A Relationship between the Mevalonate Pathway and Isoprenoid Production in Actinomycetes

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Most Streptomyces strains are equipped with only the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway for the formation of isopentenyl diphosphate. In addition to this pathway, some Streptomyces strains have the mevalonate pathway to produce terpenoid antibiotics. We have previously shown that a gene cluster for biosynthesis of terpentecin, a diterpene antibiotic, was located in adjacent the mevalonate pathway gene cluster. In this study, a mevalonate pathway gene cluster was cloned from Actinoplanes sp. strain A40644, an isoprenoid antibiotic BE-40644 producer, to examine whether the mevalonate pathway genes and isoprenoid biosynthetic genes are clustered in genomic DNA. By sequencing flanking regions a probable BE-40644 biosynthetic gene cluster was found in the downstream region of the mevalonate pathway gene cluster. Heterologous expression of a 9-kb fragment confirmed that a set of the BE-40644 biosynthetic genes was involved in the fragment. This result suggested that the presence of the mevalonate pathway might be a good landmark to detect the production of isoprenoid compounds by actinomycetes.

Isoprenoids are the largest single family of compounds found in nature with over 23,000 known examples1). All isoprenoid compounds are derived from the five-carbon precursors, isopentenyl diphosphate (IPP)2,3). In eukaryotes and Archaebacteria, the mevalonate pathway for the formation of IPP is well-established2,3). It has recently been revealed, however, that 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 plants4). Although most Streptomyces strains have only the MEP pathway for the formation of IPP4), we previously and clearly showed that both the mevalonate and MEP pathways were operating in some Streptomyces strains such as Kitasatospora griseola (terpentecin producer)5), Actinoplanes sp. strain A40644 (BE-40644 producer)6), Streptomyces sp. strain CL190 (naphterin producer)7), Streptomyces sp. strain KO-3988 (furaquinocin producer)8) and Chainia rubra (napyradiomycin producer)9).  

Interestingly, these strains were proved to produce isoprenoid compounds mainly through the mevalonate pathway4-9) (Fig. 1A). These facts suggested that the presence of the mevalonate pathway is closely related to the production of isoprenoid compounds in actinomycetes. In contrast, some actinomycetes possessing only the MEP pathway such as Streptomyces sp. strain UC5319, Nocardia brasiliensis and Streptomyces argenteolus produce the isoprenoid compounds, pentalenone10), brasiliocardin A11) and KS-505a12), respectively (Fig. 1B).  
In order to better understand the biological significance of the presence of the mevalonate pathway in actino-
mycetes, we have sought to clone mevalonate pathway gene clusters and isoprenoid biosynthetic gene clusters from actinomycetes. We have recently shown that a terpentecin biosynthetic gene cluster was located in the adjacent region to the mevalonate pathway gene cluster\(^\text{12}\). This fact led us to investigate whether mevalonate pathway genes and isoprenoid biosynthetic genes are always clustered in strains possessing both the mevalonate and MEP pathways. In this study, therefore, mevalonate pathway genes and their flanking regions were cloned from *Actinoplanes* sp. strain A40644, an isoprenoid BE-40644 producer\(^\text{13}\), to examine the relationship between BE-40644 biosynthetic genes and the mevalonate pathway genes.

**Materials and Methods**

**Chemicals**

\[\alpha^{-32}\text{P}]dCTP and (R)-[2-\text{14C}]mevalonic acid lactone (CFA. 660) were obtained from Amersham. Other chemicals were all analytical grade.

