Ornithine Lipids and Other Amino Acid-Containing Acyloxyacyl Lipids

6

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Contents

Abstract

Ornithine lipids (OLs) are phosphorus-free membrane lipids relatively common in eubacteria, but apparently absent from archaea and eukaryotes. It has been predicted that about 50% of the sequenced bacterial species have the capacity to synthesize OLs at least under certain growth conditions. Structurally, they are composed of a 3-hydroxy fatty acid amide bound to the α-amino group of ornithine and of a second fatty acyl group ester linked to the 3-hydroxy position of the first fatty acid forming an acyloxyacyl structure. This basic structure of OLs can be modified by hydroxylations in different positions, by *N*-methylation, or by taurine transfer. The presence of OL and/or modified OLs often seems to form part of a stress response to (changing) environmental conditions. OL modification allows the bacteria to adjust membrane properties by converting already existing membrane lipids into membrane lipids with distinct properties without de novo synthesis. In addition to ornithine, other amino acids (and dipeptides) such as

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glycine, serineglycine, glutamine, and lysine have been described as headgroups of these lipids in some bacterial species.

1 Introduction

A large variety of membrane lipid structures have been shown to exist in bacteria. Usually, glycerophospholipids such as phosphatidylethanolamine (PE), phosphatidylglycerol, phosphatidylcholine, or cardiolipin are the primary components of membranes, but other amphiphilic lipids are also present in the lipid bilayer. One way to classify these alternative membrane lipids is by their structure: Some, such as diacylglycerol-based glycolipids, the sulfolipid sulfoquinovosyl diacylglycerol, or the betaine lipid diacylglyceryl-*N*,*N*,*N*-trimethylhomoserine (DGTS) share a diacylglycerol (DAG) backbone (Hölzl and Dörmann 2007; Geiger et al. 2010), but others such as hopanoids, ornithine lipids, or sphingolipids lack this DAG backbone (Geiger et al. 2010; Sohlenkamp and Geiger 2016). In general, the synthesis pathways of glycerophospholipids are relatively well explored, whereas often, neither the biosynthesis nor the function of the alternative membrane lipids is understood.

One important class of these alternative membrane lipids is formed by amino lipids containing an acyloxyacyl structure: α-amino acids are *N*-acylated with a primary 3-hydroxy fatty acyl residue and a secondary fatty acid, also called "piggy-back" fatty acid, is ester-bound to the hydroxyl group of the first fatty acid. The most common amino acid present in this type of lipid is ornithine (Geiger et al. 2010; Sohlenkamp and Geiger 2016) (Fig. 1). Other headgroups that have been identified in this type of lipids are lysine, glycine, glutamine, serineglycine, and taurineornithine (Moore et al. 2016). Up to now, this type of lipids has been found only in eubacteria, but not in archaea or eukaryotes. In the last 15 years, a lot has been learned about the synthesis and modification of different OLs, but we know much less about the other amino lipids. In this chapter, I will focus on the synthesis of OL and other amino acyl lipids presenting an acyloxyacyl structure and discuss what is known about their formation and possible functions.

2 Formation of Amino Acid-Containing Acyloxyacyl Lipids

2.1 Synthesis of Ornithine Lipid

Although the capacity to synthesize ornithine lipids (OLs) is widespread among eubacteria, a pathway for OL formation has been described only relatively recently by Geiger and coworkers using the nodule-forming α-proteobacterium *Sinorhizobium meliloti* as a model. A population of chemical mutants was screened under conditions of phosphate limitation to identify mutants deficient in the

