LIPIDOMICS AS A TOOL FOR FUNCTIONAL GENOMICS

LIPIDOMICS AS A TOOL FOR FUNCTIONAL GENOMICS IN *SINORHIZOBIUM MELILOTI*

By

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Abstract

This thesis focused on the development of comprehensive, rapid and simple methodologies for the analysis of fatty acids by gas chromatography mass spectrometry (GC/MS) and intact lipids by electrospray ionization tandem mass spectrometry (ESI/MS/MS). The methodologies were applied as a tool for functional genomics in the soil bacterium *Sinorhizobium meliloti*. The effects of inorganic phosphate (P_i)-starvation and acidity on lipid composition were studied.

A micro-scale, one-vial method for the analysis of fatty acids as their fatty acid methyl esters by GC/MS was developed. The method required small sample sizes, involved minimum handling and avoided tedious extraction steps, which increased sample throughput. A series of quality controls were included to measure losses due to handling, derivatization efficiencies and the extent of side reactions. The method was suitable for the analysis of sensitive bacterial fatty acids such as cyclopropane fatty acids.

A shotgun lipidomics approach was developed for the analysis of intact lipids by ESI/MS/MS. Fatty acid distributions were obtained for eight lipid classes and up to 58 individual lipids were identified in crude lipid extracts without sample cleanup or chromatography. For the first time, fatty acid distributions were provided for non-phosphorus containing lipids using shotgun lipidomics. Fatty acid distributions within lipid classes suggested that phospholipids and 1,2-diacylglyceryl-3-*O*-4'-(*N,N,N*-trimethyl)-homoserine lipids (TMHSs) were both synthesized from phosphatidic acid while sulfoquinovosyldiacylglycerol (SLs) had a different biosynthetic origin.

The methodologies were applied to study knockout mutants of five genes thought to participate in lipid metabolism in *S. meliloti*. It was demonstrated that: (1) *cfa2* gene coded for the main cyclopropane fatty acyl synthase; (2) the *plsC* gene coded for a fatty acyl transferase specific for C16 fatty acids in the *sn*-2 position of phospholipids; (3) a metabolic phenotype was revealed for knockout mutants of *dme* and *tme* genes (DME and TME, malic enzymes) when succinate was the carbon source.

Preface

This thesis presents the results of the work conducted by the author during five years of research. This thesis describes the development of comprehensive and rapid analytical methodologies for the analysis of fatty acids and intact lipids in the soil bacterium *Sinorhizobium meliloti* and their application to the study of genes of unknown function. This thesis is the result of a collaboration with Drs. Turlough M. Finan and Rahat Zaheer (Biology Department, McMaster University) during a Genome Canada project intended to investigate genes of unknown function in the recently sequenced genome of *S. meliloti*. The bacterial cultures and mutants of target genes employed in this thesis were kindly provided by Drs. Turlough M. Finan and Rahat Zaheer.

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Big achievements are rarely the work of one person, that's how I feel about these 6 years of research. First, I would like to thank my supervisor Dr. Brian E. McCarry for sharing his knowledge and experiencie with me, for his patience and for offering me the opportunity to improve myself as a scientist and as a person. Secondly, I'm inmensely greatful for having had the chance to collaborate with Dr. Turlough Finan and Dr. Rahat Zaheer. My project is the product of what I would call a synergistic collaboration and successful team work. I thank particularly Rahat, she is not only a good scientist but also a great human being. I also would like to thank Dr. Kirk Green for all his support and help; his friendly and pleasant nature creates a hospitable environment in the mass spectrometry facility. I also thank my previous and current lab partners: Julia Jia, David Dam, Tarlika Persaud, Sujan Fernando, Uwayemi Sofowote and Catherine Amoateng. They are all very diverse, have come from different countries and speak different languages; however, they share common dreams. It takes courage and determination to pursue dreams in life, that is one of the many reasons why I admire them.

I also count myself as a very lucky person for the unconditional love and support from family and friends. Mark Hewitt, my husband, deserves a special place for loving me and for believing in me. I consider myself a very fortunate woman to have someone like him to share my life with. My most sincere gratitude goes to both of my parents, Orledo Saborido Rios and Gisela Basconcillos Quintana. I dedicate this work to them, they are always in my thoughts. I thank my family in Canada, Larry and Millie Hewitt, they have earned a very special place in my heart. Not only am I lucky to have a wonderful husband, loving parents and even fantastic in-laws; I also have a very rare and precious treasure, a true friend: my cousin Sofia Putkowski. To my family and friends, thank you.

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List of Abbreviations

2D-HPTLC: two dimensional high performance thin layer chromatography
ACP: acyl carrier protein
ANOVA: analysis of variance
CC: column chromatography
CFA: cyclopropane fatty acids or cyclopropane fatty acyl synthase
CID: collision induced dissociation
CL: cardiolipin
CoA: coenzyme A
DHB: 2,5-dihydroxybenzoic acid
DMPE: dimethylphosphatidylethanolamine
ESI: electrospray ionization
FA: fatty acid
FAB: fast atom bombardment
FAMEs: fatty acid methyl esters
GC/MS: gas chromatography coupled to mass spectrometry
HPLC: high performance liquid chromatography
HPTLC: high performance thin layer chromatography
LPS: lipopolysacharide
m/z: mass-to-charge ratio
MALDI: matrix-assisted laser desorption
MMPE: monomethylphosphatidylethanolamine
MS/MS: tandem mass spectrometry
MSTFA: N-methyl-N-(trimethylsilyl)trifluoroacetamide
NL: neutral loss
NP-HPLC: normal phase high performance liquid chromatography
OL: ornithine lipid
PA: phosphatidic acid
PC: phosphatidylcholine
PCA: principal component analysis
PE: phosphatidylethanolamine
PG: phosphatidylglycerol
PHB: poly(3-hydroxybutyrate)
P_i: inorganic phosphate
PI: phosphatidylinositol
PlsB: glycerol-3-phosphate acyltransferase
PlsC: 1-acylglycerol-3-phosphate acyltransferase or lysophosphatidic acyl transferase
PS: phosphatidylserine
QqQ: triple quadrupole mass analyzer
RP-HPLC: reverse phase high performance liquid chromatography
SL: sulfoquinovosyldiacylglycerol

TLC: thin layer chromatography
TMHS: 1,2-diacylglyceryl-3-*O*-4'-(*N,N,N*-trimethyl)-homoserine lipid
TMS: trimethylsilyl esters
TOF: time-of-flight mass analyzer

Chapter 1

Introduction

In the last decade the genomes of more than 700 organisms have been sequenced, including the human genome [1, 2]. Bacterial genomics has received much attention in recent years, driven mainly by the antibiotics resistance developed by many pathogenic bacteria [3, 4]. The complete genomes of more than 600 bacterial organisms have been published and currently there are more than a 1000 ongoing bacterial genome projects [2]. However, knowledge of complete genome sequences is not sufficient to understand the functioning of biological systems and their responses to environmental stressors. An understandi ng of gene function and how organisms regulate biological processes is necessary for applications to the diagnosis of human diseases, to biomarker discovery and to drug development [5-7]. Functional genomics has emerged as a new and exciting field that seeks to unravel gene function using integrated strategies [8]. A common approach used in functional genomics is the disruption of genes of unknown function and the measurement of gene expression products at three levels: messenger RNAs, proteins and metabolites [9].

While the genome is defined as all the genes of an organism, all the proteins, mRNAs and metabolites are defined as the proteome, the transcriptome and the metabolome, respectively. Metabolites, the final products of gene expression, provide an integrated picture of cellular function; metabolites define the phenotype of the biological system and their levels change in response to genetic manipulations or environmental stresses [10, 11]. The complexity of the metabolome is considerably greater that the genome, the proteome or the transcriptome. In addition, the chemical diversity of metabolites is enormous with a wide range of molecular masses, chemical structures and stereochemistries. Thus, the determination of all components of the metabolome with the current analytical techniques is not achievable [11]. The analysis of a subset or class of metabolites is a more realistic and attainable goal for metabolomics studies. Lipidomics is an emerging area of metabolomics that focuses on the analysis of all the lipids in a biological system, defined as the lipidome [12, 13].

Although the lipidome is less complex than the metabolome, its analysis is still a challenging task due to the vast diversity of lipid classes and molecular species within classes. The lipidome comprises numerous lipid classes that include fatty acids, phospholipids, neutral lipids, sphingolipids, glycosphingolipids, sterols, glycolipids, etc. In addition, the variation of fatty acyl chains within lipid classes or subclasses contributes to the high number of molecular lipids, for example a single cell can contain thousands of molecular lipid species [14]. The enormous complexity of the lipidome and the key

biological functions that lipids play, have only been recently recognized [12]. The need for a global and integrated approach to study lipids in cells has fueled the creation of the consortium LIPID MAPS [15]. LIPID MAPS aims to characterize the global changes in lipid metabolites as a response to stimuli and disease [15]. Lipidomics, although a new science, has already proven to be a valuable tool for the study of biochemical processes, drug development, biomarker discovery and human diseases [16-18]. Yet lipidomics applications are not common and even less so in the field of functional genomics [12]. The research presented in this thesis focused on the development of a lipidomics approach as a tool for functional genomics.

Analytical methods for the comprehensive analysis of lipids must provide information regarding: (i) lipid classes; (ii) molecular species within lipid classes caused by variations of the fatty acyl chains; and (iii) the relative locations of the fatty acyl chains in individual lipids (*sn*-1 and *sn*-2). In addition, the fatty acyl chains of bacterial lipids are very diverse and complex due to the presence of structural features such as double bonds, hydroxyl groups and cyclopropane rings [19]. In the past, lipid analysis was laborious since these biomolecules were not volatile enough for gas chromatography (GC) and mass spectrometric detection with electron impact ionization. Typically, lipids were extracted from the matrix, isolated and fractionated using tedious chromatographic schemes, degraded into simpler components (e.g. fatty acids) which could be made amenable for GC analysis by derivatization [20]. Currently, most methods for fatty acid analysis require relatively large sample amounts, involve extensive sample manipulation and liquid-liquid extraction steps; thus these methodologies are tedious and laborious.

In the last decade, the development of soft ionization mass spectrometry techniques such as fast atom bombardment (FAB), matrix-assisted laser desorption (MALDI) and especially electrospray ionization mass spectrometry (ESI/MS) has facilitated the analysis of lipids [20-23]. The technological improvements in mass spectrometry coupled with powerful separation techniques such as high performance chromatography (HPLC) has immensely advanced the field of lipidomics. Currently, most lipidomics applications employ HPLC coupled to electrospray ionization mass spectrometry (HPLC-ESI/MS) or electrospray ionization tandem mass spectrometry (HPLC-ESI/MS/MS), although older chromatographic techniques such as thin layer chromatography (TLC) are still widely used in lipid analyses [24]. However, lipids can be analyzed directly from lipid extracts without the need for chromatographic separation using a combination of tandem mass spectrometric techniques; this approach is termed shotgun lipidomics [14]. Shotgun lipidomics approaches are simple, rapid, easy to automate and hence are ideal for the analysis of high volumes of samples generated in functional genomics studies.

In this thesis, the principles and techniques of lipidomics were applied as a tool for functional genomics to study specifically the soil bacterium *Sinorhizobium meliloti*. The complete genome of *S. meliloti* was sequenced in

2001, however more than 40% of the genes are of unknown function [25-27]. *S. meliloti* infects the roots of plants such as alfalfa forming nodules and establishing a beneficial symbiotic relationship. Bacteria convert atmospheric nitrogen into a useful source of nitrogen for plants which in turn provide nutrients to bacteria in the form of dicarboxylic acids of 3-4 carbon atoms; this process is called nitrogen fixation [28, 29]. Most of the functional assignments of genes have been based on comparisons with the genomes of model organisms such as *Escherichia coli* [30, 31]; however, there are multiple cases in which the paradigm of *E. coli* does not apply [32, 33]. In the last decade, extensive research has been conducted to understand the biochemistry and genetics of nitrogen-fixing bacteria such as *S. meliloti* and their symbiosis with leguminous plants [34-36]. These efforts are driven by the prospects of enhancing nitrogen fixation through genetic manipulations, thereby reducing fertilizer applications and contributing to improved agricultural sustainability [37, 38].

In *S. meliloti*, lipid biosynthesis and fatty acid biosyntheses are thought to occur similarly as in *E. coli*. Consequently, the biochemical models for fatty acid and lipid biosyntheses are based on the existence of genes in the genome of *S. meliloti* that have homology with genes that participate in these processes in *E. coli* [30]. Lipid biosynthesis in *S. meliloti* has been studied to some extent by Geiger and coworkers however there are still numerous knowledge gaps [30, 39]. For example, reasonable homologues of genes which are essential for lipid biosynthesis in *E. coli* cannot be found in the genome of *S. meliloti* [30]. In addition, fatty acid biosynthesis has hardly been studied in *S. meliloti* and there is no information regarding fatty acyl variation in lipids [30, 40]. Lipidomics is one of the proposed strategies to improve our understanding of metabolism and gene function in a biological system [12, 13]. This thesis has two main goals: (i) first, the development of simple and robust analytical methodologies for the analysis of the lipidome (fatty acids and intact lipids) in *S. meliloti*; and (ii) second, the application of the analytical methodologies to study genes of unknown function thought to participate in lipid metabolism in *S. meliloti*.

Environmental conditions are known to alter the lipid and fatty acid composition of membranes in bacteria [30, 41]. Modifications of the fatty acyl chains of lipids are a common strategy used by bacteria to regulate membrane properties as a response to various environmental stressors (e.g. temperature, acidity) [41-43]. Soil bacteria face numerous environmental stressors which include increased soil salinity, acidic pH conditions and limited inorganic phosphate (P_i) [44-46]. Thus, the biochemical mechanisms that enable *S. meliloti* to survive and establish successful symbiosis under unfavorable conditions are of great interest [47-50]. Under P_i-limiting conditions, *S. meliloti* partially substitutes membrane phospholipids by phosphorus-free lipids as a survival strategy [51]. Hence, in this work the effect of two environmental stressors (P_i-starvation and acidic conditions) on fatty acid and lipid compositions in *S. meliloti* was explored. Shotgun lipidomics methodologies have been developed for the analysis of common phospholipids but not for non-phosphorus containing lipids, which are

important lipid classes in *S. meliloti* under P_i-limiting conditions. Therefore, an additional objective of this research was to expand the principles of shotgun lipidomics to the analysis of non-phosphorus containing lipids in *S. meliloti*.

In summary, this thesis focused on the development of a lipidomics approach as a tool in functional genomics in *S. meliloti*. The development of simple and rapid methodologies for the analysis of fatty acids by GC/MS and intact lipids by ESI/MS/MS was the starting point. Chapter 2 describes a comparison of common analytical methodologies for the analysis of bacterial lipids of *S. meliloti* as their fatty acid methyl esters (FAMEs) by GC/MS. These methodologies were particularly evaluated for the analysis of sensitive bacterial cyclopropane fatty acids. A micro-scale, one-vial method suitable for high sample throughput for fatty acid analysis was developed. In chapter 3, shotgun lipidomics approaches were applied to the analysis of intact lipids in *S. meliloti* and a method was developed using ESI/MS/MS. The combination of GC/MS and ESI/MS/MS methodologies provided a comprehensive view of the lipidome given by total fatty acid composition and fatty acid distributions within lipid classes. Fatty acid and lipid biosyntheses are coordinated and highly related processes; thus, fatty acid distributions in lipid classes can provide important clues into biosynthesis and gene function. Chapters 4-6 describe the application of these methodologies to study knockout mutants of genes hypothesized to participate in lipid metabolism, thereby providing insights into gene function.

The introduction of the thesis is divided in three sections: (i) classifications of lipids and fatty acids; (ii) a summary of the most common analytical methodologies for the analysis of fatty acids and intact lipids, with a focus on the challenges for the analysis of bacterial fatty acids and lipids; (iii) a background of the current knowledge in fatty acid and lipid biosyntheses as well as their known biological functions in *S. meliloti*. In this thesis, fatty acids were represented using the short hand designation system recommended by the IUPAC [52], while lipids were represented following the system recommended by LIPID MAPS [15].

1.1. Classifications of Lipids and Fatty Acids

Lipids can be divided in two major groups: simple or complex lipids [53]. Simple lipids upon hydrolysis yield up to two types of products (e.g., glycerol and fatty acids). Simple lipids include glycerides, steroids and amino acid-containing lipids (Figure 1.1a). Glycerides, the most common simple lipids, are composed of fatty acids linked via ester bonds to the hydroxyl groups of glycerol [53]. Glycerides are further classified into monoacylglycerols, diacylglycerols and triacylacylglycerols when one, two or three fatty acids are esterified to the hydroxyl functions of glycerol. Triacylacylglycerols are abundant lipids in animals and plants but are not abundant components in bacteria. Steroids are based on the saturated tetracyclic hydrocarbon sterane (cyclopentanoperhydrophenanthrene). Sterols are a subclass of steroids which can be found in their free form or with the hydroxyl function esterified to fatty acids

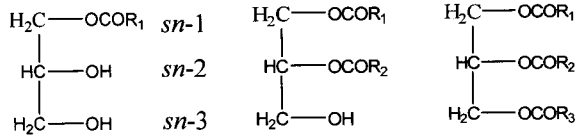
(sterol esters). Cholesterol is the most abundant sterol in verterbrates but it is not present in plants or bacteria [54]. Plants, yeasts and fungi contain a different set of sterols; for example, stigmasterol is the most abundant in plants while ergosterol is the most abundant in fungi and yeasts [53]. Amino acid-containing lipids typically include amino acids such as such as serine, ornithine and lysine which are linked to fatty acyl chains via amide or ester bonds; these lipids are commonly found in bacteria [54].

Complex lipids produce three or more types of products when hydrolyzed [53]. Complex lipids are classified into glycerophospholipids (phospholipids), sphingolipids and glycolipids (Figure 1.1b). Glycerophospholipids contain phosphoric acid attached via an ester bond to the hydroxyl group in the *sn*-3 position of glycerol and fatty acids esterified to the *sn*-1 and *sn*-2 positions; a lipid with such structure is the simplest of phospholipids known as phosphatidic acid (PA). Variations of the X moiety known as the polar head group of phospholipids originate the various phospholipid classes (Figure 1.1b). More than 90% of total lipids in bacteria are phospholipids [40]. Phosphatidylglycerols (PGs), phosphatidylethanolamines (PEs) and cardiolipins (CLs) are ubiquitous components of bacterial membranes. Phospholipids are termed lysophospholipids when one fatty acyl chain is missing from either the *sn*-1 or the *sn*-2 position. Sphingolipids contain long-chain bases linked via an amide bond to a fatty acid and via the terminal hydroxyl group to complex carbohydrate or phosphorus-containing moieties [55]. Long-chain bases are aliphatic amines of 12-22 carbon atoms containing 2-3 hydroxyl functions. Sphingolipids such as sphingomyelins, are only present in mammals [53, 56]. Glycolipids contain one carbohydrate group linked via a glycosyl bond to either diacylglycerol (glyceroglycolipids), to the phosphate group in the *sn*-3 position of phospholipids (phosphoglycolipids) or to a ceramide backbone (sphingoglycolipids).

The high number of molecular lipid species in biological systems is caused by variations of the fatty acyl chains in lipid classes and subclasses. The fatty acids (FAs) found in nature can be odd or even numbered, branched, saturated or unsaturated. Unsaturated FAs can be monoenoic, dienoic or polyenoic according to the number of double bonds present, and they can also be conjugated or non-conjugated. Fatty acids present in plants and bacteria contain 1 to 3 double bonds while those in algae and animals can contain up to 6 double bonds [53]. FAs can also exist as *cis* or *trans* isomers according to the spatial disposition of the double bonds; however, most FAs in nature exist in a *cis* configuration [53]. In addition to unsaturations, fatty acyl chains can include other structural features such as hydroxyl groups, epoxy and keto groups, cyclopropane and cyclopropene rings. Cyclopropane-containing fatty acids, branched fatty acids and 3-hydroxy fatty acids are characteristic of bacterial lipids [19].

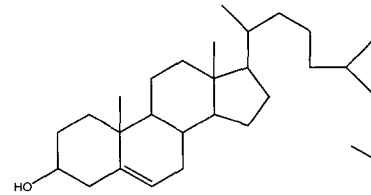
a. Simple Lipids

a.1. Glycerides



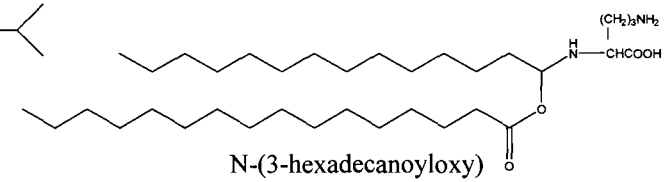
Monoacylglycerols Diacylglycerols Triacylglycerols

a.2. Steroids (Sterols)



Cholesterol

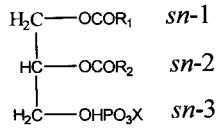
a.3. Amino acid-containing lipids



N-(3-hexadecanoyloxy) hexadecanoyl)-ornithine

b. Complex Lipids

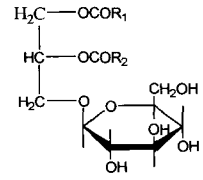
b.1. Phospholipids



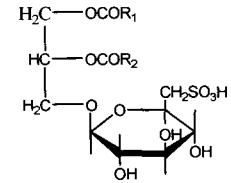
X	Phospholipid Classes
-H	Phosphatidic acid (PA)
$-CH_2CH_2NH_2^+$	Phosphatidylethanolamine (PE)
$-CH_2CH_2N(CH_3)_3^+$	Phosphatidylcholine (PC)
$-CH_2CH(COO^-)NH_3^+$	Phosphatidylserine (PS)
$-CH_2CH(OH)CH_2OH$	Phosphatidylglycerol (PG)
	Phosphatidylinositol (PI)
$-CH_2CH(OH)CH_2-PO_3-CH_2-CO(OCR')-CH_2O(COR'')$	Cardiolipin (CL)

b.2. Glycolipids

Glyceroglycolipids



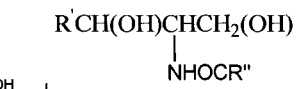
Monogalactosyl-diacylglycerol



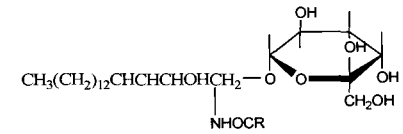
Sulfoquinovosyl-diacylglycerol

b.3. Sphingolipids

Ceramides



Sphingoglycolipids (Galactosylceramide)



Sphingophospholipids (Sphingomyelin)

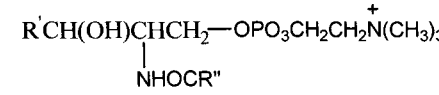


Figure 1.1. Classifications of lipids. R_1, R_2, R_3 represent fatty acyl chains.

1.2. Fatty Acid and Lipid Composition of *Sinorhizobium meliloti*

The cell walls of gram-negative bacteria such as *E. coli* and *S. meliloti* are complex structures formed by three layers: (i) the inner membrane or cytoplasmic membrane, (ii) the periplasm and (iii) the outer membrane (Figure 1.2) [57].

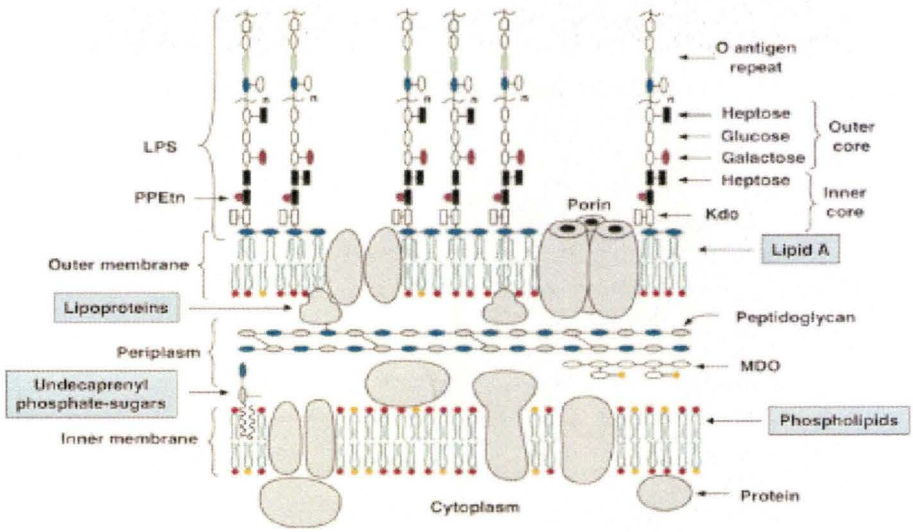


Figure 1.2. Schematic representation of the cell wall of *E. coli* [59].

The inner membrane is formed by a phospholipid bilayer while the outer membrane contains phospholipids as part of the inner leaflet and lipopolysacharides (LPSs) in the outer leaflet [57]. LPSs are complex molecules which can be divided into three regions: (i) the O-chain polysaccharide, (ii) core oligosaccharide and (iii) the lipid regions known as lipid A [40]. The lipid A consists of a disaccharide sugar backbone involving glucosamine residues which are both O- and N-fatty acylated. The lipid A serves to anchor the entire LPS to the bacterial outer membrane. LPSs are minor lipid components (<1% of dry weight) in *S. meliloti* [58] while phospholipids comprise more than 90% of all lipids [40]. Lipid A in *E. coli* and other pathogenic bacteria is associated with the infection process, therefore inhibitors of lipid A biosynthesis are targets in antibiotic research [59]. In *S. meliloti*, lipid A is crucial for symbiosis, however its function is not understood [40]. Poly(3-hydroxybutyrate) (PHB) is also present in *S. meliloti* in the form of granules in the cytoplasm. PHB can represent up to 50% of dry weight in *S. meliloti* cells under certain growth conditions [60].

The lipid composition of *E. coli* cell membranes is very simple and includes three phospholipids: phosphatidylethanolamines (PEs, 75%), phosphatidylglycerols (PGs, 20%) and cardiolipins (CLs, 5%) [56]. In contrast, lipid composition of *S. meliloti* membranes is more complex, containing up to

seven lipid classes. Phospholipids are the major components, including phosphatidylcholines (PCs, 60%), mono-methylphosphatidyletanolamines and phosphatidylethanolamines (MMPEs and PEs, 20%), di-methylphosphatidylethanolamines (DMPEs, 2%), PGs (9%) and CLs (5%) [51]. In addition, other lipid classes such as ornithine lipids (OLs) and sulfoquinovosyldiacylglycerols (SLs) are present and account for 5% of total lipids. Under P_i-limiting conditions, phospholipids in *S. meliloti* are partially substituted by non-phosphorus containing lipids (SLs, OLs and 1,2-diacylglyceryl-trimethylhomoserine lipids (TMHSs)) which can represent up to 70% of total lipids (Figure 1.3) [51].

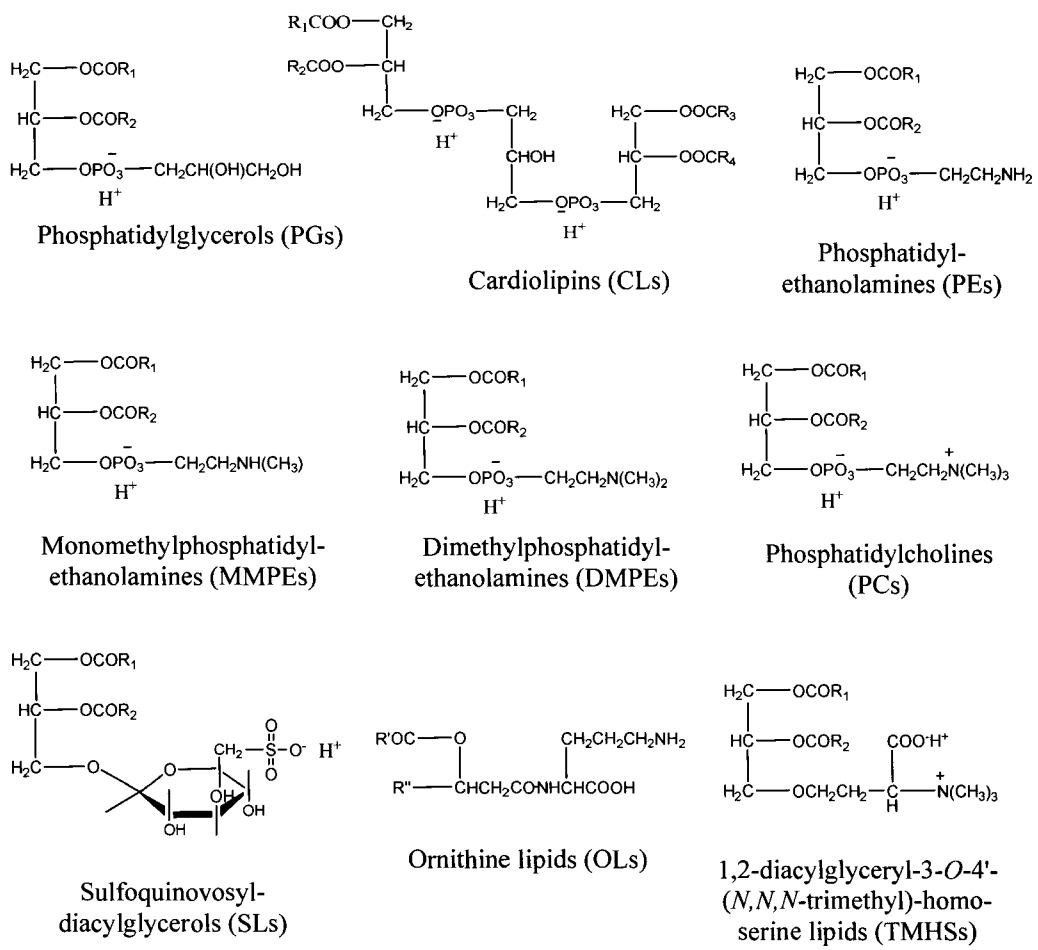


Figure 1.3. Structures of lipids in *S. meliloti*.

Fatty acids in bacterial lipids, exist as esters or amides and their occurrence as free fatty acids is limited [19]. Recently, detailed fatty acid analysis of phospholipids in *E. coli* revealed a total of sixteen fatty acids where hexadecanoic acid (16:0) was the major component. Other abundant fatty acids included tetradecanoic acid (14:0), *cis*-9-hexadecenoic acid (16:1(9)), *cis*-9,10-methylenehexadecanoic acid (17:0cyclo(9,10)) and octadecanoic acid (18:0). *Cis*-11-octadecenoic acid (18:1(11)) and *cis*-11,12-methyleneoctadecanoic acid (19:0cyclo(11,12)) were minor species [61]. In *E. coli*, 16:0 is located exclusively in the *sn*-1 position in phospholipids while unsaturated fatty acids such as 18:1(11) and 16:1(9) are located predominantly in the *sn*-2 position [62].

In contrast, the major fatty acid in phospholipids of *S. meliloti*, was 18:1(11) while 19:0cyclo(11,12) and 16:0 were abundant components [63]. Minor fatty acids in phospholipids included 14:0, 17:0, 18:0, 16:1(9) and 17:0cyclo. Fatty acid analysis of whole cells of *S. meliloti* used for chemotaxonomic identification revealed the presence of 3-hydroxy fatty acids [64, 65]; these fatty acids in bacteria are usually forming part of LPSs [19]. 3-Hydroxy tetradecanoate (3-OH-14:0) is the major hydroxyl fatty acid in LPSs of *E. coli* [56] and *S. meliloti* [40, 58]. In *S. meliloti*, the fatty acid distributions in phospholipids and fatty acids relative positions (*sn*-1 and *sn*-2) in individual lipids are not known since there are no studies that link fatty acyl variation within a lipid class [30].

1.3. Analysis of Fatty Acids and Intact Lipids in Bacteria

Lipidomics as a tool in functional genomics applications requires analytical methodologies for comprehensive lipid analysis. Methods for comprehensive lipid analysis should provide information regarding: (i) lipid classes (defined by the polar head groups in glycerophospholipids), (ii) fatty acyl chains in individual lipids and (iii) their relative positions (*sn*-1 and *sn*-2 in glycerophospholipids). Fatty acid and intact lipid biosyntheses are highly coordinated processes; thus methodologies for comprehensive lipid analysis should provide fatty acid distributions within lipid classes. Knowledge of fatty acid distributions in lipid classes can provide insights into their biosynthetic origin. Additionally, analytical methods for functional genomics applications should be rapid, simple and require small sample amounts.

Traditional methods employed in the analysis of bacterial lipids, generally use TLC to separate lipid classes. In order to obtain fatty acid distributions within lipid classes, isolated lipid classes on TLC spots must be extracted and hydrolyzed to obtain free fatty acids which are further derivatized to increase their volatility for analysis by GC/MS. Although it has been used for the past several decades, fatty acid analysis of bacteria by GC/MS still remains a useful and common methodology used in chemotaxonomy and many other applications [19]. In the last decades, the development of soft ionization techniques in mass spectrometry such as fast atom bombardment (FAB), matrix-assisted laser desorption (MALDI) and electrospray ionization (ESI) has facilitated the analysis of lipids directly [20, 22, 23]. Consequently, lipids can now be analyzed without

the need of hydrolysis or derivatization reactions. Information regarding lipid classes and fatty acyl chains in lipids can be obtained. The next sections will summarize the most common methodologies for the analysis of bacterial fatty acids and for intact lipids by GC/MS and by mass spectrometry, respectively.

1.3.1. Methods for Fatty Acid Analysis

Fatty acids have been analyzed using GC/MS [19], HPLC [66], ESI/MS/MS [20] and capillary electrophoresis [67]. However, the most common technique used in fatty acid analysis is GC/MS. Fatty acid analysis by GC/MS requires the derivatization of fatty acids in order to increase their volatility and improve their chromatographic properties [68]. Common fatty acid derivatives prepared for GC/MS analyses are fatty acid methyl esters (FAMEs) and trimethylsilyl esters (TMSs). By far, the most common derivatives are FAMEs so methods to prepare these derivatives will be reviewed along with some fundamental aspects of fatty acid analysis with an emphasis on the analysis of bacterial fatty acids.

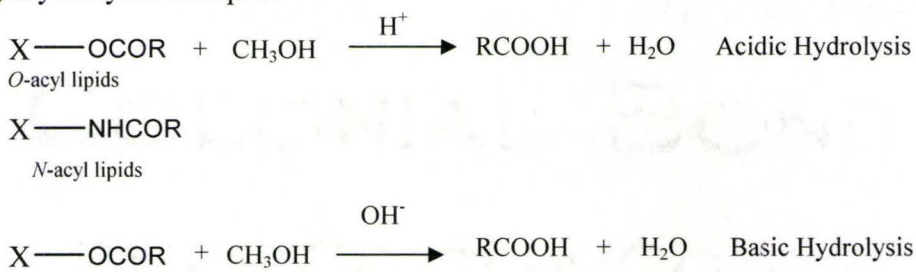
The first step in fatty acid analysis is the preparation of lipid extracts typically by liquid-liquid extraction of bacterial cultures or wet cells using the Bligh and Dyer method [69]. Briefly, aqueous samples (e.g., a suspension of bacterial cells, 1 mL) are extracted with $CHCl_3$:MeOH (3.75 mL, 2:1, v/v), vortexed for 15 minutes, extracted with $CHCl_3$ (1.25 mL) and H_2O (1.25 mL), the aqueous phase is discarded while the $CHCl_3$ phase containing the lipids is retained for analysis. Numerous variations of the Bligh and Dyer method have been developed to improve the extraction efficiencies of certain lipid classes; however, a mixture of $CHCl_3$:MeOH (2:1, v/v) efficiently extracts most lipid classes [70].

The preparation of fatty acid derivatives may require a hydrolysis step necessary to release fatty acids from lipids [19, 71]. Fatty acids bound to lipids via an ester bond (e.g., phospholipids) can be released using either acid or base-catalyzed hydrolysis while fatty acids bound via amide bonds (e.g., ornithine lipids) can only be released using acid-catalyzed hydrolysis (Figure 1.4a). TMS esters are prepared by reaction of the free fatty acids with a silylation reagent such as N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) [72]. FAMEs can be prepared from free fatty acids by acid-catalyzed methylation or by reaction with diazomethane but they can also be prepared directly from lipids by transmethylation (Figure 1.4b-c). Transmethylation reactions can be either acid or base-catalyzed. Water, if present, can act as a nucleophile and compete with methanol to produce free fatty acids thus reducing the yield of methyl esters [71]. FAMEs can also be directly generated from bacterial cells without lipid extraction via transmethylation; these procedures are known as "in situ" methods [73]. "In situ" procedures require the use of dried bacterial cells to avoid the interference of water [74].

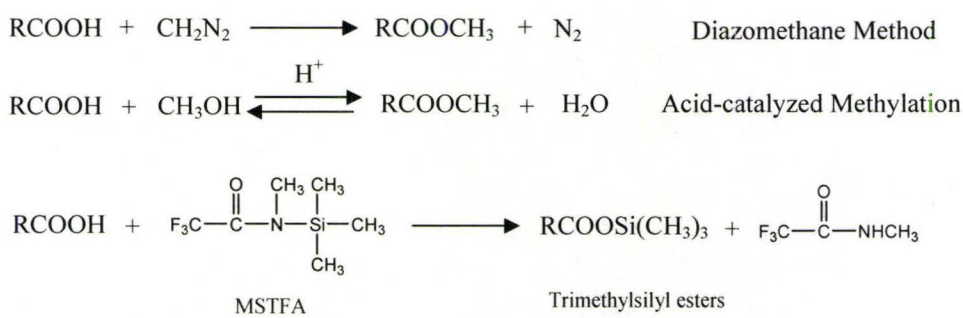
Acid-catalyzed procedures for the methylation of free fatty acids employ either 5% anhydrous hydrogen chloride or boron trifluoride in methanol, while sulfuric acid in methanol is employed for transmethylation reactions [71]. The

most popular reagent for transmethylation is sulfuric acid in methanol since it reacts with both free fatty acids and lipids and it is easy to prepare [73]. Base-catalyzed transmethylation reactions are conducted using sodium methoxide or potassium methoxide in methanol [71].

(a) Hydrolysis of Lipids



(b) Derivatization Reactions of Fatty Acids



(c) Transmethylation Reactions

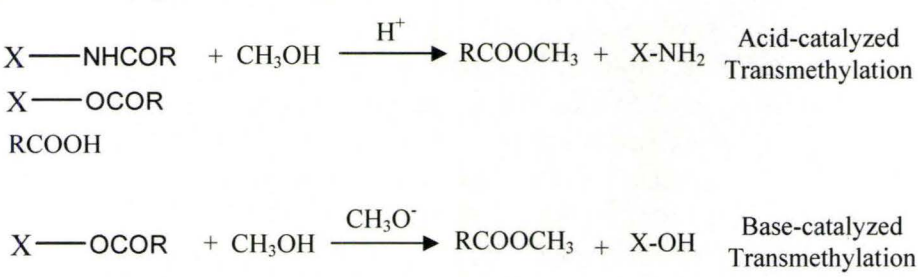


Figure 1.4. Three derivatization approaches used for fatty acid analysis by gas chromatography.

An advantage of acid-catalyzed transmethylation reactions is that lipids, free fatty acids and amide lipids react to produce FAMEs directly [73]. However, acid-catalyzed transmethylations require heat and long reaction times (>3 h). Unfortunately under these conditions, cyclopropane-containing fatty acids and hydroxy fatty acids can be degraded [73]. Alternatively, base-catalyzed transmethylations only require minutes for completion and do not affect sensitive species such as cyclopropane fatty acids. A shortcoming of this approach is that free fatty acids and amide lipids do not react [71]. In addition, the presence of traces of water will generate hydroxide ions which can hydrolyze methyl esters to carboxylate ions [71, 73]. While these methodologies have been extensively validated and tested for large sample sizes (up to gram amounts) mostly for food industry applications, the analysis of small amounts of lipids (micrograms) still remains a challenge. When working with small lipid amounts, traces of water present in solvents or glassware could significantly reduce the yield of fatty acid derivatives [71]. Overall, these methods are tedious and laborious due to multiple extraction steps and the handling of relatively large volumes of reagents and solvents (> 1 mL).

Currently, pyrolytic methods are very promising for the analysis of bacterial fatty acids. With these methods, lipids can be transesterified directly from bacterial cells with minimum sample preparation an require small (μg) sample amounts [75, 76]. The most common reagents used by these procedures are tetramethylammonium hydroxide or trimethylsulfonium hydroxide [19]. However, the application of pyrolytic methods as routine procedures is limited since special modifications are required to the injection system of GC instruments and samples need to be loaded manually.

In summary, the analysis of bacterial fatty acids presents special challenges associated with the presence of hydroxyl and cyclopropane-containing fatty acids [19, 74]. The use of acidic conditions is not recommended for the analysis of bacterial lipids since cyclopropane fatty acids can be degraded [19, 74, 77]. However, numerous applications continue to use acidic reagents for the analysis of bacterial fatty acids [78-81]. Therefore, one goal of this research was to compare and evaluate common literature procedures to the analysis of bacterial lipids of *S. meliloti* and to determine the optimal method. Furthermore, the development of a robust small-scale methodology that required minimal sample handling and small reagent volumes was also a motivation. Since sample sizes and reagent volumes would be significantly reduced in a micro-scale procedure, the occurrence of side reactions such as hydrolysis and incomplete derivatizations were significant concerns. In order to address these problems, adequate quality control protocols need to be designed to monitor recoveries, completion of reactions and losses due to side reactions.

1.3.2. Intact Lipid Analysis

Most methods for intact lipid analysis require the fractionation of lipid extracts into lipid classes by a chromatographic method followed by detection with mass spectrometry using ESI or MALDI [22, 24]. HPLC is the most popular form of chromatography for intact lipid analysis, though numerous applications continue to employ TLC and column chromatography (CC) for the fractionation of lipid extracts [24]. Recently, it has been demonstrated that phospholipids can be analyzed directly from crude lipid extracts without the need for chromatography using a combination of positive and negative ionization electrospray ionization (ESI/MS) and tandem mass spectrometry methods; known as "shotgun lipidomics" [82]. The next sections will offer an overview of the most important aspects of intact lipid analysis. First, the application of ESI and MALDI in lipid analysis will be discussed with emphasis on the advantages and limitations of each technique. Second, the fragmentations of phospholipids under collision-induced dissociation (CID) will be discussed since tandem mass spectrometry is of vital importance in the analysis of complex lipid mixtures. Third, chromatography-based methods and shotgun lipidomics approaches will be summarized.

1.3.2.1. Ionization Techniques in the Analysis of Intact Lipids

Approximately a decade ago, FAB was the most common ionization technique for lipid analysis. However, the application of FAB to lipid analysis decreased markedly with the introduction of ESI and MALDI. Lipid analysis using FAB required large sample amounts in comparison with ESI and MALDI, and undesired lipid fragmentations occurred [83]. Due to the limited application of FAB in lipid analysis at present, this ionization technique will not be discussed here. MALDI is widely employed in lipid analyses thus by far ESI is currently the most common MS-based technique. A short description of the principles of MALDI and ESI will be provided, as well as their advantages and limitations in lipid analysis.

Matrix-assisted Laser Desorption Ionization

In MALDI, the sample is mixed with an ultraviolet-absorbing matrix and deposited on a plate. Upon irradiation with a pulsed UV-laser, the analyte and matrix molecules are converted into the vapor phase. Analyte ions are produced by protonation or deprotonation due to collisions with the matrix ions to form positively or negatively charged analyte ions. The matrix in MALDI has two main functions: (i) to absorb energy from the laser beam and transfer it to the analyte, and (ii) to act as a solvent for the sample to minimize the analyte-analyte interactions [84]. The mechanism for ion formation in MALDI is still not completely understood [85]. The pulsed nature of MALDI to generate ions can be easily orchestrated with the pulsed nature of scanning of time-of-flight (TOF) mass analyzers, thus MALDI-TOF are the most common instruments [84]. Not surprisingly, most phospholipid applications have been performed with MALDI-

TOF instruments [83]. In the last five years, applications to lipid analysis have increased thanks to the development of hybrid instruments such as MALDI/TOF/TOF and MALDI/QqTOF [20, 83, 86, 87].

A homogeneous mixture of sample and matrix, is the most important variable to achieve good and reproducible spectra. The most common matrix employed in phospholipid analysis is 2,5-dihydroxybenzoic acid (DHB) [24]. In positive ionization, DHB produces spectra where phospholipids are observed as a mixture of $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$ ions as well as adducts with the matrix. In negative ionization, acidic lipids are detected as their $[M-H]^-$ ions but sensitivity is considerably lower than for lipids in positive ionization [83]. In complex lipid mixtures, the presence of lipid classes such as PC and SM causes ion suppression of other classes [83, 88]. In general, MALDI produces complicated spectra caused by the presence of multiple phospholipid adducts, background ions from the matrix and phospholipid-matrix adducts [83, 89]. The addition of CsCl to DHB has been employed as a strategy to obtain simpler spectra in positive ionization by affording mostly $[M+Cs]^+$ adducts of phospholipids [88]. Other matrices have also been explored as alternatives to DHB; for example, *p*-nitroaniline (PNA) facilitated analyses in both ionization modes and provided good sensitivity for $[M-H]^-$ ions of acidic lipids [88]. Other options such as ionic liquid matrices have been explored; these matrices produced good quality mass spectra of phospholipids with significantly less matrix background or phospholipid-matrix adducts in comparison to DHB [90].

Lipid analyses using MALDI/TOF are rapid and require minimal sample preparation [83]. Phospholipids have been analyzed directly from intact bacterial cells; however best results are obtained using lipid extracts rather than with whole cells [83, 89]. Lipid analysis using MALDI/TOF has two disadvantages; (i) first, poor reproducibility since sample-matrix mixtures should yield identical ionization of lipids, thus lipid quantitation is still difficult. (ii) Second, the analysis of complex samples with multiple lipid classes is difficult due to the overlap of $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, lipid-matrix adduct ions and high matrix background [83].

MALDI/TOF/TOF and MALDI/QqTOF instruments have extended the capabilities of MALDI to the analysis of complex lipid mixtures since accurate mass measurements and MS/MS experiments can be performed [20, 91, 92]. One disadvantage of these types of instruments is that neutral loss scans are difficult to perform. Under MS/MS conditions, phospholipids yield unique neutral losses which are typical of polar head group thus neutral loss scans are extremely useful for the analysis of complex samples [20, 23]. Finally, the high cost of these instruments at present limits their use for routine applications.

Electrospray Ionization

In ESI, a solution is infused through a capillary which is held at high potential producing a spray of highly charged droplets. These droplets are reduced in size by solvent evaporation and by Coulomb explosion due to the high charge

density to afford desolvated ions in the vapor phase [93]. Paradoxically speaking, ions are not produced in ESI but rather transferred from solution to the vapor phase; however, ions can be formed from neutral molecules in ESI by electrochemical oxidation since the capillary acts as an electrolytic cell [93].

Phospholipids normally exist in solution as ions and consequently are readily detectable under ESI conditions. Zwitterionic lipids such as PCs and PEs are detected in the positive ionization mode as a mixture of $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$ ions [22, 23]. Thus, the presence of multiple adducts per lipid component in samples that contain multiple lipid classes affords complex mass spectra. To reduce this complexity, the addition of Li^+ or Na^+ ions has been recommended to produce only $[M+Na]^+$ or $[M+Li]^+$ adducts similar to the addition of Cs^+ in MALDI [94]. Anionic lipids such as PGs, CLs and PAs form readily $[M-H]^-$ ions and are easily detected in the negative ionization mode. Therefore, an advantage of ESI over MALDI is greater sensitivity for the analysis of acidic lipids in the negative ionization mode. Overall, ESI is considered to be more sensitive compared to MALDI for the analysis of phospholipids [14, 24].

The sensitivities of detection for different lipid classes vary in ESI. Usually, sensitivity in the positive ionization mode is $[PC+H]^+ > [PE+H]^+$ while in the negative ionization mode is $[PG-H]^- > [PI-H]^- > [PA-H]^- > [PS-H]^- > [PE-H]^- > [CL-2H]^{-2}$ [94]. Response factors within lipid classes decrease with the length of the fatty acyl chains [94]. In addition, unsaturations in the fatty acyl chains result in increased response factors while this is not observed in MALDI [83, 94]. The response factors within lipid classes do not vary as a function of fatty acyl chain and unsaturation in dilute lipid solutions (0.1-10 pmol/μL per lipid) [94, 95]. Thus for quantitative analyses, the use of dilute lipid solutions or the inclusion of multiple internal standards for each lipid class has been recommended [94]. However, the inclusion of multiple internal standards per lipid class may not be a practical approach for the analysis of complex lipid samples. For applications in which a control sample is compared to a treated sample (stressed sample or mutant), the use of one internal standard per lipid class is appropriate [96].

The most common combinations of ESI and mass analyzers include triple quadrupole instruments (QqQ) or single quadrupole in tandem with a time-off-flight mass analyzer (QqTOF). In addition, ESI is compatible with HPLC systems which have also contributed to the extensive application of ESI in lipid analysis [24].

1.3.2.2. Fragmentation of Phospholipids using Collision Induced Dissociation

Phospholipids upon CID produce characteristic fragment ions corresponding to the polar head group and the fatty acyl chains [23]. The fragmentations of phospholipids have been well characterized; this section will provide the most relevant aspects of MS/MS of those phospholipid classes which are found in bacterial membranes mainly PCs, PEs and PGs. The fragmentations of non-phosphorus containing lipids have hardly been studied since analytical

standards are not commercially available. The characteristic fragmentations of phospholipids under MS/MS are used as a strategy for the analysis of complex mixtures containing multiple lipid classes. Three types of MS/MS experiments are employed:

A. Product ion scan or product ion scan

A precursor ion of a given m/z value is selected in the first mass analyzer, fragmented upon collision with an inert gas in the collision cell and the fragment or product ions are detected by the second mass analyzer [84]. This type of MS/MS experiment can be conducted using a number of instruments e.g., QqQ, QqTOF and TOF/TOF. Product ion scans are also possible using MALDI/TOF instruments since a precursor ion can undergo metastable dissociation in a process known as post-source decay [84].

B. Precursor ion scan or precursor ion scan

The first mass analyzer is scanned while the second one is at a fixed m/z value for a particular fragment ion; in this case the first mass analyzer is interrogated for precursor ions that upon fragmentation yield the selected fragment ion [84]. These types of experiments can be conducted with QqQ, QqTOF and TOF/TOF instruments.

C. Constant neutral loss scan

The first and third mass analyzers are scanned simultaneously at a constant mass difference. Using this type of experiment, information regarding precursor ions that undergo a particular neutral loss (NL) upon fragmentation can be obtained. The three MS/MS experiments described can be performed easily with QqQ instruments and in particular NL scans are unique to these instruments [20]. NL scans are key experiments for the analysis of samples containing multiple lipid classes [20, 23].

Anionic Lipids

CID of $[M-H]^-$ ions formed from acidic lipids (e.g., PGs, PAs, lysoPAs) under ESI conditions produce characteristic product ion spectra that contain a common fragment ion at m/z 153 ($[HO_3POCH_2C(OH)CH_2]^-$) corresponding to the polar head group and intense carboxylate fragment ions [23]. Thus, a precursor ion scan of m/z 153 is a useful strategy to identify PGs, PAs, LysoPAs and CLs in complex mixtures [14]. Alternatively, in negative ionization precursor ion scans targetted to specific $[RCOO]^-$ ions can be used to identify lipids that contain a particular fatty acid [14].

The fragmentation of $[PG-H]^-$ ions under CID has been studied in detail by Hsu and coworkers [97]. The product ion spectra of PGs contain intense $[RCOO]^-$ ions and low abundance $[PG-H-RCOOH]^-$ and $[PG-H-RCH=CHCO]^-$ fragment ions derived from the neutral losses of the fatty acids and the ketenes, respectively (Figure 1.5). Fragment ions $[PG-H-RCOOH]^-$, $[PG-H-RCH=CHCO]^-$ and

[RCOO]$^-$ originated from the loss of fatty acids in the *sn*-2 position are sterically favored and consequently more abundant than the equivalent ions originated from *sn*-1 fatty acids. Since *sn*-2 [RCOO]$^-$ ions are more abundant than *sn*-1 [RCOO]$^-$ ions, the fatty acids relative position in PGs can be assigned [97]. The fragmentation mechanism is complex and involves both charge remote fragmentations and charge direct fragmentations [97]. In charge remote fragmentations, the hydrogen atoms of the glycerol backbone participate, while in charge direct fragmentations the exchangeable hydrogen atoms of the phosphate group are involved (Figure 1.6). Carboxylate ions are formed by nucleophilic attack of the anionic phosphate to the *sn*-1 or *sn*-2 carbon of the glycerol backbone to expel *sn*-1 [RCOO]$^-$ or *sn*-2 [RCOO]$^-$ ions. The same phenomenon is observed in product ion spectra of PAs where *sn*-2 [RCOO]$^-$ ions are more intense than *sn*-1 [RCOO]$^-$ ions [98].

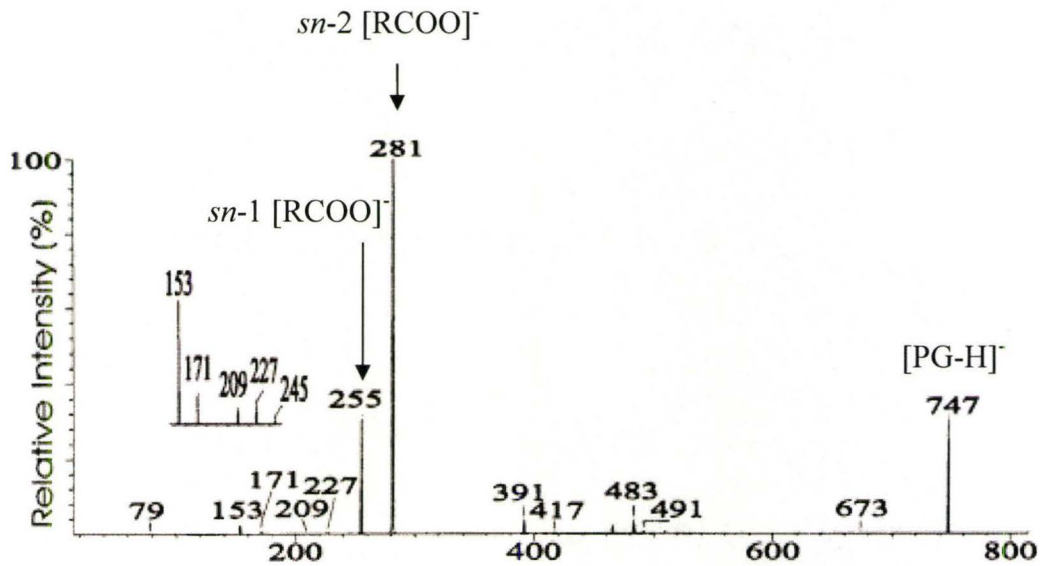


Figure 1.5. Product ion spectrum in negative electrospray ionization of 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-1-glycerol (PG(16:0/18:1)) [97].

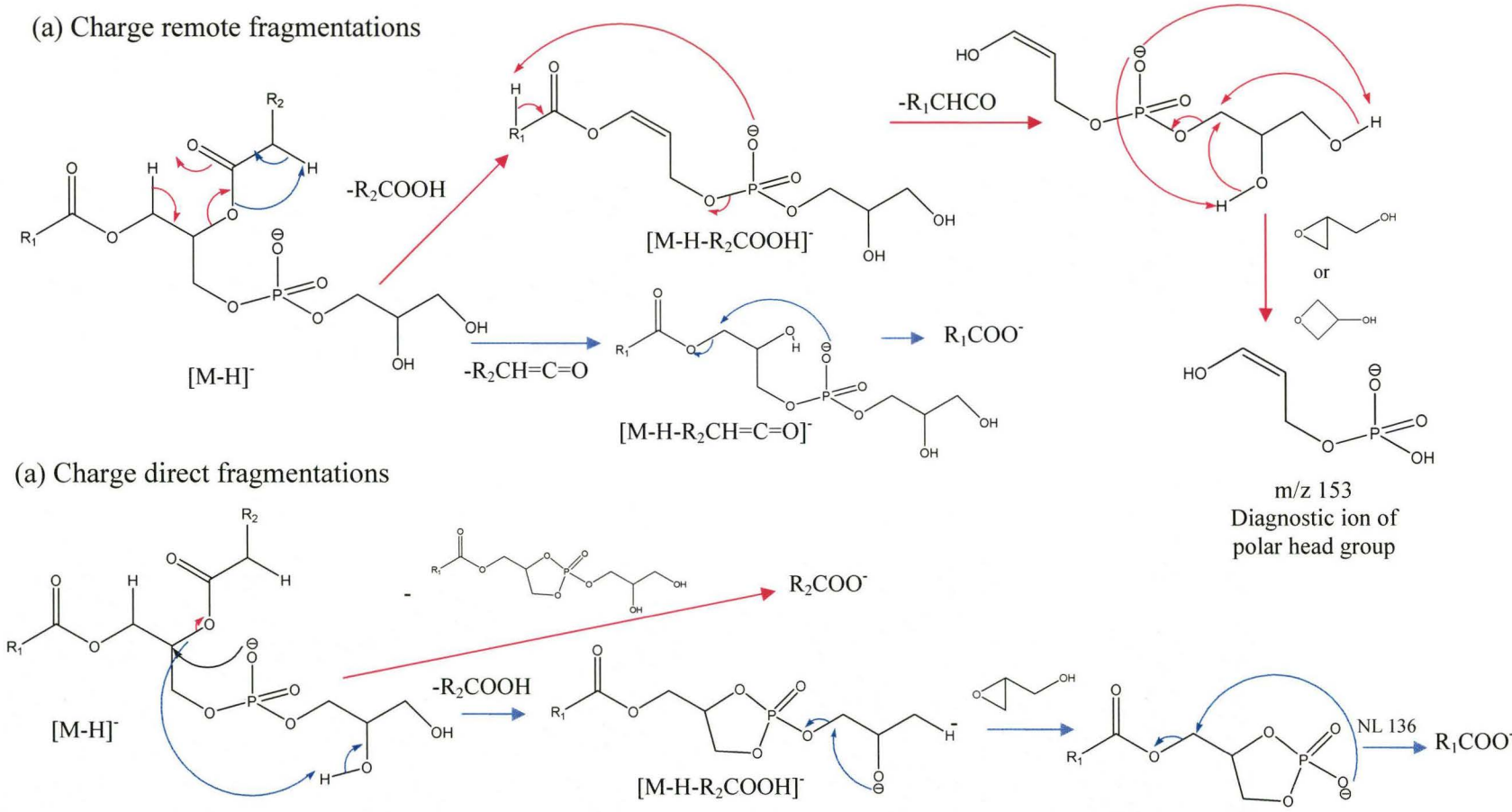


Figure 1.6. (a) Charge remote and (b) charge direct fragmentation pathways of $[PG-H]^-$ under CID to produce $[M-H-R_2COOH]^-$, $[M-H-R_2CH=C=O]^-$ and $[RCOO]^-$ ions [97].

