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Genetic evidence of branching in the isoprenoid pathway for the production of isopentenyl diphosphate and dimethylallyl diphosphate in *Escherichia coli*

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Abstract An alternative mevalonate-independent pathway for isoprenoid biosynthesis has been recently discovered in eubacteria (including *Escherichia coli*) and plant plastids, although it is not fully elucidated yet. In this work, *E. coli* cells were engineered to utilize exogenously provided mevalonate and used to demonstrate by a genetic approach that branching of the endogenous pathway results in separate synthesis of the isoprenoid building units isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). In addition, the IPP isomerase encoded by the *idi* gene was shown to be functional in vivo and to represent the only possibility for interconverting IPP and DMAPP in this bacterium.

Key words: 1-Deoxy-D-xylulose 5-phosphate reductoisomerase; Dimethylallyl diphosphate; Isopentenyl diphosphate; Isopentenyl diphosphate isomerase; Isoprenoid biosynthesis; Mevalonic acid

1. Introduction

Isoprenoids (also called terpenoids) are the most chemically diverse family of compounds found in nature. They are present in all organisms and have essential roles in membrane structure, redox chemistry, reproductive cycles, growth regulation, signal transduction and defense mechanisms. In spite of their diversity of functions and structures, all isoprenoids derive from the common building C_5 isoprene units isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Isoprenoid synthesis is accomplished by a head to tail addition of IPP units to DMAPP by various prenyltransferases to form prenyl diphosphates of different chain length. Further dimerization and cyclization reactions yield the final carbon skeleton of isoprenoids [1,2].

Following the discovery of the mevalonate pathway in the 1950s, it was believed that all isoprenoids derived from mevalonic acid (MVA) synthesized by the condensation of three acetyl-CoA units via aceto-acetyl-CoA and 3-hydroxy-3-methylglutaryl-CoA. Subsequent phosphorylation and decarboxylation of MVA catalyzed by mevalonate kinase (MVK), phosphomevalonate kinase (PMK) and mevalonate diphosphate decarboxylase (MDD) yield IPP, which is then converted to DMAPP by the enzyme IPP isomerase [3]. Recent experiments (reviewed in [4–6]) have demonstrated, however, that the isoprenoids synthesized in plant plastids and many eubacteria, including *Escherichia coli*, derive from IPP and DMAPP produced by a different pathway first described by Rohmer et al. [7] (Fig. 1). The initial reaction of this MVA-independent pathway involves the formation of 1-deoxy-D-xylulose 5-phosphate (DX5P) by condensation of (hydroxyethyl)thiamine diphosphate derived from the decarboxylation of pyruvate with the C1 aldehyde group of D-glyceraldehyde 3-phosphate [8,9]. This reaction is catalyzed by 1-deoxy-D-xylulose 5-phosphate synthase, a novel type of transketolase encoded by the *dxs* gene in *E. coli* [10–12]. In the second step, the enzyme DX5P reductoisomerase (encoded by the *dxr* gene, previously known as *yaeM* in *E. coli*) synthesizes 2-*C*-methyl-D-erythritol 4-phosphate (ME4P) by intramolecular rearrangement and reduction of DX5P [13–15]. Then, ME4P can react with CTP to form 4-diphosphocytidyl-2-*C*-methyl-D-erythritol (CDP-ME), in a reaction catalyzed by the enzyme specified by the *ygbP* gene from *E. coli* [16,17]. Recently, it has been shown that *ychB*, another *E. coli* gene reported to be involved in the pathway, encodes a kinase that phosphorylates the 2-hydroxy group of CDP-ME [18]. The resulting product, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol 2-phosphate, can be then converted by the product of the *ygbB* gene to 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate, the proposed next intermediary of the pathway [19]. The remaining enzymatic reactions leading to the synthesis of the C_5 isoprene unit have not yet been fully elucidated.

