UNIVERSITÉ DU QUÉBEC À MONTRÉAL

EFFECT OF THE DEUTERATION CONDITIONS ON THE LIPID PROFILE OF *ESCHERICHIA COLI* AND *BACILLUS SUBTILIS*

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EFFETS DES CONDITIONS DE DEUTÉRATION SUR LE PROFIL LIPIDIQUE DES BACTÉRIES *ESCHERICHIA COLI* ET *BACILLUS SUBTILIS*

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DEDICATION

To my parents,

and

To my husband, Sajjad

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LIST OF ABBREVIATIONS, ACRONYMS, AND SYMBOLS

^{12}C	Carbon-12
^{13}C	Carbon-13
^{19}F	Fluorine-19
^{2}H	Deuterium
^{15}N	Nitrogen-15
^{31}P	Phosphorus-31
AMP	Antimicrobial peptides
B. subtilis	*Bacillus subtilis*
B_0	Magnetic Field
B_1	Effective Magnetic Field
C14:0	Tetradecanoic Acid
C15:0	Pentadecanoic Acid
C16:0	Palmitic Acid
C17:0	Heptadecanoic Acid
C18:0	Octadecanoic Acid
C18:1	Oleic Acid
$CDCl_3$	Deuterated chloroform
CL	Cardiolipin
CSA	Chemical shift anisotropy
DNA	Deoxyribonucleic acid
DPA	Diaminopimelic acid
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
FA	Fatty acid
fadL	Long-chain fatty acid transport protein
FID	Free Induction Decay
FT-IR	Fourier transform infrared
GC-MS	Gas Chromatography-Mass spectroscopy
Gram-	Gram negative
Gram+	Gram positive

I	Spin
ICU	Intensive care unit
LB	Luria Broth
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
lysilPG	lysil-phosphatidylglycerol
MeOH	Methanol
NaCl	Sodium chloride
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
NMR	Nuclear Magnetic Resonance
NSERC	Natural Science and Engineering Research Council
OA	Oleic Acid
OD	Optical Density
PA	Palmitic Acid
PA	Phosphatidic Acid
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PS	Phosphatidylserine
pH	Hydrogen Potential
Phospholipids	PLs
POPE	2-Oleoyl-1-palmitoyl-sn-glycero-3-phosphoethanolamine
POPG	2-Oleoyl-1-palmitoyl-sn-glycero-3-phospho-rac-(1-glycerol)
POPS	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine
RF	Radio frequency
RNA	Ribonucleic acid
S	Saturated
S. aureus	Staphylococcus aureus
TAs	Teichoic acids
Tween 20	Polyethylene glycol sorbitan monolaurate
U	Unsaturated
UQÀM	Université du Québec à Montréal
WTA	Wall teichoic acid
γ	Gyromagnetic ratio
μ	Magnetic moment

LIST OF UNITS

µl	Unit of (microlitre)
h	Unit of time (hour)
Hz	1/s
min	Unit of time (minute)
ml	Unit of volume (milliliter)
mM	Unit of concentration (millimolar)
nm	Unit of length (nanometer)
ºC	Unit of temperature (degree Celsius)
rpm	Unit of rotational speed (rotation per minute)
s	Unit of time (second)

RÉSUMÉ

La résistance aux antibiotiques, selon les rapports publiés par différentes organisations, est un obstacle entravant l'efficacité d'un large éventail d'antibiotiques. En effet, les microorganismes sont capables de s'adapter pour résister aux antibiotiques disponibles, ce qui nécessite le développement de nouveaux agents thérapeutiques. Les peptides antimicrobiens (AMPs) sont une avenue intéressante car ils ont une activité antimicrobienne qui pourrait être utilisée comme nouvelle génération d'agents antibiotiques. Pour étudier le mécanisme d'action des AMPs contre les causes infectieuses notre laboratoire a développé une approche par résonance magnétique nucléaire du deutérium (RMN-^{2}H). Pour ce faire, les membranes bactériennes doivent être marquées au moyen d'acides gras deutérés. Pour suivre les impacts du marquage, la caractérisation du profil lipidique des bactéries est nécessaire. L'objectif de cette étude était de caractériser les phospholipides dans la membrane d'*E. coli* (Gram -) et de *B. subtilis* (Gram +) lorsque le milieu de croissance des bactéries varie.

Les deux bactéries ont été cultivées dans trois milieux différents : 1) échantillon de contrôle, 2) en présence d'acide palmitique (AP), et 3) en présence du mélange d'AP et d'acide oléique (AO). Par le marquage des chaînes acyles des acides gras de la membrane d'*E. coli*, les acides gras ont été incorporés dans le détergent Tween 20. Pour cultiver *B. subtilis*, quatre concentrations différentes d'AP ont été utilisées. Ensuite, les têtes polaires des membranes bactériennes ont été identifiées et quantifiées par la RMN du ^{31}P en solution. Enfin, les acides gras ont été caractérisés par chromatographie en phase gazeuse couplée à la spectroscopie de masse (GC-MS).

Nos résultats montrent que le profil des têtes lipidiques était peu affecté par un marquage à l'AP par *E.coli*. Cependant la proportion de chaines palmitiques augmente tandis que celle des chaines 18 :1 diminue. L'addition d'AO permettrait d'obtenir un profil en chaines d'acides gras plus des bactérie natives. Ce profil a été modifié et induisait lorsque les bactériens croissaient dans un milieu AP ou AP/AO, avec notamment une augmentation de la proportion de cardiolipine (CL). Pour *B. subtilis,* le marquage avec l'AP induisait une modification du profil des têtes polaires. Notamment, une plus grande proportion de CL a été observée. Le pourcentage de C15:0 et C17:0 est resté quasi constant lorsque les bactéries sont cultivées en présence d'AP, avec et sans AO. En augmentant la concentration d'AP de 0 à 0,19 mM, le pourcentage de

C15:0 et C17:0 a considérablement diminué, au profit du C16:0. Le protocole développé est une approche rapide et simple pour caractériser la composition lipidique des membranes bactériennes dans diverses conditions de croissance en utilisant la RMN du solution ^{31}P en solution combiné avec GC-MS. Si les bactéries sont marquées par de l'acide palmitique deutéré, notre protocole suggère de réduire fortement la quantité d'acide palmitique exogène dans le milieu de croissance ainsi que de choisir avec soin le temps de croissance correspondant aux bactéries.

Mots clés : ^{31}P RMN, GC-MS, *Bacillus subtilis, Escherichia coli,* Résistance aux Antibiotiques, Peptides Antimicrobiens.

ABSTRACT

Antibiotic resistance, according to published reports by different organizations, is an impeding obstacle threatening the effectiveness of a wide range of antibiotics. Indeed, microorganisms are able to adapt to resist available antibiotics; this requires development of new therapeutic agents. Antimicrobial peptides (AMPs) are of interest as they have antimicrobial activity that can be used as a new generation of antibiotic agents. To study the mechanism of action of AMPs against the infectious causes, our laboratory has developed a ^{2}H-NMR approach. For this purpose, the bacterial membranes must be labeled by deuterated fatty acids. By tracing the effects of labeling, the characterization of the lipid profile of bacteria is inevitable. The objective of this study was to characterize the existing phospholipids in the membrane of *E. coli* (Gram -) and *B. subtilis* (Gram +) when bacterial growth diet was varied.

The two bacteria were cultivated in three different diets: 1) control sample, 2) in the presence of Palmitic acid (PA), and 3) in the presence of the mixture of PA and Oleic acid (OA). By labeling the acyl chains of fatty acids of *E. coli* membrane, the fatty acids were incorporated in Tween 20. To grow *B. subtilis*, four different concentrations of PA were applied. Then, the polar heads of bacterial membranes were identified and quantified by the means of ^{31}P solution-state NMR. Finally, the fatty acids were characterized by gas chromatography coupled with mass spectroscopy (GC-MS).

Our results show that labeling slightly affected the profile of polar heads of *E. coli*.; however, the proportion of palmitic chain increased while 18:1 chain decreased. The addition of OA would allow to obtain a profile of fatty acid chains more similar to the natural bacteria. This profile was modified and induced when the bacteria grew in PA or PA/OA medium, by increasing of the proportion of CL. For *B. subtilis*, Labeling with PA induced a modification of the polar head profile. In particular, a higher proportion of CL was observed. The percentage of C15:0 and C17:0 remained almost constant when bacteria was cultured in the presence of PA, with and without OA. By increasing the concentration of PA from 0 to 0.19 mM, the percentage of C15:0 and C17:0 decreased considerably in favour of C16:0. The developed protocol is a fast and straight-forward approach to characterize the lipid composition of bacterial membranes under various growth conditions by using solution ^{31}P NMR combined with GC-MS. If the bacteria are labeled with deuterated palmitic acid, our protocol suggests to

strongly reduce the the amount of exogenous palmitic acid in the growth medium as well as to carefully choose the growth time corresponding to the bacteria.

Keywords: ^{31}P NMR, GC-MS, *Bacillus subtilis, Escherichia coli,* Antibiotic Resistance.

INTRODUCTION

Antimicrobial resistance is a fast-growing issue in the use of antibiotics and threatens the effectiveness of existing therapeutic agents. It therefore necessitates the development of new antimicrobial agents with new modes of action. Antimicrobial peptides (AMPs) generally intervene by changing the size, shape, permeability, and cell stress response of the bacterial membrane which leads to cell lysis and death. They are thus considered as a potential therapeutic avenue. Therefore, it is of great importance to study the interaction of bacterial cell membranes with AMPs (Epand & Epand, 2009; Kohanski, Dwyer, & Collins, 2010), and *in vivo* solid-state nuclear magnetic resonance (SS-NMR) is a promising analytical technique for this purpose.

^{31}P NMR has been used many times to identify and quantify the phospholipids in membranes. The most common phospholipids in bacterial membranes are phosphatidylethanolamine (PE), phosphatidylglycerol (PG), , and cardiolipin (CL) (Peterson & Cummings, 2006). There are two major types of bacteria , i.e., Gram(+) and Gram(-). Gram(+) bacteria have a relatively thick cell wall composed of peptidoglycan (20-80 nm), while Gram(-) bacteria have a thin layer of peptidoglycan (2-3 nm) conjugated to an outer membrane (Madigan, Martinko, Bender, Buckley, & Stahl, 2014).

Following the work was done by Davis in 1979 (Davis, Nichol, Weeks, & Bloom, 1979), ^{2}H SS-NMR has been used to study the composition and structure of bacterial membranes and to understand their dynamics and interactions with AMPs (Booth, Warschawski, Santisteban, Laadhari, & Marcotte, 2017; Bouhlel et al., 2019; Laadhari,

Arnold, Gravel, Separovic, & Marcotte, 2016; Pius, Morrow, & Booth, 2012; Tardy-Laporte et al., 2013; Warnet, Laadhari, Arnold, Marcotte, & Warschawski, 2016). To deuterium label the membrane of *E. coli*, Davis grew the bacteria in the presence of exogenous labeled fatty acids (FAs) at a concentration of 0.19 mM micellized in Brij 58 and harvested it at the mid-log phase. In 2012, the similar parameters were applied to mutant bacteria that their FAs cannot be synthesized (Pius et al., 2012). Our group has done an extensive work to replace the Brij by other detergents namely dodecylphosphocholine (DPC) or polyoxyethylenesorbitan monolaurate (Tween 20) (Booth et al., 2017; Tardy-Laporte et al., 2013). They demonstrated that it is necessary to supplement the non-mutant *E. coli* by equal amount of palmitic acid (PA or C16:0) and oleic acid (OA or C18:1) to maintain the level of its unsaturated lipids (Warnet et al., 2016). It has been also shown that the acyl chain profile of *Vibrio splendidus*, which is a slow growing bacterium, corresponds the growth condition (Bouhlel et al., 2019).