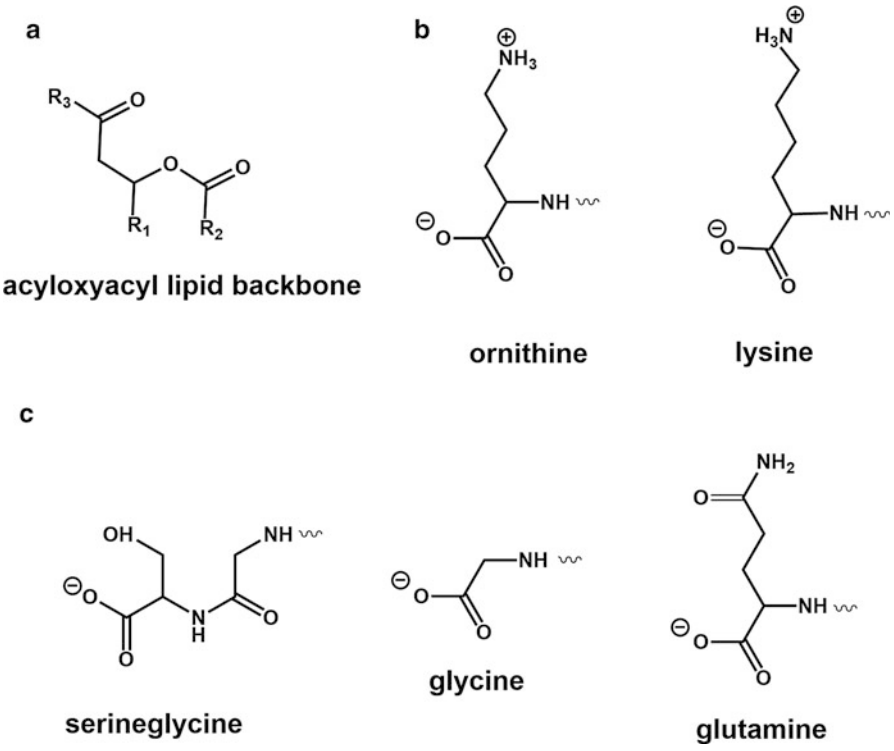

Fig. 1 Structures and headgroup variation of amino acid-containing acyloxylacyl lipids. Depending on which amino acid is present in the headgroup, the resulting lipid can be zwitterionic (ornithine, lysine) or anionic (glycine, serineglycine, glutamine). (**a**) acyloxyacyl backbone; (**b**) headgroups of zwitterionic lipids; (**c**) headgroups of anionic lipids. R_1 alkyl chain of the amide-bound 3-hydroxyfatty acid; R_2 alkyl chain of the ester-bound fatty acid; R_3 headgroups (shown in **a** and **b**)

synthesis of OL (Weissenmayer et al. 2002). Two genes encoding acyltransferases were found to be necessary to form OL in *S. meliloti*. The first step in OL synthesis is catalyzed by the *N*-acyltransferase OlsB which is responsible for the formation of lyso-OL (LOL) from ornithine and 3-hydroxyacyl-AcpP (Gao et al. 2004) (Fig. 2, reaction 1). The second step in OL synthesis is catalyzed by the *O*-acyltransferase OlsA, which is responsible for the formation of OL from LOL and acyl-AcpP (Weissenmayer et al. 2002) (Fig. 2, reaction 2). The OlsBA pathway is present mainly in α-, β-, and a few γ-proteobacteria and in some Gram-positive bacteria such as *Mycobacterium* and *Streptomyces* (Geiger et al. 2010; Sandoval-Calderón et al. 2015; Sohlenkamp and Geiger 2016). Based on the frequency of the presence of genes encoding OlsB, it has been estimated that about 25% of the sequenced bacteria have the OlsBA pathway for OL synthesis. A decade later, the bifunctional enzyme OlsF was identified in *Serratia proteamaculans* (Vences-Guzmán et al. 2015) (Fig. 2, reaction 3). The C-terminal domain of OlsF is responsible for its

