Formation of Isoprenoids

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Abstract

Isoprenoids, also known as terpenoids, are a diverse group of metabolites produced in all free-living organisms. They play an indispensable role in a wide variety of essential processes but also contribute to a better adaptation to the environment in the form of specialized secondary metabolites. In spite of their notable structural and functional diversity, all isoprenoids are synthesized from the same metabolic precursors, which are then converted into prenyl diphosphates of increasing length. Such basic prenyl diphosphate intermediates represent the starting point of downstream pathways leading to the formation of the vast diversity of end products. Here we present an overview of isoprenoid biosynthesis in microbes from the three kingdoms of life, namely, bacteria, archaea, and eukaryotic microorganisms (mainly microalgae and yeast), with a special emphasis on the research conducted during the last decade. We also discuss the main functional classes of isoprenoids occurring in these microorganisms by focusing in the representative model organisms of each kingdom. Finally, we examine key research needs in this field. This includes expanding our understanding of secondary isoprenoid metabolism in microbes, examining the evolutionary relationships between the two core biosynthetic pathways and improving our ability to engineer production of industrially useful isoprenoids in microbes.

1 Introduction

Isoprenoids are evolutionarily one of the oldest known classes of biomolecules. They were originally identified as key components of cell membranes and were widely used as taxonomic markers, even in fossil history (Chappe et al. 1979; Langworthy and Pond 1986). Today they represent the largest family of natural products, with more than 70,000 different compounds described (Dictionary of Natural Compounds: http://dnp.chemnetbase.com). Isoprenoids, also known as terpenoids, are produced in all free-living organisms. As a group, they are extraordinarily diverse structurally and functionally. They play essential primary roles in fundamental processes, including respiration (quinones), photochemical conversion (chlorophylls, bacteriochlorophylls, bacteriorhodopsins, carotenoids, plastoquinones), electron transport (cytochromes), protein translation and modification (prenylation), membrane integrity (sterols, bactoprenols, hopanoids, ether-type lipids in archaea), and antioxidant protection (carotenoids, quinones, isoprene) (Gershenzon and Dudareva 2007). These groups of compounds are highly

conserved in the evolutionary scale. Isoprenoids also have myriad secondary metabolic functions, and many of them are also industrially relevant compounds, having uses as biofuels, fragrances, drugs, pigments, or nutraceuticals (Vickers et al. 2015).

Plants are the organisms that provide most of the structural and functional diversity found in the isoprenoid family, primarily due to their very complex secondary metabolism for the interaction of the plant with the environment. Prokaryotes and eukaryotic microorganisms tend to have a less complex secondary isoprenoid metabolism. In model microbes such as *E. coli* and yeast, isoprenoid diversity is relatively limited, with isoprenoid products being mostly primary metabolites. In other classes of microbes, a more extensive secondary isoprenoid metabolism is present. For example, some non-photosynthetic bacteria and fungi produce nonessential carotenoids (which in photosynthetic organisms are usually considered as essential auxiliary pigments of the photosynthetic light-harvesting apparatus) to protect from photodynamic reactions and act as scavengers of oxygen radicals (Frank 1999); and some actinomycete and streptomyces strains produce isoprenoid-based antibiotics (Dairi 2005). However, sometimes the line between primary and secondary isoprenoids becomes rather blurry. This is the case, for instance, for isopentenyl tRNA. Addition of an isopentenyl molecule to some tRNA increases the efficiency of translation and reduces the sensitivity to the codon context both in eukaryotes and prokaryotes reducing the mutation rate (Persson et al. 1994). This provides the cell with a competitive advantage that is not environment-specific; is this a primary or secondary function?

2 Biosynthesis of Isoprenoids

Isoprenoid biosynthesis can be divided into a three-stage process that includes two relatively conserved core sets of reactions (1 and 2 below), and a third stage encompassing a set of variable reactions leading to the enormous variety of isoprenoid end products (Fig. 1):

1) Biosynthesis of the C5 prenyl phosphate universal isoprenoid precursors
2) Assembly of polyprenyl diphosphate backbones
3) Addition of functional groups and modification/decoration of the carbon backbone

These three stages take places in all organisms, but the third is especially diverse and extended in plants and support a tremendous variety of reactions including cyclization, dimerization, oxidation, or isomerization among others. This stage is more limited among prokaryotes, which mainly, but not only, produce functional isoprenoids through removal of the diphosphate and addition of the prenyl backbone to a target moiety (e.g., condensation to quinone head groups).

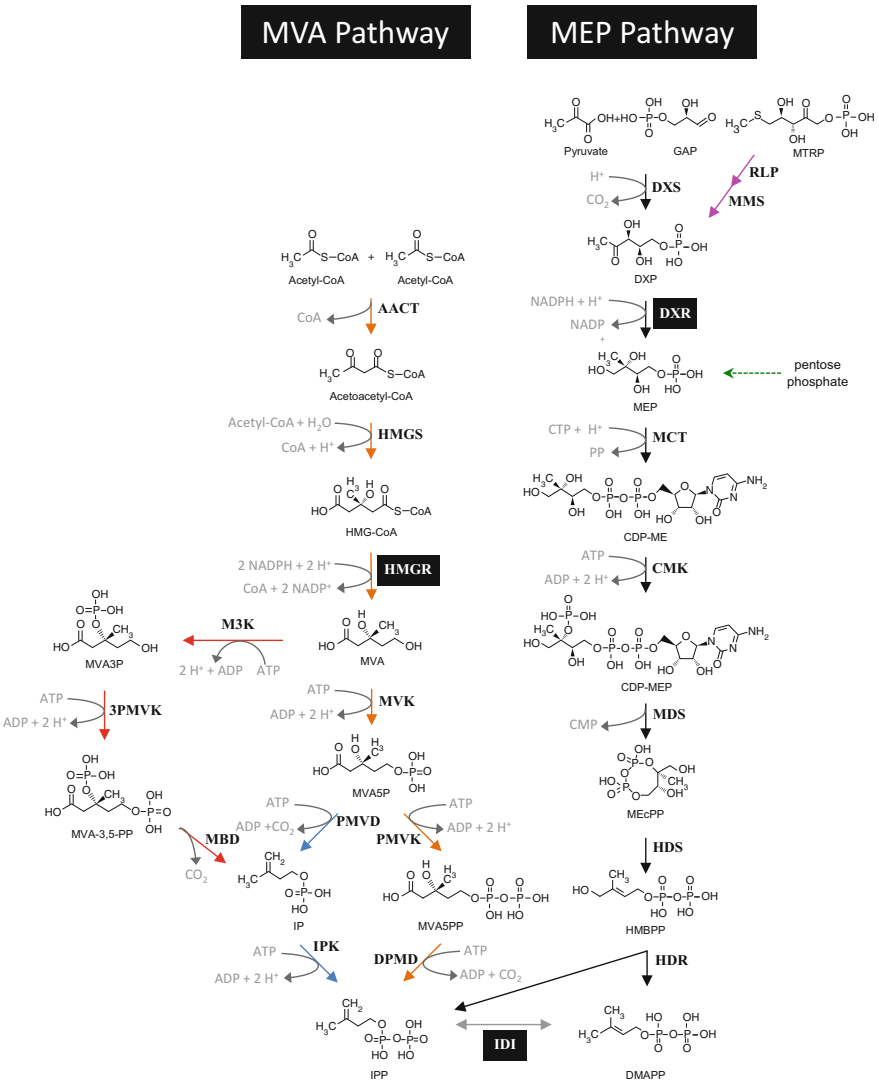

Fig. 1 Pathways for the biosynthesis of isoprenoid precursors in microbes. The canonical MEP pathway steps are shown in *black arrows* (*GAP* D-glyceraldehyde 3-phosphate, *DXP* 1-deoxy-D-xylulose 5-phosphate, *MEP* 2-C-methyl-D-erythritol 4-phosphate, *CDP-ME* 4-diphosphocytidyl-2-C-methyl-D-erythritol, *CDP-MEP* 4-diphosphocytidyl-2-C-methyl-D-erythritol-2-phosphate, *MEcPP* 2-C-methyl-D-erythritol 2,4-cyclodiphosphate, *HMBPP* 4-hydroxy-3-methylbut-2-enyl diphosphate, *IPP* isopentenyl diphosphate, *DMAPP* dimethylallyl diphosphate). The alternative MEP pathway initiation discovered in *Rhodospirillum* is shown in *purple arrows* (MTRP, 5-methylthio-D-ribulose 1-phosphate), and the alternative pathway initiation proposed for *Synechocystis* is shown in *green dashed arrow*. The canonical or eukaryotic MVA pathway is presented in *orange arrows* (*HMG-CoA* 3-hydroxy-3-methylglutaryl-CoA, *MVA* mevalonic acid, *MVA5P* 5-phosphomevalonate, *MVA5PP* 5-diphosphomevalonate). The alternative steps of the archaeal MVA pathway are shown in *blue arrows* (*IP* isopentenyl phosphate), and the alternative

