Isopentenyl diphosphate isomerase (idi) and farnesyl diphosphate synthase (ispA) genes were overexpressed in *Escherichia coli*. The resulting transformant showed 6.8-fold higher production of farnesol (389 g/l). In a similar manner, overexpression of idi and mutated ispA led to high production of geranylgeraniol (128 g/l).

Key words: farnesol; geranylgeraniol; lipoate; pyruvate; terpenoid

More than 23,000 isoprenoid compounds with 5-carbon isoprene (2-methyl-1,3-butadiene) basic structural units have been identified to date as secondary metabolites in various organisms.1,2 Farnesyl diphosphate (FPP) is a common intermediate for several essential components of living organisms, such as sterols, dolichols and quinones related to the mitochondrial respiratory chain. Geranylgeranyl diphosphate (GGPP) is essential to the biosynthesis of vitamin A, tocopherol, geranylgeranyl anchors, and the ether-type lipid membranes of archaea. FPP and GGPP are dephosphorylated by prenyl pyrophosphatase to the alcohol-type derivatives farnesol (FOH) and geranylgeraniol (GGOH) respectively.2) These prenyl alcohols are widely used as fragrances in essential oils, and also recently as starting materials in the chemical synthesis of various pharmaceuticals.

The precursor of FPP and GGPP, isopentenyl diphosphate (IPP), is synthesized from acetyl-CoA via the mevalonate pathway. Recently, IPP was found to be synthesized through a different route, the 2-C-methyl-D-erythritol pathway. Recently, IPP was found to be synthesized through a different route, the 2-C-methyl-D-erythritol pathway. This alternative pathway starts with a condensation of one DMAPP molecule and two IPP molecules to form 1-deoxy-D-xylulose 5-phosphate (DXP). This isomeroreductase, MEP cytidylyltransferase, 4-(cytidine 5'-diphospho)-2-C-methyl-d-erythritol kinase, 2-C-methyl-d-erythritol 2,4 cyclo diphosphate synthase, 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase, and 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase. IPP is further converted to dimethylallyl diphosphate (DMAPP) by isopentenyl diphosphate isomerase. The *E. coli* isomerase is 20-fold less active than its *Saccharomyces cerevisiae* counterpart, and overexpression of the IPP isomerase-encoding genes (idi) isolated from the yeast *Phaffia rhodozyma* and the green alga *Haematococcus pluvialis* with the carotenoid biosynthesis genes of *Erwinia* in *E. coli* enhanced lycopene production.5,6) Successive head-to-tail condensation of one DMAPP molecule and two IPP molecules leads to the formation of FPP, while that of one DMAPP molecule and three IPP molecules generates GGPP.7

From an industrial perspective, combining a pyruvic acid-producing strain of *E. coli* with overexpression of isoprenoid biosynthesis genes might be expected to increase prenyl alcohol production. In the present study, we examined prenyl alcohol production by *E. coli* transformants with overexpression of isoprenoid biosynthesis genes.

*E. coli* W1485, a wild-type strain, ATCC 12435, was used. FPP synthase (ispA) and its Y81M mutant (ispAm) genes were overexpressed in *E. coli* using the pAlter-Ex2 vector (Promega, Madison). The mutation Y81M leads to efficient accumulation of GGPP as a final prenyl alcohol.8) Two primers (ispA-F1, 5'-ATGGCCGAG-CTTCCAGTTGAAAGTCTC-3'; ispA-R1, 5'-TTATTGGTTCGGGCAGGACC-3') were designed for amplification of the isPA and ispAm genes.