**Bacterial Strains**

*Actinoplanes* sp. strain A40644, a BE-40644 producer, was used for the cloning experiment. Media and growth conditions for strain A40644 were as described by Torigoe *et al.*\(^\text{13}\). *Streptomyces lividans* TK23\(^\text{14}\) and pWHM3\(^\text{15}\) were used for the heterologous expression of the mevalonate pathway genes and the BE-40644 biosynthetic genes. *E. coli* JM110 \{rpsL thr leu thi lacY galK ara tonA tsx dam dcm supE44/F' [traD proAB lacP lacZΔM15]\} (Toyobo)

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Fig. 1. Structures of isoprenoids produced by actinomycetes.
and a cosmid pWE15 (Toyobo) were used for preparation of a genomic library. E. coli JM110 and plasmids, pUC118 and pUC119, were used for sequencing analysis. E. coli M15/pREP4 and a plasmid pQE30 (Qiagen) were used for expression of the His-tagged proteins. An E. coli mutant DYM1, in which the dxr gene (yaeM gene) encoding 1-deoxy-D-xylulose 5-phosphate reductoisomerase was disrupted\(^{16}\), was used for experiments to examine whether the DNA fragment cloned in this study would indeed encode the genes for the mevalonate pathway. The strain DYM1 was cultivated in the presence of 2-C-methyl-D-erythritol (0.01%) because disruption of the dxr gene was lethal for E. coli. If necessary, ampicillin (100 μg/ml) and kanamycin (25 μg/ml) were added to the medium.

DNA Isolation and Manipulation

Plasmids from E. coli were prepared using a Qiagen Plasmid Kit (QIAGEN, Inc., CA, U.S.A.). All restriction enzymes, T4 ligase, and calf intestinal alkaline phosphatase were obtained from Toyobo (Osaka, Japan) and used according to the manufacturer's 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). The transformation protocol for S. lividans was essentially the same as described by HOPWOOD et al.\(^{14}\) Other general procedures were performed as described by MANIATIS et al.\(^{17}\)

Sequence Analysis

A cosmid clone, pBE033, was screened by colony hybridization using as a probe that had been cloned from Streptomyces sp. strain CL190 and confirmed to carry the 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase gene. Several restriction enzymes-digested fragments, which hybridized to the probe, were subcloned in pUC118 or pUC119. After construction of a series of plasmids, sequencing was carried out with an automatic DNA sequencer (Li-Cor, model 4000L).

Expression of the Mevalonate Pathway Genes in S. lividans

The entire mevalonate pathway gene cluster containing a putative polyprenyl diphosphate synthase (farnesyl diphosphate synthase), 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'-GCCTGCGGCCGCGGTCGTCGAC-3' and 5'-TCGCGATCCACCCAGCAGCGG-3'. To facilitate the cloning, an additional restriction site (underlined) was incorporated into both primers. After sequence confirmation, the BamHI-HindIII fragment was inserted into the same sites of pWHM3 to give pBE-MEV1. Approximately 5 μg of cell free extract of S. lividans harboring pBE-MEV1 were incubated with (R)-[2-14C]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), and radio-labeled products were detected with BAS-1000 (Fujiﬁlm).

Expression of the BE-40644 Biosynthetic Genes in S. lividans

A DNA fragment carrying a putative BE-40644 biosynthetic gene cluster was ampliﬁed by PCR with BE-N (5'-ACCAAGCTTAGGTTACCCGGCGGACCCCGG-3') and BE-C (5'-TGCTCTAGATCACACCGTACGCCAGGACCG-3') primers. Other procedures were the same as those for construction of pBE-MEV1. The plasmid thus constructed was designated pWHM-BE1. S. lividans harboring pWHM-BE1 was grown in 300-ml Erlenmeyer flasks containing SK-no. 2 medium\(^{12}\) (30 ml) and thiostrepton (10 μg/ml). Fermentation was carried out for 4 days at 30°C with agitation (200 rpm). Metabolites from 5 liters of culture broth were puriﬁed as described by TORIGOE et al.\(^{13}\) The purity of the metabolite was examined by reverse-phase HPLC. Analytical conditions were as follows: C\(_18\) reverse-phase column (Merk Mightisil RP-18 column (250×20 mm)); column temperature of 30°C; detection at 254 nm; mobile phase of 55% acetonitrile for 0 to 20 minutes, a linear gradient from 55% to 100% acetonitrile for 20 to 50 minutes, and 100% acetonitrile for additional 50 minutes; flow rate of 1 ml/minute.