Zwitterionic Lipids

PCs and PEs lipids are generally analyzed as their $[M+H]^+$ ions in positive ionization mode. The product ion spectra of $[M+H]^+$ ions of PCs are dominated by an intense fragment ion at m/z 184 which is characteristic of the phosphocholine head group (Figure 1.7) [23]. CID of $[M+H]^+$ ions of PEs produce product ion spectra dominated by a NL of 141 mass units corresponding to the loss of the polar head group phosphoethanolamine group $[(OH)_2P(O)O(CH_2)_2NH_2]$ [23]. Thus, precursor ion scans of m/z 184 and NL scans of 141 mass units are common tactics for the analysis of PCs and PEs, respectively [23].

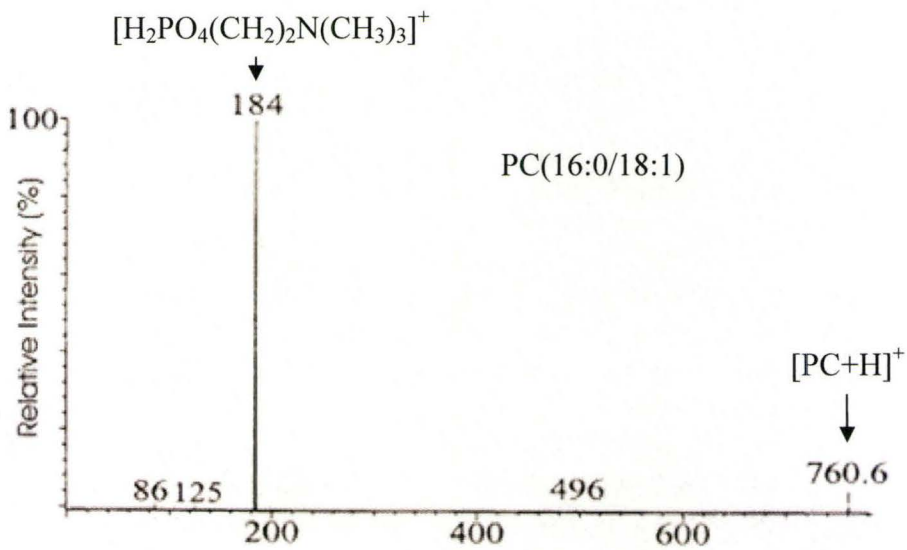


Figure 1.7. Product ion spectrum in positive electrospray ionization of $[M+H]^+$ adduct formed by 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine [101].

However, product ion spectra of $[M+H]^+$ ions formed by PCs and PEs are uninformative regarding fatty acid composition and their relative positions in the glycerol backbone [20, 23, 99]. As an alternative, PEs can be analyzed in the negative ionization mode as $[M-H]^-$ ions. $[PE-H]^-$ ions fragment under CID to produce mainly fragment ions at m/z $[RCOO]^-$ ions and m/z 153 similarly as discussed for PGs [100].

In the product ion spectra of $[PE-H]^-$, $[RCOO]^-$ fragment ions originated from the loss of fatty acids from the *sn*-2 position are more intense than those ions formed by losses of the *sn*-1 fatty acids. Therefore, the identity of the fatty acyl substituents and their relative positions in PEs can be determined [100]. PEs form weak $[M-H]^-$ ions in negative ESI; consequently, in complex lipid mixtures the

quality of product ion spectra might not be sufficient to clearly identify fatty acyl groups and their relative positions.

Fragmentations of phospholipid adducts with metals ions including Na^+, K^+ and Li^+ in ESI have been explored in the search for informative mass spectra of both polar head group and fatty acyl substituents [102]. The product ion spectra of $[PC+Na]^+$ ions produced mainly fragment ions at m/z $[PC+Na-59]^+$ due to the NL of triethylamine but were uninformative regarding fatty acyl groups. Conversely, the lithiated adducts of PCs and PEs, produce product ion spectra that contain diagnostic fragment ions of both, the polar head group and fatty acyl substituents [22, 23].

Fragmentation of $[M+Li]^+$ adducts of phospholipids under CID conditions has been extensively studied by Hsu and Turk [101, 103, 104]. The product ion spectra of $[PC+Li]^+$ ions produced abundant fragment ions at m/z $[PC+Li-183]^+$, $[PC+Li-189]^+$ and $[PC+Li-59]^+$ due to the NL of the protonated $[HPO_4(CH_2)_2N(CH_3)_3]$, lithiated phosphocholine group $[LiPO_4(CH_2)_2N(CH_3)_3]$ and triethylamine $[N(CH_3)_3]$, respectively (Figure 1.8). Fragment ions at m/z $[PC+Li-189]^+$ are characteristic of all lithiated PCs and are formed by the sequential losses of triethylamine (59 mass units) and lithium *O,O'*-dimethylenephosphate (130 mass units) (Figure 1.9a). In addition, diagnostic fragment ions of fatty acid composition were also observed at m/z $[M+Li-59-RCOOH]^+$, $[M+Li-RCOOH]^+$ and $[M+Li-59-RCOOLi]^+$ (Figure 1.8). These fragment ions, particularly ions of the type $[M+Li-59-RCOOH]^+$, originated from the loss of fatty acids in the *sn*-1 position were more abundant than fragment ions derived from losses of the *sn*-2 fatty acids. This peculiar feature of product ion spectra of lithiated PCs facilitates identification of fatty acids and their relative positions in lipids [99, 101]. The mechanism for the formation of these ions involved a nucleophilic attack on the *sn*-1 carbon or *sn*-2 carbon of the glycerol backbone to expel *sn*-1 RCOOH or *sn*-2 RCOOH, respectively (Figure 1.9b) [101].

Similarly to PCs, fragmentation of $[PE+Li]^+$ ions produce intense fragment ions at m/z $[M+Li-43]^+$, $[M+Li-141]^+$ and $[M+Li-147]^+$ corresponding to the neutral losses of $NH(CH_2)_2$, $(OH)_2P(O)O(CH_2)_2NH_2$ and $(LiO)(OH)P(O)O(CH_2)_2NH_2$, respectively [103]. These ions are characteristic to lithiated PEs thus the use of NL scans of 43, 141 and 147 has been suggested to identify these lipids in complex lipid mixtures. The product ion spectra of $[PE+Li]^+$ also produced fragment ions informative of the fatty acyl chains at m/z $[PE+Li-43-RCOOH]^+$ and $[PE+Li-RCOOH]^+$. These fragment ions were more abundant for *sn*-1 fatty acids than for *sn*-2 fatty acids which allowed identification of the relative positions of fatty acyl groups in PEs [103].

The fragmentations of non-phosphorus containing lipids, such as OLs and TMHSs, are not well studied due to the lack of analytical standards. OLs have been detected as their $[M+H]^+$ ions in positive electrospray ionization and under CID conditions typically produce an intense fragment ion at m/z 115 [51, 105, 106]. TMHSs have been detected also in positive ionization as $[M+H]^+$ ions,

however given their similarity to PCs, most likely these lipids may also form adducts with metal ions. Under CID, $[M+H]^+$ adducts of TMHSs have been reported to produce fragment ions at m/z 59 due to the loss of $N(CH_3)_3$ and neutral losses of the fatty acids [51].

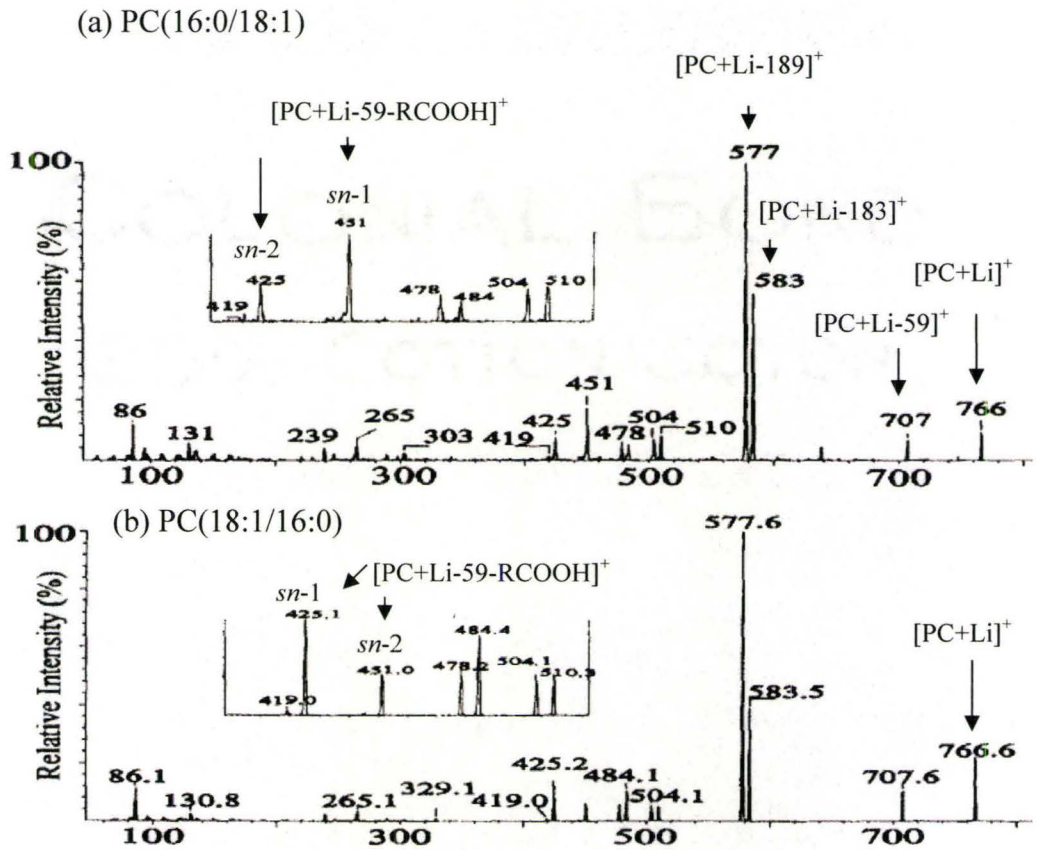


Figure 1.8. Product ion spectra of $[M+Li]^+$ adducts of (a) 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine and (b) 1- oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine [99].

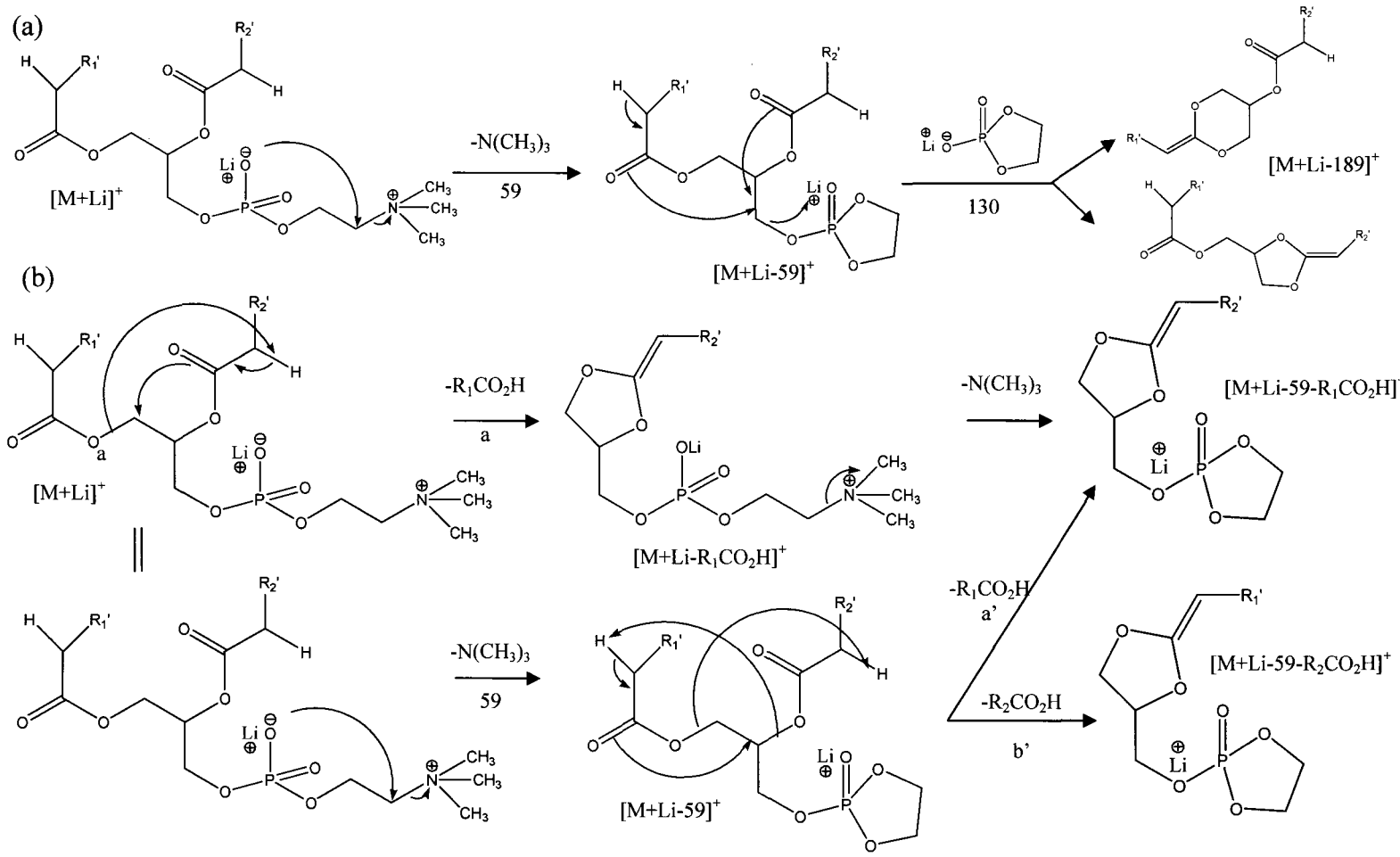


Figure 1.9. Fragmentation of lithiated PC under CID conditions [101].

1.3.2.3. Chromatographic Methods

Several types of chromatographic techniques have been applied to lipid analyses [24, 107]. TLC is a simple and inexpensive technique that can separate a wide diversity of lipids. The historical use of TLC in lipid separations continues to the present day [108]. For instance, the existing literature methods for the analysis of non-phosphorus containing lipids such as SLs, TMHSs and OLs, are TLC-based protocols [105, 109-114]. High performance thin layer chromatography (HPTLC), particularly two dimensional HPTLC (2D-HPTLC), can separate a wide array of lipid classes and is still widely employed [24, 108]. Geiger and workers were able to separate eight lipid classes in lipid extracts of *S. meliloti* using a 2D-HPTLC protocol [51]. Typically, lipid extracts are applied on to silica gel plates and developed in the first dimension using a solvent system of $CHCL_3$:MeOH:H_2O (14:6:1, v/v). TLC plates are rotated 90° and developed in the second dimension using a mixture of $CHCl_3$:MeOH:HAc(conc) (13:5:2, v/v) [115]. Lipid analysis is generally conducted by scrapping off the TLC spot, extraction of the silica with $CHCl_3$:MeOH and analysis of the lipid extract by ESI/MS or MALDI/MS [113, 116]. In order to determine the fatty acid distributions in lipid classes, the extract obtained from a TLC spot is hydrolyzed to release the free fatty acids which are further derivatized for GC/MS analysis [109, 110]. Open column chromatography procedures are still employed mainly as a preliminary fractionation in combination with TLC or HPLC [70, 113].

Drawbacks of TLC include ; (1) oxidation and degradation of lipids on the plate by contact with air; (2) poor recoveries of lipids since TLC spots must be scrapped off and extracted for analysis; (3) irreproducibility caused by changes in solvent equilibration, temperature and humidity; and (4) the inability of TLC to be used for high throughput analyses [23, 24]. To improve recoveries and increase throughput of analyses, direct analysis of lipids on TLC plates has been performed using MALDI/TOF [117].

HPLC is by far the most common chromatographic technique used in lipid analysis since it does not have the difficulties of TLC-based methodologies. HPLC methods require small sample amounts and lipids are not exposed to the environment, minimizing their oxidation [24]. Lipid separations are conducted using both normal phase (NP) and reverse phase (RP) chromatography. In NP-HPLC, lipids are separated on the basis of polarity which is determined by the polar head groups of the different lipids classes. Separation occurs by lipid classes normally in the elution order of PGs, CLs, PEs and PCs [24, 118]. In RP-HPLC, lipids are separated on the basis of their hydrophobic character which is determined by the fatty acyl chains, allowing for the resolution of molecular lipid species [24, 119]. However, when multiple lipid classes are present, PCs and PEs species with various fatty acyl combinations usually coelute [23]. Therefore for efficient separation of both lipid classes and their individual molecular species, typically NP-HPLC or TLC are used in combination with RP-HPLC [23, 24, 120].

HPLC is of great utility in lipid analysis however for the analysis of complex samples containing multiple lipid classes considerable overlap of molecular species still occurs [23, 24, 119]. For efficient separation of lipid classes and individual molecular species, complicated and long separation schemes are required. In most cases, tandem mass spectrometry is needed to differentiate individual molecular species in which case the need for complicated chromatographic protocols is questionable [119-121]. Chromatography-based methodologies are laborious and long; separation schemes typically exceed 60 minutes per sample [24, 120]. In addition, chromatography has been shown to affect molecular distributions of lipids and reduce recoveries by 30% or 50% for HPLC and TLC procedures, respectively [122].

1.3.2.4. Shotgun Lipidomics Methods

Shotgun lipidomics approaches employ the selective ionization of lipids under ESI conditions and their characteristic fragmentation patterns upon CID; thus lipids can be directly analyzed from crude lipid extracts without chromatography using a combination of positive and negative ionization ESI/MS and MS/MS [82]. There are three types of approaches to analyze lipids directly from crude extracts:

A. Multidimensional mass spectrometry

This approach involves a combination of MS/MS techniques: (i) multiple neutral loss scans to detect lipid classes, (ii) multiple precursor ion scans to detect lipids that contain specific fatty acyl chains and (iii) product ion scans to identify the relative positions of fatty acids (*sn*-1 and *sn*-2) in lipids [82]. This approach produces simplified mass spectra, reduces noise and low abundance lipid components can be detected. However, fragmentation of lipids under MS/MS is different for lipid classes and is highly dependent on collision energy [82]. This approach can be easily implemented for routine applications using a triple quadrupole mass spectrometer which is a relatively inexpensive instrument. In addition, multiple NL scan experiments can be implemented with these instruments allowing the analysis of isobaric species from different lipid classes.

Most methodologies detect PCs and PEs as their $[M+H]^+$ adducts using precursor ion scans of m/z 184 and neutral loss scans of 141 while PGs, PAs and CLs are analyzed as $[M-H]^-$ ions using precursor ion scans of m/z 153 [21]. Another approach is multiple precursor ion scanning of carboxylate ions of naturally occurring fatty acids which can be implemented using QqTOF instruments. This technique allowed the analysis of six phospholipid classes and more than 200 molecular species in crude lipid extracts [123]. One problem with this type of approach is the generation of large volumes of data that requires specialized software packages are required for data processing and interpretation. Another difficulty is the analysis of lipid classes that do not readily form $[M-H]^-$ ions in negative ionization such as PCs, since lipid detection is based on the presence of $[RCOO]^-$ ions formed upon CID of $[M-H]^-$ adducts.

B. High mass resolution analyses

In this approach, lipid analysis and detection is conducted based on the measurement of high accurate m/z values usually using Fourier Transform Mass Spectrometry (FTMS) [124, 125]. Using this approach hundreds of lipid species can be identified, however isobaric lipid species cannot be differentiated. In addition, FTMS instruments are relatively expensive instruments.

C. Intrasource separation

A combination of positive and negative ESI/MS and MS/MS is employed. Complex lipidomes can be analyzed using small lipid concentrations (100 μL solutions with lipid concentrations of ≤ 1 pmoL/mL) in 30 minutes. Intrasource separation of lipids is a simple strategy which has been pioneered by Han and Gross [82, 126, 127]. In a first stage, anionic phospholipids (PGs, CLs, PAs and PEs) are analyzed in negative ionization as their $[M-H]^-$ and product ion spectra or precursor ion spectra of common $[RCOO]^-$ are obtained to identify fatty acyl substituents. LiOH is added to analyze PCs as $[M+Li]^+$ adducts using neutral loss scans of 59 , 183 and 189 [14]. In positive ionization, $[M+H]^+$ and $[M+Na]^+$ adducts coexist with $[M+Li]^+$, thus the addition of at least one internal standard per lipid class is crucial [14].

In general, shotgun lipidomics has allowed the analysis of over 20 lipid classes, hundreds of molecular species and more than 95% of the lipidome without the need of chromatography [14, 82]. These methods are simple and offer the possibility of automation for high-throughput analyses. The use of internal standards is a key requirement for accurate lipid quantitation. However, the analysis of very low abundance lipids might require concentration of the target analytes or in some cases the development of specific methods [14]. Shotgun lipidomics approaches have been applied for the analysis of lipid classes found in eukaryotic cells (e.g., phospholipids, spingolipids) and have yet to be applied to the analysis of non-phosphorus containing lipids of bacterial origin such as SLs, TMHSs and OLs; this is most likely due to the lack of analytical standards of these lipid classes.

Due to the great potential of shotgun lipidomics for the comprehensive analysis of lipids, one of the goals of this research was to develop a shotgun lipidomics approach for the analysis of lipids in *S. meliloti*. In addition, we pursued to extend shotgun lipidomics principles and strategies to analyze two additional phospholipid classes (MMPEs and DMPEs) as well as non-phosphorus containing lipids which are important lipid components in *S. meliloti*.

1.4. Biosynthesis of Fatty Acids and Lipids in Bacteria

1.4.1. Fatty Acid Biosynthesis

Fatty acid biosynthesis in bacteria has been the subject of intensive research as potential targets for the development of antibiotics [128, 129]. Fatty acid biosynthesis has hardly been studied in *S. meliloti* or in *Rhizobiaceae* and it

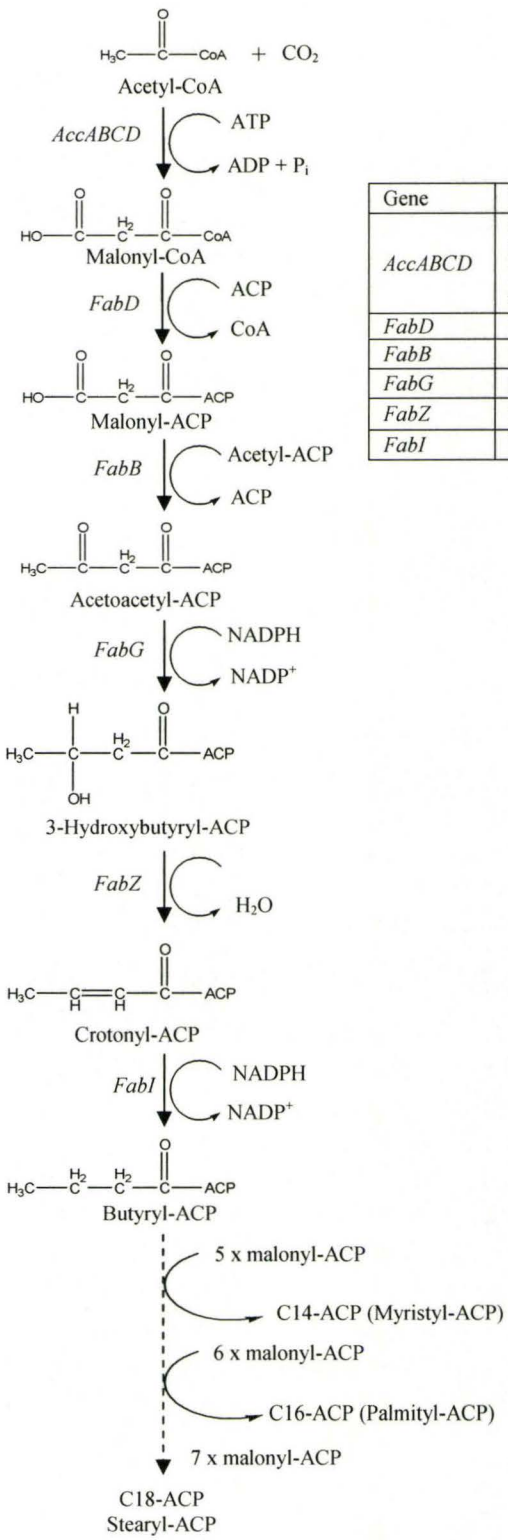
is thought to occur similarly to *E. coli* [40]. Fatty acid biosynthesis in *E. coli* occurs in two stages: (i) initiation and (ii) elongations reactions (Figure 1.10).

The first reaction in the initiation stage involves carboxylation of acetyl-coenzymeA (CoA) to produce malonyl-CoA. Next, the malonyl group of malonyl-CoA is transferred to acyl carrier protein (ACP) producing malonyl-ACP. The elongation cycle is initiated by the condensation of the acyl-ACP chain with malonyl-ACP catalyzed by the elongation-condensing enzymes (FabB or FabF). Acetoacetyl-ACP is reduced to form 3-hydroxybutyryl-ACP which is further dehydrated to enoyl-ACP to produce butyryl-ACP [128, 129]. The process continues by addition of malonyl-ACP to butyryl-ACP and successive cycles of the reactions previously described to form fatty acids of various chain lengths.

Cyclopropane Fatty Acids

Cyclopropane fatty acids (CFAs) commonly form part of bacterial lipids; these fatty acids occur as a postsynthetic modification of lipids in membranes during the stationary phase of growth [77]. Cyclopropane fatty acyl (CFA) synthases are enzymes that catalyze the addition of a methylene group from S-adenosylmethionine (AdoMet) across *cis* double bonds of monounsaturated fatty acyl chains in phospholipids (Figure 1.11) [77]; the CFA synthase of *E. coli* has been the most studied [130, 131].

The genome of *S. meliloti* contains two genes thought to code for CFA synthases. These genes have been annotated as *cfa1* and *cfa2*, but no genetic or metabolite analyses have been conducted to support this functional assignment. Three CFAs naturally occur in bacteria: *cis*-9,10-methylene hexadecanoic acid, *cis*-11,12-methylene octadecanoic acid and *cis*-9,10-methylene octadecanoic acid. These CFAs are synthesized by adding a methylene group across the double bonds of the fatty acyl chains in lipids that contain *cis*-9-hexadecenoic acid, *cis*-11-octadecenoic acid and *cis*-9-octadecenoic acid [77]. CFAs are minor components in *E. coli* where *cis*-9,10-methylene hexadecanoic acid (3.5%) is the most abundant of the CFAs [61, 79]. On the other hand, CFAs are abundant components of lipids in *S. meliloti*; *cis*-11,12-methylene octadecanoic acid is the major CFA while *cis*-9,10-methylene hexadecanoic acid is a minor component. *Cis*-11,12-methylene octadecanoic acid can account for more than 20% of total fatty acids in *S. meliloti* [63, 64].



Gene	Protein Name
AccABCD	Carboxyltransferase subunit (accA) Biotin carboxy carrier protein (accB) Biotin carboxylase (accC) Carboxyltransferase subunit (accD)
FabD	Malonyl-CoA:ACP transacylase
FabB	β-Keto-acyl-ACP synthase I
FabG	β-Keto-acyl-ACP reductase
FabZ	β-Hydroxyacl-ACP dehydrase
FabI	Enoyl-ACP reductase

Figure 1.10. Fatty acid biosynthesis in *E. coli*.

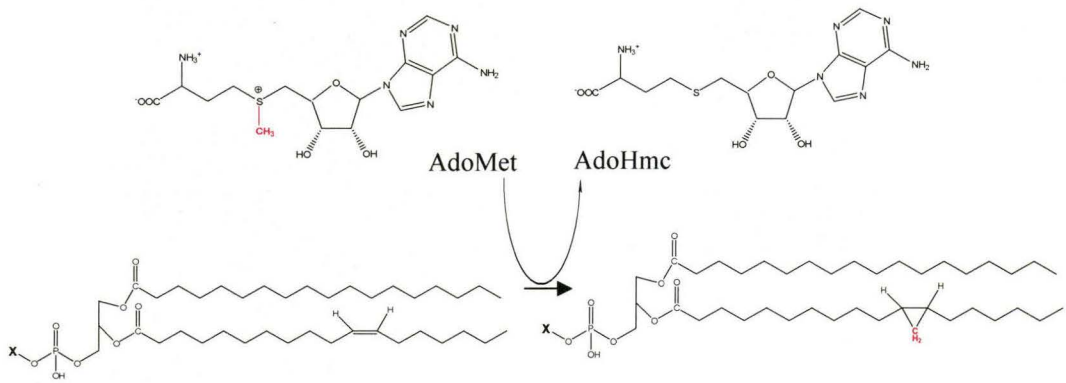


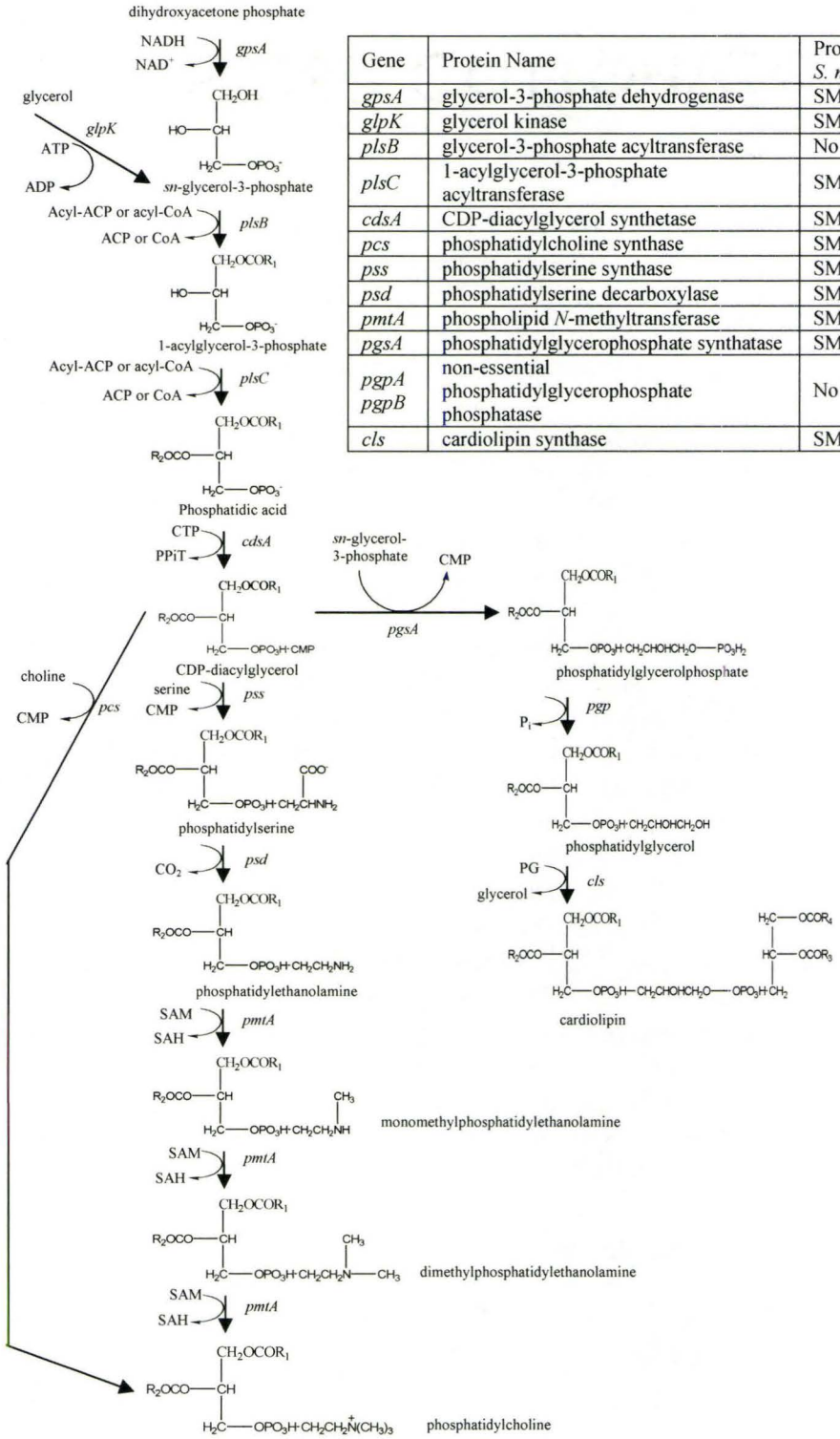
Figure 1.11. Cyclopropanation of phospholipids in bacteria. X: polar head group; AdoMet: S-Adenosyl-L-methionine; AdoHmc: S-Adenosyl-L-homocysteine; CFA: Cyclopropane fatty acid synthase.

1.4.2. Lipid Biosynthesis

Phospholipids

The model for phospholipid biosynthesis in *S. meliloti* is shown in Figure 1.12. This model is based mostly on the existence of genes in the genome of *S. meliloti* with homology to those genes that are involved in lipid biosynthesis in *E. coli* [30]. In *E. coli*, phospholipids are synthesized by modifications of the polar head group of phosphatidic acid (PA), making PA the key intermediate in the synthesis of phospholipids [132].

There are two pathways for the synthesis of PA: (i) the glycerol-3-phosphate pathway and (ii) the dihydroxyacetone phosphate pathway (Figure 1.12) [132]. In bacteria, PA biosynthesis occurs through the glycerol-3-phosphate pathway while the dihydroxyacetone phosphate pathway is restricted to yeast and mammalian cells. PA synthesis from glycerol-3-phoshate occurs in two steps; first, a fatty acyl residue is transferred to the *sn*-1 position in glycerol-3-phosphate catalyzed by glycerol-3-phosphate acyltransferase encoded by the *plsB* gene. In *E. coli*, PlsB has been studied extensively and it transfers preferentially saturated fatty acyl residues to the *sn*-1 position [132, 133]. In the second step, a fatty acyl residue is transferred to the *sn*-2 position of lysophosphatidic acid by 1-acylglycerol-3-phosphate acyltransferase encoded by the *plsC* gene to produce PA. PlsC has also been well studied in *E. coli* and it transfers unsaturated fatty acyl residues to the *sn*-2 position [134, 135]. This reactions are known as the PlsB/PlsC system.



Gene	Protein Name	Protein in *S. meliloti*
gpsA	glycerol-3-phosphate dehydrogenase	SMc03229
glpK	glycerol kinase	SMb21009
plsB	glycerol-3-phosphate acyltransferase	No homologue
plsC	1-acylglycerol-3-phosphate acyltransferase	SMc00714
cdsA	CDP-diacylglycerol synthetase	SMc02096
pcs	phosphatidylcholine synthase	SMc00247
pss	phosphatidylserine synthase	SMc00552
psd	phosphatidylserine decarboxylase	SMc00551
pmtA	phospholipid *N*-methyltransferase	SMc00414
pgsA	phosphatidylglycerophosphate synthatase	SMc00601
pgpA *pgpB*	non-essential phosphatidylglycerophosphate phosphatase	No homologue
cls	cardiolipin synthase	SMc02076

Figure 1.12. Model for phospholipid biosynthesis in *S. meliloti* [30].

However, the paradigm of *E. coli* is not universal since the genomes of most bacteria, including *S. meliloti*, do not contain homologues of the *plsB* gene of *E. coli* [30, 32, 136, 137]. Only recently, has an alternative pathway for PA biosynthesis called the PlsX/PlsY pathway been discovered [33, 138]. This system uses acyl-phosphate (Acyl-PO_4) intermediates derived from acyl-acyl-carrier protein (acyl-ACP) by PlsX and transferred to glycerol-3-phosphate by PlsY. Therefore it is most likely that *S. meliloti* employs the PlsX/PlsY system to synthesize lysophosphatidic acid since a homologue to *plsB* cannot be found in the genome. PlsC, on the other hand is universally present in bacteria and in *S. meliloti* SMc00714 protein (PlsC) is thought to code for a putative lysophosphatidic acyl transferase [30]. Recently, it has been demonstrated that some bacteria contain multiple acyl transferases with overlapping functions and lysophospholipid acyl transferases [32, 136, 139-142]. In *S. meliloti* SMc00714 (putative PlsC) has not been studied.

PA is activated to CDP-diacylglyceride by CdsA which further reacts with serine to produce phosphatidylserine by the action of phosphatidylserine synthase (Pss); the gene coding for Pss in *S. meliloti* has been characterized [143]. PE is formed by decarboxylation of phosphatidylserine by phosphatidylserine decarboxylase (Psd). PE is methylated three times using the methyl donor S-adenosyl-L-methionine (SAM) by phospholipid N-methyltransferases (PMTs) to afford MMPE, DMPE and PC. This sequence of reactions is known as the methylation pathway for PC biosynthesis and in *S. meliloti* the gene that codes for PMT (*pmtA*) has been studied [144]. Usually, bacteria can only synthesize PC via the methylation pathway, however *S. meliloti* can synthesize PC using a second pathway [145, 146]. In this alternate pathway, PC is synthesized by direct condensation of choline and CDP-diacylglycerol catalyzed by phosphatidylcholine synthase (Pcs) [147].

The anionic lipids, phosphatidylglycerol (PG) and cardiolipin (CL) are synthesized from CDP-diacylglyceride (Figure 1.12). Phosphatidylglycerolphosphate is formed by phosphatidylglycerophosphate synthatase (PgsA), PG is formed by release of phosphate from phosphatidylglycerolphosphate by phosphatidylglycerophosphate phosphatases (Pgp). Homologues for the *pgpA* and *pgpB* genes of *E. coli* cannot be found in *S. meliloti* as in the case of *plsB* [30].

Non-phosphorus Containing Lipids

Under P_i-limiting conditions *S. meliloti* synthesizes three non-phosphorus containing lipids: SLs, OLs and TMHSs [30]. Figure 1.13 shows the proposed models for their biosyntheses.

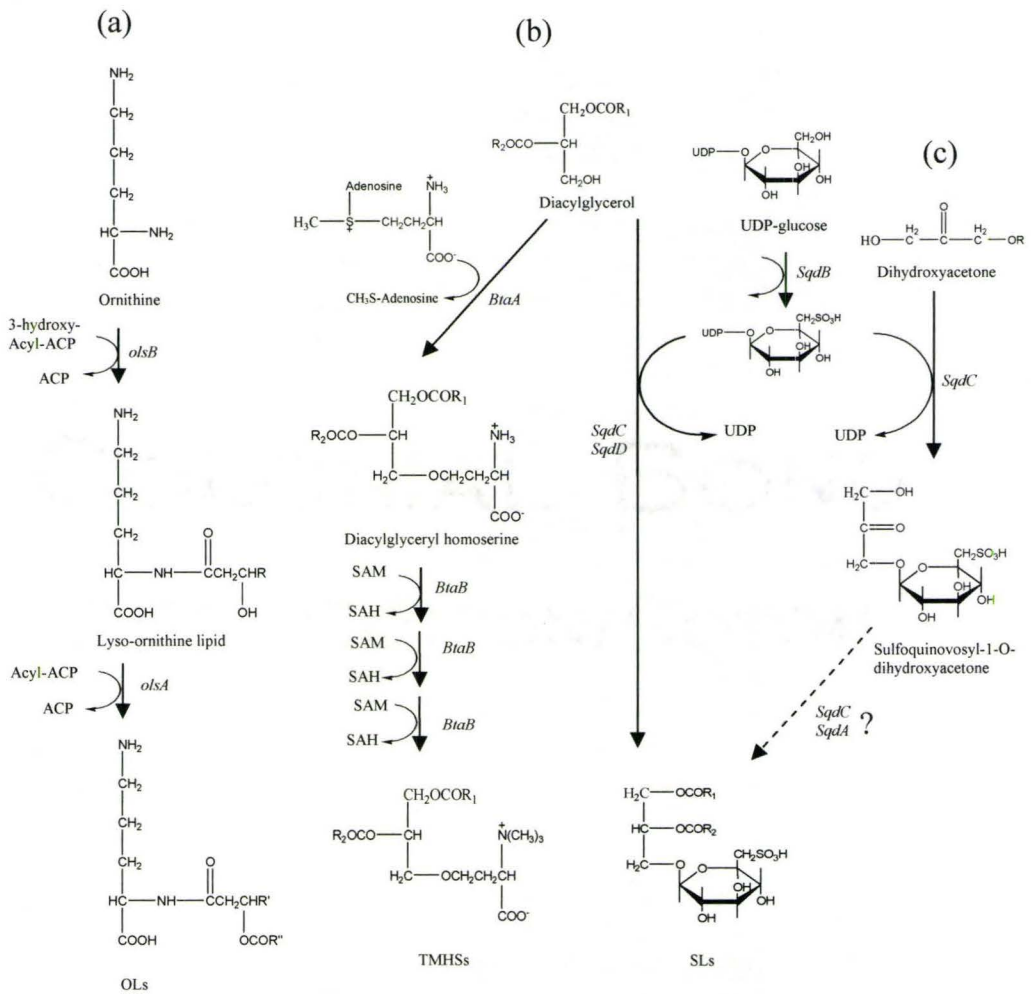


Figure 1.13. Model for the biosynthesis of (a) ornithine lipids, (b) 1,2-diacylglyceryl-trimethylhomoserine lipids and (b) sulfoquinovosyldiacylglycerols from diacylglycerol or (c) from dihydroxyacetone.

SLs are typically present in plants and photosynthetic organisms [148]. The proposed pathway for their biosynthesis in plants and bacteria is called the sugar-nucleotide pathway and involves two steps; (i) first, uridine 5-phosphate sulquinovose (UDP-SQ) is synthesized from UDP-glucose and sulfite by uridine 5'-diphosphate (UDP)-sulfoquinovose synthase encoded by the *sqdB* gene; in *S. meliloti* this gene has been characterized [149]. (ii) Second, the enzyme diacylglycerol sulfoquinvosyltransferase encoded by the *sqdX* gene transfers UDP-SQ to diacylglycerol [148, 150]. The biosynthesis of SLs in bacteria is not

well understood and in the photosynthetic organism *Rhodobacter sphaeroides* at least four genes (*sqdA*, *sqdB*, *sqdC*, *sqdD*) are thought to participate [151]. For example, deletion of the *sqdC* gene in *R. sphaeroides* caused the accumulation of sulfoquinovosyl-1-O-dihydroxyacetone suggesting that SqdC transferred sulquinovose onto dihydroxyacetone (or dihydroxyacetone phosphate) instead of onto diacylglycerol. The proteins encoded by s*qdC* and *sqdA* genes are similar to a reductase and acyltransferase respectively, thus these enzymes could transform sulfoquinovosyl-1-O-dihydroxyacetone to produce SLs [151].

The biosynthesis of OLs in *S. meliloti* is a two step process; (i) reaction of the carboxyl group of a 3-hydroxy fatty acid with the α-amino acid group of ornithine forming an amide bond, and (ii) transfer of a second fatty acyl group to the hydroxyl function of the 3-hydroxy fatty acid forming an ester bond [30]. The first reaction forming lysornithine lipids as intermediates is catalyzed by an N-acyl transferase enzyme coded by the *olsB* gene [106] while the second reaction is catalyzed by an acyl transferase enzyme coded by the *olsA* gene [152]. In some bacteria OlsA is a bifunctional enzyme involved in both OLs and phospholipid biosynthesis, with lysophosphatidic acyl transferase activity similar to PlsC of *E. coli* [153].

TMHSs are synthesized in *S. meliloti* exclusively under P_i-limiting conditions [51]. Biosynthesis of TMHSs in bacteria occurs by ether formation between diacylglycerol and a homoserine residue followed by three subsequent methylations of the intermediate diacylglyceryl homoserine [30, 111]. The genes required for TMHSs synthesis in *S. meliloti* named *btaA* and *btaB* have been identified [154].

1.5. Biological Functions of Lipids in Bacteria

The main biological function of lipids is to form the cell membrane, providing a barrier to bacteria from the environment [62]. Lipids also determine the topology of integral proteins within membranes and influence their function [155]. Both the nature of the phospholipid head group and the fatty acyl chains determine membrane fluidity and thus membrane permeability [32]. The variation of fatty acyl chains in lipids in response to changing environmental conditions (e.g., temperature, pH, etc) as a response to regulate membrane fluidity has been studied extensively [41, 42, 80]. However, the mechanisms that regulate the synthesis of phospholipids and the mix of headgroup species within the membrane are not well understood [32].

Fatty Acyl Chain Variations in Lipids

Fatty acyl modifications can be of three types: (i) desaturation, (ii) *cis/trans* isomerization and (iii) cyclopropanation [41]. The first type, desaturation has been widely studied and involves the introduction of double bonds into the acyl chains usually as a response to temperature changes. The second type, *cis/trans* isomerization, involves the conversion of *cis* unsaturated fatty acids to *trans* isomers which results in better packing of the phospholipid acyl chains.

Cis/trans isomerization seems to be a mechanism of adaptation to high temperatures providing means to decrease membrane fluidity and achieve better growth; the mechanism by which this process occurs is unknown [41]. The third type, cyclopropanation is widely observed in bacteria but its physiological role is not understood [41, 77]. Increased cyclopropanation of lipids seems to protect bacteria from adverse conditions such as acidity [79, 156-158], freeze drying [81], desiccation [78] and exposure to pollutants [159-161]. There is some debate as to whether an increase in cyclopropane content in bacterial membranes leads to a decrease in membrane fluidity [80, 81]. Furthermore, cyclopropane fatty acids are involved in the pathogenesis of *Mycobacterium tuberculosis* [162, 163] while in antagonistic *Fluorescent Pseudomonas* these fatty acids are associated with antifungal activity [164]. CFAs are of current interest as targets in the development of new drugs for the treatment of antibiotic resistant strains of tuberculosis [3, 165, 166].

Phospholipids and Non-phosphorus Containing Lipids

Most of our understanding of phospholipid function comes from genetic manipulation studies of lipid genes in *E. coli* [32, 167, 168]. Anionic lipids such as PGs and CLs were thought to be indispensable in *E. coli* as they participated in DNA replication and protein translocation in membranes [167]; however, recently it has been shown that phosphatidic acid can substitute PGs and CLs in *E. coli* mutants of *pgsA* [169]. Under P_i-limiting conditions, SLs is an anionic lipid which is thought to serve as a surrogate of PGs in plants and bacteria [150]. In photosynthetic organisms, PGs and SLs play an important role in photosynthesis [150]. However, in *S. meliloti* the biological function of SLs are not known since *sqdB* knockout mutants deficient in SLs were able to establish symbiosis and did not show a particular phenotype under laboratory conditions [149].

PEs, a zwitterionic lipid class, is required for activity of the lactose permease in *E. coli*, demonstrating the effect that lipids exert on the function of membrane proteins [155]. PCs on the other hand, is not synthesized by *E. coli* but is present in a variety of pathogenic and symbiotic bacteria. This observation has raised some debate whether about PCs could participate in the interaction between pathogen and host [145, 170]. PC-deficient mutants of *S. meliloti* were unable to form root nodules, therefore this phospholipid was vital for symbiosis and consequently nitrogen fixation [30]. TMHSs, a non-phosphorus containing lipid with similar chemical structure to PCs, is thought to substitute PCs in membranes thus having a similar biological function [30, 111]. OLs have been hardly studied; although, it was recently shown that this lipid is vital in the production of some extracytoplasmatic proteins in the bacterium *Rhodobacter capsulatus* [114]. Overall, in *S. meliloti* it has been shown that none of the phosphorus free membrane lipids were required for symbiosis [154].

1.6. Objectives of the Thesis

This research had three major goals: (i) first, the development of simple small-scale methodologies for the analysis of fatty acids by GC/MS; (ii) second, the development of comprehensive and simple methodologies for the analysis of intact lipids in *S. meliloti* by ESI/MS and MS/MS; (iii) third, the application of these methodologies to study specific knockout mutants of genes thought to be involved in lipid metabolism in *S. meliloti*. In more detail, the objectives of this research are summarized below:

A. Development of GC/MS-based Methodologies for the analysis of fatty acids
A.1. Comparison of common methodologies for the analysis fatty acids as FAMEs for bacterial lipids of *S. meliloti*.
A.2. Inclusion of appropriate quality control standards to measure and differentiate losses due to handling, completion of transformation/derivatization reactions and extent of side reactions.
A.3. Development of small-scale procedures that require minimum handling and no water/solvent extraction steps. These procedures were to be optimized to assure integrity of sensitive bacterial fatty acids such as 3-hydroxy and cyclopropane fatty acids.

B. Development of ESI/MS and ESI/MS/MS Methodologies for the analysis of intact lipids
B.1. Application of shotgun lipidomics principles to the analysis of phospholipids and non-phosphorus containing lipids of *S. meliloti*.
B.2. Description of fatty acid distributions in lipid classes and identification of fatty acids in individual lipids as well as their relative positions (*sn*-1 and *sn*-2).

C. Application of fatty acids and intact lipid analyses in functional genomics
C.1. Evaluation of the effects of P_i-starvation and acidity on fatty acids and intact lipids profiles in *S. meliloti*.
C.2. Study of knockout mutants of two genes hypothesized to code for cyclopropane fatty acyl synthases (CFA).
C.3. Study of knockout mutants of genes that code for the malic enzymes to investigate their involvement in fatty acid and lipid metabolism.
C.4. Study of knockout mutants of a gene hypothesized to code for a lysophosphatidic acyl transferase (PlsC).

1.7. Thesis Layout

Chapter 2 presents a comparison of three common methodologies used in the analysis of fatty acids as FAMEs by GC/MS. Quality control standards were introduced to monitor the recoveries, the completion of transformation reactions and the extent of side reactions. These methodologies were applied to the analysis of bacterial lipids of *S. meliloti* to determine the best methodology. A one-vial microscale method that employed sodium methoxide was developed for the

analysis of bacterial fatty acids. This method involved minimal sample manipulation and did not require water extraction steps. The methodologies were applied to the analysis of fatty acids of *S. meliloti* cultures grown under inorganic phosphate starvation.

Chapter 3 illustrates the development of a shotgun lipidomics approach for the analysis of phospholipids and non-phosphorus containing lipids in *S. meliloti*. A combination of neutral loss scans and precursor ions scans in both positive and negative electrospray ionization was used. Fatty acid distributions were obtained for each lipid class which provided valuable insights into the biosynthesis of lipids. In total, eight lipid classes and approximately 58 molecular species were detected in bacterial lipid extracts without chromatography.

Chapters 4-6 describe the application of the methodologies presented in chapters 2-3 to the study of specific knockout mutants of genes hypothesized to participate in lipid metabolism in *S. meliloti*. Chapter 4 describes a study of fatty acid and intact lipid profiles of *S. meliloti* under P_i-starvation and acidic conditions with particular interest on the effect on the cyclopropanation of lipids. In addition, the fatty acid and intact lipid profiles of knockout mutants of two putative genes hypothesized to code for cyclopropane fatty acyl synthases were obtained under the growth conditions studied.

In chapter 5, the involvement of the malic enzymes, DME and TME, in fatty acid and lipid metabolism, was explored. The fatty acid profiles and intact lipid profiles of knockout mutants of *dme* and *tme* genes grown in two carbon sources were examined in search for a metabolic phenotype. Chapter 6 describes the study of knockout mutants of a gene hypothesized to code for a putative lysophosphatidic acyl transferase (PlsC of *E. coli*) in *S. meliloti*. Finally, chapter 7 provides a summary of the main contributions of each chapter as well as future research directions derived from this work.

Chapter 2

Comparison of Three GC/MS Methodologies for the Analysis of Fatty Acids in *Sinorhizobium meliloti*: Development of a Micro-scale, One-Vial Method

Libia Saborido Basconcillo, Brian E. McCarry

Abstract

Three protocols for fatty acid analysis in *Sinorhizobium meliloti* were improved by addition of a number of standards/controls and a silylation step which allowed the determination of recoveries, extents of conversion of lipids to fatty acid methyl esters (FAMEs) and extents of side reactions. Basic hydrolysis followed by acid-catalyzed methylation and transmethylation with sodium methoxide, were the best for the analysis of 3-hydroxy- and cyclopropane fatty acids, respectively. A micro-scale, one-vial method that employed sodium methoxide/methanol was equally efficient and on a 1000-fold smaller scale than standard methods. Because this method avoids aqueous extractions, 3-hydroxybutanoic acid was detected as its trimethylsilyloxy methyl ester along with FAMEs.

Keywords: fatty acid methyl esters, Sinorhizobium meliloti, cyclopropane fatty acids, 3-hydroxy fatty acids, 3-hydroxybutanoic acid, poly(3-hydroxybutanoate), hydrolysis

The work in this chapter has been published previously in an article under the title: L. Saborido Basconcillo and B.E. McCarry, 2008, Journal of Chromatography B, 871: 22-31.

1. Introduction

Fatty acid analysis in bacteria can be used as an approach to understand metabolic networks and the function of genes involved in fatty acid metabolism. The soil bacterium *Sinorhizobium meliloti* is found in root nodules and fixes nitrogen for plants such as alfalfa. The association between the bacterium *S. meliloti* and alfalfa is considered one of the leading model systems for nitrogen fixation and symbiosis studies [27, 37]. This work was driven by the need for simple, comprehensive analytical fatty acid methods that could be applied in functional genomics studies of *S. meliloti*.

Fatty acid analyses are routinely performed by conversion of lipids into fatty acid methyl esters (FAMEs), typically followed by gas chromatographic

analysis [19, 107], although methods using high performance liquid chromatography (HPLC) and capillary electrophoresis (CE) have also been reported [66, 67]. FAMEs are normally generated from lipids by either basic hydrolysis (saponification) followed by methylation of the free fatty acids or by acid or base-catalyzed transesterification of lipids [171]. Each of these approaches has their own advantages and disadvantages. First, it is often assumed that the transformation of lipids into FAMEs is quantitative and uniform across lipid classes. Second, recovery and internal standards are rarely used in these methods so there is no way to evaluate the completeness of transformation reactions or losses due to side reactions. Hydrolysis to afford fatty acids is widely considered an important side reaction in the preparation of FAMEs, due to the presence of traces of water, leading to poor recoveries of FAMEs; yet the extent of hydrolysis is rarely measured directly or taken into account [73, 172-174]. Hydrolysis is reported to be the main cause of why well-established methodologies fail when sample size and reagent volumes are scaled down [175]. Third, the protocols for preparing FAMEs from lipids require one or more aqueous extraction steps to isolate FAMEs after methylation or transmethylation reactions [74, 171, 176-178]. Moreover, most methods have been developed and validated for applications in the food industry where sample size (10-100 mg) is not a limitation. Although recent pyrolitic methods using μg amounts of lipids have been reported, their application as routine procedures is limited since manual sample loading and special apparatus are required [75, 76]. Finally, the analysis of bacterial fatty acids poses additional challenges due to the presence of labile hydroxylated and cyclopropane-containing fatty acids [19, 73, 77].

Fatty acid compositions of *S. meliloti* and other species of the genus *Rhizobium* have been used to establish taxonomic relationships between species and for the chemotaxonomic identification of unknown strains [64, 179]. The fatty acid composition of *S. meliloti* was obtained using GC with flame ionization detection (GC/FID) based on the Sherlock Microbial Identification System (MIS) [179]. Fatty acid identification using this system is based solely on retention times since FID is a non-specific detector [180]. Additionally, fatty acid analyses were conducted using *S. meliloti* strain Rm1021, commonly used as wild type [64, 179]. However, it has been demonstrated recently that wild type strain Rm1021 carried a single nucleotide C-deletion mutation in the *pstC* gene [181]. Thus, this mutation has been repaired in *S. meliloti* strain Rm1021 and the resulting strain, RmP110, is now used as a wild type [34, 50]. Therefore, we used in our study the corrected *S. meliloti* wild type (strain RmP110) for which fatty acid composition has not been reported. We employed GC analysis with mass spectrometric detection (GC/MS) thus structural information of fatty acids in *S. meliloti* can be obtained. GC/MS analysis of fatty acids is preferred over GC/FID since the electron impact mass spectra of FAMEs provide structural information; thus identification can be conducted using retention times and mass spectral information [180, 182].

In this study, we investigated the three most commonly used methodologies in the preparation of fatty acid methyl esters: (a) basic hydrolysis of lipids followed by acid-catalyzed methylation (employed by the MIS system), (b) acid-catalyzed transmethylation using sulfuric acid in methanol and (c) base-catalyzed transmethylation using sodium methoxide in methanol. The aims of the current work were: (1) to determine the best method for the analysis of bacterial fatty acids in *S. meliloti*, particularly cyclopropane fatty acids, (2) to determine the relative transformation efficiencies for major lipid classes for each method and (3) to introduce appropriate analytical controls to monitor derivatization efficiencies, recoveries and losses due to side reactions. An important motivation was to develop a small-scale, one-vial method for the analysis of bacterial fatty acids to avoid the need for tedious extraction and sample handling steps. A comparison of these methods was conducted with *S. meliloti* samples grown in the presence and absence of inorganic phosphate (P_i).

2. Experimental

2.1. Reagents and Materials

Fatty acid standards (9:0-25:0) and N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) were obtained from Sigma-Aldrich (Saint Louis, MO) and fatty acid methyl esters of saturated fatty acids (Me-9:0-Me-25:0) were obtained from Alltech Associates (Deerfield, IL). All solvents used were HPLC grade (Caledon Labs, Caledon, ON). Phospholipid standards [1,2-dinonadecanoyl-*sn*-glycero-3-phosphocholine (PC(19:0/19:0)), 1,2-diheptadecanoyl-*sn*-glycero-3-phosphoethanolamine (PE(17:0/17:0)), 1,1',2,2'-tetramyristoyl cardiolipin (CL(14:0/14:0/14:0/14:0), sodium salt), 1,2-dilauroyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (PG(12:0/12:0), sodium salt) were purchased from Avanti Polar Lipids (Alabaster, AL). N-Octadecanoyl-sphingosylphosphorylcholine (SM(18:0/18:0)) was obtained from MJS BioLynx (Brockville, ON). Glyceryl tri(hexadecanoate-d_{31}) was purchased from CDN Isotopes Inc (Pointe-Claire, QC). 3-Hydroxy fatty acids (3-hydroxytetradecanoic acid (3-OH-14:0), 3-hydroxyhexadecanoic acid (3-OH-16:0), 3-hydroxyoctadecanoic acid (3-OH-18:0) and their fatty acid methyl esters were obtained from MJS BioLynx (Brockville, ON). Cyclopropane-containing fatty acids, *cis*-9,10-methylenehexadecanoic acid (17:0cyclo(9,10)) and *cis*-9,10-methyleneoctadecanoic acid (19:0cyclo(9,10)) and their fatty acid methyl esters were obtained from MJS BioLynx (Brockville, ON) while *cis*-11,12-methylenoctadenoic acid (19:0cyclo(11,12)) was obtained from Cedarlane Laboratories Limited (Hornby, ON). 3-Hydroxybutyric acid and methyl 3-hydroxybutyrate were obtained from Sigma-Aldrich (Saint Louis, MO).