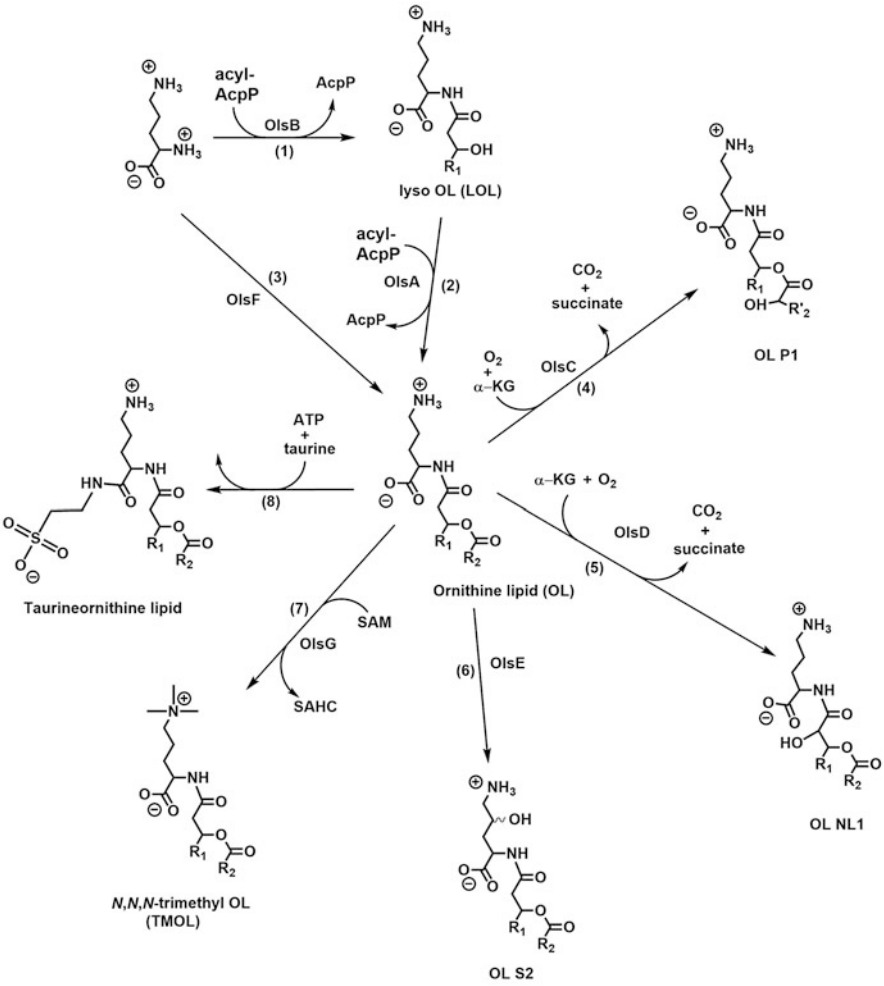

Fig. 2 Synthesis of OLs and their derivatives in bacteria. This figure shows the different known modifications of OL starting from the unmodified OL in the center of the figure. In many cases, OLs can also be subject to multiple modifications giving rise to structures not shown in the figure. The names of the OLs *S1* (substrate 1) and *S2* (substrate 2) originally described their roles as substrates in OlsC-dependent OL modifying reactions in *R. tropici*, whereas the *P1* described its role as a product of an OlsC-dependent OL modifying reaction. The lipid *NL1* (new lipid) described an unknown lipid in *B. cenocepacia*. *AcpP* constitutive acyl carrier protein; *α-KG* alpha-ketoglutarate; *SAM S*-adenosylmethionine; *SAHC S*-adenosylhomocysteine

N-acyltransferase activity and is distantly related to the *N*-acyltransferase OlsB. The N-terminal domain of OlsF is responsible for the *O*-acyltransferase reaction, but it is unrelated to the *O*-acyltransferase OlsA. Genes encoding OlsF homologues are present in about 25% of the sequenced bacterial species, mainly in γ-, δ-, and ε-proteobacteria and in bacteria of the CFB (Cytophaga-Flavobacterium-

Bacteroidetes) group. This means that about 50% of sequenced bacterial species are predicted to have the capacity to form OL at least under certain growth conditions. This number still might increase, because apparently there are OL-forming bacteria lacking genes encoding OlsBA or OlsF homologues, one example being the plantomycete *Singulisphaera acidiphila* (Moore et al. 2013; Escobedo-Hinojosa et al. 2015).

When searching sequence databases for the presence of genes encoding OlsBA or OlsF, the hits almost exclusively come from eubacterial genomes. One of the few exceptions is found in the genome sequence of the ant *Lasius niger*, where a gene encoding a good OlsF homologue can be detected. However, having a closer look at this case, it becomes clear that the contig from which this gene sequence is derived contains only bacterial sequences. The gene sequence encoding an OlsB homologue therefore most probably is derived from the genome of an endosymbiont or from contaminating bacterial DNA.

Diercks et al. (2015) showed recently that OL in *M. loti* contains 80–90% of D-ornithine. It is unclear where the D-ornithine is coming from and if D-ornithine is also present in OLs of other bacteria.

