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Cloning of the Gene Cluster Responsible for Biosynthesis of KS-505a (Longestin), a Unique Tetraterpenoid

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KS-505a (longestin), produced by *Streptomyces argenteolus*, has a unique structure that consists of a tetraterpene (C40) skeleton, to which a 2-*O*-methylglucuronic acid and an *o*-succinyl benzoate moiety are attached. It is a novel inhibitor of calmodulin-dependent cyclic-nucleotide phosphodiesterase, which is representative of a potent anti-amnesia drug. As a first step to understanding the biosynthetic machinery of this unique and pharmaceutically useful compound, we cloned a KS505a biosynthetic gene cluster. First we searched for a gene encoding octaprenyl diphosphates, which yielded a C40 precursor by PCR, and four candidate genes were obtained. Among these, one was confirmed to have the expected enzyme activity by recombinant enzyme assay. On the basis of an analysis of the flanking regions of the gene, a putative KS-505a biosynthetic gene cluster consisting of 24 ORFs was judged perhaps to be present on a 28-kb DNA fragment. A gene disruption experiment was also employed to confirm that the cluster indeed participated in KS-505a biosynthesis. This is believed to be the first report detailing the gene cluster of a cyclized tetraterpenoid.

Key words: isoprenoid cyclase; tetraterpene; KS-505a (longestin); *Streptomyces*; octaprenyl diphosphate synthase

Isoprenoids are the largest group of natural products found in nature, with over 24,000 known compounds.[1,2] They have biologically important functions that are derived from their structural diversity, such as hormones (gibberellins, brassinosteroids, and abscisic acid in plants, and cholesterol, androgen, estrogen in animals), prenylated moieties (quinones in electron transport chains and some eukaryotic proteins), phytol and carotenoid pigments in photosynthesis, and phytoalexins in plants.[1,2] Isoprenoid biosynthesis begins with the condensation of isopentenyl diphosphate (IPP) into its isomer DMAPP by polyprenyl diphosphate (PPD) synthases to form elongated linear PPDs, among which are GDP (C_{10} compound), FDP (C_{15} compound), and GGDP (C_{20} compound), precursors of mono-, sesqui- and diterpenes respectively.[3–5] In many cases, isoprenoid cyclases transform these common linear PPD intermediates into a wide variety of cyclic products. These compounds are often modified further by oxidation, reduction, and glycosylation to form a greater structural variety. The first committed reactions that yield a diversity of isoprenoid compounds are therefore catalyzed by isoprenoid cyclases. To date, many mono- and sesquiterpene cyclase genes and several diterpene cyclase genes have been cloned from eukaryotes.[3–5] Many triterpene cyclase genes have been cloned from eukaryotes and prokaryotes.[6–8] The cyclization mechanisms of their products have been extensively studied using recombinant enzymes. However, as for tetraterpenes (C40), there have been no reports on tetraterpene cyclase that catalyze the cyclization of octaprenyl diphosphate (OPD; C40), although the acyclic end of lycopene, which is formed by tail-to-tail condensation of two molecules of GGDP, is known to be cyclized in carotenoid biosynthesis.[9]

In contrast to eukaryotes, prokaryotes are known to produce a limited number of isoprenoids. Among prokaryotes, actinomycete strains have recently been found to produce relatively many types of isoprenoids, and some of these compounds have been reported to have cyclized structures.[10] From the producers of such cyclized isoprenoids, pentalenene, germacradienol (germacrene D), and (+)-*epi*-isozizaene synthases, all of which are sesquiterpene cyclases, have been identified by Cane and co-workers.[11–13] We have also cloned several diterpene cyclase genes from *Streptomyces* strains.[14–16] These isoprenoid cyclases, found in prokaryotes, have been found to be different from those in eukaryotes with respect to the number of amino acid residues and their primary structures.

Previously, KS-505a was isolated from the culture

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Abbreviations: PPD, polyprenyl diphosphate; OPD, octaprenyl diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GDP, geranyl diphosphate; FDP, farnesyl diphosphate; GGDP, geranylgeranyl diphosphate

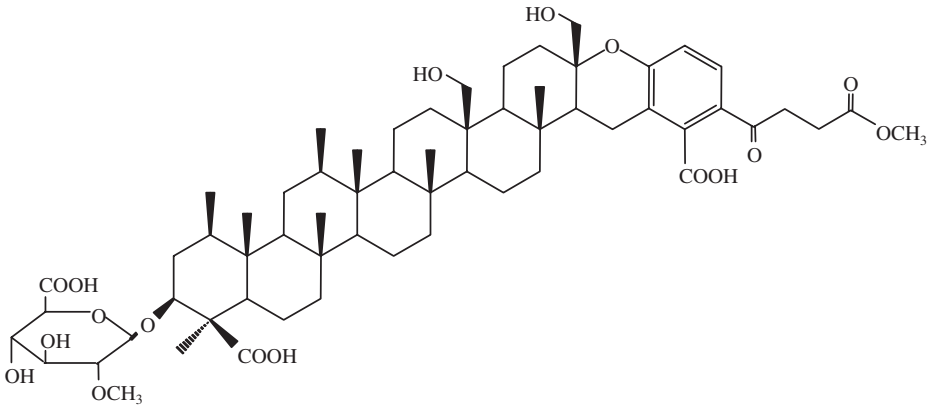


Fig. 1. Structure of KS-505a (longestin).

broth of *Streptomyces argenteolus*, and it was the first example of the compound with a heptacyclic isoprenoid skeleton (Fig. 1).[17] We have confirmed by a tracer experiment that the isoprenoid moiety is derived from OPD,[18] which suggests that a tetraterpene cyclase is involved in the biosynthesis of KS-505a. Here in this study we tried to clone the KS-505a biosynthetic gene cluster as a first step in establishing which kind of cyclase is involved in the biosynthesis of KS-505a. A gene disruption experiment to determine whether a putative tetraterpene cyclase gene found in the cluster is indeed responsible for KS-505a biosynthesis was also performed.

