An unusual isopentenyl diphosphate isomerase found in the mevalonate pathway gene cluster from *Streptomyces* sp. strain CL190

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A gene cluster encoding five enzymes of the mevalonate pathway had been cloned from *Streptomyces* sp. strain CL190. This gene cluster contained an additional ORF, *orfD*, encoding an unknown protein that was detected in some archaebacteria and some Gram-positive bacteria including *Staphylococcus aureus*. The recombinant product of *orfD* was purified as a soluble protein and characterized. The molecular mass of the enzyme was estimated to be 37 kDa by SDS-polyacrylamide gel electrophoresis and 155 kDa by gel filtration chromatography, suggesting that the enzyme is most likely to be a tetramer. The purified enzyme contained flavin mononucleotide (FMN) with the amount per tetramer being 1.4 to 1.6 mol/mol. The enzyme catalyzed the isomerization of isopentenyl diphosphate (IPP) to produce dimethylallyl diphosphate (DMAPP) in the presence of both FMN and NADPH. The *Escherichia coli* plasmid expressing *orfD* could complement the disrupted IPP isomerase gene in *E. coli*. These results indicate that *orfD* encodes an unusual IPP isomerase showing no sequence similarity to those of IPP isomerases identified to date. Based on the difference in enzymatic properties, we classify the IPP isomerases into two types: Type 2 for FMN- and NAD(P)H-dependent enzymes, and type 1 for the others. In view of the critical role of this isomerase in *S. aureus* and of the different enzymatic properties of mammalian (type 1) and *S. aureus* (type 2) isomerases, this unusual enzyme is considered to be a suitable molecular target for the screening of antibacterial drugs specific to *S. aureus*.

Isoprenoids play important roles in all living organisms; they function as steroid hormones in mammals, carotenoids in plants, and ubiquinone or menaquinone in bacteria (1). All of these isoprenoids are synthesized by consecutive condensations of the five-carbon precursor isopentenyl diphosphate (IPP) to its isomer dimethylallyl diphosphate (DMAPP). Two distinct pathways for the IPP biosynthesis are known. One is the mevalonate pathway that operates in eukaryotes, archaebacteria, and the cytosols of higher plants. The other is the nonmevalonate pathway (Fig. 1), which is generally used by many eubacteria, including *Escherichia coli* and *Bacillus subtilis*, green algae, and the chloroplasts of higher plants (2, 3). The initial step of this pathway is the formation of 1-deoxy-D-xylulose 5-phosphate (DXP) by condensation of pyruvate and glyceraldehyde 3-phosphate catalyzed by DXP synthase (4–7). In the second step, DXP is converted to 2-*C*-methyl-D-erythritol 4-phosphate (MEP) by DXP reductoisomerase (8–11). MEP is then cytidylylated by MEP cytidylyltransferase to give 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol (CDP-ME) (12, 13), which is phosphorylated by CDP-ME kinase to yield 2-phospho-4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol (CDP-ME2P; refs. 14, 15). Next, CDP-ME2P is converted to 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate (MECDP) by the action of MECDP synthase (16, 17). However, the subsequent reactions leading to the formation of IPP from MECDP remain to be elucidated. Recently, Boronat and coworkers (18) demonstrated that IPP and DMAPP were independently biosynthesized in the nonmevalonate pathway.

IPP isomerase (EC 5.3.3.2) catalyzes an essential reaction in the biosynthesis of isoprenoids by converting IPP to DMAPP (Fig. 1; ref. 19). Many IPP isomerase genes (*idi*) have been cloned from various organisms such as humans (20), *Saccharomyces cerevisiae* (21), *E. coli* (22), and *Rhodobacter capsulatus* (23). Interestingly, however, database searches using the highly conserved amino acid sequences in IPP isomerases identified to date failed to detect IPP isomerase in archaebacteria and some eubacteria that possess the mevalonate pathway. This failure to detect IPP isomerase in these organisms has suggested the existence of an unusual type of IPP isomerase the amino acid sequence of which shares no similarity to those of IPP isomerases identified to date.

We previously cloned a gene cluster encoding enzymes of the mevalonate pathway from *Streptomyces* sp. strain CL190 and demonstrated that the mevalonate pathway could function in an *E. coli* strain transformed with this gene cluster (24, 25). In addition to the known genes of the mevalonate pathway, this gene cluster contains an additional ORF, *orfD*, encoding a protein with unknown function (25). This *orfD* has recently been detected in some archaebacteria, some Gram-positive bacteria, including *Staphylococcus aureus* (26), and in the carotenoid biosynthetic gene cluster of *Erwinia herbicola* Eho10 (27). These facts led us to assume that *orfD* encodes a previously uncharacterized type of IPP isomerase.

