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Cyclization Mechanism for the Synthesis of Macrocyclic Antibiotic Lankacidin in *Streptomyces rochei*

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Summary

The lankacidin biosynthetic gene cluster in *Streptomyces rochei* strain 7434AN4 was found to span 31 kb of the giant linear plasmid pSLA2-L and contain a polyketide synthase (PKS)/nonribosomal peptide synthetase (NRPS) hybrid gene (*lkcA*), type I PKS genes, and pyrroloquinoline quinone (PQQ) biosynthetic genes (*lkcK-lkcO*). Feeding of PQQ to a *pqq* mutant restored the lankacidin production, suggesting its crucial role in an oxidation process. However, formation of the 17-membered macrocyclic ring was not catalyzed by PQQ-dependent dehydrogenase (Orf23), but was by flavin-dependent amine oxidase (LkcE). Compound LC-KA05 isolated from an *lkcE* disruptant was an acyclic intermediate lacking the C2-C18 linkage. These results suggested a cyclization mechanism for the synthesis of the lankacidin macrocyclic skeleton.

Introduction

The filamentous soil bacteria, *Streptomyces* species, are well known by a linear chromosome, complex morphological differentiation, and an ability to produce many bioactive secondary metabolites including antibiotics. A prominent class of secondary metabolites, polyketides, contain important compounds for pharmaceutical and agrochemical uses such as erythromycin, rapamycin, FK506, lovastatin, and avermectin [1]. In the biosynthesis of polyketides, short chain carboxylic acids are assembled and modified in a programmed order by multifunctional polyketide synthases (PKSs). These biosynthetic genes are intriguing targets for genetic engineering to create rationally designed novel polyketides.

Antibiotic biosynthetic genes in *Streptomyces* species usually form a condensed gene cluster on the chromosome. At this moment, however, three large linear plasmids coding an antibiotic gene cluster(s) are known: SCP1 in *Streptomyces coelicolor* A3(2) carries the methylenomycin biosynthetic genes [2–5]; pPZG103 in *Streptomyces rimosus* has the biosynthetic genes for oxytetracycline [6]; and pSLA2-L in *Streptomyces rochei* has those for lankamycin and lankacidin [7–10].

Streptomyces rochei strain 7434AN4 contains three linear plasmids (pSLA2-L, -M, and -S), and produces two structurally unrelated polyketide antibiotics, 17-membered macrocyclic lankacidins (Figure 1, lankacidin C, **1**; lankacidinol A, **2**) [11–13] and a 14-membered macrolide lankamycin (**3**) [14]. Lankacidin antibiotics exhibit significant antibacterial activities against a wide variety of bacteria [15]. Lankacidin A (7-O-acetyl-lankacidin C) is commercially used in livestock industries for the treatment of porcine infected with *Serpulina* (*Treponema*) *hyodysenteriae*, an anaerobic spirochete [16]. Lankacidins also possess potent in vivo antitumor activity against L1210 leukemia, etc. [17]. Therefore, creation of novel lankacidin derivatives by gene modification is important for pharmaceutical and agricultural uses.

Correlation between the plasmid profiles and the antibiotic production in the mutants derived from the parent strain 7434AN4 suggested that the largest plasmid pSLA2-L is involved in the production of both antibiotics [7]. Southern hybridization analysis using *eryAI* and *actI* revealed that pSLA2-L contained homologous regions to these typical type I and type II PKS probes [8]. Targeting and partial sequencing experiments confirmed that two *eryAI*-homologous regions on PstI fragment A (Figure 2A) are parts of a large type I PKS gene cluster for lankamycin [9]. Finally, the lankacidin biosynthetic gene cluster has been identified by the complete nucleotide sequencing of pSLA2-L [10].

It was found that pSLA2-L is a linear plasmid of 210,614 bp and carries 143 open reading frames (ORFs). The most striking feature of pSLA2-L is that three-quarters of its DNA are occupied by secondary metabolism-related genes. Namely, it contains two type I PKS gene clusters for lankacidin (*lkc*) and lankamycin (*lkm*), a type II PKS gene cluster (*roc*), a carotenoid biosynthetic gene cluster (*crt*), and many regulatory genes including the homologs of *afsA*, *arpA*, *adpA*, and *strR* in the A-factor regulatory cascade in *Streptomyces griseus* [18, 19].

In this paper, we delimited the range of the *lkc* gene cluster by extensive gene disruption experiments. In particular, we analyzed the disruptants of *orf7*, a pyrroloquinoline quinone (PQQ) biosynthetic gene and *orf14*, an amine oxidase gene. Structural elucidation of an intermediate isolated from the latter disruptant and its bioconversion to lankacidin C revealed a novel cyclization mechanism to the 17-membered macrocyclic skeleton. In addition, we proposed a possible gross biosynthetic route to lankacidin, which hypothesizes a modular-iterative mixed polyketide biosynthesis.