Analysis of the fate of the hydrogen atoms of deuterium-labeled 2-*C*-methyl-D-erythritol (ME) and 1-deoxy-D-xylulose (DX) added to the culture medium of *E. coli* cells [20,21], and studies on the stereochemistry of *E. coli* IPP isomerase encoded by the *idi* gene and farnesyl diphosphate (FPP) synthase encoded by the *ispA* gene [22–24] have led to the proposal of the presence of two different routes towards IPP and DMAPP starting from a common intermediate derived from ME4P (Fig. 1). Consistent with this hypothesis, the disruption of the *idi* gene encoding IPP isomerase is not lethal in *E. coli* [25]. Isoprenoids are required for essential functions in *E. coli*, including respiration (ubiquinone) and cell wall biosynthesis (dolichol). Therefore, the normal growth of cells with a disrupted, non-functional *idi* gene indicates that either there are two independent routes for the synthesis of IPP and DMAPP

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or that a second possibility to interconvert both C_5 units is present in *E. coli*. In this paper we have engineered *E. coli* cells to utilize exogenously provided MVA for the production of IPP and used a genetic approach to demonstrate that branching of the isoprenoid pathway occurs, resulting in separate synthesis of IPP and DMAPP. We also show that *idi* functions in converting IPP to DMAPP in vivo and that it is the only gene encoding an enzyme with such activity in *E. coli*.

2. Materials and methods

2.1. Plasmid constructs

Plasmids pUC19 (Pharmacia), pBR322 (Gibco-BRL) and pGEM-T (Promega) were used as vectors for the preparation of constructs to disrupt the *dxr* and *idi* genes in *E. coli*. A genomic region containing the *dxr* gene and extending from the 5′-end of *frr* to the 3′-end of *yaeS* (Fig. 2A) was amplified by polymerase chain reaction (PCR) using primers d1 (5′-GCACACTTCCACTGTGTGTG-3′) and d2 (5′-CCGCATAACACCGCCAACC-3′), digested with *Bgl*II and *Sph*I (Fig. 2A) and cloned in pUC19 previously digested with *Bam*HI and *Sph*I to produce plasmid pMJ4. The *dxr* gene in pMJ4 was disrupted by the substitution of a 0.7 kb *Eco*RI–*Nco*I fragment with the coding region of the marker gene conferring resistance to tetracyclin (*TET*; Fig. 2A) released from pBR322 after digestion with *Eco*RI and *Pfl*MI to produce plasmid pMJ5. A *Sma*I–*Hin*dIII fragment from pMJ5, containing the *Bgl*II–*Sph*I genomic region with the *dxr::TET* disruption, was then cloned in the *Acc*B71–*Hin*dIII sites of pBR322, generating pMJ6.

For disruption of the *idi* gene, the flanking chromosome region was amplified by PCR using primers iF (5′-CCCAAGCTTCATGGTTTGCGATTGTTACGC-3′, annealing on the *ygfU* gene) and iR (5′-CGGGATCCTCTCTGGTATTCGC-3′, annealing on the *lysS* gene) (Fig. 2B) and the resulting 2.5 kb fragment was cloned into pGEM-T to produce pCM1. The *CAT* gene, conferring resistance to chloramphenicol, was excised from plasmid pCAT19 [26] by *Bam*HI digestion and cloned into the *Bcl*I site at the 5′-end of the *idi* gene in pCM1. A PCR fragment amplified with primers i*Pst*I (5′-GGATGGTGCTGCAGGCGACAAATCG-3′, annealing on the 3′-end of the *idi* gene; Fig. 2B) and SP6 (annealing on the *SP6* promoter region of pGEM-T) from pCM2-4, in which the *CAT* gene was cloned in the same transcriptional direction as the rest of the genes in the region (Fig. 2B), was cloned back into pCM2-4 after digestion of both the plasmid and the PCR product with *Pst*I to generate pCMa2, in which the *idi* gene (except the 3′ last 60 nucleotides) was replaced by the *CAT* gene (Fig. 2B).

The synthetic operon to express the coding region of *Saccharomyces cerevisiae ERG12* (MVK) and *ERG19* (MDD) genes and the human *PMK* cDNA under the control of the arabinose-inducible P_{BAD} promoter was constructed in a modified version of plasmid pBAD-GFPuv (Clontech) to create pAB-M2 (Campos et al., in preparation).