Putting to gether the summary of the current works, it is known that bacteria adapt their lipid composition to external growth conditions, but this has not been thoroughly studied in the context of membrane labeling. Also, it seems worth to investigate a standard protocol for the ^{2}H-labeling conditions in order to accurately determine the lipid composition of bacteria. Therefore, the objective of this master project is to characterize the existing phospholipids in the membrane of *E. coli* and *B. subtilis* grown under various labeling conditions as well as to optimize the analysis conditions. Here, we propose a new routine protocol in which the whole lipid composition of bacteria is revealed.

This master thesis is divided into four chapters. The first chapter presents the critical review over the emergence of antibiotic resistance and the related aspects to the use of antimicrobial peptides. Then, *E. coli* and *B. subtilis*, which respectively belong to the Gram(-) and Gram(+) bacterial groups, are presented. In the second chapter, we present the detailed protocol for bacterial growth with different diets. A concise theoretical

presentation of NMR and GC-MS are also described. In the third chapter, the result of measurements and experiments are presented, and they are discussed and compared with the available literature. In the fourth chapter, a general conclusion and recommendation with regard to the future of this project is presented. The appendices include the comprehensive details of the protocols.

CHAPTER I

LITERATURE REVIEW AND RELATED THEORY

1.1 Antibiotic Resistance

Antibiotics are one of the most efficient types of medications in the treatment of infection. Some antibiotics kill the bacteria (e.g., penicillin), while some others stop their multiplication. There are several pieces of evidence demonstrating the use of infection treatment in ancient Egypt, Greece, and China (Ventola, 2015). The rate of reported deaths by bacteria has become a significant life threat in the developing world as new kinds of diseases arise such as *Staphylococcus aureus* (*S. aureus*) infection (Kapoor, Saigal, & Elongavan, 2017a). The era of the emergence of modern antibiotics begins with the discovery of penicillin by Sir Alexander Fleming in 1927 (Gould & Bal, 2013; Ventola, 2015). This antibiotic provided numerous advantages to the health care system by decreasing the mortality caused by infectious diseases.

Recently, bacterial resistance has become one of the most critical issues in the global health system and resulted in increasing infections caused by resistant bacteria. Antimicrobial resistance is a situation when antibiotics lose their ability against bacteria or they cope with the use of medications reducing the effectiveness of the agents. Antimicrobial resistance can occur when microorganisms undergo genetic evolution or they acquire the resistance ability from other inherently resistant in the environment (Kapoor et al., 2017a; Ventola, 2015). Bacterial resistance not only

decreases decreases the usefulness of the antimicrobial medications but also increases the risk of nosocomial infections (Ventola, 2015).

The misuse or inappropriate use of antibiotics are two primary root causes associated with the microbial resistance. The incorrect choice of antibiotic, duration of therapy, and indication may result in bacterial resistance with a reported rate from 30% to 60% (Luyt, Bréchot, Trouillet, & Chastre, 2014; Ventola, 2015). It is estimated that 40% to 75% of antibiotic prescriptions worldwide are inappropriate (Avorn & Solomon, 2000; Pestotnik, 2005). The reports in Canada revealed inappropriate use of antibiotics in almost 50% of cases out of 25 million patients who annually took those therapeutic agents (Conly, 2002; Dellit et al., 2007).

Cross-resistance refers to the situation where a microorganism becomes resistant to an antibiotic, as well as to another antibiotic or a class of pharmacological antibiotics Treatment of the simply infected patients, as well as empiric therapy, are two examples of improper use of antibiotics which potentially increase the risk of resistance. It is reported that 30% to 75% of patients with pulmonary infiltrates received antibiotics for a non-infectious cause (Avorn & Solomon, 2000; Pestotnik, 2005). Treatment by antibiotics when it is not necessary can cause harm to the patients as well as increase the risk of antimicrobial resistance. Cultural pressure which enforces medical professionals to prescribe antibiotics is another example of inappropriate use of antibiotics. On the other hand, lack of access to antibiotics, intake a low dosage of medication, or use of inappropriate antibiotic to a bacterium can also increase the risk of resistance. Resistance to Gram(+) and Gram(-) bacteria is, thus, a major growing concern in the health care system, especially in hospitals. Patients who are in the intensive care unit (ICU) are more vulnerable as often contamination of the intravenous catheter, urinary catheter, ventilation system, or nutrition system can increase the risk of infection. Therefore, the consequences of infection caused by Gram(+) and Gram(-)

bacteria can endanger the patients' life in ICU or those who have undergone surgical procedures.

S. aureus, a Gram(+) bacterium, is responsible for skin or soft-tissue infection (Baron, 1996). Since 1960, several hospital-associated penicillin- and methicillin-resistance cases have been reported. *S. aureus* infections were rapidly developed during the 1980s in the majority of hospitals in Canada with the resistance. The Canadian Nosocomial Infection Surveillance Program reported that methicillin-resistant *S. aureus* increased from 2.8 to 3.17 cases per 10,000 patients from 2012 to 2017 (Canadian Antimicrobial Resistance Surveillance System, Update 2018: Executive Summary).

1.2 Action mechanism of antibiotics

Antibiotics use different action mechanisms, giving them antimicrobial ability, depending on the target (Figure 1-1). Some antibiotics, e.g., penicillins, carbapenems, and cephalosporins, disturb the synthesis of the peptidoglycan (Josephine, Kumar, & Pratt, 2004) in the bacterial cell wall. Some antibiotics may breakdown the cell wall. For example, daptomycin disrupts the calcium-dependent cell membrane by depolarization. Antibiotics also may interrupt the nucleic acid synthesis by blocking the replication and transcription of DNA. Preventing protein synthesis, e.g. by ribosome inhibition, is another mode of action of antibiotics. Drugs such as clindamycin, lincomycin, and tetracycline impair the translation or elongation of the proteins, which are primary molecules in any living microorganism (Douthwaite, 1992; Patel et al., 2001). Some antimicrobial agents such as sulphonamides and trimethoprim can attach to enzymes and simulate the role of normal substrate. This leads to blockage of the normal metabolism in which amino acids and nucleic acids are produced (Talaro, 2002).

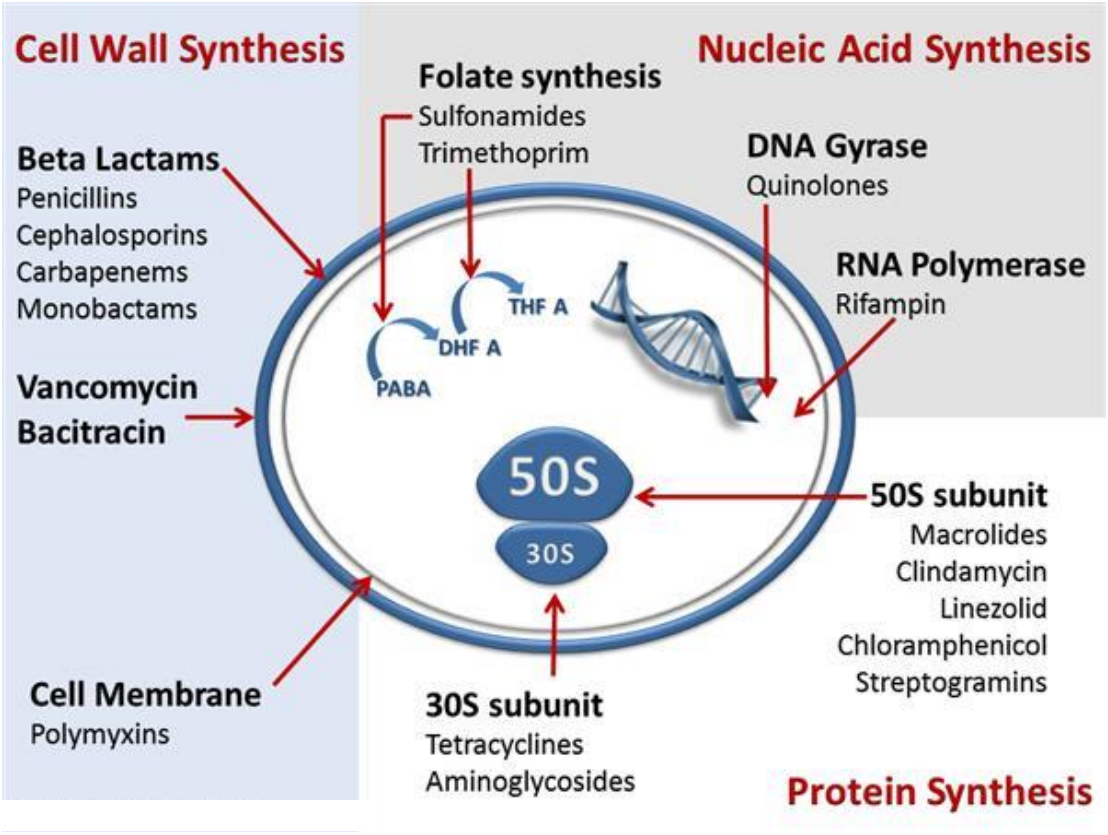


Figure 1-1 The action mechanism of antibiotics (Kapoor, Saigal, & Elongavan, 2017b)

1.3 Antimicrobial peptides

Antimicrobial peptides (AMPs) are natural or synthetic protein molecules usually made of 11 to 50 amino acids (Sitaram & Nagaraj, 2002). There exist numerous AMPs found in a variety of sources such as plants, insects, mammals, and viruses. They are multifunctional molecules that play a fundamental biological role, particularly in the elimination of pathogenic microorganisms (Lai & Gallo, 2009). AMPs provide defense against and counteract the diseases caused by various microorganisms such as Gram(-)

bacteria and Gram(+) bacteria, fungi, and viruses. Various action mechanisms of AMPs against bacteria increase their efficacy with a potentially lower risk of resistance tendency (Thacker, 2013). The discovery of new antimicrobial agents is necessary to overcome the difficulties associated with antibiotic resistance, and AMPs are a promising novel therapeutic solution. This is why AMPs will be presented with more details in the following sections.

1.3.1 Classification of AMPs

AMPs can be classified into five groups including linear cationic peptides with α-helices (e.g. magainin) (Boman, 1998), peptides which are rich in specific amino acids such as indolicidins (rich in tryptophan) and drosocins (rich in proline and arginine) (Bulet, Hetru, Dimarcq, & Hoffmann, 1999), peptides with cysteine residues forming disulfide bridges (e.g. defensins) (Bulet et al., 1999), peptides derived from larger proteins (e.g. casocidin), and anionic peptides (e.g. dermicidins) (Brogden, 2005). In general, peptides with less than 40 amino acids, characterized by the absence of cysteine residues, are unstructured in water and adopt a helical conformation in the membrane.

