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Presence of Copalyl Diphosphate Synthase Gene in an Actinomycete Possessing the Mevalonate Pathway

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We have previously shown that gene clusters for biosyntheses of terpentecin and BE-40644, a diterpene antibiotic and a sesquiterpene antibiotic, respectively, were located in the adjacent mevalonate pathway gene clusters. In this study, a mevalonate pathway gene cluster was cloned from *Streptomyces* sp. strain KO-3988, which was known to produce furaquinocin A, employing a hybridization experiment using a 3-hydroxy-3-methyl glutaryl CoA (HMG-CoA) reductase gene, which had been previously cloned from the strain KO-3988, as a probe. By sequencing flanking regions, we found four open reading frames that could encode a putative cytochrome P450 (ORF1), an isoprenoid cyclase (ORF2), an unknown protein (ORF3), and a polyprenyl diphosphate synthase gene (ORF4) in the upstream region of the mevalonate pathway gene cluster, though we did not find any genes related to furaquinocin A biosynthesis. The two ORFs (ORF2 and 4) were expressed as recombinant enzymes in *E. coli* and used for studies to investigate functions of these products. The ORF4 product was confirmed to be a geranylgeranyl diphosphate (GGDP, C_{20}) synthase. The ORF2 product proved to catalyze a conversion of GGDP into copalyl diphosphate, the first example of an enzyme with this function of prokaryotic origin. These results again showed that actinomycetes possessing the mevalonate pathway usually produce an isoprenoid and that its biosynthetic gene cluster exists in adjacent the mevalonate pathway gene cluster.

All isoprenoid compounds are derived from the five-carbon precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP)[1,2]. In eukaryotes and archaebacteria, IPP is supplied *via* the mevalonate pathway[1,2]. On the other hand, IPP is synthesized through the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway in most bacteria, green algae and in the chloroplasts of higher plants[3]. We have been studying biosyntheses of isoprenoid antibiotics produced by actinomycetes for the following reasons. Most *Streptomyces* strains have only the MEP pathway for the formation of IPP[3]. Examples are *Streptomyces* sp. strain UC5319, *Nocardia brasiliensis* and *Streptomyces argenteolus* that produce the isoprenoid compounds, pentalenolactone[4], brasilicardin A[5] and KS-505a[3], respectively. On the other hand, we have previously showed that both the mevalonate and MEP pathways were operating in some *Streptomyces* strains such as *Kitasatospora griseola* (terpentecin producer)[6,7], *Actinoplanes* sp. strain A40644 (BE-40644 producer)[7~9], *Streptomyces* sp. strain CL190 (naphterpin producer)[7,10,11],

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Abbreviations: DMAPP, dimethylallyl diphosphate; IPP, isopentenyl diphosphate; FDP, (*E*,*E*)-farnesyl diphosphate; GGDP, (*E*,*E*,*E*)-geranylgeranyl diphosphate; CPS, copalyl diphosphate synthase; ORF, open reading frame.

Streptomyces sp. strain KO-3988 (furaquinocin A producer)[7,12,13] and *Chainia rubra* (napyradiomycin producer)[7,14]. Interestingly, these strains were proved to produce isoprenoid compounds mainly through the mevalonate pathway[6~14]. Among them, we have cloned the mevalonate pathway gene clusters from producers of naphterpin[11], terpentecin[15] and BE-40644[9]. By sequencing flanking regions of each cluster, terpentecin[16] and BE-40644[9], biosynthetic gene clusters were revealed to exist adjacent to the mevalonate pathway gene cluster. These facts suggested that the presence of the mevalonate pathway is closely related to the production of isoprenoid compounds in actinomycetes. We had previously cloned a 3-hydroxy-3-methyl glutaryl CoA (HMG-CoA) reductase gene from *Streptomyces* sp. strain KO-3988 and showed its nucleotide sequences were homologous to those of HMG-CoA reductase genes from naphterpin, terpentecin, and BE-40644 producers[13]. In this study, therefore, we report cloning of an entire mevalonate pathway gene cluster from the strain KO-3988 and analysis of flanking regions of the cluster. We identified a copalyl diphosphate synthase (CPS) gene in the region just upstream of the mevalonate pathway gene cluster.

