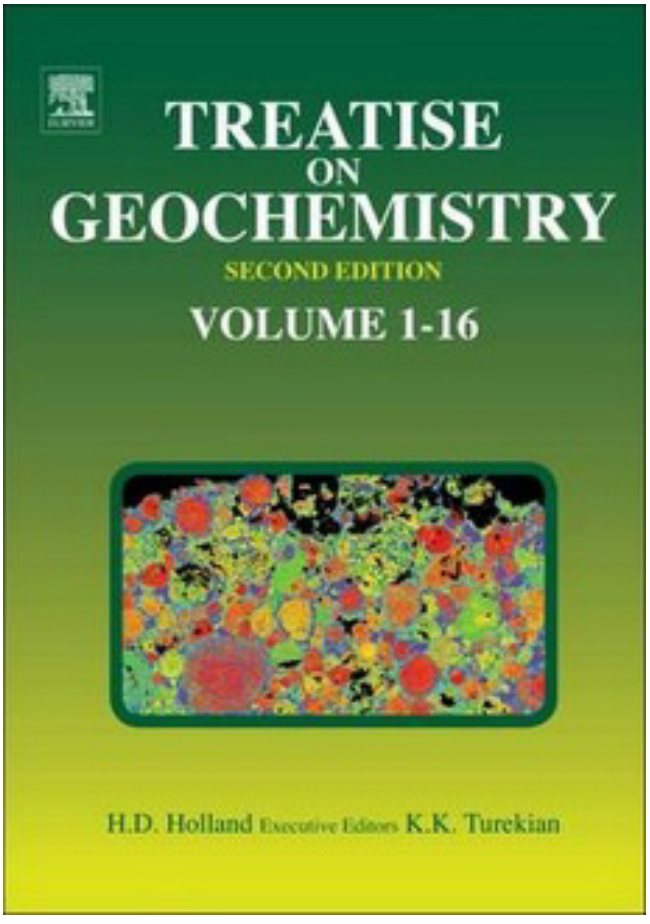

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12.11 Lipidomics for Geochemistry

A Pearson, Harvard University, Cambridge, MA, USA

12.11.1 Introduction

The field of *lipidomics* unifies investigations of the great diversity of lipids, including their cellular profiles, genes for their biosynthetic pathways, and the enzymes these genes encode. Lipidomics is a word that appears frequently in the biomedical literature, where it is associated with metabolite profiling in the characterization and treatment of disease (e.g., Wenk, 2005). In parallel, geochemists have applied many of the same approaches to investigate the occurrence of lipid biosynthetic genes and enzymes in organisms that have relevance to ecosystem function, Earth history, and paleoclimatology.

Some of the earliest history of an integrated approach to lipidomics can be credited to studies of the cyclization of squalene. In a telling memoir, Teruo Ono recalls a statement by Konrad Bloch; the latter received the Nobel Prize for his studies of sterol biosynthesis, including discovery of the role of molecular oxygen in the reaction (Tchen and Bloch, 1957). Professor Bloch is reported to have said as early as 1976, "... one can argue that any advantageous O_2-dependent or O_2utilizing reactions – and sterol biosynthesis is one of these – must [have involved an] aerobic prokaryotic partner. For this reason the capacity of the methanotrophic bacteria to synthesize sterols assumes special significance" (Ono, 2002). The geologic and evolutionary implications of an O_2-dependent lipid biosynthetic pathway were apparent to Professor Bloch, but it took several decades to determine that sterol biosynthesis has a common history and requirement for O_2 in both prokaryotes

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and eukaryotes. Decoding the enzymatic underpinning of the initial oxidative step required cloning the genes and expressing the epoxidase enzymes from yeast (Jandrositz et al., 1991) and rat (Sakakibara et al., 1995), as well as solving the structure of the sterol cyclase from humans (Thoma et al., 2004). Nearly in parallel, the squalene–hopene cyclases of *Alicyclobacillus acidocaldarius* (Ochs et al., 1990; Wendt et al., 1997) and *Methylococcus capsulatus* (Tippelt et al., 1998) also were characterized and were confirmed to cyclize squalene to hopanoids in the absence of external oxidants. Based on comparative analysis of these results, the mysterious presence of both hopanoids and steroids in *M. capsulatus* (Bird et al., 1971) now is understood as resulting from the presence of genes for both the oxidative (steroid) and direct cyclization (hopanoid) pathways in the genome of this organism (Lamb et al., 2007; Rohmer et al., 1980b), negating the alternative suggestion that a single pathway might perform both functions in the bacterium (Tippelt et al., 1998). Together, this framework illustrates how a geologically relevant biochemistry – namely, a requirement for O_2 that is preserved in a recalcitrant biomarker – is understood via chemical and molecular methods.

Such approaches are experiencing a rapid increase in popularity in association with the DNA sequencing revolution. In particular, the observation that >99% of known microbial diversity remains uncultured (Hugenholtz and Pace, 1996) has accelerated the application of lipidomic approaches to geochemical problems, especially in the realm of geomicrobiology. What lipids do these unstudied organisms make? How do we link lipids and unknown taxa with contemporary environmental conditions and with the geologic record, given the vastness of microbial diversity? These questions can be explored using the explosion of publicly available genomic data. To date, there are >2000 sequenced microbial species (http://www.ncbi.nlm.nih.gov/); ~350 environmental metagenomes (the largest of which remains the Global Ocean Sequencing project; Rusch et al., 2007), including multiple examples using high-throughput sequencing methodologies (e.g., Dinsdale et al., 2008); and a few applications using single-cell genomics (e.g., Grindberg et al., 2011). The biosynthetic potential encoded by this wealth of DNA data can provide tremendous insight into the phylogenetic distribution of lipid biosynthetic pathways.

Geochemical studies that use lipidomics generally have one or more of the following objectives:

1. Determine if a specific biosynthetic step or pathway is present in a given organism.
2. Determine the phylogenetic diversity of a biosynthetic step or pathway within a given ecological or environmental setting.
3. Link the presence of a biosynthetic step or pathway to the taxonomic identity of the organisms expressing that step or pathway.
4. Link the expression of a biosynthetic pathway to a physiological function or an ecological niche for the species containing the lipid product.

In all cases, the goal is to generate a better understanding of the sources and processes controlling the lipid distributions that are preserved in sediments and sedimentary rocks. However, the crossover of molecular and biochemical approaches into the milieu of geochemists faces substantial challenges. In particular, there are gaps in the ability to achieve objectives 3 and 4 that are difficult to address. Molecular genetics experiments (e.g., Welander et al., 2010) and metagenomic approaches (e.g., Pearson and Rusch, 2009) promise to aid these efforts, as does a more frequent application of concomitant isotopic measurements – both at natural levels and in tracer experiments (e.g., Waldbauer et al., 2011).

This chapter is not meant to be a comprehensive review of all lipidomic approaches. Rather, it is intended to serve as an introductory text. It describes the enzymes involved in some of the major lipid biosynthetic pathways, it provides a framework for lipidomics-based approaches to geochemical questions, and it reviews several recent examples of case studies that use these approaches. The biosynthesis section provides the essential underpinning for understanding relationships between enzymatic pathways and taxonomic groups. The applications section gives examples of lipidomics in practice, showing the potential of these approaches to achieve a better understanding of the origins of hydrocarbon 'biomarker' signatures preserved in the geologic record.

12.11.2 Lipid Biosynthetic Pathways

The two major families of lipids are called *acetogenic* and *isoprenoid* in reference to their respective monomeric units, acetate and isoprene. Isoprenoids are subdivided further into those that are synthesized via the mevalonic acid (MVA) pathway (Bloch et al., 1959; Lynen et al., 1958) and those that are synthesized via the methylerythritol phosphate (MEP) pathway (Rohmer et al., 1993). All cellular lipids contain core hydrocarbon structures that are homogeneous polymers of one of these precursors. (*N.b.*: In this context, the important tetrapyrrole classes of environmental biomarkers (e.g., porphyrins) are not lipids, as their pyrrole units derive from amino acid precursors: they are effectively modified protein. However, many of the principles discussed here could be applied equally to them.)

12.11.2.1 Acetogenic Lipids

Complex lipids containing a polar (often glyco- or phospho-) head group, glycerol backbone, and two esterified *n*-alkyl tails are the primary components of nearly all cell membranes. Important exceptions occur in some bacteria, which substitute ether linkages for the ester bonds, yielding glycerol mono- and diethers (Caillon et al., 1983; DeRosa et al., 1988; Huber et al., 1992; Langworthy et al., 1983) and/or diglycerol tetraethers (Damsté et al., 2007, 2011). Additional exceptions are ubiquitous in the Archaea in which not only are the glycerols ether-linked but the fatty tails are also isoprenyl (Kates et al., 1965; Koga et al., 1993; Langworthy, 1977).

Most steps in the biosynthesis of the fatty acid (FA) tails of membrane lipids are identical among bacteria and eukaryotes (White, 2000). The process begins with the carboxylation of acetyl-coenzyme A (CoA) to yield malonyl-CoA, with subsequent transfer of the malonyl group to acyl carrier protein (ACP). Condensation of the original acetate unit to a separate 'primer' molecule of acetyl-CoA – with concomitant decarboxylation – provides chain extension in units of C_2 (enzymes ***1–6***; **Table 1**) (**Figure 1(a)**). The resulting FA is even-numbered, and the most common lengths are C_{16} (palmitic acid) and C_{18} (stearic acid).

Table 1 Enzyme names, gene names, and Enzyme Commission numbers for lipid biosynthetic pathways discussed in the text

	Enzyme name	*Gene name*	*EC number*
	Fatty acid pathway		
(i)	Acyl carrier protein	*acpP*	–
1	Acetyl-CoA carboxylase	*accABCD*	6.4.1.2
2	Malonyl-CoA:ACP transacylase	*fabD*	2.3.1.39
3	3-Oxoacyl-ACP synthase	*fabB*, *fabF*, *fabH*	2.3.1.41,179,180
4	3-Oxoacyl-ACP reductase	*fabG*	1.1.1.100
5	3-Oxoacyl-ACP dehydrase	*fabA*, *fabZ*	4.2.1.58-61
6	Enoyl-ACP reductase	*fabI*, *fabK*, *fabL*	1.3.1.9-10
7	Acyl-ACP desaturase	*desA*	1.14.19.2
	MVA pathway		
8	Acetyl-CoA acetyltransferase (AAT)	*ERG10*	2.3.1.9
9	Hydroxymethylglutaryl-CoA synthase (HMGS)	*ERG13*	2.3.3.10
10	Hydroxymethylglutaryl-CoA reductase (HMGCR)	*HMG1*; *HMG2*	1.1.1.34,88
11	Mevalonate kinase (MK)	*ERG12*	2.7.1.36
12	Phosphomevalonate kinase (PMVK)	*ERG8*	2.7.4.2
13	Diphosphomevalonate decarboxylase (MVD)	*MVD1*	4.1.1.33
14	IPP-DMAPP isomerase (IDI)	*IDI1*	5.3.3.2
15	Putative archaeal phosphomevalonate decarboxylase	–	–
16[a]	Archaeal isopentenyl phosphate kinase (IPK)	*ipk*; *MJ0044*	2.7.4.-
17[a]	Archaeal IPP-DMAPP isomerase (IDI)	*idi2*; *MJ0862*	5.3.3.2
	MEP pathway		
18	Deoxy-xylulose 5-phosphate synthase (DXPS)	*dxs*	2.2.1.7
19	Deoxy-xylulose 5-phosphate reductase (DXR)	*dxr*	1.1.1.267
20	2-Methyl-erythritol-phosphate cytidylyltransferase (CMT)	*ispD* (*ygbP*)	2.7.7.60
21	4-(Cytidine 5′-diphospho)-2-methyl-erythritol kinase (CMK)	*ispE* (*ychB*)	2.7.1.148
22	2-Methyl-erythritol-2,4-cyclodiphosphate synthase (MECPS)	*ispF* (*ygbB*)	4.6.1.12
23	4-Hydroxy-3-methylbut-2-enyl diphosphate synthase (HMBPS)	*ispG* (*gcpE*)	1.17.7.1
24	4-Hydroxy-3-methylbut-2-enyl diphosphate reductase (HMBPR)	*ispH* (*lytB*)	1.17.1.2
	Glycerol diesters and diethers, *bacteria and eukaryotes*		
25	Glycerol-3-phosphate 1-*O*-acyltransferase	*plsB*	2.3.1.15
26	Acyl-ACP:phosphate transacylase	*plsX*	2.3.1.-
27	Acylphosphate:glycerol-3-phosphate acyltransferase	*plsY*	2.3.1.-
28	1-Acyl-*sn*-glycerol-3-phosphate 2-*O*-acyltransferase	*plsC*	2.3.1.51
29	Dihydroxyacetone phosphate acyltransferase	*GPT2*	2.3.1.42
30[b]	Alkyl-dihydroxyacetone phosphate synthase	*agpS*	2.5.1.26
31	NADPH:alkyl-DHAP oxidoreductase	*AYR1*	1.1.1.101
32	*O*-1-Alkyl-2-acylglycero-3-phospholipid (plasmanyl) desaturase	–	1.14.99.19
	Di- and tetraethers of Archaea		
33[c]	Geranylgeranyl diphosphate synthase (GGPPS)	*idsA*, *gdS*	2.5.1.29
34[d]	Glycerol-1-phosphate dehydrogenase (G1PDH)	*egsA*	1.1.1.261
35[c]	Geranylgeranyl glycerol phosphate synthase (GGGPS)	*MTH552*	2.5.1.41
36[c]	Digeranylgeranyl glycerol phosphate synthase (DGGGPS)	*MTH1098*	2.5.1.42
37	CTP:2,3-DGGGP cytidylyltransferase (CDP-archaeol synthase)	–	2.7.7.67
	Polar head groups		
38	CTP:phosphatidate cytidylyltransferase (CdsA)	*cdsA*	2.7.7.41
39	CDP-diacylglycerol:*sn*-glycerol-3-phosphate 3-phosphatidyltransferase	*pgsA*	2.7.8.5
40	Phosphatidylglycerophosphate phosphohydrolase	*pgpA*, *B*, *C*	3.1.3.27
41	CDP-diacylglycerol:L-serine 3-*sn*-phosphatidyltransferase	*pssA*	2.7.8.8
42	Phosphatidylserine decarboxylase	*psd*	4.1.1.65
43[e]	Phosphatidylethanolamine *N*-methyltransferase	*pmtA*	2.1.1.17
44[f]	UDP-galactose:1,2-diacylglycerol 3-beta-D-galactosyltransferase (MGD)	*mgd1*, *mgd2*	2.4.1.46
45[f]	Digalactosyl diacylgylcerol synthase (DGD)	*dgd*	2.4.1.241
46[g]	UDP-glucose:1,2-diacylglycerol 3-beta-D-glucosyltransferase (b-MGlcD)	*sll1377*	2.4.1.157
47[h]	UDP-galactose:1,2-diacylglycerol 3-beta-D-galactosyltransferase (MgdA)	*mgdA*	2.4.1.46
48[i]	Processive UDP-glucose diacylglycerol glucosyltransferase	*ypfP*	2.4.1.157
49[j]	UDP-glucose:1,2-diacylglycerol 3-alpha-D-glucosyltransferase (a-MGlcD)	*bgsB*, *alMGS*	2.4.1.-
50[j]	Diglucosyldiacylglycerol synthase	*bgsA*	2.4.1.-

(*Continued*)

Table 1 (Continued)

	Enzyme name	*Gene name*	*EC number*
51[e]	Sulfite:UDP-glucose sulfotransferase	*sqdB*	3.13.1.1
52,53[e]	UDP-sulfoquinovose:diacylglycerol sulfoquinovosyltransferase	*sqdC*, *sqdD*	2.4.1.-
54[k]	UDP-sulfoquinovose:diacylglycerol sulfoquinovosyltransferase	*sqdX*	2.4.1.-
55[e]	SAM:diacylglycerol 3-amino-3-carboxypropyl transferase	*btaA*	–
56[e]	SAM:diacylglycerylhomoserine-*N*-methyltransferase	*btaB*	–
57[l]	Ornithine *N*-acyltransferase	*olsB*	2.3.1.-
58[l]	Ornithine *O*-acyltransferase (OlsA, similar to PlsC)	*olsA*	2.3.1.51
	Linear polyprenes		
59	(*E*,*E*) Farnesyl diphosphate synthase (FPPS)	*fdps*; *ispA*	2.5.1.10
60	Geranyl diphosphate synthase (GPPS)	*gps1*	2.5.1.1
(33[c])	Geranylgeranyl diphosphate synthase (GGPPS)	*crtE*; *idsA*	2.5.1.29
61	Squalene synthase	*ERG9*; *hpnD*	2.5.1.21
62[e]	Phytoene synthase	*psy*; *crtB*	2.5.1.32
	Hopanoids and steroids		
63[m]	Squalene-hopene cyclase (SqhC; SHC)	*shc*	5.4.99.-
64[n]	SAM:hopene 5′-deoxyadenosyl transferase	*hpnH*	2.5.1.-
65[n]	(probable nucleoside phosphorylase)	*hpnG*	–
66[m]	SAM:hopene 2-methyltransferase	*hpnP*	2.1.1.-
67[o]	SAM:hopene 3-methyltransferase	*hpnR*	2.1.1.-
68	squalene monooxygenase (ERG1, SQMO)	*erg1*, *sqmo*	1.14.13.132
69	2,3-Oxidosqualene-lanosterol cyclase (LAN, OSC, ERG7)	*erg7*, *osc*, *lan1*	5.4.99.7
70	2,3-Oxidosqualene-cycloartenol cyclase (CAS, OSC)	*osc*, *cas1*	5.4.99.8
71	Sterol 14-demethylase (CYP51)	*cyp51*	1.14.13.70
72	4-Methylsterol monooxygenase (ERG25)	*erg25*	1.14.13.72
73	Sterol 24-methyltransferase (ERG6, SMT1)	*smt1*	2.1.1.41
74	24-Methylenesterol C-methyltransferase (SMT2)	*smt2*	2.1.1.143

Gene names are from *Escherichia coli* or *Saccharomyces cerevisiae* (yeast) unless otherwise noted. Exceptions are (a) *Methanocaldococcus jannaschii*, (b) *Mycobacterium tuberculosis*, (c) *Methanothermobacter thermoautotrophicus*, (d) *Sulfolobus solfataricus*, (e) *Rhodobacter sphaeroides*, (f) *Arabidopsis thaliana*, (g) *Synechocystis* sp. 6803, (h) *Chlorobium limicola*, (i) *Bacillus subtilis*, (j) *Enterococcus faecalis*, (k) *Synechococcus* sp. 7942, (l) *Rhodobacter capsulatus*, (m) *Rhodopseudomonas palustris*, (n) *Methylobacterium extorquens*, and (o) *Methylococcus capsulatus*.

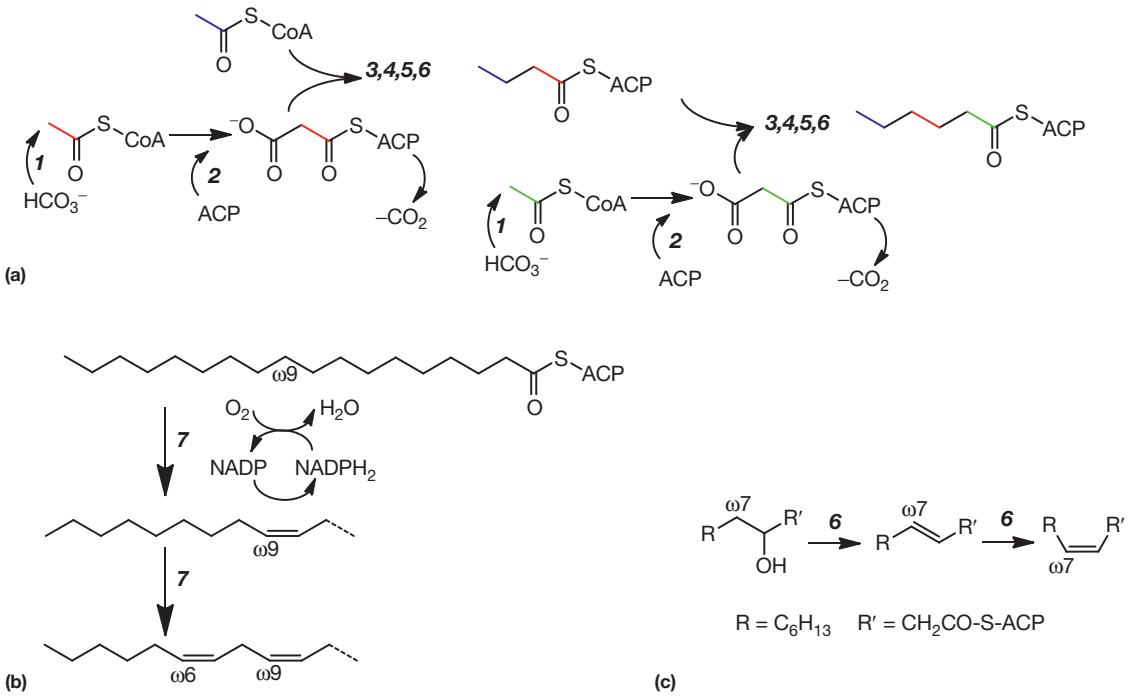

Figure 1 (a) Synthesis of fatty acids from acetate (adapted from White D (2000) *The Physiology and Biochemistry of Prokaryotes*. New York: Oxford University Press). (b) Aerobic desaturases of eukaryotes and bacteria require O_2 and most often place bonds 3 units apart, for example, ω3, ω6, and ω9. (c) Anaerobic desaturation in bacteria is achieved by isomerization during the reduction step (enzyme ***6***).

The genes encoding enzymes for transfer of malonyl-CoA to ACP (*fabD*), condensation (*fabF,B,H*), and the first reduction step (*fabG*) often can be found together in an operon in bacteria. In contrast, the four components of the acetyl-CoA carboxylase complex, *accABCD*, usually are separated, with *accB* + *accC* (biotin carboxylases) forming an operon, while *accA* and *accD* (transcarboxylases) are found remotely. This may reflect the importance of regulating the production of biotin (Abdel-Hamid and Cronan, 2007). Similarly, dehydration and reduction (*fabA,Z; fabI,K,L*) often are remote to the *fab* operon and may include multiple copies to facilitate up- and downregulation of the degree of unsaturation and/or the synthesis of lipopolysaccharide (DeMendoza and Cronan, 1983; Heath and Rock, 1996).

When the original 'primer' molecule instead is derived from leucine or isoleucine, rather than acetate, the result is a C_{15} or C_{17} *iso*- or *anteiso*-methylated FA, respectively (Kaneda, 1977). Correspondingly, there are FA synthases (enzyme ***3***, **Table 1**) specific for utilization of these branched-chain primers (Kaneda and Smith, 1980). Synthesis of branched chains is, however, only one of several strategies used to control membrane fluidity and maintain homeostasis (Cronan and Gelmann, 1975; Kaneda, 1972; McElhaney, 1974). In eukaryotes and in many bacteria, the primary strategy is the insertion of double bonds in the ω9 position (9 positions in from the methyl end) with optional additional polyunsaturation occurring in multiples of C_3 (ω6 and ω3) (**Figure 1(b)**), or occasionally in other patterns (cf. Russell and Nichols, 1999). The reaction is obligately aerobic and is mediated by a desaturase complex that acts after the FA chain has reached its full length. FA desaturases (*fasA*, enzyme ***7***; **Table 1**) transfer electrons to O_2 via NAD(P)H_2 (Bloomfield and Bloch, 1960; Fulco, 1974; Wada et al., 1990). The low homology between the forms found in animals, fungi, plants, and bacteria suggests that the various FA desaturases may have evolved independently (Shanklin and Somerville, 1991). In a simpler approach, desaturation in anaerobic bacteria capitalizes on inherent isomerase activity of the biosynthetic dehydratase (*fabA*) to yield a *cis* configuration that bypasses the *trans*-specific reductase (Heath and Rock, 1996; Kass and Bloch, 1967; Scheuerbrandt et al., 1961; **Figure 1(c)**). This presumably ancestral strategy necessarily occurs during chain elongation and can yield only monounsaturated FAs, presumed always to be at the ω7 position. Surprisingly, the aerobic and anaerobic pathways for unsaturation sometimes co-occur in the same species (Wada et al., 1989).

Because of the biochemical uniformity of these pathways among bacteria and eukaryotes, acetogenic compounds rarely are used as taxonomic biomarkers in geochemistry, although there are notable exceptions (e.g., ω8 FAs generally are diagnostic for methanotrophic bacteria; Bodelier et al., 2009; Nichols et al., 1985). These limitations soon may be circumvented, however, as knowledge of the full syntheses – including glycerol ester versus ether linkages and polar head-group combinations – should lead to opportunities to view genomic content predictively, providing target genes for experimental manipulation and/or detection in the environment.

12.11.2.2 Isoprenoids – MVA Pathway

In contrast to lipids derived from acetate building blocks, the membrane lipids of archaea, as well as carotenoid pigments and a variety of polycyclic molecules such as steroids, are made from polymerized isoprenyl (C_5) groups. The synthesis of isoprene from acetate occurs via an MVA intermediate (Bloch et al., 1959; Lynen et al., 1958). The route to MVA was elucidated from early isotope-labeling experiments, which identified acetate as the carbon source (Ottke et al., 1951; Rudney and Ferguson, 1959) and showed the participation of phosphorylated intermediates (Tchen, 1957). For decades, MVA was believed to be the only precursor to isoprene. Indeed, this is the pathway used by all animals, fungi, archaea, many protozoa, and in the cytosol of most plants and algae. Recently, it has been suggested that MVA is the ancestral pathway, perhaps originating within the last universal common ancestor (LUCA) and consistent with a very ancient origin of isoprenoids (Lombard and Moreira, 2011).

In the MVA pathway, the condensation of two acetyl-CoA units to acetoacetyl-CoA is mediated by an acetyl transferase (enzyme ***8***, **Table 1**). The product is joined with another acetyl-CoA using HMG-CoA synthase (enzyme ***9***) to yield the first essential intermediate, hydroxymethylglutaryl-CoA (**Figure 2 (a)**). In eukaryotes, the product is reduced via a type I HMG-CoA reductase (enzyme ***10***; dependent on NADPH; EC 1.1.1.34) and then twice phosphorylated (enzymes ***11***,***12***), yielding diphosphomevalonate. It is worth noting that HMG-CoA reductase is the enzyme that is blocked by statin drugs in the pharmaceutical inhibition of cholesterol synthesis (Endo, 1992); thus, statin treatment also affects the synthesis of all metabolic isoprenoids, including the steroid hormones and respiratory quinones. Most bacteria and some archaea use an alternative type II HMG-CoA reductase that instead uses NADH as a cofactor (EC 1.1.1.88). Regardless, decarboxylation of diphosphomevalonate (enzyme ***13***) yields isopentyldiphosphate, which isomerizes with dimethylallyldiphosphate (enzyme ***14***; gene *idi*) (**Table 1**); both products are essential for condensation and chain elongation of terpenoids.