1.3.2 Action mechanism of AMPs

Although it was believed that permeabilization of the cell membrane is the only mechanism of action of AMPs, it is now known that bacteria have different (alternative) modes affecting the cell membrane (Jenssen, Hamill, & Hancock, 2006; Sierra, Fuste, Rabanal, Vinuesa, & Vinas, 2017). Nevertheless, all these activities depend on the interaction of AMPs with the surface of cell membranes (Jenssen et al., 2006). The first

step is an electrostatic interaction between positively charged AMPs, and the negatively charged lipids in the membrane, namely PG and CL. Then, AMPs insert intothe membrane via hydrophobic interaction. The action of AMPs on the bacterial membrane can be described by four main mechanisms, namely “toroidal pores,” “barrel-staves,” “carpet,” and “sinking-raft” (Figure 1-2) (Sierra et al., 2017; Yeaman & Yount, 2003).

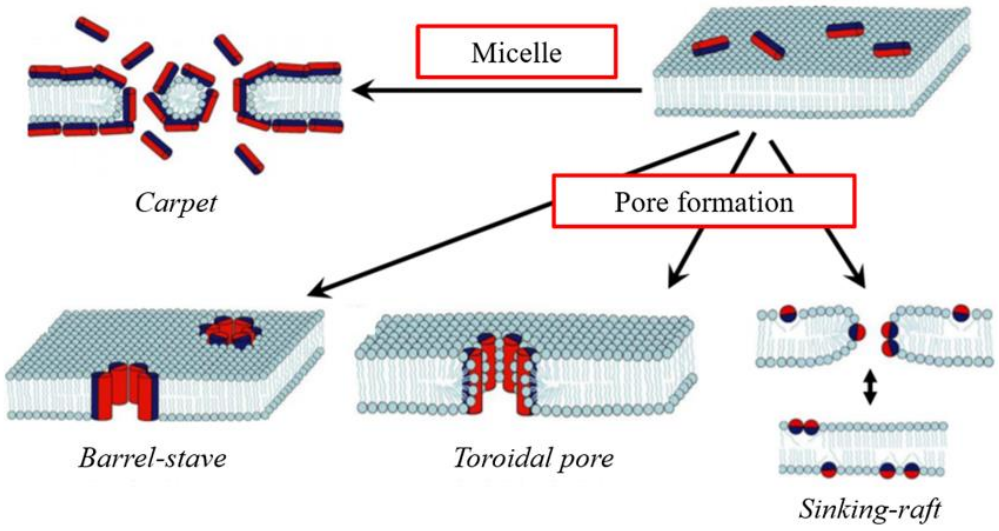


Figure 1-2 Mechanism of destabilization of bacterial membranes by antimicrobial peptides: barrel-staves, toroidal pore, sinking-raft, and carpet (Brogden, 2005)

The formation of a toroidal pore by an AMP first requires that the peptides rest parallel to the membrane, the hydrophobic surface interposed between the polar heads of the lipids, in the hydrophobic environment of the membrane. When the concentration of peptides is sufficient, they will gradually come to induce curvature in the lipid bilayer to form a pore. This type of pore is particular since the lipids of the membrane are part of the pore by having polar heads interposed between the peptides (Yang, Harroun,

Weiss, Ding, & Huang, 2001). For the barrel-stave model, pore formation begins as the previous model, with peptides inserting into the membrane. Subsequently, the peptides will form aggregates, which will then form a transmembrane tunnel. In this mechanism, the hydrophobic regions of the peptide are found outside, in contact with the lipid chains. The hydrophilic regions are found inside the “barrel,” in contact with the water (Yang et al., 2001). Like the other two mechanisms, the "sinking-raft" begins with an accumulation of peptides parallel to the surface of the membrane. For the rest, this mechanism is very different. In the sinking-raft, the peptides will always remain parallel to the membrane. The pore is formed when the peptides change its membrane surface. This temporary pore will be induced by the curvature of the membrane that will induce the peptide to “flow” in the membrane (Pokorny & Almeida, 2004). Finally, the“carpet-like” mechanism needs a very large concentration of peptides that will all remain parallel to the membrane. The peptides are going to make a carpet of molecules that will cover the entire membrane, thus solubilizing it by forming micelles. Like the other mechanisms, this mechanism requires a peptide with a certain amphiphilicity (Shai, 1999).

The exact mechanism by which antimicrobial peptides succeed in killing the cell is not fully known, but several hypotheses have been raised over the years. Indeed, it seems that not all AMPs act in the same way, although they all induce pores in the membrane of bacterial cells. As described above, the interaction of AMPs and bacteria depends on the negative electrical charge of the surface of the bacterial cell which in turn depends on the proportion of (Sierra et al., 2017). To better understand how AMPs interact with the cell membrane, solid-state NMR is a promising technique (Booth et al., 2017).

1.4 Cell membrane

The cell membrane (plasma membrane) is a complex biological structure that separates the cytosol and extracellular space while ensuring the selective permeability of the cell (Shan & Wang, 2015). This protective wall regulates the molecular transport inside/outside of the cell as well as communication with the other cells. The primary organic molecules in the cell membrane are lipids and proteins (Figure 1-4). The composition of the primary molecules of the cell membrane depends on the type of cell and the role that each cell plays in a biological environment.

1.4.1 Diversity of lipids

Lipids are mostly a group of organic molecules made of FAs bound to a polar headgroup.. phospholipids in the cell membrane have a hydrophilic head and hydrophobic tails, which together conferamphipathic behavior in aqueous solutions. Indeed, the heads of phospholipids interact with the surrounding water while the tails converge and make a bilayer construct. This self-assembly phenomenon results in the formation of a continuous spherical membrane.

Lipids serve three main functions of the cell membrane. Triglycerides can effectively store and release energy. Some lipids (e.g., steroids such as estrogens and testosterone) act as intracellular receptors to transmit signals with the extracellular matrix. Glycerophospholipids (also called phospholipids), sphingolipids, and sterols are three essential lipids in the cell membrane (Figure 1-3) (Luckey, 2008; van Meer, Voelker, & Feigenson, 2008) as will be presented below.

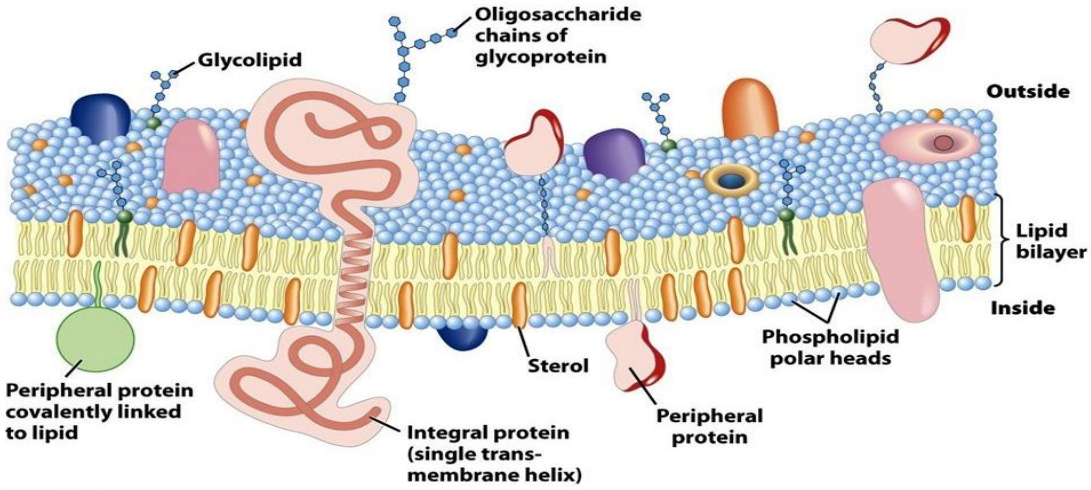


Figure 1-3 Representation of a cell membrane (Geoffrey M., 2000).

1.4.1.1 Phospholipids

Phospholipids are a group of lipids existing in cell membranes with prominent metabolic and structural functions. They are made of a phosphate group, glycerol, and fatty acid chains. The hydrophilic polar head of phospholipids is composed of a phosphate group and the glycerol. The tail of phospholipids consists of FAs with a neutral electrical charge conferring water-insolubility and hydrophobicity (Figure 1-4) (Boyer, 2005).

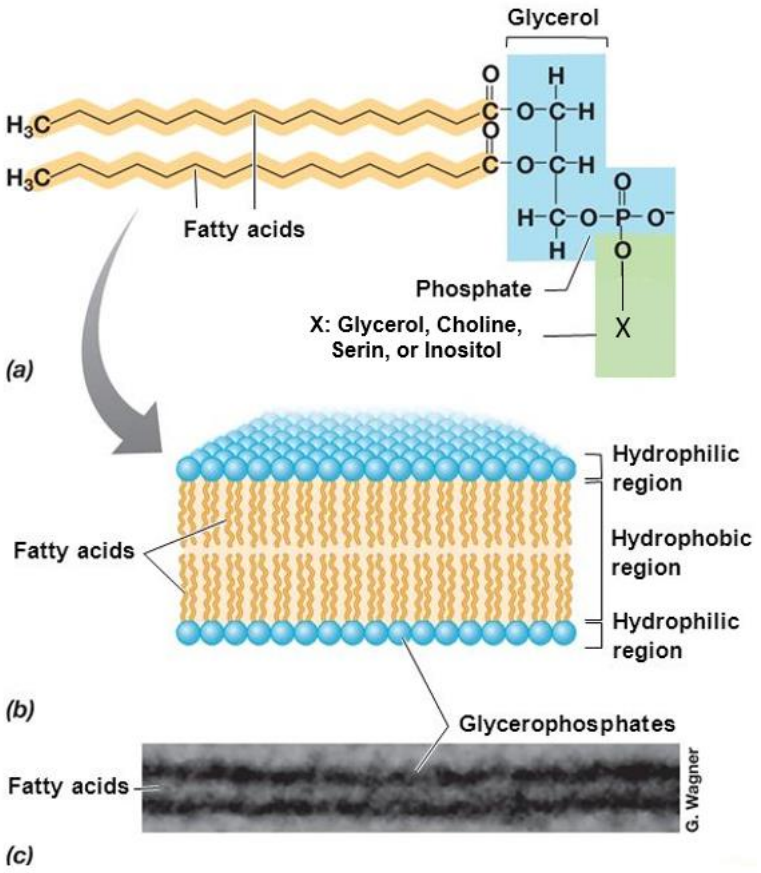


Figure 1-4 Phospholipid bilayer membrane. (a) Structure of the phospholipids. (b) Structure of the bilayer membrane. (c) Transmission electron micrograph of a membrane (Madigan et al., 2014).

The nomenclature system of phospholipids is based on their headgroups. The most common phospholipids in the biological membranes are phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), and cardiolipin (CL) (Figure 1-5) (Peterson & Cummings, 2006).

