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Cloning and Characterization of 1-Deoxy-D-Xylulose 5-Phosphate Synthase from *Streptomyces* sp. Strain CL190, Which Uses both the Mevalonate and Nonmevalonate Pathways for Isopentenyl Diphosphate Biosynthesis

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In addition to the ubiquitous mevalonate pathway, *Streptomyces* sp. strain CL190 utilizes the nonmevalonate pathway for isopentenyl diphosphate biosynthesis. The initial step of this nonmevalonate pathway is the formation of 1-deoxy-D-xylulose 5-phosphate (DXP) by condensation of pyruvate and glyceraldehyde 3-phosphate catalyzed by DXP synthase. The corresponding gene, *dxs*, was cloned from CL190 by using PCR with two oligonucleotide primers synthesized on the basis of two highly conserved regions among *dxs* homologs from six genera. The *dxs* gene of CL190 encodes 631 amino acid residues with a predicted molecular mass of 68 kDa. The recombinant enzyme overexpressed in *Escherichia coli* was purified as a soluble protein and characterized. The molecular mass of the enzyme was estimated to be 70 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and 130 kDa by gel filtration chromatography, suggesting that the enzyme is most likely to be a dimer. The enzyme showed a pH optimum of 9.0, with a V_{max} of 370 U per mg of protein and K_ms of 65 μM for pyruvate and 120 μM for D-glyceraldehyde 3-phosphate. The purified enzyme catalyzed the formation of 1-deoxyxylulose by condensation of pyruvate and glyceraldehyde as well, with a K_m value of 35 mM for D-glyceraldehyde. To compare the enzymatic properties of CL190 and *E. coli* DXP synthases, the latter enzyme was also overexpressed and purified. Although these two enzymes had different origins, they showed the same enzymatic properties.

Isoprenoids found in all organisms play important roles, such as steroid hormones in mammals, carotenoids in plants, and ubiquinone or menaquinone in bacteria (18). All isoprenoids are synthesized by consecutive condensations of the five-carbon monomer isopentenyl diphosphate (IPP). It was generally believed that IPP is only synthesized by condensation of three molecules of acetyl coenzyme A through the mevalonate pathway (Fig. 1A). However, it has recently been revealed that not all living organisms possess this ubiquitous pathway and that IPP is synthesized through a mevalonate-independent pathway (nonmevalonate pathway) in many bacteria, green algae, and the chloroplasts of higher plants (Fig. 1B) (4, 13, 14, 17, 21, 22). The initial step of this nonmevalonate pathway is the formation of 1-deoxy-D-xylulose 5-phosphate (DXP) by condensation of pyruvate and glyceraldehyde 3-phosphate catalyzed by DXP synthase. The *dxs* gene homologs encoding DXP synthase have been cloned from *Escherichia coli* (12, 25), peppermint (*Mentha* × *piperita*) (10), and pepper (*Capsicum annuum* L.) (1). No detailed studies on the enzymatic properties of the DXP synthases from these organisms, however, have been reported. To our knowledge, only K_m values for pyruvate and glyceraldehyde 3-phosphate of the recombinant DXP synthase from pepper were characterized (1).

Harker et al. and our group have independently found that ubiquinone production in *E. coli* was enhanced by overexpression of DXP synthase or DXP reductoisomerase, the enzyme catalyzing the second step of the nonmevalonate pathway (7; M. Harker and P. M. Bramley, Abstr. 4th Eur. Symp. Plant Isoprenoids, p. 22, 1999; H. Motoyama, K. Miyake, S. Hashimoto, A. Ozaki, H. Seto, T. Kuzuyama, and S. Takahashi, unpublished data). We have also found that overexpression of DXP synthase was more effective in this enhancement than that of DXP reductoisomerase. Although these data suggested that DXP synthase functioned as the rate-limiting enzyme in the nonmevalonate pathway, theoretical predictions have never been made on the basis of kinetic parameters such as the catalytic efficiency (k_{cat}/K_m) for enzymes involved in the pathway. In order to gain insight into the rate-limiting enzyme for the nonmevalonate pathway, it is important to determine the kinetic parameters of the enzymes responsible for this pathway. Therefore, we investigated the enzymatic properties of DXP synthase from *E. coli*, from which DXP reductoisomerase has also been cloned and characterized in our laboratory (26).

Unlike plants and fungi, *Streptomyces* spp., which are eubacteria, produce very few isoprenoids as secondary metabolites. Based on the results obtained by feeding experiments using ^{13}C-labeled precursors indicating that some isoprenoids of *Streptomyces* origin, such as terpentecin (8), naphterpin (23), and napyradiomycin (24), were synthesized by the mevalonate pathway, all *Streptomyces* species were assumed without doubt to employ the same pathway for isoprenoid biosynthesis. We have recently demonstrated, however, that *Streptomyces* sp. strain CL190 possesses both the mevalonate and nonmevalonate pathways (21). Interestingly, the organism utilized the nonmevalonate pathway at the early growth stage but replaced it with the mevalonate pathway at the later stage of fermentation. The presence of these two pathways for isoprenoid biosynthesis in this organism raises a question about their roles in primary and secondary metabolite biosynthesis (16, 21).