2.2 Modification of OLs

Many bacteria have the capacity to modify OL once it has been synthesized (Knoche and Shively 1972; Tahara et al. 1976b; Kawai et al. 1988; Asselineau 1991; Galbraith et al. 1999; Rojas-Jiménez et al. 2005; Lewenza et al. 2011; Moore et al. 2013; Vences-Guzmán et al. 2012; Sohlenkamp and Geiger 2016). So far hydroxylations in three different positions of OL have been described, in addition to the *N*-methylation of the δ-amino group and the modification of OL with taurine (Tahara et al. 1976b; Rojas-Jiménez et al. 2005; Gónzalez-Silva et al. 2011; Vences-Guzmán et al. 2011; Moore et al. 2013). It is not clear what the functional implications of these OL modifications might be, but one possible explanation is that modifying already existing membrane lipids allows for a quick response and adaptation to changing environmental conditions without de novo synthesis of lipids.

The 2-hydroxylation of the ester-bound fatty acid had been described already several years ago in different bacterial species such as *Gluconobacter cerinus*, *Burkholderia cenocepacia*, and *Flavobacterium* sp. (Fig. 2, reaction 4), and this is possibly the most widespread OL modification (Knoche and Shively 1972; Kawai et al. 1988; Asselineau 1991; Galbraith et al. 1999; Vences-Guzmán et al. 2012). During a search for genes involved in the response to acid stress in *Rhizobium tropici*, Rojas-Jiménez et al. (2005) identified the OL hydroxylase OlsC which is a homologue to the lipid A-hydroxylase LpxO from *Salmonella typhimurium* (Gibbons et al. 2000). Both enzymes belong to the same family of Fe^{2+}/O_2/α-ketoglutarate-dependent oxygenases. OlsC from *R. tropici* is responsible for the 2-hydroxylation of the ester-bound fatty acid in OL in this organism. The gene/enzyme responsible for introducing the same modification in *B. cenocepacia* and *Flavobacterium* sp. has not been identified yet.

Gónzalez-Silva et al. (2011) identified the OL hydroxylase OlsD from *Burkholderia cenocepacia* which is responsible for the hydroxylation of the amide-bound fatty acid (Fig. 2, reaction 5). OlsC from *R. tropici* and OlsD from *B. cenocepacia* are homologues. In addition to *Burkholderia* genomes, genes encoding OlsD homologues can be found in the genomes of *Serratia* sp. and *Mesorhizobium* sp. (Diercks et al. 2015; Vences-Guzmán et al. 2015). Diercks et al. (2015) showed that *M. loti* presents an OL hydroxylated in the 2-position of the amide-bound 3-hydroxy fatty acid.

Vences-Guzmán et al. (2011) identified the OL hydroxylase OlsE from *R. tropici* that is responsible for introducing a hydroxyl group into the ornithine headgroup of OLs (Fig. 2, reaction 6). The exact position of this hydroxylation is not known yet. OlsE belongs to the di-iron fatty acyl hydroxylase superfamily (cl01132) which includes fatty acid and carotene hydroxylases as well as sterol desaturases. Apart from *R. tropici,* OlsE activity has been detected in *Agrobacterium tumefaciens* (Vences-Guzmán et al. 2013).

Moore et al. (2013) had described a novel *N*-trimethylated OL in a few planctomycete species that had been isolated from *Sphagnum* bog. By expressing several candidate genes in an OL-forming *E. coli* strain, Escobedo-Hinojosa et al. (2015) identified the gene Sinac_1600 encoding the *N*-methyltransferase OlsG (Fig. 2, reaction 7). OlsG is a homologue of the PE *N*-methyltransferase PmtA from *S. meliloti* (de Rudder et al. 2000). Interestingly, OlsG is able to *N*-methylate PE in addition to OL when expressed in *E. coli*, thereby causing phosphatidylcholine formation.

