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Hopanoid lipids: from membranes to plant–bacteria interactions

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Abstract

Lipid research represents a frontier for microbiology, as showcased by hopanoid lipids. Hopanoids, which resemble sterols and are found in the membranes of diverse bacteria, have left an extensive molecular fossil record. They were first discovered by petroleum geologists. Today, hopanoid-producing bacteria remain abundant in various ecosystems, such as the rhizosphere. Recently, great progress has been made in our understanding of hopanoid biosynthesis, facilitated in part by technical advances in lipid identification and quantification. A variety of genetically tractable, hopanoid-producing bacteria have been cultured, and tools to manipulate hopanoid biosynthesis and detect hopanoids are improving. However, we still have much to learn regarding how hopanoid production is regulated, how hopanoids act biophysically and biochemically, and how their production affects bacterial interactions with other organisms, such as plants. The study of hopanoids thus offers rich opportunities for discovery.

Lipids are the forgotten molecules of the microbial sciences. Despite their central role in defining cellular membranes, lipids have been routinely understudied, largely because they cannot be measured, synthesized or modified as easily as nucleic acids and amino acids. Although recent technical advances are beginning to simplify these tasks (for example,

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LIPID MAPS¹), much of the functional importance of the rich chemical diversity of bacterial lipids remains a mystery.

Here, we introduce a particular class of membrane lipids — the hopanoids — as a model for modern lipid research. Hopanoids are found in diverse bacteria and in a handful of lichens and plants. Similar to eukaryotic sterols, hopanoids are planar, polycyclic hydrocarbons containing five rings compared with the four rings in sterols, and they have a variety of polar and nonpolar side chains². This structural analogy underlies their functional similarities. Both lipid classes modulate the fluidity and permeability of membranes, and they have other, subtler biological functions that are beginning to be elucidated.

Unusual for biological molecules, hopanoids were first discovered in ancient rocks rather than in living cells. Hopane skeletons, which are pentacyclic carbon backbones lacking complex side chains, are produced by hopanoid degradation during rock formation, and they were recognized as components of oil shales many years before their biological source was found³. These ‘fossil’ hopanoids are now known to be staggeringly ubiquitous in rocks. They are common elements of sedimentary rocks formed as early as 1.64 billion years ago⁴ and are estimated to have a greater mass than all other natural products from living organisms combined⁵.

The landmark discovery of hopanoids in the bacteria *Methylococcus capsulatus* and *Alicyclobacillus* (formerly *Bacillus*) *acidocaldarius*^{6,7} sparked a new field of hopanoid research. Since then, much work in this field has been motivated by a geobiological question: how can understanding the distribution and the biology of hopanoids in living microorganisms inform our interpretations of ancient bacteria on the basis of hopane biomarkers in the rock record? So far, a simple answer has proved elusive. As the number of sequenced bacteria and the availability of genetic approaches have expanded, it has become clear that remarkably diverse organisms produce hopanoids. It is therefore difficult to attribute hopanes in the rock record to the advent of a specific phylogenetic group, metabolic process or environmental context. Reviews of the efforts to interpret hopanoids in the rock record are available in the literature^{8,9}.

Here, we discuss the state of hopanoid research from a biological and biochemical perspective. First, we summarize what is known about the structural diversity and biosynthesis of hopanoids. Work on these topics has led to the characterization of the genes that are required to make hopanoids. Identifying these genes is a necessary first step for understanding the distribution of hopanoid production among bacteria and has provided a handle for genetic manipulation of hopanoid biosynthesis. In the second section, we discuss the functional consequences of disrupting hopanoid biosynthesis from the membrane scale to the population level and for bacteria in host-associated environments. In the latter context, we focus on how hopanoids may facilitate beneficial plant–bacteria interactions, an agronomically and ecologically important issue.

Hopanoid structure and biosynthesis

The terpenoids, also known as isoprenoids, are formed by condensation of two or more isoprene units. They are one of the world's largest groups of natural products¹⁰ and occur in all domains of life. In some organisms, terpenoids regulate essential metabolic processes, including growth, photosynthesis and respiration. More commonly, terpenoids form secondary metabolites that mediate the interactions between organisms and their environments.

Hopanoids belong to the cyclic triterpenoid subclass, a structurally diverse group of molecules synthesized by the cyclization of the branched terpenoid hydrocarbon squalene and its oxygenated derivative 2,3-oxidosqualene¹¹ (FIG. 1). These chemically complex reactions are catalysed by the triterpenoid cyclase superfamily of enzymes. Over a dozen triterpenoid cyclase families exist, which produce various classes of monocyclic and polycyclic triterpenoids^{11,12}.

The largest group of triterpenoid cyclases contains the epoxysqualene or oxidosqualene cyclases (OSCs). This family of triterpenoid cyclases is highly specific for the oxygenated squalene intermediate 2,3-oxidosqualene¹³, and as a result, its products bear signature oxygen groups on ring A (FIG. 1). Collectively, the OSCs can produce a broad spectrum of chemically distinct cyclic triterpenoids, and they are usually divided into subfamilies based on their primary end products. Virtually all OSC subfamilies are unique to eukaryotes, with the exception of the sterol synthases, which have been identified and characterized in a handful of aerobic methanotrophs and myxobacteria. Recent analyses of environmental samples suggest that sterol production in bacteria is more widespread than previously thought¹⁴.

Another major family of triterpenoid cyclases contains the squalene-hopene cyclases (SHCs). These enzymes can synthesize cyclic triterpenoids directly from squalene in an oxygen-independent process; they can also cyclize 2,3-oxidosqualene *in vitro*¹⁵, although the organisms containing SHCs generally do not produce this substrate. Similarities in the sequences and structures of the active sites of OSCs and SHCs have suggested that these enzyme families are evolutionarily related^{16–18}. Although the higher substrate promiscuity of SHCs than of OSCs has been used to suggest that OSCs evolved from SHCs¹⁹, some evolutionary models of SHC and OSC relatedness have favoured the idea that SHCs and OSCs diverged from a common ancestor²⁰.

Whereas SHCs can accommodate more diverse substrates than OSCs, their known products are more limited. SHCs primarily synthesize the hopanoids diploptene and diplopterol, which are also known as C₃₀ hopanoids (FIG. 1). In a few bacterial genera, SHC enzymes can also produce tetrahymanol through an additional tetrahymanol synthase enzyme. This pathway of tetrahymanol synthesis is unique to bacteria and predominantly occurs in the Bradyrhizobiaceae family of nitrogen-fixing legume symbionts²¹.

Roughly 10% of bacteria can produce hopanoids (that is, they encode *shc*)^{22–24}. The group with the highest conservation of *shc* genes is the Proteobacteria, particularly the alphaproteobacterial Bradyrhizobiaceae family and the betaproteobacteria genus

Burkholderia, which includes both plant-associated members and human pathogens; *shc* genes are also well conserved among the Firmicutes, especially the plant-associated and human-associated Bacilli as well as the Frankiaceae family of plant-symbiont Actinobacteria. Other groups in which *shc* genes are common include the Cyanobacteria, Acidobacteria and Planctomycetes.