In this paper, we demonstrate that the *orfD* product expressed in *E. coli* catalyzes the isomerization of IPP to produce DMAPP in the presence of both flavin mononucleotide (FMN) and NADPH. Based on the enzymatic properties of this enzyme, which are different from those of known IPP isomerases, we propose that this *orfD* product is an unusual type of IPP isomerase the sequence of which has no similarity to those of known IPP isomerases. This paper also describes the identification of this unusual IPP isomerase in archaebacteria and some Gram-positive bacteria such as *S. aureus*, *Streptococcus pneumoniae*, *Streptococcus pyrogenes*, and *Enterococcus faecalis*. This finding suggests that the IPP isomerase of *S. aureus* represents an effective target for the screening of anti-staphylococcal drugs.

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Abbreviations: IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; ME, 2-*C*-methylerythritol; CDP-ME, 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol; CDP-ME2P, 2-phospho-4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol; MECDP, 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate; MVA, mevalonate; PMVA, phosphomevalonate; DPMVA, diphosphomevalonate.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AB046667 (*Streptomyces* sp. strain CL190) and AB047344 (*S. aureus* ATCC25923)].

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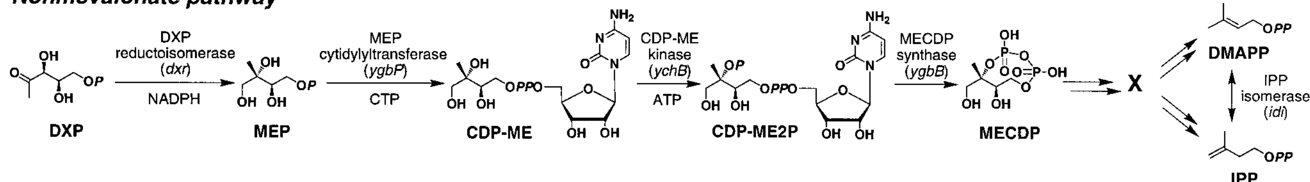

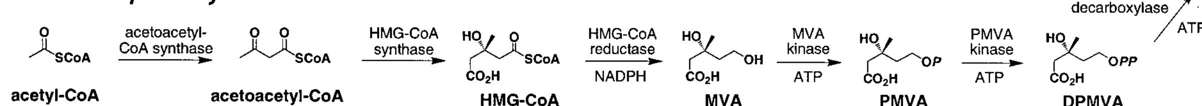

Fig. 1. IPP biosynthetic pathway in the *E. coli* transformants. The subsequent reactions leading to the formation of IPP or DMAPP from MECDP remain to be elucidated. X, unidentified biosynthetic intermediate of the nonmevalonate pathway.

Materials and Methods

Strains and Plasmids. We previously cloned a gene cluster containing *orfD*, and genes encoding 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) synthase, HMG-CoA reductase, mevalonate (MVA) kinase, phosphomevalonate (PMVA) kinase and diphosphomevalonate (DPMVA) decarboxylase from *Streptomyces* sp. strain CL190 (24, 25). The expression plasmid, pUMV19, contains all of these genes (25). The pUMV19-derived plasmids and *E. coli* strains used in this study are listed in Table 1. Because *E. coli* W3110 was not able to maintain the pUC118-derived plasmids, probably because of the high-level expressions of the mevalonate pathway genes from the *lacZ* promoter in pUC118, a pTTQ18 vector harboring *lacI^q*, which encodes the *lac* repressor, was used to introduce these genes into this strain. The pTMV20S contained *orfD* in addition to genes encoding the three enzymes of the mevalonate pathway, MVA kinase, PMVA kinase, and DPMVA decarboxylase, present in pTMV20SΔM.

Construction of the Plasmid for Overexpression of *orfD* in *E. coli*. Based on the entire nucleotide sequence of *orfD* from *Streptomyces* sp. strain CL190 (accession no. AB037666), two oligonucleotide primers, 5′-GGGGATCCACCAGCGCCCAACGCAAGGACG-3′ (5′ of *orfD*) and 5′-GGGGATCCTCGTGTGCTTCCCGTCGTCTGG-3′ (3′ of *orfD*), including a *Bam*HI restriction site (underlined) were synthesized (Amersham Pharmacia) and used together with total DNA from *Streptomyces* sp. CL190 to amplify *orfD*. By using *Taq* DNA polymerase (Roche Molecular Biochemicals) and the protocol recommended by the supplier, a 1.1-kb fragment was amplified. The PCR fragment was digested with *Bam*HI and cloned into the multicloning site of the expression vector pQE30 (Qiagen, Chatsworth, CA) to give pQCLD41.