Materials and Methods

Chemicals. [α-^{32}P]dCTP was obtained from GE Healthcare UK Ltd. (Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA, England). IPP, DMAPP, FPP, and GGDP were purchased from Sigma-Aldrich (St. Louis, MO). The other chemicals were all analytical grade. Authentic KS-505a was kindly provided by Kyowa Hakko Kogyo, Co., Ltd. (Tokyo, Japan).

Bacterial strains. S. argenteolus was used in the cloning experiment. Media and growth conditions were as described by Nakanishi *et al.*[17] *Escherichia coli* JM110 and plasmids pUC118 and pUC119 were used for sequencing analysis. When necessary, ampicillin was added to the medium to a final concentration of 100 μg/ml.

DNA isolation and manipulation. Plasmids from *E. coli* were prepared using a Qiagen plasmid kit (Hilden, Germany). All restriction enzymes, T4 ligase, and calf intestinal alkaline phosphatase were obtained from Toyobo (Osaka, Japan), and used according to the manufacturers' protocols. Transformation of *E. coli* with plasmid DNA by electroporation was performed under standard conditions using a BTX ECM 600 electroporation system (Biotechnologies and Experimental Research, San Diego, CA). Other general procedures were performed as described by Maniatis *et al.*[19]

Cloning of PPD synthase genes by PCR. Three sets of primers were designed based on the amino acid sequences around the conserved first aspartate rich motif (FARM) and the second aspartate rich motif (SARM) of PPD synthases, which have been identified in terpentecin,[20] BE-40644,[21] and furaquinocin A[22] producers: PDS-1-5, 5′-BTSCACGACGACBTSATS-GACVRS-3′; PDS-1-3, 5′-SASGTCGTCSMSSRRCTG-GAASGC-3′; PDS-2-5, 5′-YTSYTSCACGACGACGT-SRTSGACGMS-3′; PDS-2-3, 5′-GTCGTCSAYSADC-TGGAASGCSRTSCC-3′; PDS-3-5, 5′-GCSATCYTSG-CSGGSGACBTSYT-3′; and PDS-3-3, 5′-SASGTCG-TCSMSSRRCTGGAASGC-3′. These primers were used in PCR experiments to amplify PPD synthase genes with genomic DNAs of KS-505a producer as a template. The standard PCR amplification was performed with the Takara LA PCR kit (Takara Shuzo, Kyoto, Japan) under the following conditions: denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and elongation at 72 °C for 1.5 min, for 30 cycles. When necessary, annealing temperature and the type of DNA polymerase were varied. The amplified DNA fragments were inserted into pGEM-T easy vector (Promega, Madison, WI) and used sequencing analysis. A cosmid library of KS-505a producer previously constructed was screened by colony hybridization with the ^{32}P-labeled amplified fragments as probes,[23] which perhaps encode a PPD synthase, by sequencing analysis. Positive cosmids hybridizing each of the probes were selected, and DNA fragments, which hybridized to the probe by Southern blot analysis, were subcloned into the same sites of pUC119. After construction of a series of plasmids, sequencing was done with an automatic DNA sequencer (Li-Cor, Model 4000L, Lincoln, NE).

Overproduction of OPD synthase and GGDP synthase. The following two sets of primers and a cosmid

carrying the KS-505a biosynthetic gene cluster were used to amplify the *opd* gene (*lon*12) and the *ggdp* gene (*lon*22) respectively: 5′-GGGGGATCCCACGGGTACGTGGCGAACGCGCGTCG-3′ (forward) and 5′-ACCAAGCTTTCAGGCGCGGCGGTCACGGGCGAGGT-3′ (reverse); and 5′-GGGGGATCCACAGACAGTTCACTCCAACCACGGGC-3′ (forward) and 5′-ACCAAGCTTTCATCTGTCACGATCGGTAATGGCGA-3′ (reverse). For amplification of the OPD synthase gene of *E. coli*, one set of primers, 5′-GGGGGATCCAATTTAGAAAAAATCAATGAGTTAAC-3′ (forward) and 5′-ACCAAGCTTTTAACGATCGCGTTGAACAGCGATGT-3′ (reverse), and genomic DNA of *E. coli* K12 W3110 were used. PCR was carried out under standard conditions. After sequence confirmation, the *Bam*HI/*Hin*dIII fragments were inserted into the same site of pQE30. pQE30-lon12, pQE30-lon22, and pQE30-eco-opd, in which recombinant proteins were expressed as *N*-terminal His_6-tagged fusion proteins, were selected.