Although hopanoids are often assumed to be bacteria-specific, many molecules sharing the pentacyclic ring structure and core methylation sites of hopanoids have been isolated from various tropical trees, grasses, lichens and ferns^{25,26}. Some of these structures are identical to bacterial hopanoids. For example, several members of the Polypodiales (polypod) order of ferns encode an SHC enzyme²⁴ that seems to exclusively produce the C₃₀ hopanoid diplopterol in vitro²⁷. It is likely that these ferns acquired *shc* by horizontal transfer from bacteria²⁸. Other hopanoid-like molecules in plants contain additional accessory groups not observed in bacterial hopanoids (FIG. 1). The most common of these accessory groups is a C3 oxygen group, indicating that these molecules are 2,3-oxidosqualene derivatives^{26,29}. Given the paucity of *shc* genes in plants, we speculate that plants encode an as-yet-uncharacterized OSC subfamily member that produces hopanoid-like lipids as a primary product.

Side chain formation.

In most organisms, the *shc* gene is part of a conserved *hpn* (hopanoid biosynthesis) operon that encodes enzymes for upstream terpenoid biosynthesis and downstream hopanoid structural modifications³⁰. The most common hopanoid modification is the addition of hydrocarbon side chains by the enzyme hopanoid biosynthesis-associated radical SAM protein HpnH, which is found in almost all hopanoid-producing bacteria³¹. Hopanoids made by HpnH are known as the extended or C₃₅ hopanoids, and they are formed by the addition of a C₅ ribose moiety to C₃₀ hopanoids^{32,33} (FIG. 1). These groups can be processed to produce diverse side groups by other *hpn* gene products, although the fitness advantages conferred by specific side chains are generally unknown^{31,34–36} (FIG. 1).

Core modification.

Both C₃₀ and extended hopanoids can be methylated at either the C2 position by the enzyme hopanoid 2-methyltransferase HpnP³⁷, forming the 2-methyl-hopanoids (2Me-hopanoids), or at the C3 position by the enzyme hopanoid C3 methylase (HpnR)³⁸, forming the 3-methyl-hopanoids (3Me-hopanoids) (FIG. 1). These methylation sites are more geostable than hopanoid side chains, and they have therefore occupied a central position in hopanoid geobiology. Genes encoding HpnP are largely restricted to the Cyanobacteria, Proteobacteria and Acidobacteria³⁹, whereas HpnR is even less common and is present in only a handful of species among the Proteobacteria, Nitrospirae and Actinobacteria³⁸. Given that 2Me-hopanoids and 3Me-hopanoids have non-overlapping distributions, they may be functionally redundant.

In a few organisms, desaturation of one or more bonds in the pentacyclic hopanoid core also occurs, although the enzymes responsible are not known^{40–44}. In general, little attention has been paid to hopanoid catabolism

Biological functions of hopanoids

Unlike proteins and nucleic acids that can be easily probed with fluorescent dyes, antibodies or hybridization, detection of lipids in live cells can be technically complex. Most lipids can be extracted from cells using organic solvents and partially purified with chromatography⁴⁵, and protocols for hopanoid purification are well established. Great progress has also been made in identifying specific hopanoid structures by high-temperature gas chromatography⁴⁶ or reverse-phase liquid chromatography⁴⁷ coupled to mass spectrometry. Although it remains challenging to precisely quantify the abundance of hopanoids, synthetic isotope-labelled standards for some hopanoids have been developed⁴⁸. Based on the results of these approaches, hopanoids seem to constitute less than 1% up to 90% of bacterial membranes, depending on the species and growth condition, and they are present in peripheral, intracellular and extracellular membranes, such as the outer membranes of cyanobacterial akinetes, cyanobacterial thylakoid membranes and the nitrogen-fixing vesicles of *Frankia* spp.^{49–53}. In Gram-negative bacteria, hopanoids are enriched in the outer membrane by the hopanoid biosynthesis-associated RND transporter-like protein HpnN^{54–56} (FIG. 2), although other factors regulating hopanoid localization are unknown so far.

Membrane fluidity.

Owing to their planar, hydrophobic structures, eukaryotic sterols and hopanoids can intercalate into lipid bilayers and interact with other lipids to alter the biophysical properties of membranes (FIG. 2). Early experiments in synthetic membranes and liposomes demonstrated that both extended hopanoids and cholesterol can condense, thicken and decrease the permeability of membranes, although with different efficiencies^{57–64}. These data have shown that, although the membrane-modifying behaviours of sterols and hopanoids are broadly similar, they are not functionally interchangeable. Indeed, although certain hopanoids can compensate partially for the absence of cholesterol in *Mycoplasma mycoides*, the growth rate of these cells is markedly impaired, indicating that the functions of hopanoids and cholesterol are not identical⁶⁵.

Just as hopanoids differ from cholesterol, extended hopanoids with distinct side chains can also differ in their membrane-modifying capacities^{66,67}. These structural differences between hopanoids may modulate their ability to interact with other lipids in the bilayer and, perhaps, their distribution in bacterial membranes. Molecular dynamics simulations have been performed to model the orientation of the C₃₀ hopanoid diploptene and the extended hopanoid bacteriohopanetetrol (BHT) in membranes in silico⁶⁷. In these simulations, BHT adopts an upright orientation inside the lipid bilayer, where it can interact with other membrane lipids to condense the membrane (FIG. 2). By contrast, diploptene localizes between the two leaflets, partitioning to the midplane. This latter positioning is expected to decrease membrane permeability by providing an added layer of protection to the cell. In vivo analyses of how different hopanoids affect membrane fluidity partially support these molecular dynamics results. In synthetic vesicles, diplopterol, which differs from diploptene by the addition of a single hydroxyl group, had no effect on membrane fluidity, whereas BHT decreased fluidity to a similar degree as cholesterol⁵³.

The effects of hopanoid methylation on membrane fluidity are less clear and less widely studied than the effects of extended hopanoids. In synthetic membranes, 2-methyl-diplopterol outperformed cholesterol in decreasing membrane fluidity, suggesting that methylation is the most relevant modification for condensing the bilayer⁵³. Yet this finding was not supported by measurements in live cells. In the purple non-sulfur bacterium *Rhodospseudomonas palustris* TIE-1, membranes from the *hpnP* mutant, which lacks all 2Me-hopanoids, are equally as fluid as wild-type membranes⁵³. It has been shown that hopanoid mutants overproduce other membrane-condensing lipids^{68–70}, which may explain this discrepancy. Regardless, factors affecting membrane fluidity in vivo are clearly more complex than what can be captured in vitro.

Lipid rafts.

Another well-noted feature of sterols is their ability to interact with sphingolipids to promote the formation of a liquid-ordered (L_o) phase in eukaryotic membranes^{71–73} in which lipids are packed into a highly ordered organization but can still move freely throughout the bilayer. This property is important because it creates a platform for the formation of lipid rafts, which are specialized membrane microdomains that compartmentalize cellular processes by recruiting specific lipids and membrane-associated proteins. Although the existence of lipid rafts was once hotly debated, it is now accepted that lipid rafts in eukaryotes have important roles in signal transduction, endocytosis, ion homeostasis and cell growth as well as in lipid–protein and protein–protein interactions at the membrane^{73,74} (FIG. 2).