2.1 Two Nonhomologous Pathways Produce the Universal Isoprenoid Precursors IPP and DMAPP

Despite their astonishing diversity both at the structural and functional level, all isoprenoids are derived from the same C5 universal building blocks, isopentenyl diphosphate (IPP), and its isomer dimethylallyl diphosphate (DMAPP). In nature, two chemically unrelated pathways are responsible for the synthesis of IPP and DMAPP. The mevalonate (MVA) pathway is present mainly in eukaryotes and archaea, whereas the 2-methyl 3-erythritol-4-phosphate (MEP) pathway is found in most bacteria (including many important pathogens). In plants both pathways coexist, but in different subcellular compartments: the MVA pathway is located in the cytosol, and the MEP pathway is located in the plastids. IPP is the final product in the MVA pathway, whereas the last step of the MEP pathway yields both IPP and DMAPP simultaneously (Fig. 1).

2.1.1 MVA Pathway

The MVA pathway was discovered during the 1960s and was the first pathway identified for the biosynthesis of isoprenoids (Lynen 1967). For many decades, it was considered to be the only pathway producing IPP/DMAPP and was therefore thought to be ubiquitous in all living organisms. The "canonical" MVA pathway is considered to be the pathway as it was elucidated in eukaryotic organisms, though plasticity is present in other organisms (see below for details; Fig. 1). In the first two steps of the canonical or eukaryotic MVA pathway, the sequential condensation of three molecules of acetyl-CoA produces acetoacetyl-CoA and then 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), catalyzed by the enzymes acetoacetyl-CoA thiolase (AACT) and HMG-CoA synthase (HMGS), respectively. HMG-CoA is then converted to MVA in an irreversible reaction that represents the first committed step of the pathway and is catalyzed by the enzyme HMG-CoA reductase (HMGR). MVA is sequentially phosphorylated to 5-phosphomevalonate by mevalonate kinase (MVK) and then to 5-diphosphomevalonate by 5-phosphomevalonate kinase (PMVK). Finally, decarboxylation of 5-diphosphomevalonate catalyzed by 5-diphosphomevalonate decarboxylase (DPMD) generates IPP. DPMD catalyzes a two-step reaction involving an ATP-dependent phosphorylation at a 3-OH position of the mevalonate moiety prior to the decarboxylation. An isomerase (IDI) forms DMAPP from IPP in a reaction that is required to complete a fully functional MVA pathway (Fig. 1). The enzymes involved in the "canonical" MVA pathway have been recently reviewed (Miziorko 2011).

This canonical pathway is also found in a few eubacteria, including three gram-positive cocci, *Staphylococcus aureus*, *Streptococcus pneumonia*, and *Enterococcus*

Fig. 1 (continued) thermoplasma-type MVA pathway is shown in *red arrows* (*MVA3P* mevalonate 3-phosphate, *MVA-3,5-PP* mevalonate 3,5-bisphosphate). Boxed enzymes correspond to those with more than one type described. Enzyme acronyms (*in bold*) are described in the text

faecalis and the spirochete *Borrelia burgdorferi* (Lombard and Moreira 2011). In addition to the canonical MVA pathway, some eubacterial species (e.g., *Listeria monocytogenes* and some *Streptomyces* strains) also contain the MEP pathway. In *Listeria monocytogenes* despite both pathways contribute to the synthesis of IPP/DMAPP, it has been described that HMGR is an essential gene, and the MEP pathway cannot support cell growth by itself (Heuston et al. 2012). However, MEP pathway mutants are impaired in infection of mice. Interestingly, the closely related nonpathogenic *Listeria innocua* contains the MVA pathway and functional genes for the four initial steps of the MEP pathway but lacks the last two, which have been lost during evolution possibly during adaptation to a nonpathogenic life cycle (Begley et al. 2008). In the case of *Streptomyces*, the MEP pathway is used mainly for the synthesis of essential isoprenoids, whereas the MVA pathway is used to produce specialized secondary metabolites such as antibiotics due to its transcriptional regulation activated during the late phase of growth (Hamano et al. 2002).

Archaea also harbor an MVA pathway but with variation from the canonical pathway. The increasing availability of fully sequenced archaeal genomes has allowed to conclude that this kingdom of life only uses the MVA pathway, as no single representative has been found to date that also contains the MEP pathway. However, gene analyses for the full set of enzymes of the MVA pathway systematically failed to identify the genes for the last two steps of the canonical pathway, PMVK and DPMD. These two genes are absent in most archaea with only a few exceptions including halophilic archaea and *Thermoplasma* relatives, which contain a DPMD-like gene and some thermoacidophilic archaea showing ortologues for both PMVK and DMPD (Lombard and Moreira 2011).

Recent experiments have demonstrated that IPP in most archaea is produced by switching the order of last phosphorylation and decarboxylation steps. In this alternative route, 5-phosphomevalonate is converted to isopentenyl phosphate (IP) by 5-phosphomevalonate decarboxylase (PMD) and subsequently phosphorylated by IP kinase (IPK) to yield IPP (Fig. 1). IPK was first identified in *Methanocaldococcus jannaschii* (Grochowski et al. 2006). More recently, the alternative MVA route was completed with the identification of a PMVD-encoding sequence in green non-sulfur bacteria (Dellas et al. 2013). Even more recently, a phosphomevalonate decarboxylase together with an isopentenyl phosphate kinase were identified and characterized in *Haloferax volcanii*, showing how the archaeon decarboxylation step occurs prior to phosphorylation (Vannice et al. 2014).

Furthermore, in 2014 a second alternative MVA pathway was suggested in *Archaea* (Azami et al. 2014). Very recent experiments have demonstrated the existence of a thermoplasma-type MVA pathway suggested to be unique among the archaeal order thermoplasmatales (Vinokur et al. 2016). In this new alternative route, MVA is sequentially phosphorylated to mevalonate 3-phosphate by mevalonate kinase (M3K) and then to mevalonate 3,5-bisphosphate by mevalonate 3-phosphate 5-kinase (3PMVK). Finally, an ATP-independent decarboxylation of mevalonate 3,5-bisphosphate catalyzed by mevalonate 3,5-bisphosphate decarboxylase (MBD) generates IP, which in turn is phosphorylated by the previously mentioned IPK to produce IPP (Fig. 1). M3K was first identified and characterized

in *Thermoplasma acidophilum* (Azami et al. 2014) and immediately after in another extreme acidophile, *Picrophilus torridus*. 3PMVK and MBD (Vinokur et al. 2016) have also been identified in *Thermoplasma acidophilum* to complete a fully functional alternative MVA pathway. The identification of this new pathway in the most acid-tolerant organisms has been associated to an evolutionary adaptation to extremely acidic environments.

2.1.2 2-C-Methyl-D-Erythritol 4-Phosphate Pathway (MEP)

Over the years following discovery of the MVA pathway, inconsistencies in labeling experiments led to significant confusion, as the labeling patterns could not be explained by the known biochemistry of the MVA pathway. In the early 1990s, new labeling experiments revealed the existence of an alternative pathway for IPP and DMAPP production from pyruvate and glyceraldehyde 3-phosphate (Rohmer et al. 1993). The MEP pathway, also known as the non-mevalonate, Rohmer or DXP pathway, comprises seven enzymatic steps (Fig. 1; (Frank and Groll 2017)) that are best characterized in the gram-negative model organism *Escherichia coli*. The first step involves the condensation of pyruvate with the C1 aldehyde group of D-glyceraldehyde 3-phosphate to produce 1-deoxy-D-xylulose 5-phosphate (DXP) and eliminate one molecule of CO_2 in a reaction catalyzed by DXP synthase (DXS). In *E. coli*, DXP represents a branch point of the pathway as DXP is also used for the production of pyridoxal phosphate (vitamin B6) and thiamine diphosphate (vitamin B1). In the next step DXP is converted to MEP by the enzyme DXP reductoisomerase (DXR) in a reaction that involves an intramolecular rearrangement and posterior reduction and represents the first committed step of the pathway. In the next three successive steps, cytidylation, phosphorylation, and cyclization, reactions are catalyzed by the enzymes MEP cytidylyltransferase (MCT), 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol kinase (CMK), and 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (MEcPP) synthase (MDS), leading to the formation of MEcPP, a cyclic diphosphate molecule with an unusual structure cyclized through the phosphate groups (Fig. 1). In the sixth step, 4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP) synthase (HDS) catalyzes the opening reduction of the ring to form HMBPP. In the last step of the pathway, HMBPP is transformed simultaneously into a mixture of IPP and DMAPP at a 5:1 ratio by the enzyme HMBPP reductase (HDR). The simultaneous production of IPP and DMAPP by HDR makes the isomerase (IDI) a nonessential step of the MEP pathway. Despite this, it is well established that overexpression of IDI results in an increase in isoprenoid end products. This suggests that the enzyme can play an important role in balancing the availability of the universal building blocks for the production of downstream compounds. The MEP pathway has been reviewed elsewhere (Rodriguez-Concepcion and Boronat 2002). During the elucidation and characterization of the pathway, different nomenclatures were adopted to designate orthologous bacterial and plant genes leading to some confusion. A unified nomenclature for the MEP pathway genes, enzymes, and intermediates was proposed to mitigate this problem (Phillips et al. 2008).