Expression and Purification of the Recombinant *orfD* Product. *E. coli* M15 containing pREP4 [*neo*, *lacI*] (Qiagen) was used as a host for the expression of *orfD*. M15(pREP4, pQCLD41) was cultured at 18°C in 1 liter of LB medium containing 25 μg/ml kanamycin (Nacalai Tesque, Kyoto) and 200 μg/ml ampicillin (Sigma) for 6 h with the addition of 2 mM isopropyl β-D-thiogalactopyranoside (IPTG) when an optical density at 660 nm of 0.7 was reached. Cells were harvested by centrifugation and resuspended in Buffer A composed of 100 ml of 0.1 M Tris·HCl (pH 8.0) and 5 mM DTT. After sonication, the lysate was centrifuged at 10,000 $\times$ *g* for 20 min and the supernatant retained. The crude extract was applied on a Ni-nitrilotriacetic acid agarose column (Qiagen) previously equilibrated with Buffer A. The resin was washed with 50 mM imidazole in Buffer A and then the protein bound to the resin was eluted with 200 mM imidazole in Buffer A.

Cloning, Expression, and Purification of the Recombinant *S. aureus* IPP Isomerase. On the basis of the entire nucleotide sequence of the *orfD* homolog, which was located in Contig111, from *S. aureus* (http://www.sanger.ac.uk/Projects/S aureus/), two oligonucleotide primers, 5′-GGGATCCAGTGATTTTCAAAGAGAACAGAG-3′ (5′ of the *S. aureus orfD* homolog) and 5′-GGGATCCTCCTCGATGTATATTCAAGTTACG-3′ (3′ of the *S. aureus orfD* homolog), including a *Bam*HI restriction site (underlined) were synthesized and used together with total DNA from *S. aureus* ATCC25923 to amplify the IPP isomerase gene. Cloning, expression, and purification of the recombinant

Table 1. Bacterial strains and plasmids used in this study

E. coli strain and plasmid	Description	Reference or source
Strains		
E. coli DK223	W3110 *dxr*::Km^r	This study
E. coli DK331	W3110 *dxr*::Km^r and *idi*::Cm^r	This study
Plasmids		
pTTQ18	cloning vector; *lacI^q*, Amp^r	S. Matsuyama (University of Tokyo)
pGEM-11zf(+)aad-1	contains 2.0-kb Spm^r cassette	H. Ikeda (Kitasato University)
pTTQ18S	pTTQ18 with 2.0-kb Spm^r cassette from pGEM-11zf(+)aad-1 inserted at the *Sca*I site in the Amp^r gene	This study
pUMV19	pUC118 with the 6.8-kb *Sna*BI-*Sna*BI fragment (accession no. AB037666)	25
pUMV20	pUMV19 with a deletion of the 0.7-kb *Sse*8387I-*Sse*8387I fragment	This study
pUMV20ΔM	pUMV20 with a deletion of the 0.4-kb *Mlu*I-*Mlu*I fragment	This study
pTMV20S	pTTQ18S with the 6.1-kb *Xba*I-*Hin*dIII fragment from pUMV20	This study
pTMV20SΔM	pTTQ18S with the 5.7-kb *Xba*I-*Hin*dIII fragment from pUMV20ΔM	This study

S. aureus IPP isomerase were done in the same way as for CL190 *orfD*. An expression plasmid for *S. aureus* IPP isomerase was named pQSAU39.

Determination of Molecular Mass. The molecular masses of the overexpressed proteins were estimated by gel filtration using a Superdex 200 (1.6 × 60 cm) column (Amersham Pharmacia), which had been equilibrated with 20 mM sodium phosphate buffer (pH 7.1) containing 0.15 M NaCl. As the standard proteins, ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), and BSA (66 kDa) were used.

Reconstitution of the *orfD* Product with FMN. The *orfD* product (2 mg) in a volume of 1 ml was incubated with 100 μM FMN at 4°C for 12 h. After incubation, the protein solution was desalted to remove free FMN by gel filtration using a PD-10 column (Amersham Pharmacia). Absorption spectra of each 0.5 ml of fraction were recorded by using a UV-visible spectrophotometer (U-3210; Hitachi, Tokyo). It was confirmed that the fractions containing protein and those containing free FMN were well separated using this column.