Fewer studies have been performed on the formation of lipid rafts in bacteria, and the topic remains controversial^{75–77}; however, there is evidence that hopanoids could support this process^{78–80}. In hopanoid-producing Gram-negative bacteria, hopanoids typically are abundant in the outer membrane, of which lipid A is the major component (FIG. 2). Inspired by the structural similarities between lipid A and the eukaryotic sphingolipid sphingomyelin (that is, amide-linked acyl chains and hydroxyl groups), Sáenz et al. showed that diplopterol can interact with the saturated chains of lipid A to induce the formation of a L_o phase in synthetic membranes^{78,79}, which promotes a fluid but mechanically robust membrane (FIG. 2). The ability of the hopanoids to modulate outer membrane properties through their interactions with lipid A suggests that they facilitate membrane compartmentalization into functional domains. Additionally, hopanoids appear to localize to membrane microdomains in vivo. In the marine cyanobacterium *Crocospaera watsonii*, hopanoids are present in insoluble, detergent-resistant membrane fractions, as is common for raft-forming lipids in eukaryotes⁸⁰. Microanalysis of isotope-labelled hopanoids in the cyanobacterium *Nostoc punctiforme* and in *R. palustris* TIE-1 also showed that hopanoids preferentially localize to regions of high membrane curvature, where high degrees of membrane tension may require thicker, more condensed membranes⁸¹. Although these collective data are promising, further biophysical analyses of live membranes will be needed to determine whether hopanoids indeed form lipid rafts in bacterial cells.

Stress tolerance.

Many studies in diverse bacteria show that hopanoids facilitate bacterial survival under stress (for example, at high temperatures, low pH and high osmotic pressures and during antibiotic or detergent treatments), although the specific subset of conditions for which hopanoids are beneficial varies between bacterial strains^{82–87}. Under these conditions, hopanoid content is typically increased, and it has long been proposed that hopanoids are regulated by canonical stress response pathways⁸⁸. The alphaprotobacterial general stress response pathway regulates the *hpnP* gene in *R. palustris* TIE-1, although *shc* appears to be unregulated in this organism⁸⁹. Regulation of hopanoids in other bacteria has not been studied.

The relative contributions of hopanoid structural modifications to survival under stress vary. 2Me-hopanoids seem to be generally associated with pH stress in diverse bacteria but have negligible or very subtle effects in the presence of other stressors, depending upon the bacterial strain^{37,86,89–91}. Extended hopanoids are more important in this context, as they improve growth during exposure to bile salts, high temperatures, low pH, detergents, osmotic stress and antimicrobial peptides^{34,86}. In *Bradyrhizobium diazoefficiens*, extended hopanoids are also essential for hypoxic growth in rich media⁸⁶.

The role of hopanoids in stress tolerance is particularly apparent in bacteria that form specialized, stress-associated cell types. In *N. punctiforme*, hopanoids do not affect growth of vegetative cells under stress but primarily localize to stress-associated akinete cells^{52,90}. Similarly, in *Streptomyces coelicolor*, hopanoids are not detectable in vegetative cells but are massively upregulated during sporulation⁹². Tetracyclic isoprenoids, which are called sporulenes, are synthesized during spore formation in *Bacillus subtilis* and provide resistance to oxidative stress⁹³; it is possible that hopanoids may have a similar role.

It is unknown whether the role of hopanoids in stress tolerance is simply a consequence of their impact on membrane fluidity and permeability or whether more specific stress response mechanisms are at play. In the case of hopanoid-dependent pH tolerance, it was suggested that lowered membrane permeability due to hopanoids prevents leakage of cations and protons across the membrane⁸⁷. Similarly, the high content of hopanoids in membranes of thermotolerant and thermophilic bacteria⁸⁸ likely reflects their ability to reinforce the membrane bilayer against thermal disorder or thermolysis while maintaining its fluidity. Molecular dynamics simulations show that BHT has a strongly temperature-dependent effect on lipid tails, whereas its effect on the polar heads is negligible⁹⁴.

Other evidence suggests that hopanoids can promote the stress response beyond membrane stabilization. In *Methylobacterium extorquens*, a hopanoid-deficient mutant is impaired in multidrug efflux, hinting that hopanoids may modulate the action of membrane-associated protein complexes, possibly in subcellular membrane compartments^{79,95}. Hopanoids also facilitate the formation of intracellular nutrient storage granules in *N. punctiforme*, a process that does not have an obvious, direct requirement for a more stable membrane but may result from an indirect metabolic effect⁹⁰.

Nitrogen fixation.

Many hopanoid-producing bacteria are capable of nitrogen fixation, including *Anabaena* spp., *Azotobacter* spp., *Beijerinckia* spp., *Bradyrhizobium* spp., *Burkholderia* spp., *Frankia* spp. and *Nostoc* spp.^{96–99}. It has long been suggested that hopanoids facilitate this metabolism⁹⁶, though few direct tests of this hypothesis have been performed. Almost all nitrogen-fixing hopanoid producers associate with plants; some, such as the endosymbiotic non-photosynthetic *Bradyrhizobium* spp., are wholly reliant on their hosts for cofactors that are essential for nitrogen fixation¹⁰⁰. Others, such as *Nostoc* spp., *Anabaena* spp. and *Frankia* spp., are plant symbionts but can also fix nitrogen in the free-living state. A few of these nitrogen fixers are exclusively free living, most notably *Azotobacter vinelandii*.

Nitrogen fixation typically occurs under a narrow range of environmental conditions. Oxygen irreversibly inhibits the nitrogenase enzyme, and as a result, nitrogen fixation typically occurs in a low oxygen atmosphere. Nitrogen fixation is less often observed at higher oxygen concentrations, as this requires sophisticated mechanisms for nitrogenase protection. During bacterial nitrogen-fixing symbioses with plants, the hosts can provide this protection. For example, in the root nodule symbioses that occur between rhizobia and legumes (BOX 1), plants express leghaemoglobin molecules that maintain low-nanomolar levels of free oxygen¹⁰¹.

In other cases of nitrogen fixation under high oxygen concentration, the mechanisms of nitrogenase protection are less clear. The free-living obligate aerobe *A. vinelandii* is presumed to both consume oxygen rapidly and create an extracellular alginate barrier to prevent oxygen diffusion into the cell¹⁰². Uniquely in *Frankia* spp., nitrogenase is sequestered into vesicles with a multi-layered lipid envelope that is presumed to form an oxygen diffusion barrier, as the thickness of the vesicle envelope is directly correlated to the oxygen concentration^{103,104} (FIG. 3). Up to 90% of these vesicle envelopes consist of BHT and a phenylacetyl monoester of BHT (phenylacetic acid (PAA)-BHT), at an estimated PAA-BHT:BHT ratio between 30:70 and 50:50 (REFS 51,105). Purified PAA-BHT and BHT seem to optimally stabilize membranes at those relative abundances in vitro, and so this mixture of PAA-BHT and BHT may promote vesicle stability⁶⁶. Intriguingly, PAA-BHT in *Frankia alni* can also be cleaved to release PAA, which is a plant auxin involved in development^{106,107}. It is thus possible that during the interaction of *Frankia* spp. with plants, hopanoids also have indirect roles by releasing symbiotic signals.

Plant–bacteria association.

Beyond *Frankia* spp., the correlation between hopanoid biosynthesis and plant-associated nitrogen fixation may reflect a more general role for hopanoids in promoting bacterial survival in the rhizosphere, regardless of whether cells are free-living in the soil or associated with plants. Bacteria encounter diverse stressors in these habitats, and teasing out the specific selective pressures that underpin this correlation is a priority for future research. Intriguingly, an analysis of *hpnP* and *shc* distributions in metagenomes found that hopanoid production is enriched in plant-associated environments and is correlated with markers for nitrogen fixation as well as methanol utilization, another metabolic activity common in plant-associated bacteria¹⁰⁸. Hopanoid production is also common among beneficial

members of the Bacilli class²⁴, which do not fix nitrogen but are nonetheless known for promoting plant growth¹⁰⁹.