Results and Discussion

Location and Range of the Lankacidin Biosynthetic (*lkc*) Gene Cluster

To locate the *lkc* gene cluster on pSLA2-L, we carried out large deletion experiments using the ordered cosmid library. Cosmid C10 covers a 40.6 kb DNA (nt 11,258–51,820 of the pSLA2-L sequence, accession number AB088224) extending over the PstI-E1, C, and B regions (Figure 2A). Digestion of cosmid C10 with

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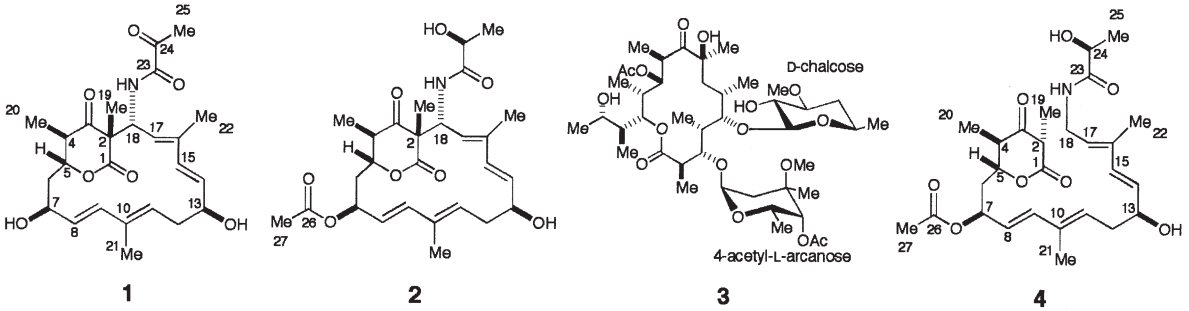

Figure 1. Chemical Structures of Lankacidin C (**1**), Lankacidinol A (**2**), Lankamycin (**3**), and LC-KA05 (**4**), Produced by *Streptomyces rochei* 7434AN4 and its Disruptants

BamHI and insertion of a kanamycin resistance gene cassette [20] gave plasmid pTI-C02, which retained only the left (1.3 kb) and right (2.5 kb) ends of the original insert. Replacement of the vector part of pTI-C02 by the shuttle vector pRES18 [21] generated a targeting plasmid pTI-C03. By using this plasmid for gene replacement, we succeeded in deleting a 36.8 kb DNA (nt 12,541–49,309) extending over *orf12–orf30*. An obtained disruptant TI-C10 did not produce lankamycin or lankacidin (Figure 3A, lane IV), which indicated that the *lkc* gene cluster is located on the left side of the *lkm* gene cluster.

To reveal what ORFs are included in the *lkc* cluster, the following ORFs were selected and subjected to gene inactivation experiments (Table 1). The Orf3 protein has a considerable similarity to StrR, a pathway-specific transcriptional regulator in streptomycin biosynthesis [22]. Five genes (*orf4–orf8*) form a gene cluster for the synthesis of PQQ [23], a newly recognized vitamin necessary for oxidoreductases. The *orf9* gene encodes an ATP binding cassette (ABC) transporter. The *orf18* gene encodes a PKS/NRPS fused protein, which was speculated to be involved in the initial condensation reaction of glycine and malonate. Orf19, Orf20, Orf21, and Orf22 show moderate similarities to dehydrogenase, tetracycline-efflux transporter, peptide synthetase, and polyketide synthase, respectively.

These genes were separately inactivated except for *orf20–22*, which were disrupted simultaneously. As shown in Figure 3A, disruptants of *orf7* (lane V), *orf9* (lane VI), and *orf18* (lane VII) did not produce lankacidin but produced lankamycin. On the other hand, disruptants of *orf3* (lane VIII), *orf19* (lane IX), and *orf20–22* (lane X) produced both antibiotics. Against our expectation, disruption of the regulatory gene, *orf3*, did not have an effect on antibiotic production, while the *pqqC* gene (*orf7*) was necessary for lankacidin production. Since *orf4–orf8* form a *pqq* gene cluster, we concluded that the left border of the *lkc* cluster is located at *orf4*. The result of the *orf9* disruptant suggested that this ABC transporter functions in the self-resistance against lankacidin. The PKS/NRPS fused gene (*orf18*) was indispensable for lankacidin production, but none of the four genes on its right side (*orf19–orf22*) were, delimiting the right border at *orf18*. Therefore, the *lkc* gene cluster was deduced to contain 15 ORFs (*orf4–orf18*) and extend over 31.0 kb. The genes in the *lkc* gene cluster were named *lkcA–lkcO* in the leftward direction from *orf18* to *orf4* (Figure 2B and Table 1).

PQQ Is Essential for Lankacidin Production

As described above, the *pqq* mutant FS7 (*lkcL* disruptant) did not produce lankacidin. To confirm the function of PQQ, feeding experiments were performed. When PQQ was added to mutant FS7 at a final concentration of 2 μg/ml, the lankacidin production was recovered to the level of the wild-type strain (Figure 3B, lane II). PQQ is a novel prosthetic group of quinopro-