E. coli M15 [pREP4] harboring pQE30-lon12, pQE30-lon22, or pQE30-eco-opd was grown at 37 °C in Luria broth with appropriate antibiotics. Expression of the recombinant protein was induced by adding 0.1 mM of isopropyl-β-D-thiogalactoside when OD_{600} reached about 0.8. Cultivation was continued for an additional 12 h at 18 °C. Purification of His-tagged recombinant proteins was performed according to the manufacturer's protocol (Qiagen). Purified proteins were analyzed by SDS–PAGE on 10% gels and used in an *in vitro* enzyme assay to determine the product chain length. Assay conditions and product analysis were the same as described previously.[23]

Disruption of the cyclase gene. A 1.2-kb DNA fragment that contained an internal region of the cyclase gene was amplified by PCR with the following two primers: 5′-CGCGGATCCGCCGAGTAGCAAGAGCGGCACCGCCT-3′ (forward) and 5′-ACCAAGCTTCCCCTCCGTGTGGTCCGCGCCGGCAG-3′ (reverse). After sequence confirmation, the *Bam*HI/*Hin*dIII fragment was inserted into the same sites of pK18mob[24] to construct pCyc-DIS. The plasmid was used for disruption of the chromosomal *cyc* (*lon*15) gene by insertional inactivation *via* a single crossover recombination. Introduction of the plasmid into the KS-505a producer was performed by transconjugation, and kanamycin-resistant colonies were selected.[25] Genomic DNAs were prepared from the transformants and subjected to PCR analysis with the following primers: 5′-GTGTCGATTTCCTGTACGCGCGCCAGCA-3′ and 5′-CTAGTCCACCCGCGTTCTGCGCAGCCAGC-3′, which corresponded to the *N*-terminal and *C*-terminal coding region of the cyclase gene. The productivity of KS-505a of the disruptant was examined as follows: The disruptants were cultivated by the methods described by Nakanishi *et al.*,[17] except for the addition of kanamycin (25 μg/ml) to the medium. After full growth cultivation, the culture broth was centrifuged, and the materials were extracted by ethyl acetate from the supernatant. Since KS-505a exists as a hemiketal/keto mixture,[17,18] the extract was treated with (trimethylsilyl)diazo-methane to derive methylated compounds. The derivatives thus obtained were analyzed by HPLC (Merck Mightysil RP-18 column, 250 × 4.6 mm; column temperature, 30 °C; detection at 230 nm; 40% acetonitrile for 0 to 10 min, and a linear gradient from 40% to 100% for an additional 20 min; flow rate, 1 ml/min).

Nucleotide sequence accession number. The DNA sequence of the KS-505a biosynthetic gene cluster was deposited in the DDBJ, EMBL, and GenBank databases under accession no. AB307968.

Results

Cloning of OPD synthase genes by PCR

We have found that biosynthetic genes of isoprenoids produced by actinomycetes are located at the flanking regions of the gene encoding PPD synthase,[14,21,22,26] which supplies a direct precursor of isoprenoid compounds by condensation of IPP. Here, first we tried to clone a gene encoding octaprenyl diphosphate (OPD) synthase, which supplies a C40 precursor from the KS-505a producer. To date, many deduced amino acid sequences of PPD synthase have been reported. Comparison of the primary structures has revealed that several domains, such as FARM and SARM, both of which are known to be essential to substrate binding, are conserved in these enzymes.[27] Here we tried to clone PDS genes by PCR with three sets of primers based on the conserved amino acid sequences of FARM and SARM. Considering that two actinomycete strains, *Streptomyces coelicolor* A3(2) and *Streptomyces avermitilis* MA-4680, the entire genome sequencing of which has been completed, have at least five and six putative PPD synthase genes,[28,29] the KS-505a producer belonging to *Streptomyces* has also been suggested to have several PPD synthase genes. Hence, we tried to clone as many PPD synthase genes as possible by varying the PCR conditions, such as annealing temperature and type of DNA polymerase.

We amplified and cloned by sequencing analysis four types of DNA fragments (*opds*1-*opds*4), all of which are thought to encode enzymes similar to PPD synthases. Among these, *opds*3 was found to be the same as one gene that had previously been cloned from the KS-505a producer, and was confirmed to be an FDP synthase.[23] Hence, we analyzed the flanking regions of the *opds*1, *opds*2, and *opds*4 genes. Although we did not find any genes related to isoprenoid biosynthesis in the flanking regions of the *opds*4 gene, examination of the others (*opds*1 and *opds*2) suggested that they constituted operons with genes that are probably related to isoprenoid biosynthesis. *opds*2 was located next to a putative isoprenoid cyclase gene, in a manner similar to that in *S. coelicolor* A3(2) (SCO6763) and *S. aver-*

mitilis MA-4680 (SAV1651). Moreover, the cyclase had high sequence similarities (85% identity) to the squalene–hopene cyclases of *S. coelicolor* A3(2) (SCO6764) and *S. avermitilis* MA–4680 (SAV1650). Taken together, the results suggest that *opds*2 is a farnesyl diphosphate (C15) synthase that provides a precursor for squalene–hopene biosynthesis. In contrast, *opds*1 perhaps formed an operon together with 4-hydroxybenzoate octaprenyl transferase (Lon13), FAD-monooxygenase (Lon14), and a putative isoprenoid cyclase (Lon15) (Fig. 2), all of which are perhaps involved in KS-505a biosynthesis.