Materials and Methods

Chemicals

[α-^{32}P]dCTP, [1-^{14}C]IPP (CFA 476), and (*R*)-[2-^{14}C]mevalonic acid lactone (CFA 660) were obtained from Amersham. IPP, DMAPP, FDP, and GGDP were purchased from Sigma-Aldrich. The other chemicals were all of analytical grade.

Bacterial Strains

Streptomyces sp. strain KO-3988 was used for the cloning experiment[17]. Media and growth conditions for the strain KO-3988 were as described by KOMIYAMA *et al.*[17] *Streptomyces lividans* TK23[18] and pWHM3[19] were used for the heterologous expression of the mevalonate pathway gene cluster. *E. coli* JM110 {*rpsL thr leu thi lacY galK ara tonA tsx dam dcm supE44* /F′ [*traD proAB lacI*^{q} *lacZ*ΔM15]} (Toyobo, Osaka, Japan) and plasmids, pUC118 and pUC119, were used for sequencing analysis. An *E. coli* mutant DYM1, in which the *yae*M gene encoding 1-deoxy-D-xylulose 5-phosphate reductoisomerase was disrupted[20], was cultivated in the presence of 2-*C*-methyl-D-erythritol (ME, 0.01%). *E. coli* M15/pREP4 and a plasmid pQE30 (QIAGEN, Inc., CA, U.S.A) were used for expression of the His-tagged proteins. A cosmid library of DNA of the strain KO-3988 had been constructed previously[13]. Ampicillin (100 μg/ml) and kanamycin (25 μg/ml) were added to the medium as required.

DNA Isolation and Manipulation

Plasmids from *E. coli* were prepared using a Qiagen Plasmid Kit (QIAGEN). All restriction enzymes, T4 DNA ligase, and calf intestinal alkaline phosphatase were obtained from Toyobo 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, Inc., San Diego, CA). Other general procedures were performed as described by MANIATIS *et al.*[21]

Sequence Analysis

A 1.6 kb *Bam*HI fragment that had been previously cloned from *Streptomyces* sp. strain KO-3988 and confirmed to carry a HMG-CoA reductase gene was prepared from pHMGRSF1[13] and used for colony hybridization as a probe. Ten cosmids that hybridized to the probe were randomly selected and subjected to Southern blot analysis using the same probe as that for colony hybridization. Since the strain KO-3988 was suggested to have two distinct HMG-CoA reductase genes as described below, a cosmid containing the *Bam*HI fragment used for the probe was selected and used for sequencing analysis. Sequencing was carried out with an automatic DNA sequencer (Li-Cor, model 4000L).

Expression of the Mevalonate Pathway Genes in *E. coli* and *S. lividans*

The entire mevalonate pathway gene cluster containing putative mevalonate kinase, mevalonate diphosphate decarboxylase, phosphomevalonate kinase, type 2 IPP isomerase, HMG-CoA reductase and HMG-CoA synthase genes, was amplified by PCR. The 5′ and the 3′ primers had the respective sequences, 5′-TGCTCTAGAGG-GTGGCCGGAGCATGACGCGACACG-3′ and 5′-ACC-AAGCTTCTAGCGCGCCTGGTAGATGCGCTTGT-3′. To facilitate the cloning, an additional restriction site (underlined) was incorporated into both primers. After sequence confirmation, the *Xba*I-*Hin*dIII fragment was inserted into the same sites of pWHM3[19] to give pFQ-MEV1, in which the inserted DNA was expressed under the control of the *lac* promoter. The *yae*M defective strain DYM1 was transformed with pFQ-MEV1, and the growth of the transformants on the LB agar plate supplemented

with or without ME was examined.

The plasmid was also introduced into *S. lividans*. Approximately 5 μg of cell free extract of *S. lividans* harboring pFQ-MEV1 were incubated with (*R*)-[2-^{14}C]mevalonic acid lactone for 2 hours at 30°C. The reaction products were spotted on cellulose TLC sheets (Merck, Art.1.05628) and developed in ethanol, ammonia and water (80 : 12.5 : 15, Rf values, mevalonic acid lactone; 0.90, mevalonic acid 5-phosphate; 0.51, IPP; 0.39, mevalonic acid diphosphate; 0.28). The sheets were exposed to an imaging plate (Fujifilm, Tokyo Japan), and radio-labeled products were detected with BAS-1000 (Fujifilm)[9].

