*Bbr*PI fragment from pKAR1038 replaced with PCR fragment	This study
pKAR1040	3.5-kb *Stu*I-*Pst*I fragment from pKAR1039 cloned into pRES18 at *Sma*I-*Pst*I region	This study
Primers		
KAR-1301	5′-GTGCTTAATTAACACGGGGATGACCCATGG-3′	This study
KAR-1303	5′-AACCCGGGAATTCAGTCCGGACTGCAGCATCGATCCAC-3′	This study
KAR7505f1	5′-GGCAGATCTCGATTATAACAGCTCGGATTC-3′	This study
KAR7505r1	5′-GTTGAATTCGTTTAAACGAGCTCGGTACCC-3′	This study
KAR-1801	5′-CAGAAATGCATATGACGGAGCGTCAGGCAA-3′	This study
KAR-1802	5′-GATCTAGAGCTAGCGAACTGCAGCAGGCTC-3′	This study
KAR-1803	5′-GCCTTAATTAAGATATCGCCGGATCCACCG-3′	This study
KAR1312link02	5′-GCGAATTCACGTGTCCGTTGGAGC-3′	This study
KAR1312link03	5′-GAACCGGTTCTGGTTCGATATCGGAATGGA-3′	This study

digestion with *Pac*I-*Sma*I, this fragment was cloned into pKAR1025 to give pKAR1029. The 5.2-kb *Pst*I-*Nhe*I fragment (nt 16,616∼22,293) was cloned into pKAR1029 to give pKAR1030, the 2.8-kb *Eco*RI-*Bsp*EI fragment (nt 4,659∼7,450) was introduced into pKAR1030 to afford pKAR1032, and then the 9.2-kb *Bsp*EI-*Pst*I fragment (nt 7,450∼16,616) was introduced into pKAR1032 to give pKAR1033. The vector part of pKAR1033 digested with *Pac*I-*Sma*I was replaced by pKAR1026-4 digested with *Pac*I-*Pme*I to afford pKAR1034. Plasmid pKAR1026-4 was constructed as follows. The insert of pHGF7604 [11] was replaced by the 1.5-kb *Eco*RI-*Hin*dIII fragment of pHGF7505 [11] to give pKAR1026, and its cloning sites were modified using primers KAR7505f1 and KAR7505r1 to give pKAR1026-4.

In-frame Gene Fusion of *lkcF* and *lkcG*

Primers KAR1312link02 and KAR1312link03 were designed to obtain a fused *lkcFG* sequence (Fig. 5). Using these primers and cosmid B10 as a template, a 1.3-kb PCR product was obtained. After digestion with *Age*I-*Bbr*PI, this fragment was substituted for the original *Age*I-*Bbr*PI

fragment of pKAR1038 to give pKAR1039. The vector region of pKAR1038 was replaced by pRES18 to afford pKAR1040. This plasmid was transformed into strain 51252, plasmid-integrated strains were selected by thiostrepton (10 μg/ml), and thiostrepton-sensitive double-crossovered strains were identified by Southern hybridization to give the *lkcFG*-fusant KA49 (Fig. 5C).

Isolation and Detection of Metabolites

Three-days culture broth was extracted with EtOAc and analyzed by TLC, HPLC, and electrospray ionization mass spectrometry (ESI-MS). HPLC analysis was carried out on a COSMOSIL 5C_{18}-MS-II column (4.6×250 mm, Nacalai Tesque, Japan), which was eluted with a 3 : 7 mixture of acetonitrile and 10 mM sodium phosphate buffer (pH 8.2) at a flow rate of 1.0 ml/minute. **1** and **2** were detected at 8.5 and 14.8 minutes, respectively, by a Jasco MD-2010 multiwavelength photodiode array detector. Production yields of **1** and **2** were determined from their peak intensities on HPLC chromatogram using authentic samples. ESI-MS spectra were obtained by an ALLIANCE2690/ZQ2000 mass spectrometer (Waters, USA).

Acknowledgments We thank Prof. Dr. Heinz G. Floss (University of Washington) and Dr. Tin-Wein Yu (Louisiana State University) for plasmids, pHGF7505 and pHGF7604. This work was supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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