Specificity of the opds*1 (*lon*12) product*

To confirm that *opds*1 did indeed encode an OPD synthase, a recombinant enzyme was expressed in *E. coli* as an *N*-terminal His-tagged fusion protein, and an *in vitro* assay was performed. Soluble protein extracts from *E. coli* harboring pQE30-lon12 were analyzed by SDS–PAGE. The His-tagged recombinant protein, with a mass of about 35 kDa, which was in good agreement with the value calculated from the deduced amino acid sequence of the enzymes, was expressed at a high level. The expressed proteins were then purified (Fig. 3A) and used in the enzyme assay. When the assay was carried out using FDP and [1-^{14}C] IPP as substrates, OPD (C40) and nonaprenyl diphosphate (C45) were detected as major products by TLC analysis (Fig. 3B, lane 3).

Analysis of flanking regions of the opds*1 (*lon*12)*

Considering that *opds*1 (*lon*12), which was confirmed to encode OPD synthase, constituted an operon together with genes related to isoprenoid biosynthesis, and that antibiotic biosynthesis genes cloned from actinomycetes are usually clustered in the genomic DNA region, all genetic information for the biosynthesis of KS-505a was expected to exist in this region. Hence the nucleotide sequences of both the upstream region of *opds1* (*lon*12) and the more downstream region of the putative isoprenoid cyclase genes (*lon*15) were determined (Fig. 2). Computer analysis of the DNA sequence by frame analysis showed 24 ORFs (Lon1-24) in a 28-kb DNA fragment.

To determine the functions of individual ORFs deduced by DNA sequencing, we searched the databases for their translated products by means of the sequence similarity search programs FASTA and BLAST. As shown in Fig. 2, the cluster included putative KS-505a biosynthetic genes, such as the *o*-succinylbenzoate synthase gene (*lon*3) and the isochorismate synthase gene (*lon*9) for *o*-succinyl benzoate biosynthesis, the glycosyl transferase gene (*lon*7) for the attachment of glucuronic acid, two P450 genes (*lon*11 and 16) for hydroxylation, and three genes for methylation (*lon*10, 20, and 23), in addition to the *opds*1 (*lon*12) and the isoprenoid cyclase genes (*lon*15). Interestingly, one additional PPD synthase gene (*lon*22, see below) existed in the cluster, although we could not amplify this gene by PCR screening. Moreover, two non-mevalonate pathway genes, a 1-deoxy-D-xylulose-5-phosphate synthase gene (*lon*4) and 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase gene (*lon*5), were also included in the cluster.

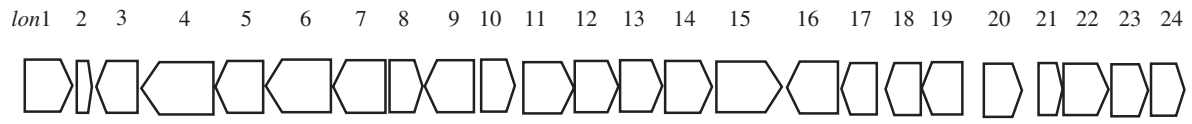
*Disruption of the cyclase gene (*lon*15)*

We constructed a mutant in which the cyclase gene (*lon*15) was disrupted by insertional inactivation to confirm that the gene cluster was responsible for KS-505a biosynthesis. pCyc-DIS was constructed by inserting the internal region of the cyclase gene into pK18mob, a transconjugation plasmid.[24] After the introduction of the plasmid into the KS-505a producer by transconjugation, kanamycin-resistant colonies were selected. Three strains were randomly selected and their genomic DNAs were prepared and used as templates for PCR analysis to study gene disruption. As shown in Fig. 4A, integration of pCyc-DIS into the corresponding genomic region by a single crossover was clearly confirmed. Hence, the disruptants and wild-type strain were cultivated in production medium, and the productivities of KS-505a were examined. None of the disruptants produced KS-505a, in contrast to the wild type strain, which produced sufficient KS-505a (Fig. 4B), showing that the cluster, including the putative cyclase gene (*lon*15), was indeed involved in the biosynthesis of KS-505a.

*Characterization of additional PPD synthase gene (*lon*22)*

Besides the OPD synthase gene (*lon*12), another PPD synthase gene (*lon*22) existed in the cluster. To date, the OPD synthase identified in *E. coli* has been studied using a recombinant enzyme, and FDP has been found to be the allylic substrate in a condensation reaction.[30] Considering that Lon12 also uses FDP as an allylic substrate, and that antibiotic biosynthetic genes are usually clustered in actinomycetes, *lon*22 was hypothesized to encode an FDP synthase to supply FDP to OPD synthase (Lon12).