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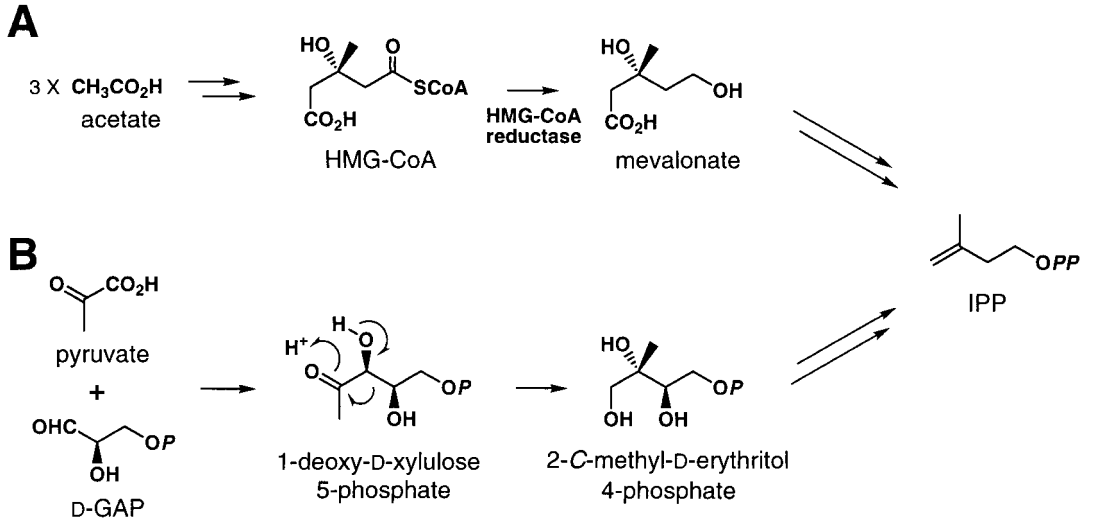

FIG. 1. IPP biosynthesis via the mevalonate pathway (A) and via the nonmevalonate pathway (B). HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; D-GAP, D-glyceraldehyde 3-phosphate. The pathway leading to IPP from 2-*C*-methyl-D-erythritol 4-phosphate is undefined.

As the first approach to answer the question by detailed analyses of the enzymes and genes involved in the two metabolic pathways, we have purified and cloned 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate-limiting enzyme in the mevalonate pathway, from *Streptomyces* sp. strain CL190 (27). Our attention was then directed to the cloning of genes responsible for the nonmevalonate pathway from CL190, an organism that utilizes both the mevalonate and nonmevalonate pathways for IPP biosynthesis.

In this paper, we report the cloning of the DXP synthase gene, *dxs*, from *Streptomyces* sp. strain CL190. The gene was overexpressed in *E. coli*, and its recombinant DXP synthase was purified to homogeneity and characterized in detail. In addition, the *E. coli* DXP synthase was overexpressed and purified, and its enzymatic properties were compared with those of CL190 DXP synthase.

MATERIALS AND METHODS

PCR amplification of a *dxs* gene probe and cloning of the *dxs* gene from the CL190 genome. Several homologous regions of DXP synthase homologs were found in *E. coli* (accession no., AF035440), *Haemophilus influenzae* (accession no., P45205), *Bacillus subtilis* (accession no., P54523), *Rhodobacter capsulatus* (accession no., P26242), *Synechocystis* sp. strain PCC6803 (accession no., S75175), and *Arabidopsis thaliana* (accession no., Q38854). Two amino acid sequences, Trp Asp Val Gly His Asn and Ile Ala Glu Asn His Ala, were highly conserved among them, and thus the corresponding forward oligonucleotide primer, pCDXS1 (5′-TGGGACGTSGGSCACCAG), and the reverse primer, pCDXS2 (5′-ACSGCGTGCTGCTCSGCG), were synthesized (Amersham Pharmacia Biotech). The letter S in these primers stands for G or C. PCR was carried out in 20 μl (total volume) of PCR buffer (Boehringer) containing 50 ng of total DNA from *Streptomyces* sp. strain CL190, a 0.2 mM concentration of each deoxynucleoside triphosphate, 2.5 pmol of each primer, and 1.8 U of *Taq* polymerase (Boehringer) for 25 cycles (0.5 min at 95°C, 0.5 min at 50°C, and 1 min at 72°C). In this PCR, a DNA fragment of 0.9 kb was amplified and then used as the DNA probe for the colony hybridization method. By using this 0.9-kb DNA fragment, a 2.9-kb *Sph*I-*Sph*I fragment was obtained from the CL190 genome. The sequence of this 2.9-kb DNA fragment was determined as described below.

