

## Diversity in isoprene unit biosynthesis: The methylerythritol phosphate pathway in bacteria and plastids\*

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*Abstract:* The long-overlooked methylerythritol phosphate (MEP) pathway represents an alternative to the mevalonate route for the formation of isoprene units. It is found in most bacteria as well as in the plastids of all phototrophic organisms. A selection of significant steps of its discovery and elucidation are presented in this contribution, as well as a complete hypothetical biogenetic scheme for the last reduction step.

*Keywords:* methylerythritol phosphate; mevalonate; phototrophic; mevalonate-independent pathway; biosynthesis of isoprenoids.

### INTRODUCTION: THE UNPROGRAMMED DISCOVERY OF A NOVEL METABOLIC PATHWAY

#### Mevalonate pathway

Life is not always simple. The reducing approach, confirmed by the trends of molecular biology, consisting in unifying the essential processes of life in one single scheme valid for all living organisms suffers indeed many exceptions. In addition, it significantly impedes the interpretation of unexpected experimental results. This is nicely exemplified by the discovery of the mevalonate-independent pathway for biosynthesis of isoprenoids [1,2]. This class of natural products presents the unique feature of a carbon skeleton formally derived from the assemblage of C<sub>5</sub> subunits possessing the branched isoprene skeleton. The biological equivalents of isoprene were identified as isopentenyl diphosphate **9** (IPP) and dimethylallyl diphosphate **10** (DMAPP) (Fig. 1). Investigations on cholesterol biosynthesis in liver tissues and of ergosterol in yeast led to the discovery of the mevalonate pathway [3]. In this pathway, the isoprene units are derived from acetyl coenzyme A, much like the fatty acids. The committed step is the reaction catalyzed by the hydroxymethylglutaryl coenzyme A reductase yielding mevalonate, which was accepted as the universal precursor of all isoprenoids.

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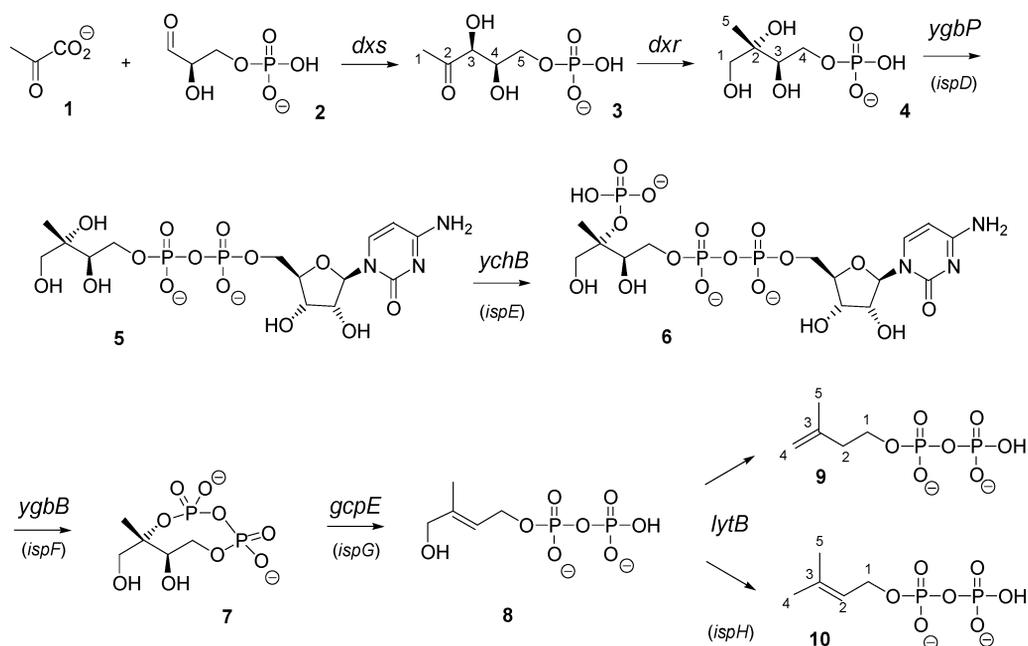


Fig. 1 MEP phosphate pathway for isoprenoid biosynthesis.

### Experimental results in contradiction with the mevalonate pathway

The results from many labeling experiments performed first on plant isoprenoids and later on bacterial isoprenoids were hardly interpretable in the frame of the mevalonate pathway, if not in complete contradiction. For instance,  $^{14}\text{C}$ -labeled mevalonate was only poorly incorporated into the chloroplast terpenoids (e.g., carotenoids or phytol from chlorophylls) [4,5] or in mono- and diterpenoids, whereas phytosterols were always significantly labeled [6,7].  $^{14}\text{C}$ -labeled carbon dioxide was in contrast readily incorporated into the pigments (carotenoids, phytol) from spinach chloroplasts [6,7]. The statins are powerful inhibitors of the hydroxymethylglutaryl coenzyme A reductase and thus prevent the formation of mevalonate. Applied to plant systems, mevinolin efficiently inhibits the biosynthesis of phytosterols in plant cells, but had no activity on the formation of carotenoids and chlorophylls containing the diterpenoid phytol [8,9]. All of these results were interpreted in terms of lack of permeability of the chloroplast membrane toward mevalonate and mevinolin.