To examine this possibility, Lon22 was expressed as a recombinant enzyme and used in an *in vitro* assay to determine the chain length of the product. pQE30-lon22 was constructed to obtain an *N*-terminal His-tagged recombinant protein. Expression of the recombinant enzyme in soluble form was confirmed by SDS–PAGE (Fig. 5A). The expressed protein was then purified and used in an enzyme assay. When the assay was carried out using DMAPP and [1-^{14}C]IPP as substrates, to our surprise, geranylgeraniol (C20) was detected as a major product by TLC analysis (Fig. 5B), which suggests that *lon*22 encodes a GGDP synthase. Hence we hypothesized another possibility, that Lon12 uses GGDP as an allylic substrate, in addition to FDP. To investigate this possibility, enzyme assays using DMAPP, GDP, FDP, or GGDP as an allylic substrate were performed, and the product was analyzed by TLC. As shown in Fig. 3B,



lon Gene	Amino acids	Homologous proteins and their accession numbers
1	336	Hypothetical protein of *Frankia* sp. EAN1pec (40% identity over 86 amino acids), Q3WIA6
2	133	ATP synthase beta subunit (Fragment) of *Erwinia americana* (29%/64), Q0GKR5
3	308	*o*-Succinylbenzoate synthase of *Propionibacterium acnes* (51%/297), dad AE017283-890
4	599	1-Deoxy-D-xylulose-5-phosphate synthase of *Mycobacterium leprae* (53%/590), sp Q50000
5	376	4-Hydroxy-3-methylbut-2-en-1-yl diphosphate synthase 1 of *Streptomyces avermitilis* (86%/377), sp Q82K43
6	502	Hypothetical protein of *Bacillus halodurans* (31%/63), prf 2624317BFW
7	416	Glycosyl transferase of *Rhodopirellula baltica* (36%/422), dad BX294152-215
8	258	2,3-Dihydro-2,3-dihydroxybenzoate dehydrogenase of *Stigmatella aurantiaca* (51%/256), dad AF299336-3
9	431	Isochorismate synthase of *Photobacterium profundum* (38%/294), dad CR378680-231
10	277	Macrocin-*O*-methyltransferase of *Herpetosiphon aurantiacus* (51%/279), Q0LKJ2
11	418	P450 of *Streptomyces avermitilis* (39%/404), prf 2724351FT
12	330	Polyprenyl diphosphate synthase of *Methanobacterium thermoautotrophicum* (38%/289), prf 2022153B
13	326	4-Hydroxybenzoate octaprenyl transferase of *Pyrococcus furiosus* (27%/284), dad AE010289-12
14	411	Oxigenase (mtmOI) of *Streptomyces argillaceus* (29%/396), prf 2708358N
15	545	Squalene-hopene cyclase of *Rhodopirellula baltica* (25%/641), dad BX294143-86
16	403	P450 of *Streptomyces atroolivaceus* (41%/403), dad AF484556-36
17	258	Hypothetical protein of *Nocardia farcinica* prf (33%/254), 3024436FBP
18	271	ABC transporter (CinH) of *Streptomyces cinnamoneus* (30%247), dad AJ536588-9
19	344	ABC transporter of *Pyrococcus abyssi* (40%/329), dad AJ248285-35
20	318	Methyltransferase of *Mycobacterium bovis* (45%/248), dad BX248337-273
21	206	*lmb*U protein of *Streptomyces lincolnensis* (48%/185), dad X79146-29
22	360	Polyprenyl diphosphate synthase of *Actinoplanes* sp. (41%/357), dad AB113568-1
23	287	Methyltransferase of *Streptomyces griseus* (41%/262), dad AB040071-7
24	292	Hypothetical protein of *Streptomyces coelicolor* (38%/289), dad AL939132-271

Fig. 2. Octaprenyl Diphosphate Synthase Gene and Its Flanking Regions in *Streptomyces argenteolus*.
ORFs deduced by sequencing analysis are shown. Dad, DNA Data Bank of Japan; sp, Swissplot; prf, Protein Research Foundation.

almost the same amount of product was formed with FDP and GGDP, and slightly fewer products were formed with GDP, although no products were formed with DMAPP, which suggests that the GGDP synthase encoded by the *lon*22 gene supplies GGDP for the biosynthesis of OPD.

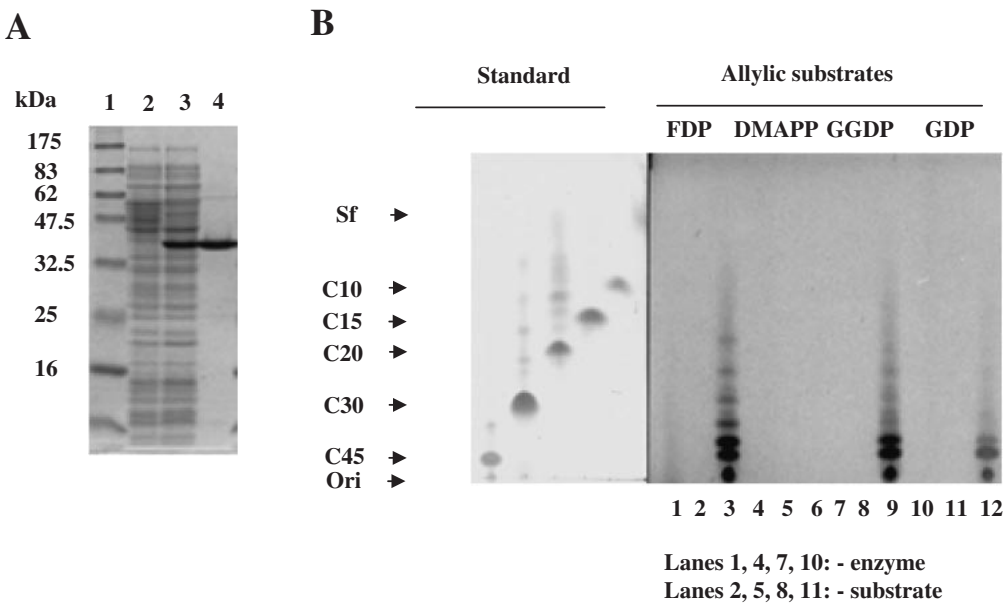


Fig. 3. Electrophoresis of the Overproduced and Purified Lon12 Product (A) and TLC Autoradiography of the Alcohols Obtained by Hydrolysis of the Products (B).