2.2. Growth conditions

All strains were grown at 37°C in Luria broth (LB) medium supplemented with tetracyclin (7.5 μg/ml, for selection of the *dxr::TET* disruption), chloramphenicol (17 μg/ml, for selection of the *idi::CAT* disruption) or/and ampicillin (100 μg/ml, for selection of cells carrying the pAB-M2 plasmid). When indicated, the growth medium was supplemented with 0.5 mM ME (synthesized as described [27]), 0.02% (w/v) arabinose or/and 1 mM MVA prepared from a stock of mevalonolactone (Sigma) hydrolyzed by incubating 1 volume of 1 M mevalonolactone with 1.02 volumes of 1 M KOH at 37°C for 30 min.

2.3. Bacterial strains

Some of the bacterial strains used in this work are listed in Table 1. For the construction of a *dxr*-defective strain, plasmid pMJ6 was linearized and used to transform strain JC7623 cells, in which the *recB21*, *recC22* and *sbcB15* mutations allow transformation by linear DNA [28]. Transformed cells with the chromosomal *dxr::TET* disruption were selected on LB medium containing tetracyclin and ME. The disruption was confirmed in colonies showing both tetracyclin resistance and ME auxotrophy by PCR analysis with primers d3 (5′-CTCTGGATGTCATATGAAGCAACTC-3′) and d2 (Fig. 2A). Bacteriophage P1 lysates obtained after infection of one of the selected JC7623 *dxr::TET* clones was used to transduce the disruption into strain MC4100 [29] (Table 1) as described [30]. The resulting MC4100 *dxr::TET* strain was designated EcAB1-2.

A JC7623 *idi::CAT* strain was constructed after transformation of JC7623 cells with a purified PCR product amplified from plasmid pCMa2 with primers iF and iR. Transformed cells harboring the disrupted *idi* gene were selected on LB medium containing chloramphenicol. The disruption was confirmed by PCR analysis with primers iFo (5′-GCTAGCATCATTCAGGTGG-3′) and iR (Fig. 2B) and transduced into strain MC4100 using bacteriophage P1 lysates obtained from JC7623 *idi::CAT* cells. The resulting MC4100 *idi::CAT* strain was designated EcAB1-3. The same JC7623 *idi::CAT* lysate was also used to transduce the *idi::CAT* disruption to strain EcAB1-2, creating the MC4100 *dxr::TET idi::CAT* strain EcAB1-4. Strains EcAB1-2(pAB-M2) and EcAB1-4(pAB-M2) were constructed after transformation of EcAB1-2 and EcAB1-4 cells, respectively, with plasmid pAB-M2 (Table 1).

3. Results and discussion

A genetic approach was taken in order to elucidate the metabolic framework for the synthesis of IPP and DMAPP in *E. coli*. Basically, the approach was to (i) create an *E. coli* strain impaired in the synthesis of ME4P and thus unable to synthesize IPP and DMAPP through the endogenous MVA-independent pathway, (ii) engineer it to synthesize IPP from exogenously supplied MVA and then (iii) evaluate the role of the IPP isomerase encoded by the *idi* gene in the conversion of the MVA-derived IPP into DMAPP.

A complete block of isoprenoid production was accomplished in a strain with a disruption of the *dxr* gene, encoding the enzyme responsible for the production of ME4P, the proposed first precursor specific of the pathway (Fig. 1) [15]. For the construction of the *dxr*-defective strain, an *Eco*RI–*Nco*I fragment from the central region of the *dxr* gene was replaced with a selectable marker conferring resistance to tetracyclin (*TET*; Fig. 2A). The corresponding construct was linearized and used to transform *E. coli* strain JC7623, in which the *recB*

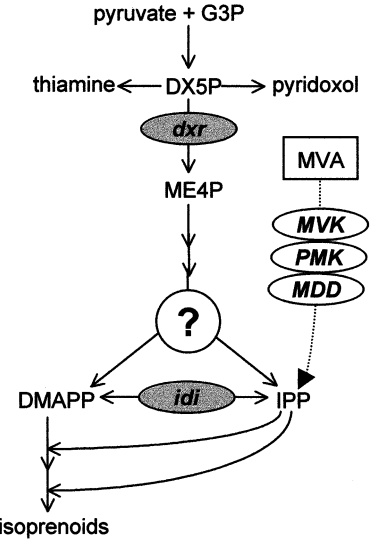

Fig. 1. Isoprenoid biosynthetic pathway in *E. coli* and engineered synthesis of IPP from exogenously supplied MVA. G3P, D-glyceraldehyde 3-phosphate; DX5P, 1-deoxy-D-xylulose 5-phosphate; *dxr*, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; ME4P, 2-*C*-methyl-D-erythritol 4-phosphate; DMAPP, dimethylallyl diphosphate; IPP, isopentenyl diphosphate; *idi*, IPP isomerase; MVA, mevalonic acid; MVK, mevalonate kinase; PMK, phosphomevalonate kinase; MDD, mevalonate diphosphate decarboxylase.