The role of hopanoids in beneficial plant–bacteria interactions has been studied best in legume–rhizobia root nodule symbioses. Hopanoid production occurs predominantly in rhizobia that associate with tropical legumes^{110–112}, and in the association of *Bradyrhizobium* spp. with tropical *Aeschynomene* legumes, they seem to support rhizobial survival within hosts (BOX 1). In the photosynthetic *Bradyrhizobium* sp. BTAi1, a *shc* mutant that lacked hopanoids showed severe defects in outer membrane integrity and was both slower growing under optimal growth conditions and less resistant to stresses mimicking the root nodule microenvironment (such as low pH, hypoxia, high osmolarity and antimicrobial peptides) than wild-type bacteria¹¹³. In association with *Aeschynomene evenia*, this *shc* mutant induced nodules showing normal development and wild-type nitrogen fixation activity but a shorter lifespan, prematurely triggering a plant senescence programme in which the symbiotic tissue is digested by the host¹¹³. This may reflect an inability to maintain a persistent host infection, perhaps owing to a long-term survival defect.

Information on the roles of different hopanoid modifications during root nodule symbiosis has been obtained in *B. diazoefficiens* USDA 110 (formerly named *Bradyrhizobium japonicum*¹¹⁴) in association with *Aeschynomene afraspera*⁸⁶. In this symbiosis, loss of 2Me-hopanoids (*hpnP*) has no discernible impact, while loss of extended hopanoids (*hpnH*) induces nodules that fix nitrogen poorly and display morphological disorders such as disorganization of the nodule tissue and triggering of plant defence responses. Given that extended hopanoids contribute strongly to survival under stress for free-living *B. diazoefficiens*, we suggest that the *hpnH* mutant is similarly challenged inside hosts.

Interestingly, hopanoids seem to be irrelevant for the symbiosis between the nitrogen-fixing cyanobacterium *N. punctiforme* and the (non-hopanoid-producing) fern hosts *Azolla* spp.⁹⁰. In this symbiosis, bacteria associate with the plant surface only and do not experience such harsh changes in their environment; it is likely that the promotion of stress survival by hopanoids is not relevant in this context. However, it is also possible that the relevance of hopanoids in *Bradyrhizobium* spp. symbioses can be attributed to the presence of an unusual lipid A structure: hopanoid-lipid A (HoLA), in which lipid A is covalently attached to an extended hopanoid polyol^{113,115,116} (FIG. 4). Although the physiological benefit of the attachment of hopanoids to lipid A remains to be determined, the impact of HoLA on the physicochemical properties of model membranes is known. The very long chain fatty acids (VLCFAs) of HoLA span the entire bilayer, helping tightly link the two membrane leaflets and inducing compact packing of the lipid tails of the bilayer, which increases the mechanical strength of the membrane¹¹³. The covalently linked hopanoic acid is positioned within the inner leaflet, further enhancing the mechanical robustness of the outer membrane¹¹³. Yeasts and archaea likely adopt similar strategies to regulate their membrane properties, as they also have very long lipid chains^{117,118}. Archaeal membrane lipids consist of saturated isoprenoid chains between 20 and 40 carbons long, which span the bilayer and contribute to the thermal stability of the membrane¹¹⁸. In yeast, sphingolipids containing hydroxylated VLCFAs are interdigitated into the opposing leaflet of the bilayer and interact

with ergosterol¹¹⁷. Despite these intriguing in vitro findings, identifying the enzyme that joins hopanoids to lipid A will be essential for assessing HoLA functions and distribution within bacteria and for assessing whether this molecule is sufficient to explain the phenotype of hopanoid iosynthesis mutants during symbiosis with legumes.

Outlook

Hopanoid biology represents a frontier for the microbial sciences. Although hopanoid biosynthetic pathways are known, much remains to be discovered about their biochemistry, biophysics and how they impact bacterial fitness in the context of plant and animal hosts. We have paid particular attention to the relationship between hopanoids and plants in this Review, not only because hopanoid-producing bacteria are abundant in the rhizosphere but also because hopanoids are critical for the development of certain plant–microorganism symbioses. Given the need to better understand what makes these symbioses robust in light of a warming climate, it is important to identify the mechanisms whereby hopanoids mediate these relationships.

The chemical similarity between hopanoids made by bacteria and pentacyclic triterpenoids made by lichens and plants is striking (FIG. 1). It is worth exploring whether this similarity is coincidental or, perhaps more plausibly, reflects an underlying functional connection. Our expanding ability to analyse the chemistry of hopanoids has revealed a plethora of structures and triggered numerous questions. What is the functional importance of this chemical diversity? Do certain hopanoids interact with membrane proteins, enabling their proper localization and/or function? How do they enable bacterial survival within the nodule microenvironment? Can hopanoids mediate the exchange of signals or nutrients between bacteria and plants? Could it be that bacterial hopanoids and plant cyclic triterpenoids engage in crosstalk, during which one can substitute the other? Might the diversity of side-chain modifications regulate the degree to which functional exchange could occur? The fact that hopanoids themselves might serve as carriers for auxin-like compounds that affect plant development is fascinating and merits further study.

In closing, we remind the reader of the words of John F. Kennedy when explaining what was needed to send astronauts to the Moon. “We choose to go to the Moon! ... We choose to go to the Moon in this decade and do the other things, not because they are easy, but because they are hard; because that goal will serve to organize and measure the best of our energies and skills...”. Replacing the words ‘go to the Moon’ with ‘study lipids’ provides fitting inspiration for tackling one of the next great challenges in microbiology. It is time for lipids to get the attention they deserve, and the study of hopanoids is an excellent place to begin.

Note added in proof

While the proofs of this Review were being finalized, a new study¹⁴⁰ was published that identified the dominant bacterial taxa in global soils, including organisms from the *Bradyrhizobium* genus, further motivating efforts to understand whether hopanoid production by this genus contributes to its ecological success.