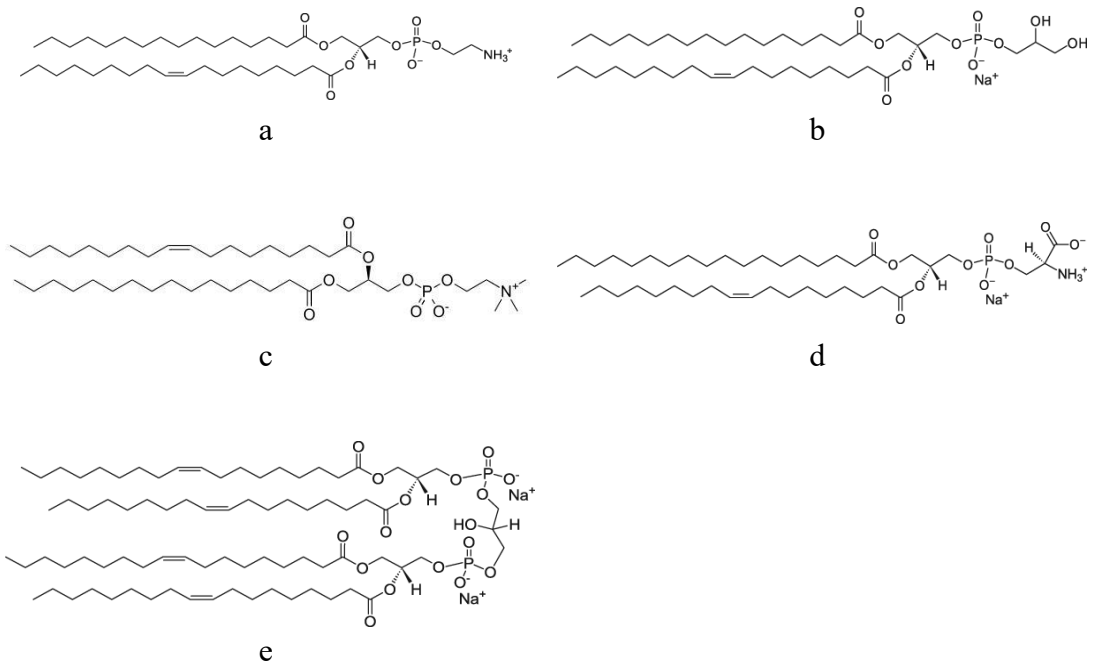


Figure 1-5 Different phospholipids existing in cell membranes: a) Phosphatidylethanolamine (PE), b) Phosphatidylglycerol (PG), c) Phosphatidylcholine (PC), d) Phosphatidylserine (PS), and e) Cardiolipin (CL).

1.4.1.2 Sphingolipids

The primary molecule in the sphingolipids structure is sphingosine, which is an amino alcohol. To make a sphingolipid, an amino functional group of the sphingosine molecule is grafted to a long chain FA, and the secondary alcohol head carries a polar group, i.e., X. Depending on the bonded X molecule, different sphingolipids may be derivatized. For example, sphingosine can be attached to the hydrogen, phosphocholine, glucose, and complex oligosaccharide derivatizes ceramide (Grosch, Schiffmann, & Geisslinger, 2012), sphingomyelin, glucosylcereboroside, and ganglioside, respectively (Boyer, 2005) (Figure 1-6).

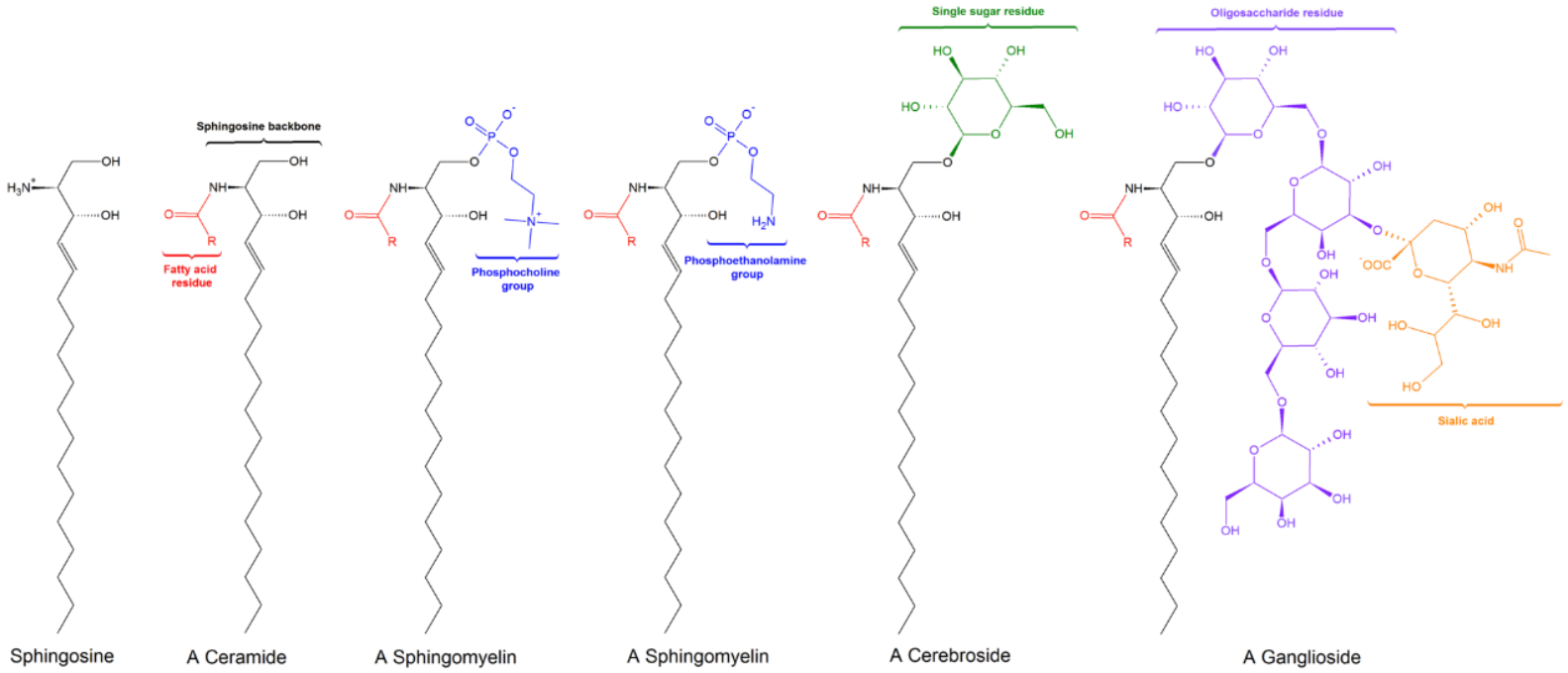


Figure 1-6 Different types of sphingolipids (Image taken from https://commons.wikimedia.org)

1.4.1.3 Sterols

Sterols are the third major group of biological lipids existing in human, plants, and fungi, but rarely found in bacterial cell membranes. They are amphiphilic molecules composed of ringed lipids, non-polar chains, bonded to the polar hydroxyl groups. Cholesterol is a common sterol in the plasma membrane of eukaryotic cells which plays a significant role in the membrane structures. Cholesterol gives the fluidity to the membrane as well as signal messengering ability. In yeast and fungi cells, ergosterol has a similar function to cholesterol (Han & Gross, 2005).

1.4.2 Fatty acids

Fatty acids are an important component of biological lipids composed of a carboxyl group (-COOH) bonded to an aliphatic chain. The carboxyl group has polar properties, while the aliphatic end is non-polar (Boyer, 2005). The latter contains C—H bonds which can eliminate the interaction of FAs with water. This hydrophobic feature of the FAs is vital in the structure of the lipids to form the bilayer cell membrane. When the carbon-carbon is a single bond, the FA is denoted as saturated, and the double or triple carbon-carbon bond in the structure of FA makes it unsaturated. Although FAs can contain between 4 (in butter) and 36 (in the brain) carbon atoms, they mostly have 12 to 24 carbon atoms in nature. Palmitate (C_{16}) and Oleate (C_{18}) are two most common FAs; Palmitate is saturated while Oleate is unsaturated with a double bond between carbons 9 and 10 causing a kink in its structure (Boyer, 2005; Luckey, 2008).

1.5 Bacteria

Bacteria, for a general audience, often imply diseases, infection, or other harmful consequences associated with these microorganisms (McFall-Ngai, 2007; Yusuf, 2015). The reported negative events caused by bacteria affect our life; for example, diseases such as tetanus, typhoid fever, tuberculosis, streptococcal pharyngitis, anthrax, plague, and cholera threaten human life, or food poisoning is also a result of bacteria (Chan, Lee, Baqui, Tan, & Black, 2013). On the other hand, many bacteria are beneficial with a broad sort of applications. Probiotic bacteria in our digestive system help against developing harmful bacteria, which subsequently decreases the risk of some diseases (Saarela, Mogensen, Fondén, Mättö, & Mattila-Sandholm, 2000). Bacteria play an important role in phenomena in the nature, for example, nitrogen cycle, or in the food processes, e.g. vinegar and yogurt production.

Bacteria are unicellular organisms, i.e., each bacterium is composed of only one cell. Bacterial cells are smaller than those of humans. They have a diameter of approximately 3µm, while human blood cells are bigger (around 10µm) (College, 2013). Several internal structures are encapsulated inside bacterial cells, although these microorganisms are very tiny. In bacteria, plasmid, genetic material (DNA), and ribosomes (protein producers) are floating in the gel-like cytoplasm. They are surrounded by the plasma membrane, cell wall, and capsule. Pilis on the surface of the cells serve the binding ability to other bacteria and attachment to the host (Figure 1-7).

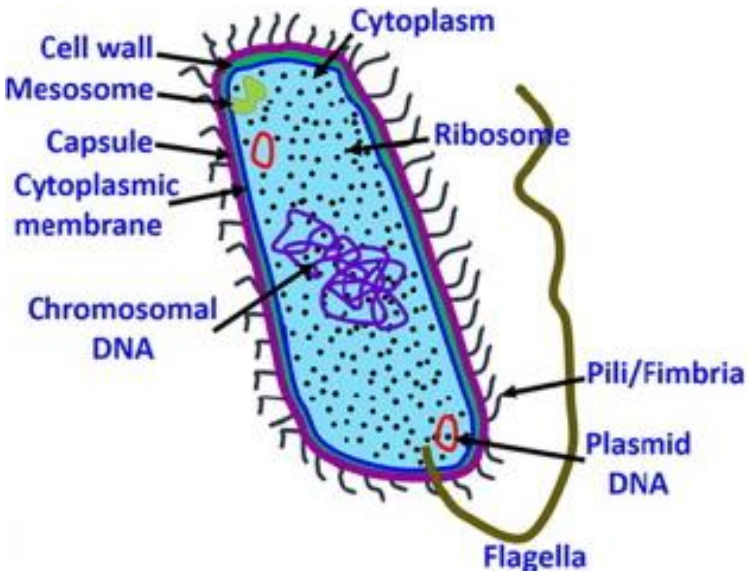


Figure 1-7 Schematic of the bacterial cell (Tripathy, Sen, Su, & Briscoe, 2017).

There are several types of bacteria around the world and which can be classified on the basis of their shapes and wall thickness. The cell wall protects the cell content, defines the cell shape, and regulates the passage of molecules in and out of the cell. Generally, bacteria are categorized into thick and thin groups according to the thickness of the cell wall. Gram staining, developed in 1884 by Danish scientist Hans Christian Gram, is a technique to discriminate bacteria by the cell thickness in two groups: Gram-positive and Gram-negative. Gram(+) bacteria have a relatively thick cell wall composed of peptidoglycan (20-80 nm), while Gram(-) bacteria have a thin layer of peptidoglycan 2-3 nm) conjugated to an outer membrane (Figure 1-8) (Madigan et al., 2014). Using different stains, Gram(+) and Gram(-) bacteria turn blue/purple and pink/red, respectively (Coico, 2005).