Sodium methoxide solutions (0.5 M) were prepared by dissolving a piece of freshly cut sodium in methanol as recommended by Christie [173]; the resulting solutions were stored in glass vials equipped with mininert® valves (Chromatographic Specialties, Brockville, ON) at 4 °C. The use of mininert®

valves protected the solutions from atmospheric moisture, facilitating routine work and solution integrity for 2-3 months.

2.2. Quality Control Standards

Isopropyl tetradecanoate-d_{27} was synthesized from tetradecanoic-d_{27} acid (CDN Isotopes Inc., Pointe-Claire, QC) using sulfuric acid in isopropanol (3%, 2 mL) at 50°C overnight as described by Christie [171]. Hexane (1 mL) and water (1 mL) were added simultaneously to the reaction mixture. The hexane layer was extracted twice more with water (1 mL); the hexane layers were combined, dried over anhydrous sodium sulfate and evaporated to dryness under a nitrogen stream. Solutions of isopropyl tetradecanoate-d_{27} (10 mg/mL) were prepared in hexane. A hexane solution containing isopropyl tetradecanoate-d_{27} (1000 ng/μL), 9-anthracenemethanol (Sigma-Aldrich, 500 ng/μL), n-eicosane (Sigma-Aldrich, 660 ng/μL), methyl tridecanoate (Sigma-Aldrich, 600 ng/μL) and methyl pentadecanoate (Sigma-Aldrich, 690 ng/μL) was prepared and an aliquot of this solution (typically 10-15 μL) was added to each sample (standards or dried bacterial lipid extracts) prior to analysis.

2.3. Bacterial Cultures

Bacterial cultures (*S. meliloti,* RmP110) were grown in MOPs buffered minimal media with glucose (15 mM) as the carbon source in the presence (2 mM) or absence of inorganic phosphate (P_i). Wet cell pellets obtained from 500-1000 mL of culture (O.D. 0.4-0.6) by centrifugation were resuspended in 2.5 or 5 mL of medium, respectively, and divided into 250 μL aliquots in Eppendorf tubes. Each tube was centrifuged, the supernatant was discarded and the wet pellets flash frozen in liquid nitrogen and stored at -80°C. The pellet equivalent to 50 mL of original culture, corresponded to 33.0 ± 4.0 mg wet weight of cells (n=15).

2.4. Lipid Extraction

Wet cell pellets were resuspended in distilled water (1 mL) and extracted with a mixture of $CHCl_3$:MeOH (1 mL, 2:1, v/v). The chloroform phase containing the lipids was separated and the aqueous phase was extracted twice more with $CHCl_3$:MeOH (1 mL, 2:1, v/v). The chloroform layers were combined and dried through a small column packed with anhydrous sodium sulphate. The solvent was evaporated using nitrogen gas and the dried lipid residue was either analyzed immediately or stored at -80°C.

2.5. Methodologies for Fatty Acid Methyl Ester Analysis

Table 2.1 summarizes the experimental conditions used in methods A, B, C and one-vial C.

Table 2.1. Experimental conditions of methods A, B, C and C one-vial method.

Experimental conditions	**Method A**	**Method B**	**Method C**	**One-vial Method C**
Reactions	1) Basic hydrolysis 2) Acid-catalyzed methylation 3) Silylation	1) Acid-catalyzed transmethylation 2) Silylation	1) Base-catalyzed transmethylation 2) Silylation	1) Equal to Method C 2) Silylation
Sample	wet cells	dried lipid extract	dried lipid extract	dried lipid extract
Reagents	1) KOH (0.1 M, 1 mL)/CH_3OH 2) HCl (6 M, 1 mL)/CH_3OH	1) 3% H_2SO_4 (1 mL)/ CH_3OH	1) $NaOCH_3$ (0.5 M, 1 mL)/CH_3OH	1) $NaOCH_3$ (0.5 M) /CH_3OH
Reaction conditions	1) 50°C, 3 h 2) 80°C, 15 min	1) 50°C, 12 h	1) 50°C, 15 min	1) 25°C, 15 min
Reagent volumes	1 mL	1 mL	1 mL	25 µL
Liquid/liquid extraction	yes	yes	yes	No

In method A, wet cells were spiked with the quality control standards (10 μL) and reacted with KOH in MeOH, followed by acid-catalyzed methylation as described by Jarvis [64]. FAMEs were extracted three times using diethyl ether:hexane (1:1, v/v, 2 mL); the organic layers were combined, dried over anhydrous sodium sulphate and evaporated to near dryness under a gentle nitrogen stream. The dried samples were treated with MSTFA (25 μL) and dry pyridine (25 μL) at 37°C for 30 min. An aliquot of the derivatization solution (5 μL) was diluted either 10-fold or 20-fold with hexane containing ethyl dodecanoate (25 ng/μL) as the internal standard; 1 μL was injected on-column for GC/MS analyses.

In method B, dried lipid extracts were spiked with the control standards and reacted with 3% sulfuric acid in methanol [71]. The reaction solution was allowed to cool and was extracted three times with hexane (1 mL); the combined hexane layers were dried over anhydrous sodium sulphate and taken to dryness under a gentle nitrogen stream. The residue was silylated and analyzed by GC/MS as described above in Method A.

In method C, dried lipid extracts were spiked with the control standards and reacted with sodium methoxide in methanol [71]. Transmethylation was stopped by the addition of glacial acetic acid (100 μL) followed by hexane (1 mL) then water (1 mL); the hexane layer was removed and the hexane extraction repeated (2 x 1 mL). The hexane layers were combined, dried over anhydrous sodium sulphate and taken to dryness carefully using a gentle nitrogen stream. The residue was silylated and analyzed by GC/MS as described above in Method A.

With the one-vial method C, dried lipid extracts were spiked with the quality control standards (15 μL) and reacted with sodium methoxide in methanol. In the method development process, various reaction temperatures and reaction times were examined. Transmethylation was stopped by the addition of a solution of acetic acid in dichloromethane (5 M, 10 μL) and the resulting solution dried using a gentle nitrogen stream. The residue was treated with MSTFA (50 μL) and pyridine (15 μL) at 37°C for 30 min. Aliquots (5 μL) were diluted 10-fold into hexane containing ethyl dodecanoate (internal standard, 25 ng/μL); 1 μL was injected on column for GC/MS analyses.

2.6. Gas Chromatography-Mass Spectrometry

Analyses were performed on a HP 5971A MSD (full scan mode) equipped with HP 5890 Series II GC, a cool on-column injector and a J&W DB-17ht column (50% phenyl/50% methyl silicone, 30m x 0.25mm x 0.15μm film) using helium as the carrier gas. The oven temperature was held at 50°C for 5 min. then programmed at 5°C/min. to 300°C and held at 300°C for 5 min. Electron impact ionization (EI^+, 70 volts) was used for all samples.

2.7. Quantitative Analyses

Calibration curves and relative response factors (RRFs) were obtained for three classes of fatty acid derivatives: (1) fatty acid methyl esters (FAMEs), (2) trimethylsilyl esters of fatty acids and (3) trimethylsilyl ethers of 3-hydroxy fatty acid methyl esters. Solutions of authentic standards of fatty acid methyl esters (Me-9:0-Me-25:0) were prepared in hexane (1-40 ng/μL). FAMEs of unsaturated fatty acids were prepared by methylation of the unsaturated fatty acids by the diazomethane method [183]. Calibration plots (R^2=0.97-0.99) were obtained using peak areas from mass chromatograms and the relative response factors were calculated from the slopes of the calibration plots. Table 2.2 summarizes the ions used for the identification and quantification of various fatty acid derivatives.

Table 2.2. M/z values of ions used for the diagnostic and quantification of various fatty acid derivatives and other compounds.

Compound	Diagnostic ions	Quantification ions	References
iPr-14:0-d_{27}	255, 297 ($M^{+\bullet}$)	105	
Me-14:0-d_{27}	91, 269 ($M^{+\bullet}$)	77 (74 in FAMEs)	
TMS-14:0-d_{27}	73, 327 ($M^{+\bullet}$)	312 $[M-15]^+$	
9-Anthracenemethanol		208	
TMS ether of 9-Anthracenemethanol	73, 191	280 ($M^{+\bullet}$)	
n-C20	57, 85, 282 ($M^{+\bullet}$)	71	[182]
Et-12:0	101, 228 ($M^{+\bullet}$)	88	
FAMEs of saturated fatty acids	87, $M^{+\bullet}$	74	[180, 182, 184]
FAMEs of unsaturated fatty acids	74, $M^{+\bullet}$	55	[180, 182, 184]
FAMEs of cyclopropane fatty acids	55, $[M-32]^+$, $M^{+\bullet}$	69	[182]
TMS ethers of 3-hydroxy-FAMEs	73, 89, $[M-15]^+$	175	[185, 186]
TMS esters of fatty acids	73, 75, 117	$[M-15]^+$	[72]

Trimethylsilyl esters were prepared by reaction of authentic standards of fatty acids with MSTFA for 30 minutes at 37°C; a mole ratio of total fatty acid:MSTFA of 1:500 was used. Solutions of the silyl derivatives were prepared by dissolving the reaction mixtures in hexane (3-50 ng/μL). Calibration plots ($R^2 \geq 0.99$) were obtained for each fatty acid derivative and the response factors were obtained from the slopes. Trimethylsilyl ethers of methyl esters of 3-hydroxy fatty acids were prepared by reaction of authentic standards of methyl esters of 3-hydroxy fatty acids with MSTFA. Solutions of the trimethylsilyl ethers of methyl esters were prepared by dissolving the reaction mixtures in hexane (3-

40 ng/μL) with a 10% MSTFA. Calibration curves (R^2=0.99) were obtained using peak areas from mass chromatograms of the m/z 175 ion.

The total mass of a given fatty acid in a sample was calculated as the sum of the methyl ester and the trimethylsilyl ester using the RRFs for each derivative and normalized by the wet weight of cells (expressed as ng fatty acid/mg wet cells). Relative percentages of fatty acids were calculated by taking the mass of a given fatty acid derivative as a percentage of the total mass of all fatty acids identified in the sample.

2.8. Statistical Analyses

All data are reported as mean values ± standard deviation; each sample was analyzed in triplicate. A statistical package (SPSS, version 15.0, SPSS Inc. Chicago, IL, USA) was used for statistical analyses. Analysis of variance (ANOVA) was used for multiple group comparisons ($P \leq 0.05$) while Student's t-test was used occasionally to evaluate two groups of data.

3. Results and Discussion

3.1. Quality Control Standards

Three types of quality control standards were introduced into the methodologies to monitor (1) recoveries due to losses in extractions, handling and side reactions, (2) the efficiencies of transmethylation or hydrolysis/methylation reactions and (3) the efficiency of silylation reactions with MSTFA. An internal standard (ethyl dodecanoate, Et-12:0) was used for quantitation of all analytes.

First, the methyl esters of tridecanoic and pentadecanoic acids (Me-13:0 and Me-15:0) were introduced as recovery standards; these fatty acids which are not present in *S. meliloti* (data not shown) will undergo transformations but should be recovered quantitatively as their methyl esters if no losses or side reactions occurred. These methyl esters have lower molecular masses and are thus more volatile than most bacterial FAMEs so lower recoveries of Me-13:0 relative to Me-15:0 would indicate losses due to volatilization. An alkane (n-eicosane, n-C20) was also added as a recovery standard. This compound does not undergo any transformation reactions under the conditions used, therefore it can be used to measure losses exclusively due to handling and volatilization. Additionally, n-C20 can be used to estimate the losses by volatilization of Me-14:0 to Me-16:0 since the normal boiling points of the n-alkane and the FAMEs are in the same range [187, 188].

Second, isopropyl tetradecanoate-d_{27} was introduced to determine the efficiency of conversion of a secondary alcohol ester into a methyl ester. Upon transmethylation or hydrolysis/methylation reactions, isopropyl tetradecanoate-d_{27} is transformed to methyl tetradecanoate-d_{27}. Both compounds are readily monitored by GC/MS and their relative amounts provide a measure of the extent of this reaction. Should small amounts of water be present, hydrolysis of the methyl and/or isopropyl esters to tetradecanoic-d_{27} acid could occur; the free fatty

acid would likely be undetected using standard analytical procedures. Derivatization with MSTFA would afford the trimethylsilyl ester of tetradecanoic-d_{27} acid, a compound readily identified by GC/MS; thus, the percentage of free fatty acid resulting from hydrolysis can be easily determined. The sum of the methyl, isopropyl and trimethylsilyl esters of tetradecanoic-d_{27} acid should be close to the recoveries of the alkane recovery standard; a sum less than this would indicate that there were losses due to side reactions other than hydrolysis.

Third, 9-anthracenemethanol was introduced to monitor the extent of silylations with MSTFA; since 9-anthracenemethanol and its trimethylsilyl derivative are readily monitored by GC/MS, they provide a direct measure of the efficiency of silylation reactions.

3.2. Derivatization with MSTFA

The silylation of any free fatty acids to their trimethylsilyl esters using MSTFA provided a direct measure of the degree of hydrolysis. This derivatization facilitated the analysis of 3-hydroxy fatty acids by conversion of the hydroxy function into the trimethylsilyl ether, a derivative which afforded narrower chromatographic peaks and better mass spectra. Under EI^+ conditions, 3-hydroxy fatty acid methyl esters produced rather uninformative mass spectra dominated by an ion at m/z 103, while the EI^+ spectra of the corresponding 3-trimethylsilyl ethers provided characteristic ions unique to these derivatives at m/z 89 and m/z 175; the $[M-15]^+$ fragment ion was used to identify individual fatty acid derivatives [185, 186]. The injection of pure derivatization mixtures produced total ion chromatograms with high backgrounds and multiple contaminant peaks from the MSTFA and pyridine. Dilution of derivatization mixtures (5 to 20-fold) produced good quality chromatograms with improved S/N ratios. Since trimethylsilyl derivatives readily hydrolyze in the presence of traces of water, all solutions for GC/MS analyses were diluted using hexane containing 10% MSTFA and analyzed immediately.

3.3. Comparison of Methods A, B and C

Mixtures of authentic standards representative of four classes of phospholipids, a triacylglycerol, an amide lipid and the quality control standards were used to evaluate the efficiencies of three standard fatty acid analysis methods (A, B and C). The quality control standards were added to each analysis to determine the recoveries, transmethylation/methylation efficiencies and the extents of hydrolysis and side reactions for each lipid class. Figure 2.1 summarizes the transformation reactions and the derivatives produced.

Each lipid produced derivatives (a FAME and possibly a trimethylsilyl ester) corresponding to the single fatty acid in each lipid standard; thus the reaction progress of each lipid could be monitored and compared to the progress of other lipids. Masses of individual lipids in these reactions were in the 10-40 μg range.

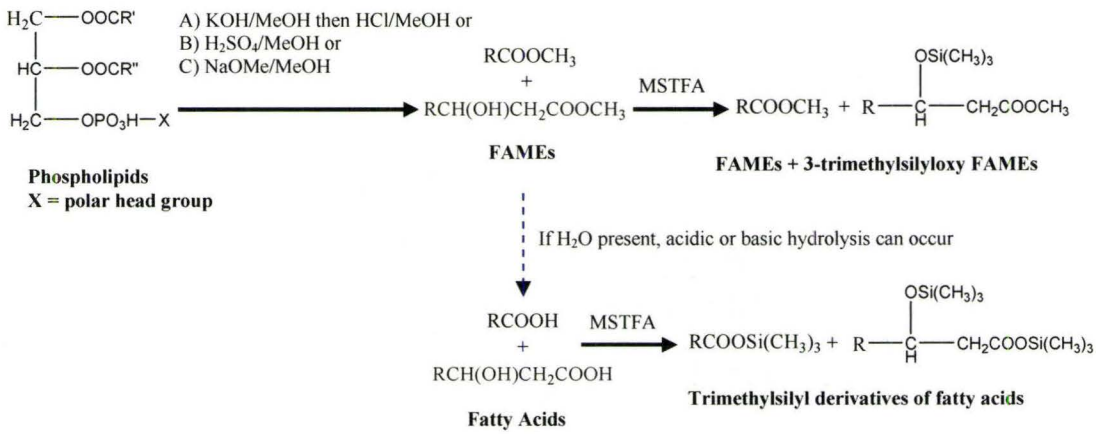


Figure 2.1. Schematic of derivatization reactions of phospholipids using methods A, B and C. Other lipid classes react under these conditions.

Table 2.3 shows the means and standard deviations obtained using the three methods for the quality control standards (Table 2.3a) and the lipid standards (Table 2.3b). The recoveries of the C20 alkane (about 90%) and Me-15:0 (81-95%) were identical for the three methods (Table 2.3a), indicating that losses due to handling and any side reactions of FAMEs were negligible. However, recoveries of Me-13:0 (60-75%) were somewhat lower than Me-15:0, probably the result of losses due to the increased volatility of Me-13:0. The recoveries of the three tetradecanoic-d_{27} acid derivatives (78-89%) paralleled the recoveries of Me-15:0 and the C20 alkane, consistent with the above results. However, the recoveries of methyl tetradecanoate-d_{27} and trimethylsilyl tetradecanoate-d_{27} showed differences between methods indicating there were different degrees of conversion of isopropyl ester to the methyl ester and different extents of hydrolysis, respectively. The overall transmethylation efficiencies were good for methods A and B (73-79%) but were lower for method C (53%) as measured by the recoveries of methyl tetradecanoate-d_{27}. The extents of hydrolysis (measured by the recoveries of trimethylsilyl tetradecanoate-d_{27}) while low (0.1-7%) were statistically different for the three methods ($P \leq 0.05$); method B had the lowest hydrolysis (0.1%) while method C had the highest (7%).

Table 2.3. Recoveries of fatty acid derivatives produced from (a) the quality control standards and (b) the lipid standards using methods A, B and C.

a)

Quality Control Standards	Fatty Acid Derivatives	Percentages of derivatives recovered from standards, Mean ± SD (n=3)		
		Method A	Method B	Method C
n-C20	-	94.6 ± 7.2	84.6 ± 5.2	90.0 ± 11.6
Me-13:0	Me-13:0	75.4 ± 7.8	60.0 ± 2.8	65.5 ± 4.9
Me-15:0	Me-15:0	95.1 ± 6.5	81.4 ± 4.6	87.7 ± 5.2
iPr-14:0-d_{27}	iPr-14:0-d_{27}	6.1 ± 0.8	14.6 ± 1.1	18.1 ± 0.9
	Me-14:0-d_{27}	79.7 ± 7.0	73.3 ± 3.3	52.8 ± 3.9 [1]
	TMS-14:0-d_{27}	2.8 ± 0.4 [1]	0.1 ± 0.1 [2]	6.7 ± 1.4 [3]
	Total 14:0-d_{27} species	88.7 ± 8.1	88.0 ± 4.5	77.6 ± 5.8

b)

Lipids	Fatty Acid Derivatives	Percentages of derivatives recovered from lipids, Mean ± SD (n=3)		
		Method A	Method B	Method C
CL-14:0	Me-14:0	87.7 ± 5.8 [a]	90.5 ± 4.3 [a]	78.8 ± 10.8 [a]
	TMS-14:0	3.4 ± 0.3	ND.	ND.
PE(17:0/17:0)	Me-17:0	82.4 ± 3.0 [a]	84.1 ± 4.8 [a]	68.7 ± 8.1 [a]
	TMS-17:0	1.5 ± 0.3	ND.	ND.
TAG(16:0-d_{31}/16:0-d_{31}/16:0-d_{31})	Me-16:0-d_{31}	67.6 ± 4.7 [b]	70.8 ± 3.1 [b]	45.4 ± 5.2 [1, b]
	TMS-16:0-d_{31}	1.1 ± 0.1	ND.	ND.
PC(19:0/19:0)	Me-19:0	61.4 ± 4.1 [b]	60.5 ± 3.7 [c]	52.9 ± 8.3 [b]
	TMS-19:0	ND.	ND.	ND.
PG(12:0/12:0)	Me-12:0	48.6 ± 8.4 [c]	37.5 ± 1.2 [d]	41.0 ± 4.2 [b]
	TMS-12:0	2.7 ± 0.3	ND.	ND.
SM(18:0/18:0)	Me-18:0	24.9 ± 1.9 [1, d]	36.4 ± 2.0 [2, d]	ND.
	TMS-18:0	1.2 ± 0.3	ND.	ND.

n-C20: n-eicosane; Me-13:0: methyl tridecanoate; Me-15:0: methyl pentadecanoate; iPr-14:0-d_{27}, Me-14:0-d_{27}, TMS-14:0-d_{27}: isopropyl, methyl and trimethylsilyl esters of tetradecanoic-d_{27} acid, respectively; CL-14:0: 1,1',2,2'-tetramyristoyl cardiolipin; PE(17:0/17:0): 1,2-diheptadecanoyl-*sn*-glycero-3-phosphoethanolamine; TAG(16:0-d_{31}/16:0-d_{31}/16:0-d_{31}): Glyceryl tri(hexadecanoate-d_{31})); PC(19:0/19:0): 1,2-dinonadecanoyl-*sn*-glycero-3-phosphocholine; PG(12:0/12:0): 1,2-dilauroyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol); SM(18:0/18:0): N-Octadecanoyl-sphingosylphosphorylcholine; ND: not detected.

[1, 2, 3] Mean values are significantly different between columns ($P \leq 0.05$).

[a, b, c, d] Mean values are significantly different within columns ($P \leq 0.05$). Means with the same letter were not significantly different within columns ($P \geq 0.05$).

ND.: not detected.

The recoveries of fatty acids produced from lipid standards are listed in decreasing order in Table 2.3b. Overall, cardiolipin (CL) and phosphatidylethanolamine (PE) showed good to adequate conversions to FAMEs (69-91%) while the conversions of the triacylglycerol (TAG), phosphatidylglycerol (PG) and phosphatidylcholine (PC) standards were poor to adequate (38-71%). Not surprisingly, conversions of sphingomyelin (SM, an amide lipid) were poor (25-36%); conversions of sphingolipids are known to require vigorous acidic conditions [173, 189]. SM was introduced since 3-hydroxy fatty acids in bacterial lipids are found in lipopolysaccharides attached via amide bonds. Conversions of a given lipid to FAMEs using different methods were statistically identical with the exception of the TAG and SM ($P \leq 0.05$); recovery of Me-16:0 from the TAG standard was particularly low for method C (45%) compared to methods A and B (68-71%).

Overall, methods A and B had similar transmethylation efficiencies for the phospholipid standards (73-80%). The conversions of lipids into FAMEs were statistically identical within lipid classes using the three methods; the sole exception was the triacylglycerol which showed poorer recoveries using method C ($P \leq 0.05$). Recoveries were adequate for the three methods (80-90%) and the extents of competing hydrolysis reactions were small ($\leq$ 7%). However, the recoveries of fatty acid derivatives from the different lipid classes were strikingly dissimilar, indicating that there must be side reactions occurring which led to differential losses of FAME analytes from different lipid classes. For those who routinely report percentage fatty acid compositions in lipid mixtures based on these methods, these results are a cautionary tale because the fatty acid composition is clearly related to the relative efficiency of methylation or transmethylation reactions for each lipid class. The addition of the suite of standards used in this protocol allows one to determine the relative degrees of conversions of lipids to FAMEs accurately in each reaction.

In reactions of crude lipid extracts of *S. meliloti* recoveries were good (total derivatives of 14:0-d_{27}, 83-103%) while losses due to hydrolysis to the free acid were minimal (TMS-14:0-d_{27}, < 1%). Method A yielded the greatest number of fatty acid derivatives (17); fourteen were positively identified as saturated, unsaturated, 3-hydroxy and cyclopropane-containing fatty acids based on comparisons of their retention times and mass spectral fragmentation patterns with authentic standards (Table 2.4).

All three methods gave essentially identical absolute levels of saturated and unsaturated fatty acids; however, there were significant differences in the levels of 3-hydroxy and cyclopropane-containing fatty acids (Table 2.4). Only method A released 3-hydroxy fatty acids from bacterial lipopolysaccharides (LPSs) in reasonable yields while method C gave the highest yields of cyclopropane fatty acids. These differences in fatty acid levels resulted in statistically significant differences in the percentage composition data between the methods (Table 2.4).

Table 2.4. Relative percentage compositions of fatty acids in *S. meliloti* using methods A, B and C.

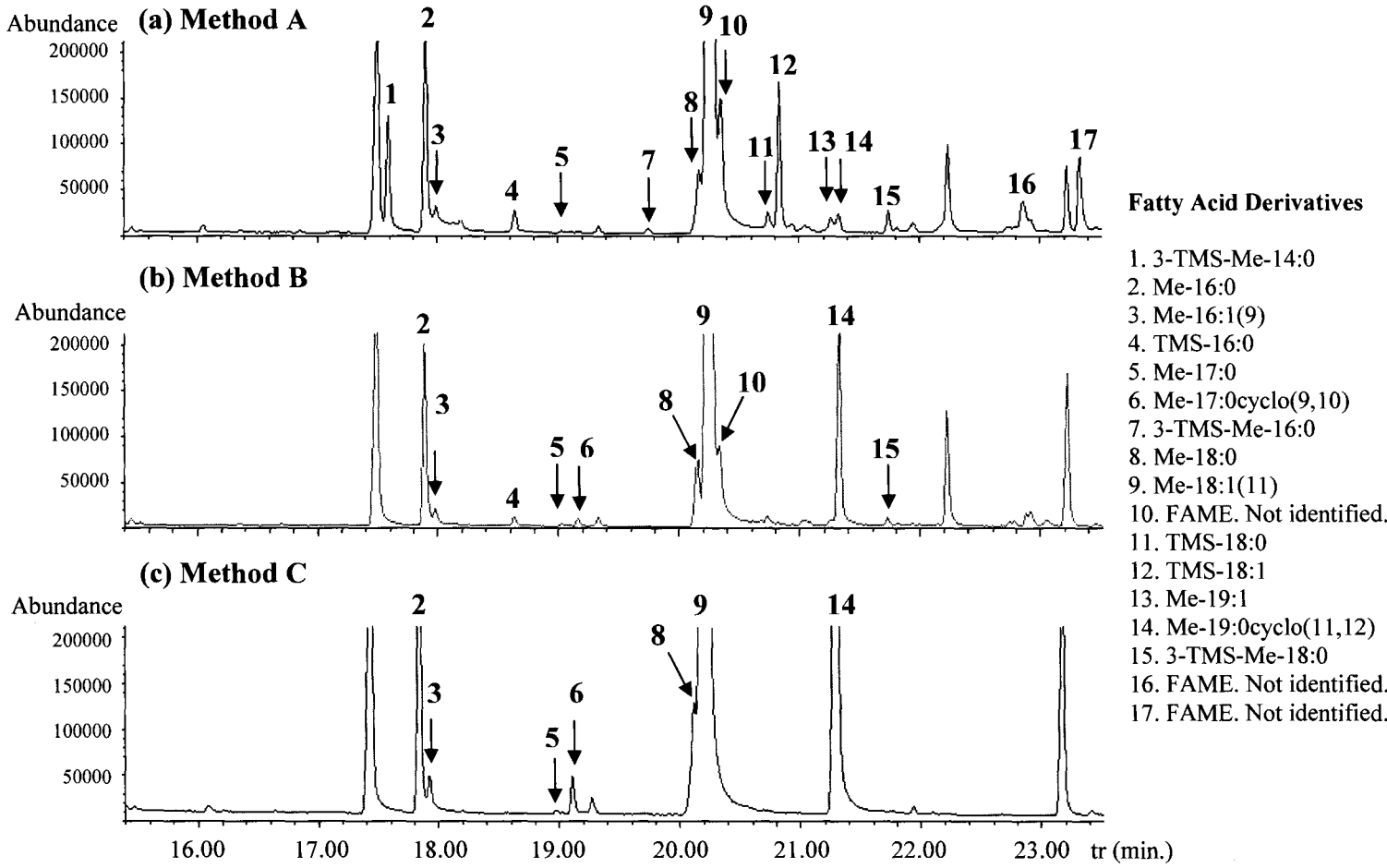
Fatty Acid Classes	**Fatty Acids** *	**Relative percentage composition of *S. meliloti*, Mean ± SD (n=5)**		
		Method A	**Method B**	**Method C**
Saturated Fatty Acids	14:0	0.28 ± 0.04	0.27 ± 0.07	0.13 ± 0.01 [1]
	16:0	13.9 ± 0.5 [1]	9.4 ± 0.9	8.4 ± 0.4
	17:0	0.16 ± 0.02 [1]	0.13 ± 0.02	0.10 ± 0.01
	18:0	6.1 ± 0.7 [1]	4.1 ± 0.4	3.6 ± 0.6
Unsaturated Fatty Acids	16:1(9)	1.1 ± 0.3	0.9 ± 0.2	0.8 ± 0.2
	18:1(11)	71.3 ± 2.2	69.6 ± 1.9	54.9 ± 1.6 [1]
	19:1(10)	1.2 ± 0.2 [1]	0.5 ± 0.1 [2]	ND.
3-Hydroxy Fatty Acids	3-OH-12:0	ND.	ND.	ND.
	3-OH-14:0	2.9 ± 0.6	ND.	ND.
	3-OH-16:0	0.09 ± 0.03	ND.	ND.
	3-OH-18:0	0.4 ± 0.2 [1]	0.09 ± 0.03 [2]	ND.
Cyclopropane Fatty acids	17:0cyclo(9,10)	ND.	0.47 ± 0.03 [1]	0.97 ± 0.08 [2]
	19:0cyclo(11,12)	2.5 ± 0.7 [1]	14.6 ± 0.9 [2]	31.0 ± 0.7 [3]
Total (%)		100	100	100

* Sum of all fatty acid derivatives observed (FAMEs and trimethylsilyl derivatives).
[1, 2, 3] Mean values are significantly different between columns ($P \leq 0.05$).
ND.: not detected.

For the analysis of cyclopropane-containing fatty acids, method C is the best choice while method A is most suitable for 3-hydroxy fatty acids. These results are not surprising since cyclopropane-containing fatty acids are labile to acidic conditions (e.g., methods A and B). Acidic conditions are reported to be the best for the release of amide-bound fatty acids from lipids, including hydroxy fatty acids [190]; however, method B has been reported to produce low recoveries of hydroxy fatty acids from bacterial lipids [19, 173]. In the current work 3-hydroxytetradecanoate (3-OH-14:0), the most abundant bacterial hydroxy fatty acid, was not observed using method B while 3-hydroxyoctadecanoate, a minor component at < 0.1% of total fatty acids, was detected. The extraction of LPSs from cells requires especial extraction procedures [191, 192]; thus lipid extraction using $CHCl_3$:CH_3OH (Methods B and C) while effective for most lipid classes, is inefficient for large molecules of various polarities such as LPSs. Thus, inadequate extraction of LPSs from cells might be the reason why 3-OH-14:0 was not detected using methods B and C. In summary, relative percentage compositions of bacterial fatty acids are highly dependent on the method used; no single method provides a comprehensive fatty acid profile due to different labilities of 3-hydroxy and cyclopropane-containing fatty acids [173].

Two cyclopropane-containing fatty acids were positively identified as *cis*-9,10-methylene-hexadecanoic acid (17:0cyclo(9,10)) and *cis*-11,12-methylene-octadecanoic acid (19:0cyclo(11,12)). Their relative percentage compositions were statistically different using the three methods (Table 2.4, $P \leq 0.05$). The mass spectra of two isomeric methyl esters, *cis*-10-nonadecenoic and *cis*-11,12-methyleneoctadecanoic acids, were virtually identical; differentiation between them was only possible by chromatographic separation (using a DB-17ht column, a 50% phenyl, 50% methyl polysiloxane phase, peaks 13 and 14 in Figure 2.2a). Derivatives such as picolinyl esters which have been reported to be useful in localizing cyclopropane rings or double bonds in fatty acids [71] were not used in this work.

Figure 2.2. Representative total ion chromatograms of fatty acid derivatives produced from *S. meliloti* using methods A, B and C.



3.4. Development of a One-vial Method for FAME Analyses

Fatty acid analysis methods are laborious procedures requiring multiple extraction steps and drying/evaporation steps prior to GC or GC/MS analysis. Method C which gave the highest recoveries of cyclopropane fatty acids was selected for development of a simplified and micro-scale version of the method. A goal of our research program is the study of genes involved in the biosynthesis of lipids, including the cyclopropanation of lipids. The scales of traditional fatty acid methods would require bacterial growth in large volumes of media. We sought to develop a micro-scale version of method C which would be amenable to small volumes of bacterial cultures, would require minimal sample handling and would be amenable to the analysis of large numbers of samples.

The one-vial method incorporated all of the quality control, recovery standards and internal standards introduced previously. A standard GC/MS vial with a 200 μL insert was used as the "one-vial" reaction vessel; the amounts of lipid introduced into the vial ranged between 10 and 100 μg, corresponding to a 100- to 1000-fold reduction in the amount of lipid needed compared to conventional methods (10-100 mg lipid per analysis). Reagent volumes were reduced 40- to 80-fold relative to conventional procedures. In a typical micro-scale analysis lipids equivalent to 30-40 μg of wet cells were injected onto the chromatographic column, making this protocol comparable in sensitivity to recently reported thermochemolysis methods [17]. We believe our procedure is more practical for most researchers than reported pyrolitic methods since a standard GC/MS instrument can be used without the need for additional apparatus or instrument modifications.

Method development data obtained using standards is reported in Table 2.5 while application of the protocol to the analysis of *S. meliloti* samples is reported in Table 2.6. Given the dramatic reduction in scale using the one-vial method, it was important from the outset to determine whether hydrolysis would be a major side reaction and whether transmethylation conditions would result in decreased reaction yields. Christie has cautioned analysts that well-established methods fail when sample size and reagent volumes are scaled down due to hydrolysis caused by the presence of traces of water in the glassware and reagents [175]. To address these issues five transmethylation reaction conditions were compared using a suite of five lipid standards (i.e., those listed in Table 2.3 except SM). Three temperatures (25°, 50° and 60°C) were compared with a fixed reaction time of 15 minutes; the remaining reactions were performed for 30 and 60 minutes at 25°C. The results are summarized in Table 2.5.

Table 2.5. Optimization of transmethylation reaction parameters for one-vial Method C.

(a)

Quality Control Standards	Fatty Acid Derivatives	Percentages Recoveries From Standards, Mean ± SD (n=3)				
		25°C, 15 min.	25°C, 30 min.	25°C, 60 min.	50°C, 15 min.	60°C, 15 min.
iPr-14:0-d_{27}	iPr-14:0-d_{27}	63.7 ± 7.4 [1]	38.6 ± 4.7 [2]	15.7 ± 0.5 [3]	0.0	0.0
	Me-14:0-d_{27}	34.2 ± 5.2 [1]	57.1 ± 5.9 [2]	78.8 ± 6.8 [3]	58.9 ± 5.1 [2]	34.1 ± 13.5 [1]
	TMS-14:0-d_{27}	ND.	ND.	ND.	0.3 ± 0.2	2.4 ± 0.9
	Total 14:0-d_{27} species	97.9 ± 12.0 [1]	95.7 ± 8.8 [1]	94.5 ± 6.4 [1]	59.2 ± 5.2 [2]	36.5 ± 14.4 [2]

(b)

Lipids	Fatty Acid Derivatives	Percentages Recoveries From Lipids, Mean ± SD (n=3)				
		25°C, 15 min.	25°C, 30 min.	25°C, 60 min.	50°C, 15 min.	60°C, 15 min.
CL(14:0/14:0/14:0/14:0)	Me-14:0	120.8 ± 9.8 [a]	108.0 ± 6.7 [a]	99.8 ± 7.5 [a]	83.7 ± 6.8 [1, a]	61.0 ± 18.5 [1]
	TMS-14:0	ND.	ND.	ND.	ND.	0.22 ± 0.26
PE(17:0/17:0)	Me-17:0	101.2 ± 13.9 [a, b]	97.4 ± 7.7 [a, b]	95.1 ± 7.9 [a, b]	75.3 ± 5.9 [a,]	53.4 ± 14.3 [1]
	TMS-17:0	ND.	ND.	ND.	0.5 ± 0.5	3.5 ± 1.1
TAG(16:0-d_{31}/16:0-d_{31}/16:0-d_{31})	Me-16:0-d_{31}	80.6 ± 12.3 [b, c]	85.1 ± 8.7 [b, c]	86.1 ± 7.3 [a]	68.3 ± 5.3 [1, a, b]	48.7 ± 17.2 [1]
	TMS-16:0-d_{31}	ND.	ND.	ND.	1.1 ± 0.8	6.1 ± 1.4
PC(19:0/19:0)	Me-19:0	89.2 ± 3.9 [b, c]	77.8 ± 8.7 [c, d]	76.6 ± 11.4 [b]	46.0 ± 6.9 [1, c]	28.2 ± 10.0
	TMS-19:0	ND.	ND.	ND.	ND.	0.3 ± 0.2
PG(12:0/12:0)	Me-12:0	69.6 ± 2.8 [c]	63.4 ± 2.2 [d]	54.1 ± 4.2 [c]	53.6 ± 7.6 [b, c]	39.1 ± 14.3 [1]
	TMS-12:0	ND.	ND.	ND.	ND.	0.04 ± 0.03

iPr-14:0-d_{27}, Me-14:0-d_{27}, TMS-14:0-d_{27}: isopropyl, methyl and trimethylsilyl esters of tetradecanoic-d_{27} acid, respectively; PE(17:0/17:0): 1,2-diheptadecanoyl-*sn*-glycero-3-phosphoethanolamine; CL(14:0/14:0/14:0/14:0): 1,1',2,2'-tetramyristoyl cardiolipin; PG(12:0/12:0): 1,2-dilauroyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol); TAG(16:0-d_{31}/16:0-d_{31}/16:0-d_{31}): Glyceryl tri(hexadecanoate-d_{31})); PC(19:0/19:0): 1,2-dinonadecanoyl-*sn*-glycero-3-phosphocholine; Me-: fatty acid methyl ester; TMS-: trimethylsilyl ester of fatty acids; ND: not detected.

[1, 2, 3] Mean values are significantly different between columns ($P \leq 0.05$). Means with the same number were not significantly different between columns ($P \geq 0.05$).

[a, b, c, d] Mean values are significantly different within columns ($P \leq 0.05$). Means with the same letter were not significantly different within columns ($P \geq 0.05$).

ND.: not detected.

The results of these experiments were truly dramatic. Recoveries of the total 14:0-d_{27} species were quantitative for the three reaction times at 25°C but decreased significantly at 50°C and 60°C (60% and 37%, respectively, Table 2.5a). The losses due to hydrolysis, as determined by the 14:0-d_{27} TMS ester levels, were insignificant to very small. The poor recoveries of FAMEs are likely the result of side reactions, probably base-catalyzed Claisen-type condensations, resulting in the formation of higher molecular mass products that are not detected by the current GC/MS method. Similar trends were observed in the FAMEs data from reactions of lipid standards; reactions at 50°C and 60°C led to statistically lower FAMEs yields that at 25°C (Table 2.5b). There were no significant differences among the three reaction times at 25°C.

These data lead to two important conclusions for fatty acid analysis: first, reaction temperatures of 50°C and 60°C result in significant losses of the target analytes (FAMEs) presumably due to unknown side reactions and not due to hydrolysis reactions; second, yields of FAMEs vary significantly with phospholipid type. Since most fatty acid analysis methods in the literature use reaction temperatures of 50°C or greater with reaction times longer than 15 minutes, the yields of FAMEs will be likely compromised and the reported percentage composition data may not accurately reflect the real compositions. Furthermore, fatty acid data from the analyses of phospholipid mixtures may be compromised due to the differences in relative yields from different phospholipid classes. The protocols described in this work could be readily used or adapted to any fatty acid analysis protocol to allow researchers to assess the relative efficiencies of conversion, degrees of hydrolysis and extents of unwanted side reactions in the protocols used in their laboratories.

It's worth mentioning that the recoveries of FAMEs from different fatty acid classes (saturated, 3-hydroxy and cyclopropane fatty acids) were studied using the same transmethylation conditions (temperatures and times). In general, the recoveries of FAMEs decreased with heating; however, this effect was more accentuated for 3-hydroxy and cyclopropane fatty acids which showed decreases of 56-74% compared to 20-45% decreases in yields for saturated fatty acids (data not shown).

The relative percentage compositions of fatty acids in *S. meliloti* determined using the one-vial method are shown in Table 2.6. Three sets of bacterial cultures, grown over a period of six months, were examined with each culture analyzed in triplicate. The one-vial method provided reproducible results with RSDs between 5% and 20% for major components and up to 60% for components with abundances below 1%. In summary, it can be concluded that complete transmethylation of lipids can be accomplished at 25°C with no hydrolysis and minimal losses due to side reactions. Our results are in agreement with those of Christie who reported complete transesterification of lipids at room temperature in a few minutes [173]. Furthermore, hydrolysis losses were negligible when working with the small volumes of reagents and small amounts of lipids (~10 μg) in the micro-scale procedure.

Table 2.6. Relative percentage compositions of fatty acids in *S. meliloti* determined using the one-vial method.

Fatty Acid Classes	Fatty Acids	Relative Percentage Composition, (%)	
		Mean ± SD (n=9)	RSD (%)
Saturated Fatty Acids	14:0	0.4 ± 0.1	27
	16:0	12.8 ± 2.2	17
	17:0	0.2 ± 0.1	38
	18:0	3.4 ± 0.7	19
Unsaturated Fatty Acids	16:1(9)	1.2 ± 0.7	60
	18:1(11)	60.4 ± 3.3	5
Cyclopropane Fatty Acids	17:0cyclo(9,10)	1.7 ± 0.2	13
	19:0cyclo(11,12)	19.9 ± 3.8	19
Total		100.0 %	

3.5. Fatty Acid Analysis of S. meliloti Cells under Two Growth Conditions using the One-vial Method

Results discussed above demonstrated that method A provided the most comprehensive fatty acid profiles while method C and the one-vial version of method C provided the best results for cyclopropane fatty acids. Therefore the one-vial format of method C and conventional method A were used to analyze *S. meliloti* cells cultured under normal and stressed growth conditions, the latter induced by phosphate starvation conditions. Isopropyl tetradecanoate-d_{27} and the recovery standards (Me-15:0, n-C20) were added to each sample to determine transmethylation efficiency, extent of hydrolysis and overall recoveries.

Recoveries of n-C20 and Me-15:0 were good (109-115%) with the one-vial method C. Recovery of methyl tetradecanoate-d_{27} (only 18%) was consistent with results presented above wherein transmethylations of the isopropyl ester were found to be significantly slower that transmethylations of lipids; the lipids were fully transmethylated under these conditions. The one-vial procedure for Method C did not show any evidence of hydrolysis (i.e., no trimethylsilyl tetradecanoate-d_{27} was detected).

Relative percentage compositions of fatty acids in *S. meliloti* grown under control and phosphate-stressed conditions are shown in Table 2.7a using the one-vial method C. The most dramatic result was a spectacular increase in the amount of 3-hydroxybutanoic acid found in the phosphate-stressed cells. 3-Hydroxybutanoic acid (often called 3-hydroxybutyric acid) is formed via hydrolysis of poly-3-hydroxybutyrate (PHB), a biopolymer commonly found in bacteria. A distinct advantage of the one-vial method C is the ability to detect 3-hydroxybutanoate directly in the GC/MS analysis; conventional fatty acid analytical procedures use one or more aqueous extraction steps which result in loss of 3-hydroxybutanoic acid to the aqueous phase. This is consistent with

results obtained when method A was applied to the analysis of phosphate-starved cells since 3-hydroxybutanoic acid was not detected (Data not shown). In contrast, this polar molecule is fully retained (and detected) using the one-vial protocol which affords the methyl ester of 3-hydroxybutanoic acid; the latter is converted by MSTFA into the readily detected methyl ester of 3-trimethylsilyloxybutanoic acid. Thus, this protocol is the only one we are aware of which allows the detection of PHB on a small scale and which allows the determination of fatty acids and 3-hydroxybutanoate simultaneously.

Previously reported methods for the analysis of PHB are tedious procedures and suffer from losses due to the water extraction steps [193-195]. While the one-vial method has great potential for the analysis of PHB in bacteria, this method has not been optimized nor have appropriate controls been developed yet. However, we are confident that the data reported here are of good quality. Given the increasing interest in the determination of PHB in a number of systems, this method should prove useful to a number of researchers.

Given the large change in 3-hydroxybutanoic acid levels it is difficult to compare the differences in the percentage compositions of the other fatty acids (Table 2.7a); by removing the 3-hydroxybutanoate data (Table 2.7b) direct comparisons of the fatty acid levels were possible. The relative percentage composition of *cis*-11,12-methyleneoctadecanoic acid, the most abundant of cyclopropane fatty acids, increased two-fold under the phosphate starvation conditions. This increase led to a modest decrease in all of the other fatty acids, particularly oleic acid, since it is the precursor of that cyclopropane fatty acid.

It should be remarked, that percentages of cyclopropane fatty acids in phosphate-starved cells were 5-8% lower using method A relative to the one-vial method C due to the acidic conditions used in method A. The distinct advantages of the one-vial protocol over standard Method C protocols are four-fold: (1) the greater ease of using the one-vial protocol, (2) the ability to do multiple samples in parallel, (3) the ability to determine 3-hydroxybutanoic acid directly and reproducibly and (4) free fatty acids generated from hydrolysis as a side reaction can be accounted for in final results.

Table 2.7. Fatty acid relative percentage composition of *S. meliloti* in the presence (control) and absence of phosphate (stressed) with the one-vial method (a). Relative percentage compositions calculated without 3-hydroxy fatty acid species for both control and stressed (b).

(a)

Fatty Acid Class	**Fatty Acid**	**Relative % Composition of *S. meliloti*, Mean ± SD (n=3)**	
		Control	**Stressed**
Saturated Fatty Acids	14:0	0.4 ± 0.1 [1]	0.1 ± 0.0 [1]
	16:0	13.8 ± 3.7 [1]	2.4 ± 1.0 [1]
	17:0	0.3 ± 0.1 [1]	0.1 ± 0.0 [1]
	18:0	2.7 ± 0.6 [1]	0.5 ± 0.1 [1]
Unsaturated Fatty Acids	16:1(9)	2.0 ± 0.5 [1]	0.3 ± 0.0 [1]
	18:1(11)	61.9 ± 3.6 [1]	13.4 ± 2.5 [1]
	19:1(10)	ND.	ND.
3-Hydroxy Fatty Acids	3-OH-4:0 (PHB)	1.2 ± 1.4 [1]	74.6 ± 4.8 [1]
	3-OH-12:0	ND.	ND.
	3-OH-14:0	0.1 ± 0.1	0.007 ± 0.002
	3-OH-16:0	ND.	ND.
	3-OH-18:0	ND.	ND.
Cyclopropane Fatty Acids	17:0cyclo(9,10)	1.7 ± 0.3 [1]	0.5 ± 0.1 [1]
	19:0cyclo(11,12)	15.8 ± 2.0 [1]	8.1 ± 1.1 [1]
Total		100%	100%

[1] Mean values are significantly different between control and stress ($P \leq 0.05$).

(b)

Fatty Acid Class	**Fatty Acid**	**Relative % Composition of *S. meliloti*, Mean ± SD (n=3)**	
		Control	**Stressed**
Saturated Fatty Acids	14:0	0.4 ± 0.1	0.3 ± 0.1
	16:0	14.0 ± 3.6	9.3 ± 2.1
	17:0	0.3 ± 0.1	0.2 ± 0.1
	18:0	2.7 ± 0.6 [2]	1.9 ± 0.2
Unsaturated Fatty Acids	16:1(9)	2.0 ± 0.5 [2]	1.4 ± 0.1
	18:1(11)	62.8 ± 3.6 [1]	52.9 ± 0.2 [1]
Cyclopropane Fatty Acids	17:0cyclo(9,10)	1.8 ± 0.3	2.0 ± 0.1
	19:0cyclo(11,12)	16.1 ± 2.3 [1]	31.9 ± 2.0 [1]
Total		100%	100%

[1] Mean values are significantly different between control and stress ($P \leq 0.05$).
ND.: not detected.

4. Conclusions

The results presented show that for fatty acid analysis in *S. meliloti* two methods were found to be useful: (1) basic hydrolysis followed by acid-catalyzed methylation was best for the analysis of 3-hydroxy fatty acids while (2) base-catalyzed transmethylation with sodium methoxide was best for determination of cyclopropane-containing fatty acids. The methods reported here included a number of recovery and internal of standards and a silylation step which not only improved the protocols but allowed, for the first time, direct determination of losses caused by sample handling or side reactions as well as the fates of acid- and base-sensitive fatty acid derivatives.

Most importantly, a micro-scale, one-vial method for fatty acid analysis was developed with sample sizes decreased by at least 100-fold compared to conventional procedures. The methodology was designed to analyze small samples sizes, to minimize sample handling, to increase sample throughput, to increase method sensitivity and to improve the overall quality of analysis; the method succeeded on all counts. This method was successfully applied to the analysis of fatty acids in *S. meliloti* and showed very few side reactions, small sample losses and excellent recoveries, identical to conventional, large scale methods. The use of a range of quality control and recovery standards allowed monitoring for completion of derivatization reactions, side reactions and recoveries. Quantitation of trimethylsilyl esters of fatty acids provided an easy and rapid way to measure the degree of sample loss due to hydrolysis. Hydrolysis side reactions were negligible when transmethylations were conducted at room temperature; reaction temperatures of 50°C or higher resulted in significant losses of fatty acids, presumably due to aldol-like condensation reactions. One unexpected outcome of these small-scale reactions was the development of a convenient, one-step method for the analysis of 3-hydroxybutanoic acid, resulting in an efficient method for the determination of poly(3-hydroxybutanoic acid) on very small scales. These procedures were applied to the analysis of *S. meliloti* grown in the presence and absence of phosphate. Analysis of phosphate-starved cultures of *S. meliloti* using the one-vial method, revealed a two-fold increased of cyclopropane fatty acids and a 73% increase in poly(3-hydroxybutanoic acid). The methodologies developed here will be applied in future studies of *S. meliloti* to investigate the effect of phosphate starvation and other stressors on the cyclopropanation of lipids and to determine the genes involved in this process.

Chapter 3

A Shotgun Lipidomics Approach in *Sinorhizobium meliloti* as a Tool for Functional Genomics

Abstract

A shotgun lipidomics approach was developed to analyze intact lipids in crude extracts of *Sinorhizobium meliloti* as a tool for functional genomics. New MS/MS transitions are reported for the analysis of two phospholipid classes (monomethylphosphatidylethanolamines, dimethylphosphatidylethanolamines) and three non-phosphorus containing lipid classes (sulfoquinovosyldiacylglycerols (SLs), ornithines (OLs), diacylglyceryl-(*N,N,N*-trimethyl)-homoserines (TMHSs)). This is the first report to use shotgun lipidomics for the analysis of bacterial non-phosphorus containing lipids. The use of unique MS/MS transitions for each lipid class allowed the analysis of isobaric species without the need for chromatographic separation. The methodology required small sample amounts and virtually no sample preparation; thus, it has excellent potential to be used as a screening tool for the analysis of large volumes of samples. While traditional methodologies that employ TLC, detected only abundant molecular species, shotgun lipidomis provided complete fatty acid distributions within lipid classes. In summary, fatty acid distributions were obtained for eight lipid classes and 58 individual lipids were identified in crude lipid extracts. This is the first report to describe fatty acid distributions in lipids and the relative positions of fatty acyl substituents (*sn*-1, *sn*-2) in phospholipids in *S. meliloti*. Fatty acid distributions in TMHSs were identical to phospholipids, which indicated a common biosynthetic origin for these lipid classes. Lipid cyclopropanation increased significantly under P_i-starvation; this effect was more accentuated in mutants deficient in the PhoB regulator protein.

Introduction

Sinorhizobium meliloti is a soil bacterium that transforms atmospheric nitrogen into a form utilizable by leguminous plants such as alfalfa while establishing a beneficial relationship; this process is called nitrogen fixation [28, 29]. The complete genome of *S. meliloti* has been annotated; however, the function of more than 40% of genes remains unknown [27, 37]. In the past decade, numerous efforts have been directed to understand gene function (functional genomics) and the mechanisms by which symbiosis and nitrogen fixation occur [35, 36, 50, 196]. Our research focuses on the study of genes in *S. meliloti* which are hypothesized to participate in lipid biosynthesis. The assignments of genes functions have been made in most cases by comparing *S.*

meliloti's genome with the genomes of other well-studied organisms such as *E. coli*.

Comprehensive lipid profiling known as lipidomics, has been proposed as a strategy to improve our understanding of metabolism and gene function [12, 13, 197]. Our approach to understand gene function, employs comparisons of metabolite (lipids and fatty acids) profiles of knockout mutants with those of wild type strains. Thus, rapid and comprehensive methods for the analysis of lipids in *S. meliloti* were needed. Analytical methods for comprehensive lipid analysis must provide information regarding: (i) lipid classes; (ii) molecular species within lipid classes caused by variations of the fatty acyl chains; and (iii) the relative locations of the fatty acyl chains in individual lipids (*sn*-1 and *sn*-2). Typically, methods for intact lipid analysis involve lipid extraction commonly by the Bligh & Dyer method followed by chromatographic separation of lipid classes and their detection by mass spectrometry.

Thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) [24, 107] are the most common chromatographic techniques although capillary electrophoresis has been also employed [198]. Two dimensional high performance TLC (2D-HPTLC) procedures have been used successfully to separate a wide spectrum of lipid classes [70]. However, to obtain fatty acid distributions within lipid classes using this approach, further analysis of the isolated lipid classes (TLC spots) is required; this is typically achieved by analyzing TLC spots by electrospray ionization mass spectrometry (ESI/MS) or gas chromatography coupled to mass spectrometry (GC/MS). TLC-based methodologies present major drawbacks such as lipid oxidation on TLC plates, poor recoveries and are inconvenient for high throughput analyses [24, 122]. On the other hand, HPLC-based methodologies separate lipids by classes or into their molecular constituents using normal phase chromatography (NP-HPLC) or reverse phase chromatography (RP-HPLC), respectively [24]. NP-HPLC separations are determined by polar head groups in lipids while the length of fatty acyl chains in lipids determines separations by RP-HPLC. When complete chromatographic separation of both lipid classes and their individual molecular species is desired, a combination of NP-HPLC followed by RP-HPLC is needed [23, 107, 199]. Yet, the separation of isobaric species of different lipid classes is a challenge and chromatographic coelution still occurs [24].

Chromatographic procedures for intact lipid analysis tend to be laborious; moreover chromatography can reduce lipid recoveries up to 50% and affect the distribution of molecular species [122]. While HPLC-ESI/MS analysis is currently the most common approach in lipid analysis, Han and Gross have demonstrated that phospholipids can be directly analyzed from crude lipid extracts under ESI conditions; this approach has been termed shotgun lipidomics [126]. Shotgun lipidomics uses a combination of MS/MS techniques to differentiate lipid classes by their fragmentation patterns under collision induced dissociation (CID) [14, 82]. This methodology required small sample amounts and no sample preparation, which are ideal features of methodologies for

functional genomics applications. Therefore, we sought to develop a shotgun lipidomics procedure for the analysis of intact lipids in crude extracts of *S. meliloti* as a tool for functional genomics studies. Intact lipids in *S. meliloti* have been previously analyzed using TLC procedures which are not amenable to high-throughput analyses. Furthermore, fatty acid distributions within lipid classes has not been reported in *S. meliloti.*

Cells membranes in *S. meliloti* are composed by phospholipids that include phosphatidylcholines (PCs, 60%), phosphatidylethanolamines and monomethylphosphatidylethanolamines (PEs + MMPEs, 20%), dimethylphosphatidylethanolamines (DMPEs, 2%), phosphatidylglycerols (PGs, 9%) and cardiolipins (CLs, 5%) [51]. Non-phosphorus containing lipids such as sulfoquinovosyldiacylglycerols (SLs) and ornithines (OLs) are also present but comprise less than 5% under normal growth conditions [51]. Under inorganic phosphate (P_i)-limiting conditions, non-phosphorus containing lipids (SLs, OLs and diacylglyceryl-*O*-(*N,N,N*-trimethyl)-homoserine lipids (TMHSs)) are important membrane components representing up to 70% of total lipids [51]. Shotgun lipidomics approaches have been developed for the analysis of lipids encountered in eukaryotic cells (phospholipids and sphingolipids). There are no reports of the use of this approach for the analysis of bacterial lipids such as MMPEs, DMPEs, SLs, TMHSs or OLs. Therefore, a goal of this work was to develop a shotgun lipidomics method suitable for the analysis of MMPEs, DMPEs and non-phosphorus containing lipids in *S. meliloti* and to provide detailed fatty acid distributions within lipid classes. In order to validate our approach, the methodology was applied to the analysis of intact lipids and fatty acids of *S. meliloti* grown in inorganic phosphate (P_i) rich medium and under P_i-limiting conditions using wild type strains and *phoB* mutant strains. PhoB was selected to validate our methodology since it is a well studied global regulator protein known to modulate the expression of genes during low-P_i stress [50, 200]. Principal component analysis (PCA) was employed to identify patterns or trends in the data sets.

Experimental

Abbreviations

Fatty acids were represented using the shorthand designation system recommended by the IUPAC [52]. Intact lipids were identified as PCs for phosphatidylcholines, di-methylphosphatidylethanolamines as DMPEs, mono-methylphosphatidylethanolamines as MMPEs, phosphatidylethanolamines as PEs, phosphatidylglycerols as PGs, cardiolipins as CLs, sulfoquinovosyldiacylglycerols as SLs, 1,2-diacylglyceryl-3-*O*-4'-(*N,N,N*-trimethyl)-homoserine lipids as TMHSs and ornithine lipids as OLs. Fatty acid composition in lipids was represented by the sum of carbon atoms in the fatty acyl chains of lipids followed by a colon and the total number of unsaturations, e.g., 1,2-dioleoyl-*sn*-glycero-3-phosphocholine was represented as PC-36:2. The relative positions of fatty acyl chains in lipids were represented by the fatty acid

in the *sn*-1 position first followed by the fatty acid in the *sn*-2 position and separated by a forward slash as recommended [201]. When the assignment of fatty acids to the *sn*-1 and *sn*-2 positions in lipids was ambiguous, the fatty acids were separated by a hyphen instead of a forward slash.