Construction of the plasmid for overexpression in *E. coli* of the *dxs* gene. On the basis of the total nucleotide sequence of the *dxs* gene from *Streptomyces* sp. strain CL190, two oligonucleotide primers, 5′-GGGAAGCTTACGATTCTGG AGAACATCCGG-3′ (5′ of the *dxs* gene) and 5′-CCCAAGCTTTGCGGGCT GCTCCTCGGCCGG′ (3′ of the *dxs* gene), including a *Hin*dIII restriction site (underlined) were synthesized (Amersham Pharmacia Biotech) and used together with total DNA from CL190 to amplify the *dxs* gene. PCR was carried out in 20 μl (total volume) of PCR buffer (Boehringer) containing 50 ng of total DNA from CL190, a 0.2 mM concentration of each deoxynucleoside triphosphate, 2.5 pmol of each primer, and 1.8 U of *Taq* polymerase (Boehringer) for 25 cycles (0.5 min at 95°C, 0.5 min at 60°C, and 1 min at 72°C). In this PCR, a single DNA fragment of 1.9 kb was amplified. The PCR fragment was cleaved with *Hin*dIII and cloned into the *Hin*dIII site in pUC118 (Takara Shuzo). *E. coli* JM109 (Takara Shuzo) was used as the recipient in this transformation. DNA sequencing as described above was used to analyze clones for correct insert DNA, and then the correct DNA fragment was cloned into the *Hin*dIII site in the multicloning site of the expression vector pQE30 (Qiagen) to give plasmid pQCDXS. pQCDXS was designed to encode a recombinant enzyme with an affinity tag consisting of six consecutive histidine residues at the N-terminal region. Ni-nitrilotriacetic acid agarose resin has a strong affinity for a protein that has such histidine residues.

Expression and purification of the recombinant DXP synthase. *E. coli* M15 containing pREP4 (*neo lacI*) (Qiagen) was used as the host for expression of the *Streptomyces* sp. strain CL190 *dxs* gene. M15(pREP4, pQCDXS) was cultured at 18°C in 100 ml of Luria-Bertani medium (19) containing 25 μg of kanamycin (Nacalai, Kyoto, Japan)/ml and 200 μg of ampicillin (Sigma)/ml for 12 h with the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside upon reaching an optical density at 660 nm of 0.8. Cells were harvested by centrifugation and resuspended in buffer A composed of 100 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, 1 mM DL-dithiothreitol, and 0.1 mM thiamine diphosphate. After brief sonication, the lysate was centrifuged at 10,000 × *g* for 20 min and the supernatant was collected. The crude extract was applied to a Ni-nitrilotriacetic acid agarose column (1.3 by 20 mm) (Qiagen) previously equilibrated with buffer A. The resin was washed with 50 mM imidazole in buffer A, and then the protein that bound to the resin was eluted with 200 mM imidazole in buffer A. The active fractions were combined and used as the purified DXP synthase in the subsequent experiments.

Determination of the molecular mass. The molecular mass of the recombinant DXP synthase was estimated by gel filtration on a Superdex 200 (1.6- by 60-cm) column (Amersham Pharmacia Biotech) which was equilibrated with 20 mM sodium phosphate buffer (pH 7) containing 0.15 M NaCl. The column was eluted at a flow rate of 0.5 ml/min, and fractions of 2 ml were collected. The molecular mass was estimated by comparing the elution of DXP synthase with that of standard proteins ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), and bovine serum albumin (66 kDa).

Assay for DXP synthase. The standard assay system consisted of 100 mM Tris-HCl (pH 8.0) containing 1 mM $MgCl_2$, 2 mM DL-dithiothreitol, 1 mM sodium pyruvate, 2 mM DL-glyceraldehyde 3-phosphate, and 150 μM thiamine diphosphate in a final volume of 0.5 ml. The reaction was initiated by adding the enzyme solution to the complete assay mixture at 37°C, and after a 10 min-incubation the reaction was halted by incubation at 100°C for 1 min. Next, the reaction mixture was treated with alkaline phosphatase (Sigma) at 56°C for 60 min to dephosphorylate completely the reaction product, DXP. Production of the resulting dephosphorylated compound, 1-deoxyxylulose (DX), was monitored by a refractive index spectrometer (model RI-71; Showa Denko, Tokyo, Japan) with a Shodex KS-801 (8- by 300-mm) column (Showa Denko), eluted with H_2O at a flow rate of 1 ml/min at 80°C. DX was eluted at 8.6 min under this condition. The amount of DX production was precisely estimated by using chemically synthesized DX as the standard. One unit of DXP synthase activity was defined as the amount of the enzyme that caused the production of 1 μmol of DXP per min at 37°C. All the assays for the calculation of K_m and V_{max} values

of both *Streptomyces* and *E. coli* enzymes were done at 37°C. These values were calculated with Lines&Kinetics software, version 1.0 (3).