Characterization of 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'-GCCTGCGGCACCTGGATCCACTCGG-3' and 5'-CGCCGATCCACCCAGCAGCGG-3', which were designed on the basis of the nucleotide sequences of the putative polyprenyl diphosphate synthase gene. The ampliﬁed PCR product was digested with BamHI, separated by agarose gel
electrophoresis, and then purified with a Gel Extraction Kit (Qiagen). After sequence confirmation, the BamHI fragment was inserted into the same sites of pQE30. In the resulting plasmid pBE-PRENYL1, a recombinant protein was expressed as an N-terminal His-Tagged protein. Assay conditions for the recombinant enzyme were the same as those described previously\(^\text{18}\).

**Analysis of the Metabolite**

MS spectrum; 360 (M+2H): \(^{13}\)C-NMR spectra; \(\delta\) 13.82, 16.72, 18.07, 18.31, 21.88, 27.20, 33.16, 33.65, 38.44, 39.63, 39.91, 41.62, 48.41, 55.06, 59.68, 79.77, 120.00, 131.69, 144.08, 153.62, 182.09, 186.67: HSQC, HMBC and DQF-COSY experiments showed that the compound produced by S. lividans harboring pWHM-BE1 was BE-40644.

**Nucleotide Sequence Accession Number**

The DNA sequences determined in this study are deposited in the DDBJ, EMBL, and GenBank\textsuperscript{TM} Data Bank with accession number AB113568.

**Results**

**Cloning of a Mevalonate Pathway Gene Cluster from a BE-40644 Producer**

We have recently shown by Southern blot hybridization that Actinoplanes sp. strain A40644, a BE-40644 producer, has a DNA region homologous to a HMG-CoA reductase gene cloned from Streptomyces sp. strain CL190\textsuperscript{19}. Therefore, a cosmid clone hybridized to the probe under the same condition used for Southern blot hybridization was selected and nucleotide sequences of a 6.3-kb SacI fragment that again hybridized to the probe were determined. Computer analysis of the DNA sequence by Frame Analysis showed 6 ORFs in the same direction. To understand the functions of each of the ORFs, we searched the databases with their translated products by BLAST and FASTA. The results are summarized in Fig. 2. In brief, each of the ORFs had a significant similarity to polyprenyl diphosphate synthase (ORF1), mevalonate kinase (ORF2), mevalonate diphosphate decarboxylase (ORF3), phosphomevalonate kinase (ORF4), type 2 IPP isomerase (ORF5), and HMG-CoA reductase (ORF6), respectively. The order of ORF2 to ORF6 was the same as those of Streptomyces sp. CL190\textsuperscript{20} and Kitasatospora griseola\textsuperscript{21}.

**Characterization of Polyprenyl Diphosphate Synthase (ORF1)**

In the terpentecin producer, a polyprenyl diphosphate synthase gene, which was confirmed to encode a geranylgeranyl diphosphate (C\(_{20}\)) synthase and was responsible for terpentecin biosynthesis, was also located in a region just upstream of the mevalonate pathway gene cluster\textsuperscript{20}. Therefore, if the polyprenyl diphosphate synthase gene (ORF1) cloned from BE-40644 producer would encode a farnesyl diphosphate synthase (C\(_{15}\)), we could expect the presence of a BE-40644 biosynthetic gene cluster in adjacent regions of the ORF1, because BE-40644 contained a C\(_{15}\) isoprene unit and antibiotic biosynthetic genes cloned from actinomycetes are usually clustered in the genomic DNA region. We therefore determined a chain length of prenyl diphosphate formed by the ORF1 product.

A plasmid, pBE-PRENYL1, was constructed to obtain an N-terminal His-tagged recombinant ORF1 product. Expression of the recombinant enzyme in soluble form was confirmed by SDS-PAGE (Fig. 3). The expressed protein was then purified and used for the enzyme assay. When the assay was carried out using DMAPP and [\(^{1-14}\)C]IPP as substrates, farnesyl diphosphate was detected as a major product by TLC analysis (Fig. 3).