Another OL modification that has been described a while ago in *Gluconobacter cerinus* is the ATP-dependent transfer of taurine to hydroxylated OL (Fig. 2, reaction 8) (Tahara et al. 1976b; Tahara et al. 1978). This lipid is also called cerilipin. The transferase responsible for cerilipin formation has not been identified yet, and the function of the taurineornithine lipid is not known. Recently, a draft genome of a *G. cerinus* strain has been published (Sainz et al. 2016), which should allow to search for candidate genes. Mass spectrometry data for cerilipin should also allow studying how widespread cerilipin formation is in bacteria (Moore et al. 2016).

2.3 Presence and Synthesis of Other Amino Acid-Containing Acyloxyacyl Lipids

OL is the most frequent of the amino acid-containing lipids presenting an acyloxylacyl structure. Several publications mainly from the 1970s and 1980s had shown that apart from OLs other structural homologues presenting other amino acid headgroups such as lysine, glycine, glutamine, and serineglycine exist. The presence of lysine lipids (LL) has been shown in *Agrobacterium tumefaciens*, *Pseudopedobacter saltans*, *Flavobacterium johnsoniae*, and *Rhodobacter sphaeroides* (Tahara et al. 1976a; Zhang et al. 2009; Moore et al. 2015a, 2016). In *P. saltans*, several hydroxylated versions of LL were detected (Moore et al. 2015a). The hydroxylation detected within the lysine headgroup would be the equivalent of the

hydroxylation introduced by OlsE into OL, and the 2-hydroxylation within the piggyback fatty acid of LL would be the equivalent of the hydroxylation introduced by OlsC into OL.

Glycine lipids (cytolipin) exist in *Cyclobacterium marinus*, *Pedobacter heparinus*, and *Cytophaga johnsonae* (Kawazoe et al. 1991; Batrakov et al. 1999; Moore et al. 2016). The presence of serineglycine lipids (flavolipin, L654) has been shown in *Flavobacterium meningosepticum*, *C. marinus* WH, *Porphyromonas gingivalis*, and *Porphyromonas endodontalis* (Kawai et al. 1988; Batrakov et al. 1998; Clark et al. 2013; Mirucki et al. 2014; Moore et al. 2016), and glutamine lipids are present in *Rhodobacter sphaeroides* (Zhang et al. 2009; Moore et al. 2016). Bacteria belonging to the CFB group seem to be most diverse with respect to the presence of amino acid diversity in the headgroup. Glutamine lipid is the only known amino acid-containing acyloxyacyl lipid that has not been detected in the CFB group yet.

To date, not much is known about the synthesis and distribution of these lipids. Recently, a study by Moore et al. (2016) confirms many of the earlier findings and detects many of these molecules for the first time by mass spectrometry (MS). MS technologies will allow a much quicker screening to learn about the presence of these lipids in other bacteria. It can be speculated that these lipids are synthesized by enzymes homologous to OlsBA or OlsF. For OlsF from *Flavobacterium johnsoniae*, it has been shown that upon expression in *E. coli*, lysine lipids (LLs) are formed in addition to OL (Vences-Guzmán et al. 2015). Moore et al. (2016) have also shown the presence of OL and LL in *F. johnsoniae*. Similarly, the fact that different hydroxylated versions of LL were identified suggests that hydroxylases such as OlsC or OlsE are responsible for LL hydroxylation. However, Moore et al. (2015a) could not identify good OlsC or OlsE homologues in the genome of *P. heparinus*. *Rhodobacter sphaeroides* is able to form glutamine lipids, OLs, and LLs and interestingly enough, it presents two genes encoding OlsB (OlsB1 and OlsB2) homologues (Geiger et al. 2010). OlsB1 might use lysine in addition to ornithine as substrate, whereas OlsB2 might be responsible for the formation of lyso-glutamine lipid. In a second step, these lysolipids would be converted to OL, LL, or glutamine lipid by an *O*-acyltransferase.