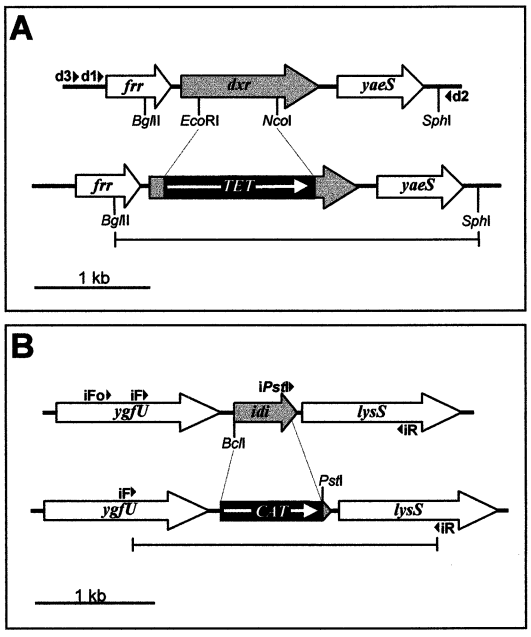

Fig. 2. Insertional disruption of the *dxr* and *idi* genes of *E. coli*. A: Genomic region flanking the *dxr* gene in wild type *E. coli* (upper scheme) and in the *dxr::TET* strains (lower scheme). Arrowheads indicate position of primers d1, d2 and d3. B: Genomic region flanking the *idi* gene in wild type *E. coli* (upper scheme) and in the *idi::CAT* strains (lower scheme). Position of primers iFo, iF and iR is shown. Arrows indicate direction of transcription and bars indicate the regions used for transformation of strain JC7623 cells.

and *recC* mutations inactivate exonuclease, preventing it from degrading the linear DNA, whereas the *sbcB* mutation restores recombination proficiency [28]. After transformation, cells in which the *dxr* gene had been replaced by the *dxr::TET* disrupted version were selected on plates supplemented with tetracyclin. Since the disruption in the *dxr* gene was designed to block the production of isoprenoids (Fig. 1) and therefore was predicted to be lethal in the absence of a source of intermediates for their biosynthesis, ME was also added to the plates. ME was used instead of ME4P since it is known that *E. coli* cells with disrupted genes of the isoprenoid pathway are capable of efficiently using free ME to synthesize isoprenoids [20]. The presence of the *dxr::TET* disruption in the bacterial chromosome was confirmed by PCR analysis. For further studies, the *dxr::TET* disruption was transferred from strain JC7623, which is not a good host for supporting replication of plasmids [28], to strain MC4100 (Table 1) by phage P1 transduction. The MC4100 *dxr::TET* strain was designated EcAB1-2. As expected, EcAB1-2 cells required ME for growth and survival (Fig. 3), confirming that the insertional disruption of the *dxr* gene prevents the synthesis of ME4P and therefore is functional in blocking the production of isoprenoid intermediates.

Table 1
Bacterial strains used in this study

Strain	Description
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 relA1 rpsL150 flbB5301 strA thi deoC7 ptsF25* [29]
EcAB1-2	MC4100 *dxr::TET*
EcAB1-2(pAB-M2)	MC4100 *dxr::TET* transformed with pAB-M2
EcAB1-3	MC4100 *idi::CAT*
EcAB1-4	MC4100 *dxr::TET idi::CAT*
EcAB1-4(pAB-M2)	MC4100 *dxr::TET idi::CAT* transformed with pAB-M2