Importantly, the steps downstream of phosphomevalonate proceed differently in many archaea (**Figure 2(b)**). Phosphomevalonate apparently is decarboxylated to isopentenyl phosphate, although the enzyme for this reaction has not been identified yet (enzyme ***15***, **Table 1**). Rather, the presence of this step was inferred from the discovery of an isopentenyl phosphate kinase (enzyme ***16***) and an alternative isomerase (enzyme ***17***; gene *idi2*) (Barkley et al., 2004; Grochowski et al., 2006). Although common, this approach is not ubiquitous among the archaea. It occurs in all known methanogens and in most thermophiles. In contrast, Thaumarchaeota and some halophiles have genes more closely resembling the rare instances of MVA that are found in bacteria (Lombard and Moreira, 2011) and are presumed therefore to make diphosphomevalonate.

Because an alternative pathway to synthesize isoprene (see succeeding text) is widespread in bacteria, it has been hypothesized that the relatively few instances of the MVA pathway in bacteria result from horizontal gene transfer from archaea or eukaryotes (Boucher and Doolittle, 2000; Lange et al., 2000). The alternative suggestion that MVA is ancestral derives from phylogenetic arguments which note that the bacterial MVA genes are monophyletic, that is, any horizontal transfer would have had to precede the wide radiation of bacteria (Lombard and Moreira, 2011). Consistent with this hypothesis, MVA operons also are organized differently between

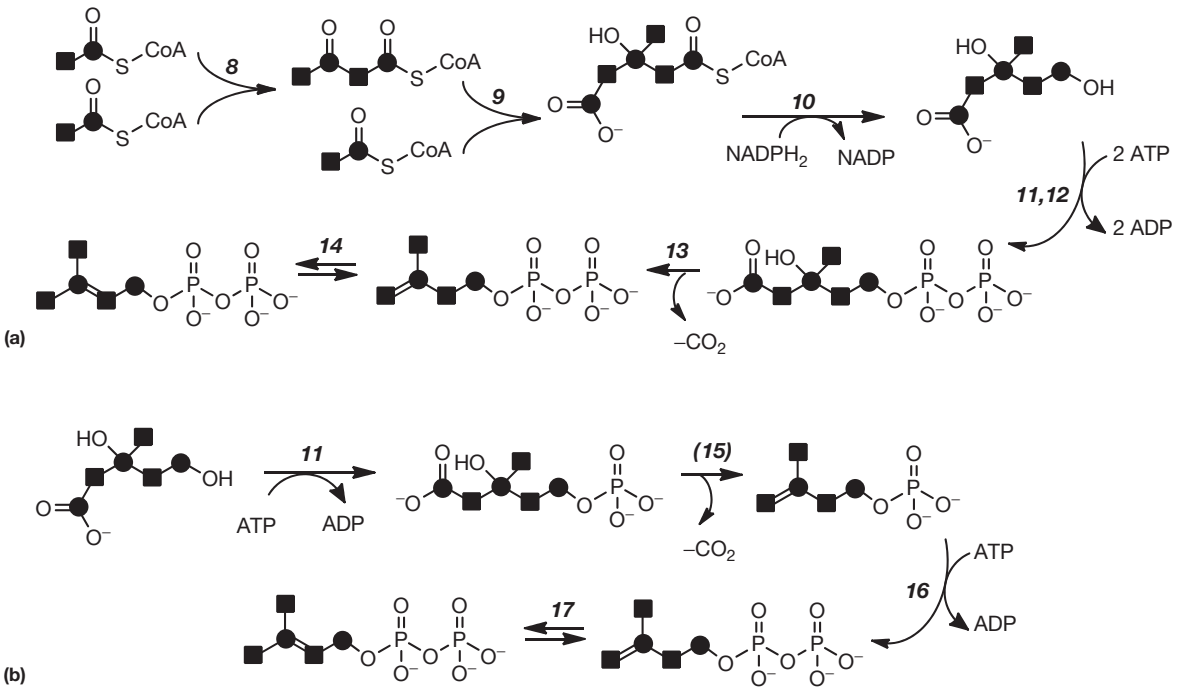

Figure 2 (a) The mevalonic acid (MVA) pathway for synthesis of isoprene units in eukaryotes and some archaea. The final step of isomerization between isopentenyl diphosphate and dimethylallyl diphosphate (enzyme ***14***) is required for chain extension to longer polyprenes (**Figure 12**). (b) Some archaea have an alternative route to IPP and DMAPP involving a putative archaeal phosphomevalonate decarboxylase (enzyme ***15***) (Grochowski et al., 2006). Black squares show carbons derived from the methyl groups of acetate, while black circles show carbons derived from the carbonyl position.

domains (Dairi et al., 2011). In bacteria, the genes encoding for the majority of steps in the MVA pathway often are present together, while in archaea, they are more likely to be scattered throughout the genome. These differences are consistent with an ancestral origin for the MVA pathway in bacteria, suggesting that the MEP pathway, in the succeeding text, is a more recent derivation. More work is needed to examine the validity of this idea, especially because the MEP pathway still must predate the endosymbiotic origin of plastids.

12.11.2.3 Isoprenoids – MEP Pathway

Between the 1950s and 1980s, a steady accumulation of confounding experimental data hinted there could be a pathway other than MVA for the synthesis of isoprene units in chloroplasts and bacteria (e.g., Goodwin, 1958; Pandian et al., 1981; Treharne et al., 1966). The critical insight came while examining the biosynthetic source of the C_5 side chains commonly added to C_{30} hopanes to yield C_{35} bacteriohopanepolyols (cf. Rohmer, 1999). While this work showed that the C_5 side chain likely derived from a ribosugar rather than from a lipid component (Neunlist et al., 1988), a secondary observation proved to be more significant: The pattern of ^{13}C isotope labeling in the hopanoid skeletal backbone was incompatible with synthesis of this isoprenyl structure from MVA pathway intermediates (Flesch and Rohmer, 1988b). Further investigations eventually revealed that the alternate pathway for synthesizing isoprene involves condensing and decarboxylating two C_3 intermediates of the glycolytic pathway (Rohmer et al., 1993). Now identified as the MEP pathway, it was found to be present in the majority of bacteria. It also is present in a few archaea, in the plastids of most eukaryotes, and as the sole source of isoprenoids in green algae (Lichtenthaler et al., 1997; Rohmer, 1999; Schwender et al., 1996).

The MEP pathway begins with the condensation of pyruvate and glyceraldehyde-3-phosphate to form 1-deoxy-D-xylulose 5-phosphate (enzyme ***18***, **Table 1**; gene *dxs*), the first dedicated intermediate of this pathway (**Figure 3**). Reduction using NADPH (enzyme ***19***; *dxr*) is simultaneous with skeletal rearrangement to the isopentyl form, yielding the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) intermediate for which the pathway is named. The remaining steps involve cyclophosphorylation (enzyme ***22***) via addition of cytidine phosphate from cytidine triphosphate (CTP) (enzyme ***20***) and phosphate from ATP (enzyme ***21***). Dephosphorylation of the cyclic phosphate yields hydroxymethylbutenyl diphosphate (enzyme ***23***), followed by a final reduction to isopentenyl diphosphate (enzyme ***24***).

The MEP and MVA pathways do not follow simple rules of taxonomic distribution, although at the gross scale, they could be defined as bacterial (MEP) and eukaryotic plus archaeal (MVA). Among the numerous exceptions and complicating factors are several known cases of both the MVA and MEP pathways occurring together in high-GC gram-positive bacteria, including in *Streptomyces* sp. CL190 (Kuzuyama et al., 2000), *Streptomyces cinnamonensis* (Bringmann et al., 2007), and *Kitasatospora griseola* (Hamano et al., 2002), although this phenomenon is not universal among the *Streptomyces* (Dairi et al., 2011). The coexistence of both pathways

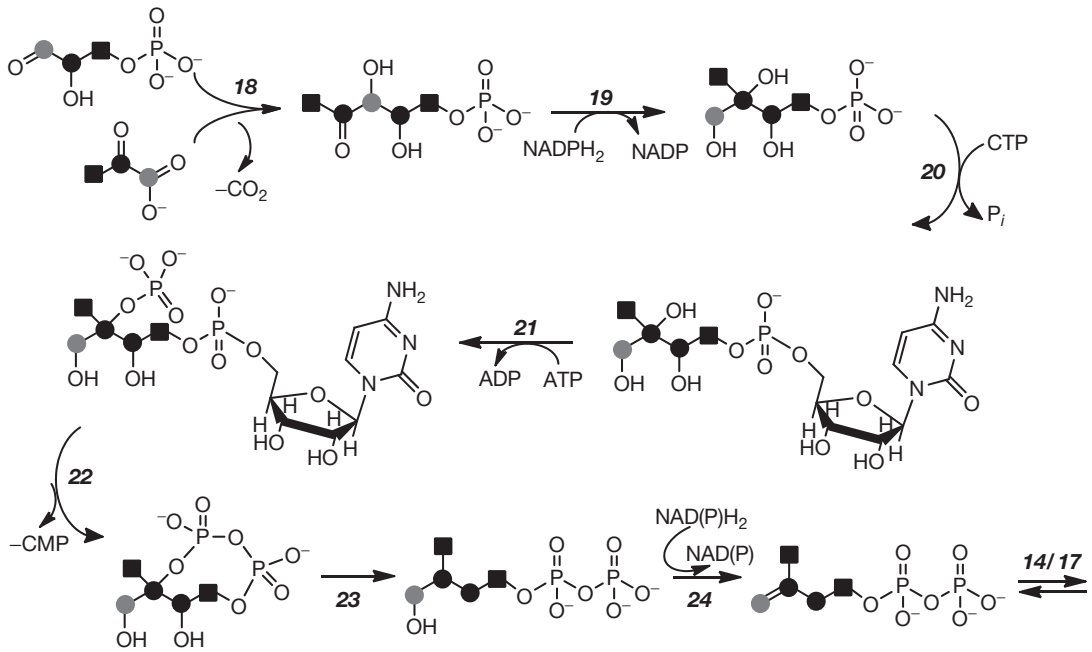

Figure 3 The methylerythritol phosphate (MEP) pathway of isoprenoid synthesis (Rohmer, 1999; Rohmer et al., 1993). The pathway begins with pyruvate and glyceraldehyde-3-phosphate, both derived from cleaving C_6 sugars. Squares mark the terminal sugar carbon positions C_1 and C_6 and black circles mark C_2 and C_5; both are equivalent to the squares and circles of acetate in **Figure 2**. Gray circles mark the central carbons C_3 and C_4. As in **Figure 2**, the final product IPP must also be isomerized to DMAPP for further chain extension.

also is the normal situation in plants. Generally, the chloroplasts of plants express the MEP pathway, which is believed to be retained from the cyanobacterial plastid ancestor. The MVA pathway simultaneously is expressed in the cytosol (Lichtenthaler, 1999; Lichtenthaler et al., 1997). Unicellular green algae are a major exception to this rule: multiple investigations spanning several genera (e.g., *Scenedesmus* and *Chlamydomonas*) repeatedly show that MEP is the pathway used for both cytosolic (sterols) and plastidic (phytol) isoprenoids (Disch et al., 1998; Schwender et al., 1996). At least some of the domain-level transfer of these two pathways probably is due to horizontal gene transfer (Boucher and Doolittle, 2000), but there also is evidence for a long, coherent history of both pathways (Lombard and Moreira, 2011). In either case, the examples in the preceding text raise the possibility that there will be more cases found where both pathways are present in a single species. Understanding how these pathways are regulated for the production of distinct pools of cellular metabolites (e.g., Hamano et al., 2002) also is benefiting attempts to bioengineer the production of natural product drugs such as the valuable antimalarial agent, artemisinin (Zeng et al., 2008).

12.11.2.4 Ether and Ester Linkages to Glycerol – Bacteria and Eukaryotes

In bacteria and eukaryotes, ester linkages are formed between fully extended fatty acyl units and the glycolytic intermediate glycerol-3-phosphate. The first step is mediated by one of two systems (**Figure 4**). Either a glycerol phosphate acyltransferase (enzyme ***25***; **Table 1**; gene *plsB*) transfers acyl-ACP or acyl-CoA to yield 1-acylglycerol phosphate (Bell, 1974) or a two-step process first transforms acyl-ACP to acyl-phosphate (enzyme ***26***, *plsX*), which then becomes the intermediate for an alternative acyltransferase (enzyme ***27***, *plsY*) (Lu et al., 2006). In all cases, the next step is addition of the second FA group via a conserved 1-acylglycerol phosphate acyltransferase (enzyme ***28***; *plsC*), which yields the simple phosphorylated *sn*-1,2-diacylglycerol-3-phosphate, an intermediate in the production of more complex intact polar lipids (IPLs) (Zhang and Rock, 2008). When *plsB* and *plsC* are found in bacteria, they tend to colocate with the *fab* cluster.

In some cases, bacteria and eukaryotes further modify their acylglycerols by transforming the ester linkages to ether linkages, forming di-*O*-alkylglycerols, 1-alkyl-2-acylglycerols, or 1-alk-1′-enyl-2-acylglycerols (**Figure 5**). The last, known as plasmalogens, are relatively common in animals and in anaerobic bacteria (Goldfine, 2010; Kamio et al., 1969), although the eukaryotic and bacterial pathways for synthesis of plasmalogens are fundamentally different.

The oxidative eukaryotic pathway requires aerobic desaturation of a glycerol alkyl ether to yield the corresponding vinyl ether. This occurs in a manner analogous to eukaryotic desaturation of FAs (cf. Goldfine, 2010), and it requires the 1-*O*-alkylglycerol as a precursor. Interestingly, this ether linkage is synthesized directly, that is, it is not produced by stepwise reduction from an ester. The pathway begins with acylation of dihydroxyacetone phosphate (DHAP), rather than glycerol-3-phosphate, to form *sn*-1-acylacetone phosphate (enzyme ***29***; **Table 1**). In the succeeding steps, the entire fatty ester side chain is replaced by a fatty alcohol (enzyme ***30***) and the ketone is reduced by NADPH:alkyl-DHAP oxidoreductase (enzyme ***31***) (**Figure 5(a)**). The second acylation, yielding the 2-acylglycerol, proceeds as indicated earlier (analogous to ***28***). Modifications of

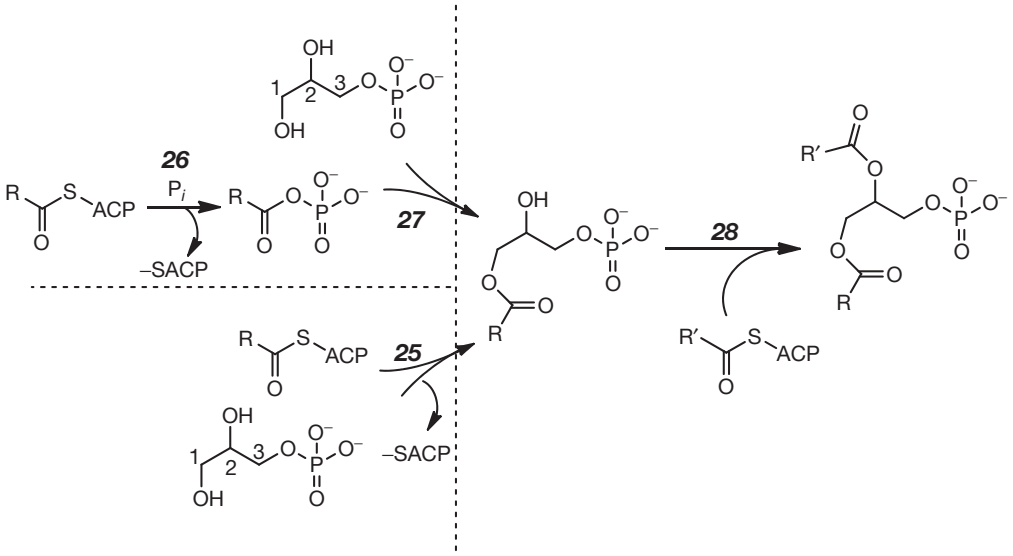

Figure 4 Formation of acylglycerols in eukaryotes and bacteria. Distinct enzymes add fatty acyl chains successively, first forming the *sn*-1, then the *sn*-2 linkage.

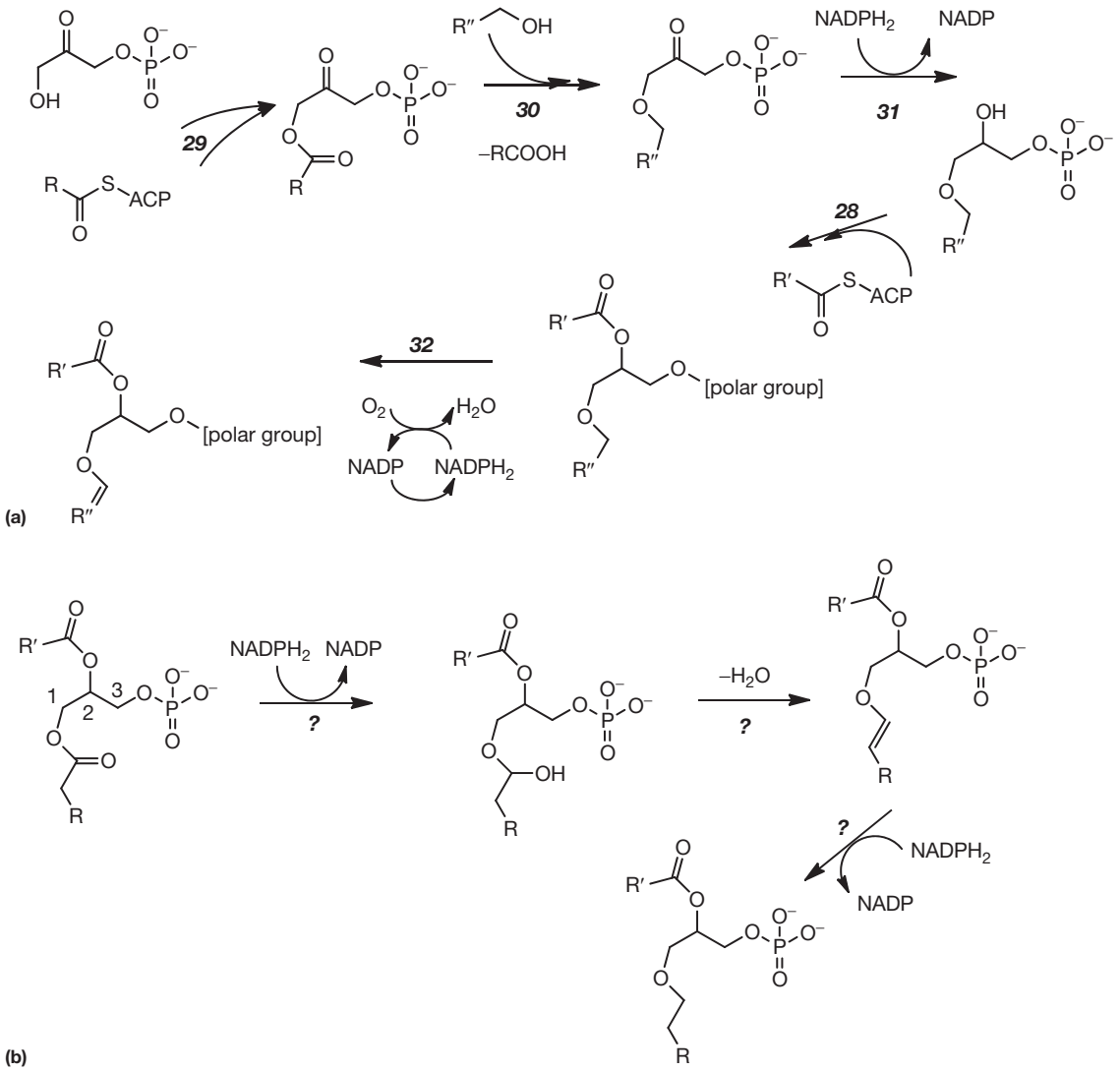

Figure 5 (a) Pathway for aerobic synthesis of plasmalogens in eukaryotes. (b) The proposed pathway in bacteria, including all anaerobes and some aerobes (Ring et al., 2006). Either or both pathways may be sources of ether-linked glycerol lipids in the environment.

the phospho- head group occur before the terminal desaturation step, which employs an O_2-dependent 1′-alkyl desaturase (enzyme *32*). Mixed ether–ester lipids are hence intermediates in the route to plasmalogens in this scheme. One could imagine that this approach might easily be copied by bacteria (either aerobes or anaerobes) to terminate at the saturated intermediate. It is not yet known if this strategy exists widely in bacteria, but important additional predictions are that mixed ether–ester lipids by this pathway always would have the configuration *sn*-1-alkyl-2-acylglycerol, and presumably, there would be no subsequent progression to form diethers unless additional enzymes were recruited.

In contrast, the anaerobic pathway in bacteria is the presumed ancient route to plasmalogens and by extension to bacterial diether lipids. This synthesis uses a quite different strategy: the original fatty ester is reduced stepwise to a vinyl ether, which then may be reduced terminally to the saturated ether (Ring et al., 2006). The plasmalogen thus is the intermediate in the formation of ether lipids. It is believed – although not yet demonstrated – that the sequence of stepwise reductions also occurs only via the *sn*-1-alkyl-2-acylglycerol (rather than *sn*-1-acyl-2-alkylglycerol) intermediate. Possible exceptions to this may occur in the Planctomycetes in which monoethers in the *sn*-2 position have been reported (Damsté et al., 2002b). The synthesis of the initial *sn*-1,2-diacylglycerol is believed to proceed through the normal route (enzymes *25–28*, in the preceding text), starting with glycerol-3-phosphate (Hill and Lands, 1970). Like in the synthesis of eukaryotic plasmalogens, the modifications to the fatty side chains may take place after head-group modifications yield the IPL forms, such as phosphatidylethanolamine (PE) (Johnston et al., 2010). The enzymes involved in all of the terminal reductive transformations remain to be discovered, and much of the reasoning in the preceding text lacks rigorous empirical evidence, but this proposed pathway is more accommodating to the synthesis of diethers than is the eukaryotic pathway.

Some further principles regarding synthesis of ether lipids may be deduced from patterns of occurrence in bacteria and from the presence of the reductive pathway in some eukaryotes. Because the reductive pathway occurs in some anaerobic protozoa (Prins and Vangolde, 1976), the primary distinction between the two routes had been suggested to be aerobic–anaerobic, rather than taxonomic (Goldfine, 2010). However, this idea conflicts with the occurrence in Myxobacteria of ether lipids apparently produced via the successive reduction pathway (Caillon et al., 1983; Ring et al., 2006; Stein and Budzikiewicz, 1987) and with the recent report of ether lipids in aerobic members of the Acidobacteria (Damsté et al., 2011). Myxobacteria are aerobic Deltaproteobacteria, and Acidobacteria are both diverse and environmentally widespread. These examples suggest that the anaerobic pathway may simply be ancestral rather than obligately associated with anaerobic physiology. The reductive 'bacterial' pathway could have migrated by horizontal gene transfer to fulfill a biosynthetic need in anaerobic eukaryotes.

As expected, mixed ether/ester and diether glycerols also have a wide phylogenetic distribution in anaerobic and microaerophilic bacteria. Although ether lipids are common among thermophiles, there is not a strict temperature-based association. Ether lipids to date have been observed in Thermodesulfobacteria (Langworthy et al., 1983); Thermotogales (Damsté et al., 2007); Planctomycetes (Damsté et al., 2002b); *Aquifex* spp. (Huber et al., 1992); Clostridiales and other Firmicutes (Clarke et al., 1980; Jung and Hollingsworth, 1994; Jung et al., 1994; Kamio et al., 1969; Klein et al., 1979; Koga and Goldfine, 1984; Prins and Vangolde, 1976); *Mycoplasma* (Wagner et al., 2000); anaerobic Deltaproteobacteria (sulfate-reducing species; Kamio et al., 1969; Rutters et al., 2001); and Actinomycetes (Pasciak et al., 2003). Among all the cases, the plasmalogen (1-alk-1′-enyl) forms are seen in Clostridiales, Actinomycetes, Myxococcales, and *Mycoplasma*, while in the other cases, only the reduced forms (1-alkyl-2-acyl- or di-*O*-alkyl) have been observed and/or the data to date are ambiguous. However, among all of these examples, the only bacteria confirmed to contain the 'eukaryotic' (oxidative) pathway are *Mycoplasma*. This pathway substitution may be related to their general ecophysiology as mammalian parasites. Most other evidence is consistent with the idea that bacteria, regardless of aerobic or anaerobic lifestyle, contain the reductive pathway. There is no prescriptive reason, however, to presume that this must be the case – in the future, more bacteria may be discovered to use the 'eukaryotic' oxidative pathway. This clearly is an area requiring further research.

An additional feature of some bacteria is that they also include among them several cases of diglycerol membrane-spanning lipids. Such monolayer lipids are especially widespread in the Thermotogales (Damsté et al., 2007), Clostridia (Thermoanaerobacter; Jung et al., 1994), *Butyrivibrio* (Klein et al., 1979; Clarke et al., 1980), *Sarcina* (Jung and Hollingsworth, 1994), and Acidobacteria (Damsté et al., 2011). In these lipids, the terminal ends of the FA units are condensed across the middle of the membrane independently from the presence or absence of ether linkages to the glycerol backbone. The membrane-spanning bonds may form after the ester–ether reduction steps, if the linking process is terminally biosynthetic. However, the balance of evidence to date makes it more likely that ester-to-ether reduction is the terminal step, especially if the common presence of easily hydrolyzable diabolic acids is an indicator of a tetraester intermediate (Damsté et al., 2011; Jung and Hollingsworth, 1994). This would suggest that the transmembrane linkage occurs either immediately before or after IPL head-group modification but generally before ester-to-ether reduction.

In bacteria, the most common central joining pattern generates vicinal dimethyl groups formed by ω-1, ω′-1 coupling of the two *n*-alkyl chains through a presumed dehydrogenation or biradical mechanism (Clarke et al., 1980; Fitz and Arigoni, 1992; Jung and Hollingsworth, 1994). The activity of this unidentified pathway is believed to be constitutive, that is, always expressed, and is thought to exploit a commonly existing – although not yet identified – enzymatic mechanism (Lee et al., 1998). In contrast, the transmembrane lipids of Acidobacteria (Damsté et al., 2011), *Thermoanaerobacter* (members of the Clostridiales; Jung et al., 1994), and possibly other, as yet unidentified environmental bacteria (Weijers et al., 2009, 2010) appear to be formed by terminal ω, ω′ coupling of *iso*-methyl branched FA units. The mechanism for this bond formation also remains unknown, but the examples earlier show it can occur in both aerobes and anaerobes. Therefore, to seek further ideas about how both glycerol ether bonds and

membrane-spanning linkages may be synthesized by bacteria, we must look to the analogous pathways in archaea.