2.1.3 Plasticity and Diversity in Isoprenoid Pathways

Since isoprenoids are essential, selection forces act to maintain isoprenoid production but not always necessarily at the individual enzyme level. Microorganisms show an astonishing biochemical and metabolic diversity (mainly in prokaryotes) as a consequence of their ability to adapt to a wide range of environmental conditions. This plasticity is also reflected on isoprenoid biosynthesis at many levels. In the last decade, extensive work has revealed the existence of a variety of alternative steps in both pathways as well as lateral gene transfer (LGT) events which transferred whole pathways and specific alternative steps between kingdoms (Perez-Gil and Rodriguez-Concepcion 2013).

The existence of two nonhomologous biochemically unrelated pathways to produce the same universal precursors represents the first and most obvious layer of diversity and plasticity. Also, as noted, the final two steps of the MVA pathway are variable in Archaea (Dellas et al. 2013), and some bacteria harbor an MVA pathway instead of a MEP pathway (Dairi 2005). However, plasticity can also be found at the enzyme level in both pathways at different enzymatic steps. These alternative steps are distributed in various phyla, but unlike the alternative archaeal MVA pathway, they are not necessarily widely distributed in one phylum.

In the MVA pathway, two different classes of homologous HMGR have been identified based on sequence alignments and phylogeny. Class 1 (HMGR-I) enzymes are found mainly in eukaryotes and archaea, whereas class 2 (HMGR-II) are predominantly found in bacteria harboring the MVA pathway. However, HMGR-II is present in some archaeal orders, including Thermoplasmatales and Archeoglobales, in what has unambiguously identified as a LGT event (Boucher et al. 2001). The two classes of HMGR enzymes, which have been proposed to arise by divergent evolution from a common ancestor, share less than 20% amino acid identity and show major differences around the active site. As a consequence of these differences, the two classes of enzymes show a different level of sensitivity to statins, a family of blockbuster drugs first isolated from fungi that inhibit HMGR activity. Statins are widely used to lower cholesterol levels in humans through strong inhibition of the activity of HMGR-I, but they show a very poor effect on HMGR-II enzymes. This might explain the substitution of HMGR-I by HMGR-II enzymes in bacteria as well as the ability of some archaea to incorporate and maintain this type of enzyme even in the presence of an active HMGR-I (Friesen and Rodwell 2004).

Different enzymes catalyzing the same reaction are also described in the MEP pathway. Two different enzymes can catalyze the first committed step of the pathway to produce MEP from DXP. Most bacteria have a canonical DXR enzyme (DXR-I) to catalyze this reaction, but recently a new type of DXR-like enzyme (DRL or DXR-II) was discovered in a heterogeneous group of MEP pathway-containing bacteria having no DXR homolog (Sangari et al. 2010). DXR-II belongs to a family of previously uncharacterized proteins with oxidoreductase features but with similarity to DXR-I only in the NADPH-binding domain and a different arrangement of the active sites (Perez-Gil et al. 2012a). Moreover, not all DXR-II proteins participate in the MEP pathway. Some organisms have a DXR-II homolog, but do not have a MEP pathway; in this case, the homolog does not in fact have DXR activity.

Phylogenetic analyses support a single origin of DXR-II and these DXR-II homologs, followed by functional divergence in different phyla. This represents a quite unique model of convergent evolution in the case of the nonhomologous DXR-I and DXR-II enzymes with DXR activity and divergent evolution in the case of DXR-II enzymes with different functions. The patchy distribution of DXR-II in specific eubacteria is best explained by gene loss (Carretero-Paulet et al. 2013). This mechanism would explain the unique presence of DXR-I in the majority of bacteria, the presence of only DXR-II in some, and the presence of both DXR-I and DXR-II in a few strains.

The last step in both MVA and MEP pathways is catalyzed by IDI. Two different types of enzymes have also been identified, IDI-I and IDI-II. Even though they catalyze the same reaction, the two classes show no sequence similarity, have unrelated structures, and have different reactions mechanisms involving different cofactors (Berthelot et al. 2012). Type I (IDI-I) enzymes, which were the first identified and studied extensively in the 1950s, are found in eukaryotes and in most bacteria. Type II (IDI-II) were first identified in *Streptomyces* 15 years ago (Kaneda et al. 2001) and then shown to be also present in archaea and some other bacteria. Type I enzymes are zinc metalloproteins utilizing a divalent metal in a well-established protonation−deprotonation reaction. In contrast, type II enzymes are flavoproteins that require a reduced form of flavin mononucleotide (FMN) produced by NADPH and Mg^{2+}. However, the mechanism of reaction is still under investigation. A radical mechanism was first proposed, but recently a protonation−deprotonation mechanism similar to the one observed for IDI-I was suggested (Berthelot et al. 2012). Interestingly, some bacteria possess either IDI-I or IDI-II, some possess both of them, but a large proportion do not contain homologs for any IDI protein. As IDI is not essential for the MEP pathway, it is feasible that they do not have an IDI activity.

In addition to enzymatic plasticity at specific nodes of the pathway, there is also some evidence that the MEP pathway can initiate from alternative central carbon precursors. The cyanobacterium *Synechocystis* PCC6803 has homologs for all the MEP pathway genes. However, no effect was observed on isoprenoid biosynthesis in photoautotrophically grown cells either when supplementing the pathway with pathway substrates/intermediates or when pharmacologically blocking the pathway (Ershov et al. 2002). This is surprising, as both approaches result in significant modification of isoprenoid production in other organisms. Conversely, feeding-labeled phosphorylated sugars of the pentose phosphate pathway resulted in incorporation of labeled IPP into isoprenoids (Perez-Gil and Rodriguez-Concepcion 2013). This suggested alternative pathway is not completely understood, but together these results suggest that photosynthesis-derived products of the pentose phosphate pathway could be used as alternative substrates for IPP biosynthesis.

A more recent but better understood alternative MEP pathway initiation route has been identified in another photosynthetic bacterium, *Rhodospirillum rubrum* (Warlick et al. 2012). In this alternative pathway, DXP is produced in two catalytic steps from 5-methylthio-D-ribulose 1-phosphate (MTRP) instead of pyruvate and G3P. MTRP is an intermediate of the methionine salvage pathway, and is isomerized

to 1-methylthio-D-xylulose 5-phosphate (MTXP) by a RuBisCO-like protein (RLP). MTXP is finally converted into DXP by MTXP methylsulfurylase (MMS). Genes encoding for RLP and MMS are present in several bacterial groups producing MTRP, making feasible a potential wider distribution of this alternative pathway initiation route. Interestingly, all these bacteria also have genes encoding DXS (the enzyme producing DXP from pyruvate and G3P), which still appears to be the main contributor to IPP/DMAPP production (Perez-Gil and Rodriguez-Concepcion 2013).

The contribution of these alternative routes linking the adenosylmethionine (SAM)-dependent polyamine metabolism or the pentose phosphate pathway with isoprenoid biosynthesis remains to be investigated. However, it is possible that bacteria harboring the canonical pathway as well as alternative routes could potentially use both pathways to synthesize their isoprenoids in response to specific environmental conditions.