3 Functions of Amino Acid-Containing Acyloxyacyl Lipids

Mutants deficient in the formation and modification of OLs have been constructed in several species, but the phenotypes are not always clear and consistent. Often (but not always) the presence of (hydroxylated) OLs has been related to stress conditions. One of the best known examples for membrane remodeling occurs in some bacterial species under phosphate-limiting conditions (1), although the presence or absence of OLs can also affect the tolerance to other forms of abiotic stress such as low pH (2) or increased temperature (3). It has been suggested that trimethylated OLs are important for plantomycetes living at the anoxic/oxic interphase in *Sphagnum* bogs (4). In addition, the presence or absence of (hydroxylated) OLs can affect the

development of pathogenic or symbiotic interactions (5). Other nonstress related phenotypes related to the presence/absence of OL also have been observed (6):

1. In a few cases, such as *Pseudomonas aeruginosa*, *Sinorhizobium meliloti*, and *Serratia proteamaculans*, it has been observed that no OLs (or only minor amounts of OLs) are formed when bacteria are cultivated in complex growth media usually containing phosphate and other nutrients in excess. When cultivating these bacteria in growth media with limiting phosphate concentration, phosphorus-containing membrane lipids such as glycerophospholipids are substituted with phosphorus-free membrane lipids (Minnikin et al. 1972; Minnikin and Abdolrahimzadeh 1974; Minnikin et al. 1974; Benning et al. 1995; Geiger et al. 1999; Lewenza et al. 2011). It has been speculated that the zwitterionic PE is replaced by the zwitterionic OL (Benning et al. 1995). This membrane remodeling is regulated by the transcriptional regulator PhoB in *S. meliloti*, and in *S. proteamaculans* the *olsF* gene is also preceded by a pho box (Geiger et al. 1999; Vences-Guzmán et al. 2015). Interestingly, in other (often closely related) bacteria, OL formation is constitutive and is not restricted to conditions of phosphate limitation (Gónzalez-Silva et al. 2011; Palacios-Chaves et al. 2011; Vences-Guzmán et al. 2011, 2013; Sohlenkamp and Geiger 2016). Nevertheless, a decreased phosphate concentration in the growth medium seems to cause an increase in the OL content (Vences-Guzmán et al. 2011). It has been suggested that this remodeling allows to liberate phosphate bound within the glycerophospholipids of the membrane and to be used for other cellular processes such as nucleic acid formation. This capacity to remodel the membrane is probably an advantage for bacteria presenting different lifestyles (Zavaleta-Pastor et al. 2010). Remodeling could improve bacterial survival in phosphate-deplete habitats, whereas under conditions of sufficient phosphate, for example, in contact with a eukaryotic host, the bacterial membrane could be remodeled and allow an adaptation of the bacteria. The adaptation of phosphate-starved bacteria to phosphate-replete conditions has not been studied, and it is not clear if OLs or other phosphate-free membrane lipids are actively degraded under these conditions.
   Surprisingly, *S. meliloti* mutants deficient in OL formation grow as the wildtype under phosphate-limiting conditions. In order to observe a growth phenotype under these conditions, the OL deficiency has to be accompanied by DGTS deficiency (López-Lara et al. 2005). Similarly, a *S. proteamaculans* mutant deficient in OlsF that cannot synthesize OL grows similarly as the wildtype under phosphate-limiting conditions (Vences-Guzmán et al. 2015).
2. In different bacteria, OLs are enriched in the outer membrane (Dees and Shively 1982; Palacios-Chaves et al. 2011; Vences-Guzmán et al. 2011). For *Thiobacillus thiooxidans*, it has been suggested that the presence of OLs in the outer membrane might be related to its acid tolerance (Dees and Shively 1982). *R. tropici* accumulates increased amounts of 2-hydroxylated OL P1 (Fig. 2) when grown at pH 4.0, and *R. tropici* mutants deficient in OlsC grew much slower than the wildtype under these conditions (Vences-Guzmán et al. 2011). For sphingolipids and lipid A which

also can be hydroxylated in the 2-position, it has been speculated that the introduction of an additional hydroxyl group increases hydrogen bonding with neighboring molecules and leads to membrane stabilization (Nikaido 2003).