12.11.2.5 Diethers and Tetraethers of Archaea

Although membrane lipids of archaea also are synthesized from glycerol backbones and hydrophobic tails, they are distinguished from those of bacteria and eukaryotes by several features. Numerous studies have established that (1) archaea form ether, rather than ester, linkages to glycerol, (2) these archaeal ethers always have *sn*-2,3-glycerol stereochemistry, and (3) the attached hydrophobic chains are isoprenoid. Diphytanyl glycerol diethers first were reported for a halophilic species of Euryarchaeota, *Halobacterium cutirubrum* (*H. salinarium*; Kates et al., 1963). The diether compound, *sn*-2,3-di-*O*-phytanyl glycerol, common in both halophiles and methanogens, is known as archaeol. The related C_{40} biphytanyl isoprenoid chains of archaea are diagnosed by their central 4′–4 isoprenoid linkage (**Figure 6**), a feature that enabled early detection of these products in geologic sediments and petroleum (e.g., Chappe et al., 1982; Michaelis and Albrecht, 1979; Moldowan and Seifert, 1979). Early references logically postulated that the presence of C_{40} biphytanyl groups implied that some archaea had joined the ends of the two phytane chains of a single archaeol to make a glycerol diether macrocycle (e.g., Derosa et al., 1974). Although in some cases this is a verified product, both in cultures and environmental samples (Comita et al., 1984; Pancost et al., 2006), Langworthy established that the vast majority of C_{40} chains are due to the presence of dibiphytanyl diglycerol tetraethers (Langworthy, 1977; Tornabene and Langworthy, 1979). These tetraether compounds, the *sn*-2,3-di-*O*-biphytanyl diglycerols, including those isomers with rings and unsaturations in the isoprenoid chains, collectively are known as caldarchaeols.

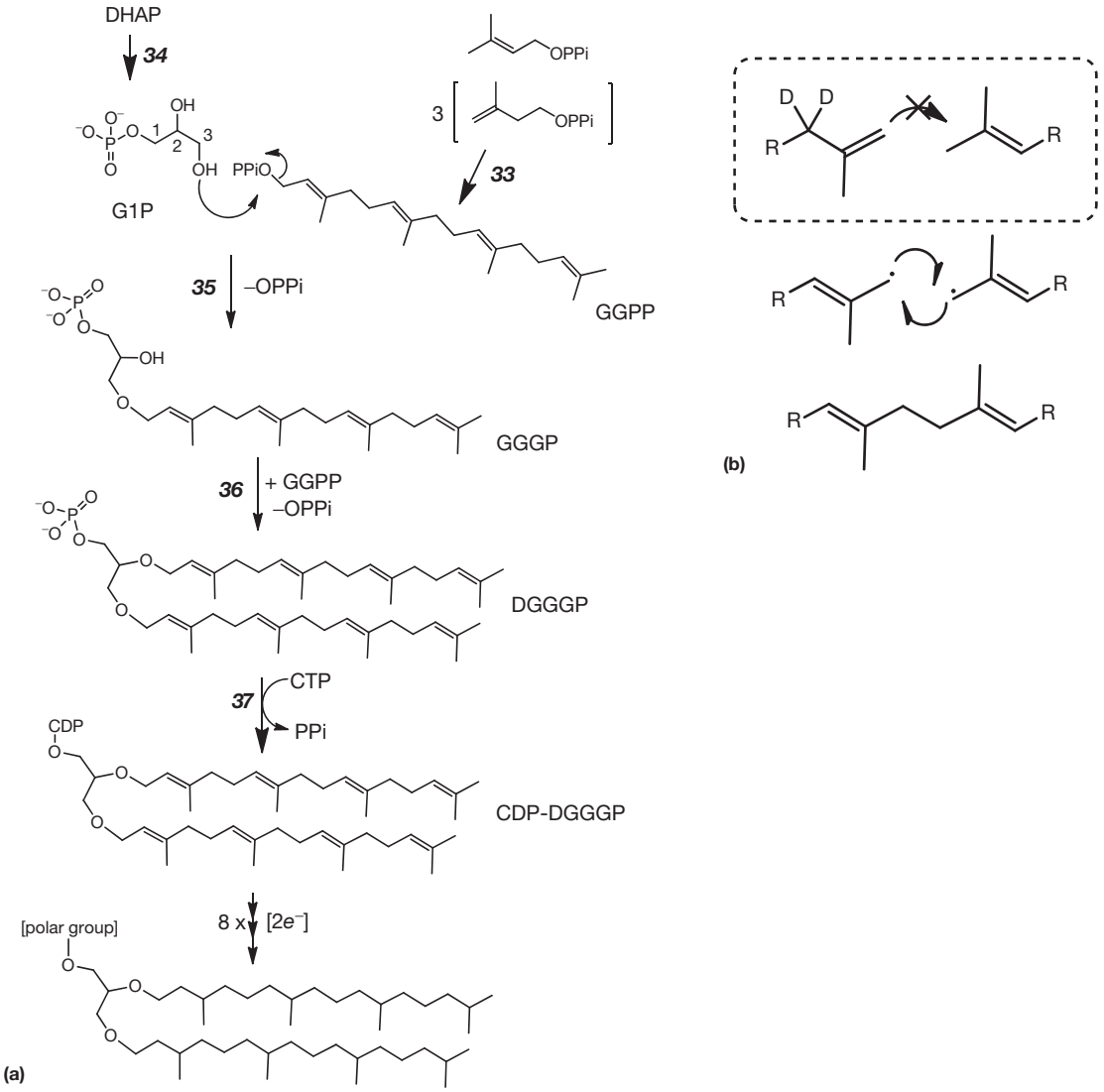

Figure 6 (a) Synthesis of archaeal diether lipids. (b) The proposed biradical synthesis of 4′–4 linkages in archaeal tetraether lipids; deuterated substrates reveal that a terminal methylene is nonreactive and therefore not an intermediate (dotted box; Eguchi et al., 2003; Kon et al., 2002).

A special cyclohexane ring-containing caldarchaeol is called crenarchaeol (Damsté et al., 2002a) for its discovery in selected Crenarchaeota, most of which now are classified as Thaumarchaeota (Brochier-Armanet et al., 2008; Spang et al., 2010). By convention, the name of this structure remains crenarchaeol. Colloquially, these compounds all are known as GDGTs (glycerol dialkyl glycerol tetraethers).

Synthesis of membrane lipids in archaea (**Figure 6**) begins with isomerization of IPP to DMAPP by the archaeal isomerase IDI2 (enzyme ***17***; *idi2*; **Table 1**), followed by chain elongation by geranylgeranyl diphosphate (GGPP) synthase (GGPPS; enzyme ***33***; gene *idsA*; Chen and Poulter, 1993, 1994; Tachibana et al., 1993). Meanwhile, the unique *sn*-2,3-di-*O*-alkyl stereochemistry of the glycerol backbone is controlled by channeling all three-carbon precursors for glycerol (whether anabolic or catabolic) through the intermediate, DHAP. DHAP then is converted to glycerol-1-phosphate (G1P) by a dehydrogenase (enzyme ***34***; Kakinuma et al., 1990b; Nishihara and Koga, 1995). Earlier suggestions that in *Sulfolobus* spp., glycerol is phosphorylated directly to G1P by a glycerol kinase (Kakinuma et al., 1990a) now are suggested to be incorrect, because *Sulfolobus* contains an active homologue of ***34*** (cf. Koga and Morii, 2007).

The components G1P and GGPP then must be joined. Importantly, the steps for prenylation of glycerol do not resemble either the eukaryotic route to ether bonds (acyl-alcohol direct substitution) or the bacterial pathway (acylation followed by reduction). Rather, this process (**Figure 6**) is akin to attachment of the phytyl group in chlorophyll biosynthesis. In archaea, the first ether bond is formed from direct nucleophilic addition of GGPP to G1P to form *sn*-3-*O*-geranylgeranyl glycerol-1-phosphate (enzyme ***35***) (Chen et al., 1993; Poulter et al., 1988; Zhang et al., 1990). There is no ester intermediate at any stage of the synthesis pathway, and the oxygen in the ether bond is the same oxygen atom that originated with the glycerol moiety (Kakinuma et al., 1990b). A separate enzyme is responsible for attaching the second side chain, forming *sn*-2,3-di-*O*-geranylgeranyl glycerol-1-phosphate (DGGGP; common name archaetidic acid) (enzyme ***36***; Zhang and Poulter, 1993). DGGGP synthase is a specific homologue of the chlorophyll synthesis enzyme ChlG and part of the UbiA prenyltransferase family (Hemmi et al., 2004). The remaining steps in the synthesis of archaeol are the addition of polar head groups and saturation of the isoprenoid side chains. Caldarchaeol requires these steps plus the additional formation of the 4′–4 central linkage. Less is known about all of these steps downstream from DGGGP, but recent reviews (e.g., Koga and Morii, 2007) and new data (Murakami et al., 2007; Sasaki et al., 2011; Sato et al., 2008) suggest a nearly complete, plausible pathway.

It is well established that the initial head-group modifications of archaetidic acid occur prior to saturation of the isoprenyl groups. Minimally, this includes conversion of phosphate to phosphatidyl glycerol phosphate (PGP) or phosphatidyl glycerol (PG) (Moldoveanu and Kates, 1988) via a common cytidyl intermediate (enzyme ***37***; Morii et al., 2000). The timing of further replacement of PGP and PG polar groups to other moieties, relative to saturation and/or formation of tetraethers, has been debated (Nemoto et al., 2003; Nishihara et al., 1989). However, it is clear that the terminal biosynthetic head groups of Archaea can include serine, ethanolamine, *myo*-inositol, and glucosyl groups (cf. Koga and Morii, 2007), while the biosynthetic 'intermediates' detected in association with the unsaturated DGGGP most often are PGP and PG. Of these IPL forms, the majority of evidence indicates that sugar-derived head groups are the terminal step of a progressive synthesis and/or are added after the saturation of side chains. This suggests that the major IPL forms at the time of the saturation step are DGGG–PGP and/or DGGG–PG.

The geranylgeranyl groups of PGP- and/or PG-archaetidic acid are saturated fully to phytanyl units by geranylgeranyl reductase (GGR) (Murakami et al., 2007; Nishimura and Eguchi, 2006), which is a flavoprotein homologous to ChlP, the enzyme responsible for reducing GGPP to phytyl-PP in chlorophyll biosynthesis in bacteria and eukaryotes. However, there are at least two important differences between archaea and the other domains: (1) in the synthesis of chlorophyll, GGPP is reduced to phytyl-PP before prenylation of chlorophyll, while in archaea, GGPP is the prenyl donor and reduction proceeds on the completed lipid skeleton (Poulter et al., 1988); (2) the final product in chlorophyll synthesis is phytyl-, while the final product in archaea is phytanyl-. Interestingly, *in vitro*, the GGR of archaea is active toward free GGPP, also yielding phytyl-PP (Sato et al., 2008), even though the preferred substrate is the digeranylgeranyl glycerol macromolecule (Murakami et al., 2007). Recent crystallization of a GGR from *Sulfolobus* may explain these observations (Sasaki et al., 2011). These authors suggest that the enzyme pocket accommodates the large anionic phospho- head group (presumably PGP or PG), and the glycerol unit sits in the space where the first double bond of the simpler substrate GGPP normally would be 'protected.' This pushes the first double bond of the side chain nearer the active site for hydrogenation. Such a mechanism suggests that general models of lipid hydrogenation that invoke partially midchain saturated isoprenoids (e.g., Chikaraishi et al., 2009) only are valid when a free prenyl phosphate is hydrogenated, that is, for chlorophyll. Such a model should not be extrapolated to an equivalent series of structural intermediates in archaea. Further caution in this regard also is warranted because all identified archaeal GGRs can use dithionite (a proxy for Fe–S clusters) as an electron donor, but among archaea, only the GGR of *Thermoplasma* is able to use NAD(P)H, implying the hydrogen donor for saturating isoprenoid lipids also is different between archaea and other organisms (Nishimura and Eguchi, 2006).

The final questions about synthesis of archaeal lipids regard the 4′–4 linkages that form tetraethers and the widespread presence of cyclopentane rings in both thermophiles and mesophiles. Also of interest are the unusual products known as H-lipids (Morii et al., 1998) and the incompletely linked, or trialkyl, structures in which only one head–head bond has been formed (e.g., Schouten et al., 2000). Simple inference suggests it must be relevant that archaeol never contains cyclopentane rings, and the only cross-linking bond for diethers occurs in association with the macrocyclic diether (DeRosa et al., 1974). Cyclopentane rings and H-lipids are unique to, and must be formed either during or after, the synthesis of tetraethers. Very little is known about any of these processes, but work by Grather and Arigoni (1995) established that the 4′–4 linkage can form indiscriminately, yielding both the parallel and antiparallel arrangement (both regioisomers).

Subsequently, it was discovered that terbinafine, an inhibitor of sterol biosynthesis, blocks the synthesis of tetraethers (Kon et al., 2002). Because the major accumulated product was the saturated diether with a PG head group, they proposed that saturation takes place before the 4′–4 linkage (Kon et al., 2002; Nemoto et al., 2003). Subsequent work has demonstrated that this idea likely is incorrect: Eguchi et al. (2003) used deuterated substrates to establish that the terminal double bond of the phytyl chain is required for tetraether formation and that this double bond does not undergo migration, that is, no terminal methylene is formed. Instead, the reaction was suggested to involve the formation of methyl radicals. This requires that 4′–4 bonds be formed before or simultaneously with the action of the isoprenyl chain reduction. A similar (methyl radical) mechanism also may be involved in synthesis of cyclopentane rings, which are noted in both archaea and bacteria to result from internal attack of methyl carbons (DeRosa et al., 1977; Weijers et al., 2006).

12.11.2.6 Glycerol Membrane Lipids: Final Comments

All of the aforementioned pathways are based on a strict division between isoprenoid and acetogenic lipid moieties as the fundamental building blocks of archaeal and bacterial (plus eukaryotic) membrane lipids, respectively. However, there is both a hypothesized mechanism and hard evidence of lipid promiscuity that together disrupt this notion. Mixed isoprenoid–acetyl chains were noted by Schouten et al. (2000) as products of the hydroiodic acid degradation of ether lipids obtained from sediments. This case of 'mixed domain' lipids cannot be dismissed, given the independent finding that *Sarcina ventriculi* (Clostridiales) can incorporate exogenous lipid chains into its membranes, apparently via the same (ω-1) chain condensation mechanism used normally by bacteria to form membrane-spanning lipids (Lee et al., 1998). Such a mechanism is not quite consistent with the hybrid structures shown by Schouten et al. (2000), which are more consistent with the *iso*-methyl linkages observed in membrane-spanning lipids of Acidobacteria and *Thermoanaerobacter* (Damsté et al., 2011; Jung et al., 1994). Regardless, Lee et al. suggest that "no new protein (and possibly no new message)… is involved in the formation of these [mixed lipids]," and they imply these reactions could occur in cell-free systems "by random and indiscriminate but catalytic tail-to-tail joining of existing [lipids]." If this phenomenon is widespread in the formation of membrane-spanning lipids – including during lipid scavenging by extant biota and/or extracellularly during diagenesis – the potential implications for sediment organic geochemistry would be frightening.

The second exception to the pathways described earlier is the case of putative membrane lipid 'recycling' in sedimentary microbial communities. This phenomenon is presumed to involve hydrolysis and reformation of the ether linkage to glycerol in archaeal tetraether lipids, because incubation with ^{13}C-labeled glucose resulted in detectable isotopic enrichment of glycerol well in excess of the incorporation of label into the isoprenoid side chains (Takano et al., 2010). Further work has discovered a possible 'intermediate' for this process. Sediments from numerous environments contain measurable amounts of glycerol dialkanol (C_{40} isoprenoid) diethers (GDDs) that appear to be related to the GDGTs of archaea (Liu et al., 2012). It is likely that these lipids are derived from breakdown of GDGTs and are not true *de novo* synthetic products, given the biosynthetic pathways known to date. The authors therefore postulate that GDDs could be intermediates for the reformation of GDGTs. If the organisms recycling GDDs are archaea, perhaps an unknown alcohol kinase generates phosphorylated intermediates for reattachment to glycerol. However, an additional difficulty would remain: the preference of GGGPS for unsaturated (geranylgeranyl) rather than saturated (phytanyl) substrates is problematic, possibly requiring an alternate synthase.

Alternatively, one could imagine that the alcohol chains might be added to a new glycerol unit by a process analogous to the eukaryotic synthesis of plasmalogens (enzyme ***30***). This would involve trans-acyl/alkyl substitutions, requiring prior action of enzyme ***29*** or the equivalent. Since no ester-forming intermediates are known in archaea, this scenario would be considered unlikely for archaea but possible for bacteria. This would suggest that bacteria might be 'stealing' lipids of archaea. However, this idea also comes with significant difficulty. Replacement of an R–OH group is believed to be specific to the *sn*-1 position, and there is no known means to add R–OH directly to *sn*-2. There is no a priori reason to exclude the possibility that bacteria might be able to make ether substitutions at both positions, but quite unusually, the end product of such a recycling reaction would be expected to have the bacterial *sn*-1,2-glycerol stereochemistry on the one end of the tetraether and normal archaeal *sn*-2,3-glycerol stereochemistry on the other end. Careful analyses certainly could test for, and potentially eliminate, the existence of such a pathway.

Assuming such mixed-stereochemistry tetraethers could be eliminated, the natural conclusion is that only archaea would be capable of recycling GDDs, although by an as yet unknown mechanism that would involve rephosphorylation of the two R–OH groups of the GDD. When taken together, the various arguments for lipid recycling have two important implications: first, that mixed isoprenoid–acetogenic membrane-spanning lipids (Schouten et al., 2000) likely are archaeal and, second, that bacteria are not likely to be able to recycle their tetraethers via GDD intermediates. This conclusion of course hinges on whether the limited observations to date are sufficiently thorough.

12.11.2.7 Polar Head Groups

The wide diversity of polar head groups of membrane lipids includes phosphorylated and sulfated moieties, neutral sugars, amino acid derivatives, and even nucleotide derivatives. Because of the wide diversity of such groups and their numerous biosynthetic pathways, only those most directly relevant to geochemistry are reviewed here: glycolipids, sulfolipids, betaine lipids, ornithine lipids (OLs), and selected phospholipids. There are numerous recent reviews (e.g., Benning, 1998; Dowhan, 1997; Geiger et al., 2010; Hölzl and Dörmann, 2007; Lopez-Lara et al., 2003), and for early history, see Kates (1964). Importantly, there is wide domain-level heterogeneity in the distribution of these structures. Several of the classes in the preceding text have been detected across all three domains, and there are many similarities in how they are synthesized.

Most cell membranes are dominated by phosphorylated diacylglycerol (DAG; diglyceride) lipids. Major classes found widely in bacteria and eukaryotes (archaea are discussed later) include phosphatidylglycerol (PG), PE, and phosphatidylcholine (PC). The primary driver of phospholipid biosynthetic diversity is thought to be their multitude of roles in cellular function. Phospholipid head groups are synthesized through a common initial pathway that branches to yield varying proportions of the final structures, a feature of biochemical unity first proposed by Kennedy (1961). The pathway universally begins with the conversion of phosphatidic acid (PA; diacylglycerol-3-phosphate) to the central intermediate, cytosine diphosphate (CDP)-diacylglycerol (CDP–DAG) (enzyme ***38***, gene *cdsA*; **Table 1**; **Figure 7**), which then is diverted to its respective fate: PG, PE, or PC. The *cdsA* gene first was cloned from *E. coli* (Icho et al., 1985) after studies already had prepared pure CdsA from both *E. coli* and yeast (Kelley and Carman, 1987; Sparrow and Raetz, 1985). In the synthesis of PG, an additional glycerol-3-phosphate is attached to form PG phosphate (PGP; enzyme ***39***, *pgsA*), with subsequent loss of phosphate to yield PG (enzyme ***40***, *pgpA*, *pgpB*, or *pgpC*). PgsA activity first was demonstrated in *E. coli* (Icho and Raetz, 1983), and *pgsA* from *Rhodobacter* is similar to the gene in *E.coli* (Dryden and Dowhan, 1996). More recently, Lu et al. (2011) showed that only one of the three phosphatases in *E. coli* (*pgpA*, *pgpB*, and *pgpC*) is needed and that many bacteria have orthologs to *pgpC* while fewer have *pgpA*; the equivalent ortholog in eukaryotes remains unknown.

In the synthesis of PE, CDP–DAG is modified to PS (enzyme ***41***, *pssA*), which then is decarboxylated to yield PE

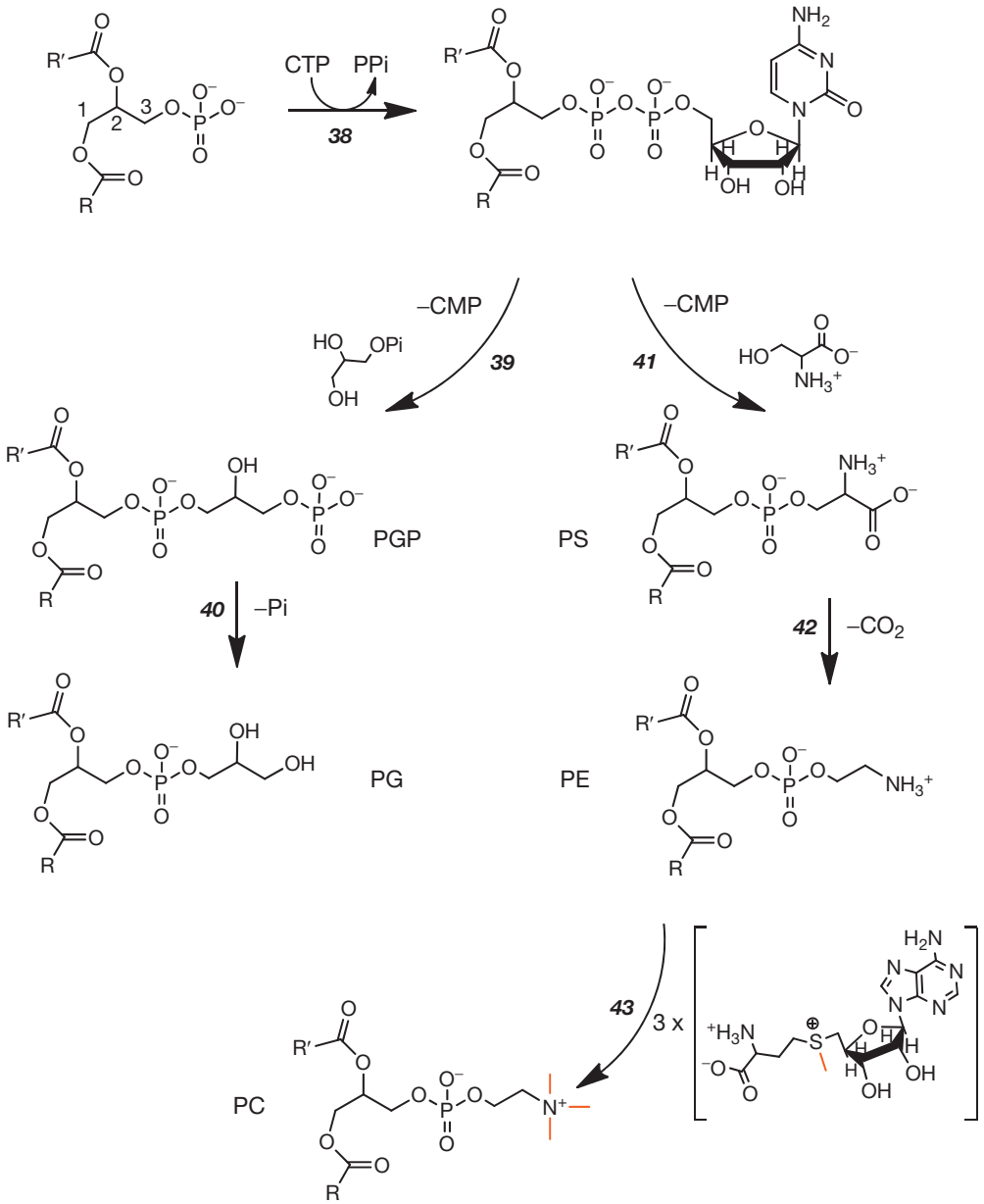

Figure 7 Synthesis of phospholipids PG, PE, and PC from diacylglycerol-3-phosphate.

(enzyme *42*, *psd*). The enzyme function of PssA has been described (Ohta and Shibuya, 1977; Raetz, 1975) and the *pssA* gene cloned in *E. coli* (Dechavigny et al., 1991). PsD activity was identified by Hawrot and Kennedy (1975) and *psd* cloned by Matsumoto et al. (1998), the latter in *Bacillus subtilis*. The resulting PE either is the final product or is modified further to PC (**Figure 7**).

PC is a major lipid of eukaryotes. There are two pathways known for synthesis of PC in eukaryotes, one based on *de novo* synthesis and one on metabolite recycling (reviewed by Kent, 1995). In the *de novo* pathway, the amine group of PE is methylated three times in succession by a PE *N*-methyltransferase (enzyme *43*, *pmtA*). Although originally thought to be rare in bacteria (Goldfine, 1982; Oliver and Colwell, 1973), PC now is recognized in a wide diversity of bacteria. Sohlenkamp et al. (2003) used both lipid analyses and genomic sequence data to estimate that >10% of bacteria can make PC, including many Alpha- and Gammaproteobacteria, Actinomycetes, Bacteroidetes, and possibly other phyla. In the earliest example of PC synthesis for bacteria, Kaneshiro and Law (1964) purified and demonstrated activity of PmtA from *Agrobacterium tumefaciens* without knowledge of the corresponding gene. The *pmtA* gene now has been cloned for *Rhodobacter sphaeroides* (Arondel et al., 1993), for yeast (Kodaki and Yamashita, 1987), and for the mammalian form (Cui et al., 1993). All PmtAs are *S*-adenosylmethionine (SAM) enzymes. However, among bacteria, there are at minimum two known families of SAM enzymes having PmtA activity, the 'Rhodobacter' and the 'Rhizobial' groups (reviewed in Sohlenkamp et al., 2003). Because of the widespread distribution and diverse functions of SAM methyltransferases across all organisms, it may be challenging to know if or how many other SAM enzymes have PmtA activity.

Glycolipids are another important class of cell membrane lipids in bacteria and eukaryotes (Hölzl and Dörmann, 2007; Kates, 1966). In particular, digalactosyl diacylglycerols are abundant and essential components of higher plant chloroplasts, eukaryotic algae, and Cyanobacteria (Benson, 1964; Nichols et al., 1965; Siebertz et al., 1979). Mono- and diglucosyl (or mixed glucose–galactose) head groups are the most common types in other bacteria (Benning et al., 1995; Brundish et al., 1966), and their proportion is governed by environmental conditions and the need to maintain cellular homeostasis (Karlsson et al., 1997). The intermediate for the formation of glycolipids is not PA, but rather is DAG. The first step is conversion to a uridine diphosphate sugar intermediate (UDP-sugar), most commonly UDP-glucose or UDP-galactose. The larger family of UDP-sugar glycosyltransferases includes both glucosyl- and galactosyltransferases, as well as sugar transferases important for cellular functions other than lipid biosynthesis (Campbell et al., 1997). Many of the lipid glycosyltransferases of bacteria, eukaryotes, and archaea share common sequence properties (Berg et al., 2001) despite their known functional differences.