Detection of DXP. Production of DXP by the DXP synthase was monitored at 195 nm by high-performance liquid chromatography with a Senshu Pak NH2-1251-N (4.6- by 250-mm) column (Senshu Science, Tokyo, Japan) eluted with 100 mM KH_2PO_4 (pH 3.5) at a flow rate of 1 ml/min (9). DXP was eluted at 8.1 min under this condition.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and native PAGE were performed in slab gels having an 8 to 25% polyacrylamide gradient with the PhastSystem (Amersham Pharmacia Biotech). Protein was visualized by Coomassie brilliant blue R-250 staining. The protein concentration was measured by the method of Bradford (2) with a protein assay kit (Bio-Rad Laboratories), using bovine serum albumin as the standard.

DNA sequence analysis. The DNA sequence was determined by the dideoxy chain termination method (20) with an automated sequencer (model 4000L; Li-cor) and the protocol of the supplier. The FASTA program (11, 15) performed a homology search of the protein databases. Amino acid sequences aligned by the GENETYX program (Software Development, Tokyo, Japan) were then edited visually to align consensus motifs.

Nucleotide sequence accession number. The nucleotide sequence of the 2,941-bp *Sph*I-*Sph*I fragment including the *dxs* gene of *Streptomyces* sp. strain CL190 has been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases with accession no. AB026631.

RESULTS

Cloning and DNA sequencing the *dxs* gene from *Streptomyces* sp. strain CL190. DXP synthase genes have been cloned from *E. coli*, peppermint, and *A. thaliana*, and at least nine amino acid sequences of DXP synthase homologs were available from the database of DNA Data Bank of Japan (DDBJ). The amino acid sequences of these DXP synthase homologs had significant similarity to one another over the entire sequences. In particular, two amino acid sequences, Trp Asp Val Gly His Asn and Ile Ala Glu Asn His Ala, were highly conserved among these DXP synthase homologs from *E. coli*, *B. subtilis*, *R. capsulatus*, *Synechocystis* sp. strain PCC6803, and *A. thaliana*. Thus, we attempted to clone the *dxs* gene from *Streptomyces* sp. strain CL190 by colony hybridization with a DNA probe which was generated by PCR with oligonucleotide primers prepared based on the highly conserved amino acid sequences just mentioned. By using these oligonucleotide primers together with CL190 total DNA, a 906-bp fragment was amplified. With this 906-bp DNA fragment as the *dxs* gene probe, a 2.9-kb *Sph*I-*Sph*I fragment was then obtained and sequenced. Sequence analysis of this 2.9-kb fragment identified one complete open reading frame (ORF) (Fig. 2). The ORF consisted of 1,896 bp starting with initiation codon GTG at position 926 and ending with termination codon TGA at position 2819 (Fig. 2). A putative Shine-Dalgarno sequence, GAAGG, was found 15 bp upstream of the initiation codon. The deduced amino acid sequence corresponding to the ORF showed significant sequence similarity to DXP synthase homologs from *E. coli* (accession no., AF035440), *H. influenzae* (accession no., P45205), *B. subtilis* (accession no., P54523), *R. capsulatus* (accession no., P26242), *Synechocystis* sp. strain PCC6803 (accession no., S75175), and *A. thaliana* (accession no., Q38854) (Fig. 3). The amino acid sequences encoded by the CL190 ORF corresponding to the highly conserved sequences were Trp Asp Thr Gly His Asn and Ile Ala Glu Asn His Ala (Fig. 3). Thus, only Thr was substituted for Val in the highly conserved sequence of the CL190 ORF product. The significant similarity suggested that the ORF encoded DXP synthase in CL190.

Enzymatic function of the ORF product from CL190. To verify the enzymatic function of the product of the complete ORF to be that of a DXP synthase, the corresponding gene was overexpressed in *E. coli*. The QIAexpress system was used because of the advantages of high-level expression and easy purification with Ni-nitrilotriacetic acid agarose resin. Incubation of the purified recombinant protein with pyruvate and DL-glyceraldehyde 3-phosphate in the presence of thiamine diphosphate at 30°C for 12 h in the assay system for DXP synthase resulted in the production of DXP, which was detected by chromatography with a Senshu Pak NH2-1251-N column. Omission of thiamine diphosphate from the reaction mixture resulted in failure of DXP production, indicating that the recombinant protein absolutely requires thiamine diphosphate for the enzymatic reaction. The enzymatic function of the product of the ORF of *Streptomyces* sp. strain CL190 was thus confirmed to be that of a thiamine diphosphate-dependent DXP synthase.