Assay for IPP Isomerase. We first used the standard assay for IPP isomerase described by Satterwhite (28). This standard assay system is based on the acid lability of DMAPP. The assay mixture consisted of 0.1 M Hepes buffer (pH 7.0), 5 mM $MgCl_2$, 1 mM DTT, 10 μM [1-^{14}C]IPP (2.0 GBq/mmol; American Radiolabeled Chemicals, St. Louis) and 40 μM IPP in a final volume of 50 μl. The reaction was initiated by adding enzyme to the assay system followed by incubation at 37°C for 10 min. The reaction was terminated by adding 0.2 ml of 25% concentrated HCl in MeOH and 0.5 ml of H_2O, followed by incubation at 37°C for 10 min. This mixture was then saturated with NaCl and subjected to extraction twice with 0.5-ml aliquots of toluene. The extracts were combined and then dried by adding Na_2SO_4. The radioactivity in the 0.3-ml aliquot of the toluene solution was determined in a scintillation solution (Scintisol AL-1; Dojin, Kumamoto, Japan) by using a scintillation counter (LS 6000LL; Beckman Coulter).

However, all attempts to detect IPP isomerase activity of the *orfD* product were unsuccessful using the standard assay system described above. We then established a new assay system for the IPP isomerase encoded by *orfD*, which is also based on the acid lability of DMAPP. The new assay mixture consisted of 0.1 M Hepes buffer (pH 7.0), 5 mM $MgCl_2$, 1 mM DTT, 10 μM [1-^{14}C]IPP, 40 μM IPP, 10 μM FMN, and 5 mM NADPH in a final volume of 50 μl. The reaction was initiated by adding enzyme to the assay system followed by incubation routinely at 37°C for 1 min. Further manipulations were the same as those used in the standard assay described above.

Product Analysis by ^{1}H Nuclear Magnetic Resonance. The reaction mixture (1 ml) consisting of 0.1 M K_2HPO_4 (pH 7.0), 5 mM $MgCl_2$, 1 mM DTT, 10 μM FMN, 2.5 mM NADPH, 1 mg of *orfD* product, and 5 mM IPP was incubated at 37°C for 12 h. After incubation, the reaction mixture was lyophilized, and the resulting residue was resuspended in 99.9% D_2O. The reaction product was then analyzed by using a ^{1}H NMR spectrometer (A-500; JEOL). As a control, a reaction mixture not containing the *orfD* product was also analyzed.

pH-Dependent Activity of IPP Isomerase. The IPP isomerase assay was conducted as described above. Assay solutions consisted of 0.1 M Mes at pH 5.0–6.5, 0.1 M Hepes at pH 6.5–7.5 or 0.1 M Tris·HCl at pH 7.0–9.5, 5 mM $MgCl_2$, 10 μM FMN, 5 mM NADPH, and 1 mM DTT.

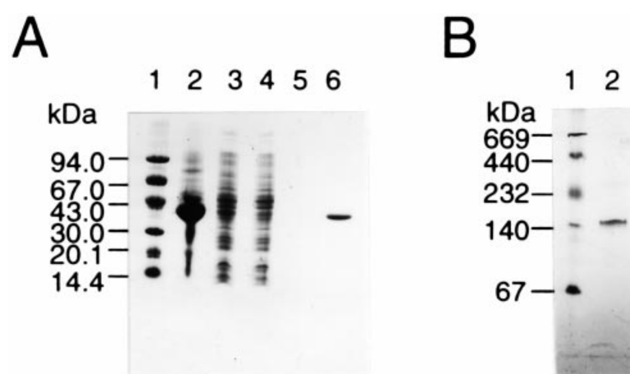

Fig. 2. Expression and purification of the recombinant *orfD* product. (*A*) SDS/PAGE of the enzyme. Lanes: 1, low molecular mass markers; 2, the pellet of the extract from *E. coli* M15 (pREP4, pQCLD41) culture after induction with isopropyl β-D-thiogalactopyranoside (IPTG); 3, the supernatant of the same extract; 4, proteins passed through an Ni-nitrilotriacetic acid agarose column; 5, the fraction eluted with 50 mM imidazole; 6, purified enzyme (1 μg) eluted with 200 mM imidazole. (*B*) Native PAGE of the purified enzyme. Lanes: 1, high molecular mass markers; 2, purified enzyme (0.7 μg).

Complementation of the Disrupted *E. coli idi* Gene. Strain DK331 (Table 1) was separately transformed with pTMV20SΔM and pTMV20S. These transformants were cultured at 37°C in LB liquid medium containing 0.005% MVA, 12.5 μg of kanamycin/ml, 17 μg of chloramphenicol/ml and 100 μg of spectinomycin/ml. As a positive control, DK223(pTMV20SΔM) was cultured at 37°C in LB liquid medium containing 0.005% MVA, 12.5 μg of kanamycin/ml and 100 μg of spectinomycin/ml. Growth of these transformants was monitored by optical density at 660 nm.