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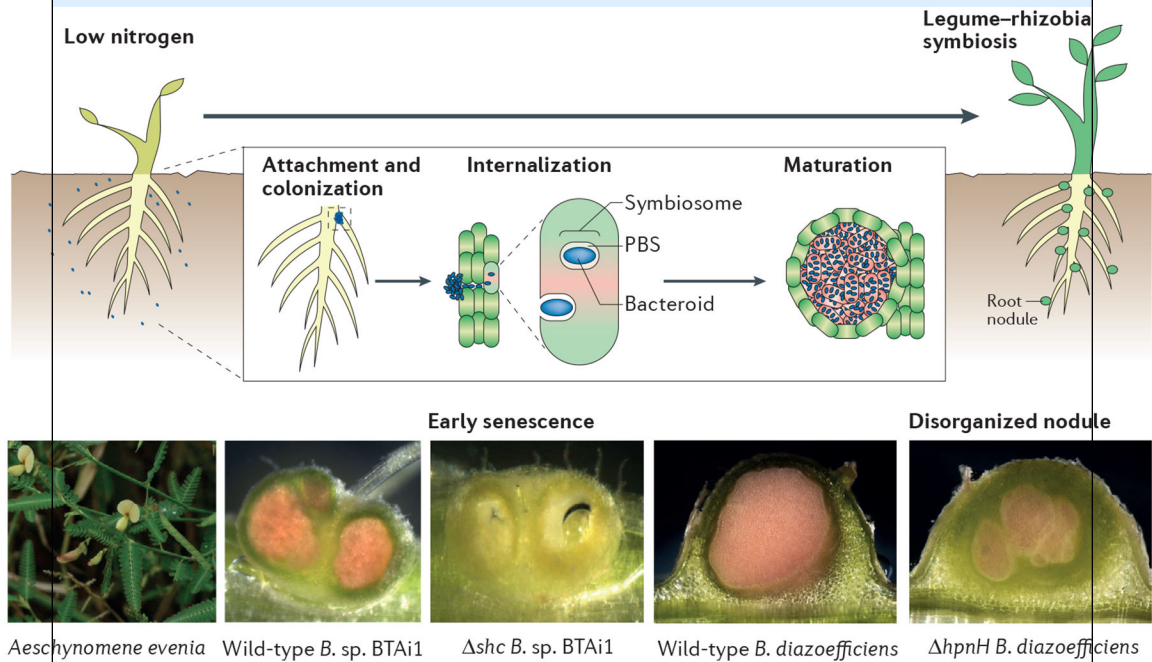
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Box 1 |**The role of hopanoids in the development of legume–rhizobia root nodule symbioses**

Nitrogen-fixing root nodule symbioses (see the figure), which catalyse the conversion of dinitrogen gas to ammonium, occur between rhizobia and most legumes as well as in a small subset of trees, shrubs and herbaceous plants. These symbioses are initiated under conditions where soil nitrogen levels are too low to support robust plant growth, resulting in the release of pro-symbiotic signals from both plants and rhizobia¹¹⁹. These signals suppress the plant immune response to allow rhizobial colonization of the root surface and, subsequently, invasion of the root¹²⁰. The simplest pathways for rhizobial invasion are through root cracks. This process is associated with reactive oxygen species that challenge rhizobial growth but are necessary to sustain the development of symbiosis¹²¹. Once the bacterial infection progresses to the root interior, host cells internalize the rhizobia through an endocytosis-like process. This creates a new organelle known as a symbiosome containing a rhizobial cell, now known as a bacteroid, surrounded by a plant-derived membrane. The region between the bacteroid and symbiosome membranes is known as the peribacteroid space (PBS). The environment of the PBS is harsh and drastically different from that of free-living rhizobia. Some of this harshness is necessary for efficient nitrogen fixation, including the creation of a low pH environment to provide protons for nitrogenase¹²² and concentrated carbon osmolytes for increased bacteroid metabolism¹²³. Other stresses result from host control mechanisms, such as antimicrobial nodule-specific cysteine-rich peptides that change the morphology, proliferation and gene expression of bacteroids to maximize their productivity^{124–126}. Bacteroid-containing host cells undergo many rounds of proliferation after the initial internalization event to form the mature root nodule. Root nodule formation occurs simultaneously across the entire root system until the supply of nitrogen to the host is sufficient to support plant growth. In some plant hosts, only a single rhizobial cell is internalized per nodule, resulting in a clonal population of bacteroids, whereas in other hosts, multiple strains of bacteria may coexist in the same nodule. It is unknown whether hopanoids can facilitate competitiveness of rhizobia under mixed-species infections; answering this question is an important next step in determining the relevance of hopanoids for real-world agricultural conditions. Hopanoid production is most conserved in root nodule symbionts from the *Bradyrhizobium* and *Burkholderia* genera, which are known to inhabit high-temperature, low-pH soils^{127,128}, suggesting that hopanoids facilitate adaptation to these conditions. In the context of global warming¹²⁹, in which temperature and soil acidity are predicted to increase globally, hopanoid-producing rhizobia may become more dominant. In the symbioses between *Bradyrhizobium* spp. and legumes, hopanoids seem to be important for maintaining a chronic infection within this stressful environment. In the symbiosis between the tropical legume *Aeschynomene evenia* and its native symbiont *Bradyrhizobium* sp. BTAi1, infection of hosts with a hopanoid-free *shc* mutant elicits nodules that initially develop normally but trigger an early onset of nodule senescence (see the figure), a process in which nodule tissue is degraded and recycled by the host. This senescence presents as air-filled cavities in the central nodule tissue and a decrease in nitrogen fixation, which is reflected by a lighter pink colour that is indicative of low

leghaemoglobin levels¹¹³. In association with *Aeschynomene afraspera*, loss of extended hopanoids (*hpnH*) results in a slight reduction in nitrogen fixation levels and a disorganization of the central nodule tissue⁸⁶ (see the figure).



Early senescence images are reproduced from REF. 113, Macmillan Publishers Limited.

Hopanoids

Pentacyclic lipids with C₆ A-D rings and a C₅ E ring, among which six core methyl groups are distributed. Hopanoids are also characterized by the formation of accessory groups at their C2, C3 and C30 positions.

Sterols

Tetracyclic lipids with C₆ A-C rings and a C₅ D ring. The parent compounds for all sterols contain oxygen groups at the C3 position.

Terpenoids

Molecules assembled from two or more C_5 isoprene units that share the core formula $(C_5H_8)_n$. They are also known as isoprenoids.

Triterpenoid

Molecules derived from assemblies of six isoprene units with the core formula $(C_5H_8)_6$ or $C_{30}H_{48}$. They may be acyclic or cyclic.

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Squalene

An acyclic triterpene with an irregular (tail-to-tail) linkage between two 3-isoprene units; a parent molecule of cyclic triterpenoids.

Triterpenoid cyclase

A superfamily of enzymes that convert acyclic triterpenoids, including squalene and 2,3-oxidosqualene, into various cyclic products.

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Oxidosqualene cyclases

(OSCs). A family of predominantly eukaryotic 3- β -hydroxytriterpene cyclases that transform 2,3-oxidosqualene into sterols and diverse other cyclic triterpenoids.

Squalene-hopene cyclases

A family of predominantly bacterial 3-deoxytriterpene cyclases that cyclize squalene to primarily form hopanoids.

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C₃₀ hopanoids

Short hopanoids containing no additional carbon atoms that are not derived from squalene.

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Tetrahymanol

A hopanoid-like compound with a C₆ E-ring. In bacteria, it is made from E-ring expansion of the C₃₀ hopanoid diploptene.

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Legume

Legumes are flowering plants of the Fabaceae (previously known as Leguminosae) family, which includes economically important crops such as soybeans, common beans and peanuts.

C₃₅ hopanoids

Extended hopanoids that contain ribose-derived hydrocarbon side chains at their C30 position.

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2-Methyl-hopanoids

(2Me-hopanoids). Hopanoids containing an accessory methyl group at the C2 position.

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3-Methyl-hopanoids

(3Me-hopanoids). Hopanoids containing an accessory methyl group at the C3 position.

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Liposomes

Spherical vesicles surrounded by one or more lipid bilayers that can be produced from cellular membranes in vivo or synthetically by sonication or extrusion of lipids into aqueous solution.

Bacteriohopanetetrol

(BHT). A common C₃₅ hopanoid containing a tetra-hydroxylated C₅ side chain.