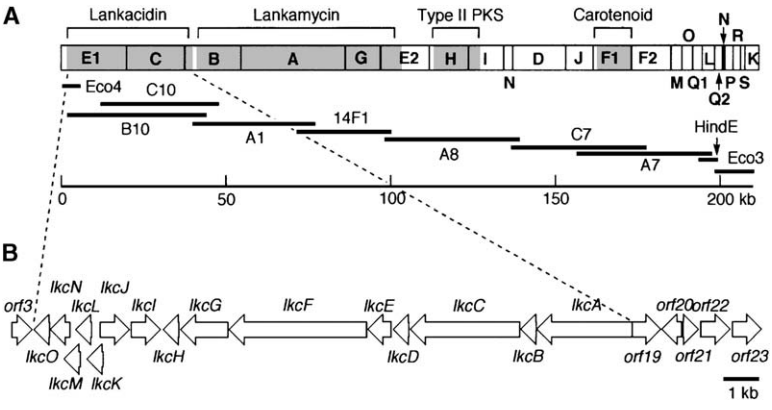

Figure 2. PstI Fragment Map and Ordered Cosmid Map of pSLA2-L and Organization of the *lkc* Gene Cluster and its Flanking Regions

(A) Fragment and cosmid map. The locations of three terminal plasmids, Eco4, HindE, and Eco3 [8] are also shown. Shadowed boxes indicate the biosynthetic gene clusters for lankacidin, lankamycin, an unknown type II polyketide, and a carotenoid.

(B) *lkc* Gene Cluster. The *lkc* genes were named in the leftward direction from *lkcA* (*orf18*) to *lkcO* (*orf4*). Arrows indicate the transcription direction of genes.

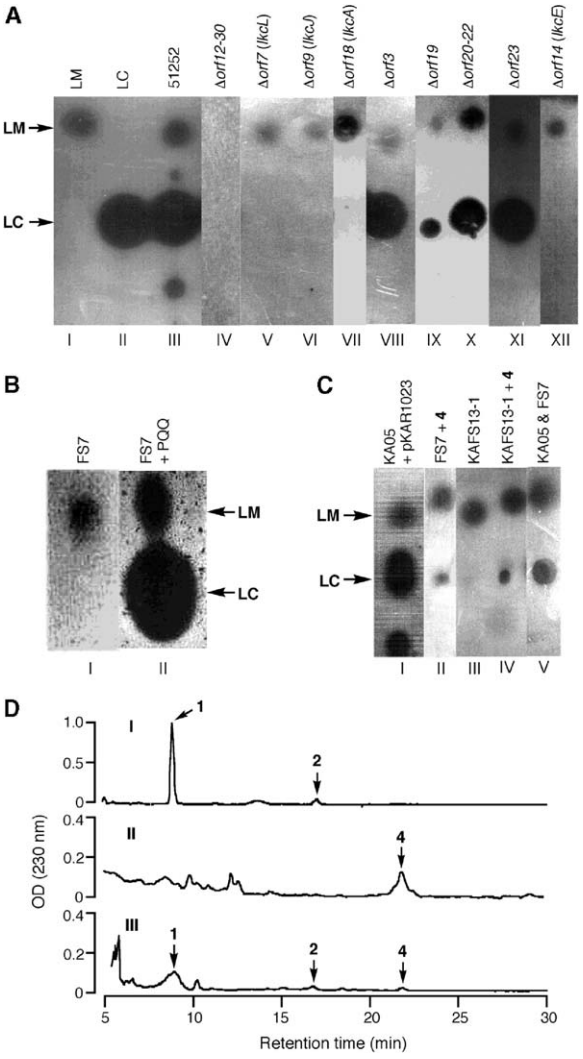

Figure 3. Analysis of Antibiotic Production by Bioautography and HPLC

(A) Bioautography of *S. rochei* strain 51252 and its gene disruptants. I, lankamycin; II, lankacidin C; III, strain 51252; IV, strain TI-C10 (Δ*orf12-30*); V, strain FS7 (Δ*lkcL*); VI, strain FS9 (Δ*lkcJ*); VII, strain FS18 (Δ*lkcA*); VIII, strain FS3 (Δ*orf3*); IX, strain FS19 (Δ*orf19*); X, strain FS2022 (Δ*orf20-22*); XI, strain KK23 (Δ*orf23*); XII, strain KA05 (Δ*lkcE*). A lower inhibitory spot on lane III shows lankacidinol A.

(B) Feeding effect of PQQ. I and II, the *pqq* mutant FS7 without and with a feeding of PQQ.

(C) Bioconversion and cofermentation. I, strain KA05 containing pKAR1023; II, strain FS7 fed with LC-KA05; III and IV, strain KAFS13-1 without and with a feeding of LC-KA05; V, cofermentaion of strains KA05 and FS7. The added thiostrepton gave an inhibitory spot at the origin (lane I).

(D) HPLC analysis. I, strain 51252; II, strain KA05; III, strain FS7 fed with LC-KA05. Lankacidin C (**1**), lankacidinol A (**2**), and LC-KA05 (**4**) were eluted at 8.5, 16.9, and 22.1 min, respectively.

teins [24, 25], which have been classified as the third oxidoreductase family following well-known pyridine dinucleotide- and flavin-dependent hydrogenases.

The *lkc* cluster and its flanking regions contain two possible oxidoreductase genes; *orf23*, on the right side of the cluster, is similar to a PQQ-dependent polyvinylalcohol dehydrogenase gene, and *lkcE* (*orf14*) in the middle of the cluster is similar to a flavin-dependent amine oxidase gene. Lupanine hydroxylase from *Pseudomonas* sp., a member of the quinoprotein family, oxidizes an amine moiety in lupanine to an imine, which is then hydrated to give 17-hydroxy-lupanine [26]. Therefore, we first speculated that Orf23 might function similarly in the oxidation reaction of an amide at C-18 to an imide in lankacidin biosynthesis. However, this was not the case, because the *orf23* disruptant produced both lankacidin and lankamycin (Figure 3A, lane XI).