Isoprenoid biosynthesis was investigated rather recently in bacteria. This is partly due on the one hand to their low concentration in bacterial cells, making them less suitable for biosynthetic studies, and on the other hand to the apparent lack of diversity of bacterial isoprenoids, at least as compared to that of plant and fungal isoprenoids. The presence of the mevalonate pathway has been confirmed for sterol biosynthesis in the gliding bacterium *Nannocystis exedens* [10] or for carotenoid biosynthesis in a bacterium assigned to a *Flavobacterium* sp. [11]. Such results could not, however, be extended to the formation of isoprenoids in other bacteria. Upon incorporation of [1- $^{14}\text{C}$ ]acetate into the ubiquinone from *Escherichia coli*, a degradation procedure was performed for determining the position of the labeled carbon atoms [12]. This resulted in a labeling pattern, which was different from the pattern expected from the mevalonate pathway. Uniformly labeled [U- $^{13}\text{C}_6$ ]glucose is often utilized as a source of doubly labeled [1,2- $^{13}\text{C}_2$ ]acetate after catabolism. Incorporation of this glucose isotopomer into the pentalenolactone from *Streptomyces* sp. UC 5319 unambiguously revealed at the level of the  $^{13}\text{C}/^{13}\text{C}$  coupling a labeling pattern in one isoprene unit that was not compatible with the mevalonate pathway: three contiguous carbon atoms were derived from a single precursor molecule [13].

This short selection of experimental data points out questions that cannot be satisfactorily answered in the frame of the sole mevalonate pathway. In fact, they represent the signature of an alternative, completely different biosynthetic route. Their interpretation will be obvious in the frame of a novel metabolic pathway.

### Discovery of the methylerythritol phosphate pathway

The discovery of a novel pathway for the formation of isoprene units was an unexpected side-product of our activity in the field of the chemistry and biochemistry of bacterial triterpenoids of the hopane series. Bacterial hopanoids are present in many bacteria [14]. They modulate membrane stability and permeability much like sterols do in eukaryotic membranes [15] and represent the most abundant natural products on earth. Their molecular fossils are found in the organic matter of any sediment, whatever its nature, origin, or age [16]. The major compounds amongst bacterial hopanoids are always the C<sub>35</sub> bacteriohopanepolyols. In contrast with most other bacterial isoprenoids, their intracellular concentration is rather high, of the same order of magnitude as that of sterols in eukaryotes (i.e., 2–30 mg/g, dry weight), making them well suited for <sup>13</sup>C NMR studies. They present also a unique feature in natural products chemistry: a C–C bond between the triterpene hopane skeleton and an *n*-alkyl polyhydroxylated moiety [17]. In order to identify the origin of this side chain, first labeling experiments were performed with [1-<sup>13</sup>C]- or [2-<sup>13</sup>C]acetate [18]. In contrast with all former labeling studies on bacterial isoprenoids reported in the literature that utilized bacterial cells grown on complex media of undefined composition, the incorporations were performed with bacteria grown on a synthetic medium containing only mineral salts and one single <sup>13</sup>C-labeled carbon and energy source. With such experimental conditions, it is sure that the labeled precursor is incorporated, which is not obvious in the case of competition between different substrates in a complex medium. It was also expected that the pathways involved in the metabolism of the substrate could be easily identified. A retrobiosynthetic analysis, starting from the labeling pattern observed on the <sup>13</sup>C NMR spectra, should allow determining the origin of the bacteriohopanepolyol side chain. Of course, nothing special was expected at the level of the isoprene units. After incorporation of <sup>13</sup>C labeled acetate, it was obvious that the side chain was a D-pentose derivative. It was synthesized via the nonoxidative pentose phosphate pathway and linked via its C-5 carbon atom to the hopane isopropyl group. Surprisingly, the labeling pattern found in the isoprene units differed completely from that expected from the mevalonate pathway [17]. There was no reason to reject a priori the mevalonate pathway for hopanoid biosynthesis. An interpretation was proposed, involving compartmentation of activated acetate pools. An alternative was a completely different biosynthetic pathway, but, from these first results of <sup>13</sup>C-labeled acetate incorporation, it was not obvious to propose another plausible hypothetical biogenetic scheme.

## ELUCIDATION OF THE METHYLERYTHRITOL PHOSPHATE PATHWAY

### Origin of the carbon atoms of isoprene units

Decisive clues were obtained by incorporation of <sup>13</sup>C-labeled glucose isotopomers into the hopanoids of the bacterium *Zymomonas mobilis*. The choice of this organism was not fortuitous. It synthesizes large amounts of hopanoids (up to 30 mg/g, dry weight). It utilizes only glucose, and to some extent other hexoses, as carbon and energy source and possesses limited enzymatic equipment with no complete tricarboxylic acid cycle. All of these features make this bacterium well suited for biosynthetic investigations on terpenoid biosynthesis.