The differential distribution of the two main pathways producing the universal precursors for the biosynthesis of isoprenoids, the MVA present in mammals (including humans), and the MEP present mostly in bacteria (including several pathogens as well as the protozoa parasite Plasmodium falciparum) along with the essential role of isoprenoids provides a new promising target for the development of much needed antibiotics (Rodriguez-Concepcion 2004). Potentially, drugs inhibiting any of the seven enzymatic steps of the MEP pathway could lead to the development of antibiotic drugs.

Additionally, the existence of different types of enzymes in some steps of both pathways provides a good opportunity for the development of highly specific drugs. Drugs targeting only HMGR-II (present in bacteria) but with no effect on HMGR-I (present in humans) could be used against eubacterial pathogens harboring this specific isoform (Friesen and Rodwell 2004). To date no good inhibitors showing specificity for HMGR-II have been identified yet. DXR-I and DXR-II represent another node species-specific. The active site of these enzymes shows a different arrangement what could be used to design highly specific antibiotics against only one of the enzyme types (Perez-Gil et al. 2012a). Most relevant would be the identification of drugs selectively targeting pathogens harboring DXR-II to develop a new generation of antibiotics without affecting beneficial bacteria in the human gut carrying DXR-I enzymes.

However, even the MEP pathway represents a promising target for the development of new antibiotics against pathogens the above-described remarkable diversity and plasticity observed in bacteria for isoprenoid biosynthesis requires special attention since represents mechanisms to overcome pharmacological blockage that apply to specific organisms. Several pieces of evidence showing the ability of bacteria to bypass inhibition of single enzymes have been already reported highlighting the requirement of accurate designs and rational development. Reduced uptake (Brown and Parish 2008), shunt pathways (Ershov et al. 2002), alternative enzymes (Perez-Gil and Rodriguez-Concepcion 2013), or spontaneous mutations (Perez-Gil et al. 2012b) are some of the mechanisms already described when inhibiting isoprenoid biosynthesis that requires higher level of attention. Currently, great effort

is focused on the identification and development of antibiotics directed against almost all of the enzymes of the MEP pathway. Despite all this effort to date, only one drug targeting the second enzyme of the MEP pathway (DXR), fosmidomycin (FSM), has reached clinical trials to fight malaria, and results show that further work is required. Phase II trials using FSM alone do not retrieve the expected cure rates, and phase III trials in combination with clindamycin also failed due to a parasite recrudescence after an initial promising high cure rate seven days posttreatment (Fernandes et al. 2015).

2.1.4 Evolution of the Pathways Producing Universal Precursors of Isoprenoids

It is often generalized that the MVA pathway is present in eukaryotes and (in slightly modified form) in archaea, whereas the MEP pathway is present in bacteria. However, a detailed exploration of the genomic data available reveals a much more patchy distribution of these pathways – in particular, among eubacteria and microalgae. In some cases, bacteria from the same phylogenetic group use different pathways; and in others, unrelated bacteria use the same pathway. Obviously, this distribution cannot be described by simple vertical inheritance of genetic information. Phylogenomic analyses have confirmed that the MEP pathway is restricted to bacteria and to the plastids of eukaryotes (Lombard and Moreira 2011). However, the canonical MVA pathway has been identified in bacterial and archaeal clades as well as the eukarya. Presence in prokaryotes has traditionally been attributed to lateral gene transfer (LGT) events among the three kingdoms of life. While LGT is not a rare phenomenon – for instance, LGT is thought to be responsible for almost 18% of *E. coli* genes (Lawrence and Ochman 1998) – LGT cannot exclusively explain the patchy distribution of the MVA pathway.

The absence of homologs for the last three steps of the classical MVA pathway in archaea was first explained by the recruitment of nonhomologous enzymes to catalyze the same reaction. This was supported by the discovery of the nonhomologous IDI-II in *Streptomyces* (Kaneda et al. 2001) that subsequently was found in most archaeal genomes. However, this is not the case for the remaining unidentified steps, which were recently identified to complete what now is generally accepted as the alternative MVA pathway in Archaea (Vannice et al. 2014). Despite sporadic cases of LGT, the alternative MVA pathway seems to be ancestral in archaea suggesting that actually three ways to produce IPP would exist – one of each for the three kingdoms of life (Lombard and Moreira 2011).

The uneven taxonomic distribution of the two major pathways has led to the proposal of two different hypotheses about their evolutionary origin. Some authors propose that the two pathways emerged in completely independent evolutionary events, one in the bacterial lineage and one in the archaeal lineage (Lange et al. 2000). However, recent phylogenetic studies suggest that not all the bacteria bearing the MVA pathway acquired the genes by LGT from archaea or eukaryotes (Lombard and Moreira 2011). This data provides the framework for a completely different evolutionary hypothesis: that the classical MVA pathway is in fact the common ancestral pathway, which was inherited in all three kingdoms and modified (in the

case of the alternate MVA pathway) or replaced (under appropriate selective conditions) by the MEP pathway following its evolution.

These two hypotheses have relevant implications with respect to the nature of the cenancestor (the last common ancestor of all organisms). An independent emergence of the two pathways implies the absence of lipid membranes in the cenancestor. In this case, the cenancestor would either be noncellular or surrounded by a mineral structure. In contrast, if the MVA pathway is in all three kingdoms, then it follows that this pathway was present in the cenancestor. This in turn implies the possibility that the common ancestor had membranes containing isoprenoids (Lombard and Moreira 2011). After being a matter of debate for many years, the presence of lipid membranes in the cenancestor is now becoming generally accepted (Lombard et al. 2012; Sojo et al. 2014).

The distribution and evolution of the two pathways in algae is even more complex. In all higher plants, the MEP pathway is always found in chloroplast, and the MVA is found in the cytosol; however, microalgae show a much more diverse distribution of the pathways. The chrysophyte *Ochromonas danica* and the red alga *Cyanidium caldarium* use both pathways, but the green algae *Scenedesmus obliquus*, *Chlorella fusca*, and *Chlamydomonas reinhardtii* produce all their isoprenoids through their chloroplastic MEP pathway. The euglenophyte *Euglena gracilis* was originally thought to produce all its isoprenoids (including plastidial isoprenoids) using the MVA pathway due to the lack of label incorporation in phytol from feeding labeled glucose or pyruvate. Later work supported a lack of label incorporation in phytol but also demonstrated label incorporation in carotenoids – demonstrating that, despite the lack of labeled phytol, the MEP pathway clearly contributes to plastidial isoprenoid biosynthesis (Lohr et al. 2012).

Evolution of eukaryotic phototrophs involved different symbiotic events that lead to the coexistence of both pathways. In the primary endosymbiosis, a heterotrophic eukaryote (harboring the MVA pathway) engulfed a cyanobacterium that ultimately became the plastid in some of the algae phyla (mostly green algae). Subsequently, another heterotrophic eukaryote (also harboring the MVA pathway) engulfed a green alga (already harboring both the MVA and MEP pathways) to become eukaryotic endosymbionts that were finally reduced to plastids (secondary endosymbiosis). Other secondary symbiotic events involving red algae have been suggested as well. In this scenario, different coexistence of the pathways has produced a variety of pathway distributions, including coexistence of the two pathways in algae. However, the overall story suggests that the MEP pathway is essential for all plastid-bearing organisms (Lohr et al. 2012).

2.2 Prenyl Transferases

Prenyl transferases (also known as prenyl diphosphate synthases) catalyze the condensation of an acceptor (that may or may not be isoprenoid-derived) to an allylic diphosphate. Three different types of prenyltransferase reactions are described. The first one catalyzes head-to-tail reactions (mainly producing *trans*-

prenyl diphosphates from DMAPP); the second catalyzes head-to-head reactions (using prenyl diphosphates to produce tri- and tetra-terpenes) or head-to-middle (leading to irregular isoprenoids); and the third involves the insertion of prenyl chains on a non-isoprenoid acceptor (alkylation), for example, in ubiquinone biosynthesis (Bouvier et al. 2005; Oldfield and Lin 2012).