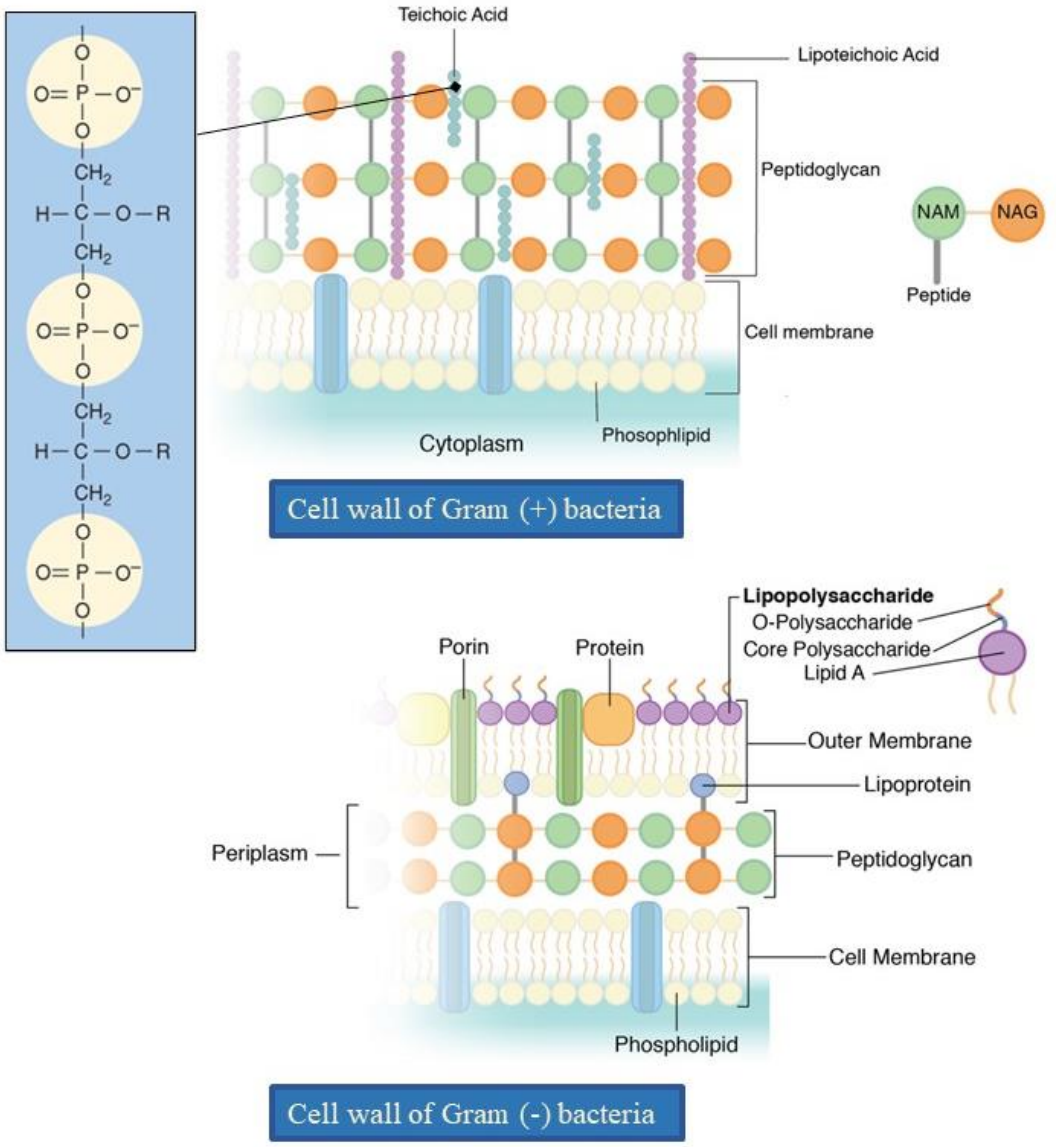


Figure 1-8 Cell wall of Gram(+) and Gram(-) bacteria. (Image taken from https://laboratoryinfo.com)

The peptidoglycan is a long-chain polysaccharide composed of glycan strand cross-linked to peptides which forms a mesh-like structure around the cytoplasm. Two alternative glucose derivatives, N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), form the glycan backbone. The glucose strands are cross-linked to another chain by tetrapeptides via the NAMs. Each peptide has four amino acids including L-alanine, D-glutamine, L-lysine or meso-diaminopimelic acid (DPA), and D-alanine. In general, D-alanine on one tetrapeptide can bind and directly cross-link to the L-lysine/ DPA on another tetrapeptide. In addition to direct cross-link between tetrapeptides, in Gram(+) bacteria, the peptide strands may be cross-bridged by a chain of five amino acids (Figure 1-9) (Coleman & Smith, 2007).

The mesh-like structure of peptidoglycan molecules has a significant role in maintaining the rigidity and shape of bacterial cells as well as for protecting the cell wall from disruption due to osmotic pressure (Schumann, 2011). Since peptidoglycan is unique in bacterial cells, it is an attractive target to inhibit their biosynthesis (e.g., by antibiotics). The mesh-like structure of this molecule also serves the ability to act as a scaffold for anchoring other components such as proteins and teichoic acids, which may maintain the cell wall integrity and shape. About 30% to 70% of the cell wall of Gram(+) bacteria is made of peptidoglycan, while it is a minor portion in Gram(-) bacteria (Schumann, 2011).

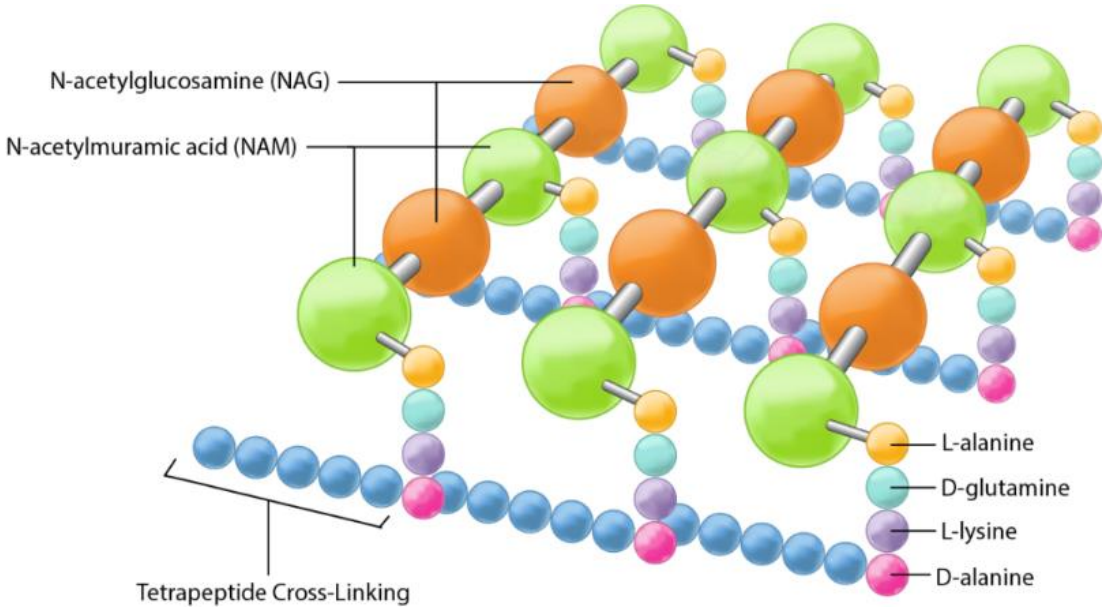


Figure 1-9 Structure of peptidoglycan (Image taken from http://library.open.oregonstate.edu)

1.5.1 Escherichia coli

Escherichia coli (*E. coli*) is a rod-shaped Gram(-) facultative anaerobic bacteria living in the intestines of healthy human or animals (Kaper, Nataro, & Mobley, 2004; Nanninga, 1998). The cell wall of this class of bacteria is made of two lipid bilayers placed on both sides of the thin peptidoglycan layer (Figure 1-8). The outer membrane of *E. coli*, which is missing in the cell wall of Gram(+) bacteria, contains lipopolysaccharide (LPS), phospholipids, and integral proteins (Figure 1-8). The LPS serve as a protection to the cell from chemical agents, and stabilizes the outer membrane by contributing to the negative charge of the cell membrane (Figure 1-10). The outermost part of LPS is O-polysaccharide chains giving antigenicity properties to the cell membrane and triggering the immune response of the host (Netea, van Deuren,

Kullberg, Cavaillon, & Van der Meer, 2002). The second part of LPS is the core polysaccharide and connects the third part of the LPS, lipid A, to the O-chains (Vance & Vance, 2008). Lipid A causes general symptoms of illness, e.g., fever and diarrhea, by acting as a toxin.

Three types of phospholipids (i.e., PE, PG, and CL) with three major types of FA lengths (C16, C17, and C18) are found in *E. coli* membranes as determined by TLC and GC-MS, respectively (Li et al., 2010; Morein, Andersson, Rilfors, & Lindblom, 1996; Pluschke, Hirota, & Overath, 1978). Pluschke et al. characterized, 75% PE, 25% PG, and traces of CL in the T2GP strainwhile, in the K12 strain, Morein et al. found 72% PE, 21% PG, and 7% CL. These two studies mainly found two fatty acids, C16:0, and C16:1 in the K1062 and K12 strains, but another study (Li et al., 2010) reported C14:0, C16:0, and cycloC17:0 chains (Table 1-1).

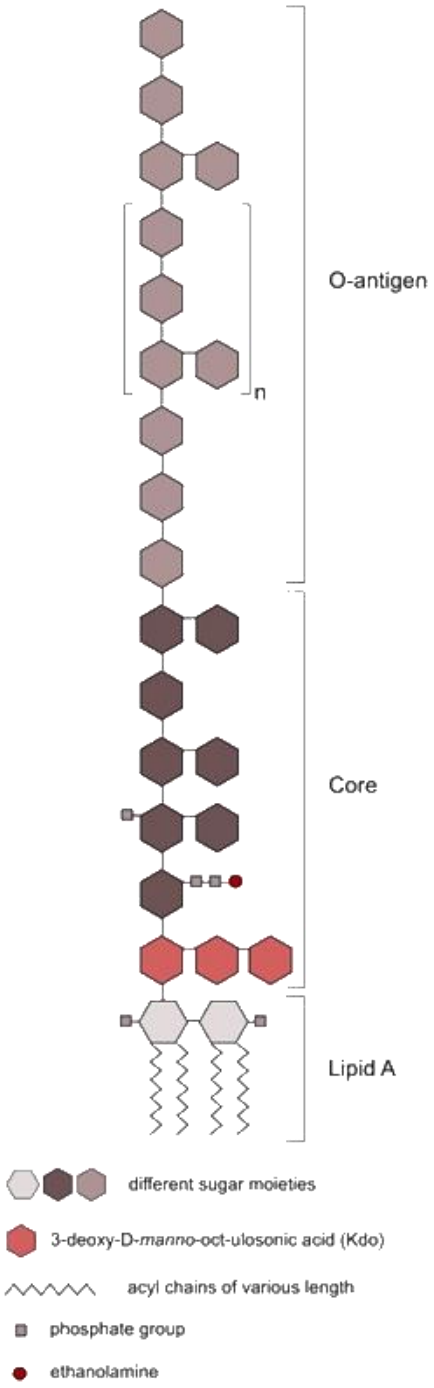


Figure 1-10 Schematic of lipopolysaccharide (LPS) of Gram (-) bacteria (Steimle, Autenrieth, & Frick, 2016).