Characterization of ORF4 (Polyprenyl Diphosphate Synthase)

To obtain the entire gene without the excess flanking region, PCR amplification was carried out. The 5′ and the 3′ primer with an additional restriction site (underlined) had the respective sequences, 5′-ACATGCATGCAGCCCCACCACCAGCCTTCA-3′ and 5′-ACCAAGCTTTCAGGTGGTCCTGAGCGTGGT-3′, which were designed on the basis of the nucleotide sequences of ORF4. The amplified PCR product was digested with *Sph*I and *Hin*dIII, separated by agarose gel electrophoresis, and then purified with a Gel Extraction Kit (QIAGEN). After sequence confirmation, the fragment was inserted into the same sites of pQE30 (QIAGEN). In the resulting plasmid pFQ-PRENYL1, a recombinant protein was expressed as an *N*-terminal His-Tagged protein. Expression, purification and assay conditions for the recombinant enzyme were the same as those described previously[22].

Characterization of ORF2 (Isoprenoid Cyclase)

To overproduce the ORF2 product as an *N*-terminal His-Tagged protein, the gene was amplified by the following 5′ and 3′ primers, 5′-ACATGCATGCAACGTGACCTCCTTCGCAGC-3′ and 5′-ACCAAGCTTTCACGCTCGTGCCTCCTTCTC-3′. Other procedures were the same as those for construction of pFQ-PRENYL1. The thus constructed plasmid was designated as pFQ-DC1. Assay conditions for the recombinant enzyme and purification procedure of copalyl diphosphate were essentially the same as those for a diterpene cyclase for terpentecin biosynthesis (Cyc1) and of terpentedienol diphosphate, respectively[23].

Characterization of ORF3 (Unknown Protein)

Two primers were used for expression of the *N*-terminal His-Tagged recombinant ORF3. The 5′ and the 3′ primer had the respective sequences, 5′-ACATGCATGCCGTGCCCGTCACCGTGTGGCGCTGA-3′ and 5′-CCCAACTTTCACATGTCGGAGGGCAGGCCGGACG-3′, which were designed on the basis of the nucleotide sequences of the ORF3 gene. Other procedures were the same as those for construction of pFQ-PRENYL1. The thus constructed plasmid, pFQ-DC2, was used for expression of the recombinant ORF3.

Analysis of the Metabolite

NMR spectra were taken on a JEOL A-500 spectrometer operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C. The sample was dissolved in D_2O. External dioxane and internal DSS were used for ^{13}C standard and ^{1}H standard, respectively.

Nucleotide Sequence Accession Number

The DNA sequences determined in this study are deposited in the DDBJ, EMBL, and GenBank™ Data Bank with accession number AB183750.

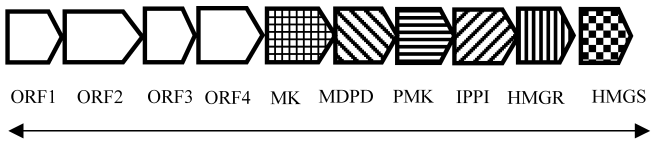
Results

Cloning of a Mevalonate Pathway Gene Cluster from a Furaquinocin A Producer

We had previously cloned an HMG-CoA reductase gene from *Streptomyces* sp. strain KO-3988, which is known to produce furaquinocin A, as a 1.6 kb *Bam*HI fragment by hybridization using an HMG-CoA reductase gene cloned from a terpentecin producer as a probe[13]. Therefore, we decided to clone and analyze regions that flanked this gene. By colony and Southern hybridizations, we isolated several positive cosmids. Among them, some cosmids were found to have the 1.6 kb *Bam*HI fragment that would be the same fragment used for the probe. A 7.2 kb *Bam*HI fragment in other cosmids also hybridized to the probe, suggesting that the strain KO-3988 might have two distinct HMG-CoA reductase genes (see Discussion). In this study, one of the cosmids containing the *Bam*HI fragment used for the probe was selected. Sequence analysis suggested that the mevalonate pathway gene cluster containing mevalonate kinase, mevalonate diphosphate decarboxylase, phosphomevalonate kinase, type 2 IPP isomerase, HMG-CoA reductase, and HMG-CoA synthase genes existed in that order (Fig. 1).