Incorporation of glucose isotopomers with single labeling at C-1, C-2, C-3, C-5, and C-6 pointed out the existence of a novel biosynthetic route to isoprene units and definitively excluded the mevalonate pathway. Isoprene units of the hopane skeleton were derived from a three-carbon subunit corresponding to a glyceraldehyde phosphate **2** derivative and from a two-carbon subunit corresponding to

decarboxylated pyruvate **1**. In addition, incorporation of doubly labeled [4,5- $^{13}\text{C}_2$ ]glucose pointed out the intramolecular rearrangement reaction allowing to insert the two-carbon subunit between the carbon atoms derived from C-2 and C-3 from glyceraldehyde phosphate [19]. Incorporation of uniformly labeled [U- $^{13}\text{C}_6$ ]glucose into the hopanoids of *Z. mobilis* and of  $^{13}\text{C}$ -labeled pyruvate or glycerol into ubiquinone of *E. coli* mutants lacking each one enzyme of the triose phosphate metabolism confirmed both the nature of the two subunits and the rearrangement reaction [20]. Similar results requiring the same interpretation were obtained with other bacteria and for other isoprenoid series, such as, for instance, for the prenyl chains of ubiquinone and menaquinone in the non-hopanoid-producing *E. coli* [19]. The formerly found labeling pattern obtained after incorporation of  $^{13}\text{C}$ -labeled acetate [18] is best explained by the insertion of acetate into the glyoxylate and tricarboxylic acid cycle to yield finally pyruvate **1** and glyceraldehyde phosphate **2**, the precursors of the isoprene units.

Independently of our work on bacterial triterpenoids, similar labeling patterns were found by Arigoni's group after incorporation of  $^{13}\text{C}$ -labeled glucose isotopomers into diterpenoids of the ginkgolide and bilobalide series from embryos of *Ginkgo biloba*, whereas sitosterol showed the labeling pattern expected from mevalonate pathway [21]. This was the first proof for the presence of the bacterial mevalonate-independent pathway in plants.  $^{13}\text{C}$ -labeled glucose incorporation established its ubiquity in phototrophic organisms for the formation of the essential isoprenoids of the photosynthetic apparatus (carotenoids, phytol from chlorophylls, and the prenyl chain of plastoquinone) [22]. All odd results concerning the biosynthesis of isoprenoids in plants and bacteria mentioned above could now be easily explained in the frame of this novel mevalonate-independent pathway.

### Toward the identification of intermediates, enzymes, and genes

#### *1-Deoxy-D-xylulose 5-phosphate and 2-C-methyl-D-erythritol 4-phosphate as intermediates*

The former labeling experiments allowed proposing the first steps of a hypothetical biogenetic scheme [19,20]. Condensation of (hydroxyethyl)thiamin diphosphate (as derived from the reaction catalyzed by pyruvate decarboxylase) on the carbonyl group of glyceraldehyde phosphate affords 1-deoxy-D-xylulose 5-phosphate **3** (DXP) in a reaction resembling that catalyzed by the transketolase. An intramolecular rearrangement of an  $\alpha$ -ketol followed by the concomitant reduction of the resulting aldehyde, much like in the biosynthesis of valine, gives 2-C-methyl-D-erythritol 4-phosphate **4** (MEP). DXP and MEP were known compounds. Free 1-deoxy-D-xylulose (DX) was found in the broth of a *Streptomyces* sp. DXP was known as an intermediate in the biosynthesis of pyridoxol phosphate and thiamin diphosphate in *E. coli*. Free methylerythritol (ME) is accumulated in many plants, and the corresponding 2,4-cyclo-diphosphate is accumulated in some gram-positive bacteria in oxidative stress conditions [1,23].

Incorporation of synthetic deuterium-labeled isotopomers of DX [24] or ME [25,26] into the prenyl chains of ubiquinone and menaquinone from *E. coli* represented the first proofs that these compounds were involved in isoprenoid biosynthesis. The gene of the first enzyme was identified by its homology with the transketolase gene [27–29]. The DXP synthase is as expected a thiamin diphosphate-dependent enzyme and catalyzes the condensation of (hydroxyethyl)thiamin resulting from pyruvate decarboxylation on glyceraldehyde phosphate. Analysis of *E. coli* mutants requiring free ME for their growth shed light on the second gene of the pathway encoding the DXP reducto-isomerase [30]. Again, as expected, this protein catalyzes the rearrangement of DXP into 2-C-methyl-D-erythrose phosphate and its concomitant reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reduction into MEP **4** [31,32]. This gene has no homology with that of the acetolactate synthase catalyzing an apparently similar reaction. The mechanism of the DXR-catalyzed reaction seems a retro-aldol/aldol reaction rather than the acido-catalyzed rearrangement of an  $\alpha$ -ketol [33]. Unlike DXP, MEP has presently no other function than that of isoprenoid precursor. This suggests that the DXR-catalyzed reaction is the committed step of the mevalonate-independent pathway. Accordingly, it was proposed at the 4<sup>th</sup> TERPNET (an international affiliation of researchers working on all aspects of terpenes and iso-

prenoids) meeting in Barcelona (April 1999) to name the pathway after this intermediate, which is the first one possessing the branched C<sub>5</sub> isoprenoid skeleton, and which may be considered as a hemiterpene.