Table 1-1 Composition of phospholipid headgroups and fatty acids in the membrane of different strains of *E. coli*

Study	Strain	Phospholipid headgroups					Fatty acids						
		Lipid analysis	PE (%)	PG (%)	CL (%)	Fatty acid analysis	C14:0 (%)	C16:0 (%)	C16:1 (%)	C17:0 (%)	cycloC17:0 (%)	C18:0 (%)	C18:1 (%)
(Pluschke et al., 1978)	T2GP	TLC, Fluorescence	75	25	Trace	GC-MS	-	-	-	-	-	-	-
	K1062		85	2	13	GC-MS	-	19-25	70-75	-	-	1-2	-
(Moreinet al.,1996)	K12	TLC	72	21	7	GC-MS	3-7	45.5	29.7	-	-	1	15
(Li et al., 2010)	W3110	TLC	-	-	-	GC-MS	20	35	6	-	17	5	6.5

1.5.2 Bacillus subtilis

Bacillus subtilis is a Gram(+), rod-shaped, aerobic bacterium found in soil and the gastrointestinal tract (McKenney, Driks, & Eichenberger, 2013; Sueoka, 1997). This bacterium is a model for investigation of other pathogenic Gram(+) bacteria such as *Staphylococcus aureus*, *Staphylococcus pneumonia* or *Listeria monocytogenes*. Gram(+) bacteria, such as *B. subtilis*, are distinguished by a thick layer peptidoglycan and a single plasma membrane (Que & Moreillon, 2015).

The plasma membrane is made of phospholipids and embedded proteins. Teichoic acids (TAs), an anionic glycopolymer in a Gram(+) bacteria cell wall, anchor the peptidoglycan layer to the lipids in the bilayer membrane (Naumova et al., 2001) (Figure 1-11). They might be covalently coupled to the peptidoglycan layer (LTA) or anchored to the lipids in the plasma membrane (WTA). TAs have significant functions in the cell wall of Gram(+) bacteria (Bhavsar, Erdman, Schertzer, & Brown, 2004). They produce a negative charge in the cell, developing a proton motive force to be used for chemical or mechanical work. They are also vital for sustaining the cell rigidity and, subsequently, to maintain the cell shape. Furthermore, they can resist adverse conditions such as increasing temperature and salt concentration, as well as β-lactam antibiotics (Campbell et al., 2011).

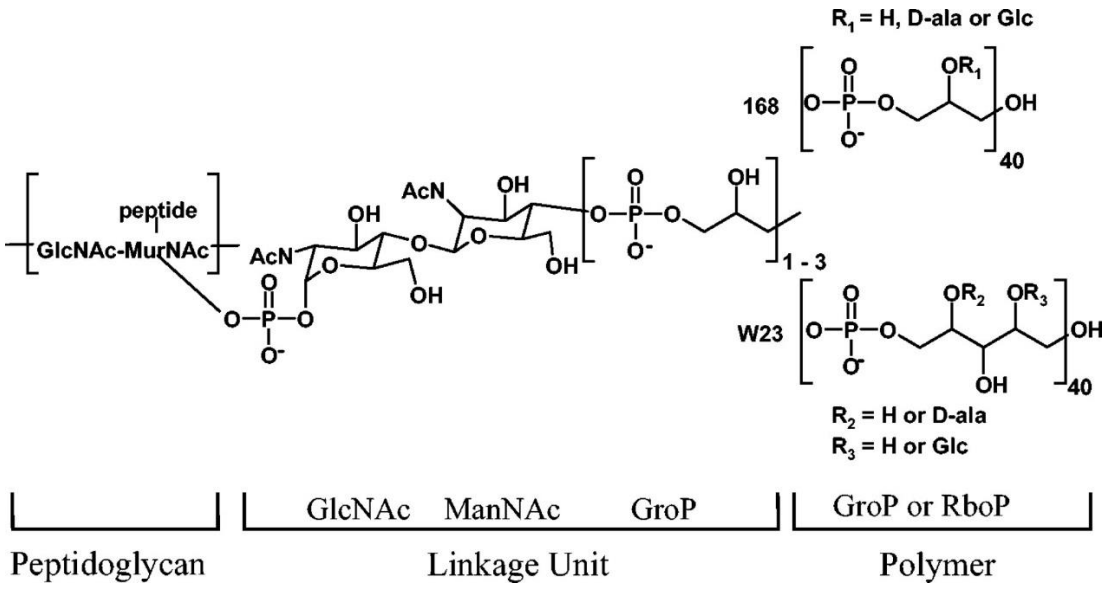


Figure 1-11 Two types of teichoic acid in the 168 and W23 strains of *B. subtilis,* Poly(glycerol phosphate) and poly(ribitol phosphate), respectively (Bhavsar et al., 2004).

Several studies (Bernat et al., 2016; Bierhanzl et al., 2016; Bishop et al., 1967; Li et al., 2010; Lopez, Alice, Heras, Rivas, & Sanchez-Rivas, 2006) were devoted to the characterization of polar heads and FAs by GC-MS (Table 1-2) of the membrane phospholipids of *B. subtilis*. Bierhanz and his colleagues characterized the PLs of SDB206 strain by TLC in the mid-log phase and reported 32% of PE, 54.2% of PG, and the remaining 9% were other lipids (Bierhanzl et al., 2016). The result of TLC experiments in the late-log phase using 168 strain of *B. subtilis* showed that the content of PE, PG, and CL were 34%, 11%, and 49%, respectively (Bishop et al., 1967). It is also confirmed that the contents of PE and PG were dependent of the growth phase while the percentage of CL and other lipids were almost constant in different phases. With the YB886 strain in the late-log *vs.* mid-log phase the contents of PE increased

from 20% to 49% while PG and CL decreased from 40% to 25% and from 22% to 9%, respectively (Lopez et al., 2006). In contrast, the contents of PE, PG, and CL were 32%, 63%, and 3.5% after 24 h of growth and they changed to 63%, 28%, and 7% after 72 h (Bernat et al., 2016). Both experiments consistently reported some other lipids mainly lysilPG.

The GC-MS is the common spectroscopic technique to characterize the FAs in the membrane of *B. subtilis*. In the mid-log phase of PY79 strain, it is reported that C 15:0 (22%), C 15:1 (11%), C 16:0 (22%), and C17:1 (11%) were the predominant FAs (Warnet et al., 2016). Other experiments in the mid-log phase with SDB206 strain reported that C 15:0 (59%), C16:0 (9%), and C17:0 (24%) were three major FAs in the membrane of *B. subtilis* (Bierhanzl et al., 2016). In the first experiment by Laddhari et al, 58% of characterized FAs were saturated while it was 97% in the second study by Bierhanz et al. In the late-log phase, C15:0 (48.8%), C16:0 (19.2%), and C17:0 (25%) were predominant FAs in the 168 strain and almost total (99.6%) of FAs were saturated (Bishop et al., 1967). Some experiments also reported the ratio of anteiso-to-iso FA compositions of different strains of *B. subtilis*. Bishop et al. reported that this ratio was 2.8 for C15:0 and 1.6 for C17:0 in the 168 strain (Bishop et al., 1967). This ratio was 2.4 and 0.7 for C15:0 and C17:0, respectively, in the B-4 strain (Kaneda, 1977).

Table 1-2 Composition of phospholipid headgroups and fatty acids in the membrane of different strains of *B. subtilis*

Study	Strain	Phospholipid headgroups					Fatty acids								
		Lipid analysis	PE (%)	PG (%)	CL (%)	Other lipids (%)	Fatty acid analysis	Saturated	unsaturated	C16:0 (%)	C16:1 (%)	C17:0 (%)	C17:1 (%)	C18:0 (%)	C18:1 (%)
(Bierhanzl et al., 2016)	SDB206	LC-MS/Ms	32	54.	-	9	GC-MS	-	-	8.7	0.7	24	-	3.3	2.3
(Bishop et al., 1967)	168	TLC	34	11	49	6	GC-MS	-	-	19.2	-	25	-	1.6	-
(Lopez et al., 2006)	YB886	TLC + HPTLC	49	25	9	16 LysilPG	GC-MS	-	-	-	-	-	-	-	-
			20	40	22	12 LysilPG		95	5	-	-	-	-	-	-
			31	25	30	12 LysilPG		-	-	-	-	-		-	-
			12	30	41	9 LysilPG		78	12	-	-	-	-	-	-
	CSL2		34	50	-	16 LysilPG		--	-	-	-	-	-	-	-
			20	78	-	1 LysilPG		97	3	-	-	-	-	-	-
			22	70	-	5 LysilPG		-	-	-	-	-	-	-	-
			12	82	-	2 LysilPG		94	6	-	-	-	-	-	-
	PGSA1		53	11	3	31 LysilPG		-	-	-	-	-	-	-	-
			60	12	3	25 LysilPG		-	-	-	-	-	-	-	-
			54	21	5	20 LysilPG		-	-	-	-	-	-	-	-
			52	33	7	8.0 LysilPG		-	-	-	-	-	-	-	-
(Bernat et al., 2016)	DSM 3257	LC-MS/MS	32	63	3.5	1 LysilPG	GC-MS	-	-	10.3	6.3	10.4	1.8	1.3	-
			43	52	3.6	1 LysilPG		-	-	-	-	-	-	-	-
			63	28	7	2 LysilPG		-	-	-	-	-	-	-	-
	l'1a		21	72	3.6	3.4 LysilPG		-	-	11.2	1.4	20	1.1	0.4	-
			20.7	71.5	5.1	2.7 LysilPG		-	-	-	-	-	-	-	-
			17	75.7	5.6	1.7 LysilPG		-	-	-	-	-	-	-	-
(Li et al., 2010)	168	Gc-MS	-	-	-	8.3	GC-MS	-	-	42.3	-	-	-	8.3	31

Deuterium solid-state nuclear magnetic resonance (^{2}H SS-NMR) has attracted more interest because of its advantages over other analytical techniques. It is a non-invasive analytical technique which allows to study the profile of lipid acyl chains as well as the dynamic of bacterial membranes. Besides, in spite of larges molecules which are used in fluorescence analytical methods, deuterium (^{2}H) in SS-NMR is a non-perturbing isotope and does not influence the lipid profile (Davis, Nichol, Weeks, & Bloom, 1979; Seelig & Macdonald, 1987; Strandberg & Ulrich, 2004). ^{2}H SS-NMR has been used repeatedly to better understand the interaction of antimicrobial agents on the membrane of bacteria (Strandberg & Ulrich, 2004). Although this technique can be adapted to investigate the interaction of exogenous molecules with the model membrane, the *in-vivo* study at the early stages should be considered because of the complex structure of biological membranes.

The membrane of wild strains of bacteria could be successfully deuterated as their lipid metabolism is known. *Escherchia coli* is an example of those bacteria which have a simple phospholipid profile including (75% PE, 20% PG and 5% CL) and three main FAs (C16, C17 and C18) (Bogdanov, Mileykovskaya, & Dowhan, 2008; Esfahani, Barnes, & Wakil, 1969). Membrane lipids of L51 and LA8 strains with deuterated FAs have been characterized by ^{2}H SS-NMR; however, the FAs became oxidized because of the mutated strains (Davis et al., 1979; Pius, Morrow, & Booth, 2012).