Reagents and Materials

N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) and ethyl dodecanoate were obtained from Sigma-Aldrich (Saint Louis, MO). All solvents used were HPLC grade (Caledon Labs, Caledon, ON). Phospholipid standards, 1,2-diheneicosanoyl-*sn*-glycero-3-phosphocholine (PC(21:0/21:0)), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (PE(14:0/14:0)) and 1,2-dilauroyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol) (PG(12:0/12:0), sodium salt) were purchased from Avanti Polar Lipids (Alabaster, AL).

Bacterial Strains and Growth Conditions

Wild type *S. meliloti* (RmP110) and *phoB* mutant RmP containing *phoB3::Tn5* (RmH838 [202] were grown in MOPS buffered minimal media [203] containing 10 ng/mL $CoCl_2$, 0.5 µg/mL biotin, and glucose (15 mM) as the carbon source in the presence (2 mM) or absence of inorganic phosphate (P_i). Wet cell pellets obtained from 500 mL of culture (O.D.=0.3-0.6) by centrifugation were resuspended in 2.5 mL of MOPS medium with no phosphate present and divided into 250 µL aliquots in microfuge tubes. Each tube was then centrifuged, the supernatant was discarded and the wet pellets were stored at -80°C following flash freezing in liquid nitrogen. The wet pellet equivalent to 50 mL from cultures with 2 mM P_i and without P_i, weighed 32.7 ± 2.3 mg (n=6) or 20.9 ± 3.1 mg (n=6), respectively.

Lipid Extraction

Wet cell pellets were resuspended in distilled water (1 mL) and extracted with a mixture of $CHCl_3$:MeOH (1 mL, 2:1, v/v). The $CHCl_3$ phase containing the lipids was separated and the aqueous phase was extracted twice more with $CHCl_3$:MeOH (1 mL, 2:1, v/v). The chloroform phases from each extraction were pooled and dried through a small column packed with anhydrous sodium sulphate (~1 g). The solvent was evaporated using a stream of nitrogen gas and the dried lipid residue was either analyzed immediately or stored at -80°C.

Fatty Acid Methyl Ester Analysis by Gas Chromatography-Mass Spectrometry

Fatty acids in lipids were analyzed as their fatty acid methyl esters (FAMEs) by GC/MS. FAMEs were prepared from dried lipid extracts using a one-vial method that employed sodium methoxide in methanol as the transmethylation reagent [63].

Adsorption Column Chromatography

Dry lipid extracts were dissolved in $CHCl_3$:MeOH (100 μL, 1:1, v/v) and applied to columns packed with silica gel (1 g, 35-75 μm). Glycolipids were eluted with acetone (10 column volumes) as described by Christie [204]. The fraction containing the glycolipids was dried under nitrogen stream and stored at -80°C for mass spectrometric analysis.

Intact Lipid Analysis by Electrospray Ionization Mass Spectrometry

Dried lipid extracts were dissolved in MeOH:$CHCl_3$ (1:1, v/v, 200 μL). For analyses in the positive ionization mode, aliquots (20 μL) were diluted 5-fold with MeOH containing PC(21:0/21:0) (32 μmol), PE(14:0/14:0) (28 μmol) and lithium chloride (2.5 mM). For analyses in the negative ion mode, aliquots (20 μL) of the lipid extract were diluted 5-fold with methanol containing PG(12:0/12:0) (3 μmol) as the internal standard. A Waters Quattro Ultima triple quadrupole mass spectrometer equipped with a microelectrospray ionization source and operating under the MassLynx software was used for the analysis of bacterial lipid extracts. The collision gas (N_2) pressure was 2 x 10^{-3} Bar and collision energies (CE) ranged between 25 and 50 eV. Lipid extracts were continuously infused at 1 μL/min using a Harvard syringe pump.

PCs, DMPEs, MMPEs and PEs were simultaneously analyzed as their lithiated adducts in the positive ionization mode using neutral loss scans of 189 [99], 175, 161 and 147 [103] mass units with a collision energy of 35 eV. TMHSs and OLs were also analyzed as $[M+Li]^+$ ions using neutral loss scan of 74 mass units (CE 40 eV) and precursor ion scan of m/z 115 (CE 35 eV), respectively. PGs and SLs were analyzed as their $[M-H]^-$ ions in the negative ionization mode, using precursor ion scans of m/z 153 [14, 20] and 225, respectively (CE 50eV). One hundred spectra were averaged in each mass spectrometry experiment. Peak areas obtained from each MS/MS spectrum were normalized to the area of an internal standard. Relative percentage compositions of individual lipid species were obtained for each lipid class using the peak areas normalized to the internal standard in precursor ion or neutral loss spectra.

High resolution mass spectra were obtained using a Global Ultima Quadrupole Time of Flight Mass Spectrometer (Waters, Manchester, UK). Lipid extracts ($CHCl_3$:MeOH, 1:1, v/v) were infused continuously (1-5 μL/min) via a microelectrospray ionization source. Argon was used as the collision gas.

Statistical Analyses

All data are reported as mean values ± standard deviation. A statistical package (SPSS, version 15.0, SPSS Inc. Chicago, IL, USA) was used for statistical analyses. Student's t-test was used ($P \leq 0.05$) to evaluate two groups of data. Principal component analyses (PCA) were performed using the SPSS software and the Multivariate Analysis Add-in for Excel, version 1.3 (Bristol Chemometrics Inc., Bristol, UK).

Results and Discussion

The combination of mass spectrometric techniques allowed complete profiling of eight lipid classes (PCs, DMPEs, MMPEs, PEs, PGs, SLs, OLs, TMHSs) in crude lipid extracts of *S. meliloti* with little sample preparation and no chromatography. Lipid extracts were prepared from 50 mL of bacterial cultures; only 1/20 of samples (corresponding to 3 mL of culture) were infused. Good signal to noise ratios were also obtained when 10-fold less material was (0.3 mL of culture). Dry lipid extracts were diluted with $CHCl_3$:MeOH and two aliquots were taken and diluted 5-fold with MeOH. One solution was infused continuously into the mass spectrometer in the negative ionization mode for the analysis of anionic lipids (PGs, SLs) as their $[M-H]^-$ ions. Lithium chloride was added to the second solution to yield $[M+Li]^+$ adducts of PCs, DMPEs, MMPE, PEs, TMHSs and OLs, their analysis was conducted in the positive ionization mode using various neutral loss scans. The analysis time per sample about 60 min. was shorter than the time required by most HPLC procedures [120, 205]. The next section provides details about the MS/MS experiments conducted and describes the profiles observed for each lipid class in *S. meliloti* wild type under normal growth conditions and under P_i-starvation. The developed methodologies were applied to mutants deficient in the PhoB protein.

Phosphatidylglycerols and Sulfoquinovosyldiacylglycerols

A typical profile of a crude lipid extract of *S. meliloti* in negative electrospray ionization in the full scan mode is presented in Figure 3.1a. An internal standard, PG(12:0/12:0), was added to lipid mixtures. The full scan mode spectrum showed $[M-H]^-$ ions corresponding to nine PGs with major ions at m/z 747.6, 773.6 and 787.6. In addition, other minor peaks that corresponded to four SLs were detected. The CID spectra of the nine PGs (m/z 719.5, 721.5, 745.5, 747.5, 759.5, 761.5, 773.5, 787.5, 801.6) afforded product ion spectra typical of PGs. All mass spectra showed fragment ions corresponding to carboxylate ions due to losses of the fatty acyl substituents and a weak ion at m/z 153 corresponding to the polar head group, $[HO_3POCH_2C(OH)CH_2]^-$ [20]. These m/z values were identified as $[M-H]^-$ ions of PGs with fatty acid compositions corresponding to PG-32:1, PG-32:0, PG-34:2, PG-34:1, PG-35:2, PG-35:1, PG-36:2, PG-37:2 and PG-38:2, respectively.

Upon CID, $[M-H]^-$ ions of PGs typically produce $[RCOO]^-$ ions from the loss of fatty acids in the *sn*-2 position which are more abundant than $[RCOO]^-$ ions originated from losses of fatty acids in the *sn*-1 position [97]. Thus, this feature of the product ion spectra of PGs was used to determine the nature of the fatty acyl chains and their relative positions (Table 3.1). Previous studies of total fatty acid composition in *S. meliloti* by GC/MS analysis, greatly facilitated the identification of possible fatty acid combinations for each lipid. The product ion spectra of anionic lipids that contain nonadecenoic acid (19:1) or methyleneoctadecanoic acid (19:0cyclo) yield a $[RCOO]^-$ ion at m/z 295.2637, with the same elemental composition of $C_{19}H_{35}O_2$. GC/MS analysis determined

that *S. meliloti* contained methyleneoctadecanoic acid and not nonadecenoic acid [63]. The assigments of fatty acids to the *sn*-1 or *sn*-2 position for some minor lipids were ambiguous.

Precursor ion scans of m/z 153 were used to provide complete profiles of PGs in crude lipid extracts of *S. meliloti* (Figure 3.1b). Upon CID fragmentation of $[PG-H]^-$ ions, the polar head group ion at m/z 153 was of low abundance. In order to improve the sensitivity of this MS/MS transition, precursor ion scans of m/z 153 were conducted at collision energies of 50 eV. In addition, 100 spectra were routinely collected to improve the S/N ratios, especially for minor PGs; detection limits were in the low picomole range. While other researchers have employed precursor ion scans of m/z 153 to profile PGs in crude lipid extracts [14], most methodologies employ chromatographic separations [119, 206, 207]. We believe that precursor ion scan of m/z 153 is a simpler and faster strategy to profile PGs in bacterial extracts in comparison with chromatographic methods.

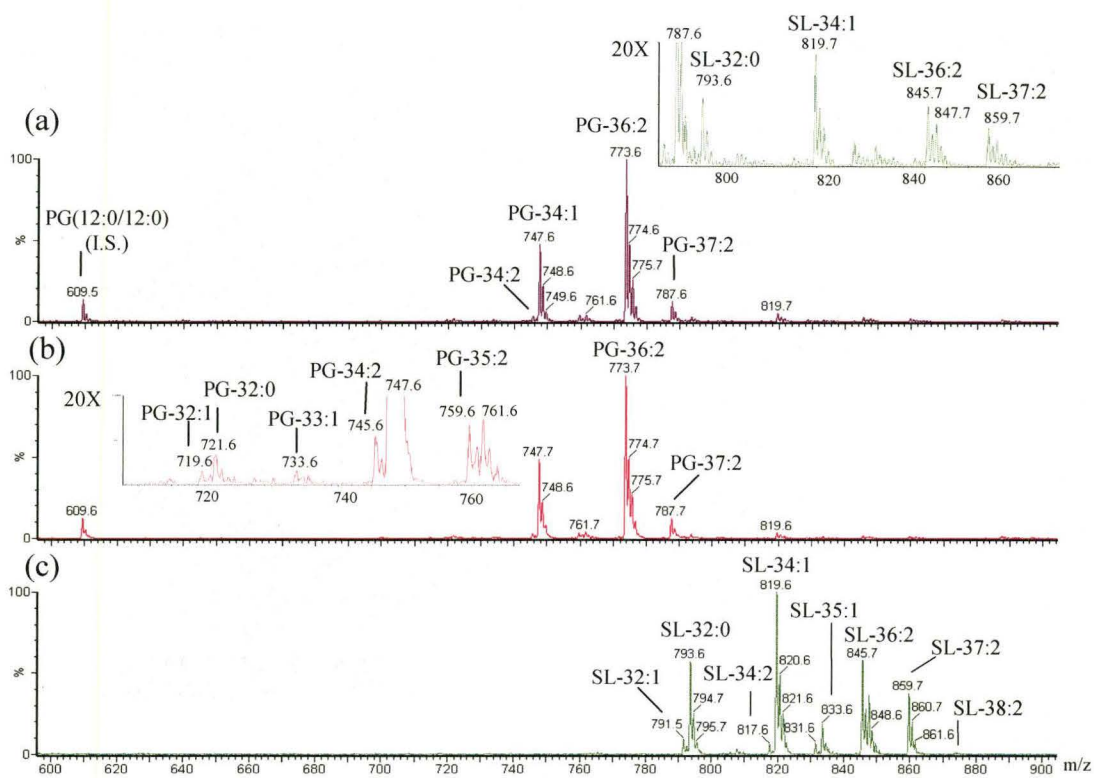


Figure 3.1. Electrospray ionization mass spectra in the negative ionization mode of crude lipid extracts of *S. meliloti*. (a) Full scan mode, (b) precursor ion scan of m/z 153, (c) precursor ion scan of m/z 225.

Table 3.1. Fatty acyl chains and their relative positions (*sn*-1/*sn*-2) in individual lipids in four lipid classes in *S. meliloti*. Fatty acids were separated by a hyphen when their assignment to the *sn*-1 and *sn*-2 positions was ambiguous. (*) Indicates possible fatty acid combinations for ornithine lipids.

Lipid	Fatty Acyl Chains in Lipids *sn*-1/*sn*-2			
	PG	SL	PC	OL
32:1	16:1-16:0	16:1-16:0		
32:0	16:0/16:0	16:0/16:0		
33:1	16:0/17:0cyclo			
33:0	17:0-16:0			
34:2	18:1/16:1	18:1-16:1	16:1/18:1	
34:1	18:1/16:0	18:1-16:0	18:1/16:0	3OH-16:0/18:1 * 3OH-18:0/16:1 *
35:2	18:1/17:0cyclo 16:1-19:0cyclo	18:1-17:0cyclo 16:1-19:0cyclo	18:1/17:0cyclo 16:1-19:0cyclo	
35:1	19:0cyclo/16:0	19:0cyclo-16:0	19:0cyclo/16:0	3OH-16:0/19:0cyclo * 3OH-18:0/17:0cyclo *
36:2	18:1/18:1	18:1/18:1	18:1/18:1	3OH-16:0/22:2 * 3OH-18:0/18:2 *
36:1		18:1-18:0		3OH-18:0/18:1
37:2	19:0cyclo/18:1	19:0cyclo-18:1	19:0cyclo/18:1	3OH-18:1/19:0cyclo *
37:1				3OH-18:0/19:0cyclo
38:2	19:0cyclo/19:0cyclo	19:0cyclo/19:0cyclo	19:0cyclo/19:0cyclo	3OH-18:0/20:2 *
39:2				3OH-18:0/21:2 *

SLs were readily detected as $[M-H]^-$ ions in negative electrospray ionization when crude lipid extracts were infused (Figure 3.1a, insert). The most abundant molecular species corresponded to SL-32:0, SL-34:1, SL-36:2 and SL-37:2 detected at m/z 793.6, 819.7, 845.7 and 859.7, respectively. Product ion spectra of these ions afforded $[RCOO]^-$ ions that matched SLs with fatty acid combinations of 16:0/16:0, 18:1/16:0, 18:1/18:1 and 19:0cyclo/18:1 for SL-32:0, SL-34:1, SL-36:2 and SL-37:2, respectively. Additionally, the product ion spectra of all SLs afforded a fragment ion at m/z 225.0. When Cedergren and coworkers characterized SLs in *S. meliloti* for the first time, they reported a fragment ion at m/z 225.0, identified as the dehydrosulfoglycosyl anion [208]. To confirm that these ions corresponded to SLs, a glycolipid fraction was prepared from lipid extracts using silica gel flash chromatography [204] and then analyzed using triple quadrupole and QqTOF mass spectrometers. Accurate mass measurements of fragment ions at m/z 80.9655 and 225.0076 in the product ion spectra of $[M-H]^-$ ions of major SLs confirmed elemental compositions of HSO_3 (80.9655) and $C_6H_9O_7S$ (225.0076), respectively. These fragment ions are typical of SLs, therefore these ions were identified as SLs [208, 209].

Precursor ion scans of m/z 225.0 were used to obtain SLs profiles in crude lipid mixtures of *S. meliloti*; ten molecular SL species were detected (Figure 3.1c). To improve the S/N ratios, collision energies of 50 eV were used to favor

fragmentation of $[M-H]^-$ ions and a 100 spectra were averaged. Normally, the analysis of SLs requires the isolation of these lipids via column chromatography or TLC [116, 149, 209, 210] and HPLC [211] prior to mass spectrometric detection. Fatty acid distributions in SLs are obtained by GC/MS analysis of a chromatographic fraction or TLC spots [109, 110, 210, 212]. These procedures are labor intensive and require milligram amounts of SLs. We demonstrated that precursor ion scans of m/z 225.0 can be used as a simple and rapid tool to obtain complete profiles of SLs in bacterial lipid extracts without chromatography.

CLs, another anionic lipid class, has been reported to occur in *S. meliloti* [51] and are normally detected in negative electrospray ionization as their $[M-2H]^{2-}$ ions [213]. CLs have been successfully analyzed in crude lipid extracts using precursor ion scans of m/z 153 with no evidence of ion suppression [14, 214]. We were not able to detect these lipids in crude lipid extracts using precursor ion scans of m/z 153. CLs in *S. meliloti* are a minor lipid class (<5%), thus is possible that these minor lipids coeluted with PEs or PGs. CLs could not be detected either in lipid extracts of *E. coli* even with the use of HPLC previous to ESI/MS analysis [119].

Phosphatidylcholines and Phosphatidylethanolamines

PCs and PEs can be detected as a mixture of $[M+H]^+$ and $[M+Na]^+$ ions upon infusion of crude extracts in positive electrospray ionization conditions (Figure 3.2.a). Under CID conditions, the product ion spectra of $[M+H]^+$ adducts of PCs and PEs showed fragments typical of the polar head groups at m/z 184 ($[H_2PO_4(CH_2)_2N(CH_3)_3]^+$) for PCs, or a neutral loss of 141 mass units ($[(OH)_2(PO)OCH_2CH_2NH_2]^+$) for PEs [20, 22]. The CID spectra of $[M+H]^+$ and $[M+Na]^+$ ions of PCs and PEs are rather uninformative regarding the nature and position of the fatty acyl substituents in lipids [99]. Table 3.2 shows the m/z values of $[M+H]^+$ and $[M+Na]^+$ adducts formed by PCs and PEs of various fatty acid compositions detected in *S. meliloti*.

In the presence of lithium salts, PCs and PEs form $[M+Li]^+$ ions which upon CID produce product ion spectra that contain diagnostic fragment ions for both the polar head group and the fatty acyl groups. Product ion spectra of $[PC+Li]^+$ contain unique fragment ions at m/z $[PC+Li-59]^+$ and $[PC+Li-189]^+$ due to neutral losses of $N(CH_3)_3$ and $[N(CH_3)_3+LiOPO_3(CH_2)_2]$, respectively [101]. Fatty acyl groups in lipids can be determined by fragment ions at m/z $[PC+Li-59-RCOOH]^+$ while their relative positions in the glycerol backbone (*sn*-1, *sn*-2) are determined by their intensities as fragment ions originated from the loss of fatty acids in *sn*-1 are more intense than those of *sn*-2 position, respectively [99, 101]. The fatty acyl chains and their relative positions in lipids (*sn*-1 and *sn*-2) are listed in Table 3.1. Similarly, product ion spectra of $[PE+Li]^+$ adducts afford fragment ions of the type $[PE+Li-43]^+$ and $[PE+Li-147]^+$ characteristic of the polar head group of PEs [103]. Fragment ions at m/z $[PE+Li-43-RCOOH]^+$ determine fatty acyl groups and their relative positions in lipids is given by their relative intensities (*sn*-1 > *sn*-2) [103].

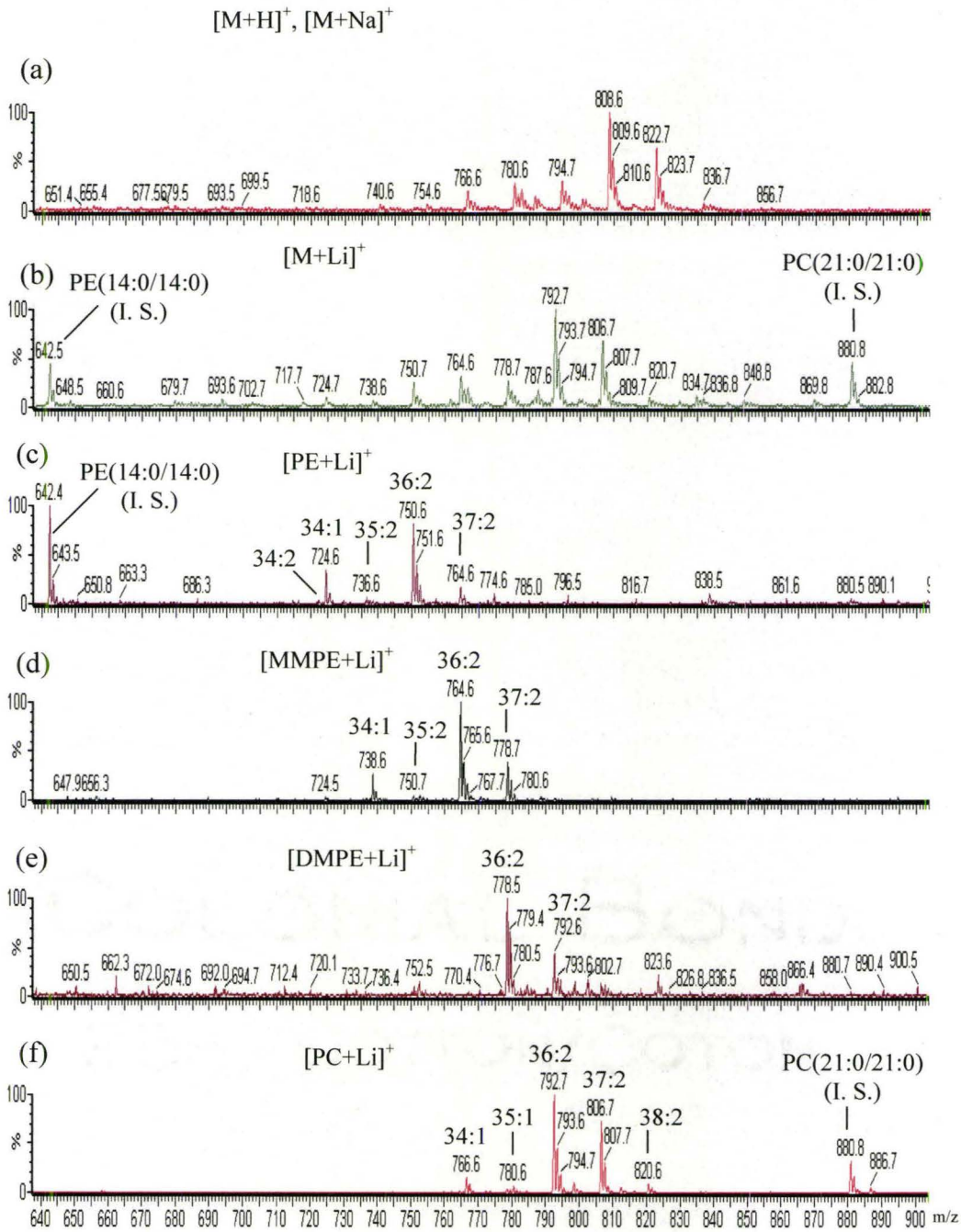


Figure 3.2. Electrospray ionization spectra in the positive ion mode of crude lipid extracts of *S. meliloti* (a) Full scan mode, (b) full scan mode, Li^+ 10 mM added, (c) neutral loss scan of 147 mass units, (d) neutral loss scan of 161 mass units, (e) neutral loss scan of 175 mass units, (f) neutral loss scan of 189 mass units.

Figure 3.2b shows the lipid profiles of extracts of *S. meliloti* in the positive ionization mode in the presence of lithium salts. Two internal standards were included, PC(21:0/21:0) and PE(14:0/14:0) (Figure 3.2b). The addition of lithium significantly simplified lipid profiles by converting the mixture of $[M+H]^+$ and $[M+Na]^+$ ions to $[M+Li]^+$ ions. This can be appreciated when comparing the high number of lipid species detected in crude extracts (Table 3.2), relative to the significantly reduced number of species detected when Li^+ was added to lipid extracts (Table 3.3). Neutral loss scans of 189 and 147 mass units, corresponding to the losses of the polar head groups of PCs and PEs respectively, afforded individual profiles for each lipid class (Figures 3.2f and 3.2b). Chromatographic methodologies require the use of TLC or NP-HPLC in combination with RP-HPLC in order to eliminate the coelution of molecular species of PCs and PEs [23].

Monomethylphosphatidylethanolamines and Dimethylphosphatidylethanolamines

MMPEs and DMPEs were detected as $[M+Li]^+$ ions in the presence of lithium salts. Under CID conditions lithiated adducts of MMPEs and DMPEs, fragmented similarly to $[PE+Li]^+$, a logical result given their chemical structures. The characteristic fragment ion of lithiated PEs at m/z $[PE+Li-147]^+$ originates due to the sequential neutral losses of $HN(CH_2)_2$ and $LiOP(O)(OH)_2$ [103]. Similarly, $[MMPE+Li]^+$ and $[DMPE+Li]^+$ afforded unique fragment ions at m/z $[MMPE+Li-161]^+$ and $[DMPE+Li-175]^+$, respectively. Most likely, these neutral losses were the result of sequential losses of $[CH_3N(CH_2)_2+LiOP(O)(OH)_2]$ and $[CH_3N(CH_2)_3 + LiOP(O)(OH)_2]$, respectively. Thus, neutral losses of 161 and 175 mass units can be related to the lithiated polar head groups of MMPEs and DMPEs, respectively. Neutral loss scans of 141 and 175 mass units afforded complete lipid profiles of lithiated MMPEs and DMPEs (Figure 3.2d-e). Unfortunately, analytical standards of MMPEs and DMPEs are not commercially available to confirm these MS/MS transitions.

In summary, unique MS/MS transitions for each lipid class originated from differences in the polar head groups of PCs, PEs, MMPEs and DMPEs, allowed their simultaneous analysis without chromatographic separation. Isobaric species of PEs, MMPEs and DMPEs can not be differentiated using high mass resolution mass spectrometry (Table 3.3).

Table 3.2. Accurate mass values calculated for $[M+H]^+$ and $[M+Na]^+$ adduct ions of PEs, MMPEs, DMPEs, PCs and TMHSs species observed in *S. meliloti*. Resolution of some pairs of ions would require mass resolutions of (a) ~6,300 and (b) ~39,000.

m/z	$[M+H]^+$				$[M+Na]^+$			
716.5230	PE-34:2							
718.5387	PE-34:1							
730.5387	PE-35:2	MMPE-34:2						
732.5543	PE-35:1	MMPE-34:1						
736.6091	TMHS-34:2							
738.5050 [a]					PE-34:2			
738.6248 [a]	TMHS-34:1							
740.5206					PE-34:1			
744.5543	PE-36:2	MMPE-35:2	DMPE-34:2					
746.5700		MMPE-35:1	DMPE-34:1					
750.6248	TMHS-35:2							
752.5206 [a]					PE-35:2	MMPE-34:2		
752.6404 [a]	TMHS-35:1							
754.5363					PE-35:1	MMPE-34:1		
758.5700 [b]	PE-37:2	MMPE-36:2	DMPE-35:2	PC-34:2				
758.5911 [b]					TMHS-34:2			
760.5856 [b]	DMPE-35:1			PC-34:1				
760.6067 [b]					TMHS-34:1			
764.6404	TMHS-36:2							
766.5363					PE-36:2	MMPE-35:2	DMPE-34:2	
768.5519						MMPE-35:1	DMPE-34:1	
772.5856 [b]	PE-38:2	MMPE-37:2	DMPE-36:2	PC-35:2				
772.6067 [b]					TMHS-35:2			
774.6013 [b]				PC-35:1				
774.6224 [b]					TMHS-35:1			
778.6561	TMHS-37:2							
780.5519					PE-37:2	MMPE-36:2	DMPE-35:2	PC-34:2
782.5676						MMPE-36:1	DMPE-35:1	PC-34:1
786.6013 [b]		MMPE-38:2	DMPE-37:2	PC-36:2				
786.6224 [b]					TMHS-36:2			
792.6717	TMHS-38:2							
794.5676					PE-38:2	MMPE-37:2	DMPE-36:2	PC-35:2
796.5832								PC-35:1
800.6169 [b]			DMPE-38:2	PC-37:2				
800.6380 [b]					TMHS-37:2			
808.5832						MMPE-38:2	DMPE-37:2	PC-36:2
814.6326 [b]				PC-38:2				
814.6537 [b]					TMHS-38:2			
822.5989							DMPE-38:2	PC-37:2
836.6145								PC-38:2

Table 3.3. Accurate mass values calculated for $[M+Li]^+$ adducts of lipid species observed in *S. meliloti*. (*) Isobaric species of different lipid classes were detected using neutral loss (NL) scans unique to each lipid class.

m/z	Lipids observed as their $[M+Li]^+$ Ions				
	NL 74	NL 147	NL 161	NL 175	NL 189
722.5312		PE-34:2			
724.5469		PE-34:1			
736.5469 *		PE-35:2	MMPE-34:2		
738.5625 *		PE-35:1	MMPE-34:1		
742.6173	TMHS-34:2				
744.6330	TMHS-34:1				
750.5625 *		PE-36:2	MMPE-35:2	DMPE-34:2	
752.5782 *			MMPE-35:1	DMPE-34:1	
756.6330	TMHS-35:2				
758.6486	TMHS-35:1				
764.5782 *		PE-37:2	MMPE-36:2	DMPE-35:2	PC-34:2
766.5938 *				DMPE-35:1	PC-34:1
770.6486	TMHS-36:2				
778.5938 *		PE-38:2	MMPE-37:2	DMPE-36:2	PC-35:2
780.6095					PC-35:1
784.6643	TMHS-37:2				
792.6095 *			MMPE-38:2	DMPE-37:2	PC-36:2
798.6799	TMHS-38:2				
806.6251 *				DMPE-38:2	PC-37:2
820.6408					PC-38:2

Diacylglyceryl-(N,N,N-trimethyl)-homoserine and Ornithine Lipids

OLs represent less than 2% of total lipids under normal growth conditions, while TMHSs are synthesized exclusively with P_i-limitation representing up to 70% of total lipids [51]. OL lipids contain two amino functions and one carboxylic group, thus $[M-H]^-$, $[M+H]^+$ or $[M+Na]^+$ ions can be observed in electrospray ionization. TMHSs are ether-linked glycerolipids with a quaternary ammonion group that resemble PCs [215]. TMHSs can be detected as mixtures of $[M+H]^+$ or $[M+Na]^+$ ions similarly to PCs and PEs in positive electrospray ionization. Geiger and coworkers, isolated TMHSs and OLs from lipid extracts of *S. meliloti* using a HPTLC-based method [51]. Using this methodology, TMHS-36:2 and TMHS-37:2, were detected as their $[M+H]^+$ ions at m/z 764 and 778, respectively [51, 154]. Also two OL lipids were detected as $[M+H]^+$ ions at m/z 679 and 693 which contained 3-hydroxyoctadecanoic acid with the hydroxyl group esterified to octadecenoic acid or methyleneoctadecanoic acid, respectively [51, 106].

The full scan mass spectrum in positive electrospray ionization of a crude lipid extract of *S. meliloti* grown under P_i-starvation conditions is shown in Figure 3.3a.. $[M+H]^+$ ions at m/z 764 and 778 for TMHS-36:2 and TMHS-37:2, respectively were not detected. On the other hand, other ions were detected at the expected m/z values for the $[M+Na]^+$ adducts of TMHS-36:2 (m/z 786.6) and TMHS-37:2 (m/z 800.5) (Table 3.2 and Figure 3.3a). Ions at m/z 786.6 and 800.5 could also correspond to $[M+H]^+$ adducts of PC-36:2, DMPE-37:2, MMPE-38:3, PC-37:2 and DMPE-38:2 lipids which were also detected in lipid extracts of *S. meliloti* (Table 3.2). If TMHSs exhibited similar fatty acid distributions as observed for phospholipids (PCs, PEs, etc), then considerable mass overlap would occur due to the coexistence of $[M+H]^+$ and $[M+Na]^+$ ions (Table 3.2). Note that mass resolutions of up to 38,600 would be required to resolve $[M+H]^+$ and $[M+Na]^+$ ions of [PCs+PEs+MMPEs+DMPEs] and TMHSs.

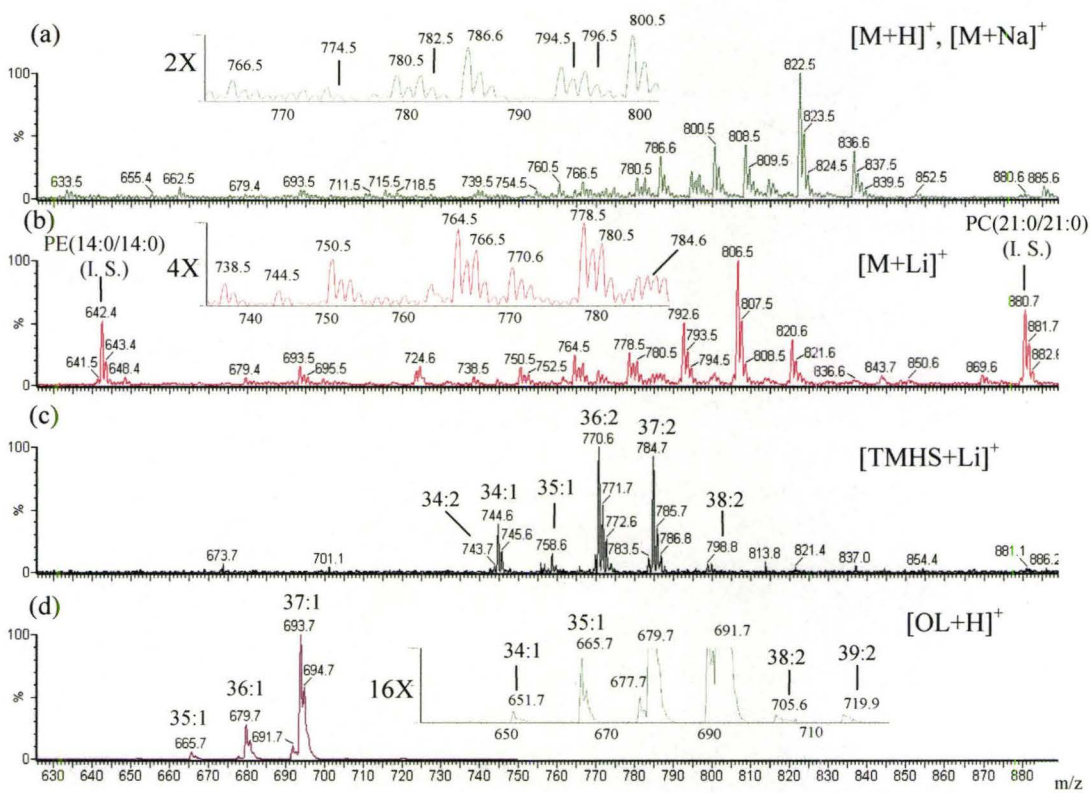


Figure 3.3. Positive ion electrospray ionization spectra of crude lipid extracts of *S. meliloti* grown under P_i-starvation conditions; (a) full scan mode spectrum with no LiCl added, (b) full scan mode spectrum with 10 mM LiCl added, (c) neutral

loss scan of 74 mass units selective for TMHSs and (d) precursor ion scan of m/z 115 selective for OLs.

The addition of lithium to lipid extracts reduced the complexity of spectra by producing only $[M+Li]^+$ adducts instead of a mixture of $[M+H]^+$ and $[M+Na]^+$ ions per lipid (Figure 3.3b and Table 3.3). Upon addition of lithium to lipid extracts, ions were detected at the expected m/z values of $[M+Li]^+$ adducts of TMHS-36:2 (m/z 770.7) and TMHS-37:2 (m/z 784.7) lipids (Table 3.3 and Figure 3.3b, insert). The product ion spectra of ions at m/z 770.7 and 784.7 showed neutral losses of 74 and 87 mass units, thus we decided to explore the use of neutral loss scans of 74 mass units. This MS/MS transition revealed an interesting pattern formed by ions at m/z 742.6, 744.6, 756.6, 758.6, 770.6, 784.6 and 798.7 (Figure 3.3c). These ions matched the calculated m/z values for $[M+Li]^+$ ions of TMHSs with fatty acid compositions of 34:2, 34:1, 35:2, 35:1, 36:2, 37:2 and 38:2, respectively (Table 3.3).

OLs were detected as low abundance ions at m/z 679.4 and 693.5 in full scan mode spectra (Figure 3.3a-b). The product ion spectra of these ions revealed abundant fragment ions at m/z 115 which are characteristic of ornithine-containing lipids [51, 105, 106]; thus ions at m/z 679.4 and 693.5 were identified as OLs. Since fragment ion at m/z 115 is common to the MS/MS spectra of OLs, we employed precursor ion scans of m/z 115 to investigate crude lipid extracts (Figure 3.3d). In addition to the major OLs at m/z 679.7 and 693.7 previously reported by Geiger, we detected other five OLs at m/z 651.7, 665.7, 677.7, 691.7, 705.6 and 719.9. These ions corresponded to protonated OLs with fatty acid compositions of 34:1, 35:1, 36:2, 37:2, 38:2 and 39:2. The fatty acid composition of these minor OL lipids could not be determined, since these ions were only observed in precursor ion spectra. *S. meliloti* contains three major hydroxy fatty acids (3-hydroxytetradecanoic, 3-hydroxyhexadecanoic, 3-hydroxyoctadecanoic acids) [63], then these OLs could be a mixture of more than one component. For example, ions at m/z 651.7 (OL-34:1) and 665.7 (OL-35:1) could correspond to amides of 3-hydroxyhexadecanoic acid with the hydroxyl function esterified to fatty acids 18:1 and 19:0cyclo, respectively. However, these lipids could also be amides of 3-hydroxyoctadecanoic acid with the hydroxyl function esterified to 16:1 (OL-34:1) and 17:0cyclo (OL-35:1) fatty acids, respectively. OLs at m/z 705.6 (OL-38:2) and 719.9 (OL-39:2) most likely are amides of 3-hydroxyoctadecanoic with fatty acids 20:2 and 21:2, respectively (Table 3.1).

In summary, the methodology described here, allowed the identification of eight lipid classes and a total of 58 individual lipids in bacterial lipid extracts without the need for chromatography. Most procedures for intact lipid analysis employ HPLC separation schemes [118-121, 205] and in many cases a combination of NP-HPLC followed by RP-HPLC is necessary to separate lipid classes and their individual molecular components [24]. Lipid analyses of *S. meliloti* using TLC protocols, did not reveal complete fatty acid distributions within lipid classes; this is probably due to poor recoveries and differential migration of molecular components in TLC plates [122]. In our experience, the

recoveries of analytical standards of phospholipids from TLC plates and silica gel columns were lower than when standards or lipid mixtures were infused directly. In this study, we demonstrated that fatty acid distributions can be obtained for non-phosphorus containing lipids (TMHSs, SLs and OLs) directly from crude extracts using neutral loss scans of 74 mass units, precursor ion scans of m/z 225 and 115, respectively. Our shotgun lipidomics approach has great potential as a methodology for the rapid screening of large volumes of samples. However, more work needs to be conducted to understand the fragmentation of $[M+Li]^+$ adducts of TMHSs and OLs in electrospray ionization. This might require the isolation of these lipids by traditional chromatographic techniques due to the unavailability of analytical standards.

Fatty Acid Distributions in Lipid classes of S. meliloti Wild Type Under Normal Growth Conditions and with P_i-starvation

The fatty acid distributions for eight lipid classes obtained from cultures of *S. meliloti* grown under normal conditions (2 mM P_i) and under P_i-starvation (0 mM P_i) are presented in table 3.4a-b. Analytical and biological variances were of 2-4% for abundant lipids (e.g., PC-36:2 and PG-36:2) when three cultures were analyzed each one in triplicate over a period of one year. The detection limits for PCs, PEs and PGs in the MS/MS mode were in the low picomole range (1.7-6.4 pmol).

The relative percentage compositions of individual molecular species within lipid classes were obtained using peak areas normalized to the peak area of the internal standard in precursor ion or neutral loss spectra. The accurate quantitation of phospholipids by ESI/MS is not a simple task since response factors vary among lipid classes and depend of the type of MS/MS transition used [95]. Additionally, response factors vary within a given lipid class with the length of the fatty acyl chains and number of double bonds [94]. The use of diluted lipid solutions and the inclusion of two or more internal standards per lipid class, have been recommended to minimize the variability of response factors across lipid classes and within lipid classes [14, 94]. The use of dilute lipid solutions (0.1-10 pmol/μL per lipid) is recommended to minimize micelle formation; micelle occurrence at higher lipid concentrations is observed as a decrease in the ESI response of lipids. In this study, concentrations for individual lipids were in the recommended range, thus micelle formation most likely is not a concern. However, a future objective of this work is the accurate quantitation of lipids; thus, the relationship between lipid concentration and ESI response must be carefully evaluated. The accurate quantitation of non-phosphorus containing lipids remains a challenge, since analytical standards are not commercially available for these lipids. Our approach, included one internal standard per lipid class (if commercially available) which is acceptable for most applications [96]. Our technique is appropriate as a screening method for large volumes of samples when control samples (wild type) are compared to treated samples (wild type in different growth conditions or mutants).

Table 3.4. Fatty acid distributions in eight lipid classes in *S. meliloti* under (a) normal growth conditions and (b) with P_i-starvation.

(a)

Lipids	Fatty Acid Distributions within Lipid classes for *S. meliloti* with 2 mM P_i, Mean ± SD (n=6)							
	PCs	PEs	MMPEs	DMPEs	PGs	SLs	TMHSs	OLs (n=3)
32:1					0.5 ± 0.2	3.1 ± 0.9		
32:0					1.5 ± 0.2	17.6 ± 0.8		
34:2	0.8 ± 0.2	2.8 ± 1.2	1.8 ± 0.4	8.9 ± 3.1	1.9 ± 0.2	1.4 ± 0.3		
34:1	8.0 ± 1.2	19.5 ± 2.7	14.0 ± 1.6	2.9 ± 1.5	29.0 ± 2.8	32.7 ± 2.0		1.1 ± 0.2
35:2	2.5 ± 0.4	2.7 ± 1.6	2.9 ± 0.9		2.0 ± 0.4	1.4 ± 0.5		
35:1	3.2 ± 0.4	4.0 ± 1.8	3.6 ± 0.9		2.7 ± 0.4	4.9 ± 0.6		3.1 ± 0.8
36:2	45.5 ± 2.1	58.4 ± 3.0	56.5 ± 3.4	58.8 ± 2.6	55.3 ± 2.3	17.3 ± 1.4		3.0 ± 0.8
36:1						11.6 ± 1.8		37.0 ± 2.3
37:2	35.9 ± 0.5	11.7 ± 2.0	19.6 ± 1.4	23.2 ± 1.6	6.8 ± 0.7	9.5 ± 1.3		5.3 ± 0.3
37:1								49.7 ± 1.2
38:2	4.2 ± 0.6	0.8 ± 0.4	1.6 ± 0.4	6.1 ± 1.8	0.3 ± 0.1	0.4 ± 0.2		0.6 ± 0.2
39:2								0.2 ± 0.2

(b)

Lipids	Fatty Acid Distributions within Lipid classes for *S. meliloti* with 0 mM P_i, Mean ± SD (n=6)							
	PCs	PEs	MMPEs	DMPEs	PGs	SLs	TMHSs	OLs (n=3)
32:1						3.3 ± 0.8		
32:0						12.7 ± 3.1		
34:2	0.5 ± 0.4	2.4 ± 1.2	1.0 ± 0.7	2.5 ± 1.6	1.1 ± 0.5	0.9 ± 0.3	1.4 ± 0.5	
34:1	4.9 ± 1.8	11.6 ± 4.7	8.8 ± 4.6	4.6 ± 1.4	15.8 ± 4.4	25.2 ± 2.8	12.4 ± 2.6	1.0 ± 0.1
35:2	2.6 ± 0.6	3.9 ± 1.0	3.4 ± 1.0		3.2 ± 0.9	2.5 ± 0.1	2.4 ± 1.7	
35:1	5.6 ± 1.2	4.0 ± 1.5	4.9 ± 0.8		4.2 ± 0.9	12.3 ± 1.3	3.2 ± 1.6	3.5 ± 0.2
36:2	24.0 ± 2.7	52.2 ± 2.5	45.1 ± 2.5	38.8 ± 4.8	54.9 ± 1.5	7.3 ± 0.6	53.4 ± 4.9	2.9 ± 0.4
36:1						6.0 ± 0.3		23.3 ± 0.4
37:2	49.0 ± 1.8	24.1 ± 7.4	33.4 ± 7.7	40.9 ± 5.2	19.5 ± 4.9	26.5 ± 5.0	24.4 ± 5.6	8.2 ± 0.2
37:1								60.4 ± 0.2
38:2	13.5 ± 3.7	1.7 ± 0.6	3.5 ±1.1	13.2 ± 2.0	1.2 ± 0.5	3.3 ± 1.2	2.8 ± 1.2	0.4 ± 0.1
39:2								0.4 ± 0.0

This is the first work to describe fatty acid distributions within lipid classes in *S. meliloti*, for both phospholipids and non-phosphorus containing lipids. The fatty acid profiles of PEs, MMPEs, DMPEs and PCs were very similar where 36:2 lipids were the most abundant components (45-60%). Lipids with fatty acid composition 36:2, contained two fatty acyl chains of *cis*-11-octadecenoic acid, which is the most abundant fatty acid in *S. meliloti* membranes [63]. The similarity in the fatty acid distributions for these lipid classes is not surprising since they are biosynthetically closely related [30]. SLs on the other

hand, showed different fatty acid distributions given by the presence of abundant species that contained hexadecanoic and hexadecenoic acid (32:1 and 32:0). Lipids with fatty acid compositions of 32:1 and 32:0 were not detected for phospholipids (PEs, MMPEs, DMPEs, PCs). Overall, lipids that contained either 16:1(9) or 16:0 fatty acids (34:2 and 34:1) were not abundant in phospholipids. PGs are most likely the closest lipid class to SLs from a biosynthetic and biological point of view [150]. Lipids that contained 16:1(9) and 16:0 were detected in PGs, however, these species were minor components (2%). Interestingly, hexadecanoic acid was also the most abundant fatty acid found in SLs in other organisms [109, 212].

PCA analysis of fatty acid distributions in lipid classes showed from a schematic point of view a biosynthetic relationship among lipid classes (Figure 3.4a). SLs were a distinctive class while phospholipids were spatially arranged following their biosynthetic order as PGs, PEs, MMPEs, DMPEs and PCs. PCA analysis of fatty acid distributions for lipid classes produced under P_i-starvation conditions showed also an interesting pattern (Figure 3.4b). The fatty acid profiles of OLs were strikingly different to all other lipid classes which was not unexpected due to the different biosynthetic origin of OL lipids [106]. However, TMHSs a distinctive lipid class synthesized exclusively under P_i-starvation, showed profiles which were highly similar to phospholipids (Figure 3.4b). In algae, TMHSs and SLs shared the same fatty acid distributions formed mostly by hexadecanoic acid [212]. In contrast, in *S. meliloti* TMHSs were closer to phospholipids such as PEs rather than to SLs.

In summary, two conclusions can be made based on the fatty acid distributions within lipid classes in *S. meliloti*: (i) TMHSs and phospholipids have a common biosynthetic origin, most likely involving phosphatidic acid as a common precursor. (ii) SLs might have a different biosynthetic origin to phospholipids or two pathways exist for their biosynthesis, one of them which does not require phosphatidic acid as a precursor. This latter hypothesis is supported by a second model proposed for SLs biosynthesis in bacteria which involves reaction of dihydroxyacetone (or dihydroxyacetone phosphate) with sulquinovose instead of diacylglycerol [148, 151].

Under P_i-starvation, a significant increase in lipids that contained cyclopropane fatty acids in all lipid classes was observed [63, 216]. Lipids that contained *cis*-9,10-methylenehexadecanoic acid and *cis*-11,12-methyleneoctadecanoic acid (35:2, 35:1, 37:2, 38:2) increased considerably (Table 3.4a-b). Ornithine lipids with fatty acid compositions of 37:2 and 37:1 increased significantly under P_i-starvation, therefore most likely these lipids contain cyclopropane fatty acids.

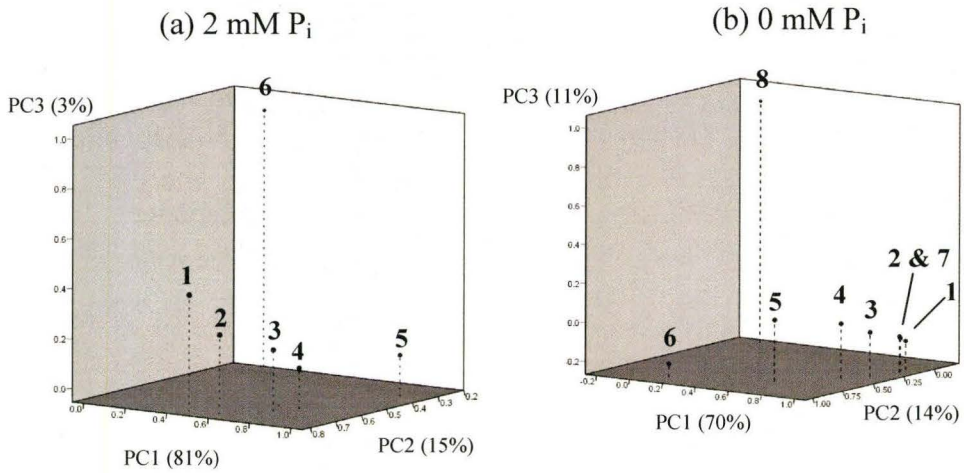


Figure 3.4. PCA of relative percentage composition in lipids of *S. meliloti* under (a) normal growth conditions and (b) under P_i-starvation. Lipid classes are represented by numbers: 1, PGs; 2, PEs; 3, MMPEs; 4, DMPEs; 5, PCs; 6, SLs; 7, TMHs; 8, OLs.

Lipid Profiles of PhoB-deficient Mutants Under Normal Growth Conditions and P_i-starvation

The regulator PhoB protein is only expressed under P_i-limiting conditions [50]; thus not surprisingly the lipid profiles of cultures of *phoB* mutant and wild type were identical under normal growth conditions (Data not shown). Analysis of total fatty acids was conducted as a complementary technique to intact lipid analyses [63]. Total fatty acid composition of *phoB* mutant and wild type were also identical under normal growth conditions (Table 3.5).

The increased cyclopropanation of lipids observed in wild type under P_i-starvation was reflected in total fatty acid composition by a significant increase of *cis*-11,12-methyleneoctadecanoic acid (Table 3.5). Furthermore, a dramatic (73-80%) increase in poly(3-hydroxybutyrate) (PHB) was detected under P_i-starvation conditions in wild type (data not shown). However, the increased levels of PHB were not a specific response towards P_i-limitation since mutants deficient in the PhoB regulator protein also showed increased levels of PHB (data not shown). The cellular role of PHB is not clear, although it has been shown to protect the cell from a diversity of stressors [217, 218].

Under P_i-limiting conditions, the lipid profiles of *phoB* mutants were different to those of wild type as expected (Figure 3.5). In *S. meliloti*, the biosynthesis of TMHSs is regulated by PhoB since PhoB-deficient mutants failed to produce these lipids [51]. TMHSs were not detected in PhoB-deficient mutants using the shotgun lipidomics methodology (Figure 3.5). Ions detected at m/z

770.5 and 784.6 for lithiated TMHS-36:2 and TMHS-37:2 lipids respectively, were not observed in lipid extracts of PhoB mutants. Furthermore, neutral loss scans of 74 or 87 mass units did not provide the profiles of TMHSs observed for cultures of wild type grown with P_i-starvation (Figure 3.3c).

Table 3.5. Percentage compositions of total fatty acids in *S. meliloti* wild type and *phoB* knockout mutants under normal growth conditions and under P_i-starvation.

Fatty Acids	Fatty Acid Percentage Compositions, Mean ± SD (n=3)			
	2 mM P_i		0 mM P_i	
	Wild Type	*phoB* Knockout	Wild Type	*phoB* Knockout
14:0	0.33 ± 0.03	0.30 ± 0.02 [3]	0.29 ± 0.06 [1]	0.43 ± 0.04 [1, 3]
16:0	11.9 ± 1.1	11.8 ± 1.5	10.2 ± 1.0	10.9 ± 1.0
17:0	0.2 ± 0.1	0.23 ± 0.02 [3]	0.25 ± 0.03 [1]	0.43 ± 0.05 [1, 3]
18:0	3.5 ± 0.3	4.0 ± 1.0	1.7 ± 0.3 [1]	3.8 ± 0.9 [1]
16:1(9)	0.9 ± 0.2	0.89 ± 0.05 [3]	0.6 ± 0.1	0.5 ± 0.2 [3]
18:1(11)	58.7 ± 2.9 [2]	57.5 ± 4.1 [3]	48.8 ± 2.6 [1, 2]	33.4 ± 2.3 [1, 3]
17:0cyclo(9,10)	1.7 ± 0.2	1.8 ± 0.3 [3]	1.7 ± 0.1 [1]	2.6 ± 0.1 [1, 3]
19:0cyclo(11,12)	22.6 ± 3.9 [2]	23.5 ± 1.7 [3]	36.5 ± 2.7 [1, 2]	47.9 ± 2.5 [1, 3]

[1] At 0 mM P_i mean values of wild type are significantly different to *phoB* mutant ($P \leq 0.05$).
[2] Mean values of wild type with 0 mM P_i are significantly different to wild type with 2 mM P_i ($P \leq 0.05$).
[3] Mean values of *phoB* mutant with 0 mM P_i are significantly different to *phoB* with 2 mM P_i ($P \leq 0.05$).

Aditionally, with P_i-starvation the cyclopropanation of lipids was more accentuated in *phoB* mutants compared to wild type (See PC-38:2 in Figure 3.5). This is also observed in total fatty acid composition of *phoB* mutants (Table 3.5). With P_i-starvation, cyclopropane fatty acids increased an additional 12% in *phoB* mutants in comparison with wild type. Cyclopropane fatty acids comprised 50% of total fatty acids in *phoB* mutants under P_i-starvation. Total cyclopropane fatty acids have also been observed to increase significantly in *S. meliloti* as a result of increased acidity [216]. The extent of lipid cyclopropanation in *phoB* mutants with P_i-starvation was more pronounced than in wild type under acidic conditions. Increased cyclopropanation of lipids is a stress response observed with P_i-starvation and acidity, however this effect was intensified in PhoB-deficient mutants with P_i-starvation.

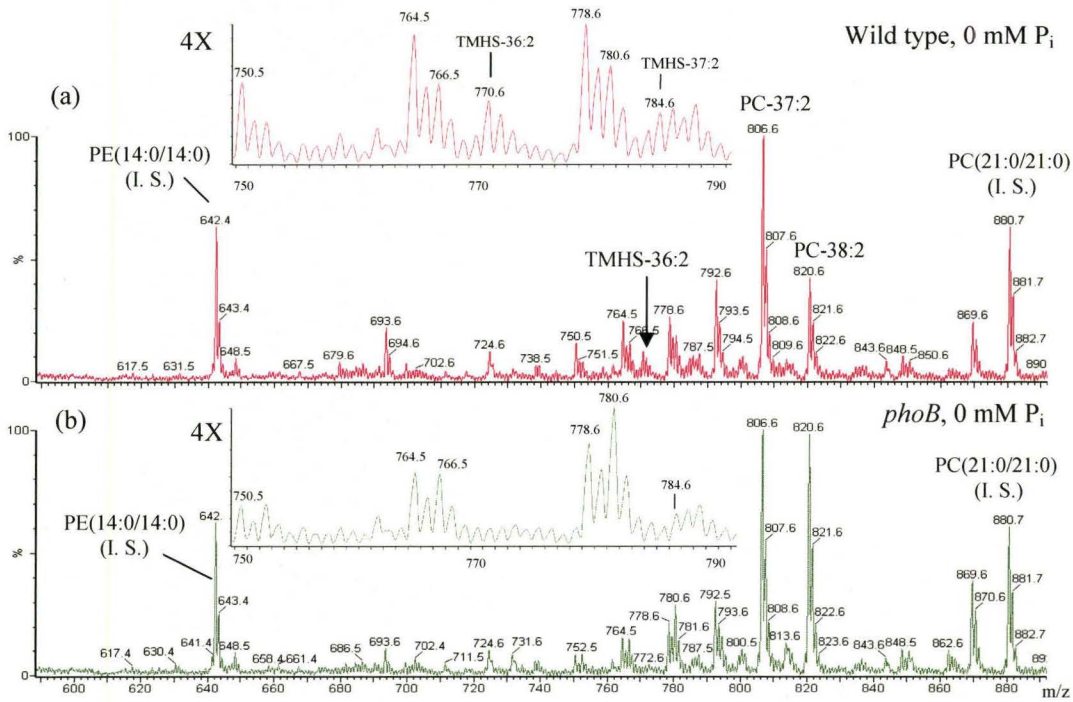


Figure 3.5. Positive electrospray ionization spectra in the full scan mode of crude lipid extracts of (a) wild type and (b) *phoB* knockout mutants under P_i-starvation conditions.

PCA analysis was used to identify trends or patterns in the fatty acid distributions within lipid classes for *phoB* mutant relative to wild type (Figures 3.6a-f). PC1, PC2 and PC3 explained 82%, 12% and 2% respectively, of the variance in the data. PC2 reflected the extent of cyclopropanation of lipids due to P_i-starvation, since 37:2 and 38:2 lipids represented by orange and red colors, respectively, clustered in the top quadrant of the scores plot of PC1 and PC2 (Figure 3.6a). This is easier to observe in the loadings plot of PC1 vs PC2 as samples were distributed along PC2 axis according to their increasing cyclopropanation levels as wild type and *phoB* 2 mM P_i, followed by wild type and *phoB* 0 mM P_i (Figure 3.6b). PC3 related to TMHSs which were only present in wild type with P_i-starvation (Figures 3.6c-f). To summarize, it can be concluded that cyclopropanation of lipids occurred independently of lipid class as no patterns were observed for the different classes represented by different shapes in the scores plots (Figures 3.6a, c, e).