We have previously demonstrated that an *E. coli* mutant DYM1, in which the *dxr* gene encoding 1-deoxy-D-xylulose 5-phosphate reductoisomerase responsible for the MEP pathway and essential for cell growth was disrupted, could be complemented by the mevalonate pathway gene cluster

Fig. 1. Mevalonate pathway gene cluster and its upstream region in *Streptomyces* sp. strain KO-3988.



ORFs	Amino acids	Most homologous proteins and their accession numbers
ORF1	462	Putative P450 of *Streptomyces avermitilis* (43% identities, AP005033-103)
ORF2	511	Diterpene cyclase (Cyc1) of *Kitasatospola griseola* (36%, AB048795-4)
ORF3	295	No significant homologous proteins
ORF4	399	Polyprenyl diphosphate synthase of *Streptomyces coelicolor* (44%, AL939106-16)
MK	343	Mevalonate kinase of *Streptomyces* sp. Strain CL190 (66% identity, AB037666-1)
MDPD	350	Mevalonate diphosphate decarboxylase of *Streptomyces* sp. Strain CL190 (76%, AB037666-2)
PMK	375	Phosphomevalonate kinase of *Streptomyces* sp. Strain CL190 (71%, AB037666-3)
IPPI	363	Type 2 IPP isomerase of *Kitasatospola griseola* (75%, AB037907-5)
HMGR	353	HMG-CoA reductase of *Streptomyces* sp. Strain CL190 (86%, AB037666-5)
HMGS	391	HMG-CoA synthase of *Streptomyces* sp. Strain CL190 (82%, AB037666-6)

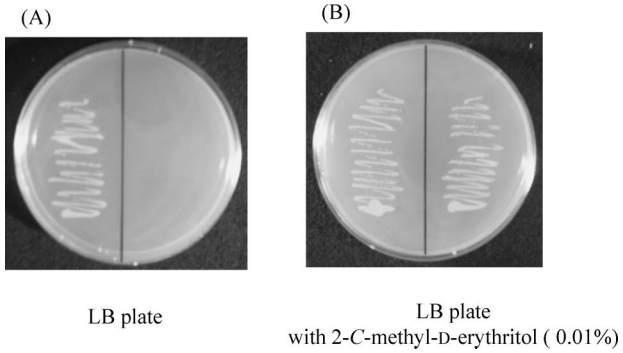
Filled bars represent the mevalonate pathway genes.

cloned from the terpentecin producer[15] and BE-40644 producer[9], because IPP needed for cell growth was supplied by the mevalonate pathway. This strategy was employed here. As shown in Fig. 2, the DYM1 mutant was complemented by pFQ-MEV1, confirming that the DNA fragment cloned in this study contained a set of the mevalonate pathway genes. The plasmid, pFQ-MEV1, was also introduced into *S. lividans*. After incubation of cell free extracts of *S. lividans* harboring pFQ-MEV1 with (*R*)-[2-^{14}C]mevalonic acid lactone, the reaction products were analyzed by TLC. Spots of mevalonic acid 5-phosphate, mevalonic acid diphosphate and IPP were clearly detected (not shown). These two heterologous expression experiments confirmed that the DNA fragment cloned in this study contained a set of the mevalonate pathway genes.

Characterization of Polyprenyl Diphosphate Synthase (ORF4)

In the region upstream of the mevalonate pathway gene cluster, four ORFs that would encode a putative cytochrome P450 (ORF1), an isoprenoid cyclase (ORF2), an unknown protein (ORF3), and a polyprenyl diphosphate synthase

Fig. 2. Phenotypes of the *E. coli* mutant DYM1 (*yae*M$^-$) and its transformant harboring pFQ-MEV1.