#### *From 2-C-methyl-D-erythritol 4-phosphate to 2-C-methyl-D-erythritol 2,4-cyclodiphosphate*

Addition of synthetic <sup>3</sup>H-labeled MEP to a crude cell-free system from *E. coli* led to the isolation of a radioactive metabolite possessing features of a nucleoside derivative of ME. A database search for genes encoding enzymes utilizing a polyol phosphate and a nucleotide triphosphate ended up in finding the *acsI* gene encoding a *Haemophilus influenzae* enzyme coupling ribitol 5-phosphate with cytidine 5'-triphosphate (CTP) yielding the cytidine 5'-diphosphate (CDP) adduct of ribitol [34]. This *acsI* gene showed homologies with the unannotated *ygbP* gene from *E. coli*, which always accompanies the *dxr* and *dxs* genes. Incubation of the recombinant YgbP protein with MEP and nucleotide triphosphates showed that CTP was the best substrate and afforded 4-diphosphocytidyl ME **5** [35]. A systematic computer search for genes accompanying the *dxs*, *dxr*, and *ygbP* genes retrieved the unannotated *yhbB* and *ygbB* genes. The latter genes respectively encode the ATP-dependent enzyme phosphorylating the C-4 tertiary hydroxyl of 4-diphosphocytidyl ME yielding 4-diphosphocytidyl ME 2-phosphate **6** [36], which is converted by the YgbB enzyme into ME 2,4-cyclodiphosphate **7** [37]. All three novel intermediates were readily incorporated into the carotenoids of *Capsicum annuum* chromoplasts.

The same genes were independently found by screening *E. coli* mutants possessing a metabolic block between MEP and IPP. Since such a mutation is lethal, an *E. coli* transformant was constructed possessing all genes required to convert mevalonic acid (MVA) into IPP. With such a transformant, mutants with an obligatory MVA requirement indicating a damage of genes implied in the isoprenoid biosynthetic pathway were prepared. This led to the characterization of the YgbP [38], YchB [39], and YgbB [40] enzymes and of their substrates and reaction products.

This three-reaction sequence between MEP **4** and ME cyclodiphosphate **7** does not modify the oxidation state of the C<sub>5</sub> skeleton of ME. This means that the last steps imply elimination and reduction reactions to yield IPP **9** and DMAPP **10**.

### **On the origin of hydrogen atoms of isoprene units**

#### *Incubation of deuterium-labeled deoxyxylulose or methylerythritol*

Knowledge of the origin of the hydrogen atoms of the isoprene units delivered interesting clues for deciphering the last two steps. They were obtained by incorporation of deuterium-labeled isotopomers of DX and ME. Free DX is usually readily incorporated into the isoprenoids from bacteria or plants after phosphorylation into DXP by a nonspecific xylulose kinase [41,42]. ME is only incorporated into isoprenoid by a few bacteria, including *E. coli*, also after phosphorylation by the sorbitol phosphotransferase system found in *Salmonella enterica* serovar *typhimurium* [43]. Complete deuterium retention in the isoprene units of the prenyl chains of ubiquinone and menaquinone of *E. coli* was observed after incorporation of labeled DX with deuterium at C-1 [44] or C-5 [24] or ME with deuterium labeling at C-1 [26,45], C-4 [26], or C-5 [45]. Striking and apparently contradictory results, depending on the investigated organisms, were obtained when deuterium labeling was at C-4 in DX or C-3 in ME. Incubation of a *Catharanthus roseus* cell culture with [2-<sup>13</sup>C,4-<sup>2</sup>H]DX resulted in <sup>13</sup>C incorporation in all isoprene units of phytol and carotenoids, indicating that the precursor was incorporated, accompanied by a complete loss of deuterium [46]. Feeding a wild-type *E. coli* with [4-<sup>2</sup>H]DX resulted in deuterium labeling in the sole isoprene unit derived from DMAPP serving as starter for the prenyl transferase with no trace of deuterium in those derived from IPP [47]. Incubation of [3,5,5,5-<sup>2</sup>H<sub>4</sub>]ME was performed with an *E. coli* mutant with a disrupted *dxs* gene. This allowed efficient incorporation, as all isoprene units had to be derived from the labeled precursor. The intact trideuterated methyl group was found intact in all isoprene units, whereas quantitative retention of the ME C-3 deuterium was only observed on carbon atoms corresponding to C-2 of DMAPP in the terminal isoprene unit derived from

DMAPP, all those corresponding to IPP being devoid of deuterium in this position [45]. A similar dual labeling pattern with differentiation between the isoprene units derived from IPP and DMAPP was found in cineol, a monoterpene from *Eucalyptus globulus*, upon incubation of [4-<sup>2</sup>H]DX [48]. Finally, a third labeling pattern was found for the plastid isoprenoids (phytoene and the prenyl chain of plastoquinone) of a tobacco Bright Yellow-2 cell culture after incorporation of [4-<sup>2</sup>H]DX. All isoprene units were deuterium labeled with the same isotope abundance on the carbon atoms corresponding to C-2 of IPP as well of DMAPP [49].