3. No increase in the formation of hydroxylated OLs can be observed when *R. tropici* is cultivated at 37 or 42 °C instead of 30 °C, but *R. tropici* deficient in the OL 2-hydroxylase OlsC grows much slower than the wildtype at 42 °C. Taylor et al. (1998) had observed an increase in the hydroxylated membrane lipids (which included hydroxylated OL and hydroxylated PE) in *B. cepacia* grown at 40 °C, supporting the idea that OL hydroxylation has a function at increased temperature. Again, the presence of an additional hydroxyl group might lead to membrane stabilization (see above).
4. Trimethylornithine lipids (TMOL) were recently described as abundant lipids in four isolates of *Sphagnum* wetland planctomycetes (*Gemmata*-like strain SP5, *Telmatocola sphagniphila*, *Singulisphaera rosea*, *Singulisphaera acidiphila*) (Moore et al. 2013). Later, Moore et al. (2015b) could show that TMOLs accumulated at the oxic/anoxic interphase in *Sphagnum* bogs and that the presence of TMOL correlated with an enrichment of the planctomycete community at this depth. This suggested that TMOLs were synthesized by planctomycetes in the bog as a response to changing redox conditions at the oxic/anoxic interphase (Moore et al. 2015b).
5. In some (but not all) cases, mutants deficient in OL formation or modification are affected during host-microbe interactions. *S. meliloti* mutants deficient in OL formation form functional nodules on their host plant alfalfa. In *Brucella*, OLs are dispensable for the development of pathogenicity (Palacios-Chaves et al. 2011). In contrast, *A. tumefaciens* mutants deficient in OL formation or OL modification show accelerated tumor formation during infection of potato tuber discs (Vences-Guzmán et al. 2013). *R. tropici* deficient in OL hydroxylation induces the formation of nodules on its host plant common bean that are defective in biological nitrogen fixation (Vences-Guzmán et al. 2011).
6. For OL and serineglycine lipid (flavolipin, L654), it has been published that they cause an immune response. The bacterial endotoxin lipid A presents an acyloxyacyl structure and is recognized by Toll-like receptor 4 (TLR4) as pathogen-associated molecular pattern. This acyloxyacyl structure is also present in OL and flavolipin/L654 (and the other amino acid-containing lipids discussed in this chapter). Lipid A binds to the co-receptor MD-2 (myeloid differentiation factor 2) and as a complex they bind to TLR4 in order to activate TLR4 signaling. The activation of the TLR4/MD-2 complex triggers the innate immune response of mammals and the biosynthesis of inflammatory cytokines such as TNF-α, IL-1, and IL-6 (Molinaro et al. 2015). Kawai et al. (1988) had shown that OL and flavolipin cause inflammatory immune responses measuring the formation of PGE_2, IL-1β, and TNF-α by macrophages. It was also shown that both molecules could be used as adjuvants and that they when injected into mice before exposure to lipid A prevent lethal effects of bacterial endotoxin (Kawai et al. 1991a, b, 1999, 2000a, b, 2002; Kato and Goto 1997). Earlier it had been suggested that the response to flavolipin is also transduced via the TLR4/MD-2 receptor (Gomi et al.

2002), but recently it was shown that serineglycine lipid L654 (which is structurally identical to flavolipin) is a ligand of human and mouse Toll-like receptor 2 (TLR2). The binding of L654 inhibits osteoblast differentiation, and it has been concluded that L654 and its corresponding lysolipid L430 have the potential to promote TLR2 dependent bone loss as reported in experimental periodontitis (Wang et al. 2015). Recently, L654 has been detected in human blood samples and interestingly multiple sclerosis patients had significant lower L654 levels than healthy patients (Farrokhi et al. 2013). In this context, L654 has been proposed as a microbiome-associated biomarker for multiple sclerosis (Farrokhi et al. 2013), and L654 administration to mice has been accompanied by an attenuation of autoimmune disease (Anstadt et al. 2016).

7. The presence of OLs in *R. capsulatus* is required for cytochrome maturation and optimal steady-state amounts of *c*-type cytochromes (Aygun-Sunar et al. 2006).
8. With the exception of the immunological studies with flavolipin/L654, nothing is known about the possible functions of (non-ornithine) amino acid-containing acyloxyacyl lipids.

4 Research Needs

Our knowledge about the synthesis and function of amino acid-containing acyloxyacyl lipids has advanced a lot in recent years. One important question to answer is: Why is there such a diversity of amino lipids? Using mass spectrometry, we can learn more about distribution of these amino lipids in the environment and in parallel possibly identify new lipids/structures. Once knowing what is out there, we can set out to discover the genes/enzymes involved in amino lipid formation and modification. Finally, mutants deficient in amino lipid formation or modification can be constructed to learn about the physiological roles of these lipids.

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