Results

Expression and Purification of the Recombinant *orfD* Product. To elucidate the *in vitro* function of the *orfD* product, *orfD* was overexpressed in *E. coli* by using the T7 promoter system and the product was purified to homogeneity as a soluble protein (Fig. 2). SDS/PAGE showed a subunit molecular mass of 37 kDa. Native PAGE gave a single protein band with a mobility corresponding to 150 kDa (Fig. 2). The apparent molecular mass of the *orfD* product was estimated to be 155 kDa by Superdex 200 gel filtration. These data indicate that the *orfD* product is most likely to be a tetramer. After purification, the protein solution was yellow, indicating that a chromophore such as flavin was present. Since it was possible that this chromophore might have been partially removed during purification, reconstitution of the protein with FMN was done. The absorption spectrum of the resulting solution (Fig. 3) demonstrates that the *orfD* product is a typical flavoprotein. Based on the extinction coefficient for free FMN (12.2 $mM^{-1}\cdot cm^{-1}$ at 450 nm), the amount of FMN per tetramer was determined to be 1.4 to 1.6 mol/mol.

FMN and NADPH Dependence of IPP Isomerase Encoded by *orfD*. We next determined whether the reconstituted *orfD* product had IPP isomerase activity. Initial efforts to detect the activity were carried out by using the standard method described by Satterwhite (28). However, these attempts were totally unsuccessful. This raised the possibility that the *orfD* product might require some cofactors. Thus, we added various cofactors into the standard assay mixture. A high level of radioactivity was detected in the toluene extract (see *Materials and Methods*) when both FMN and NADPH were added to the reaction mixture. This radioactivity could have resulted from DMAPP, suggesting that the *orfD* product catalyzed the isomerization of IPP to produce DMAPP. As described below, structure analysis of the reaction

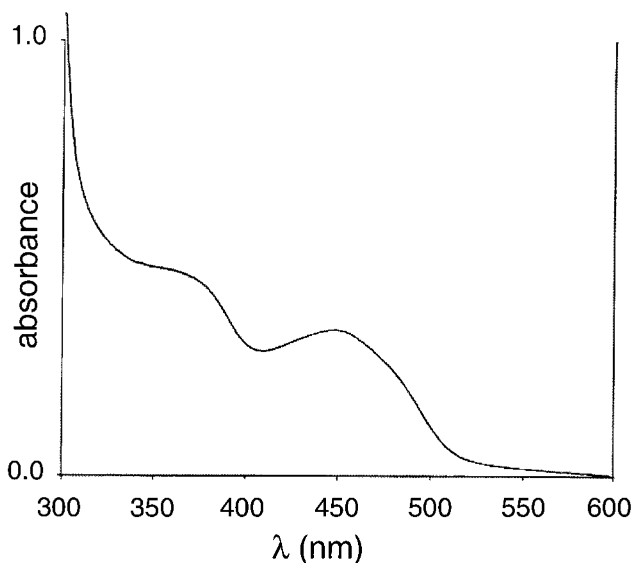

Fig. 3. Absorption spectrum of the recombinant *orfD* product reconstituted with FMN. A fraction containing the highest concentration of protein (2.8 mg/ml) is shown.

product by ^{1}H NMR spectroscopy proved that the *orfD* product converted IPP to DMAPP in the presence of FMN and NADPH.

Table 2 shows the FMN and NAD(P)H dependence of IPP isomerase encoded by *orfD*. Without addition of FMN and NAD(P)H, no activity was detected, demonstrating that IPP isomerization catalyzed by the *orfD* product was strictly dependent on both FMN and NAD(P)H. The activity was maximal when 10 μM FMN and 5 mM NADPH were added. The activity was slightly below maximal when 10 μM FMN and 5 mM NADH were used. However, replacement of FMN with FAD decreased the reaction rate to 51% of the original rate. Replacement of FMN with riboflavin resulted in the loss of IPP isomerase activity. Replacement of NAD(P)H with NAD or NADP also

Table 2. FMN and NAD(P)H dependence of the activity of the reconstituted IPP isomerase

Additions to reaction buffer*		
FMN or FAD, μM	NAD(P)H, mM	Relative activity, %
FMN	NADPH	
0	0	ND
0	0.1	ND
0	0.5	3.6
0	1	6.5
0	5	19
0	10	15
0	20	11
10	0.1	36
10	1	95
10	5	100
10	10	90
10	20	96
	NADH	
10	1	92
10	5	97
FAD	NADPH	
10	5	51

*The basal reaction buffer consisted of 0.1 M HEPES (pH 7.0), 2.5 mM $MgCl_2$, 1 mM DTT, 5 μg enzyme. ND, not detected.