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Membrane fluidity

The rotational and diffusional freedom of movement of molecules within a membrane.

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Lipid rafts

Membrane microdomains with high stability that are thought to recruit specific membrane-associated proteins to spatially regulate their functions.

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Lipopolysaccharides

(LPSs). Complex, heat-stable amphiphilic lipids that are the main component of the external leaflet of the outer membrane of Gram-negative bacteria.

Lipid A

The lipophilic moiety of lipopolysaccharides.

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Hypoxic

An environment with a low concentration of oxygen (usually less than 30% saturation).

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Nitrogen fixation

The conversion of dinitrogen gas into fixed or bioavailable nitrogen sources such as ammonia.

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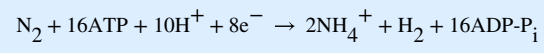
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Nitrogenase

A bacterial enzyme complex that performs the following reaction:



Root nodule

A specialized root organ that is generated by most legume plants to house nitrogen-fixing symbionts and create a specialized microenvironment to support bacterial nitrogen fixation.

Rhizobia

A paraphyletic group of nitrogen-fixing soil bacteria that can engage in symbioses with legumes.

Leghaemoglobin

An oxygen-carrying haem protein expressed in the root nodules of rhizobial host plants.

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Aeschynomene

A genus of tropical legumes that is broadly distributed globally and is used for livestock grazing.

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Hopanoid-lipid A

(HoLa). An extended hopanoid that is covalently attached to lipid A and appears to be unique to the Bradyrhizobiaceae.

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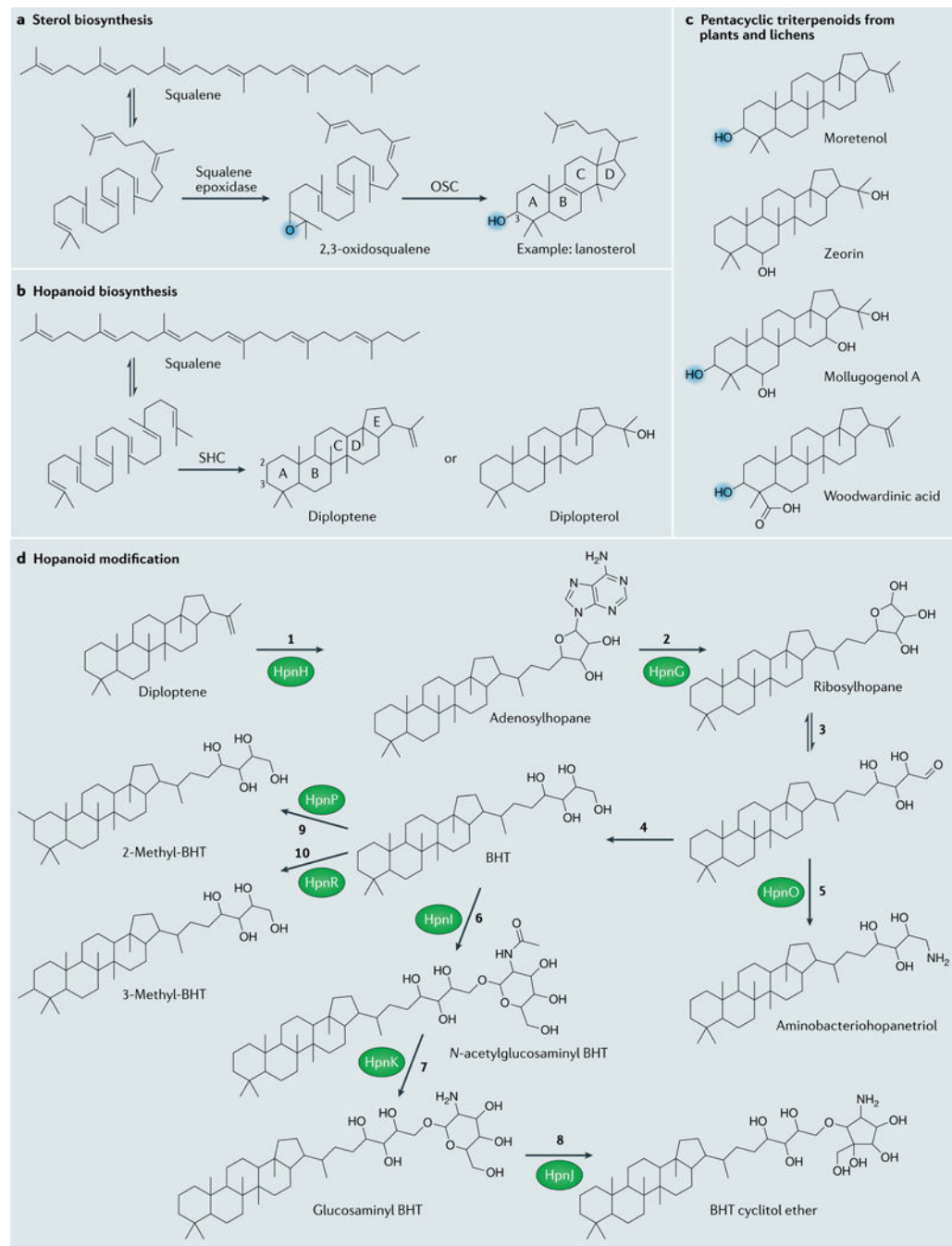


Figure 1 | Pathways of sterol and hopanoid biosynthesis.

Sterols and hopanoids are synthesized from squalene through oxygen-dependent and oxygen-independent mechanisms, respectively¹³⁰. **a** | To produce the cholesterol precursor lanosterol, squalene is oxygenated by the squalene epoxidase enzyme to form 2,3-oxidosqualene, which is then cyclized by the oxidosqualene cyclase (OSC) family member lanosterol synthase. **b** | During hopanoid biosynthesis, squalene-hopene cyclase (SHC) enzymes directly cyclize squalene to form one of two C₃₀ hopanoids — diploptene or diplopterol. **c** | Interestingly, many hopanoid-like compounds made by plants seem to

originate from a hybrid cyclization, in which 2,3-oxidosqualene cyclization results in a molecule that contains a hopanoid core with a C3 oxygen signature. **d** | Bacterial C₃₀ hopanoids, which are produced directly by SHC (as in part b), also can be modified to form C₃₅ hopanoids. The first steps of C₃₅ hopanoid formation are the addition of adenosine by hopanoid biosynthesis-associated radical SAM protein HpnH (step 1) and the removal of adenine by putative hopanoid-associated phosphorylase HpnG (step 2), which generates a ribosylhopanoid with a ribose side chain. This ribose moiety can be readily interconverted between the cyclic and acyclic forms (step 3), from which the signature-extended hopanoid bacteriohopanetetrol (BHT) can be produced by an unknown enzyme (step 4). The side chains of the extended hopanoids can be further altered by the *hpn* gene products hopanoid biosynthesis-associated glycosyltransferase protein HpnI, hopanoid biosynthesis-associated protein HpnK, hopanoid biosynthesis-associated radical SAM protein HpnJ and aminotransferase HpnO to generate *N*-acetylglucosaminy BHT, glucosaminy BHT, BHT cyclitol ether and aminobacteriohopanetriol (steps 5,6,7 and 8). Hopanoids can also be methylated at the C2 or C3 positions by hopanoid 2-methyltransferase HpnP and hopanoid C3 methylase HpnR, respectively (steps 9 and 10); although only BHT methylation is shown, it can occur for any C₃₅ or C₃₀ hopanoid. For details of triterpenoid stereochemistry, we refer the reader to REF. 131. Part d is adapted from REF. 132, CC BY.