Using these enzymes, there are at least four commonly recognized pathways for synthesis of glycolipids. (i) Plant chloroplasts use two distinct synthases, the first joining UDP-galactose and DAG to yield monogalactosyldiacylglycerol (MGDG) (enzyme *44*; **Table 1**; Shimojima et al., 1997) and the second using MGDG plus UDP-galactose to yield digalactosyldiacylglycerol (DGDG) (enzyme *45*; **Figure 8**) (Benning and Ohta, 2005; Dörmann et al., 1995). Chloroflexi also use a plant-type MGDG synthase and DGDG synthase (Hölzl et al., 2005). The amino acid sequence of *44* is most similar to bacterial MurG, an enzyme involved in peptidoglycan synthesis rather than lipid synthesis. (ii) In contrast with higher plants, Cyanobacteria begin with UDP-glucose and DAG, yielding monoglucosyl-DAG (enzyme *46*), which then is followed by an unknown epimerase to yield MGDG (Awai et al., 2006; Sato and Murata, 1982). The subsequent DGDG synthase also is unknown. (iii) The UDP-galactose diacylglyceroltransferase (enzyme *47*; MGDG synthase) in the green sulfur bacterium *Chlorobium tepidum* is not an ortholog either of *44* or *46* (Masuda et al., 2011). Discovery of this novel MGDG synthase verifies that synthesis of MGDG is essential to all known photosynthetic groups (Block et al., 1983). (iv) Perhaps most interesting are the cases of glycolipids in non-photosynthetic bacteria. *Bacillus subtilis* encodes a UDP-glucose:1,2-diacylglycerol-3-β-D-glucosyl transferase that is capable of transferring one or more sugars to create mono-, di-, or polyglycosylated diacylglycerols (enzyme *48*; gene *ypfP*; Jorasch et al., 1998). Enzymes of this type are termed 'processive glycosyl transferases' and also are found in Alphaproteobacteria and Actinomycetes (Benning et al., 1995; Jorasch et al., 2000; Tang and Hollingsworth, 1997). Other UDP-glucose transferases specific for the less common α-glucosyl linkages (enzyme *49*) are found in *Deinococcus*, Thermotogales, and the Firmicutes *Streptococcus*, *Acholeplasma*, and *Enterococcus* (Berg et al., 2001; Hölzl et al., 2005; Manca et al., 1992; Theilacker et al., 2011). The α-DGDG synthase also was identified in *Enterococcus* (enzyme *50*; Theilacker et al., 2009). As a final note, glycolipids are common in archaea but must require a dedicated 2,3-dialkylglycerol-1-glucosyl transferase. Because of this stereochemical difference, such a transferase is likely to be different from the bacterial and eukaryotic versions (Kates, 1992).

Sulfolipids are synthesized specifically in lieu of PC when cells are under conditions of phosphorous stress. Because PC is not ubiquitous in bacteria, the distribution of sulfolipids is similarly limited. The major sulfolipid, sulfoquinovosyl diacylglycerol (SQDG), is widespread in Cyanobacteria and Alphaproteobacteria. It also is found in all higher plants and in most other photosynthetic eukaryotes, where it is associated with the plastids (Benning, 1998; Lopez-Lara et al., 2003). As with other phosphorus-free IPLs (see succeeding text), synthesis of SQDG is upregulated by the activity of P-stress genes (*phoB*; Geiger et al., 1999). The appearance of SQDG in environmental samples commonly is interpreted as a bacterial signal indicating the presence of P-stressed prokaryotic autotrophs (Popendorf et al., 2011b; Van Mooy and Fredricks, 2010; Van Mooy et al., 2009). The ubiquity of the SQDG pathway among both marine and freshwater species, as well as in higher plants, has been interpreted as evidence of an ancient marine ancestry of photoautotrophs (Alcaraz et al., 2008), although given the general tendency toward P-scarcity in most environmental systems (Elser et al., 2007), this idea may need to be reexamined.

Synthesis of SQDG in *R. sphaeroides* begins with UDP-glucose, which is modified to UDP-sulfoquinovose using sulfite (enzyme *51*, gene *sqdB*; Benning and Somerville, 1992). Attachment to DAG proceeds either via a two-enzyme system

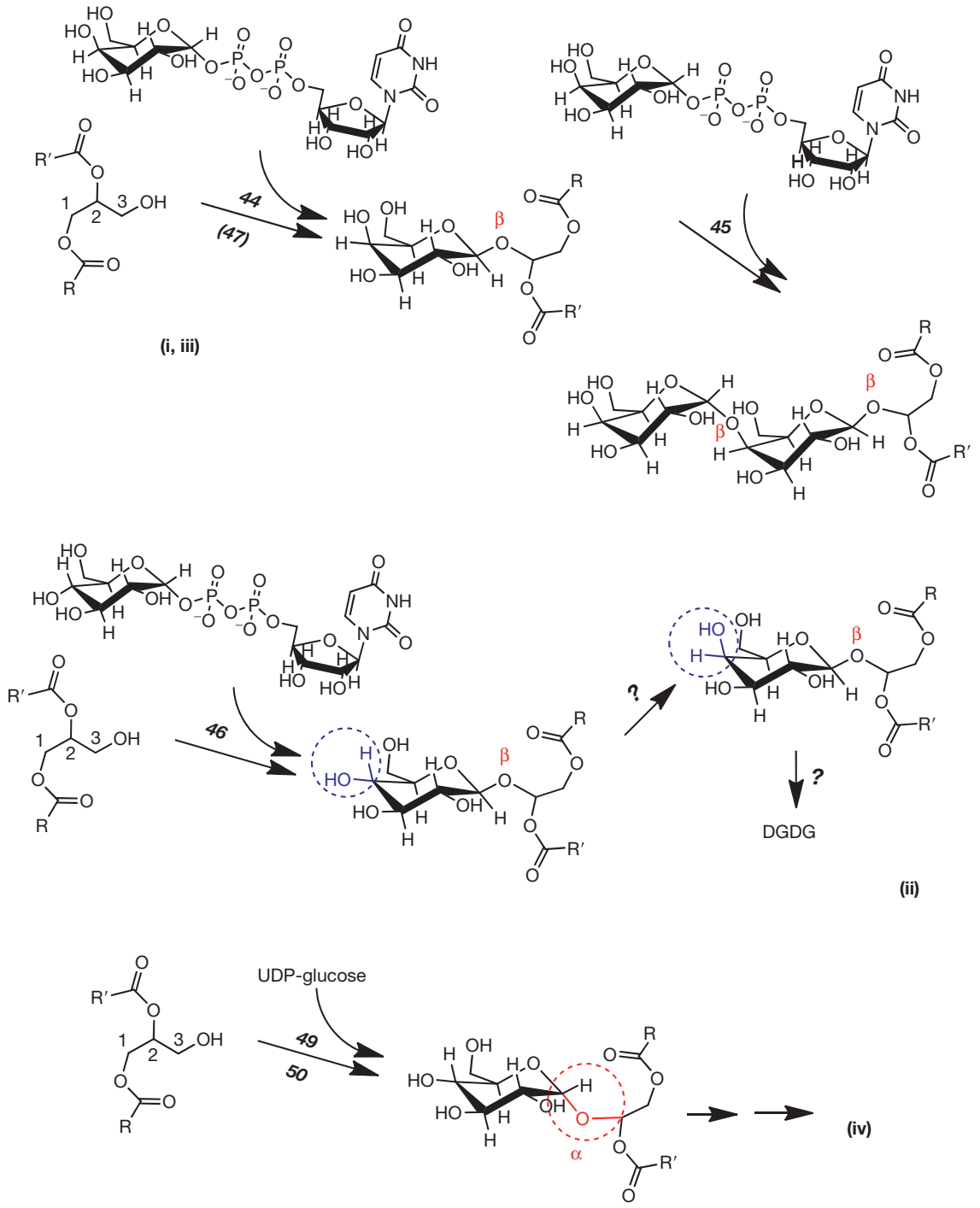

Figure 8 Synthesis of common glycolipids in (a) higher plants, (b) Cyanobacteria, (c) green sulfur bacteria, and (d) nonphototrophic bacteria. Roman numerals refer to pathway numbers described in the text.

(enzymes ***52***, ***53***; *sqdC*, *sqdD*; Benning, 1998; Rossak et al., 1995) in which SqdD is essential and its activity is enhanced by SqdC or by a one-enzyme step (enzyme ***54***; *sqdX*; Guler et al., 2000) to form the final product 6-sulfo-α-D-quinovosyl diacylglycerol (**Figure 9**). The *sqdB* gene occurs widely in photosynthetic bacteria and eukaryotes and is homologous between the domains (Lopez-Lara et al., 2003). *SqdB*, *sqdC*, and *sqdD* (or *sqdX*) commonly are organized as an operon in bacteria (Benning, 1998). A homologue encoding for SqdB activity also is found in some archaea, but the resulting UDP-sulfoquinovose in these species is believed to be used for the synthesis of *S*-layer glycoprotein, which is part of the archaeal cell wall, and not for the biosynthesis of sulfolipids (Meyer et al., 2011).

SQDG originally was discovered in higher plants (Benson et al., 1959). Synthesis of SQDG in higher plants proceeds via

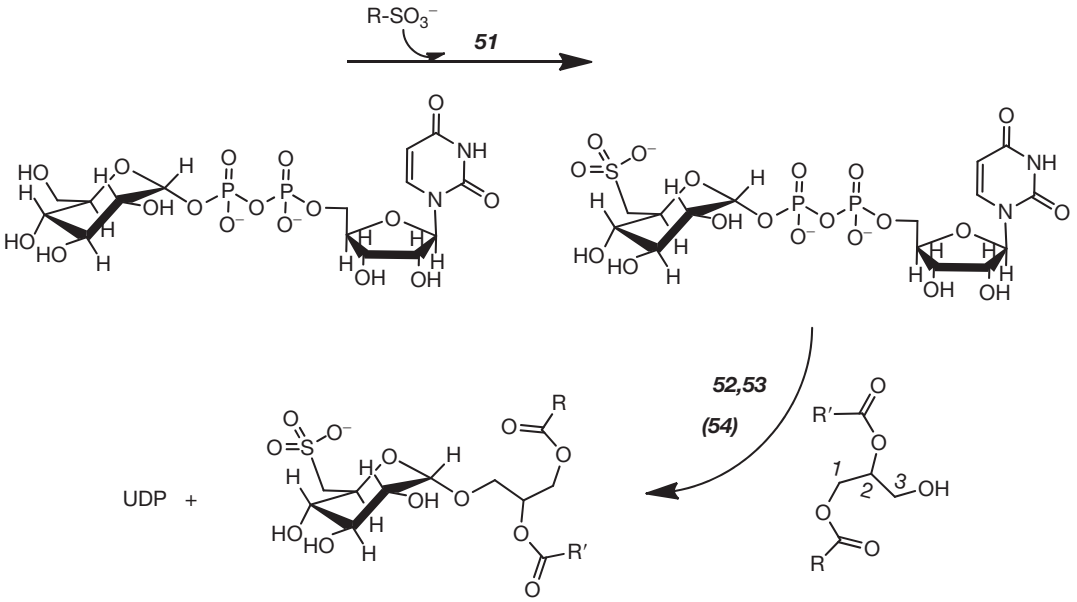

Figure 9 Synthesis of the sulfolipid SQDG from DAG (reviewed in Benning, 1998).

an identical pathway in which SQD1 is equivalent to SqdB (Sanda et al., 2001) and SQD2 is equivalent to SqdD(+C) (Yu et al., 2002). The prevalence of and specific activity of SqdB/SQD1 has been used to promote the generality that SQDG is common to all aerobic, photosynthetic species; however, SQDG also is found in anaerobic photosynthesizers and in some nonphotosynthetic organisms (Benning, 1998). Similarity searching reveals the widespread occurrence of *sqdB* homologues among Acidobacteria, Deltaproteobacteria, Chloroflexi, and Actinomycetes; but identifiable *sqdD* is less widespread (Lopez-Lara et al., 2003).

Betaine, or *N*,*N*,*N*-trimethylhomoserine lipids (BL), are diglyceride lipids in which the head group is formed from the homoserine group of SAM. They are common products of algae, bryophytes, and other 'lower plants'; some nonphotosynthetic protozoa; and some bacteria (Sohlenkamp et al., 2003). Similar to SQDG, BL are directly substituted for PC when organisms are P-limited (Benning et al., 1995; Geiger et al., 1999). They are common in nutrient-limited photic zones (Schubotz et al., 2009; Van Mooy and Fredricks, 2010; Van Mooy et al., 2009) and are believed to be primarily sourced to photosynthetic eukaryotes in these systems (Dembitsky, 1996; Kato et al., 1996; Popendorf et al., 2011a).

Although BL first were discovered in eukaryotic algae, their biosynthesis was established by studies of the bacterium, *R. sphaeroides*. The aminocarboxypropyl ('homoseryl') group of SAM is added to DAG to create DAG-*O*-homoserine (enzyme **55**), and then three additional SAM molecules donate their methyl groups (enzyme **56**; Klug and Benning, 2001; Riekhof et al., 2005a; **Figure 10**). It was originally suggested that betaine synthesis in bacteria might be limited only to Alphaproteobacteria (Klug and Benning, 2001; Lopez-Lara et al., 2003), but homologous genes are known additionally in Planctomycetes, Deltaproteobacteria, Gammaproteobacteria, and in the photosynthesizer *Chloracidobacterium thermophilum* (Geiger et al., 2010). The eukaryotic green alga *Chlamydomonas reinhardtii* has a multifunctional enzyme that mediates the transfer of both types of donor groups from the SAM cofactor, a system that apparently generalizes to all eukaryotes (Riekhof et al., 2005b).

OLs are unusual amidified 3-hydroxy fatty acyl lipids further esterified to a second fatty tail, that is, they are not glycerides. Similar to betaines and sulfolipids, OLs are formed under conditions of phosphorous stress (Minnikin et al., 1972). First characterized in *Thiobacillus thiooxidans* (Knoche and Shively, 1972), co-occurrence of OL with SQDG and BL in *Rhizobium meliloti* was established by Geiger et al. (1999). It now appears that OLs are widespread phylogenetically in bacteria, appearing in Firmicutes, Mycobacteria, a variety of Proteobacteria, and perhaps other phyla (Kawai et al., 1988; Laneelle et al., 1990; Lopez-Lara et al., 2003). To date, OLs are not known in archaea or eukaryotes (Lopez-Lara et al., 2003), and their presence in the environment has been minimally assessed, with none detected in the Sargasso Sea (Van Mooy et al., 2009), but detectable amounts in the upper anoxic region of the Black Sea (Schubotz et al., 2009).

Synthesis of OL begins with the condensation of the noncoded amino acid ornithine with a 3-hydroxy (β-hydroxy) fatty acyl group carried by acyl carrier protein (AcpP) using an *N*-acyltransferase (enzyme **57**, gene *olsB*; Gao et al., 2004). Then, an *O*-acyltransferase also requiring acyl-AcpP donates the second fatty tail (enzyme **58**, *olsA*; Weissenmayer et al., 2002) (**Figure 11**). The enzyme OlsA is so similar to the second acyltransferase (PlsC) of normal FA synthesis that, in at least one case, it is bifunctional, compensating for lack of PlsC activity in a Δ*plsC* mutant (Aygun-Sunar et al., 2007). The two genes, *olsB* and *olsA*, form an operon in many of the bacteria in which they are found, and annotations identify these genes in ≥15% of the Proteobacterial genomes currently sequenced; identifying homologues in other phyla is more difficult (Lopez-Lara et al., 2003). More recently, analogous glutamine-amidified lipids have been identified

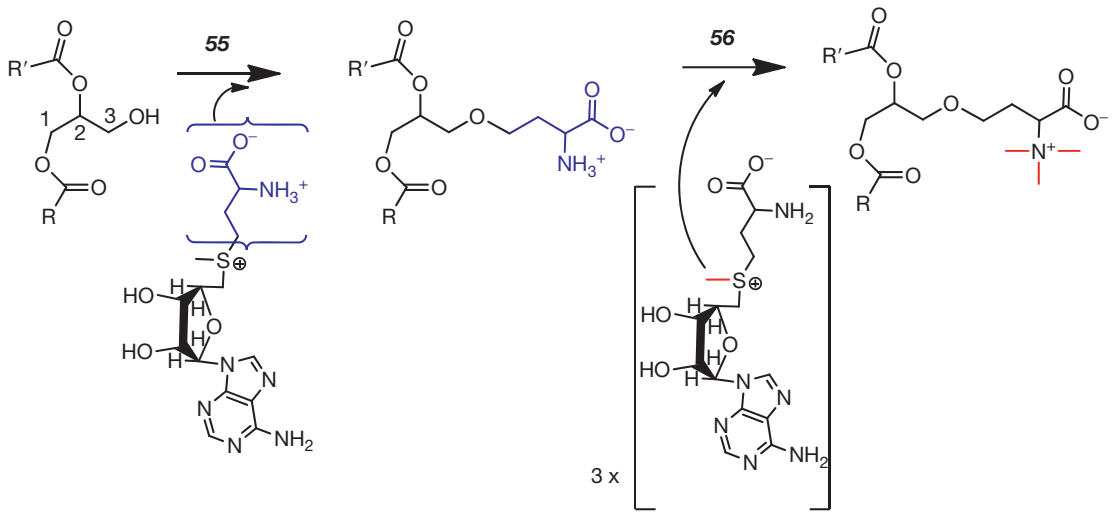

Figure 10 Synthesis of betaine lipids from DAG (Klug and Benning, 2001; Riekhof et al., 2005a).

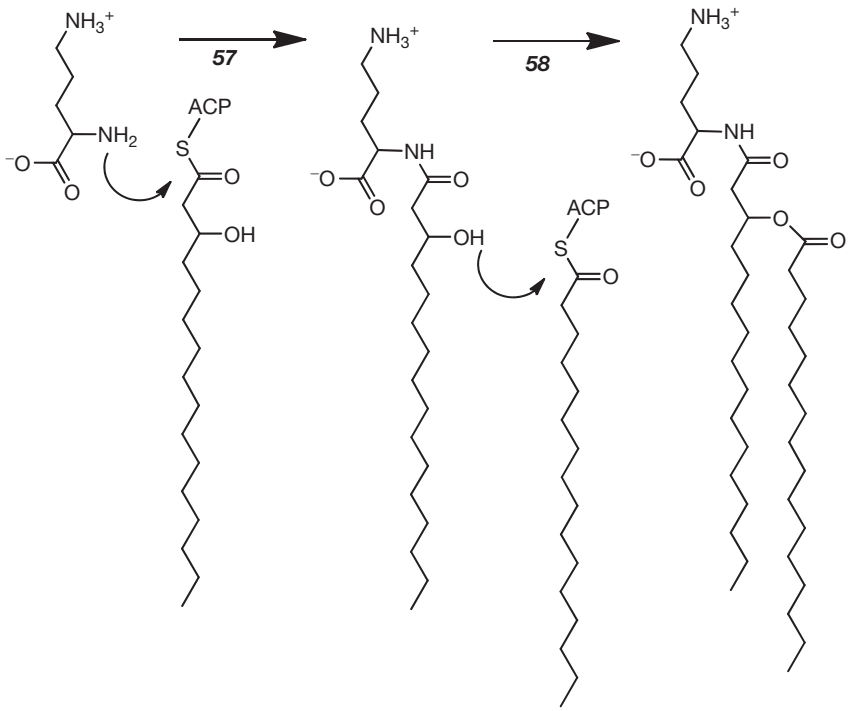

Figure 11 Synthesis of ornithine lipids (adapted from Gao JL, Weissenmayer B, Taylor AM, Thomas-Oates J, López-Lara IM, and Geiger O (2004) Identification of a gene required for the formation of lyso-ornithine lipid, an intermediate in the biosynthesis of ornithine-containing lipids. *Molecular Microbiology* 53: 1757–1770).

(Zhang et al., 2009), but it is not yet clear how widespread they are or if a distinct enzyme replaces the function of OlsB (Geiger et al., 2010).

The IPLs of archaea include no aminolipids and, with the exception of a few unusual cases in halophiles, also no sulfolipids (Kates, 1992). Instead, the balance between P-containing and P-free lipids is achieved through the widespread use of glycolipids. IPLs of archaea include the intermediates PA and PGP, and the end products PG, PE, PS, phosphoinositol (PI), and mono- and diglycosyl lipids, all pointing to the biochemical commonality of IPL head groups among the domains of life. Uniquely, archaeal membrane-spanning lipids also are found as phosphoglycolipids: structures with a mono- or diglycosidic group on the one end, and PG, PE, PS, or PI on the other. Significantly, archaea do not make phosphocholine and as such are not expected to make SQDG, betaine, or OLs as substituents. Archaea also favor glucosyl rather than galactosyl lipids. Because of the different stereochemistry in archaea (glycerol-1-phosphate instead of glycerol-3-phosphate), the enzymes involved in IPL synthesis must be different from those of bacteria and eukaryotes. Further differences concern the timing of IPL head-group synthesis relative to the joining of

diethers to form tetraethers (Section 12.11.2.5). However, in common with bacteria and eukaryotes, IPL synthesis begins with the formation of a CDP derivative, in this case CDP-archaeol (enzyme *37*, Section 12.11.2.5).

12.11.2.8 Linear Polyprenes

Long-chain polyprenes with 1′–4 and 1′–1 linkages are ubiquitous across the three domains, where in many cases they serve as substrates for the formation of more complex structures (e.g., hopanoids) or as subunits of other molecules (e.g., the phytyl ester of chlorophyll). In the synthesis of biologic isoprenoids, DMAPP is the primer and IPP is added consecutively for chain extension (Figure 12). Polyprenyl structures were recognized very early in eukaryotes (Ruzicka, 1938; Wallach, 1885), bacteria (Van Niel and Smith, 1935), and archaea (Baxter, 1960; Kates et al., 1963), primarily through the study of pigments.

All organisms contain a minimal set of polyprenyl synthases, while some contain additional, more specialized synthases. The C_{15} sesquiterpene, farnesyldiphosphate (FPP) (enzyme *59*; Clarke et al., 1987; Eberhardt and Rilling, 1975; Fujisaki et al., 1990), and the C_{20} diterpene, geranylgeranyl phosphate (GGPP) (enzyme *33*; Chen and Poulter, 1994; Math et al., 1992), are the most common extended polyprenes. The C_{10} compound, geranyl diphosphate (enzyme *60*; Burke et al., 1999), is found mainly in higher plants, where it is the precursor of monoterpene volatiles. Importantly, GPP is not a preferred exogenous precursor for FPP; rather, FPP usually is made from DMAPP + 2IPP (Poulter and Rilling, 1978; Tarshis et al., 1994). Other extended polyprenyl synthases also are essential in all species, as chain lengths of C_{40} or more are needed for the synthesis of quinones used in energy metabolism (Asai et al., 1994; Collins and Jones, 1981; Tachibana et al., 2000).

More specialized polyprenes are formed when combinations of the units in the preceding text are joined, usually in a 1′–1 linkage. This linkage is known in organic geochemistry as 'tail–tail,' but in most other chemical literature, it is referred to as 'head–head.' Of particular relevance to geochemistry are the formation of squalene from two FPP precursors and the formation of phytoene from two GGPP precursors. The former is the precursor for hopanoids and steroids, while the latter is the precursor of carotenoids. Squalene synthase (enzyme *61*, *ERG9*, *hpnD*; Jennings et al., 1991) mediates a two-step condensation of two FPPs. The reaction involves cyclopropanation (1′–2–3 coupling; Figure 13), followed by reduction by NADPH; the stable intermediate presqualene diphosphate (PSPP) does not exit the enzymatic pocket during this process (Blagg et al., 2002; Poulter, 1990). Synthesis of squalene has been characterized primarily in yeast (Beytia et al., 1973; Rilling, 1966; Sasiak and Rilling, 1988) but only recently in bacteria (Lee and Poulter, 2008). Phytoene synthase, using two GGPPs (enzyme *62*; Misawa et al., 1994), is the first committed step of carotenoid synthesis and involves a similar two-step process, although the final product is not reduced. Phytoene synthases have been widely characterized (e.g., Buckner et al., 1996; Dogbo et al., 1988; Iwata-Reuyl et al., 2003). Because of the sequence similarities between *61* and *62*, incorrect annotations are common in genomic databases. For example, *Methylobacterium extorquens* AM1, a species that is known to synthesize hopanoids, has multiple annotated phytoene synthases but no annotated squalene synthases.

12.11.2.9 Hopanoids

Hopanoid lipids were discovered as hopanes in geologic samples and in plants of the genus *Hopea*. For many years, they were regarded as 'orphan' biomarkers, because their dominant sources to sediments were not known (reviewed in Ourisson and Albrecht, 1992). These pentacyclic triterpenoids now are known as bacteriohopanepolyols, or more generically, hopanoids. Their primary bacterial origin first was detected in *Acetobacter xylinum* (Förster et al., 1973; Rohmer and Ourisson, 1976b). It was then proposed that hopanepolyols are functional analogues to eukaryotic sterols (Rohmer et al., 1979), due to their similar structures and amphiphilic properties (Ourisson et al., 1987). Hopanoids are present in a minority of bacteria and have an irregular phylogenetic distribution

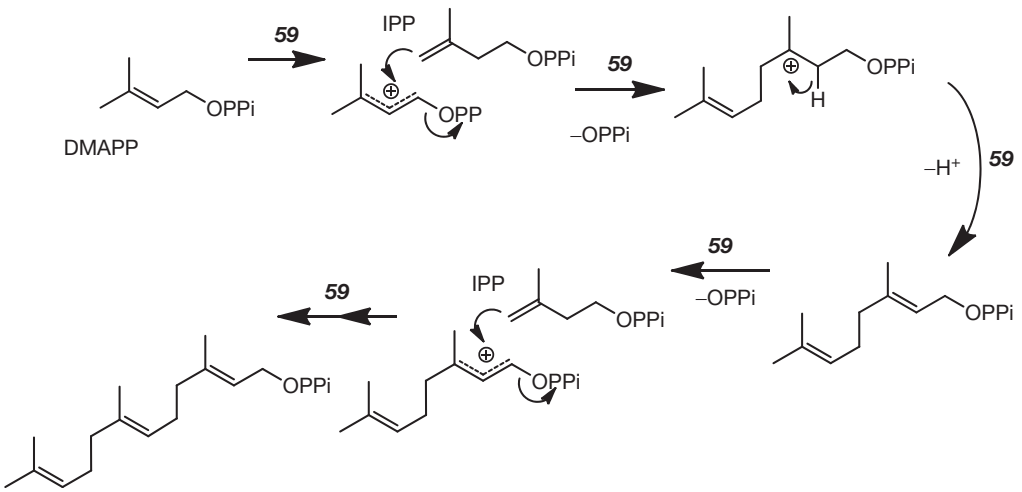

Figure 12 Synthesis of linear polyprenes from IPP and DMAPP. Extension to GPP, FPP, and GGPP is achieved by distinct enzymes optimized for specific chain length of the products; the example shown is for FPP.