Acyclic Intermediate Produced by an Amine Oxidase (*lkcE*) Mutant

In contrast to the *orf23* mutant, an *lkcE* disruptant (strain KA05) did not produce lankacidin but produced lankamycin (Figure 3A, lane XII). The defect of this mutant was complemented by introduction of a functional copy of *lkcE* on pRES18 (Figure 3C, lane I). The LkcE protein contains an FAD fingerprint motif, G-X-G-X-X-G, at the N terminus [27]. Although no antibacterial activities were detected in the lankacidin fractions of gel chromatography, a UV-absorbing compound was isolated from mutant KA05. This compound was further purified by silica gel chromatography to afford a white powder, LC-KA05: $C_{27}H_{38}NO_8$, m/z 504.2600 [M^+-H], calcd. 504.2598; λ_{max} 237 nm (ϵ 26,800) in ethanol; $[\alpha]_D^{25}$ +17.1 (*c* 0.57, ethanol).

The molecular formula of LC-KA05 indicates that it is two hydrogens larger than lankacidinol A ($C_{27}H_{36}NO_8$). ^{1}H- and ^{13}C-NMR data of lankacidin C (**1**), lankacidinol A (**2**), and LC-KA05 are listed and compared in Table 2. The latter two compounds showed 27 carbon signals, two carbons more than **1**, due to the presence of an acetoxy group (C-26 and C-27). Comparison of their DEPT spectra revealed that the tertiary carbon (C-2, 57.0 ppm) in **2** was changed to a methine carbon (50.2 ppm) in LC-KA05, and the methine carbon (C-18, 51.2 ppm) to a methylene carbon (37.1 ppm). In accordance with these changes, the C-2 carbon of LC-KA05 showed a long-range coupling with the C-19 methyl protons (1.35 ppm), but not with the C-18 methylene protons (4.02 ppm) in HMBC experiments. From these results, LC-KA05 was deduced to be an acyclic reduced compound of lankacidinol A, where the C2-C18 linkage is not present (Figure 1, compound **4**).

The ^{1}H- and ^{13}C-NMR spectra of LC-KA05 did not show any signals due to an enol form, indicating that the β-keto-δ-lactone ring exists mainly as a ketonic form. Irradiation of the C-2 proton gave a strong NOE (13%) on the C-5 proton, whereas irradiation of the overlapping methyl protons (C-19 and C-25) showed no NOE. Thus, we assigned that the C-2 methyl is *trans* (α configuration) to the C-5 proton.

To prove that LC-KA05 is a precursor of lankacidin, bioconversion experiments were performed using non-producing mutants, FS7 (*pqq* mutant) and KAFS13-1 (mutant of the *lkcF-KR1* domain; KR, ketoreductase). Bioautography clearly showed that LC-KA05 was converted to lankacidin C in both cases (Figure 3C, lanes II and IV). The bioconversion was also analyzed by a reverse-phase HPLC, where lankacidin C (**1**), lankacidi-

Table 1. Deduced Functions of ORFs in the Lankacidin Biosynthetic Gene Cluster and Its Flanking Regions

Gene	Amino Acids	Disruptant	Similar protein (origin, accession number, identity/similarity) or PKS/NRPS domains
orf3	328	FS3	Transcriptional regulator (*Streptomyces griseus* StrR, Y00459, 46/58)
lkcO (*orf4*)	296		PQQ biosynthetic gene B (*Pseudomonas aeruginosa* PqqB, AE004625, 34/47)
lkcN (*orf5*)	364		PQQ biosynthetic gene E (*Pseudomonas aeruginosa* PqqE, AE004625, 46/56)
lkcM (*orf6*)	90		PQQ biosynthetic gene D (*Pseudomonas aeruginosa* PqqD, AE004625, 39/53)
lkcL (*orf7*)	242	FS7	PQQ biosynthetic gene C (*Pseudomonas aeruginosa* PqqC, AE004625, 49/61)
lkcK (*orf8*)	34		PQQ biosynthetic gene A (*Sinorhizobium meliloti* PqqA, AL603642, 39/57)
lkcJ (*orf9*)	545	FS9	ABC transporter (*Streptomyces coelicolor* A3(2), AL133424, 64/76)
lkcI (*orf10*)	569		ABC transporter (*Streptomyces antibioticus* OleB, L36601, 44/60)
lkcH (*orf11*)	184		Isochorismatase (*Chromobacterium violaceum* ATCC 12472, AAQ61295, 31/50)
lkcG (*orf12*)	926		Type I PKS: KS (11–418), ACP (589–656), TE (725–920)
lkcF (*orf13*)	2,346	KAFS13-1	Type I PKS: KR1 (198–359), ACP1 (456–524), KS1 (605–1020), KR2 (1392–1553), ACP2 (1659–1725), KS2 (1783–2191)
lkcE (*orf14*)	438	KA05	Amine oxidase (*Agrobacterium tumefaciens* Atu1977, AAL42972, 27/43)
lkcD (*orf15*)	293		Acyltransferase (*Streptomyces atroolivaceus* LnmG, AAN85520, 44/54)
lkcC (*orf16*)	1,862		Type I PKS: KR (231–392), MT (586–782), ACP1 (821–886), ACP2 (926–991), KS (1042–1456)
lkcB (*orf17*)	276		Dehydratase (*Bacillus subtilis* PksP-DH, E69679, 24/43)
lkcA (*orf18*)	1,622	FS18	PKS-NRPS hybrid: C (8–273), A (459–856), PCP (967–1024), KS (1063–1473)
orf19	499	FS19	Dehydrogenase (*Archaeoglobus fulgidus* MaoC, A69539, 45/72)
orf20	426	FS2022	Tetracycline-efflux transporter (*Xanthomonas axonopodis* TetA, AAM39125, 23/39)
orf21	84	FS2022	Peptide synthetase (truncated) (*Streptomyces verticillus* NRPS11-10, AF210249, 47/60)
orf22	517	FS2022	Polyketide synthase (*Clostridium thermocellum* Chte1256, ZP060858, 32/50)
orf23	543	KK23	PQQ-dependent dehydrogenase (*Xanthomonas campestris* XCC4080, AAM43301, 37/51)