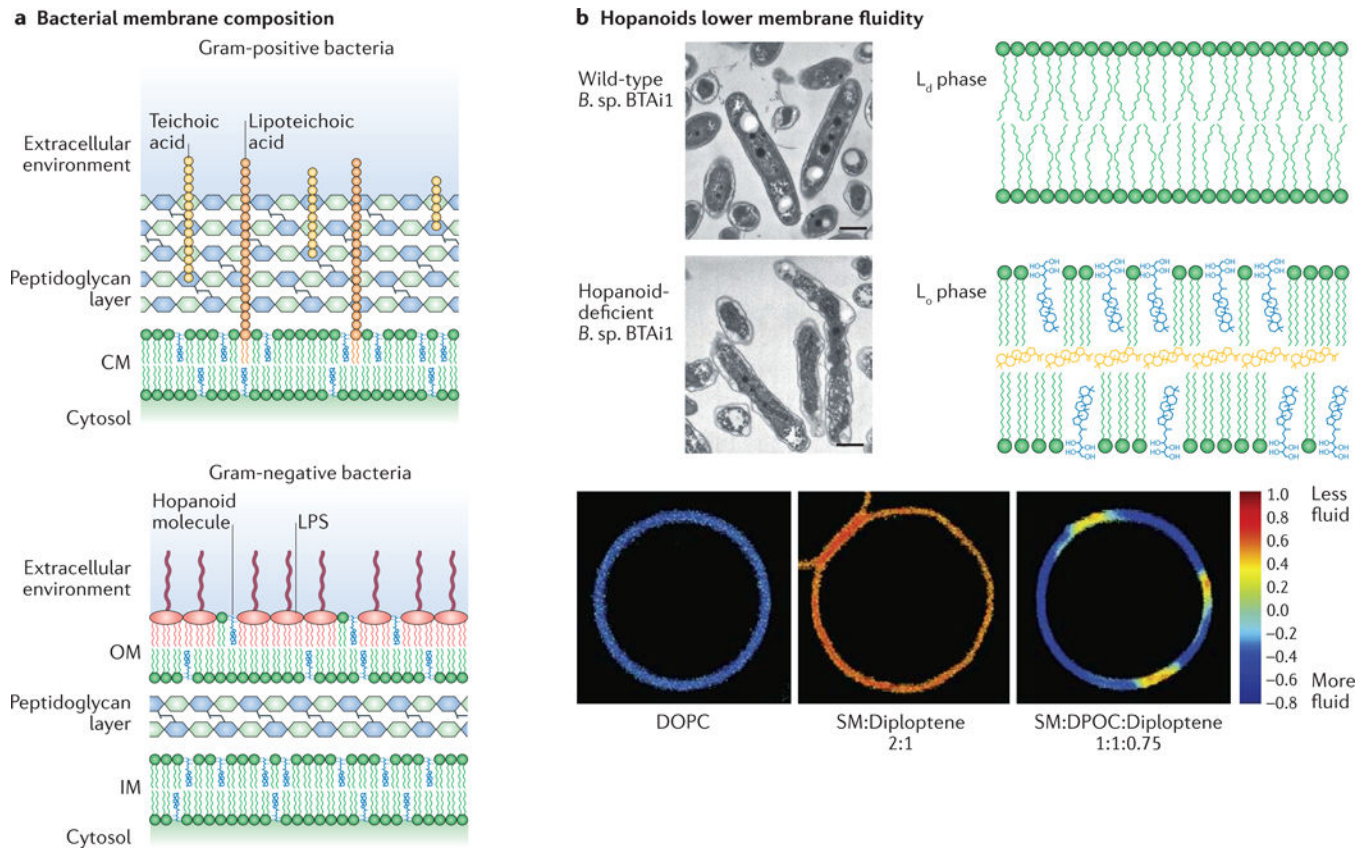


Figure 2 | Regulation of lipid raft formation by hopanoids.

a | The main constituents of bacterial membranes are amphiphilic lipids, which contain polar headgroups and hydrophobic tails that preferentially self-associate to form lipid bilayers. All bacteria have a cytoplasmic membrane (CM) that delimits the cytoplasm and is enclosed by a protective and rigid layer of peptidoglycan. In Gram-positive bacteria, a thick layer of peptidoglycan encloses the CM and forms the basis of the cell surface. In Gram-negative bacteria, the CM is also referred to as the inner membrane (IM) and the peptidoglycan is surrounded further by an additional asymmetric bilayer, the outer membrane (OM). The inner leaflet of the OM comprises glycerophospholipids, and the external leaflet mainly comprises lipopolysaccharides (LPSs), which can cover up to 75% of the cell exterior^{133,134}. LPS structures are highly strain-specific, but they can be roughly divided into three domains: a core oligosaccharide and a O-specific polysaccharide (or O-chain) that together form a hydrophilic heteropolysaccharide, and a lipophilic, membrane-anchoring domain, termed lipid A¹³⁵. The hopanoids shown in the figure are present in some, but not all, Gram-negative and Gram-positive bacteria. **b** | The distributions of specific lipid classes between, and within, the leaflets of the IM and OM determine their physical and biochemical properties. In addition to containing distinct functional groups, lipids differ in the lengths and degrees of saturation of their hydrophobic tails. These properties tune the effective molecular geometries of lipids and in turn determine their packing within the bilayer. A high proportion of unsaturated and variable-length lipids, which pack poorly in the membrane, is associated with a more fluid, liquid-disordered (L_d) phase. When more planar saturated lipids interact with cholesterol (or some hopanoids), they can self-associate within

membranes to form a less fluid, more tightly and regularly packed liquid-ordered (L_o) phase. The interactions leading to the formation of an L_o phase in model membranes are thought to provide the basis for the assembly of raft domains in eukaryotic membranes. The L_o phase is also associated with longer lipid tails, as the increased surface of hydrophobic interactions increases their stability. In Gram-negative bacteria, hopanoids are enriched in the OM where they preferentially interact with lipid A^{79,113}, and in some organisms, such as *Bradyrhizobium* sp. BTAi1, they greatly enhance the mechanical integrity of the membrane, as evidenced in transmission electron micrographs. Because hopanoids have a rigid, planar ring structure, it is presumed that they generally are found in L_o microdomains, perhaps regulating the relative position of LPSs, thereby facilitating LPS recognition during host–bacteria interactions^{136,137}. These membrane microdomains could also affect the localization and dynamics of proteins, restricting their roles in membrane trafficking, signalling and metabolism to functional sub-compartments. The ability of hopanoids to promote lipid rafts has been demonstrated in model membrane vesicles containing eukaryotic phosphatidylcholine (DOPC) and sphingomyelin (SM), in which diploptene addition results in the formation of low-fluidity microdomains. Part b is reproduced from REF. 113, Macmillan Publishers Limited, and with permission from REF. 78, Proceedings of the National Academy of Sciences.