#### *Independent synthesis of isopentenyl diphosphate and dimethylallyl diphosphate*

The different labeling pattern found in *E. coli* for isoprene units derived from either IPP or DMAPP suggested for them a different origin. This hypothesis was comforted by the fact that in *E. coli* deletion of the *idi* gene, encoding the enzyme responsible for the interconversion IPP and DMAPP, does not affect the growth of the mutant [50]. Definitive proof for a branching in the MEP pathway leading separately to IPP or DMAPP was obtained by genetic methods [51]. Deletion of the *dxr* gene can be rescued either by addition of ME to the culture medium, or by mevalonate after introduction of the genes required for the utilization of mevalonate, which is not a normal metabolite for *E. coli*, i.e., mevalonate kinase, phosphomevalonate kinase, and diphosphomevalonate decarboxylase. After deletion in this mutant of the *idi* gene, the bacteria do not grow anymore in the presence of mevalonate, indicating that the *idi*-encoded protein was the only enzyme capable of interconverting IPP and DMAPP in *E. coli*. They grew, however, normally in the presence of ME, showing that IPP and DMAPP can be separately synthesized in the MEP pathway from a yet unidentified precursor.

Satisfactory interpretation of all results presented above was only obtained once the pathway was fully elucidated and sufficient information was available on the reactions catalyzed by the last two enzymes.

#### **Last steps catalyzed by Fe/S enzymes: A unified hypothetical biogenetic scheme**

Only two genes, *gcpE* and *lytB*, accompany the formerly described genes of the MEP pathway and were thus candidates for encoding the last two enzymes. Their role in the MEP pathway was demonstrated using *E. coli* strains engineered for the utilization of exogenous mevalonate as described above. Deletion of either *gcpE* [52] or *lytB* [53] in such a mutant was rescued by the addition of mevalonate in the culture medium. This indicated that both genes are essential in isoprenoid biosynthesis. The nucleotide sequence of *gcpE* [54,55] as well as *lytB* [56] showed homologies with iron/sulfur cluster enzymes. This indication was of prime importance as most of these enzymes are quite oxygen-sensitive and must therefore be manipulated under an inert atmosphere.

#### *GcpE: The conversion of methylerythritol cyclodiphosphate into 4-hydroxydimethylallyl diphosphate*

A first indication on the reaction catalyzed by the GcpE protein was obtained with an *E. coli* strain with a deleted *dxr* gene, engineered for the utilization of mevalonate and overexpressing the *gcpE* gene. Incubation of such cells with synthetic tritium-labeled ME led to the accumulation of radiolabeled ME cyclodiphosphate **7**, suggesting that ME cyclodiphosphate was the substrate of GcpE [57]. Incubation of <sup>14</sup>C-labeled ME cyclodiphosphate with a crude cell-free system from an *E. coli* strain overexpressing GcpE in the presence of a cocktail of cofactors led to formation of 4-hydroxydimethylallyl alcohol. The formation of a nonphosphorylated product was not expected and resulted most likely from the hydrolysis of a phosphorylated derivative by the endogenous phosphatases. Addition of a phosphatase (which does not cleave ME cyclodiphosphate) resulted in the isolation of 4-hydroxydimethylallyl alcohol as the only radiolabeled compound next to the starting material [58]. A final incubation in the presence of fluoride ions as phosphatase inhibitor afforded 4-hydroxy DMAPP **8**, which was identified by comparison with synthetic material as the reaction product of the GcpE-catalyzed reaction [59]. This

first result was confirmed by enzyme tests performed with other *E. coli* cell extracts [54] and later with the purified GcpE or LytB [59,60].

*LytB: The conversion of 4-hydroxydimethylallyl diphosphate into isopentenyl diphosphate and dimethylallyl diphosphate*

A similar approach was utilized for the characterization of the *lytB* gene. In an *E. coli* strain genetically engineered for the utilization of mevalonate, deletion of the *lytB* gene was lethal and could only be rescued by addition of mevalonate to the growth medium. In such culture conditions, 4-hydroxy DMAPP **8** was detected owing to its strong immunostimulant properties. Accumulation was even sufficient for a direct identification by spectroscopic methods [61].

An elegant *in vivo* proof for the role of the GcpE and LytB enzymes was obtained by incorporation of [U- $^{13}\text{C}_6$ ]DX in *E. coli* strains overexpressing enzymes of the MEP pathway [62] and analysis of the accumulated metabolites by  $^{13}\text{C}$  NMR of the crude cell free extract. Overexpression of all genes upstream of GcpE resulted in the accumulation of ME cyclodiphosphate. Additional expression of GcpE led to the appearance of 4-hydroxy DMAPP **8** on the one hand, and of GcpE and LytB to that of IPP **9** and DMAPP **10** in a 5:1 ratio on the other hand. This comforted ME cyclodiphosphate and 4-hydroxy DMAPP as substrates of GcpE and LytB, respectively, and 4-hydroxy DMAPP and IPP and DMAPP as products of the same enzymes and showed that LytB corresponds to the branching point of the MEP pathway.