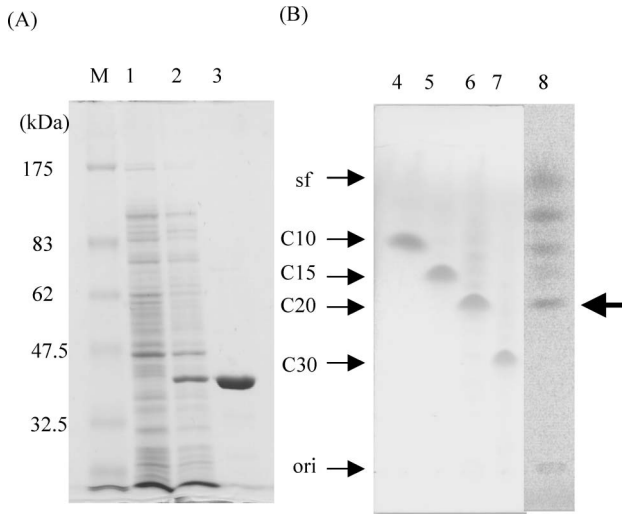


The mutant DYM1 harboring pWHM3 (vector) cannot grow on the LB plate (A; right), but can grow on the LB plate supplemented with ME (0.01%) (B; right). The DYM1 strain transformed with pFQ-MEV1 carrying the gene cluster for the mevalonate pathway enables growth on the LB plate without ME (+IPTG, 0.1 mM) (A; left).

genes (ORF4), were suggested to exist in that order (Fig. 1). In the terpentecin (diterpene) and BE-40644 (sesquiterpene) producers, polyprenyl diphosphate synthase genes, that is, a GGDP (C_{20}) synthase gene and an FDP (C_{15}) synthase gene, which supply substrates for terpentecin and BE-40644 biosyntheses, respectively, were also located in the region just upstream of the mevalonate pathway gene cluster[9,15]. Considering that the strain KO-3988 is known to produce furaquinocin A (C_{10}), the ORF4 product was suggested to encode a geranyl diphosphate (C_{10}) synthase. Therefore, the ORF4 product was expressed as a recombinant enzyme and used for *in vitro* assay to determine the chain length of the product.

A plasmid, pFQ-PRENYL1, was constructed to obtain an *N*-terminal His-tagged recombinant ORF4 product. Expression of the recombinant enzyme in soluble form was confirmed by SDS-PAGE (Fig. 3A). The expressed protein was then purified and used for an enzyme assay. When the assay was carried out using DMAPP and [1-^{14}C]IPP as substrates, followed by de-phosphorylation of the product, to our surprise, geranylgeraniol (C_{20}) was detected as a major product by TLC analysis (Fig. 3B). Although additional spots of farnesol (C_{15}) and geraniol (C_{10}) were also detected, we concluded that ORF4 would be a geranylgeranyl diphosphate synthase because the product chain lengths of polyprenyl diphosphate synthase were reported to be modulated by the reaction conditions, such as the concentrations of the substrates and the divalent cations[24,25]. This result suggested that the ORF1 to 4 would participate in the biosynthesis of a diterpene compound.

Fig. 3. Overproduction of the ORF4 product and TLC autoradiography of the alcohols obtained by hydrolysis of the products formed by the recombinant ORF4 product.



(A) Purified ORF4 product was analyzed on an SDS-PAGE (10%). Proteins were stained with Coomassie brilliant blue R-250. M, molecular mass marker; lane 1, cell free extract of *E. coli* harboring pQE30 (vector); lane 2, cell free extract of *E. coli* harboring pFQ-PRENYL1; lane 3, purified His-tagged ORF4 product. (B) The sample obtained by incubation of [1-^{14}C]IPP and DMAPP with the recombinant enzyme, followed by de-phosphorylation with an acid-phosphatase, was analyzed by TLC (lane 8). Spots of authentic standard alcohols are indicated by arrows: geraniol (lane 4); all-(*E*)-farnesol (lane 5); all-(*E*)-geranylgeraniol (lane 6); all-(*E*)-hexaprenol (lane 7). Ori., origin; sf., solvent front.