Enzymatic properties for DXP synthase from CL190. The purified recombinant DXP synthase showed a single band on SDS-PAGE gel and native PAGE gel. SDS-PAGE showed a subunit molecular mass of 70 kDa (Fig. 4). Native PAGE performed with a 8 to 25% polyacrylamide gradient gel gave a protein band with a mobility corresponding to 140 kDa. By gel filtration chromatography, the molecular mass of the enzyme was estimated to be 130 kDa. These results clearly suggested that the enzyme is most likely to be a dimer. The enzyme showed a pH optimum of 9.0. The effect of temperature on the enzyme activity was investigated over the range of 30 to 50°C. The maximum activity was observed at 42 to 44°C. The activation energy was estimated to be 99 kJ per mol by an Arrhenius plot whose curve was straight over the range of 30 to 40°C (Fig. 5). The enzyme required Mg^{2+} or Mn^{2+}, and the optimum concentration of the divalent cations was 1 mM. The enzyme activity was completely lost by an addition of EDTA. The K_m values were calculated as 65 μM for pyruvate and 120 μM for D-glyceraldehyde 3-phosphate, and V_{max} was 370 U per mg of protein. The purified enzyme catalyzed the formation of DX by condensation of pyruvate and D-glyceraldehyde as well. However, the K_m value for D-glyceraldehyde was 290-fold higher than that for D-glyceraldehyde 3-phosphate. These kinetic parameters are summarized in Table 1.

Enzymatic properties of DXP synthase from *E. coli*. Although *E. coli* DXP synthase was cloned by two independent groups (12, 25), no detailed studies of its enzymatic properties have been reported. In order to compare the enzymatic properties of CL190 and *E. coli* DXP synthases, we overexpressed and purified *E. coli* DXP synthase (9). The purified *E. coli* DXP synthase showed a single band with a molecular mass of 69 kDa (Fig. 4). The native molecular mass of the enzyme was estimated to be 117 kDa by gel filtration chromatography, suggesting that the *E. coli* enzyme is also likely to form a dimer. The enzymatic properties of *E. coli* DXP synthase were similar to those of the CL190 enzyme (Table 1). A difference between the *E. coli* and CL190 enzymes was found only in the pH optimum, with the *E. coli* enzyme showing an optimum activity at pH 7.5 to 8.0.

DISCUSSION

We successfully cloned the *dxs* gene encoding DXP synthase from *Streptomyces* sp. strain CL190 by colony hybridization with a DNA probe generated by PCR with oligonucleotide primers prepared on the basis of the highly conserved amino acid sequences among DXP synthase homologs from six genera. The *dxs* gene from CL190 encoded 631-residue DXP synthase with a predicted molecular mass of 68 kDa. The deduced amino acid sequence showed around 38% identity to the DXP synthase homologs found in the SWISS-PROT database.

In order to characterize CL190 DXP synthase, we overexpressed the CL190 *dxs* gene in *E. coli*. Moreover, in order to compare the enzymatic properties of the CL190 DXP synthase

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   1 gcatgccggtggcacaggcggtcggcgcggtgctcgaatccggcgcgccgatggtctccatcgccggcggagagccgctgatgcaccctcagatcgacgagatcgtccggcagttggtgg
       M  P  V  A  Q  A  V  G  A  V  L  E  S  G  A  P  M  V  S  I  A  G  G  E  P  L  M  H  P  Q  I  D  E  I  V  R  Q  L  V  A

 121 ccaagcggaaatacgtctttctgtgcaccaacgccctgctgctgcgcaagaagatggaactcttcacgccctcgcgctacttcgccttcgccgtgcacatcgacggcctgcgcgaacgcc
       K  R  K  Y  V  F  L  C  T  N  A  L  L  L  R  K  K  M  E  L  F  T  P  S  R  Y  F  A  F  A  V  H  I  D  G  L  R  E  R  H

 241 acgacgagtcggtggcgaaggagggcgtcttcgacgaggcggtggaggcgatcaaggaggccaagcggcgcggcttccgcgtcaccaccaactccaccttcttcaacaccgacaccccgc
       D  E  S  V  A  K  E  G  V  F  D  E  A  V  E  A  I  K  E  A  K  R  R  G  F  R  V  T  T  N  S  T  F  F  N  T  D  T  P  Q

 361 agaccgtcgtcgaggtcctgaacttcctcaacgacgacctcaaggtcgacgagatgatgatctcgcccgcctacgcctacgagaaggcccccgaccaggagcacttcctcggcgtcgagc
       T  V  V  E  V  L  N  F  L  N  D  D  L  K  V  D  E  M  M  I  S  P  A  Y  A  Y  E  K  A  P  D  Q  E  H  F  L  G  V  E  Q

 481 agacccgcgaactgttccgcaaggccttcgcgggcggcaaccggcgccgctggcggctcaaccactccccgctcttcctcgacttcctcgaaggcaaggtcgacttcccctgcaccgcct
       T  R  E  L  F  R  K  A  F  A  G  G  N  R  R  R  W  R  L  N  H  S  P  L  F  L  D  F  L  E  G  K  V  D  F  P  C  T  A  W

 601 gggcgatccccaactactccctcttcggctggcagcgcccctgctacctgatgagcgacggatacgtcccgacgtaccgcgaactgatcgaggacaccgactgggacaagtacggccggg
       A  I  P  N  Y  S  L  F  G  W  Q  R  P  C  Y  L  M  S  D  G  Y  V  P  T  Y  R  E  L  I  E  D  T  D  W  D  K  Y  G  R  G