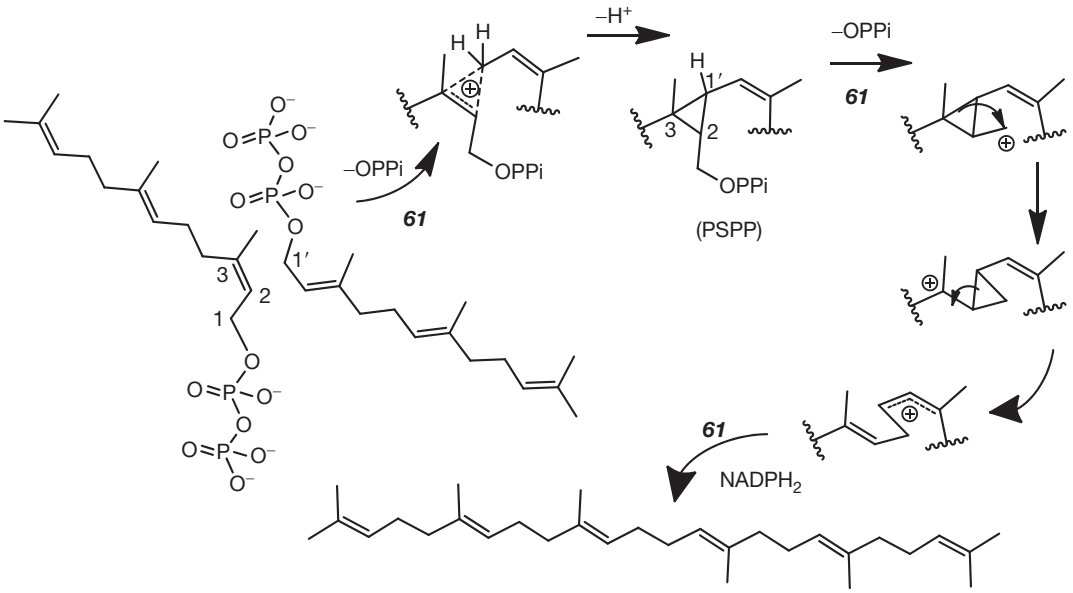

Figure 13 Synthesis of squalene via presqualene diphosphate (Lee and Poulter, 2008; Poulter, 1990). Drawings focus on the migration of electrons in the formation of specific bonds (i.e., stereochemistry, bond angles, and *E/Z* geometry are not represented accurately). For a more complete description of prenylation reactions, see Thulasiram et al. (2007) or Poulter (1990).

(Frickey and Kannenberg, 2009; Pearson et al., 2007; Rohmer et al., 1984).

Hopanoid synthesis requires the isoprenoid precursor, squalene. Squalene is cyclized in a single, concerted reaction by squalene–hopene cyclase to yield the pentacyclic backbone (enzyme ***63***; *shc*; Ochs et al., 1992; Rohmer et al., 1980a; Seckler and Poralla, 1986). Because of the similarity to synthesis of sterols, the enzymatic structure and mechanism of SqhC have been studied extensively (e.g., Abe et al., 1993; Rajamani and Gao, 2003; Wendt et al., 1997). The cyclization reaction product is a tertiary cation that is deprotonated or hydrated to yield the simple hopanoids diploptene or diplopterol, or more commonly, is modified by addition of a polyfunctionalized side chain to yield the dominant bacteriohopanepolyols. The backbone of this side chain is derived from a ribosugar (Flesch and Rohmer, 1988a; Neunlist et al., 1988) that is donated by SAM (enzyme ***64***; *hpnH*; Bradley et al., 2010); the initial product is adenosylhopane (**Figure 14(a)**). In subsequent steps, the nucleobase is cleaved by a probable nucleoside phosphorylase (enzyme ***65***; *hpnG*; Bradley et al., 2010) and then further modified with a range of polar head-group donors to yield a multitude of composite products or modified linear side chains (e.g., cyclitol ether, tetrol, and aminopentol; **Figure 14(b)**; Talbot et al., 2003a,b) resulting from an unknown number of biosynthetic and/or degradative steps.

Additional modifications to hopanoids that are critical for geobiological interpretations are the addition of A-ring methyl groups, either at the 2 position or the 3 position. 2-methylhopanoids have been interpreted – in most cases, although with some exceptions (Rashby et al., 2007) – to indicate the presence of Cyanobacteria (Summons et al., 1999), while the latter are attributed to acetic acid and aerobic, methanotrophic bacteria (Rohmer and Ourisson, 1976a; Zundel and Rohmer, 1985a). The 2-methylhopanoids are formed using a SAM methylase (enzyme ***66***; *hpnP*; **Figure 14(c)**; Welander et al., 2010), while the 3-methylhopanoids in the aerobic methanotroph *M. capsulatus* also are formed using a SAM methyl donor (Zundel and Rohmer, 1985a) distinct from ***66*** (enzyme ***67***; *hpnR*; P. Welander, personal communication). In many bacteria, the *hpn* genes are found close together in the genome in what appears to be a hopanoid synthesis operon (Perzl et al., 1998; Welander et al., 2010).

12.11.2.10 Steroids

In eukaryotes and (rarely) in bacteria, tetracyclic triterpenoids of the steroidal class are formed from cyclization of squalene epoxide (2,3-oxidosqualene) in an O_2-dependent pathway that clearly shares a common history with the hopanoid biosynthetic pathway (cf. Frickey and Kannenberg, 2009; Ourisson et al., 1987; Summons et al., 2006). The early literature on sterol biosynthesis is extensive, due to biomedical interest in the origins of cholesterol (e.g., Tchen and Bloch, 1957; Woodward and Bloch, 1953). Sterols are obligate membrane biochemicals for eukaryotes (Demel and Dekruyff, 1976), and species that cannot produce them (e.g., all insects) must ingest sterols in their diets and/or produce a physiological substitute (e.g., tetrahymanol in protists of the genus *Tetrahymena*; Conner et al., 1968; Mallory et al., 1968). Although the pathway for sterol biosynthesis is obligately aerobic, the dedicated oxygenases maintain activity at very low O_2 thresholds (Jahnke, 1986; Waldbauer et al., 2011). A select few bacteria also make sterols for unknown physiological reasons (Bird et al., 1971; Bode et al., 2003; Kohl et al., 1983; Pearson et al., 2003).

The first step in synthesis is the O_2-dependent epoxidation of squalene (enzyme ***68***; *erg1*, *sqmo*; Corey et al., 1966; Sakakibara et al., 1995; Yamamoto and Bloch, 1970) (**Figure 15(a)**). The subsequent sterol cyclases yield the biosynthetic intermediates lanosterol (enzyme ***69***; Corey et al., 1994; Dean et al., 1967; Thoma et al., 2004) and cycloartenol (enzyme ***70***; Corey

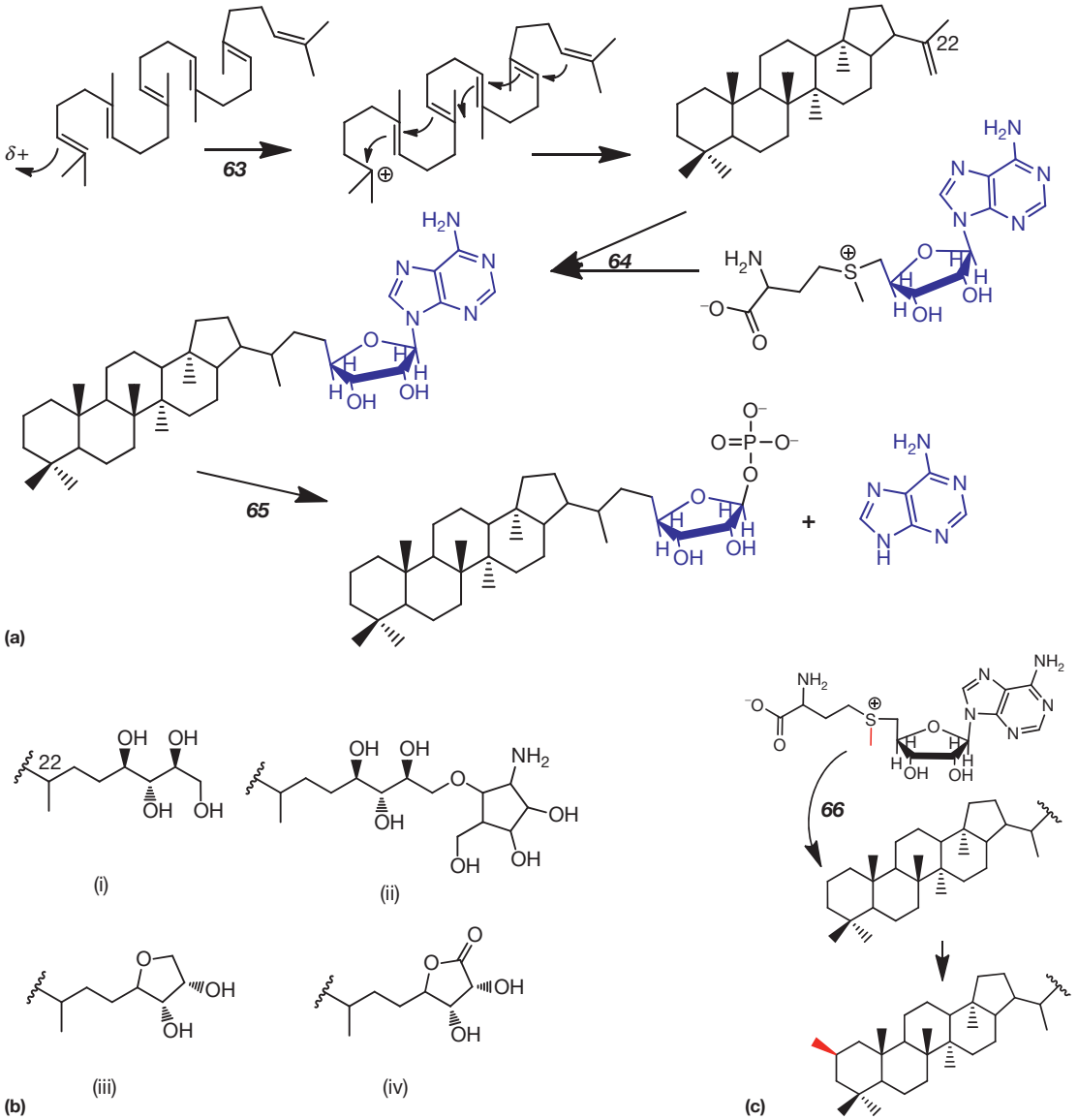

Figure 14 (a) Biosynthetic pathway for hopanoids from squalene to the proposed intermediate phosphoribohopane (Bradley et al., 2010). (b) Selected common hopanoid side chain structures, (i) bacteriohopanetetrol, (ii) bacteriohopane cyclitol ether, (iii) hopaneribonolactone, (iv) 32,35-anhydrobacteriohopanetetrol (Talbot et al., 2003a,b,c; 2008). (c) Ring A methylation, shown here for the 2-methyl position.

et al., 1993; Rees et al., 1969). These enzymes generally are phylogenetically and physiologically distinct and are found in animals/fungi and plants, respectively. An exception is the finding of bifunctional ***69***/***70*** in the plant *Arabidopsis thaliana* (Ohyama et al., 2009). SqhC (***63***) can accept 2,3-oxidosqualene as a substrate (Anding et al., 1976), but ***69*** and ***70*** cannot conversely catalyze the cyclization of squalene (cf. Schulz-Gasch and Stahl, 2003).

There are at least 19 other enzymes required to modify lanosterol and cycloartenol to the eventual end products cholesterol, ergosterol, and phytosterols. Key steps in the process include demethylation at positions C_{14}, αC_4, and βC_4. These demethylation reactions require an additional suite of oxidases (enzymes ***71–72***; *cyp51*, *ERG25*; Alexander et al., 1972; Aoyama et al., 1996; Fukushima et al., 1981). Other processes of interest are the attachment of the phytosterol methyl and ethyl groups to C_{24} (sterol methyltransferase enzymes ***73–74***; Bouvier-Nave et al., 1998; Moore and Gaylor, 1969; Nes et al., 1998) and the probable further methylation (via a ring-opening intermediate) of this side chain in the synthesis of the sponge biomarker 24-isopropylcholesterol (Giner, 1993; Stoilov et al., 1986) (**Figure 15(b)**).

12.11.2.11 Ladderanes

Ladderanes are unusual lipids believed to be essential components of the intracellular 'anammoxosome' of anaerobic, ammonia-oxidizing Planctomycetes (Jetten et al., 2009; Strous et al., 1999) and perhaps other cellular membranes (van Niftrik et al., 2008). Ladderanes contain concatenated chains

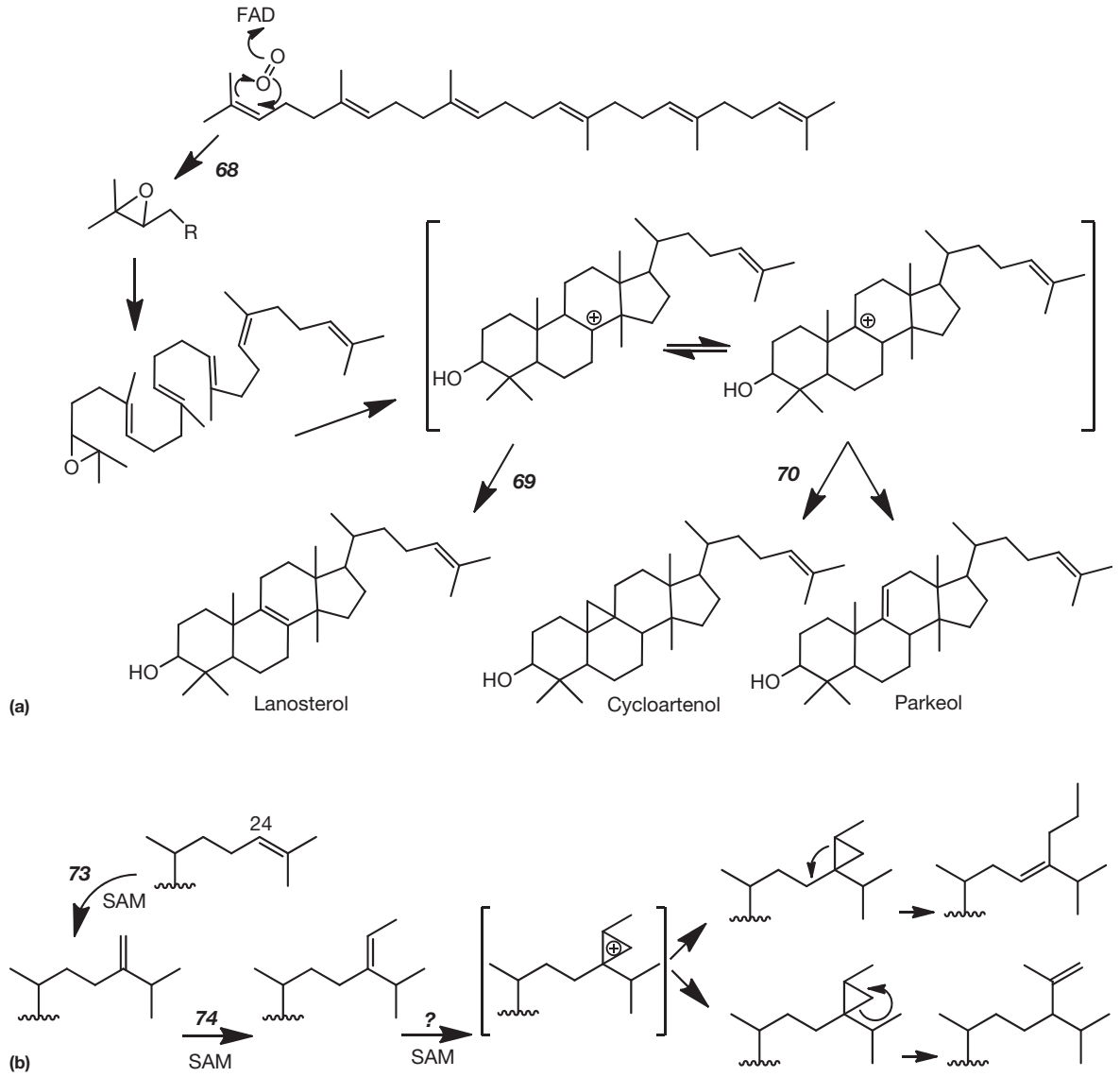

Figure 15 (a) Epoxidation and cyclization of squalene to the three possible initial intermediates, cycloartenol, lanosterol, and parkeol; the reaction requires one molecule of O_2. Subsequent demethylation reactions to remove 14α, 4α, and 4β methyl groups require additional O_2. (b) Reactions of sterol methyltransferases, including the proposed synthesis of 24-isopropylcholesterol and 24-*n*-propylcholesterol (Giner, 1993; Stoilov et al., 1986).

of cyclobutane rings (Damsté et al., 2002b). Although such strained structures might be expected to be diagenetically unstable, ladderane lipids have been recovered from sediments as old as 140 000 years and are useful markers for past anammox activity (Jaeschke et al., 2009).

The synthesis of ladderanes appears to be very unusual. The intact molecules in cells are mostly PC or PE-1-alkyl (or acyl)-2-alkyglycerols with fatty tail chain lengths of C_{18} or C_{20} (Rattray et al., 2008). These tails contain 3 or 5 cyclobutane rings, where the [3]-ladderane always is condensed to a cyclohexane ring. Isotope-labeling experiments show, however, that the tail precursor either must be derived from an exogenous moiety or must be successively decarboxylated during synthesis if derived from an FA-type pathway. A labeled 2-^{13}C acetate experiment showed that the ratio of methyl- (or exogenous) versus carbonyl-derived carbons was ≥2:1 within the polycyclic segment of [3]-ladderane (**Figure 16**; Rattray et al., 2009a). This contradicts earlier suggestions that internal cyclization of folded, polyunsaturated FAs might be the synthetic pathway (e.g., Damsté et al., 2005; Mascitti and Corey, 2006). A recently reported bicyclobutane synthase from the cyanobacterium Anabaena PCC 7120 (Schneider et al., 2007) also is unlikely to give clues to synthesis of ladderanes, as here the proposed ring opening to yield cyclobutane also would not solve the problem of unequal numbers of carbonyl- and methyl-derived carbons. Ladderane synthesis enzymes and genes to date must remain a mystery, although it remains likely that the novel pathway is related to FA synthesis, and further investigation of FA synthesis operons may shed additional light (Rattray et al., 2009b).

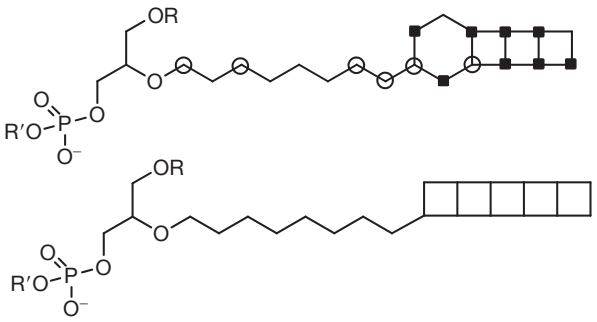

Figure 16 Structure of [5]-ladderane and [3]-ladderane lipids of Planctomycetes. Methyl-derived carbons as determined by Rattray et al. (2009a) are indicated with black squares, while carbonyl carbons are indicated with open circles. R groups are ester- or ether-linked; R′ groups are PG, PE, or PC.

12.11.2.12 Long-Chain Alkenones

Both marine and freshwater species of haptophyte algae contain a unique class of long-chain methyl and ethyl ketones having variable numbers of unsaturations (e.g., Cranwell, 1985; Marlowe et al., 1984; Volkman et al., 1980). Soon after their discovery, it was recognized that ratios of the C_{37} compounds containing 2, 3, or 4 double bonds would be a valuable sea surface paleotemperature proxy (Brassell et al., 1986; Prahl and Wakeham, 1987). Less clear is the biochemical role(s) played by these compounds in cells: they accumulate as a function of nutrient status and light exposure and are consumed – perhaps as energy storage products in lieu of triglycerides – when cells are incubated in the dark (Bell and Pond, 1996; Epstein et al., 2001). Although little is known about the synthesis of alkenones, their phylogenetic specificity is an asset when using a combination of lipid and molecular (DNA/RNA) approaches (Coolen et al., 2006, 2009).

Clues to the synthesis of alkenones come from the diversity of chain lengths of methyl and ethyl ketones, combined with analysis of positional placement of their double bonds (which also have the atypical *E* stereochemistry; de Leeuw et al., 1980; Rechka and Maxwell, 1988). Based on these patterns, and on earlier suggestions that ketones are formed by decarboxylation of β-ketoacids during the termination of fatty chain extension (Dickschat et al., 2004; Kolattukudy, 1980), Rontani et al. (2006) proposed a biosynthetic pathway (**Figure 17(a)**). Synthesis is presumed to follow the normal pathway for synthesis of FA tails, beginning with a primer of either acetyl-CoA or propionyl-CoA, and extended by malonyl-CoA (enzymes ***1–6***; **Figure 1**). After the final extension, the reaction is presumed to arrest between ***3*** and ***4*** and be followed by an unknown thioesterase and decarboxylase. The resulting products are C_{35} to C_{41} (odd-numbered) methyl ketones if the chain terminator is malonyl-CoA or C_{36} to C_{40} (even-numbered) ethyl ketones if the chain terminator is methylmalonyl-CoA. A propionate primer gives rise to the alternative series: C_{36} to C_{40} (even-numbered) methyl ketones or C_{35} to C_{41} (odd-numbered) ethyl ketones (not shown).

More interesting is the proposed mechanism for forming the unusual *E* double bonds. Rontani et al. (2006) evaluated several 'families' of alkenones according to ω (relative to terminal) versus Δ (relative to carbonyl) double bond locations. The Δ spacing reveals three systematic families: (i) the 'normal' family ($\Delta^{14,21,28}$; most commonly $\Delta^{14,21}$ diene; e.g., de Leeuw et al., 1980), (ii) the 'Black Sea' family ($\Delta^{14,19}$; Prahl et al., 2006; Xu et al., 2001), and (iii) the '*E. huxleyi* CCMP1742' family ($\Delta^{12,19}$; Prahl et al., 2006; Rontani et al., 2006) (**Figure 17(b)**). Family (i) has a regular pattern of spacing in multiples of Δ^7, as does family (iii), except that the chain terminates one C_2 unit 'too soon' at the carbonyl end ($\Delta^{12,19}$ vs. $\Delta^{14,21}$). Family (ii) is more unusual in having a multiple of Δ^7 (Δ^{14}) followed by a 5-carbon spacing (Δ^{19}). Rontani et al. (2006) suggested that this implicates a series of Δ-desaturases that have active sites a fixed distance from a carbonyl binding site; they further suggest that desaturation occurs after chain elongation is nearly complete but before the operation of the unknown thioesterase and decarboxylase. The Δ^{12} pattern of family (iii) is explained by additional chain shortening after desaturation, although it is unclear how this would be compatible with the existence of Δ^{12} ethyl ketones. Instead, it may be more likely that haptophytes employ a series of related desaturases that have active site cavities optimized for a variety of carbon chain lengths permuted from the sums of C_5 and C_7.

12.11.2.13 Highly Branched Isoprenoids of Diatoms

Highly branched C_{25} isoprenoid (HBI) lipids containing multiple double bonds are ubiquitous markers that appear to be specific for the *Rhizosolenia* and *Navicula/Haslea/Pleurosigma* groups of diatoms (Damsté et al., 1999a, 2004a,b). They were known from sediments (Gearing et al., 1976) before their discovery in diatoms (Nichols et al., 1988; Volkman et al., 1994). Structural characterization affirmed the involvement of centrally linked isoprene units as the essential feature of HBIs (Damsté et al., 1999b; Robson and Rowland, 1986; Rowland et al., 1985; Yon et al., 1982). The structures found most commonly are C_{25} and C_{30} tri-, tetra-, and penta-alkenes (Belt et al., 2000) with branching occurring at C_7 (**Figure 18(a)**).

Suggested mechanisms for HBI synthesis are surprisingly rare in the literature. Masse et al. (2004) determined that *Rhizosolenia* uses the MVA pathway and *Haslea* uses MEP for synthesis of the isoprenoid units. It remains unknown how the central HBI skeleton is joined, even though understanding what causes differences in the relative abundances of C_{25} versus C_{30} HBIs is crucial to present taxonomic interpretations. The consistent structures of HBIs do offer some clear suggestions, however. The linkage pattern invariably is consistent with joining two terpenes (farnesyl-PP + geranyl-PP or two farnesyl-PP) in a 1′–2 bond (**Figure 18(b)**). FPP synthases (enzyme ***59***, **Section 12.11.2.8**) having partial cyclopropanation activity are capable of forming the 1′–2 linkage via a cyclopropyl transition state (Thulasiram et al., 2007). This suggests that a prenyltransferase-family enzyme is involved in the synthesis of HBIs and that, in some taxa, it may be specific for adding GPP midchain to FPP, while in others, it may be flexible for adding either GPP or FPP. Based on this mechanism, the *E*/*Z* geometric isomerism at C_{9-10} (Belt et al., 2000) could be a biosynthetic consequence of the orientation of GPP (or FPP) at the point of attachment. Unfortunately, no genomes of HBI-producing diatoms have been sequenced to date to enable a search for prenyl synthase homologues.

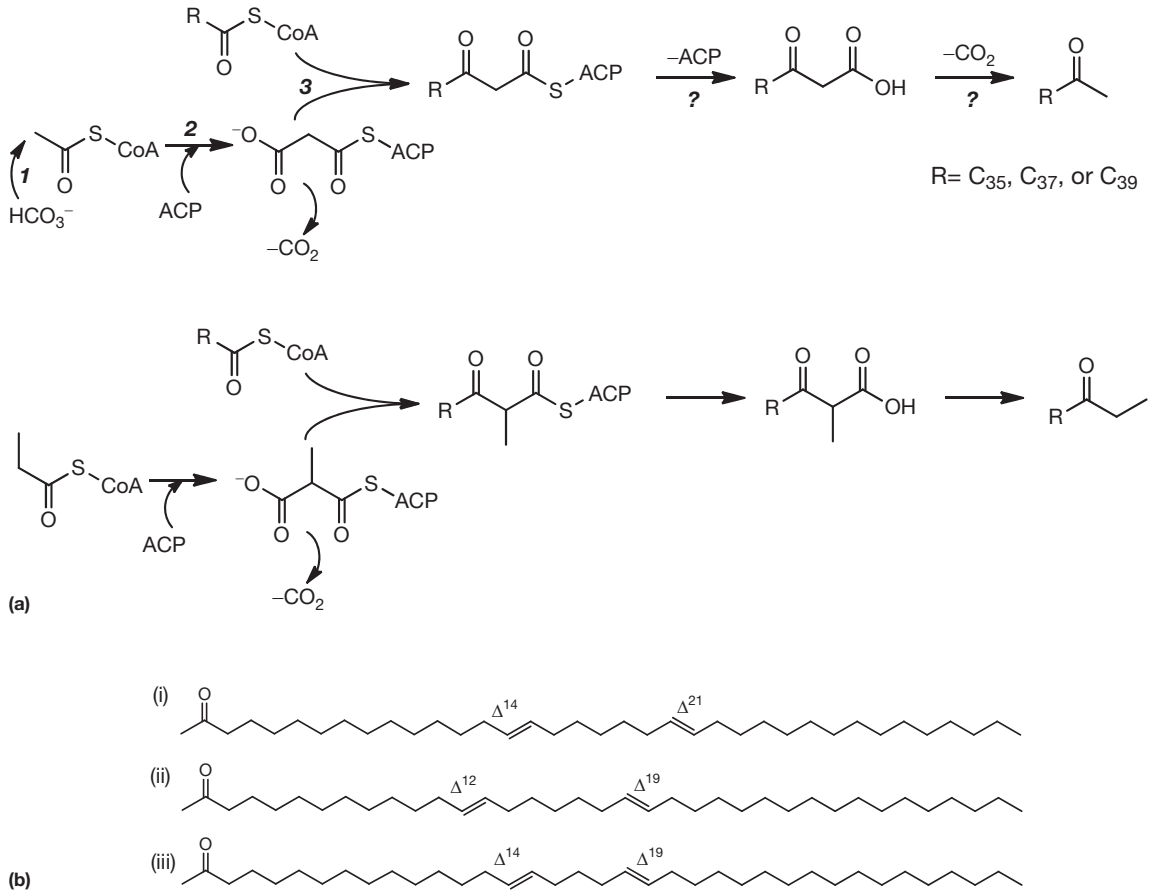

Figure 17 (a) Proposed pathway for the synthesis of long-chain alkenones in haptophytes starting from acetyl-CoA (methyl ketones) or propionyl-CoA (ethyl ketones) (Rontani et al., 2006); (b) Combinations of unsaturation patterns observed for three families of alkenones, showing permutations of 5-carbon and 7-carbon spacing between double bonds.