*GcpE and LytB: Two [4Fe-4S] enzymes*

A bioinformatic search and comparison of nucleotide sequences revealed that GcpE and LytB possess a [4Fe-4S] prosthetic group. Both enzymes are characterized by three conserved cysteines serving as anchors for the Fe/S cluster [55,56]. Like many Fe/S enzymes, GcpE and LytB are oxygen-sensitive and loose prosthetic group and activity in the presence of air. Upon reconstitution of the Fe/S cluster under an inert atmosphere and in the presence of  $\text{Fe}^{3+}$ , sulfide and dithiothreitol, both enzymes show a UV-absorption spectrum with a maximum at 410 nm characteristic for [4Fe-4S] cluster [59,63]. This cluster was directly characterized in the reconstituted GcpE by Mössbauer spectroscopy. The main component was a [4Fe-4S] $^{2+}$  cluster with three tetrahedral Fe linked to four sulfur ligands and one tetrahedral Fe linked to three sulfur and a fourth non-sulfur ligand [64]. In reconstituted LytB, the [4Fe-4S] $^{1+}$  cluster was identified after dithionite reduction by electron paramagnetic resonance (EPR) spectroscopy [63]. The as-isolated enzyme presented a signal characteristic for a [3Fe-4S] $^{2+}$  cluster representing about 10 % of the protein. The same cluster was also detected by EPR in the enzyme isolated under an inert atmosphere [65], but without the reduction step required for the identification of the [4Fe-4S] cluster, which gives only an EPR signal in its reduced form. This [3Fe-4S] $^{2+}$  cluster most probably corresponds to a degradation product in the presence of oxygen.

Both reactions catalyzed by GcpE and LytB imply elimination and reduction. The cluster itself may simultaneously act as Lewis acid, facilitating the elimination, as well as reducing agent involved in one-electron transfers. The enzyme activity of GcpE and LytB is thus only developed in the presence of an accompanying reducing system allowing the reduction of the oxidized [4Fe-4S] $^{2+}$  form into the reduced [4Fe-4S] $^{1+}$  form. This is best performed for the *E. coli* enzymes by the natural biological system flavodoxin/flavodoxin reductase/NADPH [59,63]. The flavodoxin encoding gene *fldA* has been found essential for *E. coli* and is supposed to be mainly involved in the reduction of Fe/S clusters of GcpE and LytB [66]. Chemical reducing agents such as the semiquinone radical of 5-deazaflavin [59,63] or even dithionite [67,68] are also effective. In the cyanobacterium *Thermosynechococcus elongatus*, GcpE interacts with ferredoxin and activity was found in the presence of the enzyme and the NADPH-dependent reducing shuttle system ferredoxin/ferredoxin oxido-reductase [69]. The same interaction was found between ferredoxin and LytB from *Plasmodium falciparum* [70]. Flavodoxin is absent in plant chloroplasts. The most likely surrogate for this reducing system is ferredoxin, which is implied in the electron transport chain of photosynthesis. Indeed, GcpE from *Arabidopsis thaliana* is capable of converting ME cyclodiphosphate **7** into 4-hydroxy DMAPP **8** in the sole presence of spinach

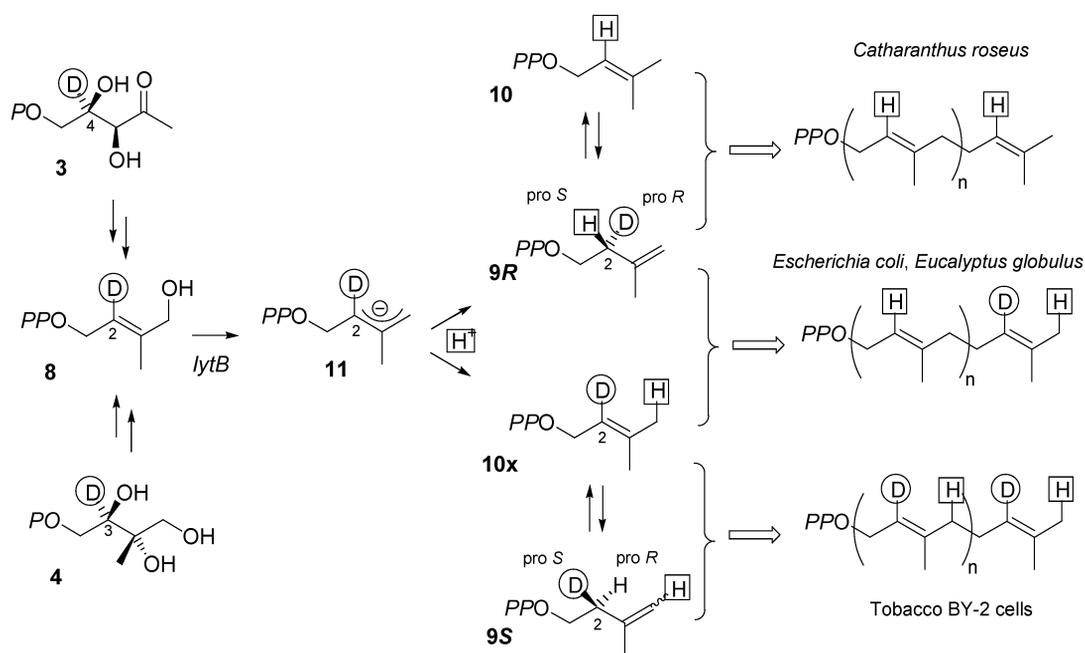
thylakoids and light and in the absence of NADPH [71]. No reaction occurs in the dark. This shows that the electrons released by the photo-oxidation of water can be directed via photosystems I and II and ferredoxin onto the Fe/S cluster of GcpE. In the dark or in nonphotosynthetic tissues such as roots, ferredoxin has most likely to be reduced by the NADPH-dependent ferredoxin oxido-reductase.