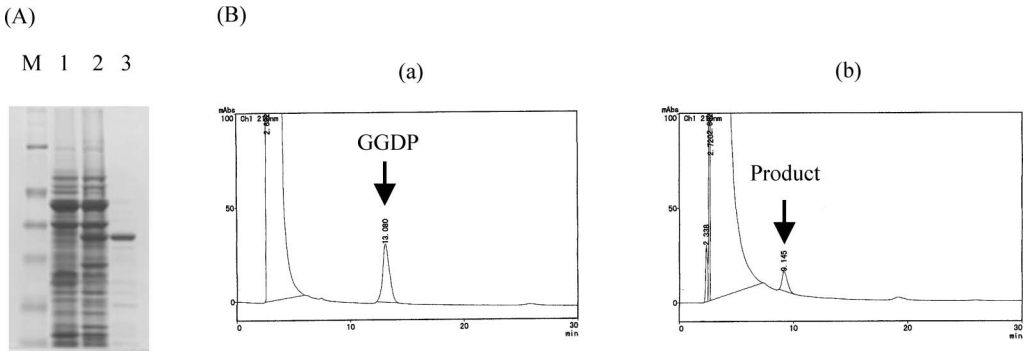
Characterization of the ORF2 Product

The ORF4 product was confirmed to be a GGDP synthase as described above. The ORF2 product homologous to isoprenoid cyclases, therefore, was suggested to encode a diterpene cyclase. Diterpene cyclases are classified into two major types with respect to their modes of cyclization[26]. One type of the reaction is initiated by ionization of GGDP to an allylic carbocation followed by cyclization and deprotonation to an olefin. The other type of the reaction is initiated by protonation at the 14,15-double bond of GGDP. The former class and the latter class of enzymes are known to possess a DDXXD motif and a DXDD motif, respectively, which mediate substrate binding by chelation to a divalent metal ion[26]. Considering that the ORF2 product is similar to isoprenoid cyclases with DXDD motifs, the ORF2 was assumed to convert GGDP into a cyclized intermediate with the diphosphate group by the protonation-initiated cyclization. To examine this possibility, we overproduced the ORF2 product as a His-tagged recombinant protein (Fig. 4A) and the purified enzyme or heat denatured enzyme was incubated with GGDP. By a reversed-phase HPLC analysis using an ion-pair reagent, the formation of a reaction product, which was eluted with a retention time shorter than that of GGDP, was specifically detected in the reaction mixture using the active ORF2 (Fig. 4B). The peak disappeared when the reaction product was incubated with phosphatase, suggesting that the product had a diphosphate group as expected.

Structure Determination of the Product Formed from GGDP by the ORF2 Product

The molecular formula of the reaction product was

Fig. 4. Overproduction of the ORF2 (A) and HPLC analyses of the products generated by the ORF2 (B).



(A) Purified ORF2 product was analyzed on an SDS-PAGE (10%). Proteins were stained with Coomassie brilliant blue R-250. M, molecular mass marker; lane 1, cell free extract of *E. coli* harboring pQE30 (vector); lane 2, cell free extract of *E. coli* harboring pFQ-DC1; lane 3, purified His-tagged ORF2 product. (B) Reaction products formed by denatured ORF2 (a) or active ORF2 (b) in the presence of GGDP were analyzed by reverse-phase HPLC using an ion-pair reagent.

determined to be $C_{20}H_{35}O_7P_2Na$ by high resolution FAB-MS (glycerol matrix, negative mode, *m/z* 471.1678, calcd. for $C_{20}H_{35}O_7P_2Na$ - H, 471.1711).

^{1}H- and ^{13}C-NMR spectral data are summarized in Table 1. The DEPT experiment revealed the presence of 4 methyl, 9 methylene, 3 methine and 4 quaternary carbons in the molecule. One olefinic carbon signal at 121.59 ppm appeared as a doublet due to long-range coupling to phosphorus ($J_{C\text{-}P}$=7.5 Hz). These carbons were connected by the use of HMBC correlations observed between methyl protons or well separated protons and relevant carbons as shown by bold lines in Fig. 5 (a). The remaining connections between C-1 and C-3, C-5 and C-7, and C-9 and C-11 were established by analysis of the COSY spectral data.