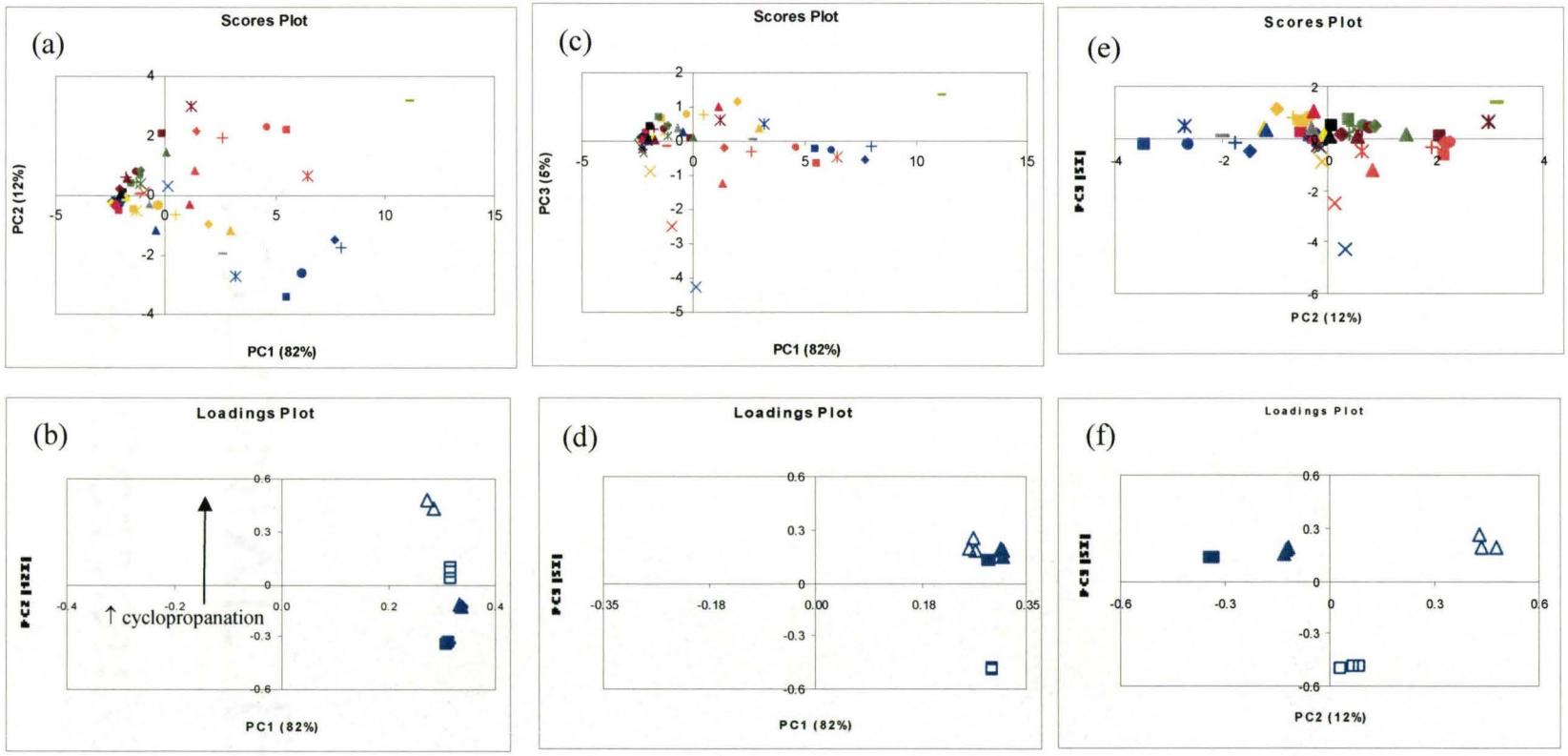


Figure 3.6. PCA plots of fatty acid distributions within lipid classes in *S. meliloti* wild type and PhoB-deficient mutant under normal growth conditions and with P_i-starvation. In the scores plot (a, c, e) lipid classes are represented by different figures, *, PCs; +, PEs; ●, MMPEs; ■, DMPEs; ♦, PGs; ▲, SLs; –, OLs; x, TMHs. Fatty acid composition in individual lipids is represented by colors, ■, 32:1; ■, 32:0; ■, 34:2; ■, 34:1; ■, 35:2; ■, 35:1; ■, 36:2; ■, 37:2; ■, 38:2. In the loadings plot (b, d, f), ■, wild type 2 mM P_i; *phoB* mutant 2 mM P_i, ▲. Samples grown under P_i-starvation are represented by hollow figures.

Conclusions

A shotgun lipidomics approach was developed to profile eight lipid classes in crude lipid extracts of *S. meliloti*. New mass spectrometric transitions were reported for the analysis of two additional phospholipids classes (MMPEs, DMPEs) and three non-phospholipid classes (SLs, TMHSs, OLs). This is the first report to use shotgun lipidomics for the analysis of bacterial non-phosphorus containing lipids. The addition of lithium salts for analyses in positive electrospray ionization provided three major advantages: (1) simplification of lipid profiles by producing only $[M+Li]^+$ ions, (2) unique MS/MS transitions for each lipid class that allowed (3) the analysis of isobaric species from different classes with no chromatography (PCs, PEs, MMPEs, DMPEs). The sensitivity and specificity of these techniques revealed fatty acid distributions in minor lipid classes such as SLs and OLs in crude extracts.

The methodology has excellent potential to be used as a screening tool for the analysis of large numbers of samples with virtually no sample preparation and small sample amounts. In contrast to TLC-based protocols which only detected major molecular species, this methodology provided complete fatty acid distributions within each lipid class. However, more work needs to be done in order to make this approach truly quantitative for phospholipid analysis. In the case of non-phosphorus containing lipids, only an approximate quantitation is possible due to the lack of analytical standards. This method is suitable for studies in which stressed/mutant samples are compared to control/wild type samples. Fatty acid analysis by GC/MS was used as a complementary technique which assisted in the identification of individual molecular species.

This is the first report to describe fatty acid distributions in lipids and the relative positions of fatty acyl substituents (*sn*-1, *sn*-2) in phospholipids in *S. meliloti*. PCA analysis of fatty acid distributions in lipid classes provided insights into the biosynthetic origin of phospholipids and non-phosphorus containing lipids. The similarities in fatty acid distributions in TMHSs and phospholipids suggest that TMHSs and phospholipids are synthesized from a common precursor, most likely phosphatidic acid. This was not case for SLs which exhibited distinct fatty acid distributions. Therefore, SLs are not synthesized from the same biochemical precursor as phospholipids and TMHSs. Finally, we found that lipid cyclopropanation increased dramatically in PhoB-deficient mutants with P_i-starvation indicated by a 25% increase in total cyclopropane fatty acids.

Chapter 4

Identification of Cyclopropane Fatty Acyl Synthase in *Sinorhizobium meliloti* using Shotgun Lipidomics

Abstract

A lipidomics approach was used to confirm the function of two putative genes, *cfa1* and *cfa2,* proposed to code for cyclopropane fatty acyl synthases in *Sinorhizobium meliloti*. Total fatty acid composition by GC/MS and fatty acid distributions within lipid classes by ESI/MS/MS were obtained for wild type and mutants of *cfa* genes under P_i-starvation and acidic conditions. Total cyclopropane fatty acids increased 10% and 15% with P_i-starvation and acidic conditions, respectively. We demonstrated that *cfa2* gene is the main gene in the cyclopropanation of lipids in *S. meliloti*. Furthermore, intact lipid analysis revealed that CFA synthases in *S. meliloti* acted on *cis*-11-octadecenoic acid located in *sn*-1 or *sn*-2 positions in phospholipids. *Cfa2* gene was also responsible for cyclopropanation in non-phosphorus containing lipids. Cyclopropanation in *sn*-2 position occurred to a larger extent with acidity than with P_i-starvation in phosphatidylcholines and sulfoquinovosyldiacylglycerols. Principal component analysis showed no differences in the cyclopropanation of four lipid classes.

Introduction

The soil bacterium *Sinorhizobium meliloti* infects the roots of plants such as alfalfa forming nodules and establishing a beneficial symbiotic relationship in which bacteria convert atmospheric nitrogen into a useful source of nitrogen for plants; this process is called nitrogen fixation [37]. In the last decade, extensive research has been conducted to understand the biochemistry and genetics of nitrogen-fixing bacteria and their symbiosis with leguminous plants with tremendous importance for a sustainable agriculture [25, 27, 36, 196]. Soil bacteria encounter numerous adverse conditions in the environment which include increased soil salinity, acidic pH conditions and limited inorganic phosphate (P_i) to mention a few stressors [44-46].

Phospholipids comprise up to 95% of lipids of *S. meliloti* including phosphatidylcholines (PCs, 60%), mono-methylphosphatidyletanolamines and phosphatidylethanolamines (MMPEs + PEs, 20%), dimethylphosphatidylethanolamines (DMPEs, 2%), phosphatidylglycerols (PGs, 9%) and cardiolipins (CLs, 5%) [51]. Other minor lipids (5%) such as ornithine lipids (OLs) and sulfoquinovosyldiacylglycerol (SLs) are also present. However under P_i-limiting conditions, *S. meliloti* substitutes partially membrane phospholipids with phosphorus-free lipids. Under these conditions, non-

phosphorus containing lipids represent up to 70% of total lipids and include 1,2-diacylglyceryl-3-*O*-4'-(*N,N,N*-trimethyl)-homoserine lipids (TMHSs), SLs and OLs [154]. In addition to the changes observed in lipid composition, we have shown recently that percentage composition of cyclopropane fatty acids increased two-fold under P_i-starvation conditions [63].

Two cyclopropane fatty acids, *cis*-9,10-methylene hexadecanoic acid (17:0cyclo(9,10)) and *cis*-11,12-methylene octadecanoic acid (19:0cyclo(11,12)) and their biochemical precursors, *cis*-9-hexacedenoic acid (16:1(9)) and *cis*-11-octadecenoic acid (18:1(11)), have been identified in *S. meliloti [63, 64].* Cyclopropane fatty acyl synthases (CFAs) are enzymes that catalyze the addition of a methylene group from S-adenosylmethionine (AdoMet) across *cis* double bonds of monounsaturated fatty acids in phospholipids [77]. Cyclopropanation of lipids is a post-synthetic modification of phospholipids which reaches a maximum during the stationary phase of cell growth [77]. The CFA synthase in *E. coli* has been the most studied CFA synthase (10, 20); there have been no studies reported as yet in *S. meliloti.* Two putative CFA synthases (Smc00358 and Smc02645) have been annotated as *cfa1* and *cfa2* genes in *S. meliloti* but no genetic or metabolite analyses have been conducted to support these functional assignments. We report the first efforts in this area.

The biological role of cyclopropane fatty acids in bacteria is not completely understood [41]. Nevertheless, it has been demonstrated that cyclopropane-containing lipids protect bacteria from adverse conditions such as acidity [79, 156-158], freeze drying [81], desiccation [78] and exposure to pollutants [159-161]. There is some debate as to whether an increase in cyclopropane content of bacterial membranes leads to a decrease in membrane fluidity [80, 81]. Furthermore, cyclopropane fatty acids are involved in the pathogenesis of *Mycobacterium tuberculosis* [162, 163] while in antagonistic *Fluorescent Pseudomonas* these fatty acids are associated with antifungal activity [164]. CFAs are of current interest as targets in the development of new drugs for the treatment of antibiotic resistant strains of tuberculosis [3, 165, 166]. Since cyclopropane fatty acids are important in bacteria-host interactions for some pathogens, then a a reasonable question was if these fatty acids were involved in the infection process of the plant by *S. meliloti* to establish symbiosis.

In this work, a lipidomics approach was used as a tool to elucidate the function of the two putative genes proposed to be involved in the cyclopropanation of lipids in *S. meliloti* (*cfa1* and *cfa2*). Since increased acidity and P_i-limitation are important environmental stressors which are associated with an increase in cyclopropane fatty acids, we studied the effects of both P_i-starvation and increased acidity. Normally, studies of *cfa* genes use fatty acid analysis of lipid extracts by GC/MS or fatty acid analysis of phospholipids from TLC spots [77, 219]. On the other hand, intact lipids can be directly analyzed from crude extracts without the need of chromatography using a combination of tandem mass spectrometric techniques (ESI/MS/MS); this approach is known as shotgun lipidomics [14, 82, 126]. We used a shotgun lipidomics strategy to

provide fatty acid distributions within five lipid classes (phospholipids and non-phosphorus containing lipids) without chromatography or hydrolysis of fatty acids in lipids. MS/MS experiments were used to determine fatty acid composition in lipids and their relative positions in lipids (*sn*-1 and *sn*-2) which provided insights about the selectivity of CFA synthases. In summary, we report total fatty acid composition and fatty acid distributions within five lipid classes in *S. meliloti* wild type and the knockout mutants of *cfa1* and *cfa2* genes. Fatty acids were analyzed as fatty acid methyl esters (FAMEs) by GC/MS using a micro-scale method [63].

Concisely, the objectives of this work were: (1) to study and compare the effects of P_i-starvation and acidic conditions on fatty acid and lipid compositions in *S. meliloti*; (2) to determine whether the putative *cfa1* and *cfa2* genes encoded for CFA synthases; (3) to investigate whether *cfa1* and *cfa2* genes respond differently under P_i-starvation and increased acidic conditions; (4) to determine whether CFA synthases exhibited any preferences for particular lipid classes and (5) to determine whether alkene-containing fatty acids located at either the *sn*-1 or *sn*-2 position in lipids underwent preferential cyclopropanation. Principal component analysis (PCA) was used as a tool to identify patterns or trends in data sets.

Materials and methods

Abbreviations

Fatty acids were represented using the shorthand designation system recommended by the IUPAC [52]. Phospholipids were abbreviated as PCs, PEs, MMPEs, DMPEs and PGs for phosphatidylcholines, phosphatidylethanolamines, phosphatidylmonomethylethanolamines, phosphatidyldimethylethanolamines and phosphatidylglycerols, respectively. Non-phosphorus containing lipids such as 1,2-diacylglyceryl-3-*O*-4'-(*N,N,N*-trimethyl)-homoserine lipids and sulfoquinovosyldiacylglycerols were represented as TMHSs and SLs, respectively. Fatty acid composition in lipids was represented by the sum of carbon atoms in the fatty acyl chains of lipids followed by a colon and the total number of unsaturations, e.g., 1,2-dioleoyl-*sn*-glycero-3-phosphocholine was represented as PC-36:2. The relative positions of fatty acyl chains in lipids were represented by the fatty acid in the *sn*-1 position first followed by the fatty acid in the *sn*-2 position and separated by a forward slash as recommended, e.g., 1,2-dioleoyl-*sn*-glycero-3-phosphocholine was represented as PC(18:1/18:1) [201]. When the assignment of fatty acids to the *sn*-1 and *sn*-2 positions in lipids was ambiguous, the fatty acids were separated by a hyphen instead of a forward slash.

Bacterial Cultures

Bacterial cultures (*S. meliloti,* RmP110) for the P_i-starvation study were grown in MOPs buffered minimal media [203] with glucose (15 mM) as the carbon source in the presence of 2mM P_i or in the absence of P_i. *S. meliloti* cells grown overnight in Luria-Bertani broth (LB containing $CaCl_2$ and $MgSO_4$, 2.5mM each) were washed with MOPs (2 and 0 mM P_i) medium, subcultured in

MOPs and grown for 36-40 hours at 30°C with shaking. Cultures used in the pH study were initially grown overnight in LBmc, washed with 0.85% NaCl and subcultured in M9 minimal growth media containing glucose (15mM), at pH 7.0 and pH 5.5 followed by an incubation of 40 hours at 30°C with shaking.

Wet cell pellets obtained by centrifugation from 500 mL of culture (O.D. = 0.6-0.9) were resuspended in 2.5 mL of medium and divided into 250 µL aliquots in Eppendorf tubes. Each tube was centrifuged, the supernatant discarded and the wet pellets flash frozen in liquid nitrogen and stored at -80°C until further use. The pellets (equivalent to 50 mL of original culture) corresponded to 61.8 ± 4.5 mg wet weight with 2 mM P_i (n=9) and 27.6 ± 1.2 mg with 0 mM P_i (n=9) from MOPs media or 40.5 ± 0.9 mg with pH 7 (n=9) and 37.0 ± 2.0 mg with pH 5.5 (n=9) from M9 media.

Construction of cfa1 and cfa2 Gene Knockouts in S. meliloti

Knockouts of *S. meliloti* genes *SMc00358* and *SMc02645*, annotated as *cfa1* and *cfa2* respectively (http://bioinfo.genopole-toulouse.prd.fr/annotation/iANT/bacteria/rhime/), were constructed in wild type strain RmP110 background [50]. For this purpose 263 bp and 271 bp fragments were PCR amplified from 5'-regions of *cfa1* and *cfa2*, respectively, using primers 5'GCTCTAGAGCTATCGCATATGATGAAGTCGTTC3' carrying XhoI restriction site and 5'TGCATGCATGTCACAACAGCTTCTGGATCGGATAGG3' containing NsiI restriction site for cfa1 and primers 5'GC**TCTAGA**GCGGGATTGTTGAATCTACTTCGC3' with XhoI and 5'CC**ATCGAT**GGTCACGCGACGATCGAATTGGTGAAGG3' with ClaI restriction site for *cfa2*. Each of these fragments was cloned into plasmid pTH1360 [50] using appropriate restriction sites and resultant plasmids from *E. coli* DH5α were recombined into *S. meliloti* RmP110 genome through triparental mating using Mt616 as a helper strain [220]. Single crossover recombinants were selected on LB plates containing 200 µg/mL streptomycin and 200 µg/mL of Neomycin. Representative colonies for each knockout type were streak-purified three times before further use.

Analytical Standards and Reagents

N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) and ethyl dodecanoate were obtained from Sigma-Aldrich (Saint Louis, MO). All solvents used were HPLC grade (Caledon Labs, Caledon, ON). Phospholipid standards 1,2-diheneicosanoyl-*sn*-glycero-3-phosphocholine (PC(21:0/21:0)), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (PE(14:0/14:0)) and 1,2-dilauroyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol) (PG(12:0/12:0), sodium salt) were purchased from Avanti Polar Lipids (Alabaster, AL).

Fatty Acid Methyl Esters Analysis by GC/MS

Fatty acids in lipids were analyzed as their fatty acid methyl esters (FAMEs) by GC/MS. FAMEs were prepared from dried lipid extracts using a one-vial method that employed sodium methoxide in methanol as the transmethylation reagent [63].

Intact Lipid Analysis by ESI/MS and ESI/MS/MS

Dried lipid extracts were prepared from wet cell pellets (50 mL culture) as described previously and dissolved in MeOH: $CHCl_3$ (1:1, v/v, 200 µL) Saborido. Aliquots were diluted 5-fold with MeOH containing the internal standards PC(21:0/21:0) (32 µmol) and PE(14:0/14:0) (28 µmol) and lithium chloride (2.5 mM) only for analyses conducted in the positive ionization mode. For analysis in the negative ionization mode, aliquots were diluted 5-fold with methanol containing PG(12:0/12:0) (3 µmol) as the internal standard. A Waters Quattro Ultima triple quadrupole mass spectrometer equipped with a microelectrospray ion source and operating under the MassLynx software was used for the analysis of bacterial lipid extracts. The collision gas (N_2) pressure was 2 x 10^{-3} Bar and collision energies ranged between 25 and 50 eV. Bacterial lipid extracts were continuously infused at 1 µL/min using a Harvard syringe pump in the positive ion mode with or without the addition of lithium chloride and in the negative ion mode (no LiCl added). PCs, PEs and TMHSs were analyzed as $[M+Li]^+$ ions using neutral loss scans of 189 [99], 147 [103] and 74 mass units [221], respectively. PGs and SLs were analyzed as their $[M-H]^-$ ions in the negative ionization mode using precursor ion scans of m/z 153 [14, 20] and 225 ions [221]. In all experiments 100 spectra were averaged to afford a single spectrum. Peak areas obtained in each MS/MS spectra were normalized to the area of the internal standard and added to obtain relative percentage composition of molecular species in each lipid class.

Statistical Analysis

Each sample was analyzed in triplicate and all data are expressed as a mean value ± standard deviation. The SPSS statistical package, version 15.0 (SPSS Inc. Chicago, IL, USA) was used for statistical analysis. Student's t-test was used to evaluate the significance of the differences when only two groups were compared whereas analysis of variance (ANOVA) was used for multiple group comparisons ($P \leq 0.05$). Principal component analyses (PCA) were performed using the Multivariate Analysis Add-in for Excel, version 1.3 (Bristol Chemometrics Inc., Bristol, UK).

Results and Discussion

Total fatty acid analyses were performed using a one-vial transmethylation method that employed sodium methoxide in methanol, the reagent of choice for the conversion of cyclopropane-containing lipids into their fatty acid methyl esters [77]. Acidic reagents are known to destroy cyclopropane fatty acids, yet

most studies of CFA synthases employed basic hydrolysis with NaOH followed by an acid-catalyzed methylation with HCl to generate FAMEs from bacterial lipids [78-81, 161]. In our experience, this acid-catalyzed methodology produced lower yields of cyclopropane fatty acids from lipid extracts of *S. meliloti* when compared with the sodium methoxide in methanol procedure [63].

The shotgun lipidomics methodology employed provided fatty acid distributions within lipid classes. Briefly, crude lipid extracts were infused directly into the mass spectrometer in the presence and absence of lithium salts for analysis in the positive and negative electrospray ionization modes, respectively. Anionic lipids (PGs, SLs) were detected as $[M-H]^-$ ions in the negative ionization mode while zwitterionic lipids (PCs, PEs, TMHSs) were analyzed in the positive ionization mode as their $[M+Li]^+$ adducts. Precursor ion scans of m/z 153 and 225 were used to analyze PGs and SLs, respectively [221]. PCs, PEs and TMHSs were analyzed using neutral loss scans which were unique to the polar head group of each lipid class [221]. This approach provided fatty acid distributions for seven lipid classes (PCs, PEs, MMPEs, PEs, PGs, SLs, TMHSs) in wild type and both *cfa* mutants under P_i-starvation and acidic conditions. In this paper we have focused mainly on four lipid classes, PCs and PGs which are the most abundant phospholipids in *S. meliloti* under normal growth conditions and TMHSs and SLs which are major membrane lipids under P_i-starvation conditions.

Effects of P_i-starvation and Acidity on Total Fatty Acid Composition

Fatty acid compositions for *S. meliloti* wild type and the knockout mutants of *cfa1* and *cfa2* in the presence of 2 mM P_i relative to 0 mM P_i and at pH 7.0 relative to pH 5.5 are presented in Table 4.1.

In *S. meliloti* under normal growth conditions (2 mM P_i and pH 7.0, Table 4.1a and 4.1b, respectively), *cis*-11,12-methyleneoctadecanoic acid (19:0cyclo(11,12), 20.9-25.8%) was the major cyclopropane fatty acid while *cis*-9,10-methylenehexadecanoic acid (17:0cyclo(9,10)) was a minor component (1.6-1.8%). The most abundant cyclopropane-containing fatty acid in other bacteria is 17:0cyclo(9,10); for example, in *E. coli* this fatty acid constitutes 4% of total fatty acids while in *Pseudomonas putida* it can represent up to 33% [79, 81]. However, these studies could have underestimated the content of bacterial cyclopropane fatty acids since acidic reagents were employed to generate FAMEs from lipids.

Under P_i-starvation conditions, in the wild type the percentage of the major cyclopropane fatty acid, 19:0cyclo(11,12), increased significantly while 17:0cyclo(9,10) showed no change (Table 4.1a). Acidic conditions (pH 7 to 5.5) caused a significant increase in both cyclopropane fatty acids in the wild type (Table 4.1b). The total increases in cyclopropane fatty acids were of 10.2% and 15% under P_i-starvation and acidic conditions, respectively (Table 4.1c). The increase of total cyclopropane fatty acids in wild type *S. meliloti* under acidic conditions (15%) was equivalent to the 17% increase reported for *E. coli* strains grown under similar conditions [79].

Table 4.1. Relative percentage compositions of fatty acids in *S. meliloti* wild type and knockout mutants of *cfa1* and *cfa2* (a) in the presence of inorganic phosphate relative to P_i-starvation and (b) at pH 7 relative to pH 5.5. Changes in the relative percentage compositions of cyclopropane fatty acids are shown in box (c).

(a)

Fatty Acids	Relative Percentage Composition of *S. meliloti* (%), Mean ± SD (n=3)					
	Wild Type		*cfa1* Knockout		*cfa2* Knockout	
	2 mM P_i	0 mM P_i	2 mM P_i	0 mM P_i	2 mM P_i	0 mM P_i
14:0	0.23 ± 0.02	0.25 ± 0.03	0.3 ± 0.1	0.3 ± 0.1	0.30 ± 0.05	0.33 ± 0.03
16:0	13.0 ± 0.5	11.9 ± 0.2	14.4 ± 0.5 [a]	10.9 ± 0.4 [2]	15.7 ± 0.1 [b]	15.3 ± 0.5 [c]
17:0	0.23 ± 0.03	0.19 ± 0.04	0.24 ± 0.05	0.20 ± 0.01	0.3 ± 0.1	0.21 ± 0.04
18:0	3.6 ± 0.3	1.8 ± 0.1 [1]	3.1 ± 0.4	1.3 ± 0.1 [2]	4.9 ± 2.5	2.4 ± 0.3
16:1(9)	1.2 ± 0.3	1.4 ± 0.2	1.5 ± 0.4	1.3 ± 0.2	3.3 ± 0.3 [a]	3.6 ± 0.1 [c]
18:1(11)	54.4 ± 1.7	46.9 ± 1.2 [1]	53.0 ± 0.2	44.8 ± 0.4 [2]	75.4 ± 2.6 [a]	78.2 ± 0.7 [c]
17:0cyclo(9,10)	1.60 ± 0.03	2.1 ± 0.2	1.9 ± 0.3	2.4 ± 0.4	N.D. [a]	N.D. [c]
19:0cyclo(11,12)	25.8 ± 1.5	35.5 ± 1.2 [1]	25.6 ± 0.9	38.7 ± 1.0 [2]	N.D. [a]	N.D. [c]
	100	100	100	100	100	100

(b)

Fatty Acids	Relative Percentage Composition of *S. meliloti* (%), Mean ± SD (n=3)					
	Wild Type		*cfa1* Knockout		*cfa2* Knockout	
	pH 7	pH 5.5	pH 7	pH 5.5	pH 7	pH 5
14:0	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
16:0	12.5 ± 1.1	12.1± 0.7	14.1± 0.4	11.1 ± 0.5	17.9 ± 1.0 [a]	12.4 ± 2.6 [3]
17:0	0.13 ± 0.03	0.44 ± 0.05 [1]	0.17 ± 0.04	0.3 ± 0.1	0.23 ± 0.01	0.4 ± 0.1
18:0	4.0 ± 0.2	2.7 ± 0.4	4.8 ± 0.2	2.6 ± 0.3	8.1 ± 2.5	2.9 ± 0.6 [3]
16:1(9)	0.6 ± 0.4	0.7 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	2.3 ± 0.4 [a]	2.2 ± 0.5
18:1(11)	59.7 ± 3.3	46.4 ± 3.4 [1]	57.2 ± 1.6	48.8 ± 0.9 [2]	71.0 ± 3.8	81.8 ± 3.9 [3, c]
17:0cyclo(9,10)	1.6 ± 0.2	2.7 ± 0.6 [1]	1.6 ± 0.2	2.3 ± 0.3	N.D. [a]	N.D. [c]
19:0cyclo(11,12)	20.9 ± 2.2	34.6 ± 4.1 [1]	20.6 ± 1.2	33.6 ± 1.4 [2]	N.D. [a]	N.D. [c]
	100	100	100	100	100	100

[1] Wild type under stressed conditions (0 mM P_i or pH 5.5) is significantly different ($P \leq 0.05$) to wild type under normal growth conditions (2 mM P_i or pH 7).

[2] *cfa1* mutant under stressed conditions (0 mM P_i or pH 5.5) is significantly different ($P \leq 0.05$) to *cfa1* under normal growth conditions (2 mM P_i or pH 7).

[3] *cfa2* mutant under stressed conditions (0 mM P_i or pH 5.5) is significantly different ($P \leq 0.05$) to *cfa2* under normal growth conditions (2 mM P_i or pH 7).

[a, b] Under normal growth conditions (2 mM P_i or pH 7), *cfa1* and *cfa2* mutants are significantly different to wild type ($P \leq 0.05$).

[c, d] Under stressed conditions (0 mM P_i or pH 5.5), *cfa1* and *cfa2* mutants are significantly different to wild type ($P \leq 0.05$).

(c)

Fatty Acids	Change in the Relative Percentage Composition of Cyclopropane Fatty Acids in *S. meliloti*			
	Wild Type		*cfa1* Knockout	
	Δ [0 mM P_i - 2 mM P_i]	Δ [pH 5.5-pH 7]	Δ [0 mM P_i - 2 mM P_i]	Δ [pH 5.5-pH 7]
17:0cyclo(9,10)	0.5	1.1	0.5	0.7
19:0cyclo(11,12)	9.7	13.7	13.1	13.0
Σ cycloFAs	10.2	14.8	13.6	13.7

The fatty acid composition data for the *cfa1* knockout mutant were statistically identical to the data for the wild type under all conditions examined. In contrast, cyclopropane fatty acids were not detected in *cfa2* knockout mutants under all conditions. Additionally, the biochemical precursor of the major cyclopropane fatty acid (18:1(11)) exhibited a significant increase. These results demonstrate conclusively that *cfa2* is the main gene for the the cyclopropopanation of lipids in *S. meliloti*. Other bacteria also present two *cfa* genes as in *S. meliloti*; for example *P. putida* contain two *cfa* genes named *cfaA* and *cfaB*. In this organism, the *cfaB* gene was specific to produce 17:0cyclo(9,10) while *cfaA* synthesized 19:0cyclo(11,12) [81]. In *S. meliloti* our results indicated that *cfa2* was the main gene involved in the synthesis of both cyclopropane fatty acids.

Enzyme Activity Assays and CFA Synthases for Nitrogen Fixation

The disruption of the *cfa1* and *cfa2* genes also created a transcriptional fusion to the *uidA* gene encoding β-glucuronidase enzyme and it was therefore possible to measure promoter activities of those genes in various growth conditions. The enzyme activity data showed that *cfa2* gene is expressed >10fold in comparison with *cfa1* in all growth conditions used in this study. This data is in agreement with the major role of *cfa2* as a gene encoding for a CFA synthase. In addition, a 4.5-fold induction of *cfa2* expression at pH 5.5 compared to growth at pH 7.0. This observation is in agreement with the 23-fold increase noted in the activity of the *Lactococcus lactis cfa* promoter in acidic medium compared to neutral pH [222]. The *cfa1* and *cfa2* knockout mutants were also tested with alfalfa plants for their nodulation and nitrogen fixation capabilities; no significant differences from wild type were recorded (data not shown), suggesting that *S. meliloti* CFA synthases are not involved in plant infection processes.

Cyclopropanation of Lipids in S. meliloti Wild Type and cfa Mutants Phospholipids

Lipid profiles of PCs and PGs are represented in Figure 4.1 for wild type and mutants of *cfa1* and *cfa2* under normal growth conditions. In lipid extracts of the wild type, ions observed at m/z 764.6, 766.7, 778.8, 780.8, 792.8, 806.8 and 820.8 corresponded to the $[M+Li]^+$ adducts of PCs with fatty acid compositions of 34:2, 34:1, 35:2, 35:1, 36:2, 37:2 and 38:2, respectively (Figure 4.1a). Similarly, in wild type samples PGs with fatty acid compositions corresponding to 34:2, 34:1, 35:2, 35:1, 36:2, 37:2 and 38:2 were present (Figure 4.1b). MS/MS experiments were conducted to identify which lipid species contained cyclopropane fatty acids and the relative positions of these fatty acids (*sn*-1 and *sn*-2) in lipids.

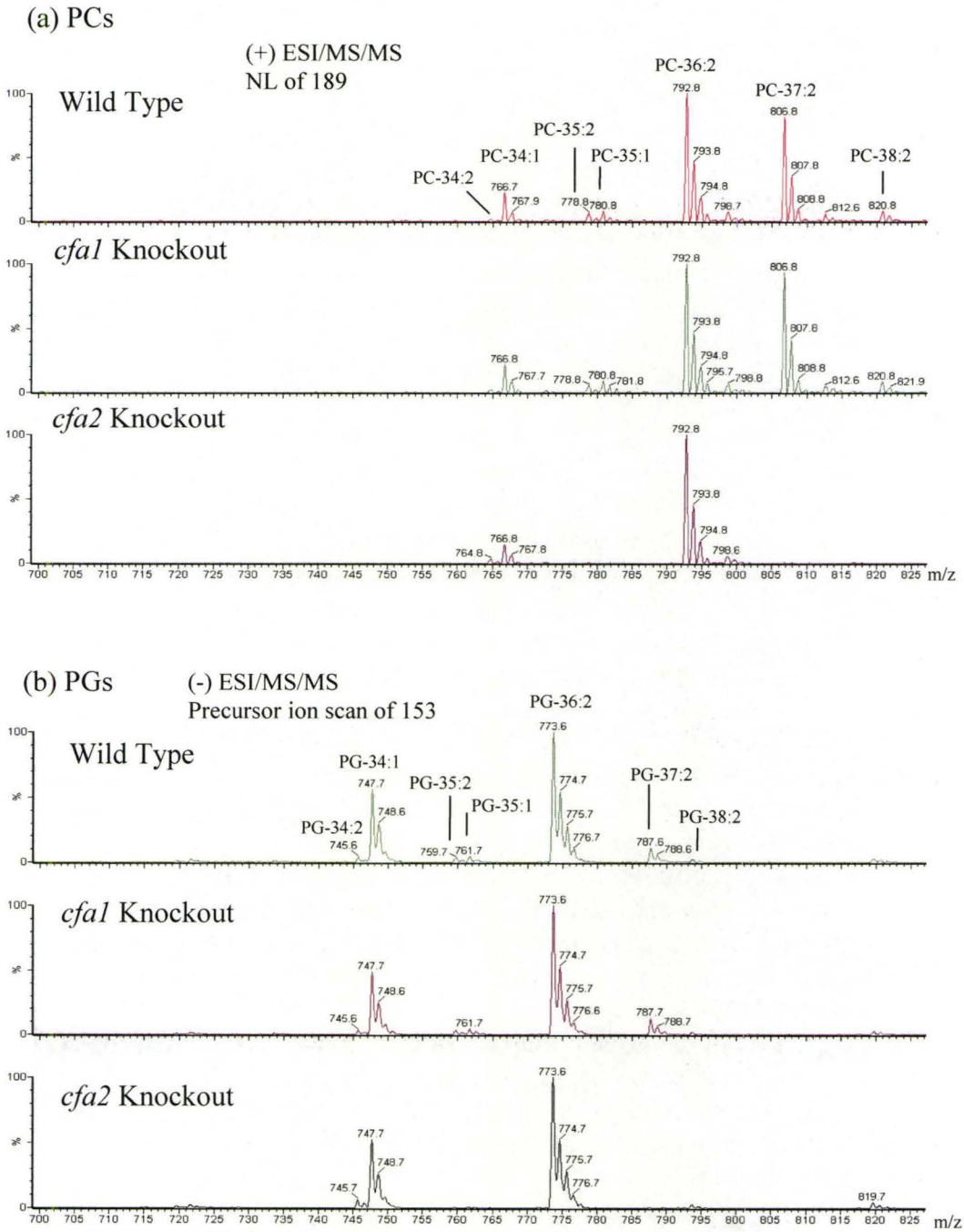


Figure 4.1. Profiles of (a) phosphatidylcholines and (b) phosphatidylglycerols in *S. meliloti* wild type and the knockout mutants of *cfa1* and *cfa2* in the presence of 2 mM P_i. NL, neutral loss scan.

Collision induced dissociation (CID) of $[M+Li]^+$ adducts of PCs typically produce fragment ions due to the neutral losses of the lithiated polar head group ($[M+Li-189]^+$) and the fatty acyl chains [99, 101]. Fragment ions due to the neutral losses of the fatty acyl substituents are observed at m/z $[M+Li-59-RCOOH]^+$, $[M+Li-RCOOLi]^+$ and $[M+Li-RCOOH]^+$. Moreover, the $[M+Li-59-RCOOH]^+$ ions produced by the loss of fatty acids from the *sn*-1 position are more intense than those produced by the loss of *sn*-2 fatty acids [101]. These features of the MS/MS spectra of lithiated PCs were used to determine which PCs were substituted with cyclopropane fatty acids at their *sn*-1 or *sn*-2 positions. The PC-35:2, PC-35:1, PC-37:2 and PC-38:2 species all contained cyclopropane fatty acids as demonstrated by the presence of fragment ions at m/z $[M+Li-59-268.2]^+$ or $[M+Li-59-296.3]^+$, corresponding to neutral losses of $[N(CH_3)_3+17:0cyclo]$ or $[N(CH_3)_3+19:0cyclo]$, respectively. The lipid PC-37:2 was the most abundant cyclopropane-containing PC and was shown to have the 19:0cyclo and 18:1 fatty acids attached to the *sn*-1 and *sn*-2 positions, respectively (Figure 4.2a). The fragment ion $[M+Li-59-RCOOH]^+$ derived from the neutral loss of 18:1 at m/z 465.3 was more intense than those corresponding to the loss of 19:0cyclo at m/z 451.4 (Figure 4.2a, insert); based on the work of Turk [101] these data were interpreted as PC-37:2 corresponded to PC(18:1/19:0cyclo). However, these ions did not provide the 2:1 to 3:1 ratio of ion intensities that were typically reported by Turk. Thus PC-37:2 may also contain some of the PC(19:0cyclo/18:1) isomer.

Product ion spectra in the negative ionization mode of $[M-H]^-$ ions of PG-33:1, PG-35:2, PG-35:1 and PG-37:2 at m/z 733.5, 759.5, 761.5 and 787.5, respectively, showed the presence of cyclopropane fatty acids indicated by carboxylate ions at m/z 267.2 and 295.3. The relative position (*sn*-1 vs. *sn*-2) of cyclopropane fatty acids in PGs could be determined since the $[RCOO]^-$ ions from fatty acids in the *sn*-2 position are more abundant than ions from *sn*-1 fatty acids [97]. The product ion spectrum of PG-37:2, clearly showed the $[RCOO]^-$ ions corresponding to 18:1 and 19:0cyclo fatty acids (Figure 4.2b). The relative intensities of these ions indicated that PG-37:2 corresponded to PG(19:0cyclo/18:1).

The profiles of PCs and PGs in samples of *cfa1* mutant showed no differences relative to the wild type (Figure 4.1). However, PCs and PGs with fatty acid compositions corresponding to 35:2, 35:1, 37:2 and 38:2 were not detected in samples of the *cfa2* knockout mutant; all of these lipids contained cyclopropane fatty acids.

In summary, it was demonstrated that PCs and PGs with fatty acid compositions corresponding to 35:2, 35:1, 37:2 and 38:2 contained primarily *cis*-11,12-methyleneoctadecanoic acid. The preferred substrate of most CFA synthases are lipids containing *cis*-9-hexadecenoic acid and not *cis*-11-octadecenoic acid [77]. However, since *cis*-11-octadecenoic acid is the major fatty acid in *S. meliloti*, it is not surprising that lipids containing this fatty acid were the principal CFA synthase substrate. MS/MS analyses of 37:2 lipids in *S. meliloti* showed that *cis*-11,12-methyleneoctadecanoic acid was mostly located in *sn*-2

position in PCs while in PGs was located at the *sn*-1 position. This is also contradictory with reports that CFA synthases prefer fatty acids in the *sn*-2 positions over *sn*-1 [77]. However, most likely PC-37:2 correspond to a mixture of both isomers were 18:1/19:0cyclo is the most abundant. *Cfa2* gene was the main gene involved in the cyclopropanation of phospholipids in *S. meliloti*.

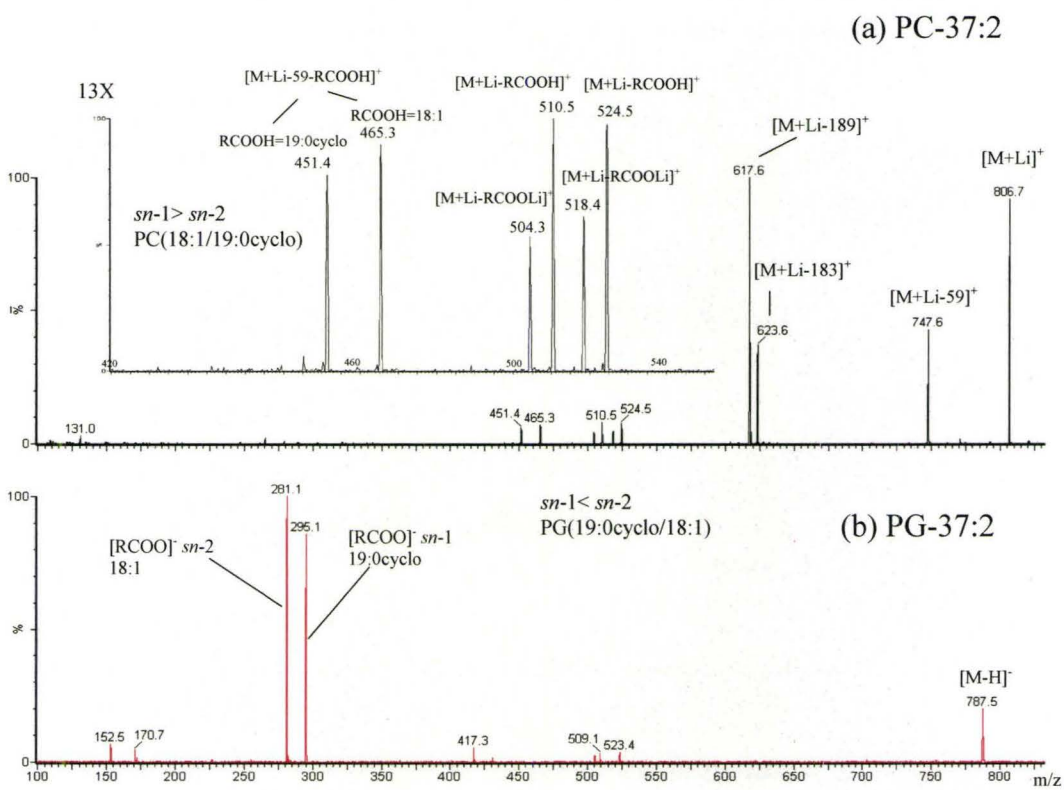


Figure 4.2. Product ion spectra of 37:2 lipids of (a) phosphatidylcholines and (b) phosphatidylglycerols in *S. meliloti*.

Non-phosphorus-containing Lipids

To the best of our knowledge, studies of CFA synthases have focused only on phospholipids. In this work we investigated the cyclopropanation not only of two major phospholipid classes (PCs, PGs) but also of two non-phosphorus containing lipid classes (SLs, TMHSs). SLs are minor lipids (1-3%) under normal growth conditions while TMHSs are synthesized by *S. meliloti* exclusively under P_i-limiting conditions [51]. Figure 4.3 shows the profiles for SLs and TMHSs derived from wild type and both knockout mutants with P_i-starvation.

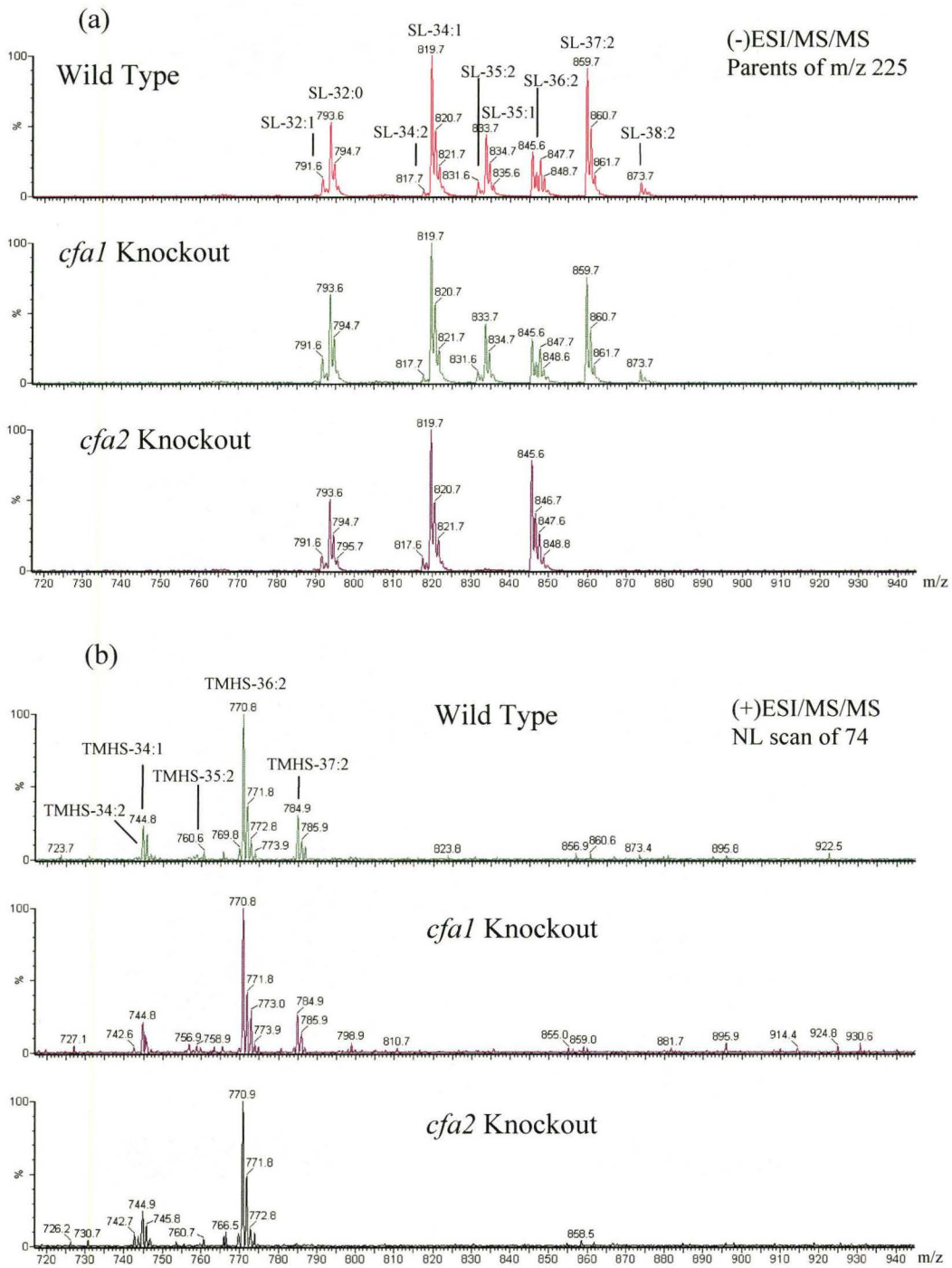


Figure 4.3. Infusion electrospray mass spectra of non-polar extracts of *S. meliloti* wild type and *cfa1* and *cfa2* knockout mutants, showing the profiles of (a) sulfoquinovosyldiacylglycerols and (b) diacylglyceryltrimethylhomoserines with P_i-starvation. NL, neutral loss scan.

The product ion spectra of $[M-H]^-$ ions of sulfolipids with fatty acid compositions of SL-35:2, SL-35:1, SL-37:2 and SL-38:2 showed that these lipids contained cyclopropane fatty acids. The presence of cyclopropane fatty acids in these lipids was confirmed by the presence of $[RCOO]^-$ fragment ions in the MS/MS spectra at m/z 267.2 and 295.3 for 17:0cyclo and 19:0cyclo, respectively. These spectra were interpreted to have fatty acid compositions for SL-35:2, SL-35:1, SL-37:2 and SL-38:2 which corresponded to fatty acid combinations of 18:1/17:0cyclo, 19:0cyclo/16:0, 18:1/19:0cyclo and 19:0cyclo/19:0cyclo, respectively. Lipid profiles of TMHSs using neutral loss scans of 74 provided profiles similar to those observed for PCs (Figure 4.3b). TMHSs detected at m/z 756.6, 758.6 and 770.6 corresponded to the 35:2, 35:1 and 37:2 lipids. The relative positions of fatty acids (*sn*-1 vs. *sn*-2) in SLs and TMHSs were not determined since the fragmentation mechanisms of these lipids in electrospray ionization mass spectrometry have not been characterized due to the lack of availability of analytical standards. Cyclopropane-containing SLs and TMHSs were not detected in *cfa2* knockout mutants while *cfa1* mutants showed no differences in lipid composition to wild type similarly as in phospholipids (Figure 4.3a-b).

In conclusion, it was demonstrated that lipid species of SL and TMHS lipids with fatty acid compositions of 35:2, 35:2, 35:1, 37:2 and 38:2 contained *cis*-11,12-methyleneoctadecanoic acid as observed in phospholipids. *Cfa2* was also the main gene in the cyclopropanation of non-phosphorus containing lipids as well as in phospholipids. Consequently, it can be concluded that cyclopropanation of lipids in *S. meliloti* occurred independent of the nature of polar head group as observed for various phospholipid classes and non-phosphorus containing lipids. Cyclopropanation occurred in fatty acyl chains located in both the *sn*-1 and *sn*-2 positions of SL lipids as demonstrated by the presence of 38:2 species.

Effects of P_i-starvation and Acidity in The Cyclopropanation of Lipids

Relative percentage compositions in wild type and *cfa* knockout mutants with P_i-starvation and acidity are presented for PCs and PGs in tables 4.2 and 4.3, respectively. Fatty acids relative positions in lipids (*sn*-1/*sn*-2) were determined for PCs and PGs by interpretation of product ion spectra [97, 101]. In some instances, one m/z value corresponded to more than one lipid demonstrated by the presence of fragment ions corresponding to more than one combination of fatty acids in the MS/MS spectra. On the other hand, the assignment of fatty acid relative position (*sn*-1/*sn*-2) in minor lipid components was not possible since the $[RCOO]^-$ ions were too low in abundance (Tables 4.2 and 4.3). Cyclopropane-containing lipids (35:2, 35:1, 37:2, 38:2) decreased significantly (87-100%) in *cfa2* knockout mutants in all lipid classes with P_i-starvation and acidity which corroborated that *cfa2* is the main gene in cyclopropanation. It is noteworthy that cyclopropane fatty acids in *cfa2* mutants were not detected by GC/MS analyses while ESI/MS/MS techniques detected small levels (<1%) of cyclopropane-containing lipids which demonstrated *cfa1*'s contribution to cyclopropanation.

The lipid profiles of other three phospholipid classes, PEs, MMPEs and DMPEs showed the same results (Data not shown).

Table 4.2. Relative percentage composition of phosphatidylcholines in *S. meliloti* wild type and *cfa1* and *cfa2* mutants with 2 mM P_i relative to 0 mM P_i (a) and at pH 7 relative to pH 5.5 (b).

(a)

Lipids	Fatty Acids in Lipids *sn*-1 / *sn*-2	Relative Percentage Composition of Phosphatidylcholines (%), Mean ± SD (n=3)					
		Wild Type		*cfa1* Knockout		*cfa2* Knockout	
		2 mM P_i	0 mM P_i	2 mM P_i	0 mM P_i	2 mM P_i	0 mM P_i
PC-34:2	16:1/18:1	0.7 ± 0.1	0.8 ± 0.4	1.9 ± 1.3	0.8 ± 0.3	3.3 ± 0.4 [a]	3.6 ± 0.1 [c]
PC-34:1	18:1/16:0	9.1 ± 0.3	6.5 ± 0.3 [1]	9.0 ± 0.2	5.8 ± 0.2 [2]	12.2 ± 0.3	10.3 ± 0.3 [c]
PC-35:2	18:1/17:0cyclo 16:1-19:0cyclo *	2.9 ± 0.1	3.1 ± 0.2	3.0 ± 0.6	3.2 ± 0.4	1.9 ± 0.3	2.0 ± 0.5
PC-35:1	19:0cyclo/16:0	3.5 ± 0.2	4.9 ± 0.3 [1]	4.2 ± 0.2	4.6 ± 0.6	1.4 ± 0.6 [a]	1.7 ± 0.3 [c]
PC-36:2	18:1/18:1	43.7 ± 0.8	26.3 ± 0.3 [1]	42.3 ± 0.5	30.9 ± 1.2 [2]	78.4 ± 4.7 [a]	79.5 ± 2.0 [c]
PC-37:2	19:0cyclo/18:1	36.2 ± 0.4	48.3 ± 1.1 [1]	35.7 ± 2.1	46.7 ± 2.1 [2]	1.7 ± 2.1 [a]	2.2 ± 2.0 [b]
PC-38:2	19:0cyclo/19:0cyclo	3.9 ± 0.6	10.1 ± 0.3 [1]	4.0 ± 0.3	8.0 ± 0.5 [2,c]	1.1 ± 1.4 [a]	0.8 ± 0.2 [d]

(b)

Lipids	Fatty Acids in Lipids *sn*-1 / *sn*-2	Relative Percentage Composition of Phosphatidylcholines (%), Mean ± SD (n=3)					
		Wild Type		*cfa1* Knockout		*cfa2* Knockout	
		pH 7	pH 5.5	pH 7	pH 5.5	pH 7	pH 5.5
PC-34:2	16:1/18:1	0.7 ± 0.4	0.5 ± 0.1	0.8 ± 0.1	0.4 ± 0.1	5.0 ± 0.4 [a]	4.7 ± 0.2 [c]
PC-34:1	18:1/16:0	7.5 ± 0.7	7.0 ± 1.3	8.5 ± 1.4	6.0 ± 1.1 [2]	9.4 ± 0.3	13.7 ± 1.2 [3,c]
PC-35:2	18:1/17:0cyclo 16:1-19:0cyclo *	2.7 ± 1.1	2.0 ± 0.2	3.2 ± 0.2	2.4 ± 0.7	N.D. [a]	N.D. [c]
PC-35:1	19:0cyclo/16:0	3.4 ± 0.3	7.8 ± 0.3 [1]	3.8 ± 0.3	7.3 ± 0.7 [2]	N.D. [a]	N.D. [c]
PC-36:2	18:1/18:1	35.4 ± 0.6	19.4 ± 1.1 [1]	37.3 ± 1.2	22.5 ± 1.4 [2]	85.6 ± 0.6 [a]	81.6 ± 1.2 [c]
PC-37:2	19:0cyclo/18:1	44.8 ± 0.9	46.7 ± 0.7	40.3 ± 1.0 [a]	46.8 ± 1.1 [2]	N.D. [b]	N.D. [c]
PC-38:2	19:0cyclo/19:0cyclo	5.6 ± 0.1	16.5 ± 1.3 [1]	6.2 ± 0.5	14.5 ± 0.9 [2,c]	N.D. [a]	N.D. [d]

[1] Wild type under stressed conditions (0 mM P_i or pH 5.5) is significantly different ($P \leq 0.05$) to wild type under normal growth conditions (2 mM P_i or pH 7).
[2] *cfa1* mutant under stressed conditions (0 mM P_i or pH 5.5) is significantly different ($P \leq 0.05$) to *cfa1* under normal growth conditions (2 mM P_i or pH 7).
[3] *cfa2* mutant under stressed conditions (0 mM P_i or pH 5.5) is significantly different ($P \leq 0.05$) to *cfa2* under normal growth conditions (2 mM P_i or pH 7).
[a, b] Under normal growth conditions (2 mM P_i or pH 7), *cfa1* and *cfa2* mutants are significantly different to wild type ($P \leq 0.05$).
[c, d] Under stressed conditions (0 mM P_i or pH 5.5), *cfa1* and *cfa2* mutants are significantly different to wild type ($P \leq 0.05$).
(*) Indicates that the assignment of fatty acids to *sn*-1 and *sn*-2 positions in lipids was ambiguous.
N.D.; not detected.

Table 4.3. Relative percentage compositions of phosphatidylglycerols in *S. meliloti* wild type and knockout mutants of *cfa1* and *cfa2* grown (a) with 2 mM P_i relative to 0 mM P_i and (b) at pH 7 relative to pH 5.5.

(a)

Lipids	Fatty Acids in Lipids *sn*-1 / *sn*-2	Relative Percentage Composition of Phosphatidylglycerols (%), Mean ± SD (n=3)					
		Wild Type		*cfa1* Knockout		*cfa2* Knockout	
		2 mM P_i	0 mM P_i	2 mM P_i	0 mM P_i	2 mM P_i	0 mM P_i
PG-32:1	16:1-16:0 *	0.7 ± 0.2	N.D. [1]	0.6 ± 0.2	N.D. [2]	1.0 ± 0.1	N.D. [3]
PG-32:0	16:0/16:0	1.6 ± 0.1	N.D. [1]	1.8 ± 0.6	N.D. [2]	2.0 ± 0.7	N.D. [3]
PG-33:1	16:0/17:0cyclo	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
PG-33:0	17:0-16:0 *	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
PG-34:2	16:1/18:1	1.9 ± 0.4	1.5 ± 0.4	2.2 ± 0.5	1.5 ± 0.4	3.9 ± 0.6 [a]	5.4 ± 0.6 [3, b]
PG-34:1	18:1/16:0	31.3 ± 1.1	19.7 ± 1.3 [1]	30.3 ± 2.8	20.4 ± 1.9 [2]	33.6 ± 2.1	27.2 ± 2.9 [3, b]
PG-35:2	18:1/17:0cyclo 16:1-19:0cyclo *	1.9 ± 0.2	2.9 ± 0.6	2.1 ± 0.2	3.3 ± 0.4 [2]	0.6 ± 0.4 [a]	N.D. [b]
PG-35:1	19:0cyclo/16:0	3.0 ± 0.5	4.2 ± 1.4	2.6 ± 0.7	3.7 ± 0.5	0.5 ± 0.3 [a]	N.D. [b]
PG-36:2	18:1/18:1	53.2 ± 0.6	55.8 ± 1.1	54.1 ± 2.3	59.1 ± 2.2	58.1 ± 3.4	67.4 ± 3.0 [3, b]
PG-37:2	19:0cyclo/18:1	6.3 ± 0.4	15.2 ± 1.8 [1]	6.3 ± 1.0	12.0 ± 0.3 [2, b]	0.3 ± 0.1 [a]	N.D. [c]
PG-38:2	19:0cyclo/19:0cyclo	N.D.	0.7 ± 0.1	N.D.	N.D.	N.D.	N.D.