#### *LytB-catalyzed reaction IPP and/or DMAPP branch for the synthesis of isoprene units*

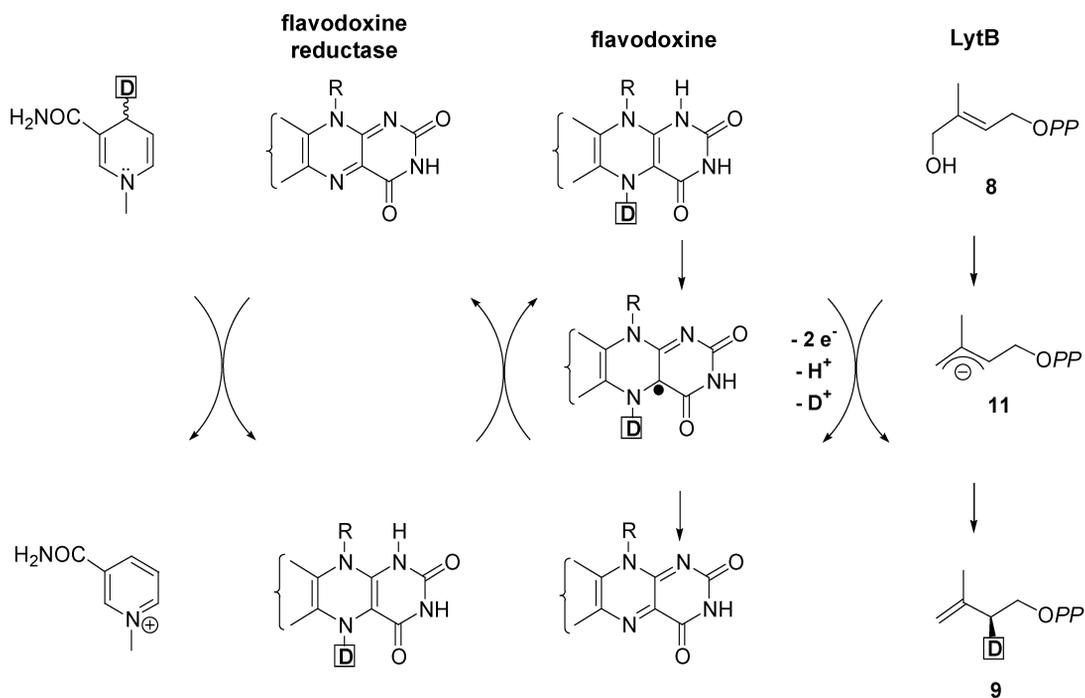
The LytB-catalyzed reaction implies elimination of the hydroxy group of 4-hydroxy DMAPP **8** and two one-electron transfers in an order to be determined. This yields the allylic anion **11** as hypothetical intermediate (Fig. 2). Protonation at C-2 affords IPP and at C-4 DMAPP. The transferred hydrogen is most likely a proton as shown by overall six-fold deuterium discrimination in favor of  $^1\text{H}$  when the enzyme test is performed in 90 % heavy water with the *E. coli* LytB [72]. In vivo, the proton donor might not be water. In an experiment designed to detect the reduction steps in the MEP pathway, the bacterium *Z. mobilis* was grown on [ $1\text{-}^2\text{H}$ ]glucose as sole carbon and energy sources. Most of the hexose is converted in anaerobic growth conditions into ethanol. This bacterium has no complete tricarboxylic acid cycle. This implies that the reducing agent pools of NAD(P)H are derived from glucose catabolism. Through the action of glucose 6-phosphate oxidase, glyceraldehyde phosphate dehydrogenase, and ethanol dehydrogenase, a pool of the two C-4 deuterated diastereomeric dihydropyridine of NAD(P) $^2\text{H}$  are synthesized in a 1:1 ratio [73]. Any NAD(P)H-dependent reduction should leave a deuterium signature on the carbon atom where the reduction occurs. On the triterpenic hopane skeleton, deuterium was thus found on all carbon atoms derived from C-4 of DMAPP and IPP corresponding to the NADPH-dependent reduction linked to DXR as well at C-11 and C-12 corresponding to the reduction occurring in the conversion of presqualenol diphosphate into squalene performed by the squalene synthase. The presence of an additional deuterium was found on carbon atoms corresponding to C-2 of IPP **9** [74]. This is precisely the site of the protonation of the allylic anionic intermediate **11** in the LytB-catalyzed reduction. This raises a puzzling question. Either the LytB-catalyzed reaction is different in *Z. mobilis* and *E. coli*, or more likely the mechanism is rather similar in the two bacteria. In the latter case, the deuterium from NAD $^2\text{H}$  has to be finally released as a  $^2\text{H}^+$ . This is possible via a system like the NAD $^2\text{H}$ /flavodoxin reductase/flavodoxin: the reduced flavin of flavodoxin releases in the presence of LytB two electrons serving to the reduction of the oxidized Fe/S cluster, one proton  $^1\text{H}^+$  and one deuterium  $^2\text{H}^+$  involved in the protonation of the allylic anion (Fig. 3).