The relative stereochemistry of the compound was determined by detailed analysis of NOESY spectral data as shown in Fig. 5 (b). The angular methyl group at C-10 (C-20) proved to be in axial orientation due to strong NOE with axial methyl protons at C-4 (C-19). The proton at C-5 showed strong NOE with equatorial methyl protons at C-4 establishing its axial orientation. Strong NOE observed between the methyl group at C-10 and the methylene protons at C-11 proved the stereochemistry of the side chain at C-9 to be equatorial. These data established the structure of the compound to be *anti*-copalyl diphosphate or its enantiomer, *ent*-copalyl diphosphate, as shown in Fig. 6. The proposed structure was supported by comparison of its ^{13}C-NMR spectral data with those of *anti*-copalol[27]. The reported ^{13}C-NMR chemical shift values of *anti*-copalol

Table 1. ^{1}H- and ^{13}C-NMR data of copalyl diphosphate

	^{13}C (multiplicity)	^{1}H (multiplicity)
1, CH_2	40.41 (t)	1.03 (dt), 1.77 (m)
2, CH_2	20.86 (t)	1.45 (bd), 1.56 (bt)
3, CH_2	43.56 (t)	1.18 (m), 1.36 (m)
4, C	34.70 (s)	
5, CH	56.71 (d)	1.14 (dt)
6, CH_2	26.05 (t)	1.31 (dd), 1.75 (m)
7, CH_2	39.74 (t)	2.00 (dt), 2.39 (bd)
8, C=	152.98 (s)	
9, CH	57.59 (d)	1.66 (dt)
10, C	41.07 (s)	
11, CH_2	23.11 (t)	1.49 (dd), 1.65 (m)
12, CH_2	39.83 (t)	1.89 (m), 2.15 (m)
13, C=	145.50 (s)	
14, CH=	121.59* (d)	5.43 (bt)
15, CH_2	64.31 (t)	4.46 (m)
16, CH_3	17.54 (q)	1.71 (s)
17, CH_2	107.38 (t)	4.61 (bs), 4.87 (bs)
18, CH_3	34.81 (q)	0.85 (s)
19, CH_3	22.96 (q)	0.79 (s)
20, CH_3	15.81 (q)	0.67 (s)

*Observed as a doublet due to $J_{C\text{-}P}$ coupling (J=7.5 Hz).

Fig. 5. (a) Important HMBC correlations, (b) Important NOE correlations.

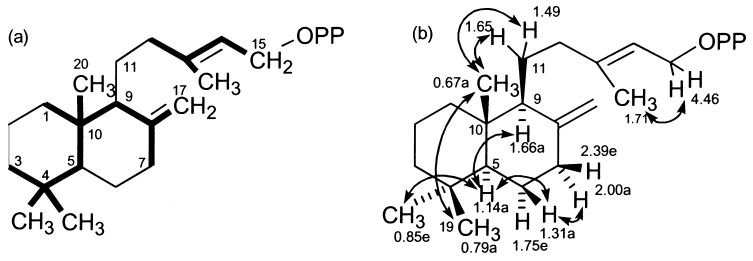
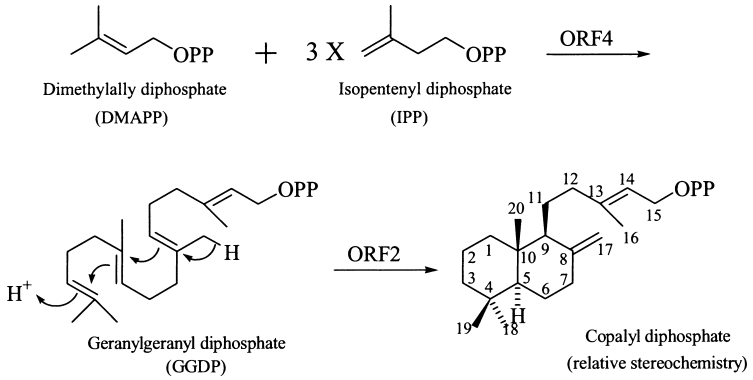


Fig. 6. Biosynthesis of copalyl diphosphate by the ORF2 and 4 products.



were smaller than those of the reaction product by approximately 1.1 to 1.2 ppm presumably due to the use of different solvent ($CDCl_3$ for copalol) and different standard. If these differences were neglected, ^{13}C-NMR chemical shifts of these two compounds were judged to be identical with regard to the two ring system carbons.

An Attempt to Characterize the ORF3 Product

The ORF3 product showed no significant similarity to any other proteins in sequence similarity search programs, BLAST and FASTA. However, considering that antibiotic biosynthetic genes cloned from actinomycetes are usually clustered in the genome, the ORF3 product was suggested to catalyze a conversion of copalyl diphosphate into a diterpene compound. To perform an *in vitro* enzyme assay with copalyl diphosphate as the substrate, we tried to express the ORF3 product as a recombinant protein in *E. coli*. However, the expressed proteins strongly formed inclusion bodies despite our several attempts to increase the soluble enzyme by decreasing the amount of IPTG added for induction and by prolonging the cultivation at low temperature. Therefore, copalyl diphosphate was incubated with washed mycelia or cell free extracts of the strain KO-3988. However, we could detect no products converted from the substrate.