(b)

Lipids	Fatty Acids in Lipids *sn*-1 / *sn*-2	Relative Percentage Composition of Phosphatidylglycerols (%), Mean ± SD (n=3)					
		Wild Type		*cfa1* Knockout		*cfa2* Knockout	
		pH 7	pH 5.5	pH 7	pH 5.5	pH 7	pH 5.5
PG-32:1	16:1-16:0 *	0.4 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.4 ± 0.2	1.0 ± 0.2 [a]	1.0 ± 0.2 [b]
PG-32:0	16:0/16:0	1.2 ± 0.1	1.7 ± 0.4	1.3 ± 0.2	1.4 ± 0.1	1.5 ± 0.3	1.9 ± 0.6
PG-33:1	16:0/17:0cyclo	0.8 ± 0.2	1.1 ± 0.2	0.6 ± 0.1	1.2 ± 0.4 [2]	N.D. [a]	0.2 ± 0.1 [b]
PG-33:0	17:0-16:0 *	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.4 ± 0.2	N.D. [a]	0.1 ± 0.1
PG-34:2	16:1/18:1	1.5 ± 0.2	1.0 ± 0.2	1.4 ± 0.1	1.2 ± 0.3	4.3 ± 0.3 [a]	6.6 ± 0.7 [3, b]
PG-34:1	18:1/16:0	28.6 ± 0.3	21.7 ± 0.9 [1]	27.9 ± 0.5	22.6 ± 1.4 [2]	29.0 ± 0.8	27.2 ± 2.3 [b]
PG-35:2	18:1/17:0cyclo 16:1-19:0cyclo *	2.5 ± 0.1	3.0 ± 0.4	2.1 ± 0.3	3.0 ± 0.8	0.2 ± 0.1 [a]	0.2 ± 0.1 [b]
PG-35:1	19:0cyclo/16:0	3.4 ± 0.5	10.0 ± 0.5 [1]	3.3 ± 0.3	6.6 ± 0.5 [2, b]	0.3 ± 0.1 [a]	0.6 ± 0.1 [c]
PG-36:2	18:1/18:1	51.5 ± 0.7	36.3 ± 1.4 [1]	53.5 ± 0.7	42.4 ± 2.0 [2, b]	63.3 ± 0.8 [a]	61.6 ± 2.6 [c]
PG-37:2	19:0cyclo/18:1	9.9 ± 0.5	24.7 ± 1.2 [1]	9.4 ± 0.2	20.8 ± 0.7 [2, b]	0.4 ± 0.1 [a]	0.5 ± 0.1 [c]
PG-38:2	19:0cyclo/19:0cyclo	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

[1] Wild type under stressed conditions (0 mM P_i or pH 5.5) is significantly different ($P \leq 0.05$) to wild type under normal growth conditions (2 mM P_i or pH 7).

[2] *cfa1* mutant under stressed conditions (0 mM P_i or pH 5.5) is significantly different ($P \leq 0.05$) to *cfa1* under normal growth conditions (2 mM P_i or pH 7).

[3] *cfa2* mutant under stressed conditions (0 mM P_i or pH 5.5) is significantly different ($P \leq 0.05$) to *cfa2* under normal growth conditions (2 mM P_i or pH 7).

[a] Under normal growth conditions (2 mM P_i or pH 7), *cfa1* and *cfa2* mutants are significantly different to wild type ($P \leq 0.05$).

[b, c] Under stressed conditions (0 mM P_i or pH 5.5), *cfa1* and *cfa2* mutants are significantly different to wild type ($P \leq 0.05$).

(*) Indicates that the assignment of fatty acids to *sn*-1 and *sn*-2 positions in lipids was ambiguous.
N.D.; not detected.

Overall, the lipid profiles of PCs and PGs in the *cfa1* mutants showed no differences to wild type under normal growth conditions (Tables 4.2 and 4.3). Increased acidity and P_i-starvation caused an increase in cyclopropane-containing species in all lipid classes. For example, PCs that contained cyclopropane fatty acids (35:2, 35:1, 37:2 and 38:2) increased significantly up to 12% under P_i-starvation in the wild type (Table 4.2a). Accordingly, species that contained the biochemical precursors of cyclopropane fatty acids (34:1 and 36:2) decreased significantly. PGs showed the same trend however only 37:2 lipids increased significantly while 35:2 and 35:1 species didn't increase as observed in PCs (Table 4.3a). Phosphatidylglycerol with fatty acid composition of 38:2 was a minor component and did not increase with P_i-starvation, while PC-38:2 species increased up to 4%. PEs and SLs that contained cyclopropane fatty acids also increased with P_i-starvation reaching a maximum of 7% and 14% for 37:2 species, respectively (data not shown).

In general, cyclopropanation increased in all lipid classes with P_i-starvation but it occurred to a larger extent in PCs and SLs than in PGs and PEs. Although 35:2, 35:1, 37:2 and 38:2 lipids increased significantly as result of both P_i-starvation and acidity, this phenomenon was more pronounced under acidic conditions. The higher extent of cyclopropanation with acidity was observed in PGs as 35:1 and 37:2 lipids increased 7% and 15% compared to 1% and 9% respectively, with P_i-starvation (Table 4.3a-b). In PCs, the increase in cyclopropanation with acidity was manifested by further transformation of 37:2 lipids to 38:2 lipids instead of a build-up of 37:2 as in PGs (Table 4.2a-b and Figure 4.4). The same pattern was observed in SLs with acidity as SL-37:2 species were further cyclopropanated to form SL-38:2 in comparison with P_i-starvation.

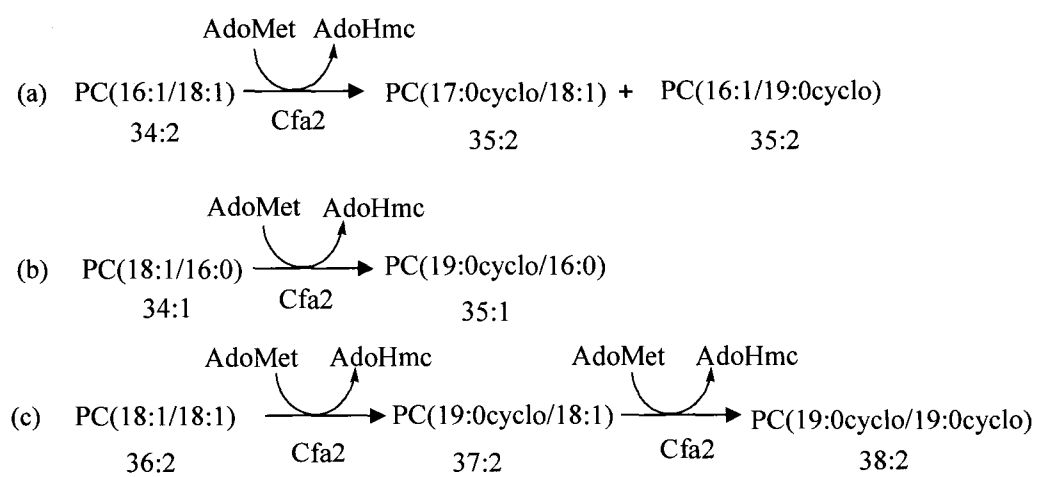


Figure 4.4. Proposed scheme for the cyclopropanation of phosphatidylcholines in *S. meliloti*. AdoMet: S-adenosylmethionine; AdoHmc: S-adenosyl-L-homocysteine.

Phospholipids are a required substrate for CFA synthases and the nature of the polar head group is thought to influence enzyme-substrate interactions [77]. In *S. meliloti*, lipid profiles for phospholipids (PCs, PEs, MMPEs, DMPEs, PGs) and non-phosphorus containing lipids (SLs, TMHSs) showed that CFA synthases acted on all lipid classes regardless of the nature of the polar head group. However, 35:1 lipids increased more in SLs than in PCs, PEs and PGs with P_i-starvation in the wild type. With increased acidity, SL-35:1 species showed a remarkable 20% increase compared to 4-7% increase for the other three lipid classes (Data not shown).

PCA was applied to the fatty acid distributions within lipid classes (PCs, PEs, PGs, SLs) in wild type and knockout mutants under all growth conditions in order to identify any patterns or trends in the data set (Figure 4.5). In the scores plots (Figures 4.5a, c), lipid classes are represented by different shapes while the fatty acid composition of individual lipids are represented by colors. PC1 accounts for 85% and PC2 for 13% of the variance in the data set. Approximately, half of the data set clustered very tightly and therefore did not contribute to the differences observed in the loadings plots (Figure 4.5b). The data points that did not cluster were grouped by color (fatty acid composition) and not by shape (lipid class). The greatest variance from the cluster was observed for lipids with fatty acid compositions of 36:2, 34:1, 37:2 and 38:2; these lipids contained either cyclopropane fatty acids or their biochemical precursors. This distribution of the data set can be interpreted as cyclopropanation was independent of lipid class. PC1 can be correlated to percentage composition of lipids while PC2 represent the extent of cyclopropanation in lipids.

In the loadings plots, samples with the highest percentage of cyclopropane-containing lipids were located in the top quadrant relative to PC2 (Figure 4.5b) corresponding to samples under acidic conditions (wild type and *cfa1* mutant) while samples with the lowest content of cyclopropane-containing lipids were located at the bottom (*cfa2* mutants).

Cyclopropanation of lipids was more accentuated with acidity than with P_i-starvation since samples at pH 5.5 were preceded by samples at 0 mM P_i along PC2. A third principal component (PC3) that contributed to 2% of the variance differentiated samples at pH 5 (WT and *cfa1*) given by an increase of 38:2 lipid species in PCs and SLs rather than an increase of 37:2 species (Figures 4.5c-d). Two main conclusions can be made from the PCA analysis: (i) acidity caused more extensive cyclopropanation of lipids than P_i-starvation and (ii) cyclopropanation of the lipids did not occur preferentially for a given lipid class.

In summary, cyclopropanation of lipids in *S. meliloti* increased as a result of P_i-starvation and acidic conditions in all lipid classes examined. Acidity had a higher impact on lipid cyclopropanation than P_i-starvation and was more accentuated in PCs and SLs compared to PGs and PEs. Higher rates of cyclopropanation in PCs and SLs were reflected by further transformation of 37:2 species to 38:2 species; in PGs and PEs conversion of 37:2 lipids to 38:2 lipids was not observed.

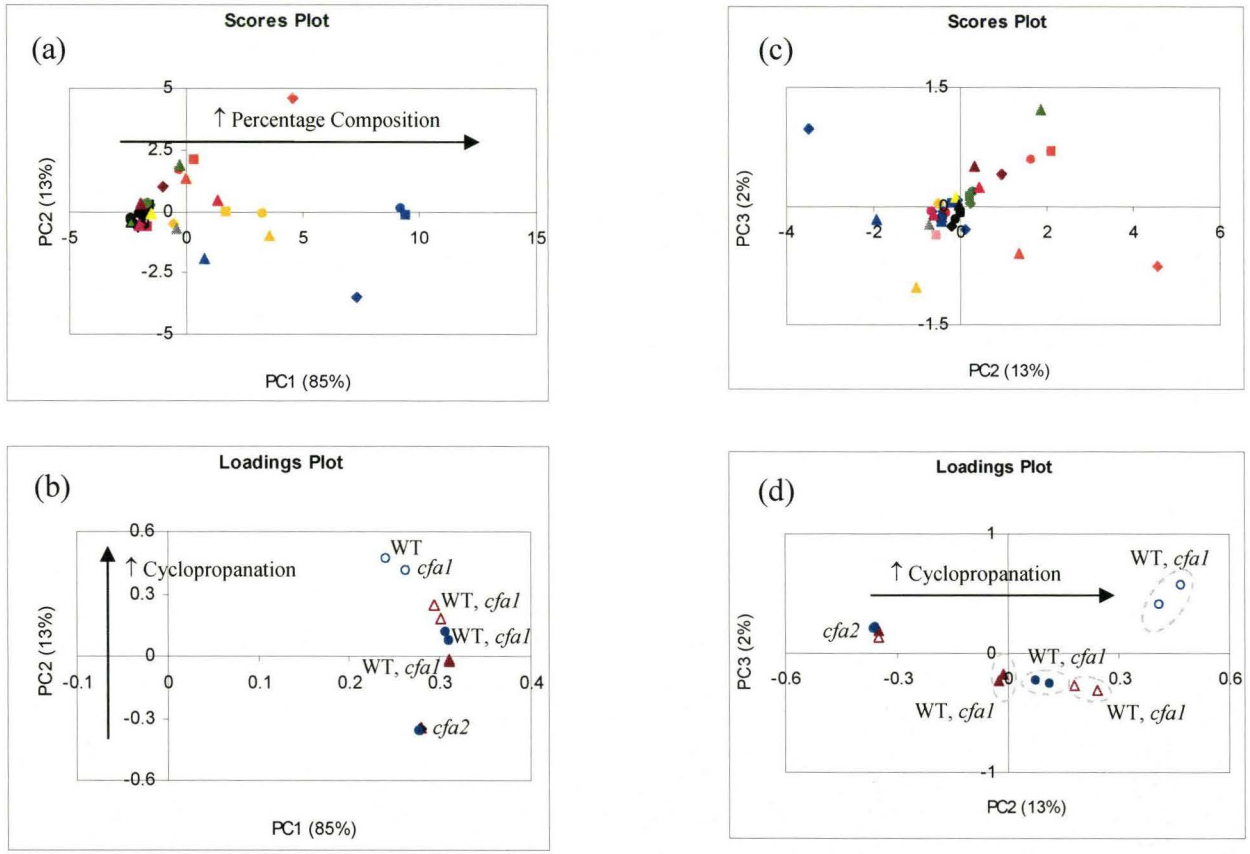


Figure 4.5. Principal components analysis of fatty acid distributions in four lipid classes in *S. meliloti* wild type and mutants of *cfa1* and *cfa2* genes with P_i-starvation and acidic conditions. (a, c) In the scores plots, lipid classes were represented by figures (♦, PCs; ■, PEs; ●, PGs; ▲, SLs). Lipid species were represented by colors (■, 32:1; ■, 32:0; ■, 34:2; ■, 34:1; ■, 35:2; ■, 35:1; ■, 36:2; ■, 36:1; ■, 37:2; ■, 38:2). (b, d) In the loadings plots, the P_i-starvation study was represented by ▲ and the acidity study by ●. Filled figures represent control conditions (2 mM P_i, pH 7) and hollow figures correspond to stressed conditions (0 mM P_i, pH 5).

Conclusions

This study showed that stress due to P_i-starvation and acidity resulted in significant increase in the cyclopropanation of lipids. We demonstrated that *cfa2* is the major gene involved in the cyclopropanation of lipids in *S. meliloti*. This is the first report about an increase in cyclopropane fatty acids under phosphate starvation.

The combination of GC/MS and ESI/MS/MS techniques for fatty acid and intact lipid analyses respectively, provided excellent insights into the biochemistry of CFA synthases in *S. meliloti*. Lipid profiles were obtained for five phospholipid classes and two non-phosphorus containing lipids in *S. meliloti*. This is also the first report of cyclopropanation of SLs and TMHSs. We showed that *cis*-11,12-methyleneoctadecanoic acid, was the major component in cyclopropane-containing lipids in *S. meliloti*. Tandem mass spectrometry experiments showed that cyclopropanation of phospholipids occurred in fatty acids located at both the *sn*-1 and *sn*-2 positions in lipids.

Fatty acid distributions within lipid classes showed that cyclopropane fatty acids were not homogeneously distributed in lipid classes as they represented 47%, 18%, 14% and 11% in PCs, PEs, SLs and PGs, respectively. Furthermore, fatty acid distributions within lipid classes showed that acidity caused a larger effect in the cyclopropanation of lipids compared to P_i-starvation conditions. This phenomenon was more accentuated in PCs and SLs as cyclopropanation of fatty acids (*cis*-11-octadecenoic acid) in *sn*-2 position proceeded to a larger extent than in PEs or PGs. However, PCA of fatty acid distributions in four lipid classes (PCs, PEs, PGs, SLs) showed that cyclopropanation occurred uniformly in lipid classes; therefore the nature of the polar head group did not determine the activity of CFA synthases.

Chapter 5

A Lipidomics Study of the Malic Enzymes in *Sinorhizobium meliloti*

Abstract

Sinorhizobium meliloti fixes nitrogen for alfalfa plants establishing a favorable symbiotic relationship. *S. meliloti* contains two distinct malic enzymes, DME (EC 1.1.1.39) and TME (EC 1.1.1.40). While both enzymes catalyze the same biochemical reaction only DME is essential for nitrogen fixation; thus it is thought these enzymes perform different biological functions however a phenotype has not been observed. It is speculated that TME could be involved in fatty acid and lipid metabolism. In this work, a lipidomics approach was used to examine both fatty acid and intact lipid profiles of null mutants of *dme* and *tme* grown in two carbon sources, succinate and glucose. The fatty acid and intact lipid profiles of both mutants were significantly different in succinate; no differences were observed in glucose. More importantly, sulfoquinovosyldiacylglycerols showed the most significant differences for *dme* and *tme* mutants when succinate was the carbon source. Principal component analysis (PCA) of fatty acid distributions in five lipid classes revealed a distinct metabolic phenotype for *tme* mutants in succinate which was determined by lipids with fatty acid composition C34:1. These results suggest the involvement of TME in fatty acid and lipid metabolism.

Introduction

Sinorhizobium meliloti is a soil bacterium which establishes symbiosis with alfalfa plants. In the symbiotic process, bacteria transform atmospheric nitrogen into a useful form for the plant which in turn provides nutrients to bacteria; this process is called nitrogen fixation [37]. In the past years, numerous efforts have been directed to gain a better understanding of the biochemistry and genetics involved in nitrogen fixation [25, 36]. A better knowledge of metabolism in *S. meliloti* would facilitate genetic engineering of the bacterium to enhance nitrogen fixation with tremendous repercussions for sustainable agriculture [196].

Dicarboxylic acids such as malate and succinate are the major sources of carbon provided by plants to nitrogen-fixing bacteria [29]. Metabolism of dicarboxylic acids in *S. meliloti* occurs via the tricarboxylic acid cycle (TCA) and is dependent of the input of acetyl coenzyme A (Acetyl-CoA) [223]. The malic enzymes catalyze the decarboxylation of malate to pyruvate which can be decarboxylated further to acetyl-CoA via pyruvate dehydrogenase, Figure 5.1 [29, 224]. The genome of *S. meliloti* contains two distinct malic enzymes: (1) TME which requires exclusively triphosphopyridine nucleotide ($NADP^+$) and (2) DME

which requires diphosphopyridine nucleotide (NAD^+) mainly but can use also $NADP^+$, as cofactors [225]. Acetyl-CoA is not only important for proper functioning of the TCA cycle and nitrogen fixation [29] but is also central in fatty acid and lipid metabolism [226]. The reaction of acetyl-CoA with malonyl-ACP catalyzed by acetyl-CoA carboxylase is the first step in fatty acid biosynthesis in bacteria [128].

DME and TME catalyze the same biochemical reaction however DME is essential for nitrogen fixation [225] while TME isn't [224]. The malic enzymes in *S. meliloti* have been cloned, sequenced and characterized [227, 228] however a clear phenotype has not been observed for their null mutants under different growth conditions [224]. The biochemical role of the malic enzymes is unknown nevertheless they seem to have different biological roles and are involved in nitrogen fixation in *S. meliloti* [223]. The major differences found between the two proteins are: (ii) Acetyl-CoA inhibits DME activity but not TME [227] and (ii) DME levels in bacteroids (bacteria in root nodules) are 10 times greater than TME [229]. It has been speculated that TME may be involved in a pathway producing acetyl-CoA for fatty acid and lipid biosynthesis [230, 231]. Furthermore, it has been demonstrated that in fungi [232, 233] and plants [234] the $NADP^+$-dependent malic enzyme (EC 1.1.1.40, TME in *S. meliloti*) is intimately involved in fatty acid biosynthesis and lipid accumulation. Given the possible implications of TME in fatty acid and lipid metabolism, in this work we focused on the analysis of fatty acids and intact lipids in null mutants of *dme* and *tme* genes in *S. meliloti*.

In *E. coli*, DME and TME activities are repressed by glucose and induced by C4-dicarboxylic acids such as succinate [230]. Furthermore, C4-dicarboxylic acids such as succinate constitute the major carbon source used by *S. meliloti* in root nodules and in nitrogen fixation [235]. We decided to explore the metabolic profiles of *dme* and *tme* mutants grown in glycolytic and gluconeogenic substrates succinate and glucose respectively, in order to detect metabolic differences which could provide insights into the biochemistry of the malic enzymes. Fatty acids were analyzed as their fatty acid methyl esters (FAMEs) using gas chromatography coupled to mass spectrometry (GC/MS) [63]. Intact lipids were directly analyzed from crude lipid extracts using a shotgun lipidomics approach [221]. Electrospray ionization mass spectrometry (ESI/MS) in positive and negative ionization modes and a combination of tandem mass spectrometric techniques (ESI/MS/MS) provided fatty acid distributions in lipid classes. Principal component analysis (PCA) was used as a tool to identify patterns or trends in the data sets.

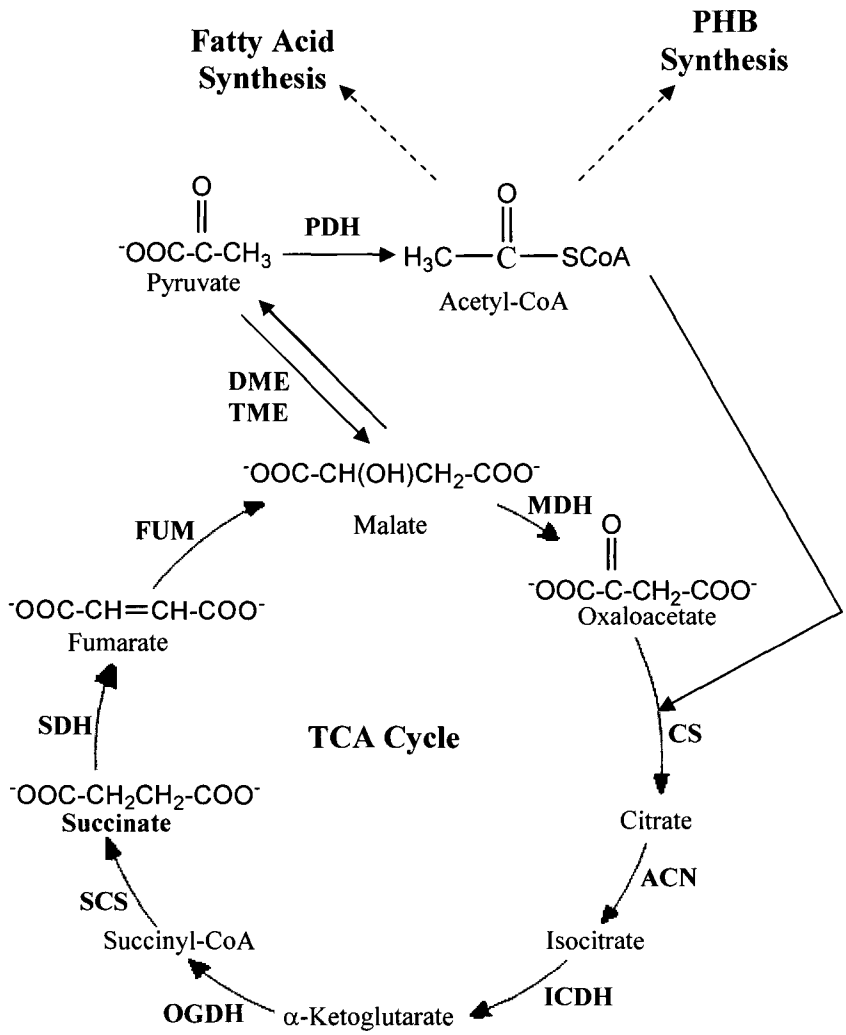


Figure 5.1. TCA cycle and related pathways. PHB, poly-3-hydroxybutyrate; MDH, malate dehydrogenase; CS, citrate synthase; ACN, aconitase; ICDH, isocitrate dehydrogenase; OGDH, 2-oxoglutarate dehydrogenase; SCS, succinyl-CoA synthetase; SDH, succinate dehydrogenase; FUM, fumarase; DME, diphosphopyridine nucleotide dependent malic acid enzyme; TME, triphosphopyridine nucleotide dependent malic acid enzyme; PDH, pyruvate dehydrogenase.

Materials and Methods

Abbreviations

Fatty acids were represented using the shorthand designation system recommended by the IUPAC [52]. Phospholipids were abbreviated as PCs, PEs, MMPEs, DMPEs, PGs and CLs for phosphatidylcholines, phosphatidylethanolamines, phosphatidylmonomethylethanolamines, phosphatidyldimethylethanolamines, phosphatidylglycerols and cardiolipins, respectively. Lysophosphatidylglycerols, phosphatidic acids and lysophosphatidic acids were abbreviated as LPGs, PAs and LPAs. Non-phosphorus containing lipids such as sulfoquinovosyldiacylglycerols were represented as SLs. Fatty acid composition in lipids was represented by the sum of carbon atoms in the fatty acyl chains of lipids followed by a colon and the total number of unsaturations, e.g., 1,2-dioleoyl-*sn*-glycero-3-phosphocholine was represented as PC-36:2.

Analytical Standards and Reagents

N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) and ethyl dodecanoate were obtained from Sigma-Aldrich (Saint Louis, MO). All solvents used were HPLC grade (Caledon Labs, Caledon, ON). Phospholipid standards 1,2-diheneicosanoyl-*sn*-glycero-3-phosphocholine (PC(21:0/21:0)), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (PE(14:0/14:0)) and 1,2-dilauroyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol) (PG(12:0/12:0), sodium salt) were purchased from Avanti Polar Lipids (Alabaster, AL).

Bacterial Cultures

S. meliloti wild type strain Rm1021 and it's *dme* and *tme* insertion mutants were used for this study. The *dme* mutant, RmG455 carries a Tn5 insertion in the *dme* gene (Rm1021, dme-3::Tn5, [225]) whereas in *tme* mutant the *tme* gene has been inactivated by the insertion of Ωspectinomycin cassette (Rm1021, *tme-4*::Ωsp, [224]). *S. meliloti* wild type and the mutant strains grown overnight in Luria-Bertani broth (LB containing $CaCl_2$ and $MgSO_4$, 2.5mM each) were washed with saline (0.85% NaCl), subcultured in M9 minimal medium using glucose or succinate as the carbon source at 15mM final concentration and grown for ~30 hours at 30°C with shaking. Cell pellets from 250 mL culture ($OD_{600} \approx 1.0$-1.2) were resuspended in 1.25mL of 0.85% NaCl and aliquots of 250 µL were made in microfuge tubes. Each tube was centrifuged, the supernatant was discarded and the wet pellets flash frozen in liquid nitrogen and stored at -80°C until further use. The pellets equivalent to 50 mL of original culture grown in succinate, corresponded to 37.7 ± 0.2 mg for wild type, 38.7 ± 0.5 mg for *dme* and 43.9 ± 0.9 mg for *tme* mutants (n=3). Wet pellets from cultures grown in glucose were 39.9 ± 0.6 mg, 46.5 ± 0.4 mg and 44.4 ± 0.2 mg for wild type, *dme* and *tme* mutants, respectively (n=3).

Lipid Extraction

Wet cell pellets were resuspended in distilled water (1 mL) and extracted with a mixture of $CHCl_3$:MeOH (1 mL, 2:1, v/v). The chloroform phase containing the lipids was separated and the aqueous phase was extracted twice more with $CHCl_3$:MeOH (1 mL, 2:1, v/v). The chloroform layers were combined and dried through a small column packed with anhydrous sodium sulfate. The solvent was evaporated using nitrogen gas and the dried lipid residue was either analyzed immediately or stored at -80°C.

Fatty Acid Methyl Esters Analysis by GC/MS

FAMEs were generated from dried lipid extracts using a micro-scale one vial method that employed sodium methoxide in methanol as described previously [63].

Intact Lipid Analysis by ESI/MS and ESI/MS/MS

Dried lipid extracts were dissolved in MeOH: $CHCL_3$ (1:1, v/v, 200 µL). Aliquots were diluted 5-fold with MeOH containing PC(21:0/21:0) (32 µmol) and PE(14:0/14:0) (28 µmol) as internal standards and lithium chloride (2.5 mM) for analyses in the positive ionization mode. For analysis in the negative ionization mode, aliquots were diluted 5-fold with methanol containing PG(12:0/12:0) (3 µmol) as the internal standard. A Waters Quattro Ultima triple quadrupole mass spectrometer equipped with a microelectrospray ion source and operating under the MassLynx software was used for the analysis of bacterial lipid extracts. The collision gas (N_2) pressure was 2×10^{-3} Bar and collision energies ranged between 25 and 50 eV. Bacterial lipid extracts were continuously infused at 1 µL/min using a Harvard syringe pump in the positive ion mode with or without the addition of lithium chloride and in the negative ion mode (no LiCl added). PCs, PEs and MMPEs were analyzed as lithiated adducts in the positive ionization mode using neutral loss scans of 189 [99], 147 [103] and 161 mass units respectively, with collision energy of 35 eV. PGs and SLs were analyzed as the deprotonated ions in the negative ionization mode using precursor ion scans of m/z 153 [14, 20] and m/z 225 [221] using 50 eV as collision energy. One hundred spectra were averaged in each mass spectrometry experiment. Peaks areas of compounds detected in negative electrospray ionization full scan mode were normalized to the peak area of the internal standard (PG(12:0/12:0)); these ratios were used to calculate relative percentage composition of compounds detected in negative electrospray ionization.

Statistical Analysis

All data are mean values ± standard deviation; each sample was analyzed in triplicate. The SPSS statistical package, version 15.0 (SPSS Inc. Chicago, IL, USA) was used for Student's t-test and analysis of variance (ANOVA). Student's t-test was used to evaluate the significance of the differences when only two

groups were compared whereas ANOVA was used for multiple group comparisons ($P \leq 0.05$). Principal component analyses (PCA) were performed using the Multivariate Analysis Add-in for Excel, version 1.3 (Bristol Chemometrics Inc., Bristol, UK).

Results and Discussion

Cells membranes in *S. meliloti* are composed of PCs (60%), PEs (20%), PGs (9%), CLs (5%) and SLs (3%) [51]. In this study, intact lipid analyses were conducted using a shotgun lipidomics approach in which crude extracts are directly infused using electrospray ionization mass spectrometry in positive and negative ionization modes without any prior chromatographic separation [126, 221]. Anionic lipids (PGs and SLs) were detected as $[M-H]^-$ in the negative ionization mode. Individual profiles were obtained using MS/MS transitions unique for each lipid class; e.g., precursor ion scans of m/z 153 and m/z 225 were used for PGs and SLs, respectively [221]. Cationic (PCs) or zwitterionic lipids (PEs, MMPEs, DMPEs) were detected as $[M+Li]^+$ adducts in the positive ionization mode upon addition of lithium salts [221]. Individual lipid profiles were obtained for $[M+Li]^+$ adducts using various neutral loss scans unique to the polar head group of each lipid class.

Intact Lipid Profiles of Wild Type, dme and tme Mutants

The negative electrospray ionization spectra in the full scan mode from crude lipid extracts of *S. meliloti* wild type and both mutants grown in succinate showed interesting differences in the region of m/z 500-700 (Figure 5.2). No differences were observed in the negative ionization spectra of cultures grown in glucose (data not shown). Full scan mode spectra obtained in positive electrospray ionization did not show differences for cultures grown in either succinate or glucose. In general, no differences were observed for cultures grown in glucose in either positive or negative electrospray ionization, similarly as observed in the fatty acid profiles of cultures grown in glucose.

The most pronounced changes were observed in the negative electrospray ionization spectra from cultures grown in succinate for peaks detected at m/z 639.6, 663.4 and 692.4 (Figure 5.2). Peak at m/z 639.6 decreased significantly (7-fold) in both *dme* and *tme* mutants relative to wild type in succinate (Figure 5.3). Peaks at m/z 663.5 and 692.4 decreased dramatically in *dme* mutants (9-fold and 11-fold, respectively) but less than 2-fold in *tme* mutants relative to wild type (Figure 5.3). Peaks at m/z 537.6, 539.6 and 563.6 also showed differences in both mutants relative to the wild type, though changes were smaller than for compounds at m/z 639.6, 663.4 and 692.4. Compounds at m/z 537.6, 539.6, 563.6, 639.6, 663.4 and 692.4 did not correspond to any lipid class known to occur in *S. meliloti*. Collision induced dissociation (CID) experiments were conducted in order to obtain information regarding the structure of these unknown compounds.

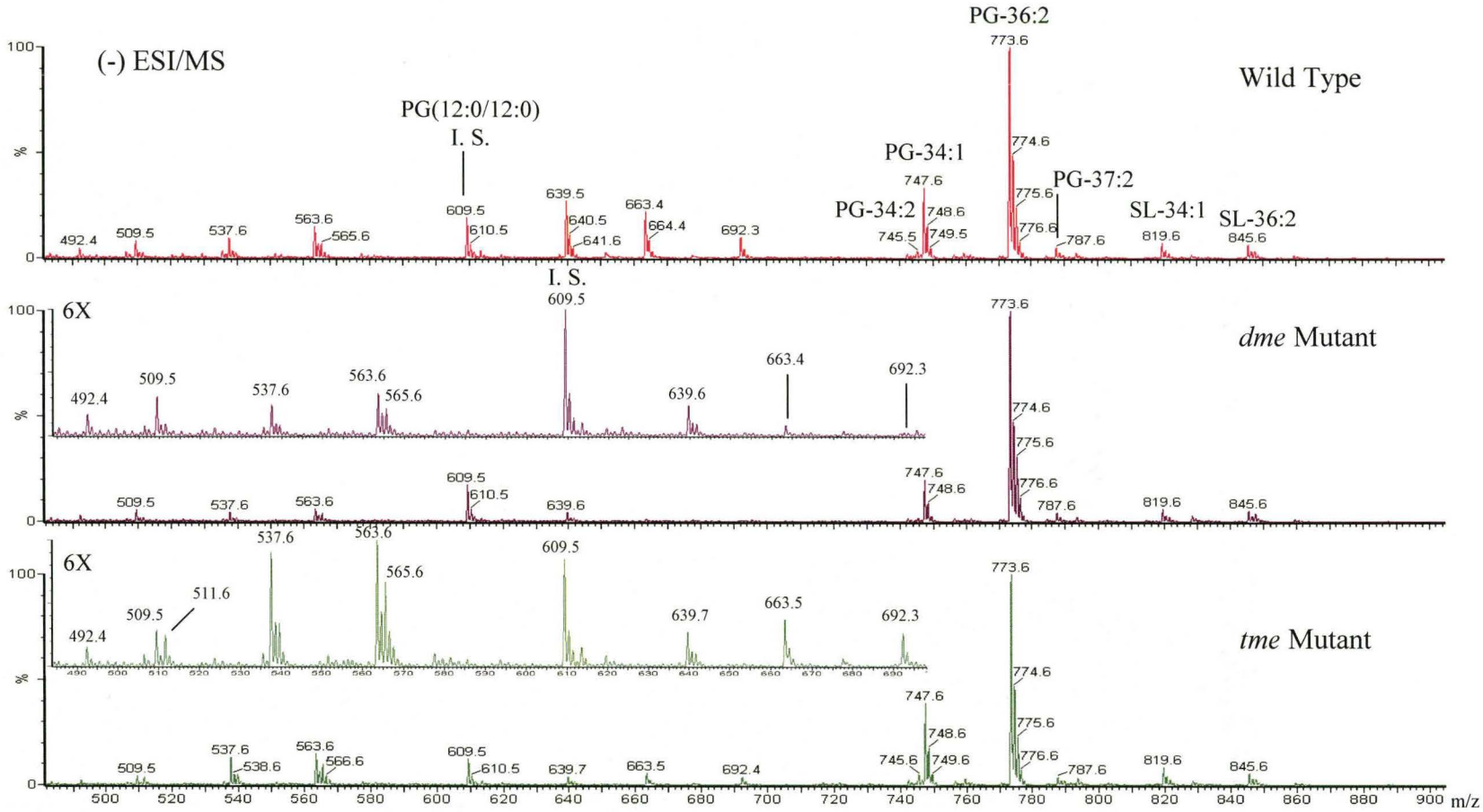


Figure 5.2. Electrospray ionization mass spectra in the negative ionization mode of crude lipid extracts of *S. meliloti* wild type, *dme* and *tme* mutants in succinate.

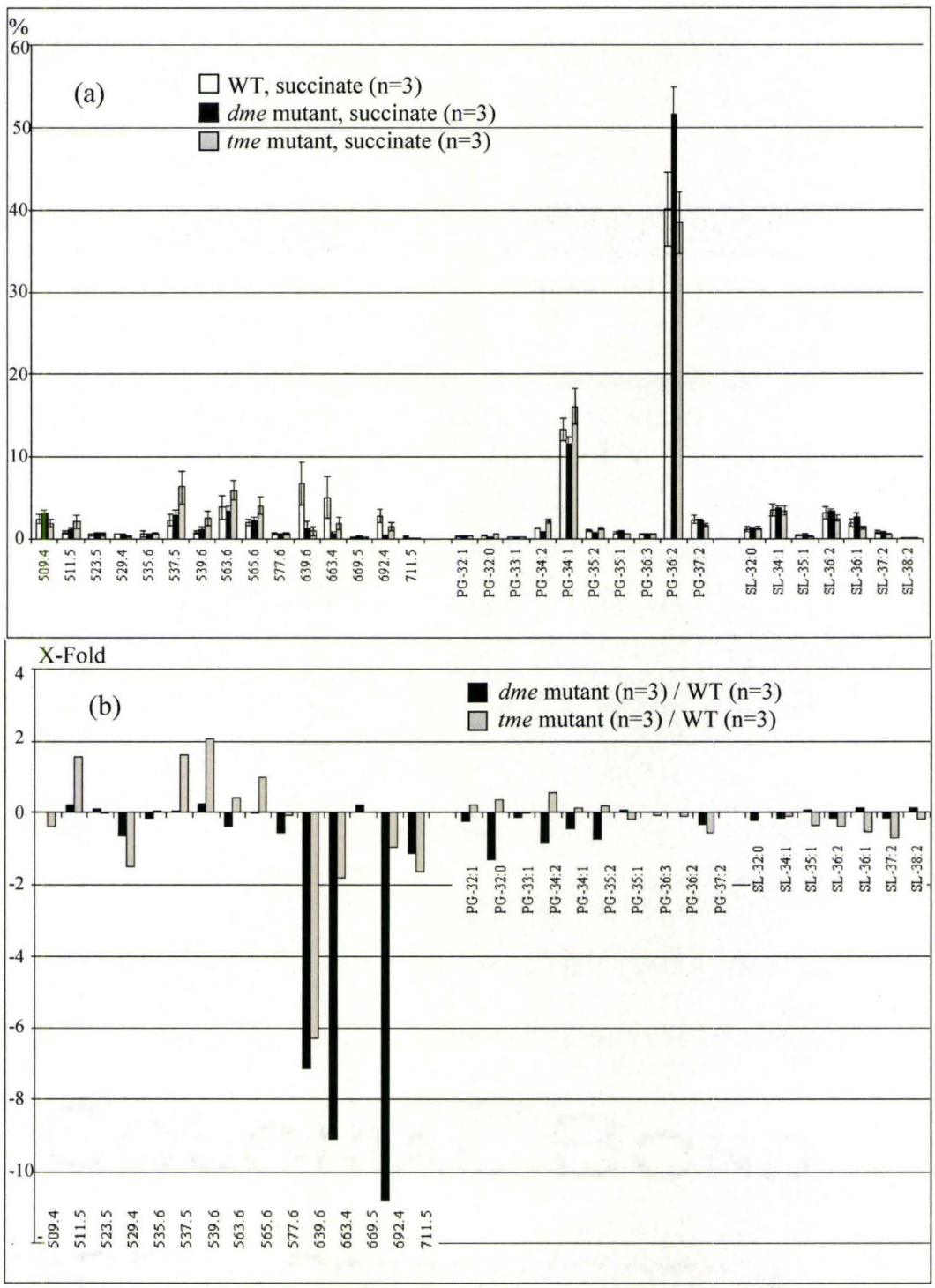


Figure 5.3. (a) Relative percentage composition of compounds detected in negative electrospray ionization in *S. meliloti* grown in succinate. (b) Fold change in relative percentage composition of these compounds in *dme* and *tme* mutants relative to the wild type in succinate.

LPGs, PAs, LPAs and CLs are lipid classes which can occur in bacteria and are readily detected as $[M-H]^-$ ions under negative electrospray ionization conditions [98, 213, 236]. If these lipids were present in *S. meliloti* their $[M-H]^-$ ions would be observed in the m/z 500-700 region if present in *S. meliloti*. Under MS/MS conditions, $[M-H]^-$ ions of LPGs, PAs and LPAs typically produce carboxylate fragment ions due to the loss of fatty acyl chains (or one fatty acyl chain for lysophospholipids) and ion $[HO_3POCH_2C(OH)CH_2]^-$ at m/z 153 corresponding to the polar head group [236]. Peaks at m/z 509.5, 537.6, 563.6 and 565.6 matched the m/z values expected for $[M-H]^-$ ions formed by LPGs with fatty acid compositions of LPG-18:1, LPG-20:1, LPG-22:2 and LPG-22:1, respectively. However, only the product ion spectrum of ion at m/z 509.5 afforded fragment ions that corresponded to LPG-18:1 demonstrated by the presence of $[RCOO]^-$ and $[HO_3POCH_2C(OH)CH_2]^-$ ions at m/z 281 and m/z 153, respectively. Small intensity $[COO]^-$ ions from other fatty acids (e.g., 14:0 and 16:1) were also present in the product ion spectrum of m/z 509.5. Therefore, peak at m/z 509 most likely corresponds to a mixture where LPG-18:1 is the most abundant component. The product ion spectra of peaks at m/z 537.6, 563.6 and 565.6 did not provide fragmentation patterns that would support their identification as LPG-20:1, LPG-22:2 and LPG-22:1, respectively.

CLs also can form $[M-2H]^{-2}$ ions in negative electrospray ionization and under MS/MS their product ion spectra is contains intense $[RCOO]^-$ ions from fatty acyl substituents and the polar head group ion at m/z 153 [213]. While the product ion spectra of peaks at m/z 537.6, 563.6 and 565.6 afforded $[RCOO]^-$ fragments from various fatty acids, these ions did not fit any possible fatty acid combination for molecular species of LPGs, PAs, LPAs or doubly charged ions of CLs. The MS/MS spectra of peaks at m/z 663.5 and 692.3 did not produce fragmentation patterns that resembled those produced by $[M-H]^-$ or $[M-2H]^-$ ions of PAs, LPGs or CLs species.

Fatty Acid Distributions in Lipid Classes

Specific MS/MS transitions were used to obtain fatty acid distributions in lipid classes [221]. Profiles of six lipid classes (PCs, PEs, MMPEs, DMPEs, PGs and SLs) were obtained for wild type, *dme* and *tme* mutants grown in succinate and glucose. The most interesting changes across lipid classes were observed for lipids with fatty acid composition of 34:1 and only in *tme* mutants (Figure 5.4). Lipids with fatty acid composition 34:1 in *S. meliloti* are formed by *cis*-11-octadecenoic acid and hexadecanoic acid as the major component [221]. The changes in the profiles of lipid classes such as PCs, PEs and PGs were minor while SLs showed the biggest differences as a class, being the most noticeable for *tme* mutants (Figure 5.5).

PCA of fatty acid distributions in SLs showed clearly no differences when cultures were grown in glucose as all replicates of wild type, *dme* and *tme* mutants clustered in the center slightly to the left relative to PC1 axis in the scores plot (Figure 5.6a). On the other hand, cultures grown in succinate showed that lipid

profiles of both mutants were different in comparison with wild type. Mutant of *dme* were slightly different to wild type but *tme* mutant was clearly different to both wild type and *dme* mutants since all replicates were located at the far right side relative to PC1 (Figure 5.6a). SLs with fatty acid compositions of 32:0, 32:1, 34:1, 34:2 and 35:2 determined the differences in the profiles of *tme* mutants in succinate as shown in the loadings plot (Figure 5.6b). These lipids contained 16:0 and/or 16:1 fatty acids as part of their structure [216].

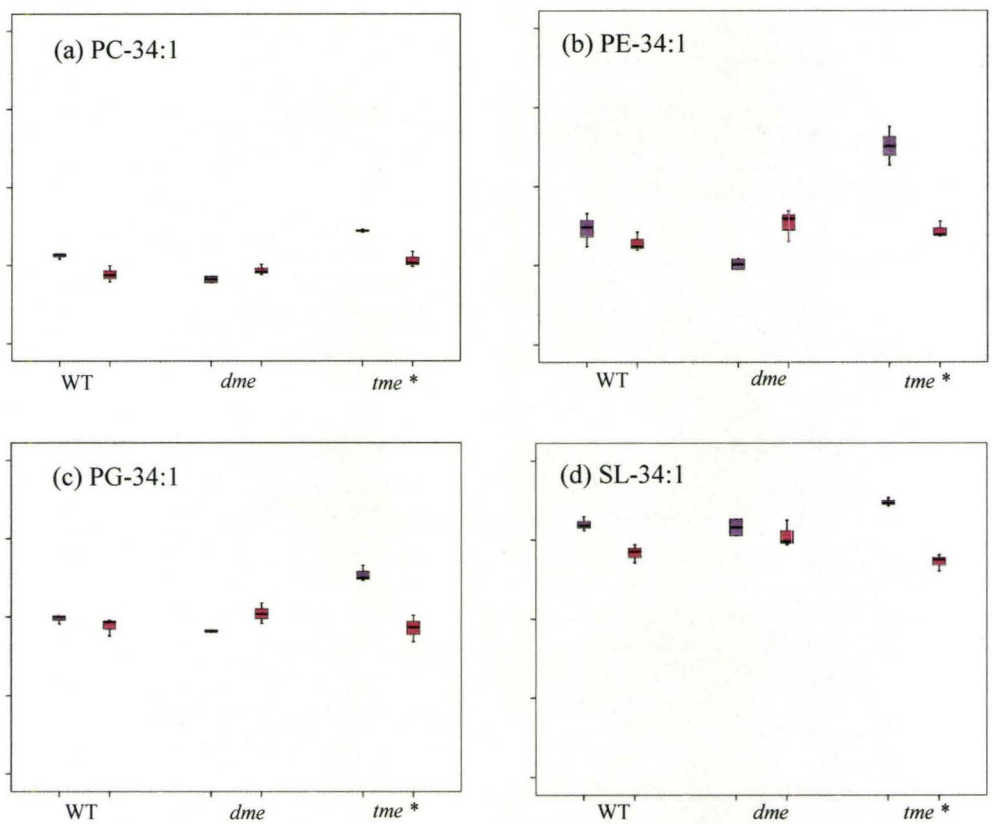


Figure 5.4. Relative percentage composition (a) PC-34:1,(b) PE-34:1, (c) PG-34:1 and (d) SL-34:1 for wild type, *dme* and *tme* null mutants grown in succinate (■) and glucose (■). PCs, phosphatidylcholines; PEs: phosphatidylethanolamines; PGs: phosphatidylglycerols; SLs: sulfoquinovosyldiacylglycerols. (*) Indicates that means in succinate are significantly different to means in glucose ($P \leq 0.05$).

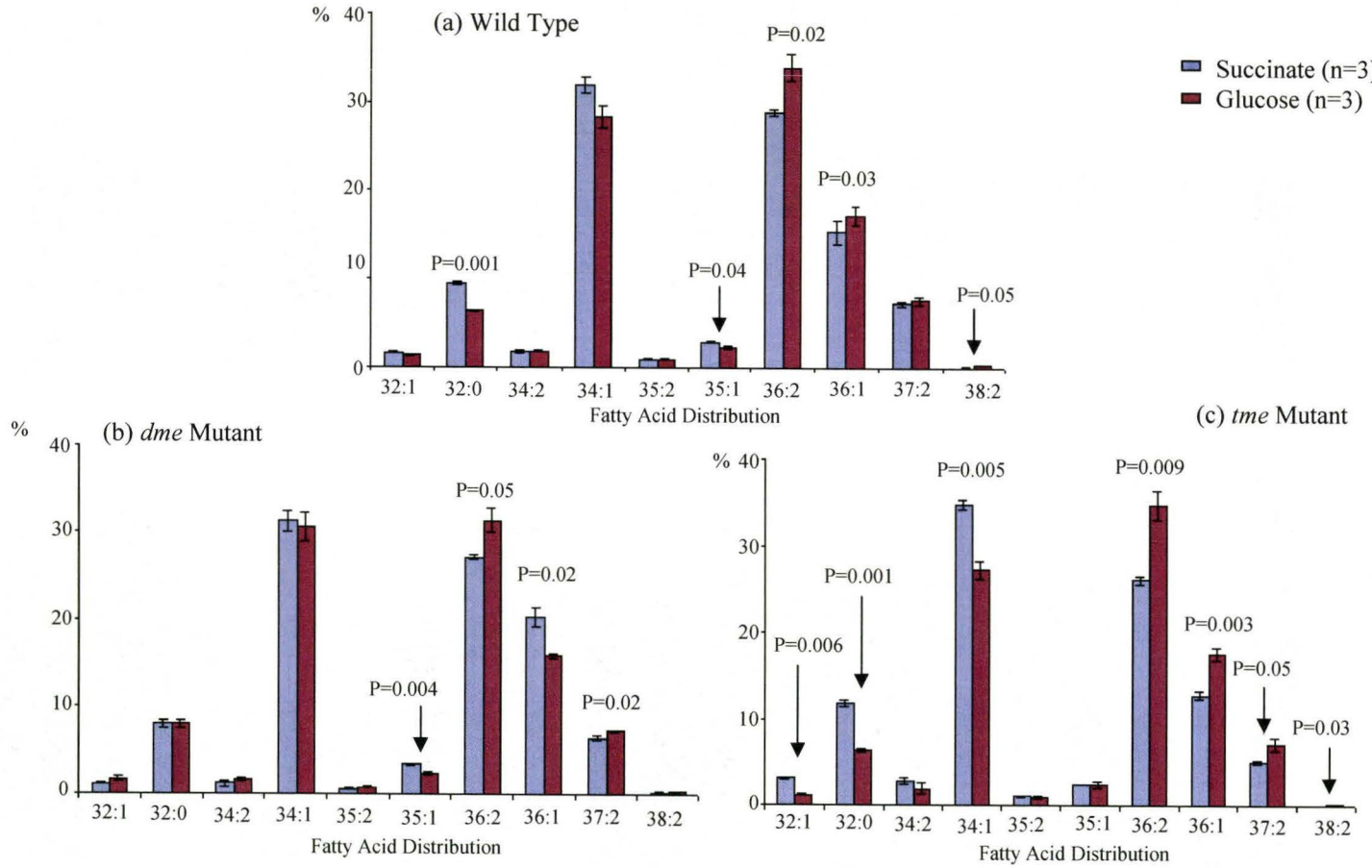


Figure 5.5. Sulfoquinovosyldiacylglycerols profiles of (a) wild type, (b) *dme* and (c) *tme* mutants in succinate and glucose.

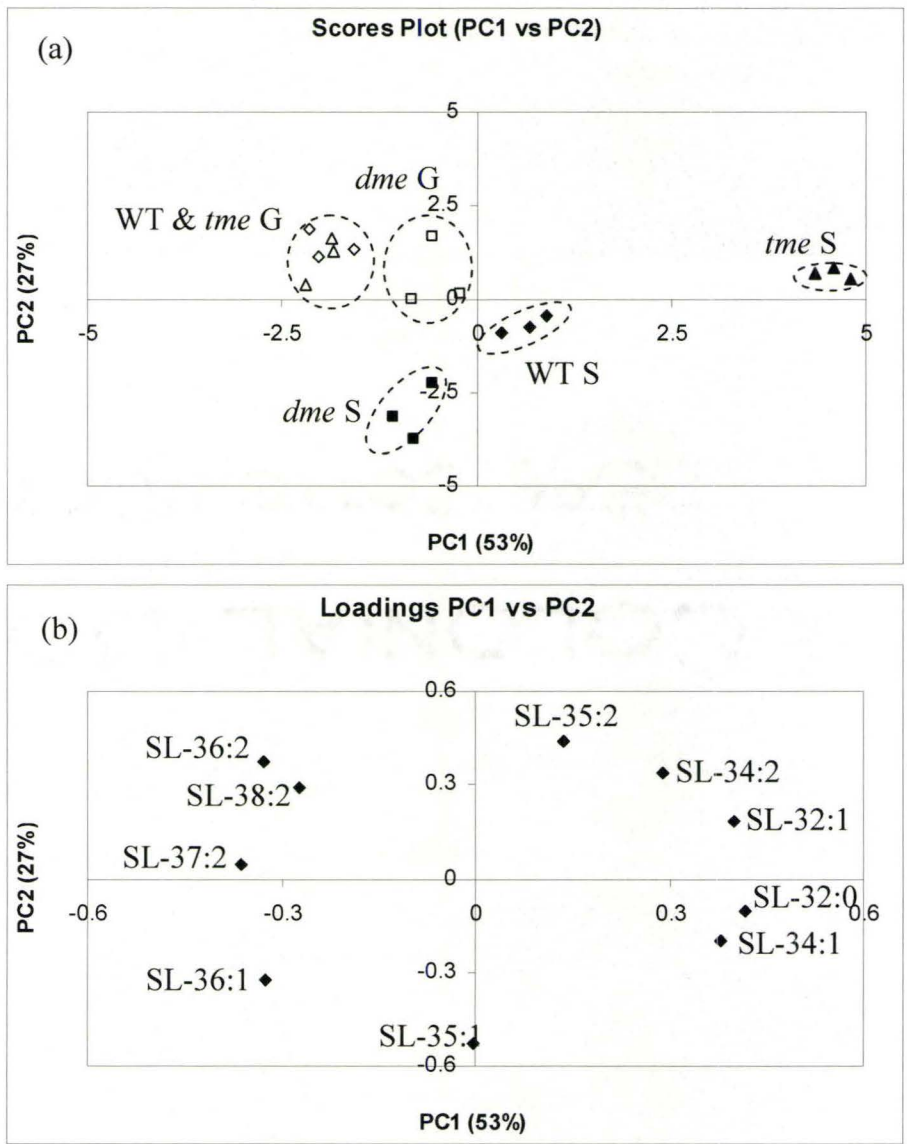


Figure 5.6. Principal component analysis of sulfoquinovosyldiacylglycerols profiles in wild type, *dme* and *tme* mutants grown in succinate and glucose.

PCA of fatty acid distributions in PCs, PEs, MMPEs, PGs and SLs (38 species in total) showed that metabolic phenotypes of cultures grown in glucose were not different demonstrated by tight clustering of wild type, *dme* and *tme* mutants in the loadings plot (Figure 5.7b). Metabolic phenotypes of *tme* mutants grown in succinate were clearly different as they were well separated relative to PC2 axis (Figure 5.7b). The differences in *tme* mutants grown in succinate can be

related to lipids with fatty acid composition of 34:1 as these species in all lipid classes were grouped in the top portion of the scores plot (Figure 5.7a).

In *E. coli*, the activities of DME and TME were repressed by glucose and induced by C4-dicarboxylic acids [230]. On the other hand, *S. meliloti* mutants of TME did not show a carbon phenotype or any differences when various carbon sources were investigated [224]. We detected a metabolic phenotype for TME deficient mutants when succinate was the carbon source. These results suggest the involvement of TME in fatty acid and lipid metabolism in *S. meliloti*.

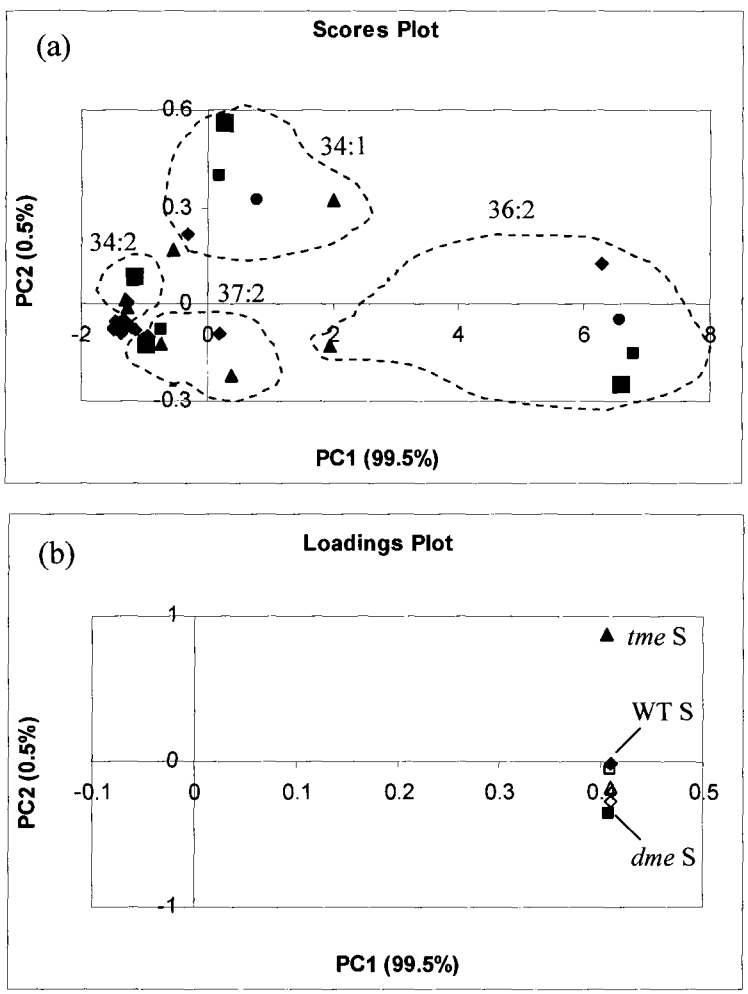


Figure 5.7. PCA of five lipid classes for wild type (WT) and mutants of *dme* and *tme* grown in succinate (S) and glucose (G). (a) In the scores plot, PCs, ♦; PEs, ■; MMPEs, ■; PGs, •; SLs, ▲. (b) In the loadings plot, different shapes represented wild type (♦), *dme* (■) and *tme* (▲) mutants. Cultures grown in succinate were represented by solid figures while cultures in glucose were represented cultures open figures.

Fatty acid Profiles of Wild Type and Mutants of dme and tme

Total fatty acids were analyzed as their FAMEs by GC/MS. A one-vial method that employed sodium methoxide in methanol was used [63]. A total of eight fatty acids were detected and positively identified which included four saturated, two unsaturated and two cyclopropane-containing fatty acids (Table 5.1).

Table 5.1. Fatty acid composition of *S. meliloti* wild type, *dme* and *tme* mutants grown in succinate and glucose. N.D., not detected. (*) Indicates that mean values in succinate are significantly different to mean values in glucose ($P \leq 0.05$).