**Analysis of Flanking Regions of the Mevalonate Pathway Gene Cluster**

Since ORF1 was confirmed to be a farnesyl diphosphate synthase, we next determined the nucleotide sequences of both the upstream and downstream regions of the mevalonate pathway gene cluster. Firstly, we analyzed a 9-kb \(Bgl\text{II}\) fragment containing the farnesyl diphosphate synthase gene and its upstream region. Although we could identify several ORFs by Frame Analysis, no genes participating in the biosynthesis of BE-40644 were evident in this region. Nucleotide sequences of a fragment carrying the HMG-CoA reductase gene and its downstream region were then determined. As shown in Fig. 2, an HMG-CoA synthase gene (ORF7) was suggested to be present in a region just downstream of the HMG-CoA reductase gene, in a manner similar to those in terpentecin and naphterpin producers\textsuperscript{20,21}. Interestingly, the nearby ORF (ORF9) has a significant similarity to 2-epi-5-epi-valiolone synthase that catalyzes a conversion of sedo-heptulose 7-phosphate into 2-epi-5-epi-valiolone and participates in biosyntheses of acarbose and validamycin A\textsuperscript{22}. Since the quinone moiety of BE-40644 was previously demonstrated to be derived from sedo-heptulose 7-phosphate by a tracer experiment\textsuperscript{8}, we
Fig. 2. Mevalonate pathway gene cluster and its flanking region in BE-40644 producer.

<table>
<thead>
<tr>
<th>ORFs</th>
<th>Amino acids</th>
<th>Homologous proteins and their accession numbers</th>
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<tr>
<td>ORF1</td>
<td>Polypropenyl diphosphate synthase of Mycobacterium tuberculosis (47% identity)</td>
<td>DAD; AE007069-11</td>
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<tr>
<td>ORF2</td>
<td>Mevalonate kinase of Streptomyces sp. CL.190 (52%), DAD; AB037666-1</td>
<td></td>
</tr>
<tr>
<td>ORF3</td>
<td>Mevalonate diphosphate decarboxylase of Streptomyces sp. CL.190 (71%), DAD; AB037666-2</td>
<td></td>
</tr>
<tr>
<td>ORF4</td>
<td>Phosphomevalonate kinase of Streptomyces sp. CL.190 (63%), DAD; AB037666-3</td>
<td></td>
</tr>
<tr>
<td>ORF5</td>
<td>Type 2 IPP isomerase of Kitasatospora griseola (71%), DAD; AB037907-5</td>
<td></td>
</tr>
<tr>
<td>ORF6</td>
<td>HMG-CoA reductase Streptomyces sp. CL.190 (80%), DAD; AB013457-1</td>
<td></td>
</tr>
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<td>ORF7</td>
<td>HMG-CoA synthase of Streptomyces sp. CL.190 (73%), DAD; AB037666-6</td>
<td></td>
</tr>
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<td>ORF8</td>
<td>3-oxoacyl-(acyl-carrier-protein) synthase of Thermoaerobacter tengcongensis, (44%), DAD; AE013105-8</td>
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<td>ORF9</td>
<td>2-epi-5-epi-valirolone synthase of Actinoplanes sp. (52%), DAD; Y18523-6</td>
<td></td>
</tr>
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<td>ORF10</td>
<td>Alcohol dehydrogenase of Pseudomonas aeruginosa (36%), DAD; AE004826-2</td>
<td></td>
</tr>
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<td>ORF11</td>
<td>Hypothetical protein of Sinorhizobium meliloti (38%), DAD; AE007260-2</td>
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<td>ORF12</td>
<td>Oxidoreductase of Salmonella enterica (47%), DAD; AL627273-164</td>
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<td>ORF13</td>
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<td>ORF14</td>
<td>1,4-dihydroxy-2-naphthoate octaprenyltransferase of Cenarchaeum symbiosum (30%), DAD; AF083071-15</td>
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<td>ORF15</td>
<td>Drug efflux protein of Bradyrhizobium japonica (32%), DAD; AP005950-245</td>
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<td>ORF16</td>
<td>Hypothetical membrane protein of Streptomyces coelicolor (38%), DAD; AL939120-22</td>
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<td>ORF17</td>
<td>Transcriptional regulator of Streptomyces noursei (29%), DAD; AF263912-17</td>
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<td>ORF18</td>
<td>Hexosyl transferase of Mycobacterium tuberculosis (46% identity), DAD; Z92669-19</td>
<td></td>
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</table>