#### *IPP and/or DMAPP branch for the synthesis of isoprene units*

A last open question remains to be addressed: the apparently contradictory labeling patterns observed in different organisms in the isoprene units after incorporation of DX labeled with deuterium at C-4 or ME with deuterium labeling at C-3. In order to propose a single coherent biogenetic scheme (Fig. 2), one has to assume that the deuterium at C-4 of DX or C-3 of ME is preserved in the whole MEP pathway and found in IPP **9** and DMAPP **10**. Taking into account the enantioselectivity of the next two enzymes found in isoprenoid biosynthesis, i.e., the IPP isomerase and the *trans*-prenyl transferase, which both remove the pro-*R* proton of IPP, the protonation of the allylic anion **11** has to occur on the *si* face in the LytB-catalyzed reaction, yielding (*2R*)-[ $2\text{-}^2\text{H}$ ]IPP **9R** and [ $2\text{-}^2\text{H}$ ]DMAPP **10** [72]. From this precursor pool for isoprene units, all observed labeling patterns could be satisfactorily explained. In *C. roseus*, isoprene units are solely derived from the IPP branch: this results in deuterium loss in DMAPP synthesized from (*2R*)-[ $2\text{-}^2\text{H}$ ]IPP **9R** by the IPP isomerase and in the IPP-derived units by the *trans*-prenyl transferase. In *E. coli* and to some extent in *E. globulus*, isoprenoids are synthesized from [ $2\text{-}^2\text{H}$ ]DMAPP resulting from the LytB-catalyzed reaction (i.e., with deuterium retention) and from (*2R*)-[ $2\text{-}^2\text{H}$ ]IPP **9R** with deuterium loss by the prenyl transferase, without significant contribution of the IPP isomerase. Finally, in the tobacco Bright Yellow-2 cells, deuterium retention in all isoprene units results from their origin from the DMAPP branch: deuterium retention occurs of course in the [ $2\text{-}^2\text{H}$ ]DMAPP-derived units as well as in those derived from (*2S*)-[ $2\text{-}^2\text{H}$ ]IPP **9S** synthesized from [ $2\text{-}^2\text{H}$ ]DMAPP by the IPP isomerase and retaining their deuterium upon chain elongation by the prenyl transferase.



**Fig. 2** Hypothetical biogenesis of IPP and DMAPP from [4-<sup>2</sup>H]deoxyxylulose or from [3-<sup>2</sup>H]ME in the MEP pathway. In the IPP isomerase and prenyl transferase-catalyzed reactions, the pro-R hydrogen of IPP is eliminated.



**Fig. 3** LytB-catalyzed reaction in the bacterium *Z. mobilis*: hypothetical mechanism for deuterium transfer from NADP<sup>2</sup>H onto IPP.

## METHYLERYTHRITOL PHOSPHATE PATHWAY: FUTURE DEVELOPMENTS

The mevalonate pathway is still the only pathway found in animal, fungi, some eubacteria, and all investigated archaea. It is also the pathway occurring in the plant cytoplasm, where it is responsible for the biosynthesis of sterols, the prenyl chain of ubiquinone, and, to some extent, of sesquiterpenes. The MEP pathway is found in most eubacteria [75], in the plastids of all phototrophs (plants, algae) [22] as well of phylogenetically related nonphototrophic organisms (e.g., *Plasmodium* spp., the parasites responsible of malaria) [76]. Interestingly, it is the only pathway found in unicellular green algae [77].

The discovery of an alternative route for isoprenoid biosynthesis opened unexpected novel trends. In plants, the dichotomy between cytoplasmic and plastidial isoprenoid metabolism is not always clear-cut. Cross-talk and intermediate exchange is regularly observed between the two compartments. In tobacco Bright Yellow-2 cells, one pathway can even replace the other in the presence of a pathway specific inhibitor [78]. Such aspects concerning the regulation of the biosynthesis of plant isoprenoids have never been considered in the past. MVA and MEP pathways respond differently for the emission of volatile terpenoids in the case of injury [79]. MEP pathway follows a nycthemeral rhythm for the emission of flower scents of terpenoid origin [80].

The MEP pathway is found in pathogenic bacteria responsible for severe diseases, in opportunistic pathogens involved in nosocomial infections as well as in the parasite responsible of malaria. It is, however, absent, in mammals and humans. This points out each enzyme of this pathway as an unexplored target for the design of novel types of antibacterial and antiparasitic drugs [75,76]. The concept has been validated by the discovery that fosmidomycin, a long-known antibiotic, is indeed an inhibitor of DXR, the second enzyme of the MEP pathway [81], and is active against bacteria [75] as well as the malaria parasite [76,82].

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