A, Molecular mass marker (lane 1), total soluble proteins from the *E. coli* harboring pQE30 (lane 2), pQE30-lon12 (lane 3), and purified Lon12 product (lane 4), were analyzed on SDS–PAGE (12.5%). Proteins were stained with Coomassie brilliant blue R-250. B, The sample obtained by incubation of [1-^{14}C] IPP and FDP, DMAPP, GDP, and GGDP with the enzymes was analyzed by TLC. Spots of authentic standard alcohols are indicated by arrows. Ori., origin; sf., solvent front.

Previously, the OPD synthase found in *E. coli* has been studied using a recombinant enzyme. In that experiment, FDP was used as the allylic substrate,[30] but Lon12 used GDP, FDP, or GGDP as an allylic substrate, as described above. Hence we expected that *E. coli* OPD synthase, which had 36% identity with the Lon12 product, might also use other allylic substrate besides FDP. Hence, we prepared recombinant OPD synthase of *E. coli*, and the reaction product was analyzed by the same method and under the same conditions as for Lon12. As shown in Fig. 6, a major product, OPD, was formed with all allylic substrates, including DMAPP.

Discussion

In order to clone the KS-505a biosynthetic gene cluster, we first cloned by PCR a gene encoding OPD synthase, which supplied a direct precursor of tetraterpene compounds, and then we analyzed the flanking regions of the OPD synthase gene. We expected that the OPD synthase and KS-505a biosynthetic genes would be clustered. Finally, we succeeded in cloning the *lon*1 to *lon*24 genes that might participate in KS-505a biosynthesis. We also confirmed by gene disruption experiments that the cluster was responsible for KS-505a biosynthesis, although we did not define the terminal region of the cluster. In the upstream region of the *lon*1 gene, an AT-rich region continued for about 800 bp, and the region appeared to contain no genes on the basis of frame analysis. In the downstream region of the *lon*24 gene, we identified a putative gene that encoded glutamyl-tRNA aminotransferase subunit A, which participates in primary metabolism. Taking these results together, we consider that *lon*1 to *lon*24 might be involved in the biosynthesis of KS-505a. However, to draw a firm conclusion, more gene disruption experiments and/or heterologous expression of the cluster are essential.

We demonstrated recently that some actinomycetes possess both the mevalonate and the MEP pathway for the formation of IPP.[10] Interestingly, in these strains, isoprenoid biosynthetic genes, such as the terpentecin,[14] BE-40644,[21] viguiepinol,[22] and furaquinocin A[26] biosynthetic gene clusters, were located just upstream and/or downstream of the mevalonate pathway gene cluster. Moreover, during production of the diterpene terpentecin, 60% of the IPP needed has been found to be supplied *via* the mevalonate pathway, and the MEP pathway contributes 40%.[31] This suggests that IPP biosynthesized *via* the MEP pathway is in short supply for the full production of terpentecin in the producer strains. On the other hand, *Streptomyces* strains that have only the MEP pathway for the formation of IPP are also known to produce isoprenoid compounds.[10] In the KS-505a producer, which has only the MEP pathway,[18] two MEP pathway genes, 1-deoxy-D-xylulose-5-phosphate synthase gene (*lon*4) and 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase gene (*lon*5), were found to exist in the cluster in this study. A *Streptomyces* strain, which has only the MEP pathway and produces phenalinolactone, a terpene glycoside, has been reported to have additional 1-deoxy-D-xylulose-5-phosphate syn-