nol A (**2**), and LC-KA05 (**4**) were separated well (Figure 3D, columns I and II). Most of the added LC-KA05 was converted to lankacidin C by mutant FS7, although small amounts of LC-KA05 and lankacidinol A were detected (Figure 3D, column III). These results confirmed that LC-KA05 is a biosynthetic intermediate to lankacidin. At the same time, it was suggested that PQQ is not involved in the oxidation process of an amide to an imide, because the *pqq* mutant FS7 was able to convert LC-KA05 to lankacidin C. This speculation was supported by a cofermentation experiment; when strain KA05 was cultured together with strain FS7, lankacidin

Table 2. ^{1}H- and ^{13}C-NMR Data of Lankacidin C (**1**), Lankacidinol A (**2**), and LC-KA05 (**4**)

	Lankacidin C (**1**)		Lankacidinol A (**2**)		LC-KA05 (**4**)	
No.	δ_C	δ_H (*J* value in Hz)	δ_C	δ_H (*J*)	δ_C	δ_H (*J*)
1	169.9 (s)	-	169.9 (s)	-	169.1 (s)	-
2	56.7 (s)	-	57.0 (s)	-	50.2 (d)	3.53 (q, 6.7)
3	210.9 (s)	-	211.2 (s)	-	203.8 (s)	-
4	46.4 (d)	2.36–2.44 (m)	46.5 (d)	2.40–2.44 (m)	46.6 (d)	2.34 (m)
5	75.6 (d)	4.43 (dt, 11.9, 3.4)	75.3 (d)	4.40 (dt, 12.5, 3.7)	76.5 (d)	4.40 (dt, 2.7, 6.3)
6	36.7 (t)	2.28 (m)	34.3 (t)	2.21–2.35 (m)	37.4 (t)	2.05–2.15 (m)
7	69.9 (d)	4.33 (dt, 6.1, 9.8)	71.5 (d)	5.43 (dt, 5.2, 10.2)	71.9 (d)	5.64 (dt, 7.8, 8.0)
8	129.2 (d)	5.79 (dd, 9.5, 15.3)	124.5 (d)	5.70 (dd, 9.5, 15.3)	123.5 (d)	5.50 (dd, 7.8, 15.6)
9	138.2 (d)	6.14 (d, 15.3)	141.0 (d)	6.27 (d, 15.3)	139.3 (d)	6.41 (d, 15.6)
10	136.4 (s)	-	136.2 (s)	-	135.1 (s)	-
11	128.6 (d)	5.31 (dd, 7.0, 9.5)	129.6 (d)	5.31 (dd, 6.7, 10.1)	129.9 (d)	5.59 (t, 7.3)
12	36.8 (t)	2.36–2.44 (m)	36.9 (t)	2.40–2.44 (m)	36.5 (t)	2.38–2.51 (m)
13	74.6 (d)	4.08 (dt, 5.5, 8.2)	74.6 (d)	4.09 (dt, 5.5, 8.2)	72.3 (d)	4.24–4.28 (m)
14	131.2 (d)	5.50 (dd, 8.2, 15.9)	131.0 (d)	5.49 (dd, 8.0, 15.6)	130.8 (d)	5.71 (dd, 6.7, 15.6)
15	134.6 (d)	5.59 (d, 15.9)	134.8 (d)	5.60 (d, 15.6)	134.6 (d)	6.25 (d, 15.9)
16	139.4 (s)	-	139.0 (s)	-	136.1 (s)	-
17	124.2 (d)	4.67 (d, 10.7)	125.0 (d)	4.68 (d, 11.0)	127.4 (d)	5.49 (m)
18	51.8 (d)	5.40 (t, 10.7)	51.2 (d)	5.54 (t, 10.4)	37.1 (t)	4.02 (dd, 7.3, 13.4)
19	20.9 (q)	1.38 (s)	20.9 (q)	1.41 (s)	7.9 (q)	1.35 (d, 6.4)
20	9.6 (q)	1.26 (d, 6.7)	9.4 (q)	1.30 (d, 6.7)	12.3 (q)	1.21 (d, 7.5)
21	12.7 (q)	1.55 (s)	12.5 (q)	1.55 (s)	12.7* (q)	1.77 (s)
22	12.9 (q)	1.91 (s)	12.9 (q)	1.90 (s)	12.6* (q)	1.81 (s)
23	159.7 (s)	-	173.7 (s)	-	174.0 (s)	-
24	196.5 (s)	-	68.5 (d)	4.24 (q, 6.7)	68.5 (d)	4.25 (q, 6.7)
25	24.5 (q)	2.47 (s)	21.5* (q)	1.38 (d, 6.7)	21.4* (q)	1.35 (d, 6.7)
26	-	-	170.2 (s)	-	170.1 (s)	-
27	-	-	21.4* (q)	2.04 (s)	21.3* (q)	2.07 (s)
N-H	-	8.09 (d, 9.8)	-	7.53 (d, 10.1)	-	6.54 (br)