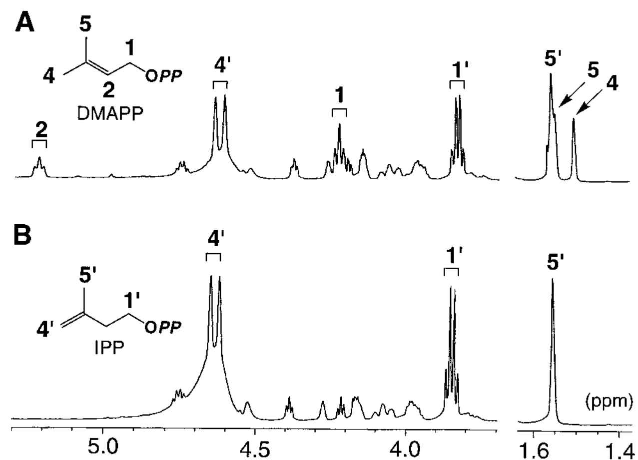

Fig. 4. 1H NMR spectra of the reaction mixtures. The reaction buffer contained 5 mM $MgCl_2$, 1 mM DTT, 10 μM FMN, 2.5 mM NADPH, and 5 mM IPP. The spectra were obtained after incubation for 12 h at 37°C without (*B*) and with (*A*) the *orfD* product (1 mg). Small signals in ≈3.9–4.8 ppm can be ascribed to NADPH.

resulted in the loss of IPP isomerase activity. The FMN- and NAD(P)H-dependence of the *orfD* product was unchanged under anaerobic conditions. The enzyme also required Mg^{2+}, Mn^{2+}, or Ca^{2+}. The activity was maximal with Mg^{2+}, but lower with Mn^{2+} and Ca^{2+}. No effects of other divalent cations such as Co^{2+}, Cu^{2+}, Ni^{2+}, and Zn^{2+} at 5 mM were detected. Addition of 5 mM EDTA resulted in the almost complete loss of enzyme activity.

The IPP isomerase encoded by *orfD* was most active in Hepes buffer. The optimum activity of the enzyme occurred at approximately pH 7.0. The effect of temperature on the enzyme activity was investigated over the range of 15–55°C, with the maximum activity being observed at approximately 35–37°C.

Product Analysis by ^{1}H NMR. The reaction catalyzed by the *orfD* product was analyzed by incubating 5 mM IPP with 1 mg *orfD* product in phosphate buffer containing 10 μM FMN and 2.5 mM NADPH. After 12 h of incubation, conversion of IPP to DMAPP was observed as evidenced by the appearance of additional signals for DMAPP at δ 5.25 (t, J = 6.4 Hz, 1H, H-2), 4.27 (d, $J_{H–H}$ = 6.4, $J_{H–P}$ = 6.1, 2H, H-1), 1.57 (s, 3H, 5-methyl), and 1.53 (s, 3H, 4-methyl) ppm and the concomitant decrease in the intensities of the signals for IPP at δ 4.68 (s, 1H, H-4a), 4.65 (s, 1H, H-4b), 3.87 (dt, $J_{H–H}$ = 6.4, $J_{H–P}$ = 1.7, 2H, H-1), 2.21 (t, J = 6.4, 2H, H-2), and 1.58 (s, 3H, 5-methyl) ppm (Fig. 4). These ^{1}H NMR spectral features were in complete agreement with those of authentic IPP and DMAPP, the spectral data of which were newly collected in this study by using a 500 MHz ^{1}H NMR spectrometer. This result unambiguously established that the *orfD* product catalyzed the isomerization of IPP to produce DMAPP. Thus, *orfD* encodes an FMN- and NAD(P)H-dependent IPP isomerase. The isomerization reaction was reversible, because the enzyme also converted DMAPP to IPP in the presence of FMN and NADPH (data not shown).

The equilibrium ratio of IPP to DMAPP in the reaction mixture was estimated to be 1.4 by comparing the integral of the IPP H-1 signal (2H) at δ 3.87 ppm with that of the DMAPP H-2 signal (1H) at δ 5.25 ppm.

Complementation of the Disrupted *E. coli* IPP Isomerase Gene. We next determined whether *orfD* could complement the disrupted *idi* gene in *E. coli*. Poulter and coworkers (22) reported that disruption of the *idi* gene was not lethal to *E. coli*; this phenom-

enon is explained by the independent formation of IPP and DMAPP in the nonmevalonate pathway without involvement of IPP isomerase (Fig. 1; ref. 18). Therefore, to determine whether *orfD* could complement the disrupted *idi* gene, it was essential to construct *E. coli* disruptants defective in the nonmevalonate pathway in addition to having a disruption of the *idi* gene. We simultaneously disrupted the *idi* gene and the *dxr* gene, which encodes DXP reductoisomerase in the nonmevalonate pathway (Fig. 1), to prepare a new disruptant named DK331 (Table 1). Despite the *idi* and *dxr* disruptions, strain DK331 grew in the presence of 2-*C*-methylerythritol (ME), a biological equivalent of 2-*C*-methylerythritol 4-phosphate that is an intermediate of the nonmevalonate pathway (Fig. 1). DK331 was then separately transformed with pTMV20SΔM and pTMV20S. The pTMV20S contained *orfD* in addition to genes encoding the three enzymes of the mevalonate pathway, MVA kinase, PMVA kinase, and DPMVA decarboxylase, present in pTMV20SΔM.