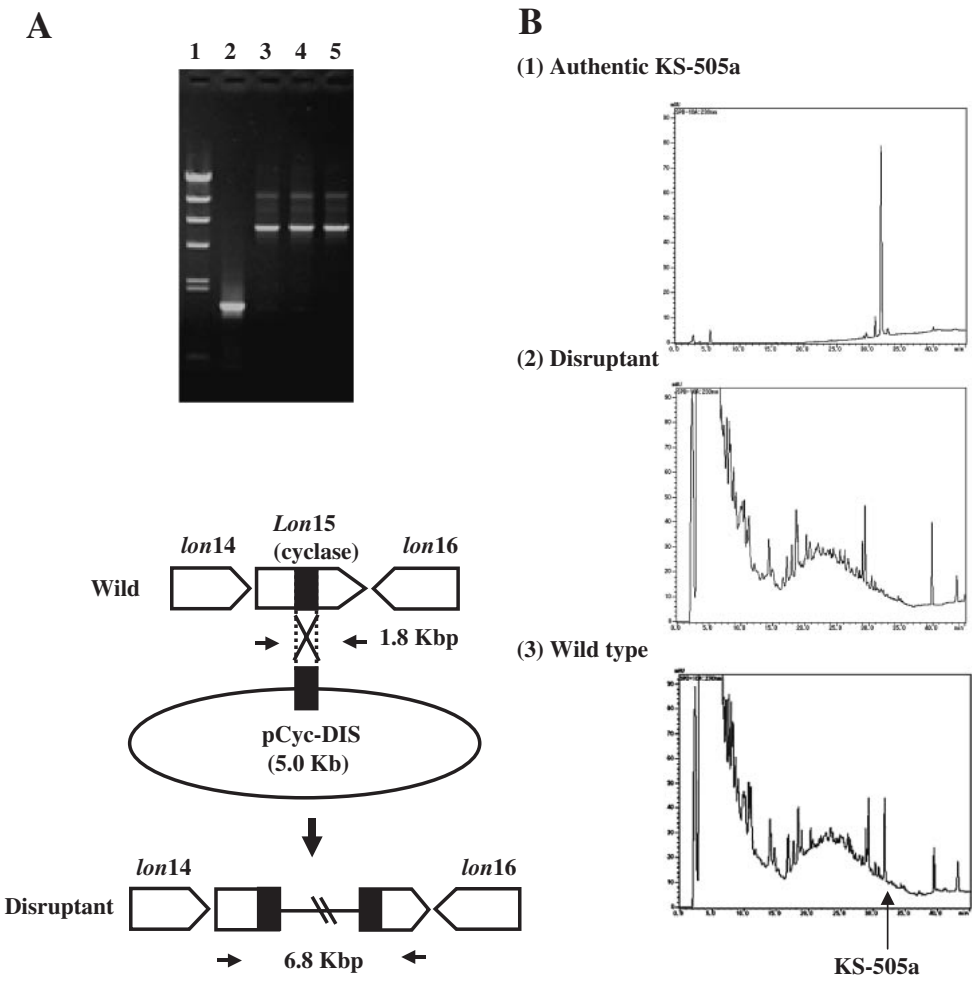


Fig. 4. PCR Analysis of the Genomic DNA of the *lon*15 (cyclase gene)-Inactivated Strains (A) and KS-505a Productivity of the Disruptants (B). A, Molecular marker (lane 1), PCR products with genomic DNA of wild-type strain (lane 2), and disruptant (lanes 3–5) as templates. The hybridized fragments are shown schematically. Arrows indicate the primers used in PCR analysis; they correspond to the *N*-terminal and *C*-terminal coding region of the cyclase gene. Filled boxes indicate the DNA fragment (1.2 Kb) used for gene disruption, which contained the internal region of Lon 15 (cyclase). B, Authentic KS505a (1), products of disruptant (2), and products of wild-type strain (3) were analyzed by HPLC.

thase and 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase genes in the phenalinolactone biosynthetic gene cluster.[32] By genome sequencing, both *S. coelicolor* A3(2) and *S. avermitilis* were found to have two 1-deoxy-D-xylulose-5-phosphate synthase genes, SCO6768 and SCO6013 in the former strain,[28] and SAV1646 and SAV2244 in the latter strain.[29] Two distinct 4-Hydroxy-3-methylbut-2-en-1-yl diphosphate synthase genes were also suggested to exist in the former strain (SCO5696 and SCO6767)[28] and in the latter strain (SAV1647 and SAV2561).[29] Interestingly, one set of the genes (SCO6768/6767 and SAV1646/1647) would constitute a cluster with the genes responsible for biosynthesis of squalene-hopene and phytoene (SCO6759 to SCO6768 and SAV1646 to SAV1654). Taking these results together, for full production of isoprenoid compounds that are produced by strains possessing only the MEP pathway, the producers might acquire the additional MEP pathway genes instead of the mevalonate pathway genes. Considering that 1-deoxy-D-xylulose-5-phosphate synthase has been shown to be a rate-limiting enzyme in the MEP pathway,[33] the existence of the additional gene is quite reasonable.

In *E. coli* and *Streptomyces* strains, OPD synthases are known to supply the C40 prenyl side chain of menaquinone essential for respiration.[34,35] Although the OPD synthase of *E. coli* accepts DMAPP, GDP, FDP, and GGDP as allylic substrates, the intrinsic substrate of the enzyme *in vivo* must be DMAPP or FDP, because *E. coli* has been reported not to synthesize GDP and GGDP.[36] On the other hand, the OPD synthase (Lon12) found in the biosynthetic gene cluster of the secondary metabolite used GDP, FDP, or GGDP, but not DMAPP, as the allylic substrate. It is very curious that the Lon12 enzyme did not use DMAPP, because it is easily and directly supplied by the MEP pathway. Considering that the GGDP synthase (Lon22) was involved in the cluster, the true allylic substrate of the enzyme *in vivo* might be

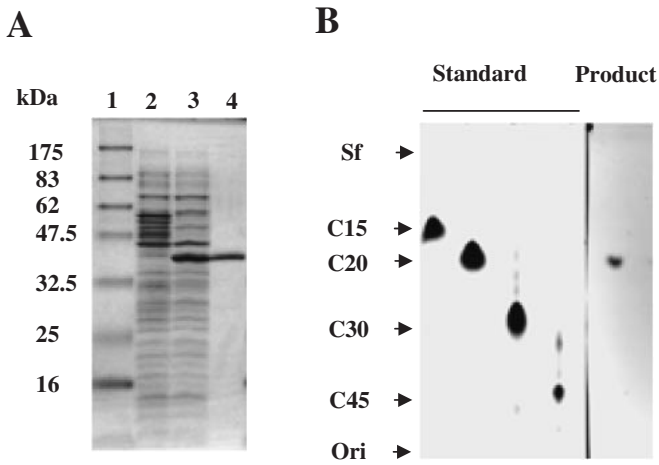


Fig. 5. Electrophoresis of the Overproduced and Purified Lon22 Product (A) and TLC Autoradiography of the Alcohols Obtained by Hydrolysis of the Products (B).