12.11.3 Case Studies and Approaches to Lipidomics

There are numerous lipidomic studies with relevance to geochemistry. In the succeeding text, I survey some recent examples that take advantage of the wealth of new analytical capabilities and/or employ novel experimental approaches to understanding the distribution of biomarker lipids. Since the invention of the polymerase chain reaction (PCR; Mullis et al., 1986), appreciation of total phylogenetic diversity – generally defined by the sequences of small subunit ribosomal RNA (SSU rRNA) genes – has grown tremendously. In 1987, the number of known bacterial phyla was 12 (Woese, 1987), while by 2007, it had grown to ~100 (including archaea) (López-García and Moreira, 2008; Pace, 1997; **Figure 19**). More recently, Jonathan Eisen and colleagues have made the suggestion that there may even be additional domains beyond the Eukarya, Bacteria, and Archaea (Wu et al., 2011). Within this diversity, >99% of species to date remain uncultivated, and there is almost no information about the rare individuals (Hugenholtz et al., 1998; Sogin et al., 2006). A similar story presently is unfolding for unicellular eukaryotes in the ocean, suggesting they may be at least as diverse as prokaryotes (Karsenti et al., 2011).

In parallel to efforts to determine rRNA diversity, large-scale metagenomic sequencing efforts are mapping the distribution of known and unknown protein families in diverse environmental settings (DeLong et al., 2006; Denef et al., 2010; Dinsdale et al., 2008; Yooseph et al., 2007). Together, these large data streams hold promise for understanding the distribution of and genetic capacity for lipid biosynthetic pathways among prokaryotes and unicellular eukaryotes. Such data can be predictive. DNA sequence data can expose the origins of lipids, either (1) by identifying the genes encoding for specific enzymes involved in lipid biosynthesis (**Table 1**) or (2), in the rare cases of a definitive physiological and/or taxonomic link between compounds and species (e.g., ladderanes of anammox Planctomycetes), by establishing a correspondence between distribution of compounds and the associated phylogenetic diversity obtained from sequences of SSU rRNA genes. In support of these efforts, the high-throughput DNA sequencing revolution (automated capillary Sanger sequencing) enabled scientists by the 1990s–2000s to obtain what seemed like a remarkable 10^5 base pairs of DNA sequence data per day. However, this number now is startlingly obsolete when viewed from the perspective of 'next-generation,' massively parallel sequencing methods such as 454 pyrosequencing (www.454.com), Illumina (www.illumina.com), and Ion Torrent (www.iontorrent.com), each of which operates at $>10^8$ base pairs per day (Mardis, 2011). Soon,

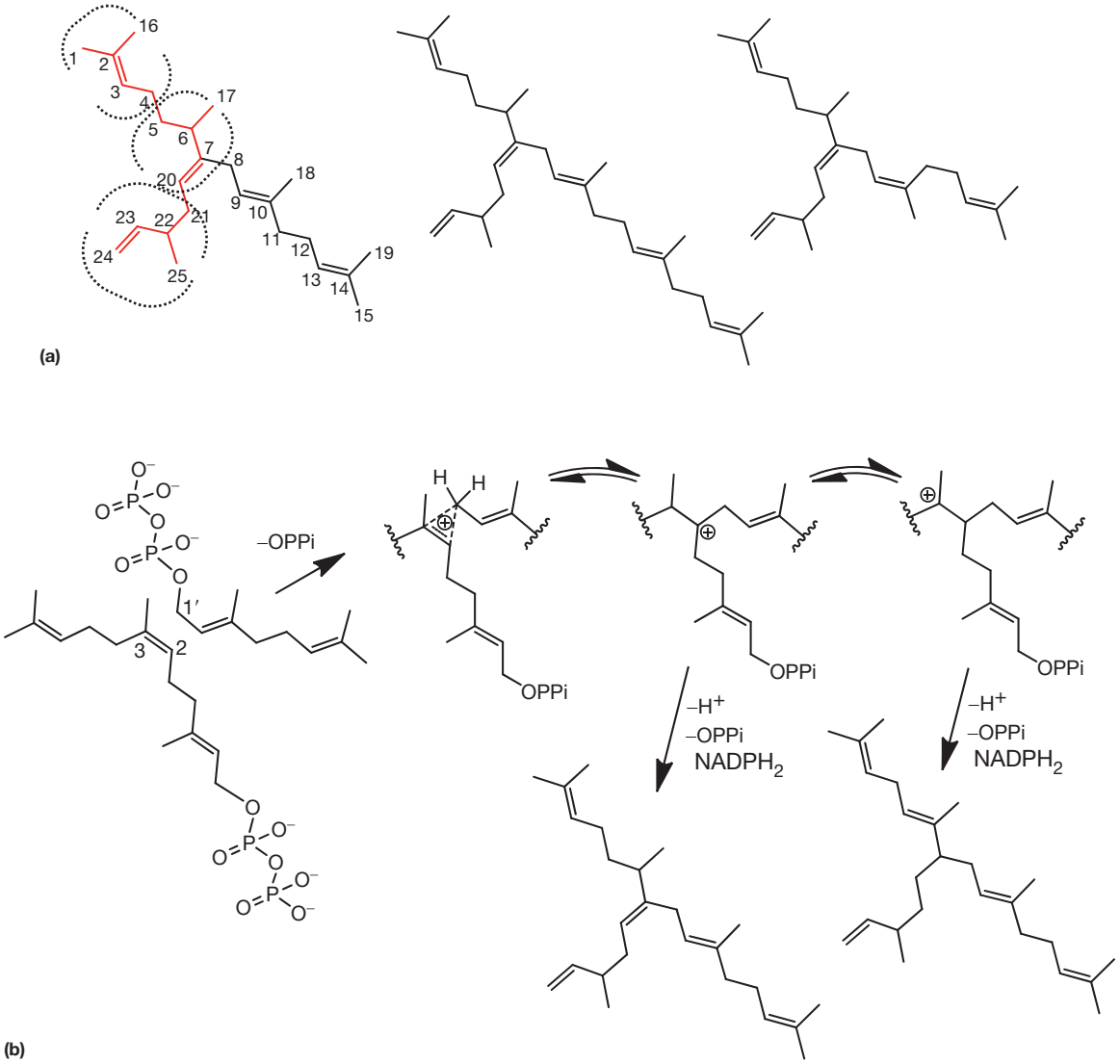

Figure 18 (a) Common structural variations in diatom HBI lipids, showing conventional numbering (Belt et al., 2000); note that $C_{20–25}$ likely are part of an original FPP chain composed of the three isoprene units marked off with brackets. (b) Proposed mechanism for the synthesis of HBI lipids, by analogy to **Figures 13** and **24**.

the high-throughput and functional genomic approaches will come together, enabling identity to be linked to functional capacity. New techniques in single-cell genomics (e.g., Martinez-Garcia et al., 2012; Ottesen et al., 2006; Stepanauskas and Sieracki, 2007; Zhang et al., 2006) now promise to link biosynthetic pathway genes definitively to the species that contain them, even for uncultivated taxa.

The examples highlighted in the succeeding text all share a fundamental underpinning of being dependent on DNA sequence data, either directly as part of the work or indirectly through reliance on this information to guide the direction of research. The examples fall broadly in the categories of molecular genetics, genome mining, environmental metagenomics, biochemical experiments, and SSU rRNA correlations. When a particular study falls into more than one category, it is grouped with others by scientific context rather than by analytical technique.

12.11.3.1 Examples Using Bacterial Genetics

The following examples primarily focus on experimental bacterial genetics. In these approaches, modern organisms with known genomes are genetically manipulated to expose the roles of specific genes in lipid biosynthetic pathways. Genetic manipulation relies on the ability to introduce DNA into the target organism either by transformation (direct uptake of foreign DNA), conjugation (transfer of DNA from a donor cell to a recipient cell), or transduction (introduction of foreign DNA to the bacterial cell from a bacteriophage). Using these strategies, specific genes are removed or introduced. To remove function, a mutation or deletion is performed. This is done by introducing a deliberately truncated segment of DNA into the bacterium, relying on DNA recombination to replace the natural gene with the foreign DNA. Similarly, function can be restored by introducing a plasmid containing a fully intact

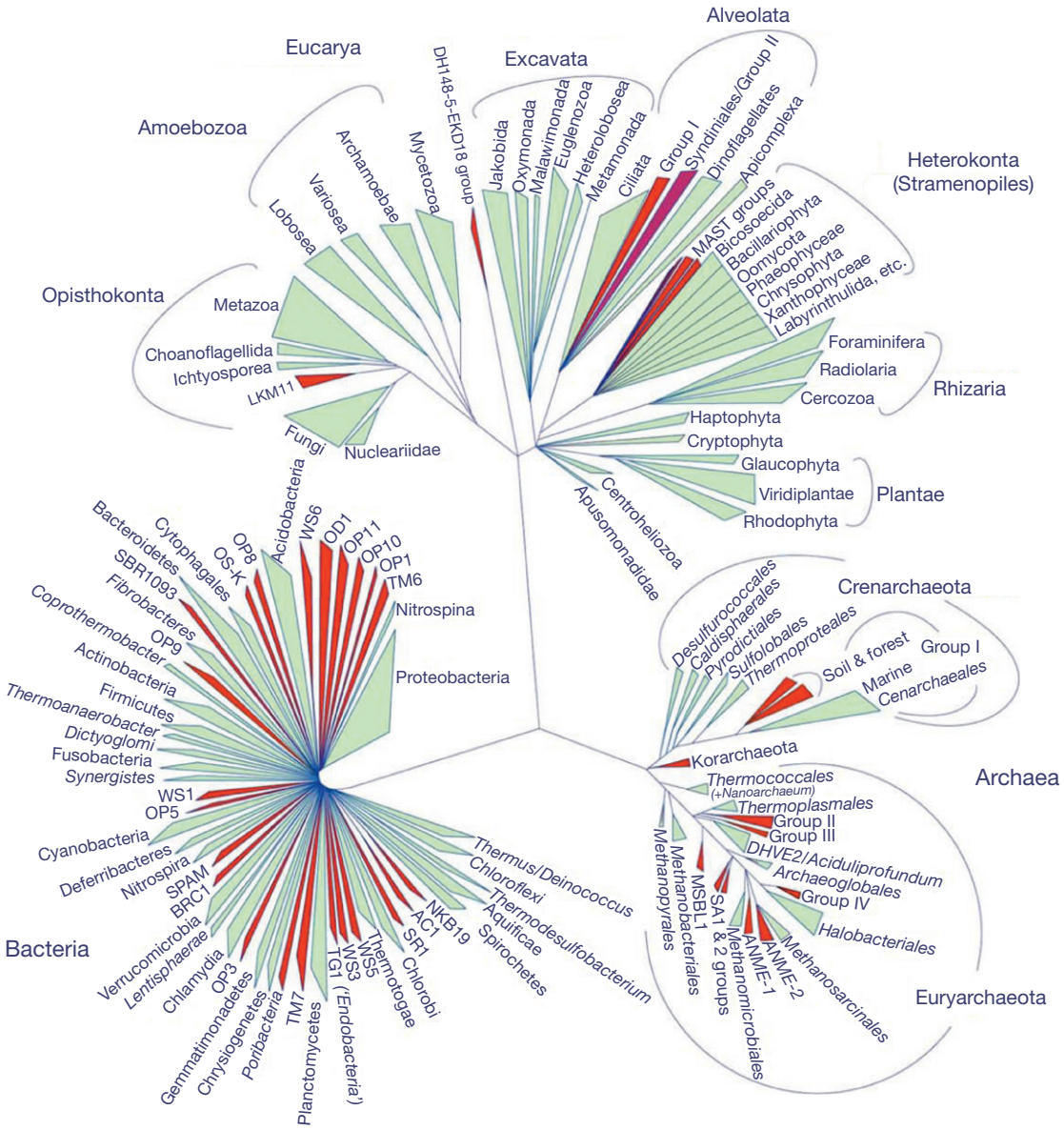

Figure 19 Phylogenetic tree of life based on current molecular knowledge (SSU rRNA and other molecular evidence). Green/light triangles represent phyla, divisions or groups of high taxonomic rank for which at least one member has been cultivated and/or properly described (e.g., many protist species); red/dark triangles represent candidate divisions or highly divergent lineages without cultivated/described species. The tree is highly simplified; only a fraction of known eukaryotic phyla are depicted, and, in the case of bacteria, only phyla and candidate divisions agreed upon by at least three classification systems (http://greengenes.lbl.gov) are shown. Reproduced from López-García P and Moreira D (2008) *Research in Microbiology* 159: 67–73, with permission from Elsevier.

copy of a gene, plus a transcriptional promoter, into the bacterium; this is known as complementation. Pure cultures are a must, and every recombinant must have its altered DNA sequence verified to confirm the fidelity of the new constructs. Understandably, such projects are laborious.

12.11.3.1.1 Role of squalene–hopene cyclase in the synthesis of hopanoid lipids

Lipid biomarkers preserved in ancient sedimentary rocks provide one of the few available windows to the record of early life on Earth. Among the most useful compounds are the terpenoid lipids known as bacterial hopanepolyols, compounds that signify the presence of their bacterial sources. The hopane carbon skeletons of these parent compounds are resistant to degradation and are abundant and ubiquitous components of sedimentary rocks and petroleums (Ourisson and Albrecht, 1992). They date at least to the Paleoproterozoic (Brocks et al., 2005). Hopanes also have been reported in rocks of Archaean age, although in the latter, their syngeneity is controversial (Brocks et al., 1999; Dutkiewicz et al., 2006; Rasmussen et al., 2008; Waldbauer et al., 2009). While information about the sources of hopanoids can shed light on early

evolution and environments, most of our present understanding of the distribution of hopanoids has been discovered empirically – through broad survey approaches to lipid screening – without prior knowledge of the genetic capacity required to make the specific structures of interest (e.g., Rohmer et al., 1984; Summons et al., 1999; Talbot et al., 2008).

Historically, some of the first applications of bacterial experimental genetics to geochemical questions focused on hopanoids. Squalene–hopene cyclase (*shc*) from *A. acidocaldarius* was the first hopanoid synthesis gene cloned and sequenced (Ochs et al., 1992). When expressed heterologously in *E. coli*, lysed cells converted exogenous squalene to the cyclized product. In quick succession, *shc* homologues from *Zymomonas mobilis* (Reipen et al., 1995), *Bradyrhizobium japonicum* (Perzl et al., 1997), and *M. capsulatus* (Tippelt et al., 1998) all were studied similarly. Once the enzymatic function was established, attention turned to searching for the physiological function of hopanoids in bacteria. Although hopanoid production long had been known to increase at high temperatures and in the presence of excess ethanol in selected species (Poralla et al., 1984; Schmidt et al., 1986), genetic manipulations could now be attempted. The first such example did not manipulate the *shc* gene but rather determined the role that SHC activity played in sporulating and nonsporulating mutants of *Streptomyces coelicolor* (Poralla et al., 2000). Hopanoids were produced only during the formation of aerial mycelia but were not necessarily associated to sporulation. More recent work on *Streptomyces scabies* contrasts with this result: hopanoid-free cells showed no detectable phenotype (*Δshc* mutants), leading the authors to conclude that in this species, "hopanoids are not required for normal growth or for tolerance of ethanol, osmotic and oxidative stress, high temperature, or low pH" (Seipke and Loria, 2009). This interpretation does not apply to all hopanoid producers, as *Δshc* deletion mutants in *Rhodopseudomonas palustris* are sensitive to extremes of pH (Welander et al., 2009). A potential function for hopanoids in protecting the N_2-fixing enzyme nitrogenase from oxygen toxicity (Berry et al., 1993) also recently has been questioned (Doughty et al., 2009; Nalin et al., 2000). It therefore remains to be seen whether there may in fact be a variety of physiological roles for hopanoids, including a possible role in cell division (Doughty et al., 2011).

12.11.3.1.2 Synthesis of A-ring methylated bacteriohopanepolyols

An example of particular interest is the case of methyl groups added to the hopanoid A-ring, either at position 2 or position 3 (**Figure 14**). Until the discovery of 2-methylbacteriohopanepolyols in the Alphaproteobacterium *Rhodopseudomonas palustris* TIE-1 (Rashby et al., 2007), synthesis of the extended side-chain (C_{35}) hopanoids with an extra 2-methyl group was known primarily from its prevalence in Cyanobacteria (Summons et al., 1999), although it also had been reported in the Alphaproteobacterium *Methylobacterium organophilum* (Renoux and Rohmer, 1985). Because these authors had a working genetic system in *R. palustris* TIE-1, in a follow-up investigation, they were able to demonstrate the origin of the methyl group. (*N.b.*, 2-methylhopanoids without polyfunctionalized side chains are more widely distributed among bacterial genera, including in other *Methylobacterium* spp. and other Proteobacteria.)

Early clues to the synthesis of A-ring methylated (2-methyl and 3-methyl) hopanoids were provided by studies showing that excess methionine stimulated the production of methylated products (Zundel and Rohmer, 1985a,b). Based on this evidence, it was hypothesized that a SAM methyltransferase might be involved. Welander et al. (2010) identified an operon-like region in the *R. palustris* genome containing several genes likely to encode for steps of hopanoid biosynthesis. Based on annotations that identified a putative SAM methyltransferase gene, they designated it *hpnP* (**Figure 20**; nomenclature from Perzl et al., 1998). To establish the role of HpnP in hopanoid biosynthesis, a deletion vector was introduced to *R. palustris* TIE-1 via conjugal transfer from *E. coli*. Homologous recombination resulted in a mutant that lacked the *hpnP* locus. Absence of any 2-methylhopanoids in the *ΔhpnP* mutant showed the involvement of this gene in the biosynthesis of these molecules; activity was restored by reintroducing the gene (complementation), confirming that *hpnP* is a SAM methyltransferase (enzyme ***66***) solely responsible for adding the methyl group in the production of 2-methylbacteriohopanepolyols.

The identification of the 2-methylbacteriohopanepolyol-specific *hpnP* methylase opens the phylogenetic window to examine all other bacteria for putative 2-methylases. By comparing the sequence of *R. palustris hpnP* to all other bacterial genomes available at the time of publication, Welander et al.

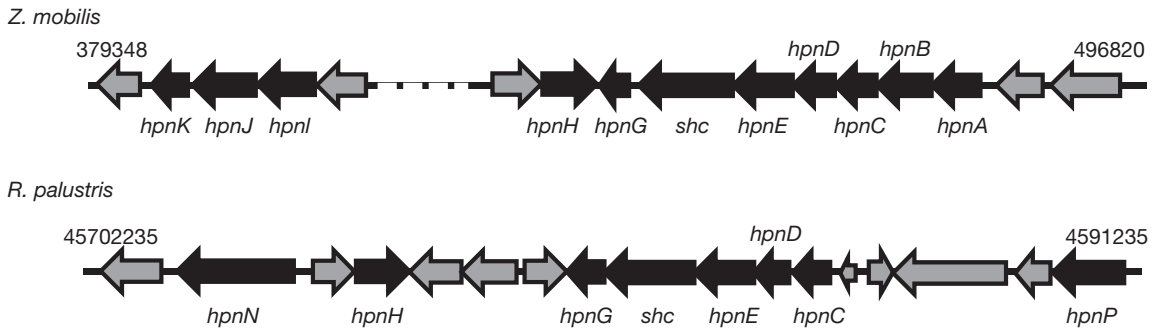

Figure 20 Regions of the genomes of *R. palustris* TIE-1 and *Z. mobilis* ATCC 10988 containing genes encoding for hopanoid synthesis enzymes (adapted from Welander PV, Coleman ML, Sessions AL, Summons RE, and Newman DK (2010) Identification of a methylase required for 2-methylhopanoid production and implications for the interpretation of sedimentary hopanes. *Proceedings of the National Academy of Sciences of the United States of America* 107: 8537–8542; Perzl M, Muller P, Poralla K, and Kannenberg EL (1997) Squalene-hopene cyclase from *Bradyrhizobium japonicum*: Cloning, expression, sequence analysis and comparison to other triterpenoid cyclases. *Microbiology* 143: 1235–1242).

(2010) suggested that homologues to this gene might belong to four groups: those of the *M. extorquens* variety (only producing simple 2-methylhopanoids without side chains), those of the Cyanobacteria/*Rhodopseudomonas* variety (also producing 2-methylpolyols), those belonging to other Alphaproteobacteria, and an unexpected occurrence in Acidobacteria. The last is a lone case that appeared in one of the few sequenced Acidobacteria at the time of publication, *Candidatus* Koribacter versatilis Ellin 345. The observation of *hpnP* homologues across three phyla prompts the additional question of how many uncultured bacterial phyla might also contain *hpnP* homologues. It also reopens long-standing questions about the fidelity of using 2-methylhopanoids from the sedimentary record as specific biomarkers for the emergence of oxygenic photosynthesis (Welander et al., 2010). A search of the HpnP protein sequence from *R. palustris* TIE-1 against all translated metagenomic sequences (NCBI database env_nr) suggests there are closely related sequences in the environment. (A. Pearson, unpublished).

12.11.3.1.3 Synthesis of hopanoid C_5 side chains

The synthesis of extended bacteriohopanepolyols involves addition of a C_5 ribosugar to the initial C_{30} hopanoid product derived from squalene (Flesch and Rohmer, 1988a; Neunlist et al., 1988). The source of this ribosugar remained unknown until recently, when it was shown that a SAM adenosyl transferase is responsible (enzyme **64**). Working with *M. extorquens* AM-1, Bradley et al. (2010) created in-frame deletion mutants of the *hpnH* gene; the gene was excised by homologous recombination, after conjugation with an *E. coli* strain into which a facile exchange plasmid had been introduced (Marx, 2008). The resulting *ΔhpnH* mutants failed to accumulate hopanoids larger than C30. Interestingly, production of 2-methyl C_{30} hopanoids was not affected by this mutation.

As part of the same study, mutants were also constructed for an adjacent gene that had been annotated in the genome as a putative nucleoside phosphorylase homologue (*hpnG*). Such an enzyme would be expected to cleave a nucleobase from its attached ribosugar, leaving behind a ribosylphosphate, in this case phosphoribohopane (Erion et al., 1997). Bradley et al. (2010) indeed showed that the absence of this gene in the Δ*hpnG* mutant causes the reaction to accumulate adenosylhopane (**Figure 14**). Whether the enzyme (**65**) truly is a phosphorylase remains to be determined.

This mechanism for synthesis of hopanoid side chains has several implications. First, the universal conclusion is that all bacterial producers of C_{35} extended structures minimally should contain *hpnH*; in fact, all bacterial genomes from species known to produce C_{35} bacteriohopanepolyols appear to contain both *hpnH* and *hpnG*. This immediately raises an interesting question: why do soil environments accumulate adenosylhopane? Adenosylhopane is abundant in terrestrial and lacustrine environments, whereas it is absent in sediments with negligible terrigenous input (Cooke et al., 2008a; Talbot and Farrimond, 2007). The presence of adenosylhopane either reflects an arrested biosynthetic pathway, that is, inhibition of HpnG for unknown reasons, or an imbalance in the rates of expression of HpnH and HpnG. Alternatively, it also could indicate the presence of uncultured/unstudied species that contain *hpnH* but not *hpnG*. It remains unknown why any of these circumstances would be more prevalent in soil-dwelling microbes. Similarly, hopaneribonolactone and 32,35-anhydrobacteriohopanetetrol (**Figure 14(b)**) have been found in oxidizing and reducing environments, respectively (Bednarczyk et al., 2005; Talbot et al., 2005). Bradley et al. (2010) suggested that both compounds are generated abiotically through oxidative or reductive cleavage of the intermediate, phosphoribohopane. The ratio of the lactone-BHP to anhydro-BHP may be a function of pH and Eh, thus carrying little phylogenetic information but perhaps a useful environmental signal. The phosphoribohopane intermediate itself has not yet been observed, and therefore, its existence remains a speculation.

Future work promises to determine the distribution of these hopanoid biosynthesis genes not only in cultured species but also in the environment. In particular, when more sequences are identified, the evolutionary relationships among them – and in relation to SSU rRNA genes – can be determined. Initial work on *hpnP* suggests the distribution of 2-methylase activity may have been affected by horizontal gene transfer (Welander et al., 2010). It is not yet known if the same applies to *hpnG* and *hpnH*, or if here the scenario is more similar to the cyclase *shc* (**63**), which primarily seems to be governed by vertical inheritance (Frickey and Kannenberg, 2009; Pearson et al., 2007).

12.11.3.2 Examples Using Genomic Data from Characterized Species

In all of the examples earlier, the tool was a specific organism that could be studied through genetic manipulation. This is distinct from genomic survey approaches that begin with in silico data mining of whole genomes or from studies that presume gene functionality and seek to detect the presence of these genes in pure cultures. Such projects are interested in discovering the putative physiological capacity to synthesize particular lipids and/or to place these lipid biosynthetic pathways in evolutionary, physiological, or environmental context.