To evaluate whether an alternative pathway for the production of IPP could rescue ME auxotrophy in EcAB1-2 cells, we engineered *E. coli* for the utilization of MVA, an isoprenoid precursor that is not synthesized in this bacterium. For the cells to be able to transform MVA into IPP, they were transformed with plasmid pAB-M2 harboring a synthetic operon containing the coding regions of *Saccharomyces cerevisiae* genes *ERG12* (encoding MVK) and *ERG19* (encoding MDD) and the human *PMK* cDNA (Fig. 1). The expression of the operon in pAB-M2 was under the control of the P_{BAD} promoter, which can be induced with arabinose. Transformation of EcAB1-2 cells with pAB-M2 yielded strain EcAB1-2(pAB-M2). This strain grew normally on LB plates containing ME, but it was also able to grow on LB plates supplemented with arabinose and MVA (Fig. 3), confirming that pAB-M2 contained a functional operon encoding the enzymes required for the synthesis of IPP from MVA in *E. coli*. In addition, the results also indicated the presence of an endogenous enzymatic activity that was active in converting the MVA-derived IPP into DMAPP.

Synthesis of DMAPP in *dxr*-defective EcAB1-2(pAB-M2) cells grown in the presence of MVA could be accomplished either by the IPP isomerase encoded by the *idi* gene, by a different enzyme encoded by another gene that could also function as an IPP isomerase or by several enzymes allowing the interconversion of IPP and DMAPP. To elucidate the role of *idi* in the synthesis of DMAPP from IPP, a derivative of strain EcAB1-2(pAB-M2) harboring a disruption in the *idi* gene was generated. Replacing the almost entire coding sequence of the *idi* gene with a selectable marker gene encoding chloramphenicol acetyltransferase (*CAT*; Fig. 2) created the *idi::CAT* disruption. JC7623 strain was transformed with the PCR-amplified linear construct and cells harboring a genomic *idi::CAT* disruption were selected on LB plates supplemented with chloramphenicol. As described above for *dxr::TET*, the presence of the *idi::CAT* disruption in the bacterial chromosome was confirmed by PCR analysis and then transduced to

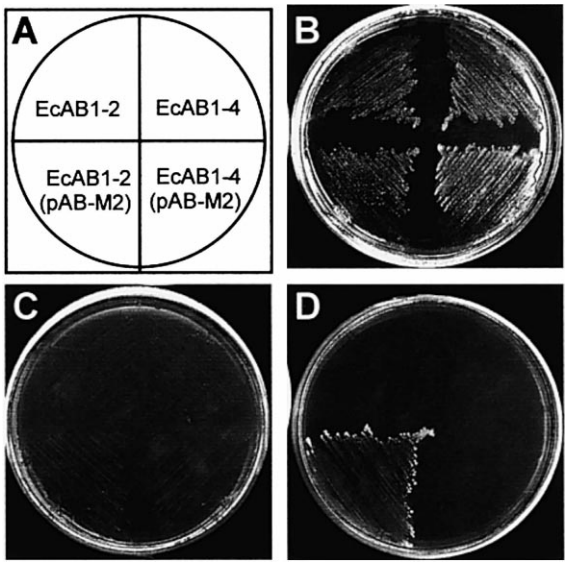

Fig. 3. Growth of the *E. coli* strains indicated in (A) on plates containing LB medium supplemented with tetracyclin and either ME (B), arabinose (C) or MVA and arabinose (D).