Having constructed the transformants DK331(pTMV20S) and DK331(pTMV20SΔM), we examined their ability to grow in the absence of ME by using DK223(pTMV20SΔM) as a control strain possessing the endogenous *idi* gene. If the *orfD* product could function as IPP isomerase in DK331, the transformant DK331(pTMV20S) should grow even in the absence of ME. As expected, DK331(pTMV20SΔM) did not grow in the absence of ME. On the other hand, DK331(pTMV20S) and DK223(pTMV20SΔM) grew in the absence of ME. The growth of DK331(pTMV20S) is explained by its ability to synthesize both IPP and DMAPP from MVA added into the growth medium. Thus, in DK331(pTMV20S), IPP was concluded to be synthesized by the actions of MVA kinase, PMVA kinase, and DPMVA decarboxylase encoded on pTMV20S, and then IPP was isomerized by the *orfD* product to give DMAPP. On the other hand, in DK223(pTMV20SΔM), IPP was synthesized as in DK331(pTMV20S) and then isomerized by the endogenous *idi* gene product to give DMAPP. These results demonstrated that the *orfD* product functioned as IPP isomerase *in vivo* and had sufficient IPP isomerase activity to complement the disrupted *E. coli idi* gene.

Streptomyces IPP Isomerase Homologs are Widespread in Archaebacteria and Gram-Positive Bacteria. Homology searches using the sequence of the *Streptomyces* IPP isomerase as a query sequence was performed by using the protein databases SWISS-PROT, of the DNA Data Bank of Japan (DDBJ), and Microbial Genomes Blast, of the National Center for Biotechnology Information (NCBI). Fig. 5 shows the multiple alignment of the amino acid sequences of the *Streptomyces* IPP isomerase and proteins with unknown functions from six organisms, *E. herbicola*, *Synechocystis* sp. strain PCC6803, *S. aureus*, *E. faecalis*, *Methanococcus jannaschii*, and *Sulfolobus solfataricus*. The *Streptomyces* IPP isomerase showed 29% to 46% identity to these six hypothetical proteins. Although only six homologs are shown in Fig. 5, this search revealed that *orfD* homologs also existed as proteins with unknown functions in many bacteria such as *Borrelia burgdorferi*, *Rickettsia prowazekii*, *B. subtilis*, *S. pneumoniae*, *S. pyrogenes*, *Deinococcus radiodurans*, *Halobacterium* sp., *Methanobacterium thermoautotrophicum*, *Archaeoglobus fulgidus*, *Aeropyrum pernix*, *Pyrococcus abyssi*, and *Leishmania major*.

Biochemical Properties of _S. aureus_ IPP Isomerase. Because the database search identified the *S. aureus* IPP isomerase, this recombinant enzyme expressed in *E. coli* was also purified and characterized. SDS/PAGE showed a subunit molecular mass of 39 kDa. Native PAGE gave a single protein band with a mobility corresponding to 150 kDa. The apparent molecular mass was estimated to be 155 kDa by gel filtration. These data indicate that the *S. aureus* IPP isomerase is also most likely to be a tetramer.

Fig. 5. Multiple alignment of the amino acid sequences of *orfD* and other *orfD* homologs. Identical amino acids among six proteins are marked by asterisks. Dashes indicate gaps introduced for the optimization of the alignment. The thick lines represent glycine-rich motifs. orfD, *Streptomyces* sp. strain CL190; Erwi, *E. herbicola* Eho10 (accession no. Q01335); Syne, *Synechocystis* sp. strain PCC6803 (accession no. P74287); Saur, *S. aureus* ATCC25923; Enfa, *E. faecalis* (http://www.tigr.org/); Meta, *M. jannaschii* (accession no. Q58272); Sulf, *S. solfataricus* (accession no. P95997).

This enzyme also catalyzed the isomerization of IPP to produce DMAPP in the presence of FMN and NADPH.