In these approaches, the nucleotide or amino acid sequence for a known lipid biosynthesis enzyme (**Table 1**) is compared to a database of completed genomes. The former is known as the 'query' and the latter as the 'targets.' The searching algorithms collectively are known as BLAST tools (Basic Local Alignment and Search Tool; Altschul et al., 1990), and although many variations exist, all utilize the same principles. The query is broken into small segments (called 'words'), and the words are compared to the target sequences. Each word is assigned a score for the quality of the match, and the cumulative score for a given larger segment of sequence is obtained by assessing the cumulative score extending up- and downstream on each side of the initial word match. The resulting output is reported as the statistical likelihood that the total score over that segment would occur by chance; this number is known as an 'expect value,' or E-value. The threshold for significance of a given E-value depends on the question being asked. Generally, it must be evaluated along with additional results such as the length of sequence over which there is significant similarity and the percent of identical matches over this same region. The most significant advantage that BLAST has over other approaches (such as pairwise or multiple alignments) is

that it is extremely fast, enabling gigabases of DNA or protein sequence to be searched in just a few seconds.

12.11.3.2.1 Bacteria capable of sterol biosynthesis

Steroidal lipids are required universally by eukaryotes, and while some eukaryotes (e.g., insects) depend on dietary sources to meet this requirement, most eukaryotes synthesize their sterols *de novo*. Such ubiquity – along with the obligate requirement for molecular oxygen (O_2) in sterol biosynthesis – makes geologic steranes excellent temporal and taxonomic markers for the emergence of eukaryotes (cf. Summons et al., 2006). The fidelity of these interpretations has been questioned on the basis of biomarker syngeneity, that is, the equivalence of lipid ages and rock ages (Rasmussen et al., 2008), and on the basis of proposed alternative pathways that could substitute for the oxidative requirement (Raymond and Blankenship, 2004). But while the issue of syngeneity remains controversial, recent work has suggested the functional oxygen requirement is so low that alternative enzyme systems may not be needed to explain the presence of sterols in early Earth environments (Waldbauer et al., 2011). However, to fully evaluate the fidelity of steroids as eukaryotic markers, a third line of questioning is necessary: how widespread is bacterial synthesis of steroids? Early reports of sterols being detected in bacteria generally were controversial, with only a few exceptions; for a recent review, see Volkman (2003). The earliest robust data showing sterols in a bacterium were reported for the aerobic methane oxidizer, *M. capsulatus* (Bird et al., 1971). This species interestingly produces both hopanoids and steroids (Rohmer et al., 1980b). Subsequently, the aerobic Deltaproteobacterium *Nannocystis exedens* (order Myxococcales) also was shown to produce sterols (Kohl et al., 1983), as do several other species of Myxococcales (Bode et al., 2003). Given that the vast majority of bacteria are uncultured and/or unexplored for their lipid contents, the nonzero occurrence of sterol synthesis in bacteria could be problematic for geochemical interpretations. To answer this question requires a broader picture of sterol synthesis and its potential distribution among bacteria.

Synthesis of the steroidal backbone from squalene first requires oxidation (enzyme ***68***) followed by cyclization (enzyme ***69*** or ***70***). The detection of genes encoding for triterpenoid cyclases (***69***, ***70***) is particularly amenable to an in silico exploratory approach. The folding and catalysis of the polyprenoid cyclization reaction requires strict conformational homology of the enzymatic active site and the structure of the enzyme 'pocket' that holds the substrate (Thoma et al., 2004). Consequently, all terpenoid cyclases share a common heritage of their amino acid sequences and structures (Frickey and Kannenberg, 2009; Oldfield and Lin, 2012), including the presence of diagnostic individual loci (Hoshino and Sato, 2002). Using the amino acid sequences for ***68*** and ***69***/***70*** from a variety of eukaryotes, translated protein nucleotide BLAST (tBLASTn) searches of all bacterial genomes available in the year 2002 revealed only one additional bacterial species that contained (for both enzymes) homologues that putatively would be functional based on their sequence similarities to known ***69***/***70*** (Pearson et al., 2003). The organism was a species of Planctomycetes, *Gemmata obscuriglobus*. The lipid products of this taxon revealed that it only produced the sterols lanosterol and the unusual product, parkeol, both of which are C_{30} compounds still containing a 14α-methyl group and which together must indicate the absence of further sterol demethylases in this species.

Initial phylogenetic trees for the amino acid sequences of ***68*** and ***69***/***70*** revealed that the bacterial sequences for *M. capsulatus* and *G. obscuriglobus* are more closely related to each other than they are to sequences of eukaryotes, suggesting they might have an ancient origin (Pearson et al., 2003). However, subsequent work shows that the cyclase in Myxococcales is related to the cycloartenol synthases of plants (***70*** rather than ***69***) and may have been obtained by horizontal gene transfer, especially since some plant versions of ***70*** are multifunctional for both cycloartenol, lanosterol, and parkeol production (Ito et al., 2011; Ohyama et al., 2009; Sawai et al., 2006; Summons et al., 2006). Together, the results are too sparse to yield a definitive answer as to whether the synthesis of sterols has its origin within the bacteria, or later, within the eukaryotes. Instead, the results may be more significant for what they do not show: in the 2237 bacterial genomes sequenced to date (as of early 2012; approximately 10 times as many as in 2002), the list of bacteria with putative capacity to produce sterols still consists only of the species mentioned earlier, plus *Fluviicola taffensis* DSM 16823, a member of the order Flavobacteriales of the phylum Bacteroidetes; *Methylomicrobium alcaliphilum*, a member of the *Methylococcales* and similar to *M. capsulatus*; and *Plesiocystis pacifica* SIR-1, another member of the Myxococcales (A. Pearson, unpublished). The finding of a putative sterol producer in a member of the Bacteroidetes is unexpected and warrants further investigation. Similarly, all publicly available microbial metagenomes of environmental samples (NCBI protein database env_nr) contain a total of only 8 homologues to ***69***/***70*** (excluding homologues to SHC, ***63***), all of which show by reciprocal BLAST searching that they come from *Ostreococcus* spp. or other microeukaryotes (A. Pearson, unpublished). This suggests that nearly all sterols obtained from the environment are indeed eukaryotic in origin. The most significant new result may instead be the recent report of putative sterol biosynthetic capacity in a sponge symbiont (Siegl et al., 2011). The authors suggest these bacteria belong to a new phylum, Poribacteria, and they further posit that the bacteria, rather than the sponge cells, may be the source of the taxonomic marker 24-isopropylcholesterol. Future work is essential to determine if this is true.

12.11.3.2.2 Anaerobes capable of synthesizing hopanoids

The seminal survey of hopanoid lipids in microorganisms revealed a widespread taxonomic distribution of these compounds (Rohmer et al., 1984). The same study also failed to detect hopanoids in any anaerobes, despite the lack of a requirement for O_2 in any of the biosynthetic steps. From this and other studies, hopanoids became known as 'aerobic biomarkers,' and consistent with this paradigm, diagenetic hopanes found in marine sediments generally were interpreted to have a marine aerobic origin. Confounding evidence subsequently appeared in the form of combined measurements of hopanoids and their compound-specific values of $\delta^{13}C$. In sediments mediating the anaerobic oxidation of methane, hopanoids often are strongly depleted in ^{13}C. The signatures are consistent with synthesis by bacteria that assimilate a

fraction of their carbon from substrates derived from oxidation products of methane, thus implying an in situ source for these lipids (Elvert et al., 2000; Pancost et al., 2000; Thiel et al., 2003). The data suggested a renewed search for anaerobes that might contribute hopanoids directly to sediments.

In the first study specifically designed to use genetic predictions to examine anaerobes for their capacity to synthesize hopanoids, the bacterial family Geobacteraceae (order Desulfuromonadales) was identified as a likely candidate (Fischer et al., 2005). The genus *Geobacter* is abundant in a wide range of sediment types (Coates et al., 1996; Lovley and Phillips, 1986). The completed genome sequences of *G. sulfurreducens* PCA and *G. metallireducens* GS-15 each contained two copies of putative *shc* genes, based on searches performed using tBLASTn. Subsequent lipid analyses confirmed the presence of hopanoids in pure cultures of these species (Fischer et al., 2005; Härtner et al., 2005), and currently, all organisms known to possess a *shc* gene of sufficient homology to known, functional *shc* genes also have been found to produce hopanoids. Presence of *shc* is therefore a good predictor of active hopanoid synthesis.

Subsequent searching of the *shc* sequences from *Geobacter* spp. against genomes of other Deltaproteobacteria revealed additional information that may be relevant to geochemical interpretations. All *Geobacter* spp. contain at least one copy of *shc*, and most contain two copies; the same is true for the closely related *Pelobacter* spp. (Desulfuromonadales) and for *Syntrophobacter* spp. (order Syntrophobacterales) (Pearson et al., 2007). Work in parallel to these studies revealed that some recently isolated strains of *Desulfovibrio* (order Desulfovibrionales) also contain abundant hopanoids (Blumenberg et al., 2006). Although *shc* genes were not investigated at the time, genome searching of recently sequenced strains confirms the presence of *shc* homologues in *Desulfovibrio africanus* Walvis Bay, and *Desulfovibrio salexigens* DSM2638 (A. Pearson, unpublished). Together, the evidence suggests that synthesis of hopanoids may be common – but not universal – in the anaerobic orders of Deltaproteobacteria (Blumenberg et al., 2009). However, it is unlikely these taxa are the exclusive anaerobic sources of environmental hopanoids: other anaerobes also have been shown to synthesize hopanoids. These include purple nonsulfur bacteria (Alphaproteobacteria such as *R. palustris*) growing as facultative anaerobes (Neunlist et al., 1985; Rashby et al., 2007; Rohmer et al., 1984) and several species of anammox Planctomycetes (Damsté et al., 2004b).

12.11.3.2.3 The distribution of phosphatidylcholine in bacteria

The traditional paradigm of phospholipid biosynthesis holds that eukaryotes produce PC, while prokaryotes (bacteria in particular) favor PG and PE (**Section 12.11.2.7**; **Figure 7**). Increased study of the diversity of polar lipid products, however, exposes the degree to which PC also is prevalent in bacteria. Among the major pieces of evidence in support of a widespread biosynthetic distribution of PC comes from mining bacterial genomes for the presence of PE *N*-methyltransferases (enzyme *43*, *pmtA*; **Table 1**). In their review of PC synthesis, Sohlenkamp et al. (2003) estimated that >10% of bacteria have *pmtA* and thus may be capable of making PC from PE. Previous culture studies had suggested that PC was confined to a small number of bacterial species, primarily concentrated within Alpha- and Gammaproteobacteria, as well as Bacteroidetes (Goldfine, 1982; Lopez-Lara and Geiger, 2001). These culture studies undoubtedly are biased by the availability and selection of organisms that are commonly utilized for lipid characterizations in laboratory experiments, and Sohlenkamp et al. (2003) noted that this factor has probably has contributed to a biased picture of the distribution of PC biosynthesis.

Although the selection of organisms for genome sequencing also suffers from many of the same biases, present databases of bacterial genomes might give a more representative picture than the limited information from culture studies. Experimental studies already had identified two families of bacterial *pmtA* sequences, the *Rhodobacter* family (Arondel et al., 1993) and the *Sinorhizobium* family (Sohlenkamp et al., 2000). Although the conserved active sites encoded by both types of sequences indicate they are each SAM methyltransferases, the *Sinorhizobium*-type sequences are homologous to rRNA methylases, while the *Rhodobacter*-type sequences are homologues of quinone biosynthesis methyltransferases. This represents two fundamentally different routes to synthesis of PC within the Alphaproteobacteria and presents a cautionary note regarding the diversity and promiscuity of the many types of SAM methyltransferases participating in lipid biosynthesis. Further laboratory work also showed that a related sequence in *Pseudomonas aeruginosa*, which by sequence similarity would appear to encode for a PmtA, did not exhibit the ability to methylate PE → PC (Wilderman et al., 2002).

The multitude of types of PmtA-encoding gene families, and the difficulty of distinguishing them from SAM methyltransferases having other functions, makes it difficult to catalogue true *pmtA* sequences by genome searching. Nevertheless, performing BLAST searches using the sequences that showed positive results in molecular experiments, the distribution of putative *pmtA* genes in bacterial genomes suggests that PE → PC methylation could be expected in ~10% of all bacteria; although the authors caution that these results need to be verified experimentally and are biased by the distribution of species in the database (Sohlenkamp et al., 2003).

12.11.3.2.4 Hypotheses about synthesis of ladderane lipids

Finally, in an example of genomic data mining to explore for an unknown lipid biosynthetic pathway, Rattray et al. (2009b) catalogued all the putative FA biosynthesis genes from *Candidatus* Kuenenia stuttgartiensis, an anammox bacterium with a completed genome (Strous et al., 2006). Hypotheses about the potential biosynthetic route to ladderanes had, at this time (e.g., Damsté et al., 2005; Mascitti and Corey, 2006), suggested that ladderane synthesis might follow a radical or cation-mediated cyclization cascade from a polyunsaturated long-chain FA precursor. Subsequent isotopic labeling experiments (Rattray et al., 2009a) have since called these proposed mechanisms into question (**Section 12.11.2.11**). However, the goal to explore lipid biosynthetic potential using genomic data mining approaches is still with merit, even if it will be more difficult to obtain fruitful results. Rattray et al. (2009b) identified all of the necessary genes for FA synthesis, plus several additional copies of 3-oxoacyl-ACP synthesis genes (β-ketoacyl-ACP synthases and reductases; *fabF*, *fabG*). The

resulting genome organization revealed that the FA synthesis genes of this species are organized into four clusters, some of which are accompanied by putative SAM enzymes. The authors suggested that one or more of these gene clusters participates in synthesis of specialized long-chain structures that are in some way involved in the synthesis of ladderanes; how this is done, and what the specific ladderane precursor may be, could not be inferred.

12.11.3.3 Examples Using Environmental Metagenomics and Functional Genomics

Similar to the previous examples, BLAST searches also can be used to query databases of DNA sequence obtained directly from the environment. The advantage of the environmental metagenomic (Handelsman, 2004) approach is that it can provide information directly about the vast numbers of uncultivated species, many of which belong to phyla or orders of prokaryotes that have not a single cultured representative. In metagenomics (or metatranscriptomics), DNA (or RNA) is extracted directly from the mixed assemblage of species that forms the natural community, and in many cases, it is sequenced directly without the need for PCR amplification and cloning steps, both of which are associated with significant biases. The resulting raw data often do not give sufficient coverage to be 'assembled' into genomes – although with the highest-throughput sequencing methods, for example, Illumina, this is changing. Regardless of assembly, the raw data are directly amenable to BLAST.

An alternative approach to characterizing the distribution of lipid biosynthesis genes from environmental samples is to accept the challenges of PCR-based approaches in order to focus directly on a specific pathway or gene. Functional gene PCR is a culture-independent approach that seeks to use conserved regions of sequence as targets for amplification; the methods are similar to studies of SSU rRNA diversity and can illuminate the distribution of important processes in the environment (e.g., ammonia oxidation by archaea (Francis et al., 2005)). An advantage of this approach over standard metagenomics is that it can increase the detection of rare genes, enabling greater numbers and diversity of sequences to be obtained.

12.11.3.3.1 Functional gene surveys – environmental shc *genes*

The work of Rohmer et al. (1984) first demonstrated that hopanoids are irregularly distributed phylogenetically, and only cyanobacteria, methanotrophic bacteria, and Alpha-, Beta-, and Gammaproteobacteria are reported to be among the most prolific producers (Kannenberg and Poralla, 1999). However, this taxonomic information mainly is based on culture studies (e.g., Rohmer et al., 1984; Summons et al., 1999) and is, thus, prone to uncertainty about the adequate representation and characterization of the vast majority of global hopanoid-producing species, which may either be uncultivable (>99%) and/or yet unknown (Hugenholtz and Pace, 1996).

The poorly understood taxonomic distribution of hopanoid synthesis contrasts with the extensive research on the complexity of bacteriohopanepolyol (BHP) structures found in both pure cultures and environmental samples (e.g., Cooke et al., 2008a,b; Talbot and Farrimond, 2007; Talbot et al., 2003c). Most of this structural diversity involves the many types of polar head groups that are attached to the hopanoid side chain. It has been proposed that these side-chain structures carry taxonomic and/or physiological information (Talbot and Farrimond, 2007). To achieve the goal of placing phylogenetic constraints on the sources of hopanoids preserved in sedimentary rocks, it will be necessary to link taxonomic groups of hopanoid producers (e.g., Alphaproteobacteria) either to specific compounds or, more likely, to patterns or assemblages of BHPs and their diagenetic products. However, when the phylogenetic distribution of putative hopanoid producers in the environment is compared to the results from culture surveys, the challenges affecting progress in this area become apparent.

The high degree of sequence conservation observed for sterol cyclases also applies to squalene–hopene cyclases (Hoshino and Sato, 2002). All SHC enzymes must conserve the catalytic site for protonation of squalene to initiate cyclization, and they must control the product stereochemistry while propagating the cyclization reaction (**Figures 14** and **15**). To fulfill these strict requirements, several amino acid sequence motifs are conserved among SHCs of nearly all bacterial phyla (Fischer and Pearson, 2007; Perzl et al., 1997). We previously used these conserved motifs to identify environmental *shc* genes through conventional PCR-cloning-Sanger sequencing approaches, using a set of highly degenerate PCR primers that cover a wide range of bacterial phyla (Pearson et al., 2007, 2009). The PCR amplicon spans from the middle of the SHC sequence nearly to the C-terminal end of the protein (**Figure 21**), providing a translated amino acid segment that includes the initial catalytic motif, as well as several of the critical aromatic amino acids that determine the final number of rings for the polycyclic product (Hoshino and Sato, 2002). Using this approach, we sought to determine the diversity and ubiquity (or paucity) of hopanoid biosynthetic capacity in uncultivated environmental communities.

An initial study comparing a freshwater lake to an open marine system revealed a higher total number and diversity of *shc* sequences in the lake (Pearson et al., 2007). Fifty-eight sequences were obtained from the lake, of which 23 were unique; 21 sequences were obtained from the ocean, of which 11 were unique. Rarefaction analysis of these results suggested in both cases that the predicted total diversity would be <100, while the SSU rRNA diversity in comparable samples is >1000, that is, it suggests that fewer than 10% of species contain genes for making hopanoids. A second study measured both the distribution of BHPs and the diversity of *shc* sequences across a transect of onshore to offshore sites in the Bahamas (Pearson et al., 2009). An upland soil site had both the greatest diversity of measured BHP structures and the greatest number and diversity of sequenced *shc* genes. Under the assumption that the more *shc*-containing organisms there are, the more potential capacity there could be to create diverse BHP head groups, it may be imagined that the diversity of *shc* sequences would predict the diversity of co-occurring BHPs. However, this relationship was not consistent throughout the samples. The data showed that the soil contained the largest number of unique *shc* genes (31) and contained 12 BHPs. A sample from an inner tidal creek was less diverse for *shc* (8) and contained only 6 BHPs. These two samples agreed with the idea that *sqhC* diversity predicts BHP diversity.

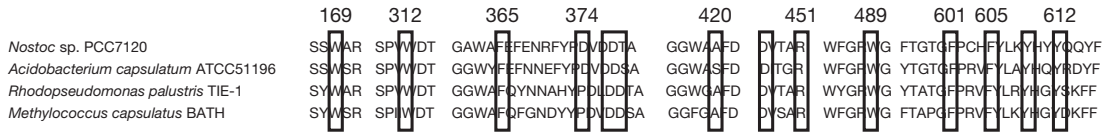

Figure 21 Representative alignment of the critical conserved amino acid residues for squalene–hopene cyclase activity. Numbering corresponds to the conventional *Alicyclobacillus acidocaldarius* reference sequence. Residues for catalytic protonation and initiation of the reaction, 374, 376, 377, 447, and 451. Substrate-binding residues, 169, 312, and 489. Residues that propagate the A-ring and B-ring carbocations, 365, 420, 609, and 612. Essential residue for C-ring carbocation, 601; essential residue for D-ring carbocation, 605 (adapted from Fischer WW and Pearson A (2007) Hypotheses for the origin and early evolution of triterpenoid cyclases. *Geobiology* 5: 19–34).

However, a lower tidal creek sample and a purely marine sample contained 15 and 4 identifiable *shc* genes, respectively, but both contained 11 BHPs. This may suggest that offshore transport influences the marine BHP composition distinct from the local *shc* diversity; or, it may suggest that BHP diversity is unrelated to *shc* but instead relates to diversity of side-chain biosynthesis genes or to local diagenetic transformations.

The phylogenetic assignments of these environmental sequences also supply another layer of information. The translated amino acid sequences of the *shc* genes from environmental samples show the presence of all critical functional residues needed for cyclase activity, suggesting these organisms would be expected to contribute to hopanoid production in the environment. However, most of the sequences have $<60\%$ overall identity at the amino acid level to their nearest relative among cultured bacterial species. This indicates that in natural systems, most bacteria that are the sources of hopanoids come from phyla, orders, or classes of bacteria that neither have been identified nor characterized metabolically; although most do appear to be affiliated (or have nearest relatives) within the Proteobacteria and/or Acidobacteria (Pearson et al., 2007, 2009). With only one exception, no environmental sequence from any PCR experiment has been identified as having $>80\%$ amino acid sequence identity to a cultivated strain, and yet these unknown species appear to be the dominant hopanoid producers in the environment. The exception is a sequence obtained from an acidic biofilm that was $>90\%$ identical to cultivated *Acidithiobacillus ferrooxidans* (Jones et al., 2012). The differences between culture studies and environmental data may raise questions about the origin of hopanoids preserved in marine sedimentary rocks. In the future, it will be important to supplement studies of *shc* with analogous approaches to expand the range of target genes to include other steps in hopanoid synthesis such as methylation (*hpnP*; Welander et al., 2010), ribosugar addition, and adenine cleavage (*hpnG*, *hpnH*; Bradley et al., 2010). Such approaches should achieve a more complete picture of the relationship between production of hopanoids and the patterns of distribution of molecular structures.

12.11.3.3.2 General metagenomic surveys – hopanoid synthesis genes

A different approach to the diversity of hopanoid producers is possible through direct assessment of environmental metagenomes, without relying on PCR amplification. A tabulation of all instances of *shc* fragments that occur in publicly available metagenomic data sets, including the Global Ocean Sampling expedition (Rusch et al., 2007; Venter et al., 2004), acid mine drainage (Tyson et al., 2004), and several other systems, reveals patterns similar to those obtained by functional PCR (Pearson and Rusch, 2009). In general, the metagenomic surveys reveal a somewhat wider diversity of *shc* sequences than are obtained with current PCR approaches. Accepting the BLAST-assigned taxonomic identities of these sequences at face value (without filtering for the quality of the assignment), the data suggest that although marine environments are poor in putative hopanoid producers overall, most of the responsible taxa are Alphaproteobacteria. In contrast, terrigenous environments are richer in both numbers and diversity of putative hopanoid producers, favoring several of the divisions of Proteobacteria and Acidobacteria. And yet because copies of *shc* genes associated with Cyanobacteria were detected only in microbial mats and not in the open ocean or other systems (Pearson and Rusch, 2009), the absence of additional cyanobacterial sequences suggests that *all* of the results may be affected by the chosen methods of sample collection. This suggests better approaches to sampling, combined with hybrid approaches of metagenomics and high-throughput sequencing of PCR-amplified *shc* genes – both of which avoid the biasing step of cloning – could bring improvements to the estimation of hopanoid biosynthetic diversity.

However, as was observed for the PCR data Section 12.11.3.3.1, the amino acid sequence identity between environmental metagenomic data and culture data again reaches an average maximum of $\sim$60–70%. Because the metagenomic data do not include a PCR step, the comparison indicates that the earlier PCR data were not greatly affected by amplification biases that might have skewed the types of sequences detected. Both types of data show that environmental producers of hopanoids are not close relatives of species in culture collections. A maximum-likelihood phylogenetic tree of all available SHC sequences – metagenomic, PCR-derived, and from culture collections – exposes the extent of this issue (Figure 22; A. Pearson, unpublished). There are several large clades, possibly representing phyla, for which there is no sequenced relative.

Finally, it should be noted that none of these gene survey approaches actually determines the expression, or activity, of hopanoid biosynthesis in the environment. Further efforts will be needed to determine if biosynthetic capacity translates to quantitatively proportional fluxes from the various species that produce hopanoids. The environmental work to date suggests that $<2\%$ to $<10\%$ of bacterial cells in most environments are capable of producing hopanoids. Which ones actually do the job remains an open question.

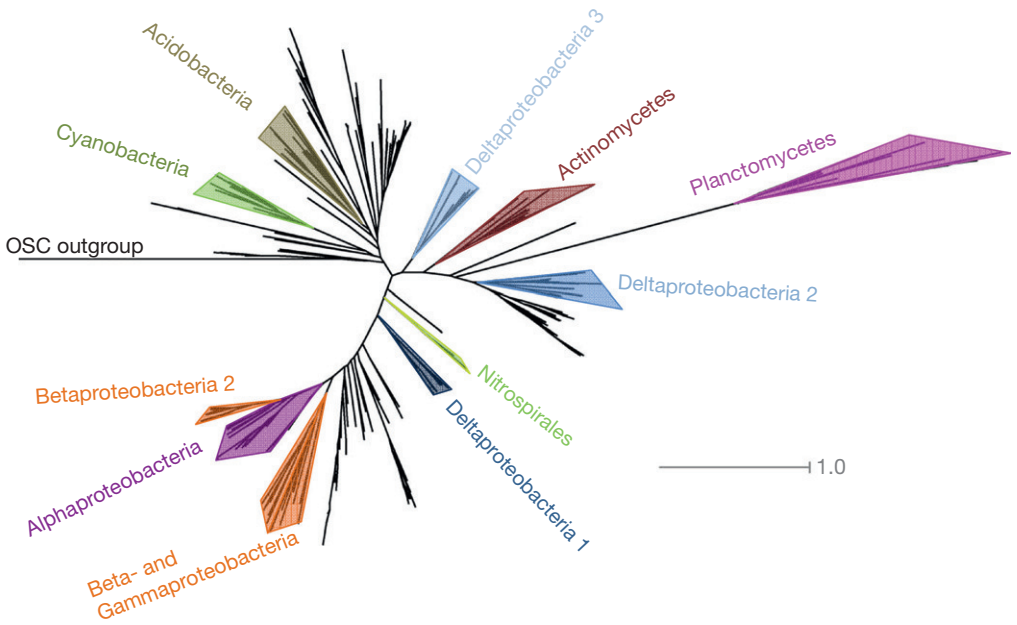

Figure 22 Phylogenetic tree of squalene–hopene cyclases, showing the presence of major clusters of sequences that have no known relatives among the species in genomic databases (A. Pearson, unpublished). All available environmental PCR amplicons, metagenomic sequences, and more than 100 cultured species are represented. The tree was prepared from an amino acid alignment of the C-terminal region of SHC, corresponding to the length of sequence obtained by PCR; alignment was performed using Opal (http://opal.cs.arizona.edu), followed by maximum likelihood calculations by RAxML (http://www.phylo.org/news/RAxML) implemented through the CIPRES portal (CyberInfrastructure for Phylogenetic RESearch).

12.11.3.3.3 General metagenomic surveys – other lipid biosynthetic pathways

A number of other recent studies have taken advantage of the opportunities offered by metagenomics and high-throughput sequencing. Lipid biosynthetic pathways that are particularly targeted include general patterns of phospholipid and FA synthesis, as well as the synthesis of lipids by archaea.