The three core prenyl transferase enzymes involved in the biosynthesis of the main backbones for the production of isoprenoid end products are the enzymes geranyl diphosphate synthase (GDS), farnesyl diphosphate synthase (FDS), and geranylgeranyl diphosphate synthase (GGDS) (Fig. 2). The reaction is initiated at the primary allylic diphosphate, DMAPP; sequential addition of IPP results in the linear all-*trans*-prenyl phosphates of increasing length, starting with the C10 geranyl diphosphate (GPP), then the C15 farnesyl diphosphate (FPP), and then the C20 geranylgeranyl diphosphate (GGPP). A new C-C bond is generated between two

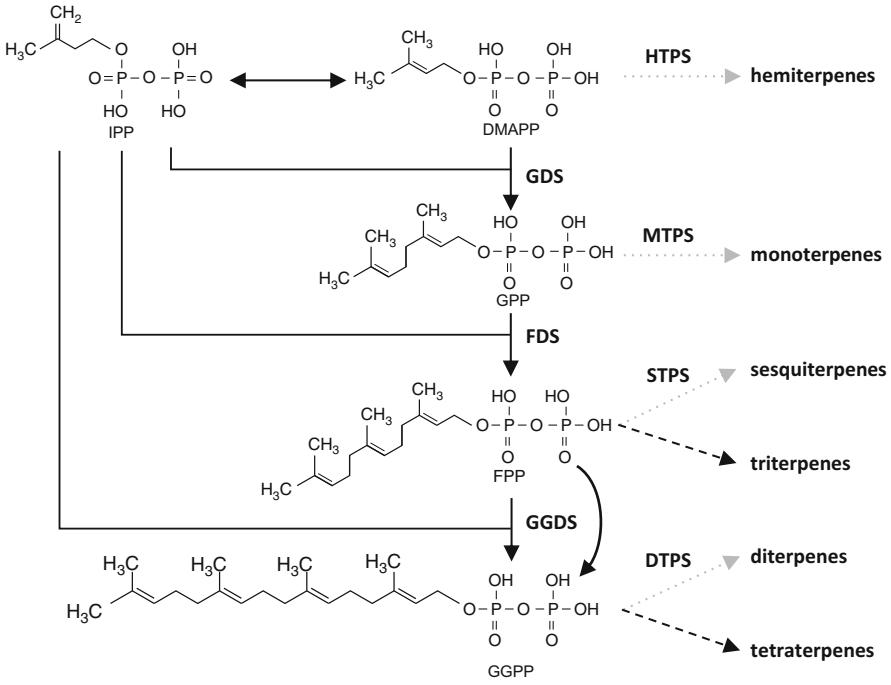

Fig. 2 Schematic representation of the biosynthesis of the isoprenoid end products' families from the universal building blocks, IPP (isopentenyl diphosphate), and DMAPP(dimethylallyl diphosphate). *Solid arrows* indicate head-to-tail condensation reactions leading to the formation of geranyl diphosphate, GPP; farnesyl diphosphate, FPP; and geranylgeranyl diphosphate, GGPP. Reactions are catalyzed by GDS, geranyl diphosphate synthase; FDS, farnesyl diphosphate synthase; and GGDS, geranylgeranyl diphosphate synthase, respectively. *Dashed arrows* indicate head-to-head condensations for the formation of triterpenes and tetra-terpenes. Hemiterpene synthases, HTPS; monoterpene synthases, MTPS; sesquiterpene synthases, STPS; and diterpene synthases, DTPS catalyze the conversion of basic backbones into isoprenoid end products, *dotted arrows*

prenyl units through a nucleophilic substitution releasing a pyrophosphate molecule (Sacchettini and Poulter 1997). In bacteria (as well as in plants), GGDS sequentially adds IPP to the allylic co-substrates DMAPP, GPP, and FPP to produce GGPP, whereas in yeast (as well as in mammals, and presumably all higher eukarya), GGDS use only FPP as a substrate. This suggests two different ways to produce GGPP involving one (GGDS) or two (FPPS/GGDS) enzymes. Prenyl diphosphates such as IPP, GPP, FPP, and GGPP are the polyprenyl backbones leading to isoprenoid end products' families of hemiterpenes, monoterpenes, sesquiterpenes, and diterpenes, respectively (Fig. 2).

2.3 Terpene Synthases

Terpene synthases (TPSs) catalyze a range of reactions (primarily dephosphorylation and cyclization) to convert the polyprenyl backbones into members of the different subfamilies of isoprenoids. These transformations may then be followed by a variety of modifications of the parental skeletal types to produce the vast family of isoprenoid metabolites described to date. In plants, the TPS family is particularly diverse and has been extensively studied (Bohlmann et al. 1998). There are various classification systems. TPS genes are generally divided into seven clades based on sequence similarity (Chen et al. 2011), whereas the proteins can be classified based on their mechanism of reaction or substrate specificity.

The two reaction mechanism groups, class I and class II, produce carbocations that ultimately lead to the formation of new carbon-carbon bonds; however, they differ in the way reactions are initiated. Class I enzymes catalyze heterolytic cleavage of an allylic diphosphate ester bond, whereas class II enzymes utilize protonation to initiate the reaction (Gao et al. 2012). Class I TPS enzymes are the most abundant and often produce multiple products, using either a single prenyl diphosphate substrate or more than one. This is due to the stochastic nature of bond rearrangements that can follow the formation of the carbocation intermediate. Alternatively, TPS enzymes can be classified based on substrate specificity for the length of the prenyl phosphate substrate. Hemiterpene synthases, monoterpene synthases, sesquiterpene synthases, and diterpene synthases use C5, C10, C15, and C20 prenyl phosphates as substrates, respectively. Most of the C5–C15, and some C20, terpenoids are volatile under normal environmental conditions.

Following the initial dephosphorylation (+/− cyclization), further alterations and decorations by oxidation, peroxidation, methylation, acylation, or cleavage may occur through the action of other enzymes, particularly cytochrome P450s. These decorations have an impact on the specific physical properties of the final compound and may be responsible for new biological activities.

Less is known about TPSs in prokaryotes, where relatively few TPSs have been identified. It has been known that bacteria produce odorous volatile compounds, presumably in the terpenoid family, since the nineteenth century; however, the first studies on these compounds only began in the 1960s, when Gerber and Lechevalier (Gerber and Lechevalier 1965) described the petrichor ("rain-wet-earth" smell)

isoprenoid, geosmin, produced by actinomycetes. It appears that it took over 50 years for the next research reporting volatile isoprenoids (isoprenoid hydrocarbons and alcohols) from bacteria to be published (Wilkins and Schöller 2009). While generally far fewer than in plants, bacteria also contain genes coding for terpene synthases. The encoded enzymes are class I TPSs (so far, no class II TPSs have been found in bacteria). However, they do not show sequence similarity with the plant or fungal genes and usually have low levels of similarity with other bacterial TPSs, making their identification through local sequence alignment challenging. Despite these differences at the primary sequence level, terpene synthases all typically display two highly conserved metal-binding motifs: an acidic amino acid-rich motif and a triad of residues located downstream that is fully conserved in all three classes of bacterial terpene synthases (mono-, sesqui-, and diterpene synthases) (Komatsu et al. 2008). Hemiterpene synthases have not been described in bacteria; instead, in the case of isoprene production in *Bacillus*, the hemiterpene isoprene is produced by HDR (also known as IspH), the terminal enzyme in the MEP pathway (Ge et al. 2016).

Based on the two conserved motifs, a search method for new TPSs was developed using hidden Markov (HMM) models and previously identified plant, fungal, and bacterial TPSs. This approach retrieved 41 new predicted synthases from the protein family (Pfam) database. A second-generation method using the new set of 41 bacterial sequences to train the HMM model leads to the identification of 140 presumptive terpene synthases. More recently, a third-generation model, trained with the previous 140 bacterial TPS, has expanded the family up to 262 genes, the biochemical function of some of which has also been determined. Results show that sesquiterpene synthases are by far the most prevalent TPS among prokaryotes (Yamada et al. 2015).

3 Functional Classes of Microbial Isoprenoids

It is currently considered that isoprenoids in microbes mainly fulfill essential biological roles in growth and survival. These processes include cell wall biosynthesis, membrane function or electron transport among others. However, some microbes, especially microalgae, also produce highly specialized isoprenoid-based secondary metabolites. Moreover, as more microbial TPS genes are discovered and the functions of their products determined, this view may change. In this section we briefly describe the main functional classes of isoprenoids identified to date focusing primarily on isoprenoid roles in the model microbial organisms for each kingdom but also examining some other interesting organisms/roles.

3.1 Biosynthesis of the Cell Wall

The bacterial cell wall is a rigid semipermeable structure surrounding the cytoplasmic membrane that maintains the cell shape and prevents deleterious effects of

internal osmotic pressure in the cell. The biosynthesis of the cell wall is a complex process that involves the transport of highly hydrophilic monomeric units across the cytoplasmic membrane that are then assembled to produce the main component of the cell wall, peptidoglycan (Scheffers and Pinho 2005). The key compound allowing this transport is an isoprenoid lipid carrier, undecaprenyl phosphate (UP), commonly referred to as bactoprenol (Fig. 3). UP is a C55 compound derived from a prenyl diphosphate produced by the sequential addition of 8 molecules of IPP onto FPP followed by dephosphorylation. This step is essential since the oligosaccharide transfer between the UDP-sugar and the lipid requires the monophosphate form of the lipid (Touz and Mengin-Lecreulx 2008).