Fatty Acids	Fatty Acid Relative Percentage Composition, % (Mean ± SD, n=3)					
	Wild Type		*dme* Mutant		*tme* Mutant	
	Succinate	Glucose	Succinate	Glucose	Succinate	Glucose
14:0	0.6 ± 0.2	0.5 ± 0.2	0.6 ± 0.1	0.6 ± 0.2	0.4 ± 0.1	0.5 ± 0.1
16:0	12.1 ± 2.8	9.2 ± 0.6	9.3 ± 1.2	10.4 ± 2.0	11.1 ± 1.7	9.2 ± 0.8
17:0	0.2 ± 0.0	0.3 ± 0.1	0.6 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
18:0	4.9 ± 0.6	5.1 ± 0.4	7.7 ± 0.5	6.2 ± 0.5	6.9 ± 1.4	5.4 ± 0.6
16:1(9)	2.1 ± 0.2	1.9 ± 0.2	1.1 ± 0.2	1.9 ± 0.1 *	3.1 ± 0.2	2.0 ± 0.1 *
18:1(11)	68.6 ± 1.6	74.4 ± 1.6 *	72.8 ± 1.7	71.6 ± 1.9	70.0 ± 3.9	74.8 ± 1.5
17:0cyclo(9,10)	1.6 ± 0.7	0.5 ± 0.9	N.D.	0.7 ± 0.7	1.3 ± 0.1	N.D. *
19:0cyclo(11,12)	9.8 ± 1.6	8.1 ± 0.8	7.9 ± 0.2	8.4 ± 1.5	7.0 ± 0.8	8.0 ± 0.5

The fatty acid profiles of wild type, *dme* and *tme* mutants were identical when glucose was used as the carbon source. In contrast, for cultures grown in succinate the fatty acid profiles of *dme* and *tme* mutants showed statistically significant differences ($P \leq 0.05$) compared to wild type (Figure 5.8). In succinate, the most noticeable change compared to wild type was a decrease in fatty acids with sixteen carbon atoms (16:1(9) and 17:0cyclo) in the *dme* mutant while in *tme* mutants 16:1(9) increased by 1%. The only other study that has explored fatty acid composition of *tme* mutants, revealed a slight increase in fatty acid unsaturation when TME was expressed in fungi [233].

The changes observed in fatty acid composition of DME and TME mutants are consistent with changes detected in the fatty acid distributions in lipids when succinate was the carbon source. However, changes in total fatty acid composition were modest while the differences observed in SLs were more noticeable. The biochemical roles and differences between DME and TME in *S. meliloti* are not understood [224]. While no other phenotype has been revealed for DME and TME mutants, the results presented here showed these mutants have a metabolic phenotype in succinate.

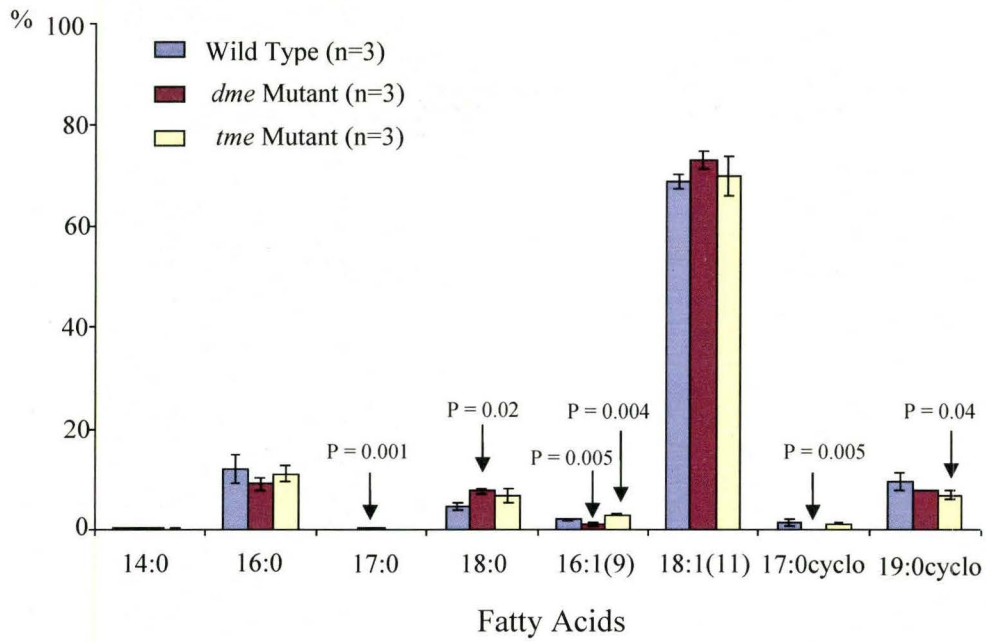


Figure 5.8. Fatty acid profiles of *S. meliloti* wild type, *dme* and *tme* mutants grown in succinate.

Other subtle differences in fatty acid composition were observed for cultures grown in succinate relative to cultures grown in glucose. For example, wild type cultures grown in succinate showed a slight decrease (6%) of *cis*-11-octadecenoic acid (18:1(11)) compared to cultures grown in glucose. Mutants of *dme* showed a statistically significant ($P \leq 0.05$) decrease of *cis*-9-hexadecenoic acid (1%) in succinate relative to glucose while *tme* mutants showed the opposite effect. No other significant differences were observed between cultures grown in succinate and glucose.

There has been some speculation that the combined actions of DME, TME and pyruvate dehydrogenase (Figure 5.1) could influence the amounts of excess acetyl-CoA in the TCA cycle and increase poly-3-hydroxybutyrate (PHB) accumulation [29]. Our analytical methodology was able to detect methyl 3-trimethylsilyloxy butanoate the derivative formed from PHB [63]. PHB accumulation was not detected in either *dme* or *tme* mutants under the growth conditions studied.

In summary, we conclude that small but significant differences were observed in the fatty acid profiles of *dme* and *tme* mutants when cultures were grown in succinate while no differences were observe in glucose. This is the first report of fatty acid and intact lipid compositions in mutants of the malic enzymes in *S. meliloti*.

Conclusions

This research presented metabolite profiles (fatty acids and intact lipids) for the first time of *S. meliloti* mutants deficient in DME and TME in two carbon sources. The fatty acid and intact lipid profiles of *dme* and *tme* mutants were different to each other and relative to wild type when succinate was the carbon source. Fatty acid analysis by GC/MS showed significant but slight differences for hexadecenoic acid in *tme* mutants in succinate. However, changes observed in fatty acid distributions in lipids were more noticeable in *dme* and *tme* mutants in succinate, particularly for sulfoquinovosyldiacylglycerols. In *tme* mutants, sulfoquinovosyldiacylglycerol lipids with fatty acid compositions of 32:0, 32:1, 34:1, 34:2 and 35:2 increased significantly in cultures grown in succinate relative to cultures in glucose. All these lipids contained hexadecenoic acid and/or hexadecanoic fatty acids. Principal component analysis of intact lipid profiles of four lipid classes showed that lipids with fatty acid composition of 34:1 contributed to the metabolic phenotype of *tme* mutants in succinate. These results indicate the involvement of TME in fatty acid and lipid metabolism in *S. meliloti* as suggested in *E. coli*. Moreover, the differences in metabolic profiles in mutants of *dme* and *tme* have revealed a metabolic phenotype for mutants deficient in DME and TME in succinate. In the future, we plan to characterize and identify the unknown compounds that exhibited significant changes in both mutants in succinate which could provide useful clues into the biochemistry of these enzymes. This study demonstrated the usefulness of combining metabolite profiling with genetic techniques as a more comprehensive approach which could provide valuable insights into metabolism and gene function.

Chapter 6

A Shotgun Lipidomics Study of a Putative Lysophosphatidic Acyl Transferase (PlsC) in *Sinorhizobium meliloti*

Abstract

A shotgun lipidomics approach was used to study knockout mutants of a putative lysophosphatidic acyl transferase (PlsC) in *Sinorhizobium meliloti*. Lipids that contained C16 fatty acids and their biosynthetically related fatty acid (*cis*-9,10-methylenehexadecanoic acid) decreased up to 93% in *plsC* knockout mutants. MS/MS experiments showed that these fatty acids were located in the *sn*-2 position of lipids. Total fatty acid composition showed up to 83% decrease of C16 fatty acids in *plsC* knockout mutants. Thus, putative PlsC in *S. meliloti* corresponded to a lysophosphatidic acyl transferase specific for C16 fatty acids in the *sn*-2 position of lipids. Lipids that contained C16 fatty acids decreased significantly in phospholipids but also in non-phosphorus containing lipids such as sulfoquinovosyldiacylglycerols and 1,2-diacylglyceryl-trimethylhomoserine lipids. This demonstrates that phospholipids and non-phosphorus containing lipids have a common biosynthetic origin most likely involving phosphatidic acid. The levels of lipids that contained C18 fatty acids were unaffected in *plsC* mutants suggesting the presence of other fatty acyl transferases in the genome of *S. meliloti*.

Introduction

Sinorhizobium meliloti is a soil bacterium that establishes a beneficial relationship (symbiosis) with leguminous plants. In this process, plants provide nutrients to these bacteria which in turn transform atmospheric nitrogen into a usable form by plants; this is known as nitrogen fixation [29]. In recent years, numerous efforts have been directed to understand and decipher the biochemical mechanisms by which symbiosis and nitrogen fixation occur with undeniable relevance to agriculture [37, 196, 237]. The complete genome of *S. meliloti* has been annotated however the function of most genes is unknown or has been assigned by comparison with the genomes of other organisms [25, 27, 36]. Functional genomics can improve our understanding of gene function, symbiosis and nitrogen fixation [35, 36]. Our research focuses on the study of genes thought to participate in lipid biosynthesis in *S. meliloti*. The comprehensive profiling of lipids in a biological system, known as lipidomics, is an approach used to study gene function and metabolism [12, 238, 239].

Phospholipid biosynthesis in bacteria occurs by successive transformations of the polar head group of phosphatidic acid (PA); thus PA is

central to lipid metabolism [32, 132]. PA biosynthesis in *E. coli* is initiated from glycerol-3-phosphate by transference of a fatty acyl residue to the *sn*-1 position; this reaction is catalyzed by an acylphosphate transferase coded by the *plsB* gene [133]. In a second stage, PA is produced by transference of a second fatty acyl residue to the *sn*-2 position of the lysophosphatidic acid. The second reaction is catalyzed by a lysophosphatidic acyl transferase coded by the *plsC* gene in *E. coli* [134, 135]. This model for PA biosynthesis in *E. coli* is known as the PlsB/PlsC system; however, the genomes of most bacteria do not contain homologues of *plsB* [136, 137]. Recently, an alternative pathway for PA biosynthesis called the PlsX/PlsY pathway has been discovered [33, 138]. This system uses acyl-phosphate (Acyl-PO_4) intermediates derived from acyl-acyl-carrier protein (acyl-ACP) by PlsX and transferred to glycerol-3-phosphate by PlsY [33]. The proposed model for phospholipid biosynthesis in *S. meliloti* is based on the assumption that the genome contains homologues to *E. coli* genes involved in lipid biosynthesis [30]. However, the genome of *S. meliloti* does not contain homologues of *plsB* [30].

In contrast to PlsB, PlsC is universally present in bacteria and in *S. meliloti* SMc00714 protein (PlsC in *E. coli*) is thought to code for a putative lysophosphatidic acyl transferase [30]. Lysphosphatidic acyl transferases are coded by essential genes in some bacteria; consequently, extensive research has focused on these genes as attractive targets for the development of new antibiotics [240]. Recently, it has demonstrated that some bacteria contain multiple acyl transferases with overlapping functions and lysophospholipid acyl transferases [136, 139-142, 241]. In *S. meliloti*, no studies have been conducted to characterize putative *plsC* (SMc00714) and support its functional assignment as a lysophosphatidic acyl transferase. The goal of this study was to determine whether the putative PlsC (SMc00714) in *S. meliloti* coded for a lysophosphatidic acyl tranferase using a lipidomics approach.

Cell membranes of *S. meliloti* are formed mostly by phospholipids (95%) and include phosphatidylcholines (PCs), dimethylphosphatidylethanolamines (DMPEs), monomethylphosphatidyletanolamines and phosphatidylethanolamines (MMPEs and PEs), phosphatidylglycerols (PGs) and cardiolipins (CLs) [51]. Non-phosphorus containing lipids such as ornithine lipids (OLs) and sulfoquinovosyldiacylglycerols (SLs) are also present, but represent minor components. Under P_i-limiting conditions, phospholipids are partially replaced by non-phosphorus containing lipids (SLs, OLs and 1,2-diacylglyceryl-trimethylhomoserine lipids, (TMHSs)) representing up to 70% of total lipids [51]. Since P_i-limitation has a marked effect on membrane lipids in *S. meliloti*, in this work both phospholipids and non-phosphorus containing lipids were studied under normal growth conditions and under P_i-starvation.

Intact lipid profiles of *plsC* knockout mutant were compared to lipid profiles of wild type to investigate the role of PlsC in *S. meliloti*. Additionally, a PlsC suppressor mutant was employed as a strategy to understand the function of PlsC and its interaction with other related genes in lipid biosynthesis. Lipid

analyses were conducted using a shotgun lipidomics approach that allowed profiling eight lipid classes directly from crude lipid extracts [221]. A combination of electrospray ionization mass spectrometry (ESI/MS) and tandem mass spectrometry (ESI/MS/MS) in negative and positive ionization modes provided fatty acid distributions within lipid classes. Principal component analysis (PCA) of fatty acid distributions in lipid classes was used to identify patterns or trends in the data. Analysis of total fatty acids by GC/MS was used as a complementary technique [63].

Concisely, the objectives of this study were: (i) to determine if putative *plsC* in *S. meliloti* coded for a lysophosphatidic acyl transferase; (ii) to determine differences in the lipid profiles of *plsC* suppressor mutants; (iii) to determine if *plsC* was involved in the biosyntheses of phospholipids and non-phosphorus containing lipids.

Experimental

Abbreviations

Fatty acids were represented using the shorthand designation system recommended by the IUPAC [52]. Intact lipids were identified as PCs for phosphatidylcholines, dimethylethanolamines as DMPEs, monomethyletanolamines as MMPEs, phosphatidylethanolamines as PEs, phosphatidylglycerols as PGs, sulfoquinovosyldiacylglycerols as SLs, 1,2-diacylglyceryl-3-*O*-4'-(*N,N,N*-trimethyl)-homoserine lipids as TMHSs and ornithine lipids as OLs. Cardiolipins, phosphatidic acids, lysophosphatidylglycerols and lysophosphatidic acids were identified as CLs, PAs, LPGs and LPAs, respectively. Fatty acid compositions in lipids were represented by the sum of carbon atoms in the fatty acyl chains of lipids followed by a colon and the total number of unsaturations, e.g., 1,2-dioleoyl-*sn*-glycero-3-phosphocholine was represented as PC-36:2. The relative positions of fatty acyl chains in lipids were represented by the fatty acid in the *sn*-1 position first followed by the fatty acid in the *sn*-2 position and separated by a forward slash as recommended, e.g., 1,2-dioleoyl-*sn*-glycero-3-phosphocholine was represented as PC(18:1/18:1) [201]. When the assignment of fatty acids to the *sn*-1 and *sn*-2 positions in lipids was ambiguous, the fatty acids were separated by a hyphen instead of a forward slash.

Bacterial Cultures

Bacterial cultures (*S. meliloti,* RmP110) were grown in MOPs buffered minimal media [203] with glucose (15 mM) as the carbon source in the presence of 2 mM inorganic phosphate and in absence of inorganic phosphate. *S. meliloti* cells were grown overnight in Luria-Bertani medium (LB, containing $CaCl_2$ and $MgSO_4$, 2.5 mM each). Cell pellets were washed with MOPs medium (0 mM P_i), subcultured in MOPs medium with 2 mM P_i or with no P_i and grown for 36-40 hours at 30°C with shaking. Wet cell pellets obtained from 500 mL of culture (O.D.=0.6-0.9) were resuspended in 2.5 mL of medium and divided into 250 µL

aliquots in Eppendorf tubes. Each tube was centrifuged, the supernatant was discarded and the wet pellets flash frozen in liquid nitrogen and stored at -80°C until further use; each cell pellet corresponded to 50 mL of original culture. The pellets obtained from cultures of wild type, *plsC* knockout mutants and *plsC* suppressor grown with 2 mM P_i afforded 34.4 ± 0.6 mg (n=3), 27.7 ± 0.8 mg (n=3) and 30.6 ± 0.4 mg (n=3) wet weight of cells, respectively. Pellets from cultures grown under P_i-starvation conditions afforded 20.0 ± 2.2 mg (n=3), 22.8 ± 0.4 mg (n=3) and 19.0 ± 0.9 mg (n=3) wet weight for wild type, *plsC* knockout mutants and *plsC* suppressor, respectively.

Construction of plsC Gene Knockout Mutants in S. meliloti

Knockout mutant of putative *plsC* gene (SMc00714) in the genome of *S. meliloti* [242] was constructed in wild type strain RmP110 background [181]. For this purpose 250 bp and fragment was PCR amplified from 5'-regions of *plsC* using forward primer 5'-GC**TCTAGA**GCTGCGTTCGATCCTCTTCAATCTGG-3' carrying *Xba*I restriction site and reverse primer 5'-CC**ATCGAT**GGTCACGCATAGGCGTCCCAGAAAGACTG-3' containing *Cla*I restriction site. The amplified fragment was cloned into a suicide plasmid pTH1360 [50] using the above described restriction sites and resultant plasmid pTH2125 in *E. coli* DH5α was recombined into *S. meliloti* RmP110 genome through triparental mating using Mt616 as a helper strain [220]. Single crossover recombinants were selected on LB plates containing 200 μg/mL streptomycin (Sm) and 200 μg/mL of Neomycin (Nm). Representative colonies for each knockout type were streak-purified three times before further use and stored in laboratory's frozen stock culture collection as RmP1702.

Plant Assays

Symbiotic proficiency was assayed on alfalfa (Medicago sativa cv. Iroquois) under nitrogen-deficient conditions in Leonard assemblies as described previously [235]. Briefly, Medicago sativa var. Iroquois seeds were surface-sterilized by immersion in a sodium hypochlorite (1%) solution for 20 min. Following repeated washes with sterile water, the seeds were germinated in the dark for two days on water agar. Eight seedlings were planted in each Leonard Jar assembly [243] containing 1:1 quartz sand:vermiculite mixture saturated with nitrogen-free Jensen's salts [244]. For each strain two such assemblies were inoculated with 10^7-10^8 cells from an overnight culture. Growth conditions were maintained at 25°C (16 h days) and 19°C (8 h nights). Plants were harvested 28 days after inoculation and shoot dry weights were determined following drying in an oven.

Reagents and Materials

All solvents used were HPLC grade (Caledon Labs, Caledon, ON). Phospholipid standards, 1,2-diheneicosanoyl-*sn*-glycero-3-phosphocholine (PC(21:0/21:0)), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (PE(14:0/14:0)), 1,2-dilauroyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol) (PG(12:0/12:0), sodium salt) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (PG(16:0/18:1), sodium salt) were purchased from Avanti Polar Lipids (Alabaster, AL).

Lipid Extraction

Wet cell pellets were resuspended in distilled water (1 mL) and extracted with a mixture of $CHCl_3$:MeOH (1 mL, 2:1, v/v). The $CHCl_3$ phase containing the lipids was separated and the aqueous phase was extracted twice more with $CHCl_3$:MeOH (1 mL, 2:1, v/v). The chloroform layers were combined and dried through a small column packed with anhydrous sodium sulphate (~1 g). The solvent was evaporated using a stream of nitrogen gas and the dried lipid residue was either analyzed immediately or stored at -80°C.

Fatty Acid Methyl Esters Analysis by GC/MS

Fatty acids in lipids were analyzed as their fatty acid methyl esters (FAMEs) by GC/MS. FAMEs were prepared from dried lipid extracts using a one-vial method that employed sodium methoxide in methanol as the transmethylation reagent [63].

Intact Lipid Analysis by Electrospray Ionization Mass Spectrometry

Dried lipid extracts were dissolved in MeOH:$CHCl_3$ (200 μL, 1:1, v/v). For analyses in the positive ionization mode, aliquots (20 μL) were diluted 5-fold with MeOH containing PC(21:0/21:0) (32 μmol), PE(14:0/14:0) (28 μmol) and lithium chloride (2.5 mM). For analyses in the negative ion mode, aliquots (20 μL) of the lipid extract were diluted 5-fold with methanol containing PG(12:0/12:0) (3 μmol) as the internal standard. A Waters Quattro Ultima triple quadrupole mass spectrometer equipped with a microelectrospray ion source and operating under the MassLynx software was used for the analysis of bacterial lipid extracts. The collision gas (N_2) pressure was 2 x 10^{-3} Bar and collision energies (CE) ranged between 25 and 50 eV. Lipid extracts were continuously infused at 1 μL/min using a Harvard syringe pump. PCs, DMPEs, MMPEs and PEs were simultaneously analyzed as their lithiated adducts in the positive ionization mode using neutral loss scans of 189 [99], 175, 161 and 147 [103] mass units with a collision energy of 35 eV. TMHSs and OLs were also analyzed as $[M+Li]^+$ ions using neutral loss scan of 74 mass units (CE 40 eV) and precursor ion scan of m/z 115 (CE 35 eV), respectively. PGs and SLs were analyzed as $[M-H]^-$ ions in the negative ionization mode, using precursor ion scans of m/z 153 [14, 20] and 225, respectively (CE 50 eV). One hundred spectra were averaged in each mass spectrometry experiment. Peak areas were obtained from each MS/MS spectra and normalized to the area of an internal standard used. These data were

used to calculate relative percentage compositions of the molecular species in each lipid class.

Statistical Analyses

All data are reported as mean values ± standard deviation. A statistical package (SPSS, version 15.0, SPSS Inc. Chicago, IL, USA) was used for statistical analyses. Student's t-test was used ($P \leq 0.05$) to evaluate two groups of data. Principal component analyses (PCA) were performed using the SPSS software and the Multivariate Analysis Add-in for Excel, version 1.3 (Bristol Chemometrics Inc., Bristol, UK).

Results and Discussion

A shotgun lipidomics approach was used to profile intact lipids in crude extracts of wild type, *plsC* knockout mutant and *plsC* suppressor under normal growth conditions (2 mM P_i) and under P_i-starvation (0 mM P_i). The suppressor mutation was employed as a tool to obtain more information regarding the function of putative PlsC in *S. meliloti*. The PlsC suppressor mutant showed no phenotype since cultures presented the same growth rates observed in cultures of wild type. In addition, PlsC suppressor mutants infected alfalfa plants and formed nodules succesfully.

In the next section, the lipid profiles of wild type and *plsC* mutants obtained in negative and positive electrospray ionization in the full scan mode are described. Anionic lipids (PGs, SLs) were detected in the negative electrospray ionization mode as $[M-H]^-$ ions. Zwitterionic lipids (PCs, DMPEs, MMPEs, PEs, OLs, TMHSs) were analyzed in positive electrospray ionization mode as $[M+Li]^+$ adducts following addition of lithium salts to lipid extracts. Fatty acid distributions in lipid classes were revealed using tandem mass spectrometric transitions which were unique to each class [221]. Profiles of PGs and SLs were obtained using precursor ion scans of m/z 153 [97] and m/z 225 [221], respectively. Profiles of PCs, PEs, MMPEs and DMPEs were achieved using neutral loss scans of 189 [99, 101], 147 [103], 161 and 175 mass units, respectively; these neutral losses corresponded to the losses of the lithiated polar head groups in each lipid class. Neutral loss scan of 74 mass units and precursor ion scans of m/z 115 provided profiles for TMHSs and OLs, respectively [221]. Total fatty acid composition of *plsC* knockout mutants and PCA analysis of fatty acid distributions in lipid classes are presented.

Lipid Profiles of Wild Type, plsC Mutant and plsC Suppresor in Negative Electrospray Ionization

A typical lipid profile in negative electrospray ionization from cultures of *S. meliloti* wild type grown under normal conditions is presented in Figure 6.1a.

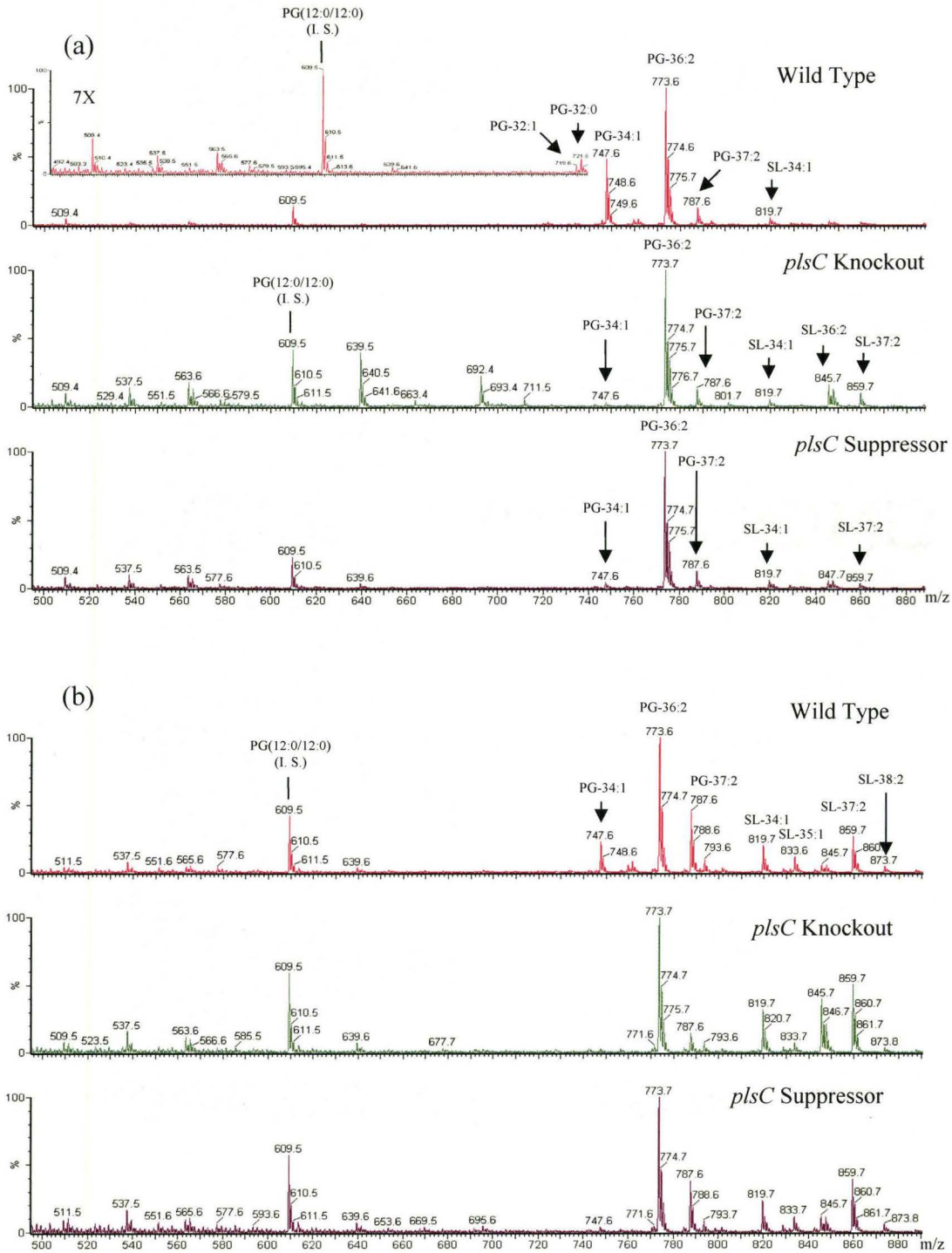


Figure 6.1. Negative ion electrospray mass spectra in the full scan mode obtained by infusion of crude lipid extracts of *S. meliloti* under (a) normal growth conditions (2 mM P_i) and (b) P_i-starvation (0 mM P_i).

The mass spectrum was dominated by $[M-H]^-$ ions of PGs with fatty acid compositions corresponding to 34:1, 36:2 and 37:2; minor PGs with fatty compositions of 32:1, 32:0, 35:2, 35:1 and 38:2 were also observed [221]. Minor ions that corresponded to $[M-H]^-$ ions formed by SLs are also normally detected in negative electrospray ionization. The negative electrospray ionization spectra of *plsC* knockout mutants revealed two major differences compared to wild type: (i) ion at m/z 747.5 corresponding to PG-34:1 decreased significantly; (ii) a new series of ions were detected in the lower mass region (m/z 500-700 region). In the mass spectrum of *plsC* suppressor mutant, PG-34:1 presented decreased levels as observed in *plsC* knockout mutant.

The most dramatic changes were observed for PG-34:2 and PG-34:1 lipids which decreased 7-fold and 25-fold respectively, in *plsC* knockout mutants under normal growth conditions compared to wild type (Figure 6.2a-b). The levels of other lipids such as PG-36:2 and PG-37:2 at m/z 773.7 and 787.6, respectively, were unchanged in *plsC* deficient mutants compared to wild type. The changes observed for *plsC* knockout mutants in the m/z 500-700 region were particularly notable for ions at m/z 639.6, 663.4, 692.4 and 711.5 which increased 13-fold, 8-fold, 35-fold and 17-fold respectively, compared to wild type (Figure 6.3b).

Under P_i-starvation conditions, the absence of PG-34:1 was the major difference observed in the lipid profiles of both *plsC* knockout mutants and *plsC* suppressor mutants in comparison to wild type (Figure 6.1b). The unknown ions observed in the m/z 500-700 region in the mass spectra of *plsC* knockout mutants under normal conditions were of low abundance and didn't show significant changes in *plsC* knockout mutants relative to wild type or *plsC* suppressor (Figure 6.3a-b). SLs increased in wild type and *plsC* mutants under P_i-starvation compared to cultures grown under normal conditions; this was not unexpected since non-phosphorus containing lipids are known to increase in *S. meliloti* under P_i-limiting conditions [51].

CID experiments confirmed that lipid at m/z 747.6 corresponded to PG-34:1 in wild type. $[M-H]^-$ ions of PGs upon CID afford MS/MS spectra that contain a weak ion at m/z 153 corresponding to polar head group $[HO_3POCH_2C(OH)CH_2]^-$ and abundant carboxylate ions originated by the losses of the fatty acyl chains [14, 97]. The product ion spectrum of lipid at m/z 747.6 provided a fragmentation pattern characteristic of PG-34:1 indicated by the presence of the polar head group ion at m/z 153 and $[RCOO]^-$ ions at m/z 255.2 and 281.1 for hexadecanoic acid (16:0) and *cis*-11-octadecenoic acid (18:1(11)), respectively (Figure 6.4). Moreover, the relative positions of the fatty acyl groups was determined since $[RCOO]^-$ fragment ions derived from the loss of fatty acids in the *sn*-2 position are more intense than losses from the *sn*-1 position [97]. Accordingly, CID spectra of PG-34:1 using three collision energies clearly demonstrated that PG-34:1 contained 18:1 fatty acid in the *sn*-1 position while 16:0 was located in *sn*-2 position.

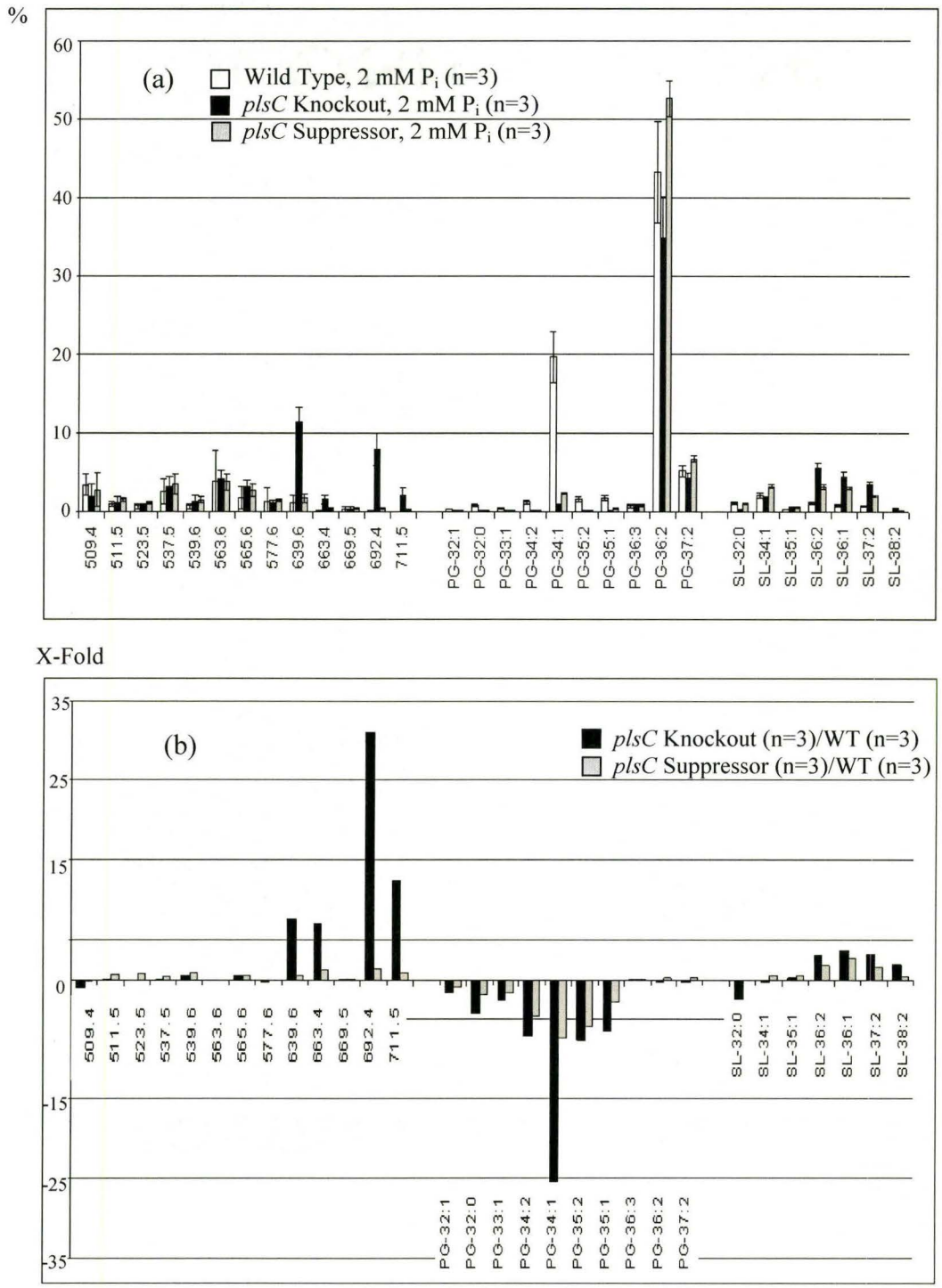


Figure 6.2. (a) Relative percentage composition of compounds detected in negative electrospray ionization in *S. meliloti* under normal growth conditions (2 mM P_i) and (b) fold change of these compounds in *plsC* knockout and *plsC* suppressor mutants relative to wild type.

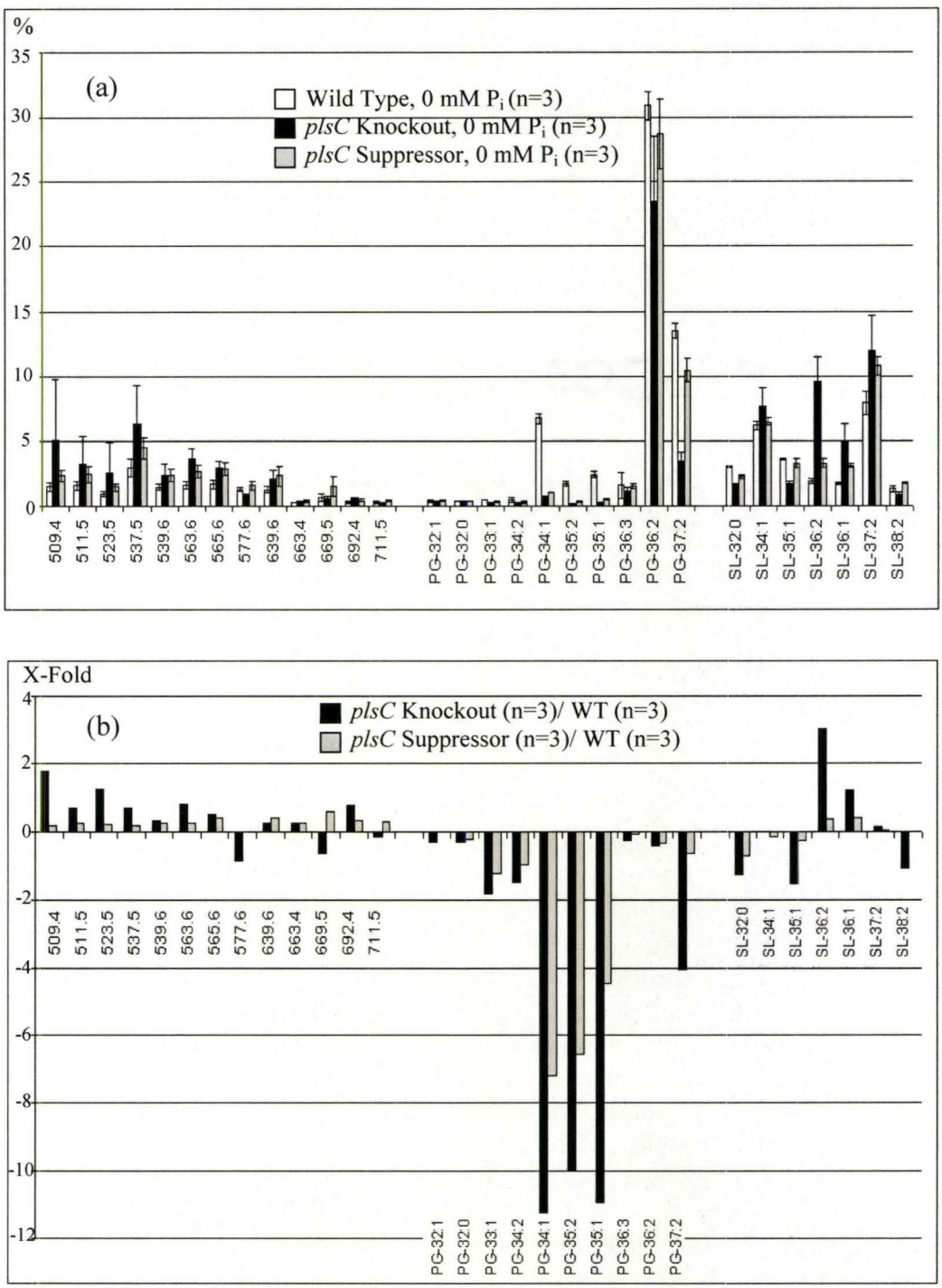


Figure 6.3. (a) Relative percentage composition of compounds detected in negative electrospray ionization in crude lipid extracts of *S. meliloti* under P_i-starvation conditions (0 mM P_i). (b) Fold change of these compounds in *plsC* knockout and *plsC* suppressor relative to wild type.

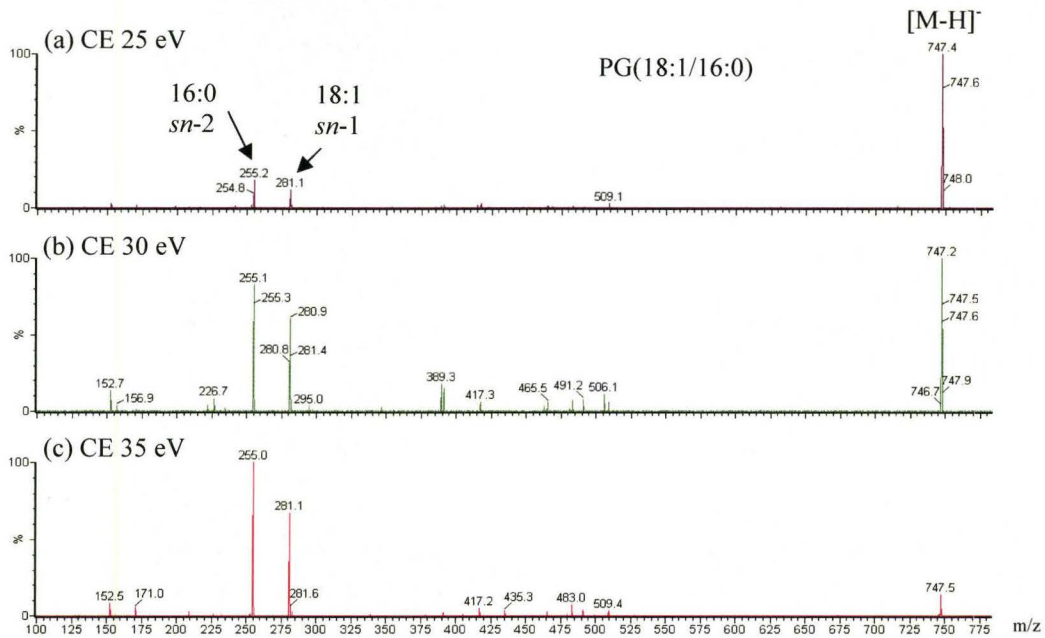


Figure 6.4. Product ion spectra at three collision energies of PG-34:1 in crude lipid extracts of *S. meliloti* wild type under normal growth conditions.

Conversely, the MS/MS spectra of weak ions at m/z 747.6 in cultures of *plsC* knockout mutant showed that 16:0 fatty acid was located at the *sn*-1 position and 18:1 at the *sn*-2 position (PG(16:0/18:1)). MS/MS spectra of an authentic standard of PG(16:0/18:1) confirmed these observations (Data not shown). This clearly demonstrated that putative PlsC in *S. meliloti* did not participate in the transfer of fatty acyl residues to the *sn*-2 position in all PG lipids. MS/MS experiments showed that PG-36:2 and PG-37:2 lipids corresponded to fatty acid combinations of 18:1/18:1 and 19:0cyclo/18:1, respectively; while PG-34:2 and PG-34:1 corresponded to 18:1/16:1 and 18:1/16:0, respectively [216]. Thus, only PGs that contained 16 fatty acids in the *sn*-2 position decreased in PlsC-deficient mutants. Under P_i-starvation conditions, PG-34:1, PG-35:2 and PG-35:1 decreased 11-fold, 10-fold, 11-fold respectively, in *plsC* knockout mutants compared to wild type (Figure 6.3b). CID experiments demonstrated that PG-35:2 and PG-35:1 had fatty acid compositions of 18:1/17:0cyclo and 19:0cyclo/16:0, respectively [221]. Note that these lipids contained fatty acids with sixteen carbons (16:0 and 16:1(9)) in the *sn*-2 position or the cyclopropane-containing fatty acid synthesized from 16:1(9).

In *E. coli*, 16:0 is located exclusively in the *sn*-1 position in phospholipids [134]; this is clearly not the case in *S. meliloti* since PG-34:1 contained primarly 16:0 in the *sn*-2 position. Moreover in PlsC-deficient mutants of *E. coli*, the synthesis of normal phospholipids (PGs, PEs, CLs) decreased significantly while

1-acyl-glycerol-3-phosphate lipids (LPAs) increased up to 30% [134]. On the other hand, PlsC-deficient mutants of other bacteria showed accumulation of monoacylglycerol and fatty acids [136]. In PlsC-deficient mutants of *S. meliloti*, a series of unknown ions were detected in the low m/z region; particularly increased levels were observed for ions at m/z 639.6, 663.4, 692.4 and 711.5. However, these unidentified compounds did not correspond to $[M-H]^-$ ions of LPAs, LPGs or fatty acids. The MS/MS spectra of $[M-H]^-$ ions of LPAs or LPGs produce characteristic fragment ions in negative electrospray ionization [98, 236]; these fragment ions were not produced by ions at m/z 639.6, 663.4, 692.4 and 711.5 upon CID. An increase of 1-octadecenoyl-glycerol-3-phosphate (PA-18:1) or 1-octadecenoyl-lysophosphatidylglycerol (LPG-18:1) was expected in *plsC* knockout mutants since PG-34:1 decreased significantly. Nevertheless, an increase of $[M-H]^-$ ions of LPG-18:1 (m/z 509.3) or LPA-18:1 (m/z 435.3) was not observed.

Fatty Acid Distributions in Phosphatidylglycerols and Sulfoquinovosyldiacylglycerols

A total of nine PGs with fatty acid compositions of 32:1, 32:0, 34:2, 34:1, 35:2, 35:1, 36:2, 37:2 and 38:2 were detected in *S. meliloti* wild type using precursor ion scans of m/z 153 (Figure 6.5a and Table 6.1a). Similarly nine SLs were present with fatty acid compositions similar to those observed in PGs (Figure 6.5b and Table 6.1b). Under P_i-starvation, cyclopropane-containing species (35:2, 35:1, 37:2, 38:2) of PGs and SLs increased significantly in the wild type with a concomitant decrease of lipids that contained their biochemical precursors [216].

The profiles of *plsC* knockout mutants and *plsC* suppressor showed a 25% decrease in PG-34:1 as discussed above. PG-32:0, PG-34:2, PG-35:2 and PG-35:1 also decreased significantly in *plsC* knockout mutants and *plsC* suppressor under normal growth conditions (Table 6.1a). In *plsC* suppressors a slight increase of PG-34:1 lipid was observed relative to *plsC* knockout mutants; PG-34:1 lipid was still significantly lower (3.7%) than the normal levels observed in wild type (27%) for this lipid. The profiles of SLs in *plsC* knockout mutants and *plsC* suppressors showed the same pattern as PGs where SL-32:1, SL-32:0, SL-34:2, SL-34:1, SL-35:2 and SL-35:1 decreased significantly (Table 6.1b). However, in *plsC* suppressor mutants SLs that contained 16:0 and 16:1 fatty acids recovered partially reaching an intermediate state between *plsC* knockout mutants and wild type; this phenomenon was not observed for PGs in *plsC* knockout mutants. For example in *plsC* suppressor mutant, SL-34:1 was restored 74% relative to its normal percentage composition in wild type. In contrast, PG-34:1 only recovered 14% relative to its normal level in wild type.

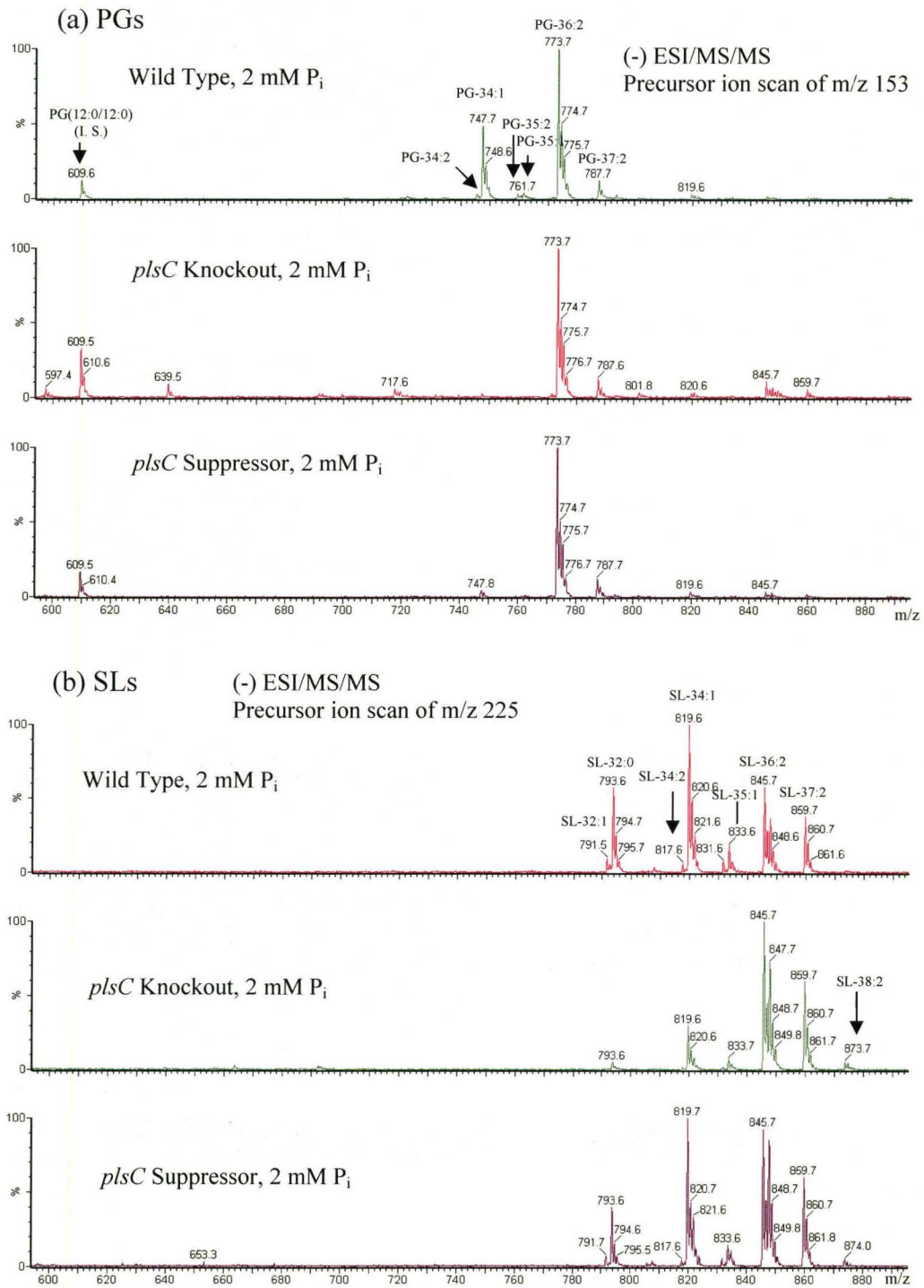


Figure 6.5. Lipid profiles of (a) phosphatidylglycerols and (b) sulfoquinovosyldiacylglycerols in *S. meliloti* under normal growth conditions (2 mM P_i).

Table 6.1. Relative percentage compositions of (a) phosphatidylglycerols and (b) sulfoquinovosyldiacylglycerols in *S. meliloti* wild type and *plsC* mutants under normal growth conditions (2 mM P_i) and under P_i-starvation (0 mM P_i). (*) Indicates that the assignment of fatty acids to *sn*-1 and *sn*-2 positions was ambiguous.

(a)

Lipids	Fatty Acids in Lipids *sn*-1 / *sn*-2	Relative Percentage Composition of Phosphatidylglycerols, Mean ± SD (n=3)					
		2 mM P_i			0 mM P_i		
		WT	*plsC* Knockout	*plsC* Supp.	WT	*plsC* Knockout	*plsC* Supp.
32:1	16:1-16:0 *	0.4 ± 0.1	2.3 ± 1.8	N.D.	N.D.	N.D.	N.D.
32:0	16:0/16:0	1.4 ± 0.4	0.7 ± 0.4 [1]	N.D. [2]	N.D. [a]	N.D. [b]	N.D.
34:2	18:1/16:1	1.8 ± 0.1	N.D. [1]	N.D. [1]	0.7 ± 0.1 [a]	N.D. [3]	N.D. [3]
34:1	18:1/16:0 16:0/18:1	26.6 ± 1.1	1.7 ± 0.1 [1]	3.7 ± 0.2 [2]	11.9 ± 0.8 [a]	2.1 ± 0.3 [3]	2.2 ± 0.4 [3]
35:2	18:1/17:0cyclo 16:1-19:0cyclo *	2.2 ± 0.5	N.D [1]	N.D. [1]	3.5 ± 1.2	N.D. [3]	N.D. [3]
35:1	19:0cyclo/16:0	2.6 ± 0.3	N.D [1]	N.D [1]	4.3 ± 0.5 [a]	N.D. [3]	N.D. [3]
36:2	18:1/18:1	57.3 ± 1.0	82.6 ± 1.7 [1]	85.4 ± 0.8 [1]	54.0 ± 1.5	84.2 ± 0.6 [3]	70.5 ± 0.6 [4, c]
37:2	19:0cyclo/18:1	7.3 ± 0.4	9.9 ± 0.5 [1]	10.0 ± 0.5 [1]	23.8 ± 1.0 [a]	11.7 ± 0.4 [3]	25.5 ± 0.9 [c]
38:2	19:0cyclo/19:0cyclo	0.3 ± 0.1	2.8 ± 0.3 [1]	0.9 ± 0.1	1.7 ± 0.3 [a]	2.0 ± 0.4 [b]	1.7 ± 0.2 [c]

(b)

Lipids	Fatty Acids in Lipids *sn*-1 / *sn*-2	Relative Percentage Composition of Sulfoquinovosyldiacylglycerols, Mean ± SD (n=3)					
		2 mM P_i			0 mM P_i		
		WT	*plsC* Knockout	*plsC* Supp.	WT	*plsC* Knockout	*plsC* Supp.
32:1	16:1-16:0 *	3.0 ± 0.7	N.D. [1]	1.3 ± 0.4 [2]	2.7 ± 0.3	0.9 ± 0.1 [3]	1.3 ± 0.1 [3]
32:0	16:0/16:0	17.2 ± 0.4	1.6 ± 0.2 [1]	8.0 ± 0.8 [2]	10.0 ± 1.0 [a]	3.5 ± 0.4 [3, b]	6.3 ± 0.3 [4, c]
34:2	18:1-16:1 *	1.4 ± 0.2	0.4 ± 0.1 [1]	0.8 ± 0.1 [1]	0.7 ± 0.1 [a]	1.1 ± 0.1 [b]	1.0 ± 0.3
34:1	18:1-16:0 *	31.9 ± 1.6	10.1 ± 0.6 [1]	23.7 ± 0.5 [2]	23.1 ± 1.9 [a]	19.1 ± 0.6 [3, b]	21.2 ± 0.7
35:2	18:1-17:0cyclo * 16:1-19:0cyclo *	1.5 ± 0.3	0.5 ± 0.1 [1]	0.8 ± 0.1 [1]	2.4 ± 0.1 [a]	1.3 ± 0.1 [3, b]	1.7 ± 0.2 [3, c]
35:1	19:0cyclo-16:0 *	5.4 ± 0.3	2.3 ± 0.1 [1]	4.0 ± 0.2 [2]	13.4 ± 0.2 [a]	4.3 ± 0.1 [3, b]	10.6 ± 0.3 [4, c]
36:2	18:1/18:1	17.8 ± 0.6	34.4 ± 1.3 [1]	22.4 ± 0.7 [2]	6.9 ± 0.2 [a]	24.2 ± 0.7 [3, b]	9.5 ± 1.0 [4, c]
36:1	18:1-18:0 *	10.8 ± 0.6	26.8 ± 0.6 [1]	22.7 ± 0.4 [2]	5.8 ± 0.3 [a]	13.1 ± 1.8 [3, b]	9.4 ± 0.7 [4, c]
37:2	19:0cyclo-18:1 *	10.5 ± 0.7	21.5 ± 0.4 [1]	15.2 ± 0.1 [2]	30.8 ± 2.3 [a]	30.8 ± 1.4 [b]	34.2 ± 1.0 [4, c]
38:2	19:0cyclo/19:0cyclo	0.5 ± 0.1	2.3 ± 0.2 [1]	1.2 ± 0.2	4.3 ± 0.9 [a]	1.9 ± 0.1 [3]	4.7 ± 0.1 [c]

[1] *plsC* knockout mutant and *plsC* suppressor are significantly different to wild type but not different from each other or [1, 2] *plsC* knockout mutant and *plsC* suppressor are significantly different to wild type and different from each other under normal growth conditions ($P \leq 0.05$).
[3] *plsC* knockout mutant and *plsC* suppressor are significantly different to wild type but not different from each other or [3, 4] *plsC* knockout mutant, *plsC* suppressor are significantly different to wild type and different from each other under P_i-starvation conditions ($P \leq 0.05$).
[a] wild type, [b] *plsC* knockout, [c] *plsC* suppressor under P_i-starvation are significantly different to wild type, *plsC* knockout and *plsC* suppressor under normal growth conditions, respectively ($P \leq 0.05$).

In general, PGs and SLs that contained fatty acids 16:0, 16:1 and 17:0cyclo decreased significantly in *plsC* knockout mutants and *plsC* suppressors compared to the wild type. In summary, we can make the following conclusions: (i) PG-34:1 in *S. meliloti* corresponded to a mixture of two lipids 18:1/16:0 and 16:0/18:1, were the first isomer was the major component; (ii) PlsC in *S. meliloti* is required and specific for the synthesis of PGs that contained 16 fatty acids in the *sn*-2 position. (iii) SLs and PGs have a common biosynthetic origin most likely involving phosphatidic acid since lipids that contained 16 fatty acids decreased significantly in both lipid classes. (iv) Other mechanisms (pathways) exist for the synthesis of SLs which do not involve phosphatidic acid; since SLs that contained 16 fatty acids were partially restored back to normal levels in *plsC* suppressor mutants.

Total Fatty Acid Composition of PlsC-deficient Mutants

Total fatty acid composition data of *plsC* knockout mutants also demonstrated that fatty acids with sixteen carbon atoms (16:0, 16:1(9), 17:0cyclo) decreased 83% and 55% under normal growth conditions and with P_i-limitation, respectively (Table 6.2).

Table 6.2. Relative percentage compositions of fatty acids in *S. meliloti* wild type and *plsC* knockout mutant under normal growth conditions (2 mM P_i) and under P_i-starvation (0 mM P_i).

Fatty Acids	Relative Percentage Composition of *S. meliloti* (%), Mean ± SD (n=3)			
	Wild Type		*plsC* Knockout	
	2 mM P_i	0 mM P_i	2 mM P_i	0 mM P_i
14:0	0.23 ± 0.02	0.25 ± 0.03	0.2 ± 0.1	0.30 ± 0.05
16:0	13.0 ± 0.5	11.9 ± 0.2	2.8 ± 0.2 [a]	6.3 ± 0.5 [2, b]
17:0	0.23 ± 0.03	0.19 ± 0.04	0.19 ± 0.01	0.16 ± 0.01
18:0	3.6 ± 0.3	1.8 ± 0.1 [1]	7.8 ± 0.1 [a]	4.9 ± 0.4 [2, b]
16:1(9)	1.2 ± 0.3	1.4 ± 0.2	N.D. [a]	0.6 ± 0.1 [2, b]
18:1(11)	54.4 ± 1.7	46.9 ± 1.2 [1]	57.3 ± 1.1	53.0 ± 0.9 [2, b]
17:0cyclo(9,10)	1.60 ± 0.03	2.1 ± 0.2	N.D. [a]	N.D. [b]
19:0cyclo(11,12)	25.8 ± 1.5	35.5 ± 1.2 [1]	31.8 ± 1.1 [a]	34.8 ± 0.6 [2]

[1] Wild type under stressed conditions (0 mM P_i) is significantly different ($P \leq 0.05$) to wild type under normal growth conditions (2 mM P_i).

[2] *plsC* mutant under stressed conditions (0 mM P_i) is significantly different ($P \leq 0.05$) to *plsC* under normal growth conditions (2 mM P_i).

[a] Under normal growth conditions (2 mM P_i), *plsC* mutant is significantly different to wild type ($P \leq 0.05$).