Kinetic Parameters of _Streptomyces_ and _S. aureus_ IPP Isomerases. The kinetic parameters of the *Streptomyces* and *S. aureus* IPP isomerases were calculated and compared with those of FMN- and NADPH-independent IPP isomerases from *E. coli*, *S. cerevisiae*, and humans (Table 3). The *Streptomyces* isomerase with a k_{cat} of 0.70 showed less activity than the human and *S. cerevisiae* enzymes with k_{cat} values of 1.8 and 8.0 s^{-1}, respectively. On the other hand, the *Streptomyces* enzyme showed almost the same activity as that of the *E. coli* isomerase. However, the K_m value of 450 μM for IPP of the *Streptomyces* isomerase was 10- to 50-fold higher than those of other isomerases. The *S. aureus* isomerase has the same catalytic efficiency (k_{cat}/K_m) of 6.8×10^4 $M^{-1} \cdot s^{-1}$ as those of the three FMN- and NADPH-independent IPP isomerases.

Discussion

We have demonstrated in this study that *orfD* in the mevalonate pathway gene cluster of *Streptomyces* sp. strain CL190 encoded an unusual type of IPP isomerase with no sequence similarity to other IPP isomerases identified to date. All of the usual IPP isomerases characterized to date have a conserved cysteine

Table 3. Comparison of enzymatic properties of IPP isomerases

	Monomer, kDa	K_m (for IPP), μM	k_{cat} (for IPP), s^{-1}	k_{cat}/K_m, $M^{-1} \cdot s^{-1}$	Divalent cation	FMN-dependence	NAD(P)H-dependence	Reference
Streptomyces sp. CL190	41	450	0.70	1.6×10^3	Mg^{2+}	yes	yes	This study
S. aureus	39	19	1.3	6.8×10^4	Mg^{2+}	yes	yes	This study
E. coli	25	7.9	0.33	4.2×10^4	Mg^{2+}	no	no	22
S. cerevisiae	26	43	8.0	1.9×10^5	Mg^{2+}	no	no	21
Human	39	33	1.8	5.5×10^4	Mg^{2+}	no	no	20

residue (boldface) in the NXX**C**XHP motif and a conserved glutamate residue (boldface) in the EX**E** motif (22). Neither of the two motifs, however, were found in the type of IPP isomerases characterized in this study. Instead of the motifs, three glycine-rich sequences, MTGG, GXGGT, and (A/G)SGG, were conserved in the new type of IPP isomerases (Fig. 5). Interestingly, these new enzymes required both FMN and NAD(P)H for enzyme reaction. Thus, *orfD* encodes an FMN- and NAD(P)H-dependent IPP isomerase. Therefore, the glycine-rich sequences may be associated with the binding of these cofactors. Based on the difference in enzymatic properties, we classify the IPP isomerases into two types: Type 2 for FMN- and NAD(P)H-dependent enzymes, and type 1 for the others. We renamed *orfD* the *fni* gene for *F*MN- and *N*AD(P)H-dependent IPP *i*somerase.

IPP isomerase encoded by *fni* was a flavoenzyme containing FMN, as evidenced by the UV/visible spectral feature (Fig. 3). Flavoenzymes are classified into six categories based on the nature of the electron acceptors and donors; dehydrogenases, oxidases, oxidase-decarboxylases, monooxygenases, dioxygenases, metalloflavoenzymes, and flavodoxins (29). However, type 2 IPP isomerases do not belong to any of these categories. How do the flavoenzymes such as the *fni* product catalyze the isomerization of IPP to produce DMAPP? In type 1 IPP isomerases, the isomerization of IPP proceeds via a carbocationic intermediate (30). In this mechanism, an electrophilic attack by a proton from water occurs on the IPP double bond to yield the carbocationic intermediate and then the C-2 *pro-R* hydrogen of the intermediate is stereospecifically eliminated (31). In a similar reaction mechanism, the carbocationic intermediate may be involved in the reaction catalyzed by type 2 IPP isomerases. Further investigations are needed to clarify this hypothetical reaction mechanism of type 2 IPP isomerases.

Database searches identified a type 2 IPP isomerase in *S. aureus*, a pathogenic bacterium that is notorious as a methicillin resistant *S. aureus* (MRSA). We cloned and overexpressed this *S. aureus* IPP isomerase in *E. coli* and demonstrated that the recombinant enzyme had FMN- and NAD(P)H-dependent IPP isomerase activity. The essentiality of the type 2 IPP isomerase for *S. aureus* growth and the difference in the reaction mechanism from that of the mammalian type 1 IPP isomerase identify the type 2 enzyme as a promising target for anti-staphylococcal drugs. *S. pneumoniae*, *S. pyrogenes*, and *E. faecalis* IPP isomerases are also considered to be molecular targets for the screening of antimicrobials specific to these Gram-positive bacteria.

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