3.2 Membrane Architecture and Fluidity

As already mentioned, cell membrane isoprenoids have a long history, both evolutionarily and academically as taxonomic markers. The cell membrane is a selectively permeable layer, basically containing lipids and proteins, which surrounds and encloses the contents of the cell. Glycerolipids are one of the main lipid components in the membrane; they are composed of a polar head and two alkane chains attached to a glycerol moiety. In archaea, the architecture of the membrane shows some unique features. Unlike any other form of life, archaeal membranes contain ether-type lipids with hydrocarbon chains bound to the glycerol moiety exclusively by ether linkages. In eukaryotes and eubacteria, fatty acyl hydrocarbon chains are linked to an *sn*-glycerol-3-phosphate backbone via an ester bond, whereas in archaea ether bonds link isoprenoid hydrocarbon chains to *sn*-glycerol-1-phosphate (Jain et al. 2014). Usually, the isoprenoid side chains are methyl-branched C20 and C25 prenyl phosphates derived from GGPP (Fig. 3).

Other isoprenoids in other microbes are also involved in the regulation of the fluidity of the membrane. In yeast, as a representative of eukaryotic microorganisms, ergosterol plays a similar role to that described for cholesterol (the most common sterol in vertebrates) or phytosterol (in plants) in the regulation of the membrane rigidity, fluidity, and permeability (Abe and Hiraki 2009). The reason for this specificity of sterols in each eukaryotic clade is not clear but must have some evolutionary rationale. In particular, the reason why fungi produce ergosterol is not clear since its biosynthesis is more energy-demanding compared to cholesterol, and structure-function studies have not been able to identify any advantage (Shrivastava and Chattopadhyay 2007). One recent explanation is that ergosterol may provide a better adaptation to the typical climatic instabilities of fungal ecological niches (Dupont et al. 2012). Interestingly, it was recently shown that some less evolutionary advanced species of fungi produce cholesterol instead of ergosterol (Weete et al. 2010). The biosynthetic pathway for ergosterol involves several catalytic steps, but the basic backbone derives from the head-to-head condensation of two molecules of FPP to produce the C30 squalene (Fig. 3).

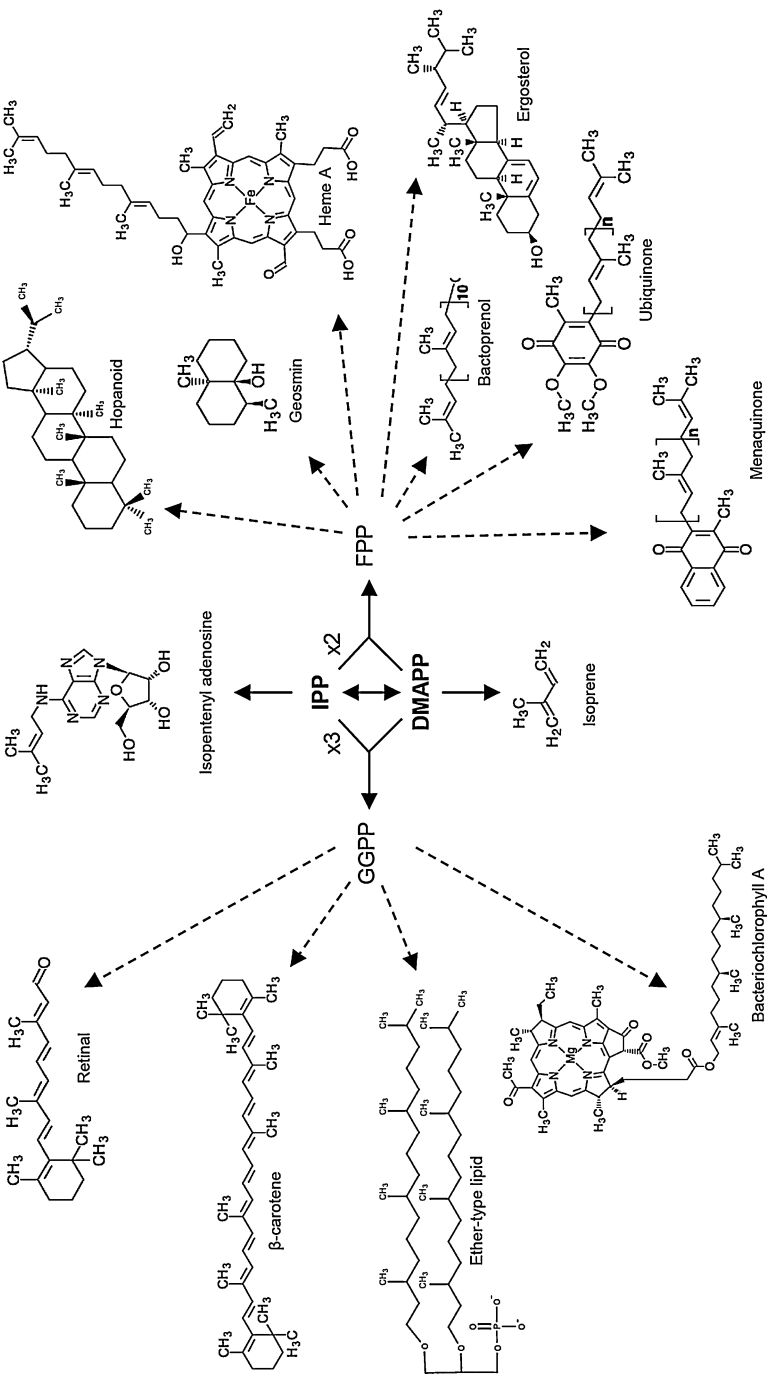

Fig. 3 Representative isoprenoids found in microorganisms encompassing the three kingdoms of life. Examples of isoprenoids derived from IPP, DMAPP, FPP, and GGPP are shown. *Solid arrows* indicate single-step reactions; *dashed lines* indicate multiple steps

In contrast, the distribution among microalgae is quite diverse with some producing mainly cholesterol, some others producing ergosterol and some of them producing a highly complex profile of sterols (Volkman 2003).

Most prokaryotes do not produce any kind of sterols. Membrane fluidity in these organisms is regulated through another set of isoprenoid-based molecules, hopanoids, which is found in about 50% of the species investigated. Hopanoids are pentacyclic triterpenoids produced from a basic skeleton of squalene showing a structure that resembles the one described for sterols (Saenz et al. 2015). Poulter and co-workers recently described an alternative path to squalene in bacteria (Pan et al. 2015).

Isoprenoids are also involved in the membrane organization by taking part on the formation of membrane microdomains as a result of the segregation of different lipids due to their physicochemical properties. Lipid rafts are better known in eukaryotes as functional membrane microdomains (FMM) enriched in particular lipids such as cholesterol (or ergosterol in yeast), which harbor and compartmentalize proteins involved in several cellular functions like signal transduction. They also contain specific proteins like flotillins, membrane-bound chaperones suggested to be responsible of the recruitment of functional proteins into the rafts. The existence of lipid rafts has been associated to the higher cellular complexity of eukaryotes. However, the existence of FMM in bacteria was recently reported. Most bacteria do not have two of the major components described for lipid rafts in eukaryotes, cholesterol, and sphingomyelin, but flotillin-like proteins have been identified both in bacteria and archaea. The existence of lipid rafts in bacteria still remains controversial, and the molecular structure of the lipids involved has not been elucidated. However, hopanoids and carotenoids have been suggested as the most plausible candidates for the assembly of FMM, whereas cardiolipin, a diphosphatidylglycerol lipid could play the role described for sphingomyelin in eukaryotes (Bramkamp and Lopez 2015).