A, Molecular mass marker (lane 1), total soluble proteins from the *E. coli* harboring pQE30 (lane 2), pQE30-lon22 (lane 3), and purified Lon22 product (lane 4) were analyzed on SDS–PAGE (12.5%). B, The sample obtained by incubation of [1-^{14}C] IPP and DMAPP with the enzymes was analyzed by TLC. Spots of authentic standard alcohols are indicated by arrows: C10, geraniol; C15, (all-*E*)-farnesol; C20, (all-*E*)-geranylgeraniol; C30, (all-*E*)-farnesylfarnesol; C45, (all-*E*)-nonaprenol. Ori., origin; sf., solvent front.

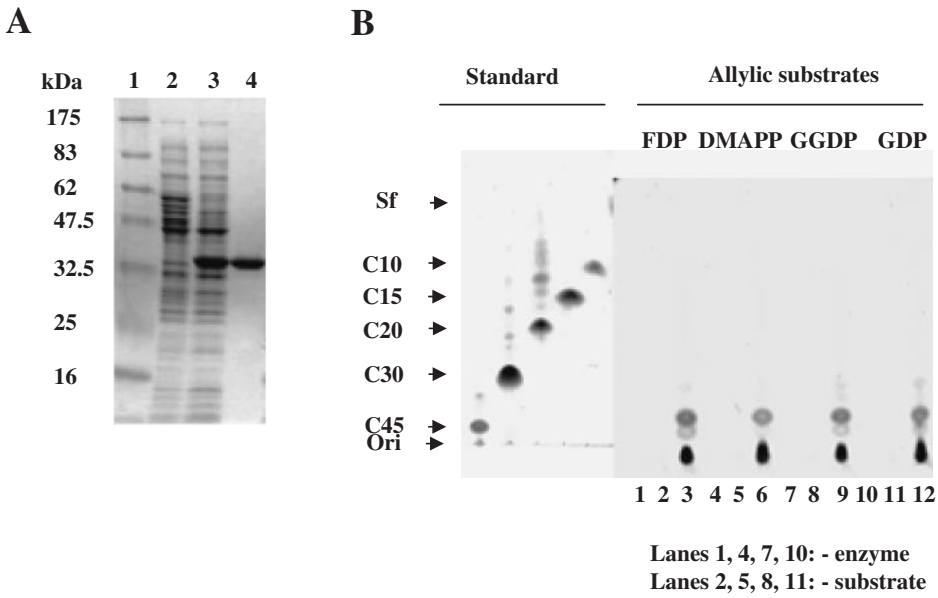


Fig. 6. Electrophoresis of the Overproduced and Purified *E. coli* OPD Synthase (A) and TLC Autoradiography of the Alcohols Obtained by Hydrolysis of the Products (B).

A, Molecular mass marker (lane 1), total soluble proteins from the *E. coli* harboring pQE30 (lane 2), pQE30-eco-opd (lane 3), and purified *E. coli* OPD synthase (lane 4) were analyzed on SDS–PAGE (12.5%). B, The sample obtained by incubation of [1-^{14}C] IPP and FDP, DMAPP, GDP, and GGDP with the enzymes was analyzed by TLC. Spots of authentic standard alcohols are indicated by arrows. Ori., origin; sf., solvent front.

GGDP. If this hypothesis is correct, two separate enzyme reactions, biosynthesis of GGDP followed by formation of OPD, are required for the biosynthesis of KS-505a. Although we are not able to estimate the biological significance of this complicated biosynthesis of OPD, one possibility is the following: Assuming that another OPD synthase essential for menaquinone biosynthesis in KS-505a producers has the same substrate specificity as that of the *E. coli* enzyme, such an enzyme might predominantly use DMAPP for menaquinone biosynthesis during the early growth phase. When the biosynthesis of KS-505a switches on at the mid-to-late log growth phase, the additional OPD required for the biosynthesis of KS-505a might be supplied by Lon12 with GGDP or FDP as the allylic substrate, the former and the latter being supplied by Lon22 and the *opds*3 product respectively.

Our ultimate goal was to identify and characterize a

tetraterpene cyclase essential to the production of KS-505a. In this study, we detected the isoprenoid cyclase gene that was confirmed by gene disruption to be essential for KS-505a production. Hence, we expressed the cyclase gene as a recombinant enzyme and used it for a preliminary *in vitro* assay using OPD as a substrate, but we did not detect any reaction products under various conditions. Although we do not know the reason why no product was formed at this stage, we suggest the following explanation: attachment of an *o*-succinyl benzoate moiety to OPD, preceded by a cyclization reaction. A study to examine this possibility is now in progress and will be reported in the near future.

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