Carbons with an asterisk are exchangeable.

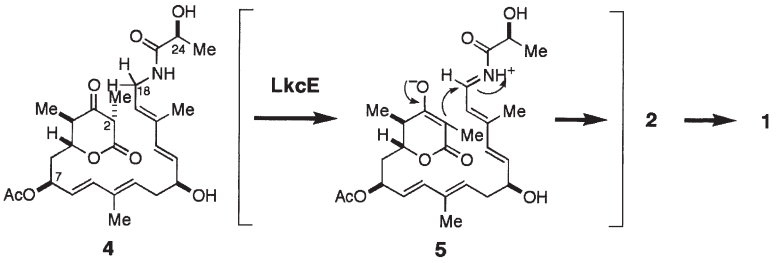

Figure 4. Macrocyclization Mechanism for the Synthesis of the Lankacidin Skeleton

The hypothetical intermediate (**5**) in a bracket has not been isolated.

production was restored (Figure 3C, lane V). Therefore, it has not yet been clarified how PQQ functions in lankacidin biosynthesis.

Macrocyclization Mechanism to the Lankacidin Skeleton

Based on the results described above, we propose a cyclization mechanism for the synthesis of the lankacidin skeleton (Figure 4). Namely, the amide intermediate (**4**) is first oxidized by the amine oxidase (LkcE) to an imide (**5**), a protonated form of which in turn accepts the nucleophilic attack of an enolate ion at C-2 to give the 17-membered lankacidinol A (**2**). Oxidation of a hydroxyl group at C-24 and cleavage of an acetyl group at C-7 are necessary for the conversion of **2** to lankacidin C (**1**), although the timing of these reactions is unknown.

Previous feeding experiments of [$^{2}H_{5}$]- and [1-^{13}C]glycine into lankacidin showed that the deuterium on C-18 was totally lost in contrast to a considerable incorporation of ^{13}C into C-17 [28]. To explain this result, a cyclopropanone intermediate consisting of C-1, C-2, and C-18 was proposed, which was then converted to the lankacidin skeleton by Favorskii-type rearrangement. This rearrangement was also suggested for enterocin biosynthesis [29]. However, the present work indicated this possibility was unlikely in lankacidin synthesis. Our preliminary experiment using ^{13}C-labeled serine suggested that glycine-hydroxymethyl transferase might catalyze the equilibrium between glycine and serine, leading to a complete loss of deuterium at C-18 (data not shown).

The novel cyclization mechanism revealed by this work has a significant meaning, because amides and 1,3-diketones are common in PKS/NRPS hybrid compounds; therefore, amine oxidases like LkcE have a potential to create novel macrocyclic compounds with various molecular architectures and ring sizes in engineered biosynthesis.

Possible Biosynthetic Route to Lankacidin

Gene inactivation experiments in this study delimited the range of the *lkc* gene cluster from *lkcA* to *lkcO*. A comprehensive view of the predicted functions of the *lkc* genes (Table 1) and the structural characteristics of lankacidin led us to propose a possible gross biosynthetic route to lankacidin (Figure 5), which is explained below.

The carbon skeleton of lankacidin is synthesized from a starter glycine molecule and eight malonate molecules [30]. All the methyl groups at C-2, C-4, C-10, and C-16 are not derived from propionate but from methionine by C-methylation. In accordance with these results, *lkcA*, the first PKS gene in the cluster, encodes a fusion protein of PKS and NRPS. PKS/NRPS hybrids have been found in many biosynthetic gene clusters from *Streptomyces*, *Xanthomonas*, cyanobacterium, and even beetles [31]. The NRPS region of LkcA has domains for condensation, adenylation and thiolation (PCP; peptidyl carrier protein), while the PKS region possesses only a ketosynthase (KS) domain. The adenylation domain contains amino acid residues, DILQTLVEAK, at the positions of 235, 236, 239, 278, 299, 301, 322, 330, 331, and 517 (nt 33,131-33,979) [32] for glycine recognition.

An interesting feature of the *lkc*-PKSs is that they do not have a linear relationship with condensation reactions, which is different from usual modular-type PKSs. However, in the last few years, similar type I PKS systems without a colinearity have been reported; for instance, the biosynthetic gene clusters for lovastatin [33], leinamycin [34], C-1027 [35], calicheamicin [36], borrelidin [37], and albicidin [38].