In an example that utilized the Global Ocean Sampling expedition metagenome (Rusch et al., 2007), Gianoulis et al. (2009) developed a multivariate correlation mapping approach to analyze what they termed 'metabolic footprints' distributed within the GOS data. Among their results was the observation that expression of lipid metabolic pathways (including isoprenoid and glycerophospholipid metabolism) varied strongly in response to salinity, temperature, and depth in the water column, while these pathways were slightly less correlated with overall water column depth or geographic location. In similar work, Gilbert et al. (2010) reported analysis of metagenomic data obtained over the span of three seasons from a coastal ocean observatory located in the Western English Channel. The data include samples collected both in the daytime and at night. Sequencing was done by 454 pyrosequencing with a reported yield of $>5 \times 10^6$ sequences, which likely is equivalent to $>10^9$ DNA bases. This is more data for a single site than for any of the GOS sites other than the initial Sargasso Sea study (Venter et al., 2004). The English Channel data were annotated and binned into protein families using the SEED database of MG-RAST (Metagenome Rapid Annotation using Subsystem Technology; Meyer et al., 2008). The results showed patterns similar to the conclusions of Gianoulis et al. (2009). As evidence of environmentally mediated control on communities, lipid biosynthesis of archaea was upregulated in winter, although the species involved were not very diverse. However, transcription in both bacteria and archaea was even more strongly associated with diel cycling rather than seasonal cycling, suggesting again that local environmental condition is the major driver of lipid biosynthesis.

In a more directly lipid-focused approach, Shulse and Allen (2011a,b) recently examined the distribution of genes specifically involved in synthesis of bacterial long-chain, polyunsaturated FAs (PUFAs) in marine communities. Some cultured bacteria, for example, *Shewanella* and *Colwellia* spp., are known to produce PUFAs, compounds which otherwise are considered to be dominantly eukaryotic products (Fang et al., 2004; Nichols, 2003). By targeting the PUFA gene *pfaA* of the previously characterized synthase complex *pfaABCD* (Allen and Bartlett, 2002), PCR primers were developed to amplify this gene from six diverse marine environments, including a coastal surface water, high- and low-chlorophyll tropical Pacific Ocean waters, surface Antarctic seawater, the deep Atlantic, and a marine sediment (Shulse and Allen, 2011a). Due to a high degree of sequence conservation, they were able to design degenerate PCR primers to identify several hundred new, diverse *pfaA* genes. In addition, they further examined metagenomic databases (GOS, HOT station ALOHA, a whale fall, and DEEPMED; DeLong et al., 2006; Martin-Cuadrado et al., 2007; Rusch et al., 2007; Tringe et al., 2005) to compare to the amplicon sequences, identifying 67 additional metagenomic reads. In total, they observed that marine habitats support an abundant and diverse genetic potential to produce PUFAs and that this capacity is widespread across a diversity of environments. The phylogenetic clustering of results

indicated that 13 of the 17 identified groups of *pfaA* genes represented uncharacterized sequences belonging to unknown species and having unknown lipid products. The four identified clusters associated with *Flavobacteria* (Bacteroidetes), *Psychromonas* spp. (Gammaproteobacteria), Desulfuromonadales (Deltaproteobacteria), and *Thraustochytriidae* spp. (marine heterotrophic protists) (**Figure 23**).

In follow-up work, the distribution and diversity of PUFA-type biosynthetic gene clusters were analyzed in a wide variety of cultured bacterial taxa chosen for their relevance to microbial oceanography. Analysis of their complete genomes showed that the potential to synthesize these long-chain lipids is widespread, appearing in "10 microbial phyla, representing 86 species across two domains of life. . .[and is] scattered throughout the bacterial domain. . .[showing that] these biosynthetic pathways are not relegated solely to a narrow groups of marine bacteria, as previously believed" (Shulse and Allen, 2011b). Given these results, the abundant FAs of chain length $>C_{18}$ that are preserved in marine sediments might have sources from bacteria and eukaryotes. Carbon isotopic data from sediments already had been used to suggest this idea in some environments (Gong and Hollander, 1997), but genetic evidence for widespread microbial sources had, to date, been lacking.

Finally, in a recent example of the power of ultrahigh-throughput sequencing, Iverson et al. (2012) obtained a complete genome for the sparsely distributed and still (to date) uncultivated marine group II Euryarchaeota. These authors assembled the genome from <2% of a >50 gigabase metagenome, reflecting the minor contribution of these cells to surface water communities. In addition to describing a consensus sequence inferred from what may be five strains of these archaea in surface waters, they were able to describe the metabolism of this taxonomic group as photoheterotrophic. The group II Euryarchaeota contain a proteorhodopsin that may be ancestral to the version common in Proterobacteria (Béjà et al., 2002). Of interest to organic geochemists is another tentative finding: the authors "identified several putative acyl-carrier-protein fatty-acid synthesis enzymes and enzyme homologs for glycerolipid biosynthesis, most similar to bacterial proteins. . .and without homologs among the sequenced Archaea" (Iverson et al., 2012). If these Archaea are capable of synthesizing ester-linked acetogenic membrane lipids – or even simple, extended acyl-chain free metabolites – such a finding would be unprecedented, as the authors note. Examining the raw data (their Table S8) and comparing to enzymes ***1–7*** (**Table 1**), this species appears tentatively to contain all of the steps for synthesis of straight-chain FAs, except that the annotated list contains no identified malonyl-CoA:ACP transacylase (nor any other malonyl-CoA transferase; **Table 2**). The need to verify this suggested presence of 'bacterial' lipids in archaea will undoubtedly prompt a major effort to obtain one of these strains in pure culture.

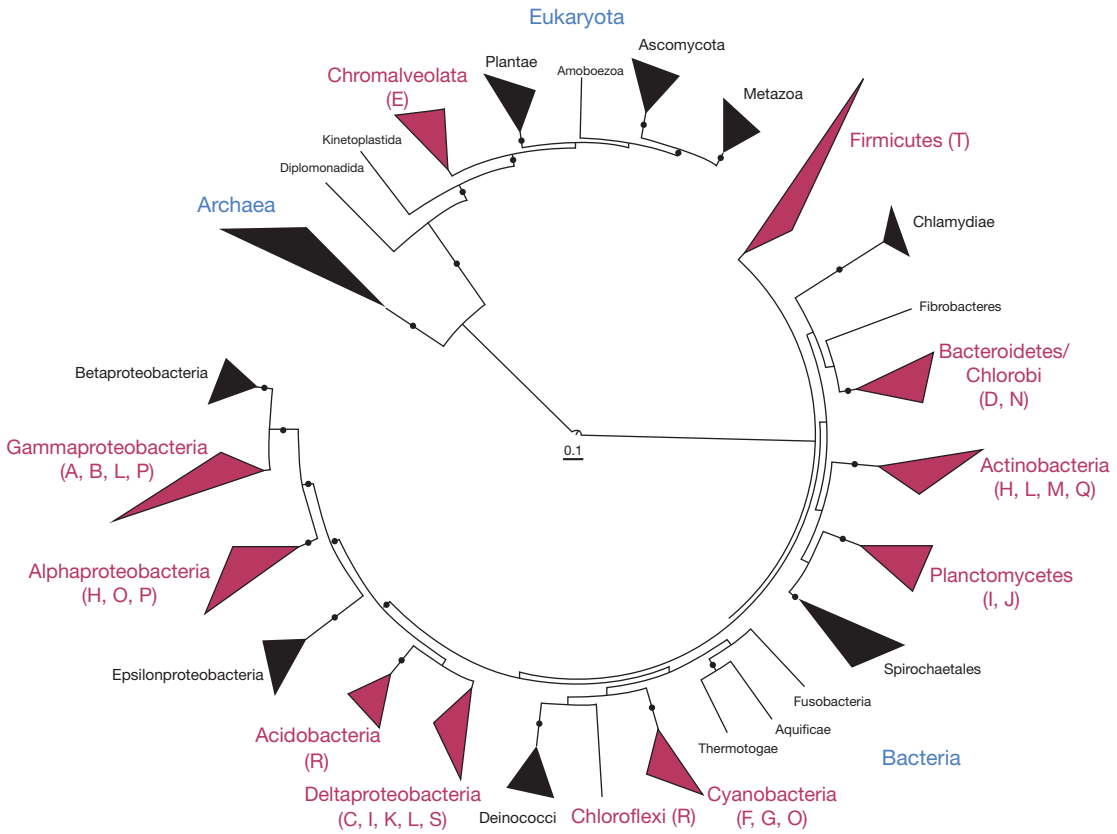

Figure 23 Phylogenetic distribution of *pfa* gene clusters, highlighting groups of bacteria identified as potential sources of PUFAs, based on the presence of extended fatty acid synthase complexes. Clades in red contain putative secondary lipid gene clusters; clusters labeled A, B, D, E, F, and P represent synthase complexes with known lipid products, while lipids produced by the other clusters remain unknown. Reprinted from Shulse CN and Allen EE (2011b). Widespread occurrence of secondary lipid biosynthesis potential in microbial lineages. *PLoS One* 6, with permission.

Table 2 Reported enzymatic potential for fatty acid biosynthesis in marine group II Euryarchaeota (Y = yes; N = no)

	Marine Group II Euryarchaeota		*Present?*
1	Acetyl-CoA carboxylase	6.4.1.2	Y
2	Malonyl-CoA:ACP transacylase	2.3.1.39	N
3	3-Oxoacyl-ACP synthase	2.3.1.41,179,180	Y
4	3-Oxoacyl-ACP reductase	1.1.1.100	Y
5	3-Oxoacyl-ACP dehydrase	4.2.1.58-61	Y?
6	Enoyl-ACP reductase	1.3.1.9-10	Y
7	Acyl-ACP desaturase	1.14.19.1	Y

Source: Iverson V, Morris RM, Frazar CD, Berthiaume CT, Morales RL, and Armbrust EV (2012) Untangling genomes from metagenomes: Revealing an uncultured class of marine Euryarchaeota. *Science* 335: 587–590.
? = not determined.

12.11.3.4 Examples Using Experimental Biochemical Approaches

The following examples were initiated based on biochemical assays or DNA hybridization and screening approaches, rather than beginning with databases of DNA sequence data. By beginning with traditional biochemical and isotope-labeling experiments, the results were able to guide subsequent analyses of functional genes, transcriptomes, or genomes to explore the observations and elucidate details of biochemical pathways.

12.11.3.4.1 Synthesis of botryococcene

Botryococcenes appear to be found only in the green algal species *Botryococcus braunii* Race B, giving them the potential for high taxonomic specificity (Maxwell et al., 1968; Metzger et al., 1985). These organisms are confined to lacustrine or brackish systems, and the associated botryococcanes are primary markers for classification of oil types (McKirdy et al., 1986; Moldowan and Seifert, 1980). Although the botryococcanes are major contributors to organic deposits only in the Tertiary (cf. Peters et al., 2005), algal remains of *Botryococcus* spp. are abundant throughout the Phanerozoic (Derenne et al., 1988; Largeau et al., 1990). It remains unknown whether the lack of ancient botryococcanes is due to poor preservation or if these compounds indeed are of younger origin than the taxonomic division in which they are found.

The suggested synthesis for botryococcene involves an unusual isoprenyl linkage (Cox et al., 1973). However, until recently, it remained unknown exactly what pathway might be involved or how this bond is formed. Additional remaining questions about botryococcene include whether the difference between *B. braunii* Race A (no botryococcene) and Race B (abundant botryococcene) is environmental or genetic, whether botryococcenes are found outside the genus *Botryococcus*, and how the timing of evolution of this lipid biosynthetic pathway evolved relative to the evolution of the genus.

Answers to some of these questions recently were provided by a biosynthetic study (Niehaus et al., 2011). The parent C_{30} botryococcene is formed from two molecules of FPP, and it was long suspected that its synthesis resembled that of squalene (Okada et al., 2000; Poulter, 1990). Squalene is produced by the 1–1′ joining of two FPPs in a mechanism that proceeds through a 1′–2–3 intermediate (**Figure 13**, in the preceding text); such cyclopropanation is a feature of known squalene synthases and other diphosphate synthases (Blagg et al., 2002; Thulasiram et al., 2007). For squalene, the reaction is a two-step process of condensation to PSPP, followed by reduction by NADPH. Both reactions are mediated by a single enzyme (***61***) containing separate domains to mediate the different parts of the reaction (Gu et al., 1998). The work of Niehaus et al. (2011) confirmed that the synthesis of botryococcene from two molecules of FPP does require enzymes homologous to known squalene synthases. But unlike in the synthesis of squalene, the botryococcene synthase complex consists of two distinct enzymes, a PSPP synthase (SSL-1) and a separate NADPH-dependent, squalene synthase-like protein (SSL-3) that functions as a reductase. The two steps are shown in **Figure 24**.

This example is notable because the investigators did not have a genome for *B. braunii* with which to initiate their targeted search for homologues of FPPase and/or squalene synthase. Instead, an authentic squalene synthase gene identified from previous work (Okada et al., 2000) was used in a relaxed-stringency hybridization protocol to screen a cDNA library prepared from *B. braunii* mRNA transcripts. By sequencing only the positive hybridization signals that were *not* exact matches to the authentic squalene synthase, they found SSL-1. However, expression of SSL-1 in yeast revealed only PSPP as a product. For further gene candidates, the investigators also performed in silico screening of a cDNA sequence library (newly obtained by 454 pyrosequencing; http://www.jgi.doe.gov/sequencing/why/botryococcus.html) to look for additional squalene synthase homologues. This approach revealed two additional candidates, SSL-2 and SSL-3. By cloning and expressing those genes in *E. coli* and in yeast, followed by assays for FPP-catalyzing activity, it was shown that the combination of SSL-1 plus SSL-3 expression yielded C_{30} botryococcene.

Knowing the genetic and enzymatic requirements for synthesis of botryococcene now makes it possible to study the evolution of this molecular pathway. Niehaus et al. (2011) suggested that gene duplication of an ancient triterpene synthase could have led to the distinct functions of SSL-1 and SSL-3. Molecular clocks could help estimate the timing of this event. Alternatively, horizontal gene transfer could have supplied either SSL-1 or SSL-3; this would warrant a search of other algal species for similar sequences.

12.11.3.4.2 Multifunctional 2,3-oxidosqualene cyclases in plants

Synthesis of sterols from 2,3-oxidosqualene involves cyclization to the first stable intermediate, lanosterol, cycloartenol, or parkeol (**Figure 15(a)**). The traditional paradigm is that animals and fungi contain lanosterol cyclases (***69***), while plants contain cycloartenol cyclases (***70***) (Abe et al., 1993; Corey et al., 1966, 1993). This generalization also applies to unicellular relatives of these more complex organisms, with the animal ancestors, choanoflagellates, choosing the lanosterol route (Kodner et al., 2008), while all photosynthetic algae, heterokont algae, and photosynthetic protists (with the exception of dinoflagellates) contain cycloartenol synthases (e.g., Anding et al., 1971; Nes et al., 1990; Raederstorff and Rohmer, 1987).

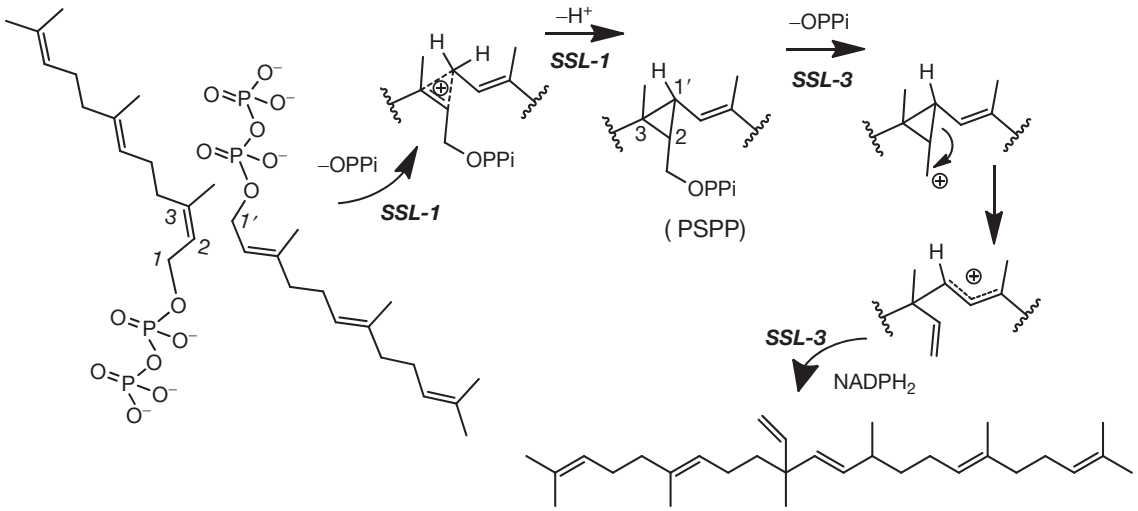

Figure 24 Biosynthetic pathway for botryococcene (Niehaus et al., 2011). Two squalene synthase-like (SSL) enzymes are required: the first, ***SSL-1***, yields the cyclopropyl intermediate presqualene diphosphate, while the second, ***SSL-3***, opens the ring and provides reducing power. Both enzymes are required to produce the final 1′–3 linked polyprene.

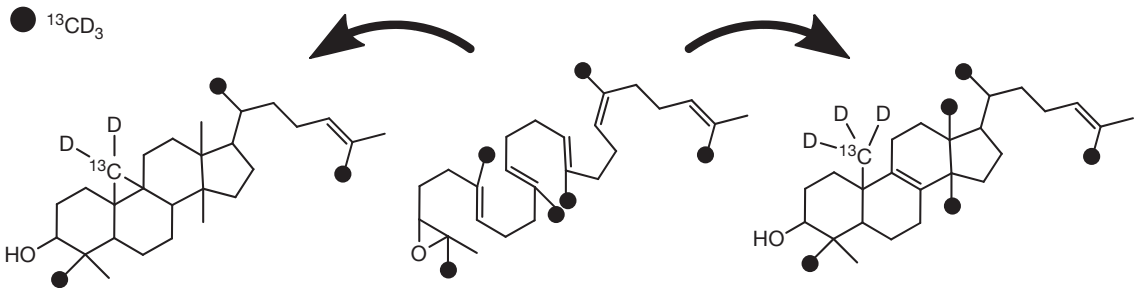

Figure 25 Isotopic labeling method to distinguish the presence of cycloartenol versus lanosterol intermediates in the synthesis of sterols. This approach allowed the identification of multifunctional 2,3-oxidosqualane cyclases in plants (adapted from Ohyama K, Suzuki M, Kikuchi J, Saito K, and Muranaka T (2009) Dual biosynthetic pathways to phytosterol via cycloartenol and lanosterol in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* 106: 725–730).

The sequence and functional similarity between lanosterol and cycloartenol synthases is apparent from studies that have employed site-directed mutagenesis to examine the sequence–structure–function relationships governing the cyclization reaction. Mutation of several individual amino acid residues of cycloartenol synthase (***70***) from *Arabidopsis thaliana* can induce ***70*** to have the activity of ***69***, yielding lanosterol and some parkeol (Meyer et al., 2002). Recognizing that such site-specific sensitivity might portend functional heterogeneity among naturally occurring cyclases, Sawai et al. (2006) identified naturally occurring lanosterol cyclase activity in *Lotus japonicus*, by using the plant gene to successfully complement a sterol-deficient yeast strain (sterol auxotroph *S. cerevisiae* GIL77). In general, plants contain numerous, divergent copies of triterpene cyclases, suggesting both that this capacity might be widespread in plants and that ancestral versions of ***70*** easily evolved to the several present-day lineages of ***69*** (e.g., Sawai et al., 2006; Xue et al., 2012).

To follow up from the biochemical perspective, Ohyama et al. (2009) took an isotopic screening approach to assess if or how much a functional lanosterol cyclase activity actually contributes to the production of terminal phytosterols, such as β-sitosterol. In an elegant experiment, they fed *A. thaliana* seedlings with isotopically labeled mevalonate (6-$^{13}CD_3$). This approach yielded isoprenoids in which all of the methyl groups were both ^{13}C-labeled and perdeuterated. However, in the case of a cycloartenol intermediate (Figure 25), ring opening of the cyclopropyl group adds a new (unlabeled) hydrogen to C_{19}, and phytosterols downstream of this route have $^2/_3$ the deuterium signal in this position relative to phytosterols generated via the lanosterol route. The results showed that normal activity of *A. thaliana* generates ~1.5% of total sterols from lanosterol. While a small number, the result demonstrates the natural plasticity of oxidosqualene cyclization in plants and the ease with which these cyclases may have mutated and evolved with time.

12.11.3.5 Examples Using SSU rRNA Combined with Taxonomic Specificity of Algal Biomarkers

The following examples take advantage of the unique lipids found in some taxa of eukaryotic algae. In these examples,

lipidomic information is not inferred from analysis of the lipid biosynthetic pathway directly but rather derives from the taxonomic diversity of SSU ribosomal RNA. The implications for paleobiology include the ability to calibrate molecular clocks against the fossil record and to examine questions of biomarker origin versus biomarker preservation (taphonomy).

12.11.3.5.1 Paleorecord of alkenone-producing haptophyte algae

The taxonomic specificity of C_{37} alkenones provides a useful window into paleoproductivity of haptophyte algae and the history of sea surface temperatures (Brassell et al., 1986). Because of the unique association of these lipid biomarkers with a specific phylogenetic group, there is promise for associating genetic diversity metrics (SSU rRNA genes) with lipid distributions. In general, it is difficult to make predictions about lipid biosynthesis simply by detecting SSU rRNA gene sequences, since it is rare that a given biosynthetic pathway can be viewed with confidence to be taxonomically exclusive. The alkenones are sufficiently constrained that they may prove to be a useful exception.

Different species of haptophytes have slightly different calibrations for the $U^{k'}_{37}$ sea surface temperature proxy. The causes include species-specific effects and effects induced by changes in nutrient concentrations and growth rate (Conte et al., 1998; Popp et al., 1998; Prahl and Wakeham, 1987; Versteegh et al., 2001). Such differences are observed most readily as a contrast between the *Isochrysis* and the *Emiliania*/*Gephyrocapsa* taxonomic clusters. Recognizing this potential for heterogeneity, Coolen et al. (2006) explored whether the community composition of haptophyte algae has changed over time in the Black Sea and whether any such changes could affect the preserved values of $U^{k'}_{37}$. Using SSU rRNA gene primers, fossil DNA extracted from Black Sea sediments was examined by PCR. The breakdown of DNA is inhibited in this system, due to the preservational effects of sulfidic pore waters (Coolen and Overmann, 1998; Rochelle et al., 1992). The record of preserved DNA extends several thousands of years. Quantitative PCR showed that although most of the total eukaryotic DNA was from other taxonomic groups (e.g., dinoflagellates), the haptophyte contribution appeared to be dominated by *Emiliania* and/or *Gephyrocapsa* spp. during Holocene stage I, with no evidence of a significant contribution from other genera. Additional experiments suggested this result was not an artifact of differential preservation or degradation of DNA among the different algal strains.

In follow-up work, these investigators more intensively studied Holocene stage II, a depositional interval predating the recent coccolith ooze. Here, the quantitative PCR approaches were refined to include PCR primer sets optimized specifically to *Isochrysis* and to *Emiliania*/*Gephyrocapsa*, and the resolution and length of the sedimentary record was extended (Coolen et al., 2009). The SSU rRNA gene abundances again were consistent with sediment stratigraphic changes in suggesting that the precoccolith stage II was dominated by noncalcifying *Isochrysis* spp. having an unreliable $U^{k'}_{37}$–SST relationship. The transition to *E. huxleyi* and related species in the more recent stage I sediments corresponded to changes in lipid profiles.

Although these interpretations are based only on abundances of rRNA genes, and not on genes associated specifically with lipid biosynthesis, the results are predictive. Episodes of noncalcareous deposition that are associated with species changes rather than with carbonate dissolution may correlate to the different pathways of alkenone production as suggested by Rontani et al. (2006). In particular, the distribution of the putative $\Delta^{(5)n}$ and $\Delta^{(7)n}$ desaturases may be phylogenetically coherent with the radiation of taxa. If so, it could be determined if the alkenone lipid biosynthetic pathways diverged at the same time the major clades of haptophyte algae radiated.

12.11.3.5.2 Highly branched isoprenoids of diatoms

Another useful example of taxonomic specificity is the association of C_{25} and C_{30} HBI lipids with the *Rhizosolenia* and *Navicula*/*Haslea*/*Pleurosigma* diatom genera (Damsté et al., 2004a). These compounds are independent tools that can be used to evaluate the fidelity of the paleontological record of diatom frustules. The apparent recalcitrance of HBIs is especially important, due to the high solubility of silica and the resulting sporadic record of frustules.

The earliest diatoms arose at least 190 Ma ago (Sims et al., 2006; Sorhannus, 2007), but their major radiations and rise to prominence occurred during the mid-Cretaceous, around 100 My. Recent phylogenetic analyses propose the divergence of the *Navicula*/*Haslea*/*Pleurosigma* group considerably more recently (<75 My; Sorhannus, 2007), supporting the suggestion that the first appearance of HBIs in the sedimentary record is associated with the emergence of *Rhizosolenia* (Damsté et al., 2004a). The record of HBIs begins abruptly in the Turonian, and the specific date of 91.5 My obtained for HBIs has been used to calibrate molecular clocks by providing a constraint on the rate of diatom evolution (Sorhannus, 2007; Sorhannus and Fox, 2012). However, Girard et al. (2009) recently reported the presence of fossilized diatoms assignable to the order Rhizosoleniales in Albian ambers dating to 98–99 My. This is older than the date specified by the HBI markers for the evolution of this taxonomic group. There are two options to reconcile the data: either (1) additional high-resolution data of sedimentary deposits in the Cenomanian and Albian might reveal low levels of C_{25} HBIs prior to 91.5 My or (2) the timing of evolution of the HBI biosynthetic pathway postdates the evolution of *Rhizosolenia* by a few million years. Because the exact physiological role of HBIs is not known, it is unclear if the taxonomic diversification bears any relationship to the presence/absence of the HBI biosynthetic pathway; there may be no requirement that the two be linked.

Answers to such questions will depend on identifying the genes encoding for HBI synthases and the ancestral proteins from which these enzymes descended. Such evolutionary inferences must be based on extant sequences. Direct identification of paleo-DNA at this timescale is improbable (for a recent debate about ancient DNA, see the counterpoints of Inagaki et al. (2005) and Damsté and Coolen (2006)). To date, there is only one study that examines the distribution of paleo-DNA specifically focused on diatoms, and it was limited to amplification of SSU rRNA from recent sediments (Coolen et al., 2007).

12.11.4 Conclusions

Lipidomic approaches provide powerful tools to decipher the relationships between geologically important lipid biomarkers and their physiological and phylogenetic origins. Much like the benefits that were gained from the advent of routine continuous-flow stable isotope measurements of carbon and hydrogen (Hayes et al., 1990; Sessions et al., 1999), analysis of biochemical pathways provides another lens, or dimension, through which we can view the biomarker record. Future decades will see the organic geochemistry community broaden its perspective through the expanded use of lipidomics.

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