3.3 Electron Transport

Isoprenoid quinones are membrane-bound compounds found in all living organisms with the only known exceptions of some obligatory fermentative bacteria and some methanogenic archaea. They are composed of a polar head, which interacts with proteins in the membrane, and an isoprenoid hydrophobic side chain that confers a lipid-soluble character and allows anchoring in the lipid layer. Isoprenoid quinones function mainly as electron and proton carriers in electron transfer chains (photosynthesis and respiration) but have also an important function as antioxidants. The reduced forms of isoprenoid quinones have antioxidant properties that protect membranes from lipid peroxidation and the effects of reactive oxygen species on membrane components. The great majority of naturally occurring isoprenoid quinones belong to the naphthoquinone family (polar head is a naphthoquinone ring) or the evolutionary younger benzoquinones (polar head is a benzoquinone group)

(Nitschke et al. 1995). In both families of compounds, the isoprenoid side chains are of a different length depending on the organism; they are usually 6–10 isoprene (C5) units although chains ranging from 1 to 14 units have been identified in different species (Fig. 3). Most often the side chain is fully unsaturated, but in some organisms it is partially or fully saturated. The reasons for the length and degree of saturation of the side chain are not fully understood, but these characteristics are often dependent on the growth temperature of specific species. It has been shown that quinones with different side-chain length can complement the absence of the endogenous ubiquinone in *E. coli* (Choi et al. 2009), suggesting that length is not critical for basic biological function.

Menaquinones (members of the naphthoquinone family) function in respiratory and photosynthetic electron transport chains of bacteria. Among benzoquinones, the most widespread and important are ubiquinones and plastoquinones. Ubiquinones participate in respiratory chains of eukaryotic mitochondria and some bacteria, whereas plastoquinones are components of photosynthetic electron transport chains of cyanobacteria. Menaquinones have low midpoint redox potential, and their appearance in early phase of evolution has been correlated with the reducing character of the ancient atmosphere. Reduced menaquinones become rapidly oxidized in the presence of oxygen; therefore these compounds cannot efficiently operate in an atmosphere containing oxygen. The evolutionary transition from menaquinones to ubiquinones (showing higher midpoint redox potential) occurred independently in some groups of prokaryotes as an adaptation to aerobic metabolism (Nitschke et al. 1995). Facultative anaerobes like *E. coli* produce ubiquinone when growing under aerobic condition but mainly produce menaquinone under anaerobic conditions.

An isoprenoid tail (hydroxyethyl farnesyl) derived form a FPP molecule is found both in HemeA and HemeO (Mogi et al. 1994), which are key components of cytochromes. Cytochromes are membrane-bound proteins containing a heme group that facilitate the movement of electrons. Heme groups are heterocyclic porphyrin rings made up of four pyrrolic groups joined together by methine bridges with a metal ion (usually iron) in the central position, bound to the four pyrrole nitrogen atoms (Fig. 3). There are different heme groups (A, B, C, D, O) showing different substitutions along the porphyrin ring. Cytochrome-based respiratory chains are present in eukaryotes, archaea and bacteria (O'Brian and Thony-Meyer 2002).

3.4 Conversion of Sunlight into Chemical Energy

Some microorganisms as well as higher plants are able to capture solar photons and convert the light energy into storage chemical energy. Two unrelated mechanisms have evolved to sense and harvest light for phototrophic growth, and both of them require isoprenoid-derived compounds. The first one is commonly known as photosynthesis and is dependent on photochemical reaction centers, and the second one is

based on membrane proteins binding retinal pigments and forming a light-driven proton pump.

In microbes there are two different types of photosynthesis, both of which use isoprenoid phytyl side chains for the light-harvesting pigments. Oxygenic photosynthesis uses chlorophylls for light harvesting and is found in algae, cyanobacteria, and some bacteria. Anoxygenic photosynthesis uses bacteriochlorophylls and is found in purple bacteria, green sulfur, and non-sulfur bacteria as well as in heliobacteria (Bryant and Frigaard 2006). The structure of these two pigments is similar, with a distinctive tetrapyrrole ring and a C20 phytyl tail derived from a molecule of GGPP that anchors the molecule to the photosynthetic membrane (Fig. 3). Major differences between these two families occur in the substitutions around the ring and substitutions on the phytyl tail, which modulates the specific wavelength of light that each pigment absorbs. Although phytyl is the most common tail in bacterial chlorophylls, other isoprenoids (farnesyl, geranylgeranyl, or 2,6 phytadienyl) have also been identified (Chew and Bryant 2007). These pigments play a central role in light harvesting and photochemistry. All chlorophototrophs also produce essential carotenoid pigments, which act primarily as photoprotective agents (protecting against reactive oxygen species that are generated during photosynthesis) but are also involved as light-harvesting helpers (Frank 1999). Carotenoids are a diverse subfamily of isoprenoids derived from the C20 backbone GGPP. Some non-photosynthetic microorganisms also synthesize carotenoids; in this case, they can be considered secondary metabolites since they are not essential but protect the organism from oxidative damage.

The second mechanism for light harvesting was originally identified in marine haloarchaea (Oesterhelt and Stoeckenius 1971). This mechanism employs membrane proteins called proteorhodopsins and bacteriorhodopsins, which bind isoprenoid-derived retinal; the retinal acts as a chromophore to harvest light, and the proteins function as proton pumps ultimately producing ATP. In addition to their originally described role, members of the diverse family of retinal-binding proteins have roles as transmembrane chloride pumps and photosensors (Fuhrman et al. 2008). Unlike chlorophylls, retinal – a relatively simple pigment – captures light without accessory pigments. During the last decades, this mechanism has also been found to be widespread among marine bacteria showing different absorption spectra (allowing them to live at different water depths where the wavelengths of light available are filtered) (Beja et al. 2000).

Retinal is best known as the vitamin A compound that forms the basis of animal vision. It is an isoprenoid-derived C20 compound (Fig. 3) produced in archaea and most bacteria by oxidative cleavage of the C40 carotenoid β-carotene (Fig. 3) at the 15,15′ bond to produce two identical molecules of retinal. In cyanobacteria retinal is also produced by the cleavage of β-carotene, but at least two carotenoid oxigenases are known to also produce retinal using apo-carotenoids with all-*trans* linear end groups as a substrate (Ruch et al. 2005; Scherzinger et al. 2006). The origin of these apo-carotenoids remains unclear, but it is likely that they are derived by asymmetrical oxidative cleavage at one end of a C40 carotenoid.

3.5 Protein Translation

Most of the bacterial and eukaryotic tRNAs that read codons starting with uracil are modified at the adenosine at position 37 (corresponding to the 3′ adjacent of the anticodon) by isopentenylation. The isopentenyl group of an IPP molecule is transferred to the amino group on position C_6 of the nucleoside (Fig. 3) to produce N^6-Δ^2-isopentenyl adenosine (i^6A). Isopentenyl adenosine (as well as other non-isoprenoid modifications at different positions) increases the efficiency of translation of the modified tRNA and reduces the sensitivity to the codon context. These chemical modifications in the anticodon loop of tRNAs alter the conformation and reduce flexibility, providing proper geometry that acts as a proofreading mechanism and allows optimal rate of protein synthesis (Persson et al. 1994).

A recent study has described the identification of geranylated RNA in some bacteria including *E. coli*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, and *Salmonella enterica* (Dumelin et al. 2012); these observations represent the first example of oligoisoprenylated nucleic acids. The C10 isoprenoid geranyl binds to the sulfur atom at the 2-thiouridine position in around 7% of the first position of anticodons for tRNAs UUC, UUU, and UUG. RNA geranylation has been suggested as an alternative to selenation at low selenium levels. Geranylation of tRNA affects codon bias and frame shifting of the open reading frame during translation.

3.6 Protein Prenylation

Prenylation of proteins is a posttranslational modification that is mainly involved in facilitating protein association to the membrane and directing subcellular localization. It has been best studied in eukaryotes (especially mammalian and plant cells) but was first identified in the basidiomycete yeast *Rhodosporidium toruloides* and, a few years later, in the ascomycete yeast *Saccharomyces cerevisiae* in mating factor peptides. Prenylated peptides contain a C-terminal cysteine thioether linked to (most commonly) a molecule of farnesyl or hydroxyfarnesyl (derived from FPP) that is essential for its biological function (Omer and Gibbs 1994). This addition is catalyzed in yeast by the enzyme farnesyl protein transferase (FTP) (Zhang and Casey 1996). Less commonly, GGPP is involved, and two types of GGPP protein transferases have been described (Zhang and Casey 1996). These activities have also been identified in other eukaryotic microorganisms such as *Plasmodium falciparum*. Protein prenylation has so far been demonstrated only in eukaryotes, but recent work has also predicted that pathogenic bacteria can access eukaryotic machinery to prenylate their own proteins. These bacteria have evolved mechanisms to direct their effector proteins to the desired subcellular localization in the eukaryotic host cell. One of these mechanisms involves secretion of proteins containing prenylation motifs that are recognized by the prenylation machinery of the host. In-host prenylation of bacterial proteins has been identified using both farnesyl (by addition of FPP) and geranylgeranyl (by addition to GGPP) (Ivanov et al. 2010). In silico analyses show that most bacterial species contain effectors with the conserved

prenylation motif. However, to date no in-cell protein prenylation has been described in prokaryotes.