The fused PKS/NRPS (LkcA) and the three *lkc*-PKSs (LkcC, LkcF, and LkcG) have a total of five KS domains, although the lankacidin skeleton requires eight condensation events. Additional type I PKS genes (*orf56-orf61*) were found in the center of pSLA2-L, disruption of which however gave no effect on lankacidin synthesis (data not shown). Thus, we hypothesized that LkcC catalyzes iteratively four chain elongation reactions. In this respect, it is noteworthy that LkcC carries two tandemly aligned ACP domains; similar domains were suggested to be involved in iterative condensations in albicidin synthesis [38]. The discrete acyltransferase (AT, LkcD) and dehydratase (DH, LkcB), and also the methyltransferase (MT) domain in LkcC may act iteratively in *trans* on each biosynthetic intermediate. In contrast, LkcA, LkcF, and LkcG may function modularly in the first and the last two condensation reactions. This modular-iterative mixed biosynthesis completely agrees with the chemical structure of lankacidin. To prove this hypothesis, we are now constructing point mutants of the three KR domains (*lkcC-KR*, *lkcF-KR1*, and *lkcF-KR2*), which are expected to accumulate crucial intermediates.

Another possibility that missing modules are fed by the adjacent *lkm* gene cluster was denied by disruption of *lkmAI*, which gave no effect on lankacidin production [9]. However, an additional possibility that missing modules are located on the chromosome has not been ruled out. Heterologous expression of the whole *lkc*

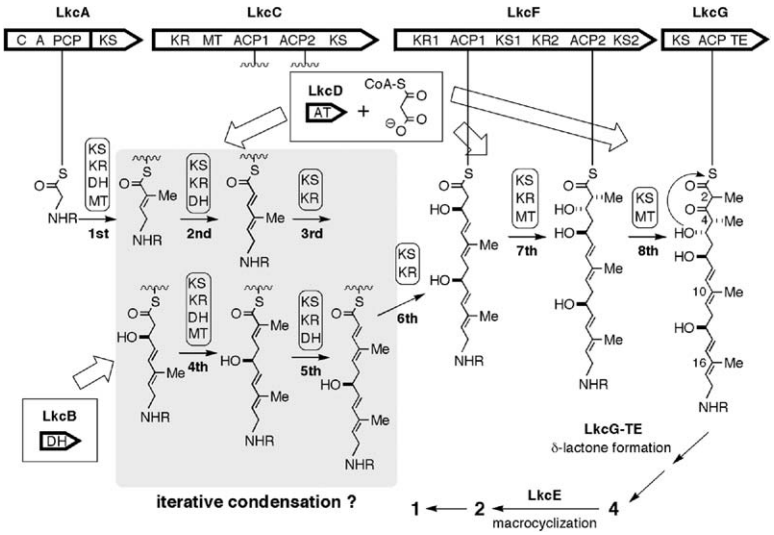

Figure 5. Possible Gross Biosynthetic Route to Lankacidin

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

gene cluster will give a final answer to this question, which is also in progress. The thioesterase (TE) domain in LkcG may catalyze the formation of a δ-lactone ring in place of a usual macrolide ring to give LC-KA05 (**4**), which is finally converted to the macrocylic lankacidin C (**1**) by the mechanism described above.

Significance

The range of the lankacidin biosynthetic (*lkc*) gene cluster on the giant linear plasmid pSLA2-L has been delimited by precise gene inactivation experiments and analysis of metabolites of the disruptants. The *lkc*-PKSs have two unique characteristics, cyclization to a macrocyclic skelton and no colinearity between modules and condensation reactions. Concerning the first characteristic, we revealed a cyclization mechanism where an amine oxidase (LkcE) oxidizes the acyclic amide intermediate (LC-KA05) to an imide, which in turn is converted to the 17-membered lankacidin skeleton. This result has opened a way to create "unnatural natural" polyketide compounds with a macrocyclic skeleton. For the second characteristic, we proposed a hypothesis in which LkcC iteratively functions four times, which enables the five KS domains in the *lkc*-PKSs to accomplish eight condensation reactions. Thus, the *lkc*-PKSs may be a mixture of modular and iterative PKSs.

Experimental Procedures

Strains and Culture Conditions

S. rochei strain 51252 [7] carrying only pSLA2-L was used to construct various *lkc* gene disruptants. All the gene disruptants constructed in this study are listed in Supplemental Table S1 (Supplemental Data). For DNA preparation, *Streptomyces* strains were grown in YEME liquid medium [39] (0.3% yeast extract, 0.5% polypepton, 0.3% malt extract, 1% D-glucose, 34% sucrose, 5 mM $MgCl_2$, and 0.25% glycine). *E. coli* XL1-Blue was used for routine cloning and construcion of targeting plasmids. *E. coli* strains were grown in Luria Bertani (LB) medium supplemented with ampicillin (100 μg/ml). YMG medium (0.4% yeast extract, 1.0% malt extract, and 0.4% glucose, pH 7.3) and TSB medium (tryptic soy broth, 30 g per liter) were used for antibiotic production and bioassay, respectively.