The ids gene was amplified with two primers (ids-F1, 5'-ATGGAAACGGAACACGTACATTTATGAGTGG-3'; ids-R1, 5'-TTATTATAGTCTGCAACGATG-AATCG-3') from *E. coli* genomic DNA by PCR, and was co-expressed with plasmid pACYC-idi derived from the expression vector pACYC177 (Nippon Gene, Tokyo).9) The transformed cells were cultivated in 300-ml flasks with 80 ml of 2×YT medium (1.6% tryptone, 1% yeast extract, and 0.5% NaCl pH 7.0) and appropriate antibiotics in the presence of 1 mM IPTG at 37 °C for 11 h. After the addition of 5 ml of a solution containing 20% glucose, 5% Na2HPO4·12H2O, and 10% KNO3, the culture was further incubated at 37 °C with vigorous shaking for 3 h, at which the cell density of each culture was approximately OD600 = 2.0. Each culture (25 ml) was separated into the supernatant (extracellular fraction) and the precipitate (cellular fraction) by centrifugation at 2,000 × g for 10 min. After incubation of the extracellular fraction (pH 7.0–
7.5) at 60 °C for 1 h for disruption of the residual cells, a 1/10 volume of 0.5 M sodium acetate buffer (pH 4.5) containing 5% Triton X-100 and 5 units of potato acid phosphatase (Sigma, St. Louis, MO) was added, and the mixture was incubated at 37 °C overnight. The prenyl alcohols, FOH and GGOH, were extracted with an equal volume of extraction solvent (methanol:pentane, 1:1, v/v). After centrifugation at 2,000 × g, the upper organic layer was concentrated to 200 μl and transferred to a vial containing 10 μl of internal standard solution (0.1% v/v undecanol in ethanol) for GC/MS. The cellular fraction was resuspended in 4 ml of TE solution (10 mM of Tris and 1 mM of EDTA, pH 8.0), incubated with 100 μl of lysozyme solution (10 mg/ml in TE) at room temperature for 30 min, and then disrupted with a UCM-201 sonicator (Cosmo Bio., Tokyo). After incubation at 60 °C for 1 h for disruption of cells suspended with 50 mM sodium acetate buffer (pH 4.5) containing 0.5% Triton X-100 and potato acid phosphatase and subsequent dephosphorylation of the cell lysate, the prenyl alcohol was analyzed as described above. FOH and GGOH were determined with an Agilent 5973 GC/MS system equipped with a HP-5MS column (Agilent Technologies Japan, Hachiioji). The initial column temperature of 115 °C was held for 1.5 min, raised by 70 °C/min to 250 °C, held for 2 min, raised by 70 °C/min to 300 °C, and held for 7 min. The amounts of FOH and GGOH present were calculated from the ratios of the relevant ion peak areas (69 mass fragments) compared to that of the undecanol.

As shown in Fig. 1, overexpression of ispA in E. coli, W1485 (1F) increased FOH production approximately 5-fold, from 57.3 to 271 μg/l, and co-expression of idi (1FI) further increased production, to 389 μg/l. Both genes are thought to encode key enzymes in the early steps of prenyl alcohol production in E. coli, together with the DXP synthase gene (dxs). This result parallels the finding that mono-terpenoid and di-terpenoid production was significantly enhanced by co-expression of ispA, idi, dxs, and various terpenoid cyclase genes. Overexpression of ispAm greatly increased the ratio of GGOH production as compared with that of FOH. Co-expression of ispAm and idi in E. coli W1485 (1GI) led to the accumulation of 128 μg/l of GGOH in the cellular fraction. This outcome was consistent with the results of an in vitro experiment involving mutant forms of prenyltransferases, in which replacement of conserved aromatic amino acids in the first aspartate-rich motif of FPP synthase with amino acids with smaller side chains led to the formation of longer-chain prenyl diphosphates. We detected no GGOH in the extracellular fraction, perhaps due to differences in the permeability of the cell membrane depending on molecular shape and hydrophobicity.

After pyruvic acid, which is involved as an early intermediate in the biosynthesis of prenyl alcohols via the MEP pathway, we tested a lipoic acid synthetase-intermediate in the biosynthesis of prenyl alcohols (data not shown). Thus, in the bacterial cytosol, the accumulation of pyruvic acid due to a deficiency of lipoic acid synthetase did not stimulate prenyl alcohol production, even when idi, ispA, and ispAm, involved in the biosynthesis of FPP and GGPP were overexpressed. E. coli W1485 lip2 strain might contain insufficient amounts of the high-energy compounds NADPH, ATP, and CTP, needed for prenyl alcohol production from pyruvic acid in the MEP pathway. There is probably an inadequate supply of these compounds under aerobic conditions in the absence of an electron-transfer system.

References
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