 721 gcaaggacccgcgctgcgccaactgcatggcgcactgcggctacgagccgaccgccgtgctggccaccatgggctccctgaaggagtccctgcgcgcgatgccgaaaccgtcaacgggaa
       K  D  P  R  C  A  N  C  M  A  H  C  G  Y  E  P  T  A  V  L  A  T  M  G  S  L  K  E  S  L  R  A  M  P  K  P  S  T  G  T

 841 ccgtgagtgacgccatgaccgccggcccgggcagggcaggcggcggtggaccgagtacaaggcacggaccgaaggggggcccgagcgtgacgattctggagaacatccggcaaccacgcga
       V  S  D  A  M  T  A  G  P  G  R  A  G  G  G  G  P  S  T  R  H  G  P  K  G  A  R  A  *
                                                                                  dxs→V  T  I  L  E  N  I  R  Q  P  R  D

 961 cctgaaggcgctgcccgaggagcagctgcacgaactgtccgaggagatcaggcagttcctggtgcacgcggtcaccagaaccggcggtcatctgggacccaacctgggggtggtggagct
       L  K  A  L  P  E  E  Q  L  H  E  L  S  E  E  I  R  Q  F  L  V  H  A  V  T  R  T  G  G  H  L  G  P  N  L  G  V  V  E  L

1081 gaccatcgccctgcaccgggtcttcgagtcgcccgtcgaccgcatcctgtgggacaccggccaccagagctacgtacacaagctgctgacgggacgtcaggacttctccaagctgcgcgg
       T  I  A  L  H  R  V  F  E  S  P  V  D  R  I  L  W  D  T  G  H  Q  S  Y  V  H  K  L  L  T  G  R  Q  D  F  S  K  L  R  G

1201 caagggcggcctgtccggctacccctcgcgcgaggagtccgagcacgacgtcatcgagaacagccacgcctccaccgccctcggctgggccgacggactcgccaaggcccgccgggtgca
       K  G  G  L  S  G  Y  P  S  R  E  E  S  E  H  D  V  I  E  N  S  H  A  S  T  A  L  G  W  A  D  G  L  A  K  A  R  R  V  Q

1321 gggggagaagggccatgtcgtcgccgtcatcggcggacgggcgctgaccggcggcatggcctgggaggccctgaacaacatcgcggccgccaaggaccagccgctgatcatcgtcgtcaa
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1441 cgacaacgagcgctcctacgcgcccaccatcggcggcctcgccaaccacctggccaccctgcgcaccaccgacggctacgagaaggtcctcgcctggggcaaggacgtcctgctgcgtac
       D  N  E  R  S  Y  A  P  T  I  G  G  L  A  N  H  L  A  T  L  R  T  T  D  G  Y  E  K  V  L  A  W  G  K  D  V  L  L  R  T

1561 ccccatcgtcggccacccccctctacgaggccctgcacggcgccaagaagggcttcaaggacgccttcgccccgcagggcatgttcgaggacctgggcctgaagtacgtcggccccatcga
       P  I  V  G  H  P  L  Y  E  A  L  H  G  A  K  K  G  F  K  D  A  F  A  P  Q  G  M  F  E  D  L  G  L  K  Y  V  G  P  I  D

1681 cgggcacgacatcggcgcggtcgagtccgcgctgcgccgcgccaagcgcttccacgggccggtgctggtgcactgcctcaccgtcaagggccgcggctacgaacccgccctcgcccacga
       G  H  D  I  G  A  V  E  S  A  L  R  R  A  K  R  F  H  G  P  V  L  V  H  C  L  T  V  K  G  R  G  Y  E  P  A  L  A  H  E

1801 ggaggaccacttccacaccgtcggcgtgatggacccgctcacctgtgagcccctctcgcccaccgacggcccgtcctggacctcggtgttcggcgacgagatcgtacggatcggcgcgga
       E  D  H  F  H  T  V  G  V  M  D  P  L  T  C  E  P  L  S  P  T  D  G  P  S  W  T  S  V  F  G  D  E  I  V  R  I  G  A  E

1921 gcgcgaggacatcgtcgcgatcaccgccgcgatgctccacccggtggggctcgccaggttcgccgaccgcttcccggaccgggtctgggacgtcggcatcgccgagcagcacgcggccgt
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2041 gtccgcggccgggctcgccaccggcggactgcacccggtcgtcgccgtctacgccaccttcctcaaccgcgccttcgaccagctcctgatggacgtcgccctgcaccgctgcggtgtgac
       S  A  A  G  L  A  T  G  G  L  H  P  V  V  A  V  Y  A  T  F  L  N  R  A  F  D  Q  L  L  M  D  V  A  L  H  R  C  G  V  T

