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 as well, with a kinetic parameter of 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. 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 13C-labeled precursors indicating that some isoprenoids of *Streptomyces* *origen*, such as terpentene (8), naphthene (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).
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, 5-GTP-carboxylase-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., AF015440), *Haemophilus influenzae* (accession no., P45205), *Bacillus subtilis* (accession no., P54523), *Rhodobacter capsulatus* (accession no., P26242), *Synechocystis* sp. strain PC6803 (accession no., S75175), and *Anaballinis thaliana* (accession no., Q58854). Two amino acid sequences, Tryp 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'-ACSGCGTGCTGCTCSGCG), 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 single DNA fragment of 1.9 kb was amplified. The PCR fragment was cleaved with *Hind*III and cloned into the *Hind*III site of 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 *Hind*III 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 600 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 MgCl₂, 1 mM 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 MgCl₂, 2 mM dithiothreitol, 1 mM sodium pyruvate, 2 mM D-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-deoxynylulose (DX), was monitored by a refractive index spectrometer (model RI-71; Showa Denko, Tokyo, Japan) with a Shodex KS-801 (6 by 300-mm) column (Showa Denko), eluted with H₂O 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 DX per min at 37°C. All the assays for the calculation of *Kₘ* and *Vₘₐₓ* values.
of both Streptomyces and E. coli enzymes were done at 37°C. These values were calculated with Lin&Kinetics software, version 1.0 (3).

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 among DXP synthase homologs from six genera. The maximal activity was observed at 42 to 44°C. The activation energy was estimated to be 99 kJ/mol by an Arrhenius plot whose curve was straight over the range of 30 to 40°C (Fig. 5). The enzyme required Mg²⁺ or Mn²⁺, and the optimum concentration of the divalent cations was 1 mM. The enzyme activity was completely lost by an addition of EDTA. The Kₘ values were calculated as 65 μM for pyruvate and 120 μM for D-glyceraldehyde 3-phosphate, and Vₘₙₐₓ was 370 U per mg of protein. The purified enzyme catalyzed formation of DX by condensation of pyruvate and D-glyceraldehyde as well. However, the Kₘ 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. 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
FIG. 2. Nucleotide sequence of the 2.9-kb SphI-SphI 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.
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 *Km* 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-<sup>5</sup>-methyl-D-erythritol 4-phosphate (Fig. 1). The catalytic efficiency, \( k_{cat}/K_m \), for *E. coli* DXP reductoisomerase was calculated to be \( 2.2 \times 10^7 \) M<sup>-1</sup> s<sup>-1</sup> in the presence of Mg<sup>2+</sup> (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 \( 2.8 \times 10^6 \) M<sup>-1</sup> s<sup>-1</sup> 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. 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.](http://jb.asm.org/)

![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-nitrotriacetic acid agarose column were analyzed by SDS–8 to 25% PAGE. Lanes: 1, molecular mass standard; 2, SDS-treated CL190 enzyme (0.2 mg); 3, SDS-treated *E. coli* enzyme (0.1 mg). Proteins were stained with Coomassie brilliant blue R-250.](http://jb.asm.org/)

![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.](http://jb.asm.org/)
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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