strain MC4100 using phage P1. The obtained MC4100 *idi::CAT* strain was named EcAB1-3. As previously reported [25], the absence of the IPP isomerase encoded by *idi* had no apparent harmful effects for growth and survival of *E. coli* EcAB1-3 cells (data not shown), confirming that *idi* is a non-essential gene. The *idi::CAT* disruption was then transferred to strain EcAB1-2 by P1 transduction to generate strain EcAB1-4 harboring a double disruption at the *dxr* and *idi* genes (Table 1). Similar to EcAB1-2, EcAB1-4 cells grew in the presence of ME (Fig. 3). These results indicate that, if there is only one route for the biosynthesis of either DMAPP or IPP in *E. coli*, isomerization is not accomplished by the IPP isomerase encoded by *idi*, thus requiring another possibility to interconvert IPP and DMAPP. The presence of a second gene encoding IPP isomerase, however, appears unlikely since no other sequences showing homology to IPP isomerases from bacterial, fungal, plant or animal sources were identified in the *E. coli* genome after databases searches [25]. Alternatively, our results could be explained by a branching point after ME4P resulting in the independent synthesis of IPP and DMAPP. This possibility has been recently proposed based on experiments of feeding of *E. coli* cells with deuterium-labeled ME [20]. The C3 deuterium of [3,5,5,5-$^2H_4$]ME was only preserved in the DMAPP-derived isoprenic units but was completely lost in all those formed from IPP, suggesting the presence of two different routes towards IPP and DMAPP in *E. coli* [20]. Previous evidence for the independent synthesis of IPP and DMAPP came from studies on the incorporation of deuterium-labelled DX in ubiquinone [21] and the stereochemistry of the *E. coli* IPP isomerase encoded by *idi* and FPP synthase encoded by *ispA* [22,24]. When [4-^{2}H]DX was fed to *E. coli* cultures, the label was incorporated exclusively at the C2 position of the ϖ isoprenoid unit in the side chain of ubiquinone, which arises from DMAPP [21]. Since both IPP isomerase and FPP synthase show the same stereochemistry observed for the eukaryotic enzymes [22,24], the DMAPP incorporated into the ϖ position of the ubiquinone can not be synthesized from IPP by the action of the isomerase encoded by *idi*. These results suggested that either IPP and DMAPP are synthesized independently from an as yet unidentified intermediate or another isomerase activity with a different stereospecificity exists in *E. coli*.

To discriminate between the two hypotheses (or confirm both), the *idi::CAT* disruption was transferred to strain EcAB1-2(pAB-M2) by P1 transduction. Cells harboring both *dxr::TET* and *idi::CAT* disruptions and the pAB-M2 plasmid were selected on LB plates supplemented with tetracyclin, chloramphenicol, ampicillin and ME. The presence of the *dxr* and *idi* insertional disruptions in the chromosome of the new strain, designated EcAB1-4(pAB-M2), was verified by PCR analysis. EcAB1-4(pAB-M2) cells grew normally in LB medium containing ME but they did not grow at all when the LB medium was supplemented with arabinose and MVA (Fig. 3). The obligatory requirement of ME for growth of EcAB1-4(pAB-M2) cells indicated that MVA-derived IPP could not be isomerized to DMAPP in the *idi::CAT* genetic background. A possibility existed, however, that pAB-M2 was not functional in EcAB1-4(pAB-M2) cells and therefore no IPP was synthesized from MVA. To confirm that pAB-M2 remained functional, EcAB1-2 cells containing the *dxr::TET* disruption were transformed with plasmid pAB-M2 isolated from strain EcAB1-4(pAB-M2). The positive clones resulting from the transformation were able to grow in medium containing MVA as the only source for the production of IPP (data not shown), similar to that reported above for strain EcAB1-2(pAB-M2) (Fig. 3). Together, these results demonstrate that the enzyme encoded by the *idi* gene represents the only possibility of interconverting IPP and DMAPP in *E. coli*. The identity of *idi* as a gene encoding IPP isomerase had been established biochemically, but the absence of other known genes for enzymes of the isoprenoid pathway in the same cluster and its non-essential function in *E. coli* pointed against an active role in the synthesis of isoprenoids [25]. Our results show, however, that *idi* can function in converting IPP to DMAPP in vivo and that it actually plays a role in isoprenoid biosynthesis. In spite of its non-essential role during normal growth and survival of *E. coli* cells, it can be speculated that the IPP isomerase encoded by the *idi* gene might have a role in certain metabolic situations. Furthermore, since no other enzyme with IPP isomerase activity exists in *E. coli*, the results obtained with strain EcAB1-4 indicate that the isoprenoid pathway produces both IPP and DMAPP after a branching point. In contrast to *E. coli*, analysis of the fate of the atoms of labeled DX5P in the bacterium *Zymomonas mobilis* [31] and plant cells [32] are consistent with the presence either of a single route or, in the case of a putative branching leading separately to IPP and DMAPP, of a largely predominant route for IPP synthesis. In the latter case, the presence of functional IPP isomerase would be essential. Therefore, it is likely that branching of the MVA-independent isoprenoid pathway might depend on the organism. In the case of *E. coli*, the existence of two separate routes for the production of IPP and DMAPP together with an IPP isomerase encoded by the *idi* gene might optimize utilization of the two central intermediates for isoprenoid synthesis.

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