2161 cttcgtcctggaccgggccggcgtcacgggcgtcgacggcgcctcgcacaacggcatgtgggacatgtccgtcctccaggtcgtgcccggcctcaggatcgccgccccgcgcgacgccga
       F  V  L  D  R  A  G  V  T  G  V  D  G  A  S  H  N  G  M  W  D  M  S  V  L  Q  V  V  P  G  L  R  I  A  A  P  R  D  A  D

2281 ccacgtgcgcgcccagctgcgggaggcggtcgccgtggacgacgcgccgacgctgatccgcttcccgaaggagtccgtcggcccgcggatcccggccctcgaccgggtcggcggcctcga
       H  V  R  A  Q  L  R  E  A  V  A  V  D  D  A  P  T  L  I  R  F  P  K  E  S  V  G  P  R  I  P  A  L  D  R  V  G  G  L  D

2401 tgtgctgcaccgcgacgagcggcccgaggtgctgctggtcgccgtgggcgtcatggcacaggtctgcctccagaccgccgagctgctccgggcccgcggcatcggatgcacggtcgtcga
       V  L  H  R  D  E  R  P  E  V  L  L  V  A  V  G  V  M  A  Q  V  C  L  Q  T  A  E  L  L  R  A  R  G  I  G  C  T  V  V  D

2521 cccgcgctgggtcaagcccgtcgaccccgtgctgcccccactcgccgccgagcaccggctcgtcgccgtcgtggaggacaacagccgggccgccggggtcggttcggcggtcgccctggc
       P  R  W  V  K  P  V  D  P  V  L  P  P  L  A  A  E  H  R  L  V  A  V  V  E  D  N  S  R  A  A  G  V  G  S  A  V  A  L  A

2641 gctcggggacgccgatgtcgacgtaccggtgcgccgcttcggcatccccgagcagttcctcgcgcacgccaggcgcggtgaggtgctcgccgacatcgggctgaccccggtggagatcgc
       L  G  D  A  D  V  D  V  P  V  R  R  F  G  I  P  E  Q  F  L  A  H  A  R  R  G  E  V  L  A  D  I  G  L  T  P  V  E  I  A

2761 cgggcggatcggcgcgagcctgcccgtgcgggaggaaccggccgaggagcagcccgcatgaccaccctcgaacccgtggccgggaccgggcagttcgacctcgccgggctcctcgccgag
       G  R  I  G  A  S  L  P  V  R  E  E  P  A  E  E  Q  P  A  *