[b] Under stressed conditions (0 mM P_i), *plsC* mutant is significantly different to wild type ($P \leq 0.05$).

N.D.: Not detected.

The levels of *cis*-11-octadecenoic acid remained unchanged in *plsC* mutants under both growth conditions while 18:0 increased significantly. A similar pattern was observed in *Pseudomonas fluorescens*, where fatty acids 16:0 and 16:1 decreased almost 50% in mutants deficient in a gene that showed homology to *plsC* of *E. coli* [139]. Moreover, lysophosphatidic acid acyltransferase (PlsC) in *Synechocystis* showed to be specific towards 16:0-ACP compared with 18:1-ACP or 18:0-ACP [142]. Our data suggests this is also the case for putative PlsC in *S. meliloti* which we will identify as $PlsC_{C16}$.

Lipid Profiles of Wild Type, *plsC* Mutant and *plsC* Suppresor in Positive Electrospray Ionization

Zwitterionic lipids (PCs, DMPEs, MMPEs, PEs, OLs, TMHSs) were detected in positive electrospray ionization mode as $[M+Li]^+$ adducts following addition of lithium salts to lipid extracts. A typical spectrum of crude lipid extracts of *S. meliloti* wild type in the presence of lithium salts is shown in Figure 6.6a. A mixture of $[M+Li]^+$ ions of isobaric species of PCs, PEs, MMPEs and DMPEs with various fatty acid combinations is normally present [221]. Under normal growth conditions, the full scan mode mass spectra of *plsC* knockout and *plsC* suppressor showed minor differences when compared to wild type (Figure 6.6a).

Under P_i-starvation conditions the profiles of *plsC* knockout mutants showed striking differences in comparison to wild type (Figure 6.6b). The profiles of *plsC* suppressor were highly similar to wild type. In wild type under P_i-limiting conditions, non-phosphorus containing lipids such as TMHSs and OLs normally increase and constitute more than 50% of total lipids [51]. We observed an increase of these non-phosphorus containing lipids in wild type as expected, but this phenomenon was highly marked in *plsC* knockout mutants. This is demonstrated by the presence in the full scan mode spectrum of two intense ions at m/z 764.7 and 770.7 that corresponded to $[M+H]^+$ and $[M+Li]^+$ adducts of TMHS-36:2. Ornithine lipids increased, particularly abundant OLs with fatty acid compositions of 36:1 and 37:1 (Figure 6.6b). Normal phospholipids such as PCs and PEs and their intermediates (DMPEs and MMPEs) were still present with P_i-starvation but were minor components. Under P_i-limiting conditions, anionic phospholipids such as PGs decreased slightly while SLs increased in *plsC* knockout mutant (Figure 6.1b); however the decrease of PGs was not so dramatic as observed for PCs and PEs.

Fatty Acid Distributions in Phosphatidylcholines and Phosphatidylethanolamines

Neutral loss scans of the lithiated polar head groups of $[M+Li]^+$ adducts of PEs, MMPEs, DMPEs and PCs provided with fatty acid distributions within each lipid class [221]. A typical profile of PCs and PEs in *S. meliloti* wild type includes lipids with fatty acid compositions of 34:2, 34:1, 35:2, 35:1, 36:2, 37:2 and 38:2 (Table 6.3a-b). Fatty acid distributions in MMPEs and DMPEs are very similar to PCs and PEs [221].

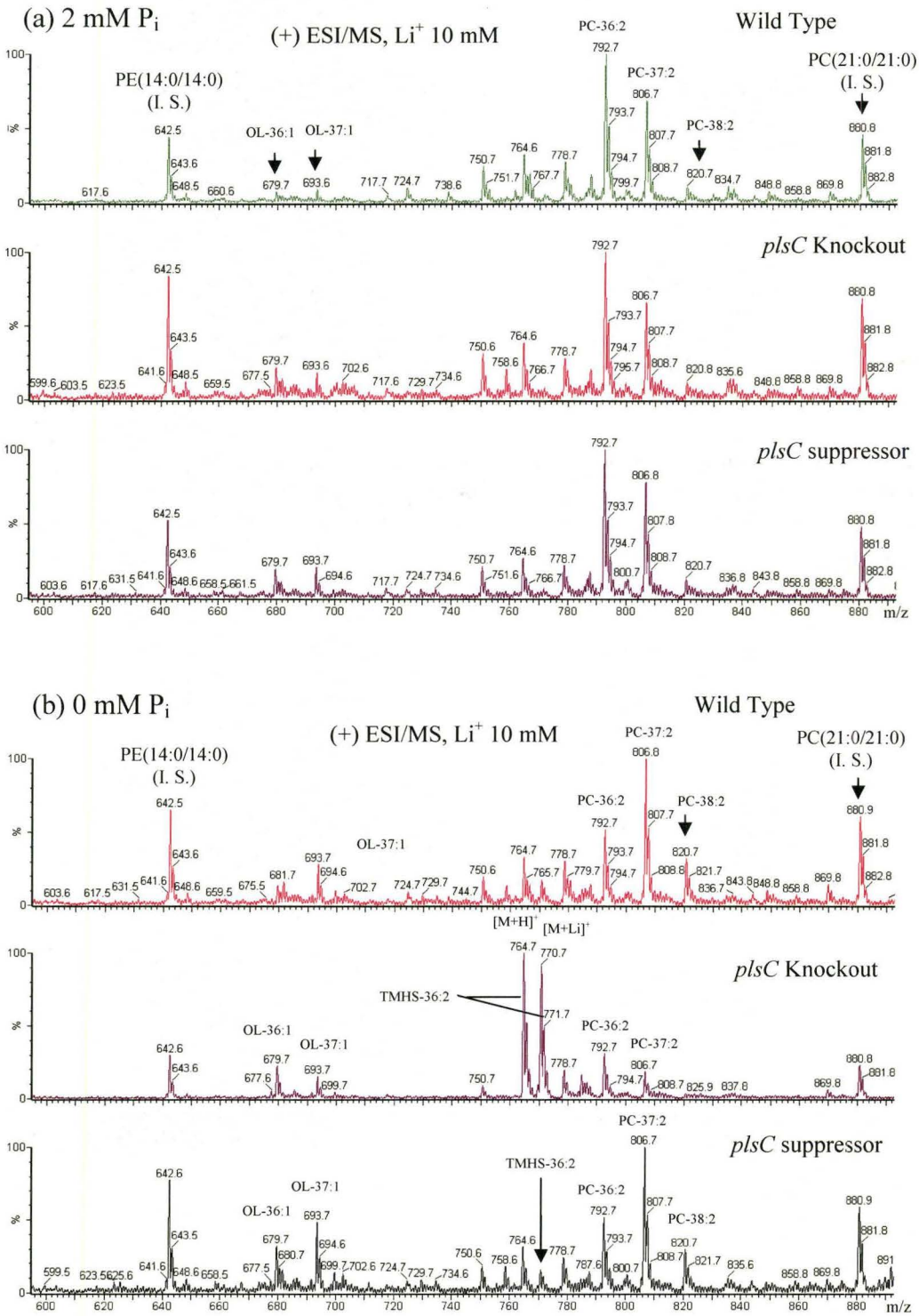


Figure 6.6. Positive electrospray ionization spectra in the full scan mode of crude lipid extracts of *S. meliloti* grown under (a) normal growth conditions and (b) P_i-starvation.

Table 6.3. Relative percentage composition of (a) phosphatidylcholines and (b) phosphatidylethanolamines in *S. meliloti* wild type and *plsC* mutants under normal growth conditions (2 mM P_i) and under P_i-starvation (0 mM P_i). (*) Indicates that the assignment of fatty acids to *sn*-1 and *sn*-2 positions was ambiguous.

(a)

Lipids	Fatty Acids in Lipids *sn*-1 / *sn*-2	Relative Percentage Composition of Phosphatidylcholines, Mean ± SD (n=3)					
		2 mM P_i			0 mM P_i		
		WT	*plsC* Knockout	*plsC* Supp.	WT	*plsC* Knockout	*plsC* Supp.
34:2	18:1/16:1	0.9 ± 0.3	N.D. [1]	N.D. [1]	0.3 ± 0.1 [a]	N.D.	N.D.
34:1	18:1/16:0	7.0 ± 0.4	N.D. [1]	1.0 ± 0.1 [1]	3.4 ± 0.8 [a]	N.D. [1]	0.7 ± 0.3 [1]
35:2	18:1/17:0cyclo 16:1-19:0cyclo *	2.1 ± 0.1	N.D. [1]	N.D. [1]	2.1 ± 0.2	N.D. [1]	N.D. [1]
35:1	19:0cyclo/16:0	2.9 ± 0.1	N.D. [1]	0.4 ± 0.1 [1]	6.3 ± 1.5 [a]	N.D. [1]	0.8 ± 0.3 [1]
36:2	18:1/18:1	47.2 ± 1.1	56.7 ± 0.9 [1]	50.3 ± 0.8	21.7 ± 1.5 [a]	55.7 ± 2.4 [1]	24.8 ± 1.2 [c]
37:2	19:0cyclo/18:1	35.5 ± 0.4	38.2 ± 0.4	42.7 ± 1.0 [2]	49.6 ± 2.4 [a]	38.9 ± 2.3 [1]	56.9 ± 1.0 [2, c]
38:2	19:0cyclo/19:0cyclo	4.4 ± 0.5	5.1 ± 0.6	5.6 ± 0.5	16.8 ± 1.1 [a]	5.4 ± 1.3 [1]	16.9 ± 1.1 [c]

(b)

Lipids	Fatty Acids in Lipids *sn*-1 / *sn*-2	Relative Percentage Composition of Phosphatidylethanolamines, Mean ± SD (n=3)					
		2 mM P_i			0 mM P_i		
		WT	*plsC* Knockout	*plsC* Supp.	WT	*plsC* Knockout	*plsC* Supp.
34:2	18:1/16:1	2.2 ± 1.0	N.D. [1]	N.D. [1]	1.5 ± 0.6	N.D. [3]	N.D. [3]
34:1	18:1/16:0	18.2 ± 2.8	N.D. [1]	2.3 ± 1.0 [1]	7.9 ± 1.4 [a]	N.D. [3]	N.D. [3, c]
35:2	18:1/17:0cyclo 16:1- 19:0cyclo *	1.8 ± 1.3	N.D. [1]	N.D. [1]	3.2 ± 0.6	N.D. [3]	N.D. [3]
35:1	19:0cyclo/16:0	3.6 ± 2.1	N.D. [1]	N.D. [1]	3.1 ± 0.1	N.D. [3]	N.D. [3]
36:2	18:1/18:1	61.0 ± 1.0	78.7 ± 3.5 [1]	79.2 ± 3.5 [1]	52.2 ± 2.8 [a]	87.5 ± 1.3 [3, b]	67.8 ± 3.4 [4, c]
37:2	19:0cyclo/18:1	12.7 ± 0.7	19.3 ± 2.9	16.7 ± 3.1	30.3 ± 2.5 [a]	12.5 ± 1.3 [3]	26.1 ± 4.6 [c]
38:2	19:0cyclo/19:0cyclo	0.5 ± 0.2	1.9 ± 0.6	1.8 ± 0.1	1.8 ± 0.4	N.D. [3, b]	6.1 ± 1.4 [4, c]

[1] *plsC* knockout mutant and *plsC* suppressor are significantly different to wild type but not different from each other or [1, 2] *plsC* knockout mutant and *plsC* suppressor are significantly different to wild type and different from each other under normal growth conditions ($P \leq 0.05$).
[3] *plsC* knockout mutant and *plsC* suppressor are significantly different to wild type but not different from each other or [3, 4] *plsC* knockout mutant, *plsC* suppressor are significantly different to wild type and different from each other under P_i-starvation conditions ($P \leq 0.05$).
[a] wild type, [b] *plsC* knockout, [c] *plsC* suppressor under P_i-starvation are significantly different to wild type, *plsC* knockout and *plsC* suppressor under normal growth conditions, respectively ($P \leq 0.05$). N.D. Not detected.

Under normal growth conditions, PCs and PEs with fatty acid compositions of 34:2, 34:1, 35:2 and 35:1 were not detected in *plsC* knockout mutants or *plsC* suppressors (Table 6.3a-b). The same pattern was observed for MMPEs and DMPEs in *plsC* mutants (Data not shown). MS/MS experiments determined that lipids with fatty acid compositions of 34:2 and 34:1 contained 16:0 and 16:1 fatty acids in the *sn*-2 position. The absence of PCs and PEs (also MMPEs, DMPEs) with fatty acid compositions of 34:2, 34:1, 35:2 and 35:1 in *plsC* deficient mutants was undetected in full scan mode spectra (Figure 6.6a).

Under P_i-starvation conditions, a significant increase of lipids that contained cyclopropane fatty acids (35:2, 35:1, 37:2, 38:2) was observed in wild type [216]. Interestingly, the profiles of *plsC* knockout mutants under normal growth conditions and P_i-starvation did not show differences; an increase in the cyclopropanation of lipids was not observed with P_i-starvation. On the other hand, a significant increase in the cyclopropanation of lipids (37:2 and 38:2 lipids) was observed in *plsC* suppressor mutants under P_i-starvation similarly as in wild type.

In summary, PCs and PEs that contained C16 fatty acids decreased significantly in *plsC* knockout mutants and *plsC* suppressor similar to PGs and SLs; these fatty acids were located in the *sn*-2 position in lipids. The lipid profiles of *plsC* suppressor were intermediate between the profiles of *plsC* knockout mutants and wild type but statistically different to both wild type and *plsC* knockout. These results demonstrate that PlsC in *S. meliloti* was specific for C16 fatty acids as shown by the fatty acid distributions in three phospholipid classes (PGs, PCs, PEs, MMPEs, DMPEs) and SLs.

Fatty Acid Distributions in Ornithine Lipids and Diacylglyceryl-N,N,N-trimethylhomoserines Lipids

OLs are a minor lipid class in *S. meliloti* under normal growth conditions but with P_i-limitation represent up to 6% of total lipids [51]. In contrast, TMHSs are not present in *S. meliloti* under normal growth conditions; these lipids are synthesized exclusively under P_i-limiting conditions representing almost 60% of total lipids [51]. $[M+H]^+$ ions formed by OLs under CID conditions in positive electrospray ionization produce a common fragment ion at m/z 115 which is typical of ornithine lipids [105, 106]. We have reported the use of precursor ion scans of m/z 115 as a simple strategy to obtain OLs profiles in crude lipid extracts of *S. meliloti* [221]. In the presence of lithium salts, complete profiles of TMHSs can be obtained from crude lipid extracts using neutral loss scans of 74 mass units [221]. These MS/MS transitions revealed fatty acid distributions for OLs and TMHSs in *S. meliloti* wild type and *plsC* mutants under normal growth conditions and P_i-starvation.

Precursor ion scans of m/z 115 revealed complete fatty acid distributions of ornithine lipids in *S. meliloti* under normal growth conditions (Figure 6.7). The most abundant OLs in *S. meliloti*, OL-36:1 (m/z 679.8) and OL-37:1 (m/z 693.8), are composed of 3-hydroxyoctadecanoic acid with the hydroxyl function esterified to octadecenoic acid or methyleneoctadecanoic acid, respectively [51,

106]. In addition, minor OLs with fatty acid compositions of 34:1, 35:1, 36:2 and 37:2 were detected. Unfortunately, fatty acid composition for minor OLs could not be determined; these compounds were only present in precursor ion scan spectra of m/z 115 and were not observed in full scan mode spectra. However, reasonable fatty acid combinations for these minor lipids are proposed on Table 6.4a based on total fatty acid composition of *S. meliloti* [63, 64]. Under P_i-starvation conditions, OLs that contained cyclopropane fatty acids (37:1) increased significantly in the wild type (Table 6.4a); this effect has been observed with P_i-starvation in all lipid classes in *S. meliloti* [216]. Under P_i-starvation conditions, the profiles of TMHSs resembled that of normal phospholipids and included lipids with fatty acid compositions of 34:1, 35:2, 35:1, 36:2, 37:2 and 38:2 (Table 6.4b).

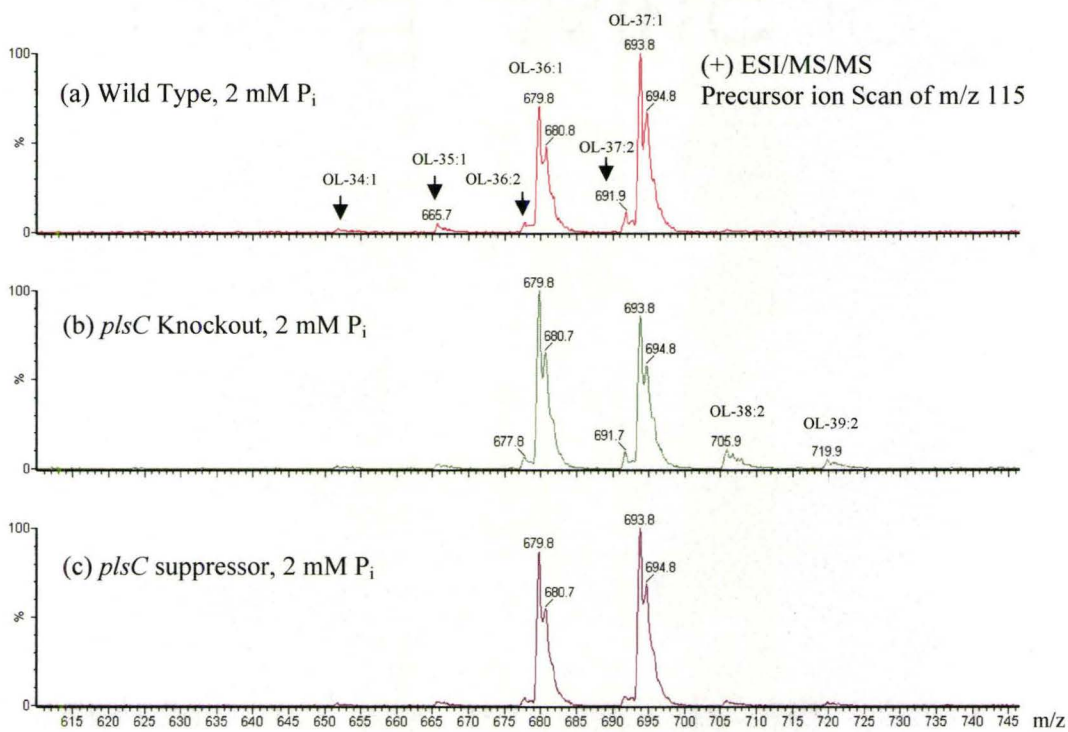


Figure 6.7. Ornithine lipid profiles of wild type, *plsC* knockout mutant and *plsC* suppressor under normal growth conditions (2 mM P_i).

Table 6.4. Relative percentage composition of (a) ornithines and (b) diacylglyceryl-(*N,N,N*-trimethyl)-homoserines in *S. meliloti* wild type and *plsC* mutants under normal growth conditions (2 mM P_i) and under P_i-starvation (0 mM P_i). (*) Indicates that the assignment of fatty acids to *sn*-1 and *sn*-2 positions was ambiguous.

(a)

Lipids	Fatty Acids in Lipids	Relative Percentage Composition of Ornithines, Mean ± SD (n=3)					
		2 mM P_i			0 mM P_i		
		WT	*plsC* Knockout	*plsC* Supp.	WT	*plsC* Knockout	*plsC* Supp.
34:1	3OH-16:0/18:1 3OH-18:0/16:1	1.2 ± 0.2	0.5 ± 0.1 [1]	0.5 ± 0.1 [1]	1.0 ± 0.1	2.1 ± 0.2 [3, b]	1.2 ± 0.1 [c]
35:1	3OH-16:0/19:0cyclo 3OH-18:0/17:0cyclo	3.1 ± 0.8	1.0 ± 0.1 [1]	1.4 ± 0.1 [1]	3.5 ± 0.2	1.7 ± 0.1 [3]	2.9 ± 1.0 [c]
36:2	3OH-16:0/22:2 3OH-18:0/18:2	3.0 ± 0.8	3.6 ± 0.8	1.9 ± 0.3	2.9 ± 0.4	7.4 ± 0.7 [3, b]	3.1 ± 0.2
36:1	3OH-18:0/18:1	37.1 ± 2.2	44.3 ± 0.6 [1]	42.5 ± 1.5 [1]	23.4 ± 0.4 [a]	48.0 ± 1.1 [3, b]	31.4 ± 1.4 [4, c]
37:2	3OH-18:1/19:0cyclo	5.3 ± 0.3	3.8 ± 0.4 [1]	2.6 ± 0.3 [2]	8.3 ± 0.2 [a]	5.4 ± 0.5 [3, b]	5.7 ± 0.7 [3, c]
37:1	3OH-18:0/19:0cyclo	49.8 ± 1.3	40.0 ± 0.9 [1]	48.7 ± 2.1	60.6 ± 0.2 [a]	32.0 ± 1.4 [3, b]	53.8 ± 1.9 [4, c]
38:2	3OH-18:0/20:2	0.6 ± 0.2	4.4 ± 0.4 [1]	1.6 ± 0.7 [2]	0.4 ± 0.1	2.3 ± 0.1 [3, b]	1.2 ± 0.2
39:2	3OH-18:0/21:2	N.D.	2.2 ± 0.2 [1]	0.8 ± 0.2 [2]	N.D.	1.1 ± 0.1 [3, b]	0.9 ± 0.1 [3]

(b)

Lipids	Fatty Acids in Lipids *sn*-1 / *sn*-2	Relative Percentage Composition of Trimethylhomoserines, Mean ± SD (n=3)					
		2 mM P_i			0 mM P_i		
		WT	*plsC* Knockout	*plsC* Supp.	WT	*plsC* Knockout	*plsC* Supp.
34:2	18:1-16:1 *	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
34:1	18:1-16:0 *	N.D.	N.D.	N.D.	10.6 ± 2.7	0.7 ± 0.1 [3]	2.1 ± 0.6 [3]
35:2	18:1-16:0 * 16:1-19:0cyclo *	N.D.	N.D.	N.D.	3.1 ± 1.7	0.2 ± 0.1	2.9 ± 1.8
35:1	19:0cyclo-16:0 *	N.D.	N.D.	N.D.	4.4 ± 1.3	0.3 ± 0.1 [3]	1.8 ± 1.0 [3]
36:2	18:1/18:1	N.D.	N.D.	N.D.	49.5 ± 2.8	82.9 ± 1.7 [3]	58.0 ± 4.3 [4]
37:2	19:0cyclo-18:1 *	N.D.	N.D.	N.D.	29.0 ± 3.6	14.3 ± 1.7 [3]	30.1 ± 6.9
38:2	19:0cyclo/19:0cyclo	N.D.	N.D.	N.D.	3.2 ± 1.7	1.6 ± 0.2	5.1 ± 2.9

[1] *plsC* knockout mutant and *plsC* suppressor are significantly different to wild type but not different from each other or [1, 2] *plsC* knockout mutant and *plsC* suppressor are significantly different to wild type and different from each other under normal growth conditions ($P \leq 0.05$).
[3] *plsC* knockout mutant and *plsC* suppressor are significantly different to wild type but not different from each other or [3, 4] *plsC* knockout mutant, *plsC* suppressor are significantly different to wild type and different from each other under P_i-starvation conditions ($P \leq 0.05$).
[a] wild type, [b] *plsC* knockout, [c] *plsC* suppressor under P_i-starvation are significantly different to wild type, *plsC* knockout and *plsC* suppressor under normal growth conditions, respectively ($P \leq 0.05$).

The fatty acid distributions of TMHSs in *plsC* knockout mutants showed the same pattern observed for normal phospholipids (PCs, PEs, MMPEs, DMPEs, PGs) where lipids that contained C16 fatty acids (34:1, 35:2, 35:1) decreased significantly (Table 6.4b). Therefore, the similarities in the profiles of TMHSs and phospholipids in PlsC-deficient mutants indicate a common biosynthetic origin. Biosynthesis of TMHSs in *S. meliloti* is speculated to occur by the ether formation between diacylglycerol and a homoserine residue [30]. Our data suggests that in *S. meliloti*, phosphatidic acid must be involved in the synthesis of both phospholipids and TMHSs. Phosphatidic acid, the precursor of phospholipids, under P_i-limiting conditions can be transformed to diacylglycerol by phosphatidic acid phosphatases and employed for TMHSs biosynthesis [150].

The fatty acid distributions in OLs revealed interesting differences in *plsC* knockout mutants compared to wild type (Figure 6.7 and Table 6.4a). Under normal growth conditions, OLs with fatty acid compositions of 36:1, 38:2 and 39:2 increased significantly in *plsC* knockout while OL-34:1, OL-35:1, OL-37:2 and OL-37:1 decreased significantly. OL-34:1 and OL-35:1 decreased 42% and 32% in PlsC-deficient mutants, these lipids most likely contain C16 fatty acids (Table 6.4a). Major OLs in *S. meliloti* contain C18 fatty acids (OL-36:1, OL-37:2) while OLs that contain C16 fatty acids are minor species. If $PlsC_{C16}$ participates in the synthesis of OLs that contain C16 fatty acids, then reasonably changes in their profiles in PlsC-deficient mutants would be small. Our results suggest that $PlsC_{C16}$ might be involved in the biosynthesis of OLs that contain C16 fatty acids.

The biosynthesis of OLs in *S. meliloti* is a two step process; (i) reaction of the carboxyl group of a 3-hydroxy fatty acid with the α-amino acid group of ornithine forming an amide bond; (ii) transfer of a second fatty acyl group to the hydroxyl function of the 3-hydroxy fatty acid forming an ester bond [30]. The second reaction is catalyzed by an acyl transferase enzyme coded by the *olsA* gene [152]. Alignments of amino acid sequences of $PlsC_{C16}$ (SMc00714) and OlsA (SMc01116) in *S. meliloti* showed that these proteins contained motifs which are characteristic of fatty acyl transferases [245]. Moreover, in some bacteria OlsA is a bifunctional enzyme involved in the biosyntheses of both OLs and phospholipids, with similar activity to PlsC [153]. In *S. meliloti*, OlsA displays similar activity as PlsC but the differences between this two enzymes is not well understood [152].

We demonstrated that putative PlsC in *S. meliloti* coded for an acyl transferase specific for C16 fatty acids ($PlsC_{C16}$) in the *sn*-2 position. Furthermore, our data suggest that $PlsC_{C16}$ might also participate in OLs biosynthesis. Two additional proteins (SMc02490 and SMc02158) that contained amino acid motifs typical of acyl transferases were found in the genome of *S. meliloti* [242]. These proteins might be involved in the transference of C18 fatty acids to the *sn*-2 position in lipids. However, OlsA might also participate as an acyl transferase in the biosynthesis of phospholipids and ornithine lipids with preference towards C18 fatty acids (also $PlsC_{C18}$). Previous lipid analyses of OlsA-deficient mutants in *S. meliloti* did not reveal differences in total lipid

composition [152]. If lipids that contained C18 fatty acids decreased in OlsA-deficient mutants, this change would not have been detected since the methodology employed did not provide fatty acid distributions in lipid classes. To test our hypothesis, the fatty acid distributions in lipid classes in OlsA-deficient mutants should be investigated using our methodology.

Effect of PlsC Deficiency in Lipid Classes in S. meliloti under Normal Growth Conditions and P_i-starvation

PCA was used to identify patterns or trends in the profiles of each lipid class in wild type and *plsC* mutants grown under normal growth conditions and under P_i-starvation. First, PCA analysis was applied to the profiles of each lipid class individually to determine: (i) Differences in the profiles of lipid classes in *plsC* knockout mutants compared to wild type; (ii) Effect of P_i-starvation in *plsC* mutants for each lipid class; (iii) Differences between *plsC* knockout mutant and *plsC* suppressor in each lipid class. Second, PCA analysis was applied to the lipid profiles of all lipid classes in wild type, *plsC* knockout mutant and *plsC* suppressor.

Figure 6.8, summarizes the scores plots obtained from the profiles of six lipid classes (loadings plots, not shown) in wild type and *plsC* mutants for both growth conditions. The scores plot for TMHSs are not shown since these lipids were only produced under P_i-starvation conditions. The following observations can be derived from these PCA plots: First, the profiles of wild type in all lipid classes under P_i-starvation were different to normal growth conditions; this phenomenon was driven by an increase of cyclopropane containing lipids under P_i-starvation. Second, the lipid profiles of *plsC* knockout mutants were clearly different to wild type under normal growth conditions and P_i-starvation in all lipid classes. Third, the profiles of *plsC* suppressor under normal conditions were intermediate between wild type and *plsC* knockout mutants for SLs and OLs, whereas for other lipid classes *plsC* suppressor was similar to *plsC* knockout mutant. Fourth, the scores plots of PEs, MMPEs and DMPEs (not shown) were identical in all cases.

PCA analysis of fatty acid distributions in seven lipid classes in wild type and *plsC* mutants is represented in Figure 6.9. Three principal components were identified in the data set; PC1 which contributed to 91% of the variance in the data set while PC2 and PC2 components contributed to 6% and 2%, respectively. In the scores plot of PC1 versus PC2, 37:2 lipids clustered in the top quadrant relative to PC2 axis while 36:2 lipids clustered in the bottom quadrant (Figure 6.9a). These clusters of lipids with fatty acid compositions of 36:2 and 37:2 were associated to the effect of P_i-starvation demonstrated in the corresponding loadings plot (PC1 versus PC2) where samples grown under P_i-starvation grouped in the top quadrant relative to the PC2 axis and samples under normal growth conditions (except *plsC* knockout) grouped in the bottom portion (Figure 6.9b). Therefore, it can be concluded that PC2 represents an increase in the cyclopropanation of lipids with P_i-starvation which was independent of lipid

class; 36:2 lipids were transformed to 37:2 lipids through cyclopropanation. It should be noted that *plsC* knockout mutants under P_i-starvation conditions did not show an increase of cyclopropane containing lipids. In general, the trends observed were independent of lipid classes since clusters were related to fatty acid composition (colors) and not to lipid classes (shapes) however SL-34:1 was clearly different to 34:1 species in other lipid classes.

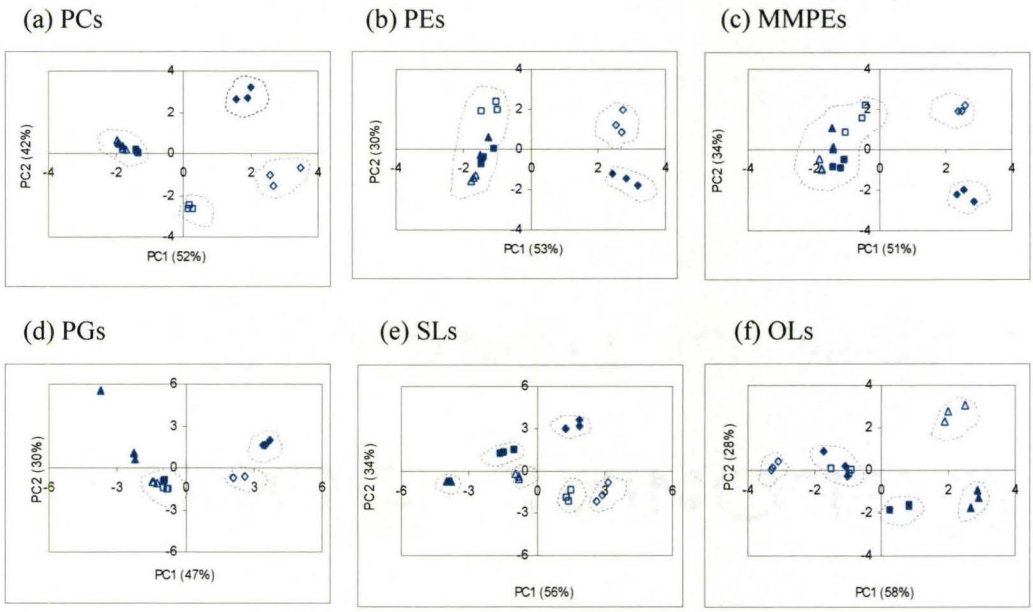


Figure 6.8. PCA scores plots of fatty acid distributions in (a) PCs, (b) PEs, (c) MMPEs, (d) PGs, (e) SLs and (f) OLs. Wild type, *plsC* knockout mutants and *plsC* suppressor mutants are represented by ♦, ▲ and ■, respectively. Filled figures represent normal growth conditions (2 mM P_i) while opened figures represent P_i-starvation conditions (0 mM P_i).

In the scores plot of PC2 versus PC3, three major clusters were observed for lipids with fatty acid compositions of 34:1, 36:2 and 37:2 (Figure 6.9c). The increase in lipid cyclopropanation with P_i-starvation along PC2 axis is observed by clusters of samples (wild type and *plsC* suppressor) grown under P_i-starvation located to the right of PC2 in the loadings plot (Figure 6.9d). Lipids with fatty acid compositions of 37:2, 38:2 and OL-37:1 contained the major cyclopropane fatty acid (19:0cyclo). The third cluster in the loadings plot corresponded to lipids that contained C16 fatty acids (mainly 34:1 lipids and SL-32:0), therefore PC3 is related to lipids that contained C16 fatty acids (Figure 6.9c). In the scores plots, wild type under normal growth conditions contained the highest percentage of

lipids that contained C16 fatty acids while *plsC* knockout mutants contained the lowest percentage of 34:1 lipids (Figure 6.9d).

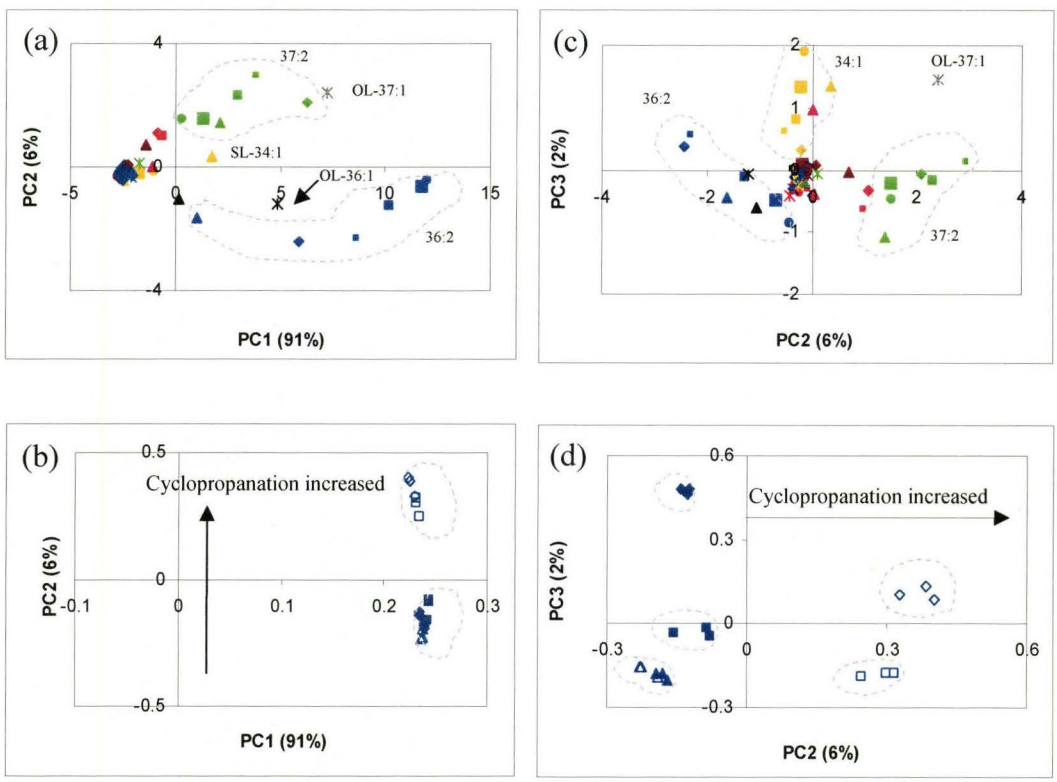


Figure 6.9. PCA of fatty acid distributions in lipid classes under normal conditions and under P_i-starvation. (a, c) In the scores plots, lipid classes are represented by shapes: ♦, PCs; ■, PEs; ■, MMPEs; ■, DMPEs; •, PGs; ▲, SLs; *, OLs. Fatty acid compositions corresponded to: ■, 32:1; ■, 32:0; ■, 34:2; ■, 34:1; ■, 35:2; ■, 35:1; ■, 36:2; ■, 36:1; ■, 37:2; ■, 37:1; ■, 38:2. (b, d) Loading plots: ♦, Wild type; ▲, *plsC* knockout; ■, *plsC* suppressor. Filled figures represent normal growth conditions (2 mM P_i) while open figures represent P_i-starvation conditions (0 mM P_i).

To summarize: (i) Under P_i-starvation, the cyclopropanation of lipids increased in wild type and *plsC* suppressor but not in *plsC* knockout mutants; (ii) Lipids that contained C16 fatty acids (mainly 34:1) decreased in *plsC* knockout mutants in all lipid classes; (iii) Lipid profiles of *plsC* suppressor mutants were intermediate between wild type and *plsC* knockout mutant; (iiii) however, this

recuperation was the most noticeable for SLs while minimum or not observed in other lipid classes.

Conclusions

A shotgun lipidomics strategy was used to study *plsC* deficient mutants in *S. meliloti* under normal growth conditions and under P_i-starvation. We demonstrated that protein SMc007714 (putative PlsC) in *S. meliloti* coded for a lysophosphatidic acyl transferase that showed specificity towards fatty acids with sixteen carbon atoms ($PlsC_{C16}$) in the *sn*-2 position of lipids. This was demonstrated by a 55-83% decrease of C16 fatty acids (16:0, 16:1, 17:0cyclo) in PlsC-deficient mutants. Lipids that contained C16 fatty acids (34:2, 34:1) decreased significantly in phospholipids (PCs, PEs, MMPEs, DMPEs, PGs) as well as lipids that contained *cis*-9,10-methylenehexadecanoic acid (35:2, 35:1). Lipids with fatty acid compositions of 35:2 and 35:1 are synthesized from 34:2 and 34:1 lipids, respectively. We also demonstrated that non-phosphorus containing lipids (SLs, TMHSs) with fatty acid compositions of 34:2, 34:1, 35:2 and 35:1 decreased significantly. Therefore, phospholipids and non-phosphorus containing lipids have the same biosynthetic origin most likely involving phosphatidic acid as a common precursor.

PG-34:1 in *S. meliloti* was composed by a mixture of 18:1/16:0 and 16:0/18:1, where the first isomer was the major component. In *plsC* knockout mutant mutant PG(18:1/16:0) decreased 25-fold while PG(16:0/18:1) was detected. This suggests the presence of another lysophosphatidic acyl transferase that catalyzed the transfer of C16 fatty acids to the *sn*-1 position in lipids. Moreover, lipids with fatty acid compositions of 36:2, 37:2 and 38:2 were present in *plsC* mutants suggesting the presence of multiple lysophosphatidic acyl tranferases in *S. meliloti*.

This study is the first to show: (1) complete fatty acid distributions in OLs in *S. meliloti*; and (2) OLs with fatty acid composition of 34:1 and 35:1 decreased significantly in *plsC* knockout mutants. OL-34:1 and C35:1 are minor lipids that might contain C16 fatty acids; thus PlsC might also participate in the biosynthesis of OLs by transferring C16 fatty acids. In other bacteria, lysornithine acyl transferase (OlsA) has been shown to participate in the synthesis of both, OLs and phosphatidic acid. The hypothesis that OlsA in *S. meliloti* could participate in phosphatidic acid biosysnthesis should not be excluded; clearly other lysophosphatidic acyl transferases catalyze the transfer of C18 fatty acids to the *sn*-2 position.

Finally, *plsC* suppressor mutant in *S. meliloti* showed partial recovery of SLs with fatty acid compositions of 34:1 and 34:2. This recuperation was almost 50% relative to wild type levels and was only observed for SLs. Research is currently underway to identify and locate in the genome the suppressor mutation of PlsC. This can provide additional information regarding the function of PlsC and its interaction with other genes involved in lipid biosynthesis.

Chapter 7

Conclusions

The work presented in thesis demonstrated the need for comprehensive lipid analysis (lipidomics) in combination with genetic techniques to understand gene function. Fatty acid analysis by GC/MS and intact lipids analysis by ESI/MS/MS were shown to be complementary techniques. A combination of tandem mass spectrometric techniques allowed the analysis eight lipid classes directly from bacterial lipid extracts and provided fatty acid distributions within lipid classes. The use of tandem mass spectrometry allowed the detection of minor lipid classes and minor lipid species within lipid classes. Fatty acid distributions were obtained for non-phosphorus containing lipids (SLs, OLs and TMHSs) for the first time using tandem mass spectrometry. Existing methods for the analysis of non phosphorus-containing lipids involve chromatographic separation typically employing HPTLC, HPLC or a combination of both separation techniques. These methods require large amounts of bacterial samples and are long and tedious. Additionally, in order to obtain fatty acid distributions within a lipid class TLC spots are extracted for mass spectrometric analysis. Lipid recoveries from TLC plates are poor, thus using these procedures only the major molecular components in a lipid class are detected. In contrast, shotgun lipidomics is a simple and rapid methodology that requires minimal sample preparation and small sample amounts. Although the accurate quantitation of lipids was not achieved in this thesis, it remains as a subject of future research. The accurate quantitation of lipids using shotgun lipidomics requires the inclusion of at least one internal standard per lipid class. Consequently, the lack of analytical standards of non-phosphorus containing lipids is a limitation.

The biosyntheses of fatty acids, phospholipids and non-phosphorus containing lipids are orchestrated and highly related processes which are influenced by environmental stresses such as acidity and P_i-limitation. The analytical methodologies presented in this thesis allowed the simultaneous analysis of various lipid classes and their fatty acid distributions. The fatty acid distributions within lipid classes provided valuable insights into the biosynthesis of phospholipids and non-phosphorus containing lipids in *S. meliloti*. This study focused on knockout mutants of specific genes in *S. meliloti*; however the methodologies can be applied to other organisms and to address different biological questions. The next two sections of this chapter provide a summary of the main contributions of each chapter and future research directions derived from this work.

7.1. Summary

Chapter 2

A combination of methods is the best approach for the analysis of bacterial fatty acids of *S. meliloti* as their FAMEs. Basic hydrolysis followed by acid-catalyzed methylation was best for 3-hydroxy fatty acids while base-catalyzed transmethylation with sodium methoxide was best for cyclopropane-containing fatty acids. Appropriate quality control standards were introduced to measure and differentiate losses due to volatilization and handling, completion of derivatization reactions and extent of side reactions. A one-vial micro-scale method that employed sodium methoxide in methanol was developed for the analysis of bacterial fatty acids; this method required minimum sample manipulation and no liquid/liquid extraction steps. Since the micro-scale method did not include water extraction steps PHB was detected as 3-hydroxybutyric acid. Fatty acid analyses of *S. meliloti* cultures grown under P_i-starvation showed a 2-fold increase of cyclopropane fatty acids and a 73% increase of PHB.

Chapter 3

A shotgun lipidomics method was developed for the analysis of intact lipids in *S. meliloti*. A combination of neutral loss scans and precursor ions scans in both positive and negative electrospray ionization mass spectrometry provided profiles of eight lipid classes without chromatography; 58 molecular species were identified in bacterial lipid extracts. Shotgun lipidomics principles were extended for the analysis of two additional phospholipid classes (MMPEs, DMPEs) and three non-phosphorus containing lipid classes (SLs, OLs, TMHSs). The sensitivity and specificity of tandem mass spectrometry allowed the analysis of minor lipid classes and low abundance molecular species in crude lipid extracts. This work provided the missing link between intact lipids and their fatty acid distributions. It was revealed that phospholipids (PCs, PEs, MMPEs, DMPEs) and TMHSs had identical fatty acid distributions; this strongly suggests that TMHSs are synthesized from the same biochemical precursor as phospholipids (phosphatidic acid). On the other hand, SLs did not have a similar fatty acid distribution as PGs or other phospholipids. This finding suggests the existence of more than one biosynthetic pathway for SLs, one of them which might not involve phosphatidic acid as a common precursor.

Chapter 4

The effect of two environmental stressors, P_i-starvation and acidity, on fatty acids and intact lipids profiles was investigated in *S. meliloti*. Fatty acids and intact lipids were analyzed using the one-vial microscale method and the shotgun lipidomics approach, respectively. Acidity is known to increase cyclopropane fatty acid content in *E. coli*; this effect was also observed in *S. meliloti* when cultures were grown under acidic conditions. P_i-starvation conditions similarly to acidity, caused cyclopropanation to increase significantly in *S. meliloti*. This is the

first work to report the effect of P_i-starvation on lipid cyclopropanation in bacteria.

Two putative genes, *cfa1* and *cfa2* thought to code for CFA synthases were studied. It was demonstrated that *cfa2* gene coded for a CFA synthase which was the was the main gene responsible for the cyclopropanation of lipids in *S. meliloti*. *Cis*-11,12-methyleneoctadecanoic acid, was the major component in cyclopropane-containing lipids in *S. meliloti*. Tandem mass spectrometry experiments showed that cyclopropanation of phospholipids occurred in fatty acids located at both the *sn*-1 and *sn*-2 positions in lipids. Fatty acid distributions within lipid classes showed that acidity caused a larger effect in the cyclopropanation of lipids compared to P_i-starvation conditions. This phenomenon was more accentuated in PCs and SLs as cyclopropanation of fatty acids (*cis*-11-octadecenoic acid) in *sn*-2 position proceeded to a larger extent than in PEs or PGs. PCA of fatty acid distributions in four lipid classes showed that cyclopropanation occurred uniformly; therefore the nature of the polar head group did not determine the activity of CFA synthases in *S. meliloti*.

Chapter 5

The combination of techniques developed for fatty acid and intact lipid analyses by GC/MS and ESI/MS/MS, was applied to investigate metabolite profiles of knockout mutants of the malic enzymes (DME and TME) in *S. meliloti*. The metabolite profiles of knockout mutants of *dme* and *tme* genes were studied in two carbon sources (glucose and succinate). It was revealed that *dme* and *tme* mutants showed different metabolite phenotypes compared to wild type when cultures were grown in succinate but not in glucose. The biggest changes were observed for SLs in *dme* and *tme* mutants grown in succinate. SLs that contained hexadecenoic acid and hexadecanoic acid increased in *tme* mutants. PCA of intact lipid profiles of four lipid classes showed that lipids with fatty acid composition of 34:1 contributed to the metabolic differences observed in *tme* mutants in succinate. These results suggest that TME might be involved in fatty acid and lipid metabolism in *S. meliloti* as suggested in *E. coli*. The different metabolite phenotypes observed in succinate for mutants of *dme* and *tme* supports the hypothesis of different biochemical functions for DME and TME in *S. meliloti*.

Chapter 6

Shotgun lipidomics demonstrated that putative PlsC coded by SMc007714 protein in *S. meliloti* corresponded to a fatty acyl transferase. *PlsC* in *S. meliloti* was specific for C16 fatty acids in the *sn*-2 position of lipids. It was revealed that TMHS and SL lipids that contained C16 fatty acids also decreased significantly in PlsC-deficient mutants; this suggested that phospholipids, TMHSs and SLs share the same biochemical precursor (phosphatidic acid). Lipids that contain C18 fatty acids were unchanged suggesting the presence of other fatty acyl tranferases in the genome of *S. meliloti*. OlsA is a fatty acyl transferase that participates in OLs

biosynthesis; this enzyme catalyzes the reaction of fatty acids with the hydroxyl function of lysornithine lipids. In this work, it was shown that OLs that most likely contain C16 fatty acids decreased significantly in PlsC-knockout mutants. Thus, it is proposed that PlsC participates in the biosynthesis of OLs by transfering C16 fatty acids to lysornithine lipids. It is also suggested that OlsA participates in phospholipid biosynthesis by transfering C18 fatty acids to the *sn*-2 position.

7.2. Outcomes and Future Work

Based on the results presented in this thesis, an integrated biochemical pathway for the synthesis of phospholipids and non-phosphorus containing lipids is proposed (Figure 7.1). This model is based on the presence of genes in the genome of *S. meliloti* that showed homology to genes of known function in other microorganisms (Table 7.1). Four hypotheses have emerged which can improve and broaden our current knowledge of lipid biosynthesis and gene function in *S. meliloti* and other bacteria.

Table 7.1. Genes proposed to participate in lipid biosynthesis in *S. meliloti*. This table was built using three data bases: the Kyoto Encyclopedia of Genes and Genomes [246], the Comprehensive Enzyme Information System [247] and *S. meliloti* strain 1021 Genome Project [242].

Gene	Protein Name	E.C.#	Protein in *S. meliloti*	Function Assignment
gpsA	glycerol-3-phosphate dehydrogenase	1.1.1.94	SMc03229	Putative
glpK	glycerol kinase	2.7.1.30	SMb21009	Probable
plsB	glycerol-3-phosphate acyltransferase	2.3.1.15	No homologue	-
plsX	phosphate:acyl-ACP transferases	-	Smc01784	Putative
plsY	acyl-phoshate glycero-3-phosphate acyltransferases	-	No homologue	-
plsC	1-acylglycerol-3-phosphate acyltransferase selective for fatty acyl chains of 16 carbon atoms	2.3.1.51	SMc00714 ($PlsC_{C16}$)	Confirmed (Chapter 5)
	1-acylglycerol-3-phosphate acyltransferase selective for fatty acyl chains of 18 carbon atoms	-	SMc01116 (OlsA) Smc02490 Smc02158	Putative Putative Putative
dgkA	Diacylglycerol kinase	2.7.1.107	Smc04213	Putative
pgp	Phosphatidate phosphatase	3.1.3.4	No homologue	-
pgpA *pgpB*	non-essential phosphatidylglycerophosphate phosphatases	3.1.3.27	No homologue	-

1. *Common Biosynthetic Origin of Phospholipids and TMHS Lipids*

TMHSs showed identical fatty acid distributions as phospholipids (PCs, PEs, MMPEs, DMPEs); thus TMHSs must be synthesized from phosphatidic acid as phospholipids. This theory is supported by evidence than in plants under P_i-limiting conditions, phosphatidic acid is transformed to

diacylglycerol to be used in the synthesis of non-phosporus containing lipids such as SLs [150]. Phosphatidic acid can be converted to diacylglycerol by the action of phosphatidate phosphatases or phosphate hydrolases (PGP). At present, a gene with homology to *pgp* is not known in the genome of *S. meliloti*. However, a gene for the reverse reaction (conversion of diacylglycerol to phosphatidic acid by DgkA) is present (Figure 7.1 and Table 7.1). The fatty acid distributions of phospholipids and TMHSs in *S. meliloti* indicate that a gene coding for phosphate hydrolases must exist in the genome. This hypothesis is further supported by the fatty acid profiles of phospholipids and TMHSs in PlsC-deficient mutants of *S. meliloti*; lipids that contained fatty acids 16:0 and 16:1 were absent in both phospholipids and non-phosphorus containing lipids indicating a common biosynthetic origin. Lipid analysis of cultures of *S. meliloti* grown under low phosphate conditions and in the presence of labeled phosphatidic acid could provide data to support this idea.

2. *Multiple Fatty Acyl Transferases in the genome of S. meliloti*

PlsC, coded by SMc007714 protein in *S. meliloti*, was shown to be specific for C16 fatty acids in the *sn*-2 position of lipids; this protein can be identified as $PlsC_{C16}$. Consequently, another PlsC-like protein that transfers C18 fatty acids to the *sn*-2 position must exist in *S. meliloti* which can be represented as $PlsC_{C18}$. Proteins that code for fatty acyl transferases contain characteristic amino acid sequences called *motifs* which are typical of this class of proteins [245]. The genome of *S. meliloti* contained three genes that coded for proteins with fatty acyl transferases *motifs*: (i) Smc01116 (also OlsA), (ii) Smc02490 and (iii) Smc02158 (Table 7.1).

(i) The first protein Smc01116, also named OlsA, participates in the biosynthesis of OLs by transfering a second fatty acyl chain to the hydroxyl function of the 3-hydroxy fatty acid of lysornithine lipids [152]. In other organisms such as *Rhodobacter capsulatus*, OlsA was shown to be involved in the biosyntheses of both, phospholipids and OLs [153]. Additionally, in this work it was found that OLs that contained C16 fatty acids decreased significantly in PlsC-deficient mutants; this suggests the participation of PlsC in the transference of C16 fatty acids to lysornithine lipids. Therefore, OlsA is a good candidate to participate in the synthesis of phosphatidic acid by catalyzing the transfer of C18 fatty acids to the *sn*-2 position; thus OlsA could be $PlsC_{C18}$. So, it is proposed that fatty acids in the *sn*-2 position of phosphatidic acid are transferred by the combined action of $PlsC_{C16}$ (SMc007714) and $PlsC_{C18}$ (Smc01116, OlsA). This hypothesis can be tested by applying shotgun lipidomics to OlsA-deficient mutants in *S. meliloti*.

(ii) The second protein, Smc02490, is a homologue of SqdA from *Rhodobacter sphaeroides*. SqdA in *Rhodobacter sphaeroides* is involved in SLs biosynthesis but also has homology with fatty acyl transferases [151].

(iii) The third protein, Smc02158, has fatty acyl transferase *motifs* but is unknown.

3. The PlsX/PlsY System in S. meliloti

PlsB in *E. coli* catalyzes the transference of the first fatty acyl residue to the *sn*-1 position of 3-phosphate-glycerol [133] but a homologue of PlsB can not be found in the genome of *S. meliloti* [30]. The alternative PlsX/PlsY pathway has been proposed for the synthesis of lysophosphatidic acid in bacteria that lack PlsB (Figure 7.1) [33]. We have found a good homologue for PlsX of *Streptococcus pneumoniae* in the genome of *S. meliloti*, however a homologue of PlsY could not be found (Table 7.1).

4. Two Pathways for SL Biosynthesis in S. meliloti

The accepted biochemical pathway for the synthesis of SLs in plants is the sugar-nucleotide pathway [150]. However, this model does not explain the accumulation of sulfoquinovosyl-1-O-dihydroxyacetone in SqdC-deficient mutants of *Rhodobacter sphaeroides* [151]. Thus, a second model has been proposed for SLs biosynthesis in bacteria which involves reaction of dihydroxyacetone (or dihydroxyacetone phosphate) with sulquinovose instead of diacylglycerol [148, 151]. The fatty acid distribution in SLs in *S. meliloti* was different to phospholipids and other non-phosphorus containing lipids such as TMHSs. Therefore, this observation suggests a biosynthetic origin for SLs which does not involve phosphatidic acid as proposed for TMHSs. However, this is contradicted by the lipid profiles obtained for PlsC-deficient mutants which showed a significant decrease of SLs that contained C16 fatty acids similarly to phospholipids. Then, is it possible that two biosynthetic routes exist for SLs biosynthesis in *S. meliloti*? It has been shown that two different pathways participate in the biosynthesis of PCs in *S. meliloti* [145]; consequently the theory of two pathways for SLs biosynthesis is plausible.

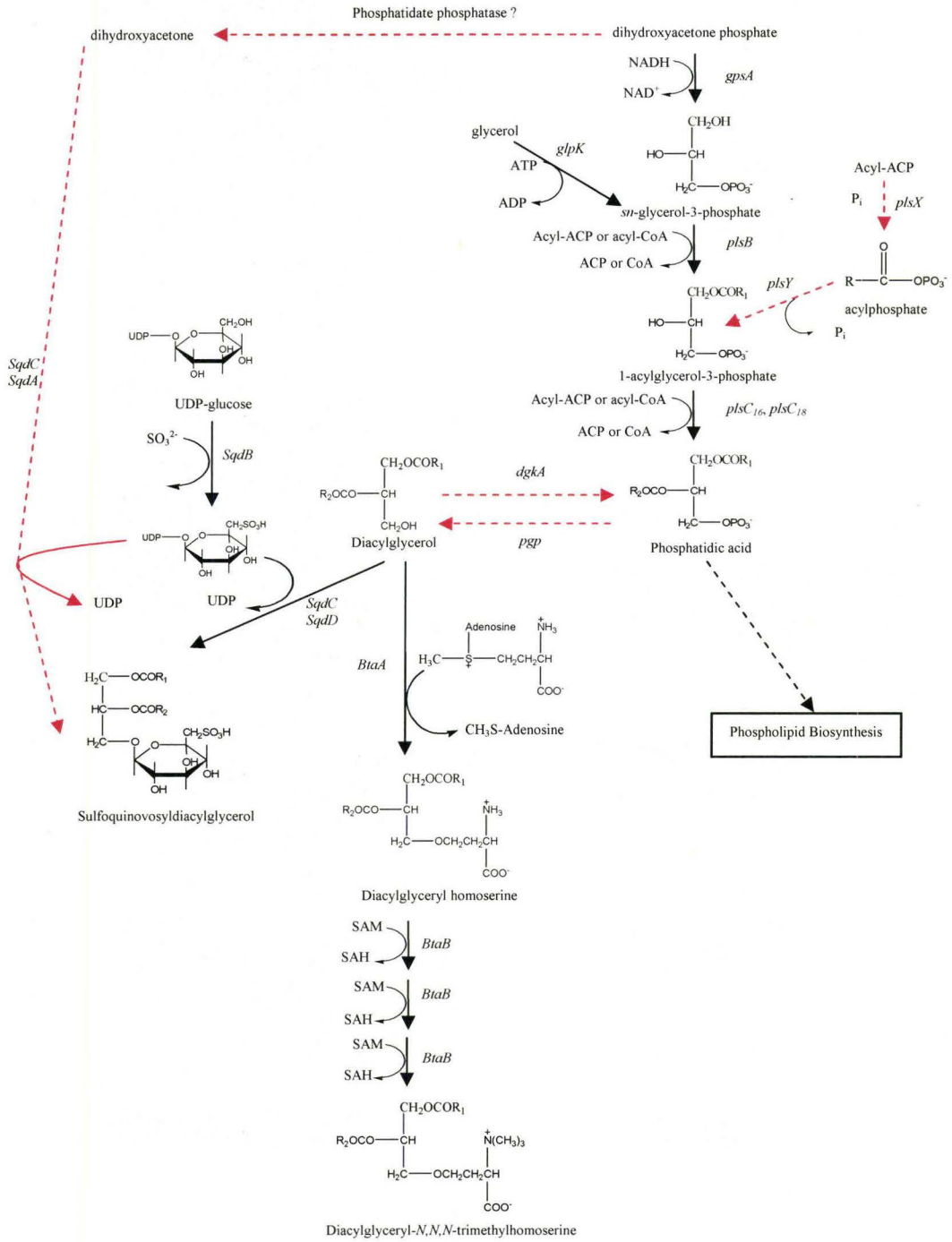


Figure 7.1. Proposed model for the biosynthesis of phospholipids and non-phosphorus containing lipids in *S. meliloti*.

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