Discussion

We had previously shown that terpentecin[16] and BE-40644[9] biosynthetic gene clusters existed in adjacent the mevalonate pathway gene clusters. In this study, the mevalonate pathway gene cluster was cloned from the strain KO-3988, which was known to produce furaquinocin A[12], by a hybridization experiment using the HMG-CoA reductase gene, which had previously been cloned from the strain KO-3988[13], as a probe. We analyzed flanking regions of the cluster to examine whether furaquinocin A biosynthetic genes might be present. As a result, we found four ORFs, two of which were confirmed to participate in the biosynthesis of copalyl diphosphate by *in vitro* studies,

in a just upstream region of the cluster, though we could not find any genes related to biosynthesis of furaquinocin A in the more upstream region of the ORF1 and downstream region of the mevalonate pathway gene cluster.

As described in the Result section, we could find by colony and Southern hybridizations that the 7.2 kb *Bam*HI fragment was also carrying a gene homologous to the HMG-CoA reductase gene used as the probe. We had previously shown by genomic Southern hybridization that two *Bam*HI fragments, whose sizes were estimated to be 1.6 kb and 7.2 kb, hybridized to the probe carrying the HMG-CoA reductase gene[7]. These results suggested that the strain KO-3988 would have two distinct HMG-CoA reductase genes. Considering that mevalonate pathway genes found in actinomycetes always clustered in the same order[9,11,15] as that revealed in this study (mevalonate kinase to HMG-CoA synthase), the other HMG-CoA reductase gene present in the 7.2 kb *Bam*HI fragment might also be clustered, and furaquinocin A biosynthetic genes might exist in flanking regions of that cluster. To examine this possibility, sequencing of the 7.2 kb *Bam*HI fragment is now in progress and will be reported in the near future.

In this study, copalyl diphosphate was confirmed to be formed from GGDP by the ORF2 product although absolute stereochemistry of the product remains unclear. *anti*-Copalyl diphosphate and *ent*-copalyl diphosphate are intermediates of abietadiene and *ent*-kaur-16-ene, respectively[26]. To estimate whether the ORF2 product might be an *anti*-CPS or *ent*-CPS, we compared amino acid similarities between the ORF2 product and the abietadiene synthase or *ent*-CPSs responsible for *ent*-kaur-16-ene biosynthesis. First, we compared the amino acid sequence of the ORF2 product with that of the *N*-terminal domain of abietadiene synthase because the *N*-terminal domain of abietadiene synthase was reported to catalyze a conversion of GGDP into *anti*-copalyl diphosphate[28]. By means of the amino acid sequence alignment programs FASTA and BLAST, the similarity between the two enzymes was 22% identity over 327 amino acids. On the other hand, the similarities between the ORF2 and *ent*-CPSs were almost the same as that between the ORF2 product and abietadiene synthase, such as *ent*-kaurene synthase from *Gibberella fujikuroi*[29] (27% identity over 481 amino acids) and *ent*-kaurene synthase A (*ent*-CPS) from *Pisum sativum*[30] (22% identity over 439 amino acids). Therefore, comparison of the amino acid sequence of these enzymes did not give any clue as to whether the ORF2 product is an *anti*-CPS or *ent*-CPS.

In contrast, the most homologous protein to the ORF2 product was the Cyc1 (36% identity over 499 amino acids), which was confirmed to catalyze a conversion from GGDP into terpentedienol diphosphate in terpentecin biosynthesis[16, 23]. Therefore, the ORF2 product would be a good partner to examine a relationship between amino acid sequences of an isoprenoid cyclase and its product specificity including stereo-specificity. Moreover, isoprenoid cyclases found in actinomycetes might have unique primary structures and enzymatic properties different from those found in eukaryotes. Therefore, actinomycetes possessing the mevalonate pathway gene clusters might be a treasure house of unique isoprenoid compounds.

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