In this research, therefore, we developed a protocol to label the acyl chains of wild-type bacteria, namely *E. coli* and *B. subtilis*, by incorporation of exogenous PA and OA (Bouhlel et al., 2019; Laadhari et al., 2016; Tardy-Laporte et al., 2013). In addition, we optimized the protocol to characterize and quantify the phospholipid headgroups and FA chains. The protocol was also engineered to identify and quantify the phospholipid headgroups and FA chains as a function of growth condition.

1.6 Research Proposition

Measurement of nuclear magnetic moment backs to 1938, when I. I. Rabi described it in nuclear beams and received the Nobel Prize in 1944 for this work. In 1970s, the development of two-dimensional experiments commenced a new era in NMR spectroscopy. This technique provides the ability to record spectra with two frequency axes while the third being the intensity axis. NMR is a class of absorption spectrometry which measures and observes the interaction of nuclear spins in the presence of a specific magnetic field. This technique relies on the fact that any compound, for example phospholipids, can absorb a certain portion of the generated electromagnetic radiation energy in the radio frequency (Becker, 1993) region. The frequency in which the absorption occurs differs for any nuclei in the structure of molecules. An important class of lipids is phospholipids, all of which have a phosphate group. NMR of phosphorus-31 (^{31}P NMR) is a method of choice in recognition of phospholipid phases, and more generally in the study of membrane systems. In an organic solution, each phospholipid can be discriminated according to the chemical shift of ^{31}P, nuclei spin ½, natural abundance 100%, and relatively high sensitivity (Gerothanassis, Troganis, Exarchou, & Barbarossou, 2002).

^{31}P NMR has been used many times to identify and quantify phospholipids in the nature. The most common phospholipids in bacterial membranes are phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL) (Peterson & Cummings, 2006). There are two major types of bacteria , i.e., Gram(+) and Gram(-). Gram(+) bacteria have a relatively thick cell wall composed of peptidoglycan (20-80 nm), while Gram(-) bacteria have a thin layer of peptidoglycan (2-3 nm) conjugated to an outer membrane (Madigan et al., 2014).

The existing PLs in the bovine liver, including phosphatidylethanolamine (PE), lysophosphatidylethanolamine (lyso-PE), phosphatidylserine (PS), lysophosphatidylserine (lyso-PS), sphingomyelin (SPH), lysophosphatidylcholine (lyso-PC), phosphatidylglycerol (PG), and cardiolipin.(CL) were characterized relative to their chemical shifts (Henderson, Glonek, & Myers, 1974). The results showed the dependency of the signals to the solvent such that it came to the resonance at higher frequency with benzene compared to chloroform-methanol (2:1) solvent. When methanol was added to chloroform, the chemical shifts of PC and PE decreased by 25 and 12 Hz while the chemical shift of lyso-PC experienced a slight increment of 3 Hz. The crude lipids which were extracted from porcine spleen were quantified by ^{31}P NMR in two solvents, namely $CHCl_3$–CH_3OH–H_2O–EDTA and triethylamine, dimethyl-formamide and guanidinium chloride (Et_3–DMF–GH^+) (Culeddu, Bosco, Toffanin, & Pollesello, 1999). The efficiency of the analytical technique in terms of variation of chemical shifts was higher with the second solvent (less than ±0.02 Hz) vs. the first solvent (less than ±0.14 Hz).

The evidences collected by ^{31}P NMR also revealed that the lipid profile and production of the bacteria depend on the cell cycle (Furse, Wienk, Boelens, de Kroon, & Killian, 2015). In the MG1655 strain of *E. coli* from B to D cell cycle, PG approximately increased by 1.7 times while CL was almost remained constant. These finding highlight the importance of the control over the production of membrane lipids at different growth stages to fulfill the success cell division. The lipid profile of extracted PLs from *S. aureus* had sensitivity to the pH in which the LPs were extracted and bulked (Rehal et al., 2017). For example, the content of lysilPG can differ between 30% to 55% of the whole lipid content in different strains of this bacterium at two pH of 5.5 and 7.4 (Rehal et al., 2017). This variation directs the attention to the important role of pH in the process of lipid characterization by spectrometry techniques, i.e. ^{31}P NMR.

Traditionally, model membranes were used to investigate the effect of AMPs, but ^{2}H SS-NMR can now be done *in vivo* on deuterated bacteria such as *E. coli*, *B. subtilis*, and *Vibrio splendidus*, developed in our laboratory (Booth, Warschawski, Santisteban, Laadhari, & Marcotte, 2017). To do so, bacteria are grown in a medium enriched with deuterated palmitic acid (PA-d_{31}) without or with oleic acid (OA). The fatty acids (FAs) are micellized in a detergent.

Previous experiments have already shown that incorporation of PA-d_{31} perturbed the saturated/unsaturated FA ratio in *E. coli* membranes (Warnet, Laadhari, Arnold, Marcotte, & Warschawski, 2016). On the other hand, adding OA to the growth culture can restore the S/U FA ratio and provide a ratio closer to the "natural unlabeled *E. coli*" (Tardy-Laporte et al., 2013). These exogenous FA chains cross the *E. coli* membrane with the help of the long-chain FA transport protein (fadL). Within the cell, the amount of FA chains is negligible because the bacteria use it to produce phospholipids through two mechanisms. The majority of exogenous FAs are transformed to acyl-Coenzyme A in the cytoplasm. Acyl-Coenzyme A can be degraded in the β-oxidation cycle or might be used for phospholipid synthesis (Byers and Shen, 2002; Jiang et al., 2010; Zhang and Rock, 2008). The second mechanism is the synthesis of PE by direct conversion of free FA chains which are coupled to acyl carrier protein (Byers and Shen, 2002). These two physiological mechanisms ensure that incorporation of exogenous FAs into phospholipids is without any elongation or modification in their structure.

As described above, lipid composition (headgroups and FAs) of bacteria has never been studied thoroughly as a function of the ^{2}H-labeling conditions.. In addition, there is no standard protocol to determine the lipid composition of bacteria accurately. Liquid chromatography combined to mass spectrometry (LC-MS) is a complex and expensive method that is not used routinely (Oursel et al., 2007), and many laboratories use gas chromatography coupled with MS (GC-MS) to determine the FA composition. High

Performance Liquid Chromatography (HPLC) is a well-established, quick, and precise, however, analysis by HPLC is time consuming, requires high amount of solvents, and has lower resolution compared to other techniques such as GC-MS. Thin-layer chromatography (TLC) is often used to identify headgroups, but determination of FAs is difficult because of low resolution and precision in its quantification (Ames, 1968; Bishop, Rutberg, & Samuelsson, 1967; Li et al., 2010). Nuclear Magnetic Resonance (NMR), rather the other spectrometry techniques, allows to characterize the structural composition, for example the lipid profile, while the sample is till intact. This technique uses different nuclei and provide the information based on the emitted energy in a magnetic field. ^{31}P NMR has been proposed as an efficient analytical tool but has mostly been used for eukaryotic cells (Estrada, Stolowich, & Yappert, 2008).

GC-MS serves as an analytical technique to characterize the FA composition of lipids. These results will hopefully allow researchers and clinicians to investigate the interaction between the cell membrane of pathogens and antimicrobial agents. Marcotte et al. developed a protocol to deuterate FA chains in *E. coli* and *B. subtilis* (Laadhari, Arnold, Gravel, Separovic, & Marcotte, 2016; Tardy-Laporte et al., 2013), which allows *in vivo* study of membrane interactions by using ^{2}H solid-state NMR.

To improve the ^{2}H-labeling conditions at the basis of ^{2}H SS-NMR studies of membrane interactions of AMPs with intact bacteria, the objective of this project was to characterize the phospholipids in *E. coli* and *B. subtilis* under various deuterium labeling conditions. The specific objectives were:

- To optimize the labeling of *E. coli* and *B. subtilis* by incorporating exogenous in PA-d_{31} and OA in the growth medium.;
- To optimize the protocol for lipid characterization and quantification (headgroups and FA chains) in a bacterial sample;

- To identify and quantify the phospholipid headgroups and FA chains in *E. coli* and *B. subtilis* membranes, as a function of the growth conditions (labeled and non-labeled).

We propose a new routine protocol in which the whole lipid composition of bacteria can be rapidly determined in a couple of days. After bacteria have been collected, we extract the lipids and identify and quantify their headgroups by ^{31}P solution NMR. Then we hydrolyze and methylate their FAs, identify and quantify them by GC-MS.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials and Methods

2.1.1 Materials

All solvents such as HPLC grade dichloromethane, methanol, and hexane, as well as palmitic acid (hexadecenoic acid) and oleic acid (octadic-9-enoic acid) were purchased from Sigma Aldrich (St. Louis, MO, USA). Peptone A from meat was obtained from Bio Basic (Markham, ON, Canada). Polyoxyethylene sorbitan monolaurate (Tween 20) was purchased from Bishop (Burlington, ON, Canada).. Deuterochloroform ($CDCl_3$) was purchased from Cambridge Isotope Laboratories, Inc (Tewksbury, MA, USA).

2.1.2 Sample preparation

All experiments were performed in triplicates.

2.1.2.1 *Escherichia coli* growth

Escherichia coli BL21 was obtained from the Department of Microbiology, Infectiology, and Immunology-University of Montreal, Canada. This bacterium was grown at 37 °C overnight on a shaker in Luria Broth (LB) culture containing peptone,

NaCl, and yeast extract in deionized water at pH 7.2. The culture of *E. coli* was collected at the exponential mid-log phase (optical density at 600 nm (OD_{600nm})) and centrifuged at 3400 rpm for 10 min to separate pellets from the nutrient solution. After washing with deionized water and centrifuging, the pellets were freeze-dried and stored at -20 °C. The mass of pellets obtained after this procedure was 60-70 mg per 300 ml of culture (Supporting information is available in Appendix A).

2.1.2.2 Incorporation of palmitic acid without or with OA membrane in *Escherichia coli*

Escherichia coli BL21 were grown in batch culture as described above, in the presence of 0.19 mM of PA without or with OA (0.19 mM) micellized in Tween 20 (0.15 mM) using three series of freeze (liquid N2) / thaw (90°C) / vortex shaking cycles. Briefly, the cultures were centrifuged to separate pellets from the nutrient solution. Then, the pellets were washed twice with nanopure water and stored at -20 °C. The mass of pellets obtained after this procedure was 60-70 mg in 300 ml of culture (Supporting information is available in Appendix A).

2.1.2.3 *Bacillus subtilis* growth

A strain of *Bacillus subtilis (B. subtilis)* 168 was obtained from the Bacillus Genetic Stock Center at Ohio State University. *B. subtilis* was grown at 37 °C overnight on a shaker in Luria Broth (LB) culture containing peptone, NaCl, and yeasts in deionized water at pH 7.2. Bacteria were collected at the exponential mid-log phase (OD_{600nm}=0.8) and centrifuged at 3400 rpm for 10 min to separate pellets from the nutrient solution. After washing twice with nanopure water and centrifuging, the pellets were freeze-dried and stored at -20 °C. The mass of pellets obtained after this procedure was 45-55 mg in 300 ml of culture (Supporting information is available in Appendix A).