Thick bars represent the DNA fragment used for the heterologous expression experiments. The bar with arrowheads and the bar with filled circles contain the mevalonate pathway gene cluster and the BE-40644 biosynthetic gene cluster, respectively. Question marks mean small putative ORFs that show no similarities to any proteins. DAD is an abbreviation for the DNA data bank of Japan.
thought that ORF9 might be involved in BE-40644 biosynthesis and that the other BE-40644 biosynthetic genes might be present in the downstream region of the ORF9. To examine this possibility, an additional downstream region was analyzed. Consequently, at least 9 ORFs were identified in this region as shown in Fig. 2. Among them, ORF10, ORF12, ORF14 and ORF15 were found to have a significant similarity to dehydrogenases, oxidoreductases, 1,4-dihydroxy-2-naphthoate octaprenyltransferase, and multi-drug efflux protein, respectively (Fig. 2), and were suggested to participate in the biosynthesis of BE-40644 based on the proposed biosynthetic pathway for BE-40644 (Fig. 4).

**Expression of the Putative BE-40644 Biosynthetic Genes in S. lividans**

To examine if the ORFs found in the downstream region of the mevalonate pathway gene cluster would encode the BE-40644 biosynthetic genes, these genes were expressed in a heterologous host, Streptomyces lividans, and production of BE-40644-related compounds was examined. A DNA fragment containing the region from ORF8 to ORF15 (Fig. 2) was amplified by PCR and inserted into pWHM3 to give pWHM-BE1. S. lividans harboring pWHM-BE1 was cultivated and the production of new compounds was investigated by HPLC analysis. A compound which was eluted with almost the same retention time as that of BE-40644 was specifically detected in culture broth of the transformant harboring pWHM-BE1 (Fig. 5). The product was purified and its structure was determined to be BE-40644 itself by mass spectrum and NMR spectral analyses, suggesting that the genes specific to the biosynthesis of BE-40644 were contained in the DNA fragment used (the ORF8 to ORF15) except for a farnesyl diphosphate synthase gene (ORF1).

**Expression of the Mevalonate Pathway Gene Cluster in Heterologous Hosts**

To examine whether the mevalonate pathway gene cluster cloned in this study would indeed encode the predicted enzymes, these genes were expressed in S. lividans and E. coli, which have only the MEP pathway for the formation of IPP. A shuttle plasmid pBE-MEV1 neighboring the region from the farnesyl diphosphate synthase gene to the HMG-CoA synthase gene (the ORF1 to ORF7) was constructed and introduced into S. lividans. After cell free extracts of S. lividans harboring pBE-MEV1 were incubated with (R)-[2-14C]mevalonic acid lactone, the products were analyzed by TLC. As shown in Fig. 6, spots of mevalonic acid 5-phosphate, mevalonic acid diphosphate...
and IPP were clearly detected. These results showed that at least mevalonate kinase, phosphomevalonate kinase and mevalonate diphosphate decarboxylase genes were included in the DNA fragment employed.

We 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 cloned from the terpentecin producer, because IPP needed for cell growth was supplied by the mevalonate pathway. This strategy was again employed to examine whether the mevalonate pathway gene cluster was contained in the DNA fragment inserted into pBE-MEV1. The DYM1 mutants were again complemented by pBE-MEV1 (not shown). These two heterologous expression experiments confirmed that the DNA fragment cloned in this study contained a set of the mevalonate pathway genes.