DNA Manipulation and Gene Inactivation

DNA manipulations for *Streptomyces* [39] and *E. coli* [40] were performed according to standard procedures. pRES18, a shuttle vector containing a thiostrepton resistant gene [21] and a kanamycin resistance gene cassette [20], were used for gene disruption. Construction procedures of targeting vectors are described in Supplemental Data. They were propagated in *E. coli* ET12567 (*dam*, *dcm*, *hsdM*) [41] and transformed into *S. rochei* 51252. *Streptomyces* protoplasts were regenerated on R1M plates [42] (K_2SO_4, 0.25 g; $MgCl_2 \cdot 6H_2O$, 4.07 g; casamino acids, 0.1 g; L-asparagine, 2 g; yeast extract, 8 g; polypepton, 5 g; glucose, 10 g; sucrose, 103 g; trace element solution, 2 ml; 5.73% TES (pH 7.3), 100 ml; 7.37% $CaCl_2 \cdot 2H_2O$, 100 ml; KH_2PO_4, 10 ml; and 2% agar per liter) and then overlaid with soft agar containing thiostrepton (final concentration of 10 μg/ml).

Thiostrepton-resistant colonies were picked up and subjected to successive liquid cultures in YEME medium containing 10 μg/ml of kanamycin to facilitate a double crossover. All the disruptants were subjected to Southern blot analysis to confirm a double crossover. Hybridization was carried out using DIG DNA labeling and a detection kit (Roche) according to the manufacturer's protocol.

Antibiotic Production and Bioautography

One milliliter of the full-grown precultures of *S. rochei* strains were inoculated into 100 ml of YMG liquid medium in a 500 ml Sakaguchi flask and reciprocally cultured at 28°C for 3 days. The broth filtrates were extracted with ethyl acetate, dehydrated with Na_2SO_4, and concentrated to crude extracts. The extracts were applied to thin layer chromatography (TLC) (Kieselgel 60 F_{254}, Merck) and developed with chloroform-methanol (15:1). The developed TLC plate was dried and contacted with a bioassay plate for 30 min, and incubated at 28°C overnight. The bioassay plate was composed of two layers; the bottom layer contained TSB medium containing 1.5% agar, while the top layer contained TSB-agar (0.8%) supplemented with 2% of the overnight culture of the indicator organism, *Micrococcus luteus*.

Isolation and Analysis of Metabolites

The crude extract was applied to Sephadex LH-20 chromatography (1 × 40 cm, Amersham Pharmacia Biotech AB) with methanol. Lankamycin was eluted in 20–25 ml fractions, while lankacidins were in 28–34 ml. The latter fractions were further purified by silica gel chromatography with chloroform-methanol (50:1–20:1). Average yields of lankacidin C (**1**) and lankacidinol A (**2**) from the parent

strain 51252 were 8.0 and 0.5 mg per liter, respectively. The yield of compound LC-KA05 (**4**) from strain KA05 was 1.5 mg per liter.

Lankacidins and LC-KA05 were also analyzed by HPLC. The crude extract was applied on a TSKgel ODS-80Ts column (5 μm diameter, 4.6 × 250 mm; Tosoh, Tokyo), eluted with a mixture (3:1) of acetonitrile and 10 mM sodium phosphate (pH 8.2) at a flow rate of 1 ml/min, and monitored by a Jasco MD-2010 multiwavelength detector.

Feeding, Complementaion, Bioconversion, and Cofermentaion Experiments

Feeding of PQQ

Two hundred micrograms of PQQ was added to a 100 ml YMG culture of the *pqq* mutant FS7 at 24 hr and the culture was stopped and analyzed at 72 hr.

Complementation of the lkcE Mutation

Plasmid pKAR1023, which contained a functional *lkcE* gene on pRES18 (see Supplemental Data), was transformed into the *lkcE* mutant KA05. The transformant was cultured in YMG medium at 28°C for 72 hr in the presence of 10 μg/ml of thiostrepton.

Bioconversion of LC-KA05

1.0 milligram of LC-KA05 was added at 24 hr to 100 ml YMG cultures of strains FS7 and KAFS13-1, respectively, and the fermentation was stopped and analyzed at 72 hr.

Cofermentation Experiment

Strains KA05 and FS7 were mixed cultured in YMG medium at 28°C for 72 hr.

Spectroscopic Instruments

Nuclear magnetic resonance (NMR) spectra were recorded on a Jeol LA-500 spectrometer equipped with a field-gradient accessory. Deuteriochloroform (99.8 atom %) was used as NMR solvent. ^{1}H NMR and ^{13}C NMR chemical shifts were recorded in δ values based on the signals of tetramethylsilane (TMS) ($\delta_H = 0$) and $CDCl_3$ ($\delta_C = 77.0$), respectively. Whole correlations in the lankacidin framework were determined by several 2D-NMR spectra (DQF-COSY, HMQC, and HMBC). The high-resolution FAB-MS spectra were monitored on a Jeol SX-102A mass spectrometer.

Supplemental Data

The construction procedures of all the gene disruptants and their Southern hybridization analysis as well as spectroscopic data of compounds **1**, **2**, and **4** are available at http://www.chembiol.com/cgi/content/full/12/2/249/DC1.

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