2881 cgcggcgccgaacgctacgaactgcacaccaggtacctcaaccaccaactcccgcgcatgc  2941
```

FIG. 2. Nucleotide sequence of the 2.9-kb *Sph*I-*Sph*I DNA fragment including the *dxs* gene from *Streptomyces* sp. strain CL190 and deduced amino acid sequence. The *dxs* gene consists of 1,896 bp starting with initiation codon GTG at position 926 and ending with termination codon TGA at position 2819. A putative Shine-Dalgarno sequence, GAAGG, was found 15 bp upstream of the initiation codon. An incomplete ORF product flanking the N terminus of the *dxs* gene product showed 24% identity in the 120-amino-acid region of overlap with the *Helicobacter pylori* putative protein (accession no., P56414) involved in the biosynthesis of the molybdopterin precursor from guanosine.

FIG. 3. Multiple alignment of the amino acid sequences of the *Streptomyces* DXP synthase and other DXP synthase homologs. Identical amino acids among the seven proteins are marked by asterisks. Dashes indicate gaps introduced for the optimization of the alignment. The amino acid sequences used for design of the PCR primers are underlined. CL190, *Streptomyces* sp. strain CL190; ECOLI, *E. coli*; HAEIN, *H. influenzae*; BACSU, *B. subtilis*; RHOCA, *R. capsulatus*; SYNY3, *Synechocystis* sp. strain PCC6803; ARATH, *A. thaliana*. For accession numbers, see Materials and Methods.

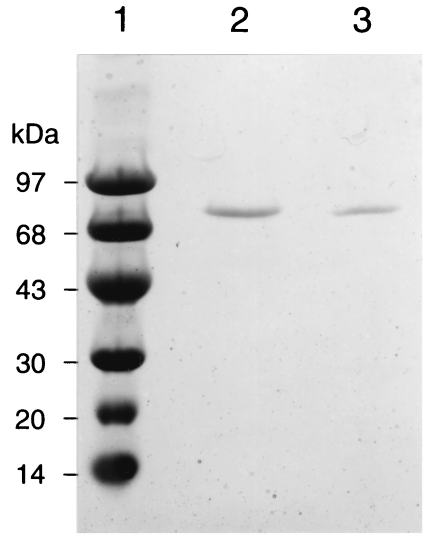

FIG. 4. Electrophoresis of the purified CL190 and *E. coli* DXP synthase overexpressed in *E. coli*. Purified DXP synthases of CL190 and *E. coli* obtained by using a Ni-nitrolotriacetic acid agarose column were analyzed by SDS–8 to 25% PAGE. Lanes: 1, molecular mass standard; 2, SDS-treated CL190 enzyme (0.2 μg); 3, SDS-treated *E. coli* enzyme (0.1 μg). Proteins were stained with Coomassie brilliant blue R-250.

with those of the *E. coli* DXP synthase, which had not been characterized in detail, we overexpressed, purified, and characterized the *E. coli* enzyme as well. The DXP synthases of both CL190 and *E. coli* were purified as soluble proteins and showed similar enzymatic properties (Table 1). On the other hand, it has been reported that the CapTKT2 gene was cloned from pepper and that the recombinant CapTKT2 gene product expressed in *E. coli* catalyzed DXP formation with K_m values of 500 μM for pyruvate and 750 μM for D-glyceraldehyde 3-phosphate (Table 1) (1). The values of the kinetic parameters of pepper DXP synthase are much higher than those of CL190 and *E. coli* enzymes (Table 1).

Recently we cloned and characterized *E. coli* DXP reductoisomerase, the enzyme for the second step of the nonmevalonate pathway (26). DXP reductoisomerase simultaneously catalyzes intramolecular rearrangement and reduction of DXP to form 2-*C*-methyl-D-erythritol 4-phosphate (Fig. 1). The catalytic efficiency, k_{cat}/K_m, for *E. coli* DXP reductoisomerase was calculated to be 2.2×10^7 $M^{-1} \cdot s^{-1}$ in the presence of Mg^{2+} (T. Kuzuyama, S. Takahashi, M. Takagi, T. Shimuzu, and H. Seto, unpublished data). On the other hand, the k_{cat}/K_m value for *E. coli* DXP synthase was estimated to be 2.8×10^6 $M^{-1} \cdot s^{-1}$ in this study. Thus this value for DXP synthase is lower than that for DXP reductoisomerase by a factor of 8. This difference suggests that DXP synthase is a rate-limiting enzyme in the nonmevalonate pathway, at least in *E. coli*. This suggestion is also supported by the finding that overexpression of DXP synthase or DXP reductoisomerase in *E. coli* resulted in an increase of ubiquinone production and that overexpression of DXP synthase was more effective in this increase than that of DXP reductoisomerase (7; Harker and Bramley, Abstr. 4th Eur. Symp. Plant Isoprenoids; Motoyama et al., unpublished data). At present it is difficult to determine the rate-limiting step of the nonmevalonate pathway, because most reaction steps of this pathway remain undefined. However, the results obtained above seem to imply that the DXP synthase reaction is the rate-limiting step of the nonmevalonate pathway.

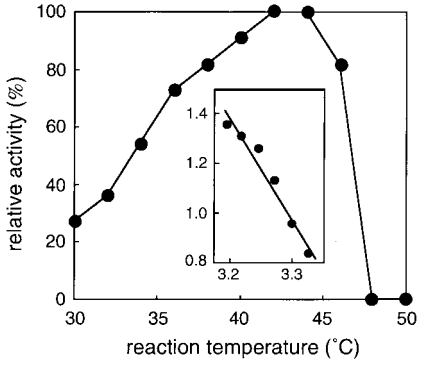

FIG. 5. Temperature dependence of the CL190 DXP synthase activity and the Arrhenius plot (insert). The DXP synthase activity of *Streptomyces* sp. strain CL190 was measured in the complete assay mixture as described in Materials and Methods except for the reaction temperature. One hundred percent activity corresponds to 0.42 U. All data are average values for duplicate determinations. The insert shows the Arrhenius plot used to estimate the activation energy of the enzyme.

TABLE 1. Comparisons of enzymatic properties among CL190, *E. coli*, and pepper DXP synthases

DXP synthase source	K_m for:			V_{max} (U/mg of protein)	Optimum temp (°C)	Optimum pH[a]	Activation energy (kJ/mol)	Divalent cations	Molecular mass (kDa) by:		Multimeric form
	Pyruvate (μM)	D-Glyceraldehyde 3-phosphate (μM)	D-Glyceraldehyde (mM)						SDS-PAGE	Gel filtration chromatography	
CL190	65	120	35	370	42–44	9.0	99	Mg^{2+}, Mn^{2+}	70	130	Dimer
E. coli	96	240	38	300	42–44	7.5–8.0	63	Mg^{2+}, Mn^{2+}	69	120	Dimer
Pepper[b]	500	750									

[a] Assay solutions consisted of 100 mM Tris-HCl at pH 7.0 to 9.5.
[b] From Bouvier et al. (1).

3-Hydroxy-3-methylglutaryl coenzyme A reductase is the rate-limiting enzyme of the mevalonate pathway in humans (6), and its specific inhibitors, pravastatin and related compounds, are used as cholesterol-lowering agents (28). If DXP synthase were the rate-limiting enzyme of the nonmevalonate pathway, its specific inhibitors would be reasonable antibacterials and herbicides with no toxicity to humans. Screening for DXP synthase inhibitors from natural products is now in progress in our laboratory.

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