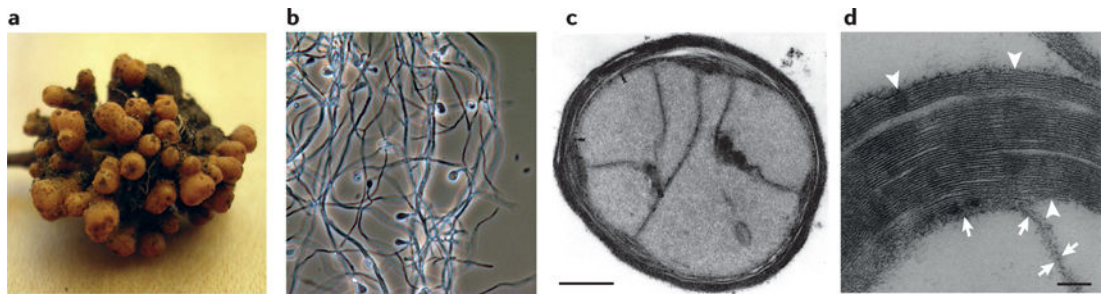


Figure 3 | Hopanoid-rich vesicles in *Frankia* spp.

a | Root nodules induced by *Frankia* spp. on *Alnus incana* subsp. *rugosa*. Sample is ~1.5 cm in diameter. **b** | Filamentous structure of *Frankia* symbionts containing spherical nitrogen-fixing vesicles. **c** | Detail of cultured *Frankia* vesicles containing nitrogenase (scale bar = 500 nm). **d** | Multilamellate, hopanoid-rich lipid envelope (arrowheads) of the *Frankia* nitrogenase vesicles (scale bar = 50 nm) (arrows show the bacterial cell wall). Parts a and b are courtesy of David R. Benson, University of Connecticut, USA, and part c is amended from *Journal of Bacteriology*, 173, 2061–2067 doi: 10.1128/jb.173.6.2061–2067.1991 (1991) with permission from American Society for Microbiology (REF. 138). Part d is reproduced with permission from REF. 139, Proceedings of the National Academy of Sciences.

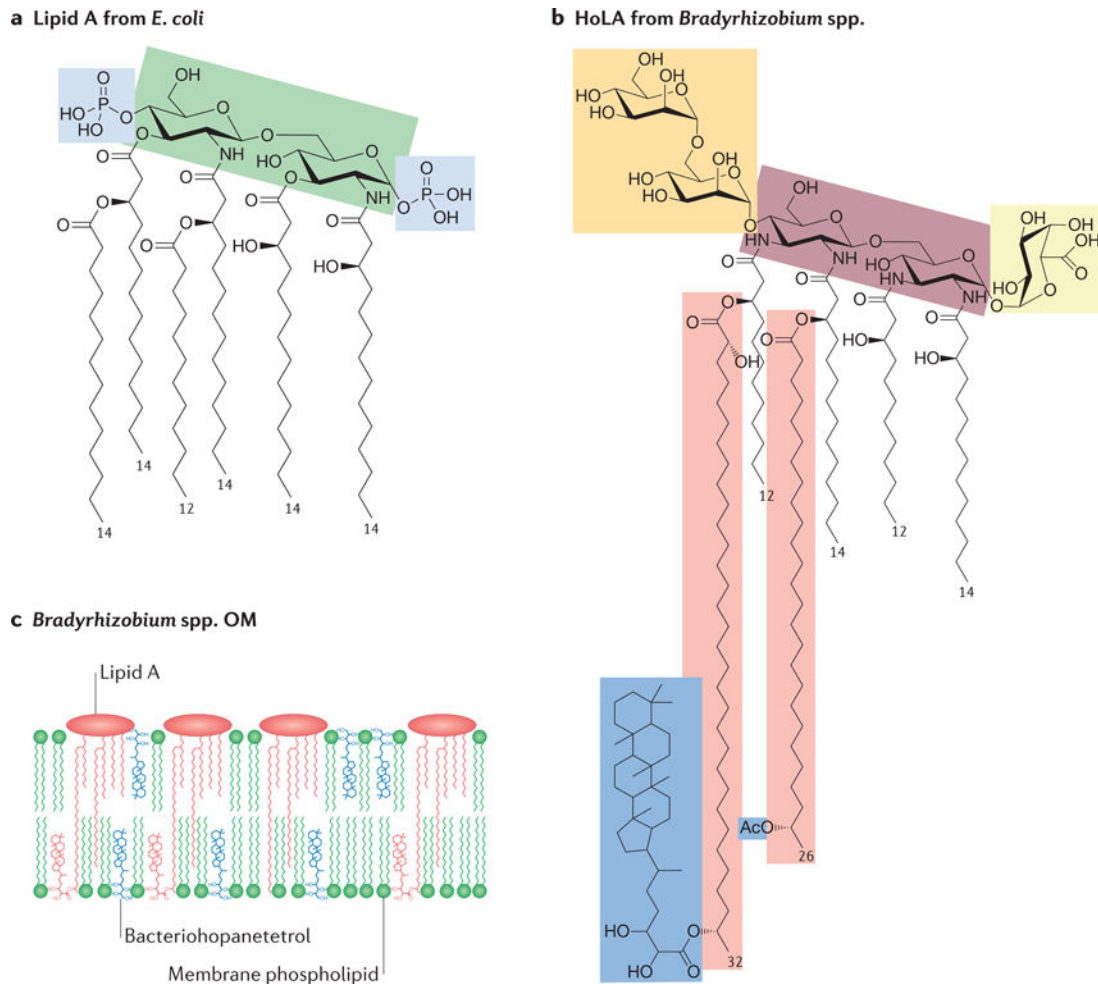


Figure 4 | Chemical structure of hopanoid-lipid A from *Bradyrhizobium* spp.

a | Hexa-acylated lipid A from *Escherichia coli* contains a bis-phosphorylated (light blue) glucosamine disaccharide backbone (green), which is asymmetrically substituted by six acyl chains. **b** | *Bradyrhizobium* spp. hopanoid-lipid A (HoLA) molecules are a mixture of penta- to hepta-acylated species, which differ in the number, length and nature of acyl substitutes on the saccharide backbone and are devoid of phosphate groups. *Bradyrhizobium* spp. lipid A has a 2,3-diaminoglucose (DAG) disaccharide backbone (violet), a galacturonic acid (GalA) residue (yellow) on the vicinal DAG and an α -(1, 6)-mannose disaccharide (orange) on the distal DAG. The acyl chains are asymmetrically distributed on the sugar skeleton; the two secondary very long chain fatty acids (VLCFAs; red), one of which is not stoichiometrically hydroxylated at C2, are substituted at the ω -1 position by a hopanepolyol acid and by an acetyl (Ac) group (blue), respectively. The primary acyl chains on the *E. coli* and *Bradyrhizobium* spp. lipid A backbone have the R configuration at the chiral centre, whereas the VLCFAs have the S configuration at the ω -1 position. The non-stoichiometric 2-hydroxylation of the fatty acids occurs with the S configuration. **c** | A conceptual scheme illustrating the outer membrane (OM) of *Bradyrhizobium* spp. is shown. Lipid A (red) insertion decreases the fluidity and increases the mechanical strength of the membrane; VLCFAs entirely span the lipid bilayer so that the covalently linked hopanoid (red) is

positioned in the inner leaflet; collectively, this would be expected to order both leaflets, with VLCFAs contributing to tightly link the two. Regular phospholipids are shown in green and free hopanoid molecules are shown in blue.

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