3.7 Secondary Metabolites

Secondary metabolites represent a diverse group of compounds playing different biological roles on the interaction with the environment and providing better fitness to the specific ecological niche. In some cases, the role of specific compounds is still not clear or still under debate. Higher plants and macroscopic fungi, rather than microbes, produce most of the terpenoid secondary metabolites known to date. The amount of these secondary metabolites found in microorganisms including bacteria, archaea, unicellular fungi (yeast), and microalgae is more restricted or, at least, less studied. Here, we will outline a few well-known examples, but these are by no means exhaustive; secondary metabolism is very complex.

Actinomycetes and specially *Streptomyces* are well known for their relatively large genome size (8–10 Mb), which correlates with a complex secondary metabolism, usually occurring at the late growth phase. Most of the secondary metabolites produced are antibiotics, and some of them are isoprenoid-derived molecules (Dairi 2005). Most *Streptomyces* produce their isoprenoids through the MEP pathway, but interestingly, some of them also possess the MVA pathway as a gene cluster on their genome. In this case, the MVA pathway genes are expressed only at very low levels during the growth phase, suggesting that the MEP pathway supports biosynthesis of essential isoprenoids. During the late phase of growth, the MVA genes are upregulated, and around 60% of cellular IPP is produced via the MVA pathway for biosynthesis of secondary metabolites such as antibiotics. Additionally, most of the genes involved in further modifications of the isoprenoid backbone are located in the flanking regions of the MVA cluster genes (Dairi 2005). However, *Streptomyces* species carrying only the MEP pathway also produce antibiotics. Actinomycetes are also well known for the production of other isoprenoid-derived secondary metabolites including some volatile terpenes, the aforementioned FPP-derived geosmin (Jiang et al. 2007) being perhaps the original and best-known example. Geosmin is also produced by other microorganisms such cyanobacteria, myxobacteria, and fungi.

All photosynthetic microorganisms produce carotenoids, which as noted play an essential role in light harvesting and photoprotection of the photosynthetic machinery (Takaichi 2011). Some produce sufficient amounts that they can provide a natural source of industrially relevant carotenoids, e.g., the chlorophytes *Haematococcus* for astaxanthin and *Dunaliella* for β-carotene (Fig. 3). However, a variety of non-photosynthetic microbes, including some yeast, a variety of bacteria, and a few archaea (mainly Halobacteriaceae (Calegari-Santos et al. 2016)), also produce carotenoids as secondary metabolites. Their main physiological function is thought to be as antioxidants, but a role has also been described as virulence factors in some eubacteria (Liu et al. 2005). Most of the prokaryotic carotenoids known to date are found in land bacteria, but marine bacteria represent a partially unexplored source of new carotenoid

structures still to be identified. Recently, a compilation of carotenoid structures and biosynthetic pathways from prokaryotes with more than 300 unique structures has been publicly released (http://bioinfo.imtech.res.in/servers/procardb/) (Nupur et al. 2016). Carotenoids are mainly C40 compounds (Fig. 3) derived from the head-to-head condensation of two molecules of GGPP (Fig. 2) that is then further modified by desaturation, cyclization, or oxidation to produce the vast number of structures identified. However, C30 structures derived from FPP (Furubayashi et al. 2014), and C50 structures produced by the addition of two molecules of IPP onto the C40 lycopene, have also been reported (Yang et al. 2015).

Another intriguing metabolite produced by many organisms including animals, plants, and bacteria is isoprene. Isoprene (2-methyl-1,3-butadiene) is the smallest C5 representative of the isoprenoid family but in terms of total production is the most important isoprenoid with a total annual production estimated at 600 Tg carbon equivalents, mostly from plants and microalgae (Guenther et al. 2006). This massive amount of isoprene affects atmospheric chemistry and contributes to local air pollution, especially in the presence of anthropogenic nitrous oxides. The physiological role of isoprene is still under debate but has been suggested to act as a protectant in response to abiotic stress in plants (Vickers et al. 2009). Isoprene is produced from DMAPP in a reaction catalyzed by the enzyme isoprene synthase (IPS) (Fig. 3). In addition to microalgae, many other microorganisms produce isoprene. Both gram-negative and gram-positive bacteria have been identified as isoprene emitters, with *Bacillus* showing the highest emission rates. However, for a long time attempts to identify an isoprene synthase responsible for the conversion of DMAPP in prokaryotes failed. Surprisingly, recent *in vitro* studies have demonstrated the ability of HDR/IspH, the last step of the MEP pathway catalyzing the simultaneous production of IPP and DMAPP from HMBPP, to produce isoprene directly from HMBPP in *Bacillus* (Ge et al. 2016).

4 Research Needs

Biosynthesis of isoprenoids is highly complex, not only in the decoration phase but also in the core IPP/DMAPP production pathways (MVA and MEP). A decade ago, we had a fairly clear idea of how isoprenoids were synthesized through the canonical MVA and MEP isoprenoid pathways – even though the MEP pathway was only fully elucidated 15 years ago (Rodriguez-Concepcion and Boronat 2002). In recent years, we have discovered a number of variations on these canonical pathways; indeed, there is an astonishing genetic and metabolic plasticity among microbes. In the future, we can probably expect that further biochemical complexity will be discovered. Moreover, decoration and modification biochemistry downstream of prenyl phosphate metabolism is still poorly understood for some classes of compounds, for example, the recently discovered strigolactone group, which was identified as a group of plant hormones only in 2008 (Umehara et al. 2008). We still have much to learn about this group and many other isoprenoids. In particular, while plant secondary isoprenoid metabolism is relatively well characterized, relatively little is

known about prokaryotic secondary isoprenoid metabolism. Recent analyses have revealed the existence of a widespread variety of putative terpene synthases in bacteria (Yamada et al. 2015) that could soon lead to the identification of new catalytic activities and new isoprenoid structures. A systematic approach to validate all these candidates is required.

The high plasticity in the pathways producing IPP/DMAPP and the patchy distribution of the pathways across the three kingdoms of life have relevant evolutionary repercussions regarding both the origin of the pathways and the relationship between the two. The case of the coexistence of both pathways in microalgae is a particularly interesting topic relevant to these issues. Although some retain the two pathways (MEP in the chloroplast and MVA in the cytosol, as described in higher plants), some others, like the model green alga organism *Chlamydomonas reinhardtii,* produce their isoprenoids solely from the chloroplast-localized MEP pathway (Lohr et al. 2012). This raises the question of whether the ancestor of green algae ever used the MVA pathway or if it was lost during evolution. Regardless, the absence of the MVA pathway indicates that mechanisms exist to transport MEP pathway-derived isoprenoids from the chloroplast for the biosynthesis of cytosolic isoprenoids. To date, very limited information about the export/import of MEP pathway prenyl phosphate intermediates is available. A better understanding of these processes could help us to understand isoprenoid biosynthesis in the cytosol of green algae.

Many isoprenoids are of industrial interest, with applications including biofuels, chemical feedstocks, industrial solvents, agricultural chemicals, essential oils, fragrances, nutraceuticals, pharmaceuticals, etc. However, availability from natural sources is often limited and insufficient for industrial applications. The structural complexity of many of these compounds makes chemical synthesis from petrochemical precursors complex, expensive, and often impossible. In this context biological platforms represent a very attractive environmentally friendly approach. However, while we understand the basic metabolic pathways allowing us to engineer production of many of these interesting targets, much less is known about the regulatory constraints that currently limit isoprenoid production titers/rates/yields at the industrial level (Vickers et al. 2012). For example, in silico simulations predict a 10% greater theoretical maximum yield for the MEP pathway compared with the MVA pathway (Gruchattka et al. 2013). However, the regulation of the MEP pathway has revealed to be highly complex (Ajikumar et al. 2010), and yields from current efforts to boost the pathway flux to optimize isoprenoid production are still far from the predictions. Most successful approaches for industrial isoprenoid production have been achieved using the MVA pathway (Paddon and Keasling 2014) or generating new synthetic circuits (Meadows et al. 2016). A better understanding of the regulatory controls on the MEP pathway might allow us to achieve the predicted increase in yields through the MEP pathway.

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