**Discussion**

A mevalonate pathway gene cluster has been cloned from a BE-40644 producer using the HMG-CoA reductase gene cloned from *Streptomyces* sp. strain CL190 as a hybridization probe. To date, we have cloned three mevalonate pathway gene clusters from actinomycetes, *Streptomyces* sp. strain CL190 (naphterpin producer)\(^{20}\), *Kitasatospora griseola* (terpentecin producer)\(^{21}\) and *Actinoplanes* sp. strain A40644 (this study). Interestingly, all of these clusters contain mevalonate kinase, mevalonate diphosphate decarboxylase, phosphomevalonate kinase, type 2 IPP isomerase, HMG-CoA reductase, and HMG-CoA synthase (Fig. 7). The order of each of the ORFs is also the same and the respective homologous ORFs have 50 to 80% amino acid identity with each other. These facts strongly suggest that the mevalonate pathway gene clusters have common ancestral genes and have evolved horizontally into the isoprenoid-producing actinomycetes.
In contrast to the high conservation of the mevalonate pathway gene clusters, a diversity of genes is distributed in their flanking regions. In the terpentecin producer, the geranylgeranyl diphosphate synthase gene is found in the region just upstream of the mevalonate kinase gene\textsuperscript{21}, with the terpentecin biosynthetic gene cluster locates at further upstream\textsuperscript{12} (Fig. 7). On the other hand, the BE-40644 biosynthetic gene cluster is located in the region downstream of the mevalonate pathway gene cluster, although the farnesyl diphosphate synthase gene is located immediately upstream region of the mevalonate kinase gene (Fig. 7). These facts suggest that the mevalonate pathway genes and isoprenoid biosynthetic genes are usually clustered in isoprenoid-producing actinomycetes and that the presence of the mevalonate pathway might be a good indicator to detect the production of isoprenoid compounds by actinomycetes. To examine this hypothesis, we are now analyzing flanking regions of the mevalonate pathway gene cluster of the naphterpin producer.

We have found 8 ORFs (ORF8 to ORF15), which were suggested to encode the BE-40644 biosynthetic gene cluster by a heterologous expression experiment, in the downstream region of the mevalonate pathway gene cluster. Although we do not know yet the function of each ORFs and can not exclude possibility that some genes of the heterologous host strain function as replacement for the native BE-40644 biosynthetic genes, we could predict the functions of some genes by comparing their deduced amino acid sequences with their sequence homologues. First, the ORF9 product has a significant similarity to the 2-epi-5-epi-valiolone synthase (50\% identity) as described above. On the other hand, ORF9 has only a low (30\%) similarity to 3-dehydroquinolinate synthases that convert 3-deoxy-D-
arabino-heptulose 7-phosphate into 3-dehydroquininate in the shikimate pathway, suggesting that ORF9 would be a BE-40644 biosynthetic enzyme rather than a shikimate pathway enzyme. Second, the quinone moiety of BE-40644 can be formed by successive dehydrations of 2-epi-5-epi-valiolone (Fig. 4). Therefore, ORF10 and ORF12, both of which have a similarity to dehydrogenases and oxidoreductases, might participate in these reactions. Third, ORF14 has 30% identity with MenA, 1,4-dihydroxy-2-naphthoate octaprenyltransferases which participates in the biosynthesis of menaquinones and catalyzes a transfer of polyisoprenyl diphosphate to 1,4-dihydroxy-2-naphthoate23). Considering that BE-40644 has a structure composed of the quinone moiety and the C15 isoprene unit, ORF14 is strongly suggested to catalyze a transfer reaction of farnesyl diphosphate to the quinone moiety. Fourth, ORF15 is suggested to encode an enzyme that mediates BE-40644 efflux because ORF15 has a significant similarity to many membrane proteins known as self-defense efflux enzymes in antibiotic-producing microorganisms. As for ORF8, ORF11 and ORF13, we cannot assign their functions because all of these ORFs have a similarity only to hypothetical proteins. However, considering that antibiotic biosynthetic genes cloned from actinomycetes are usually clustered in the genomic DNA region, these ORFs might also be responsible for steps in the biosynthesis of BE-40644 such as cyclization of the isoprenoid moiety as shown in Fig. 4. To elucidate the individual reactions catalyzed by these ORFs, in vitro studies using recombinant enzymes will be required. These studies are now in progress and will be reported in the near future.

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