2.1.2.4 Incorporation of palmitic acid in *Bacillus subtilis* membrane

In previous published experiments, a concentration of 0.19 mM of PA was added to the bacteria (Davis 1979; Tardy-Laporte 2013; Warnet 2016; Laadhari 2016). In the present study, we have tried different concentrations of PA (0.02 mM, 0.05 mM, 0.1 mM, and 0.19 mM). They were micellized with Tween 20 (0.15 mM) using series of freeze (liquid N_2) / thaw (90°C) / vortex shaking cycles, until the PA crystals had melted. *B. subtilis* 168 was grown in LB medium in the presence of PA/Tween 20 at 37 °C under shaking (220 rpm) until reaching an OD_{600nm} of 0.8 at the exponential mid-log phase. The culture was collected as described in the previous section and its mass was 45-50 mg in 300 ml of culture (supporting information is available in Appendix A).

2.1.2.5 Incorporation of palmitic acid and oleic acid in *Bacillus subtilis* membrane

Palmitic acid (0.19 mM) and oleic acid (0.19 mM) were micellized with Tween 20 (0.15 mM) using three series of freeze (liquid N_2) / thaw (90°C) / vortex shaking cycles until PA crystals had melted. Then, *B. subtilis* was grown in the presence of PA/OA/Tween 20 in the culture medium until the mid-log phase at OD_{600nm} of 0.8. The culture was collected as described in section 2.1.2.3. Bacteria were centrifuged at 3400 rpm for 10 min to separate pellets from the nutrient solution. After washing twice with nanopure water and centrifuging, the pellets were freeze-dried and stored at-20 °C. The mass of pellets obtained after this procedure was 35-40 mg in 300 ml of culture (Supporting information is available in Appendix A).

2.1.2.6 Phospholipid extraction

Cell pellets from *B. subtilis* and *E. coli* in the presence and absence of PA/OA were extracted following the Folch method (Folch, Lees, & Sloane Stanley, 1957) by adding 100 ml of dichloromethane-methanol (2:1). The dry pellet of bacteria was sonicated in the solvent for 10 min at 8W (sonicate for 1 min and put in an ice bath for 1 minute,

repeat this process five times). Then 27 ml of KCl was added (0.88% W/V) to the pellet. The two phases were separated by shaking and waiting for 20 min. The lower phases were collected and dried by heating (40 °C) under a nitrogen gas stream. Samples were then kept at -80 °C. The mass of lipids obtained after this procedure was 3-4 mg in both bacteria (labeled/unlabeled). (Detailed protocol can be found in the supporting information in Appendix B).

For solution ^{31}P NMR and identification of PL headgroups, the lipids were solubilized in an optimized organic solvent made of 500 µL of deuterated chloroform ($CDCl_3$), 200 µL of methanol, and 50 µL of *EDTA* solution (Estrada et al., 2008) as described in supporting information in Appendix B). In addition, we strived to characterize the headgroups of phospholipids in the membrane of *B. subtilis* by using thin-layer chromatography (TLC). This technique uses the fundamental concept of chromatography in which there are stationary and mobile phases. In general, when a mixture is introduced to the thin layer of the stationary phase, e.g. silica gel, its components flow at different rates depending on their affinity for the phases which enable the examiner to distinguish them. The developed protocol for TLC is presented in Appendix D.

2.1.2.7 ^{31}P NMR experiment

Spectra were acquired using a 600 MHz AVANCE III-HD solution NMR spectrometer from Bruker (Milton, On, Canada), equipped with a probe operating at 242.84 MHz for ^{31}P detection. Typically, ^{31}P NMR spectra were recorded at 298 K with a 90º pulse of 15 µs, a recovery delay (D1) of 10 s, ^{1}H decoupling. Typically, 128 scans were acquired, with a spectral width of 50 ppm and 8k data points (Figure 2-1).

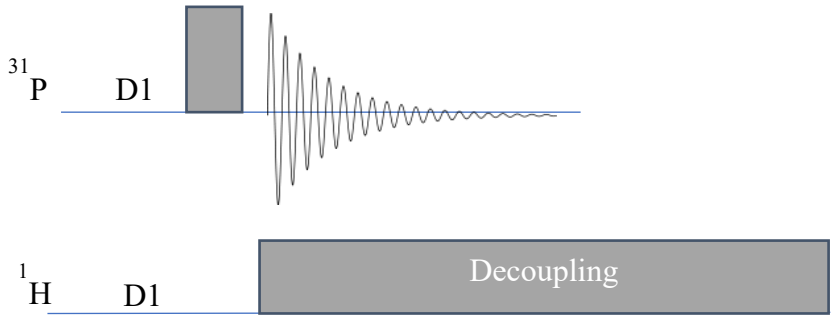


Figure 2-1 Schematic of the pulse sequence of ^{31}P NMR experiment

2.1.3 Gas Chromatography-Mass Spectroscopy

2.1.3.1 Introduction

Coupling gas chromatography-mass spectrometry is one of the most widely used techniques of analytical chemistry (Skoog, Crouch, & Holler, 2007). In this combination, gas chromatography (GC) is a physicochemical technique that has been favorably used for many years to separate volatile and semi-volatile compounds, but it cannot identify the compounds. Indeed, gas chromatography separates compounds according to their partition coefficient between two different phases (Skoog et al., 2007). Mass spectrometry (MS) is used to provide detailed structural information of compounds and also to identify and quantify components. This technique allows to precisely identify and quantify numerous substances presented in minimal quantities, even in traces.

In the procedure of GC-MS, a mixture of dissolved substances is injected into a long (generally rolled) column for which inner walls are coated with a chromatographic material. The flow of a carrier inert gas makes the compounds sweep to the outlet of

the column. Compounds which have a higher vapor pressure at low temperature, i.e., volatile, spend more of their time in the gas phase and are the first to come out of the column and to be detected. Once the separation process is completed, each substance is sent to the GC coupled mass spectrometer. In MS procedure, the substance is ionized by electron ionization (EI), producing the positively-charged molecular ion with the same chemical formula as the non-ionized substance. The resulting ions are sorted according to their masse/charge ratio by a quadrupole analyzer. A mass spectrum is a set of peaks derived from the molecular ion and its fragments, and it represents a fingerprint of an organic compound. The ability of GC-MS spectrometry to accurately detect one or more molecules in a very complex sample provides an ideal detector for the analysis of products in pharmacology, toxicology, medicine, environment, petrochemical, and organic synthesis (Sneddon, Masuram, & C. Richert, 2007).

2.1.3.2 Methylation of lipids

Although whole phospholipids are analyzed by ^{31}P NMR, GC-MS requires to separate the FA chains from the lipid headgroup by hydrolysis followed by methylation. To do so, 2 ml of H_2SO_4 (in 2% MeOH) and 800 µL of toluene were added to around 60 mg of bacterial pellet. Suspensions were heated for 10 min at 100^0 C. Then, 4 ml of nanopure H_2O and 800 µL of hexane were added to 2-3 mg of extracted lipids. Tubes were vortexed and left to stand until two phases were obviously separated. Then, the phase was transferred to a new vial. Finally, the FAMEs were dried under a nitrogen stream and suspended in 500 µL hexane to be analyzed by GC-MS. The mass of FAs was 2-3 mg for both bacteria (labeled/unlabeled). Supporting information is available in Appendix C.

2.1.3.3 GC-MS experiment

The standard FAME mix (C4-C24) and FAMEs were resuspended in hexane at a concentration of 1 mg/mL and the analyses were performed by GC-MS using an Agilent Technologies 7890 A GC/ 5975C MS system. A HP-5MS (Santa Clara, CA, USA) silica capillary of 30 m × 250 µm × 0.25 µm film thickness was used and 1 µL of the sample was injected into it under split mode (50:1) conditions. The temperature of the column was programmed to be held at 140 °C for 5 min, then increased to 300°C at a rate of 4°C/min. Helium was the carrier gas at a constant flow rate of 0.5205 ml/min. Electron ionization (EI) at 70 eV was done. In positive mode, the scan was conducted at full-scan mode with the m/z interval of 40-600. The total retention time was approximately 40 min for each sample. To identify the peaks, the retention times and the values of mass were compared with the standard FAME mix. Then, the confined area under each peak was compared to that of the FAME mix to calculate the percentage of each FA.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Effect of exogenous FAs on bacterial growth

In previous work, we have deuterated the lipid acyl chains in *E. coli* and *B. subtilis* using PA-d_{31} and OA micellized in Tween20 (Laadhari et al., 2016). We first measured the effect of these FAs on the bacterial growth at the concentrations and temperature used in our publication, i.e., 0.19 mM for both FAs, and 37 °C, respectively. Here, protonated PA was used instead of PA-d_{31} to lower the cost of the experiments.The growth curves were measured over time using a microplate reader and using a spectrophotometer, as shown in Figure 3-1 to Figure 3-4.

These two photometry techniques count the bacterial growth rate by absorbance of a light which passes through the sample. Microplate reader uses a vertical light beam whereas it is horizontally in spectrophotometer. Because of these two different principles, the absorbance for a sample might be sensitive to the photometry technique and consequently the determine growth rate may change. To confirm that the identified mid-log and late-log phases are identical, we used both techniques to validate our measurements.

The figures show little effects of the exogenous FAs on the growth of both *E. coli* and *B. subtilis*, as previously described (Tardy-Laporte et al., 2013). Moreover, no significant difference is observed between *B. subtilis* grown in the presence of 0.10 or 0.19 mM of PA (Figure 3-4).

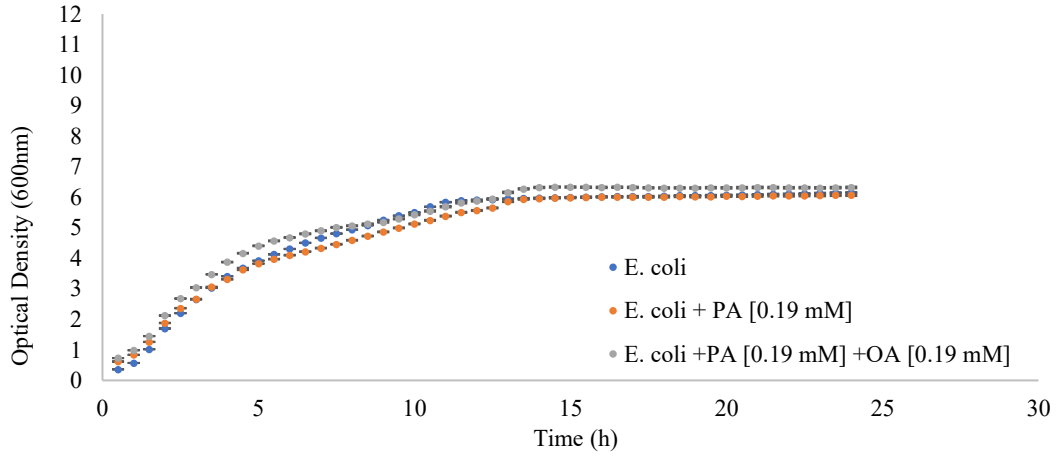


Figure 3-1 Growth curves of *E. coli* without (blue), and with PA (orange) and OA (grey) at 37ºC (measured by microplate reader). Tween 20 was used as a detergent to micellize the FAs.

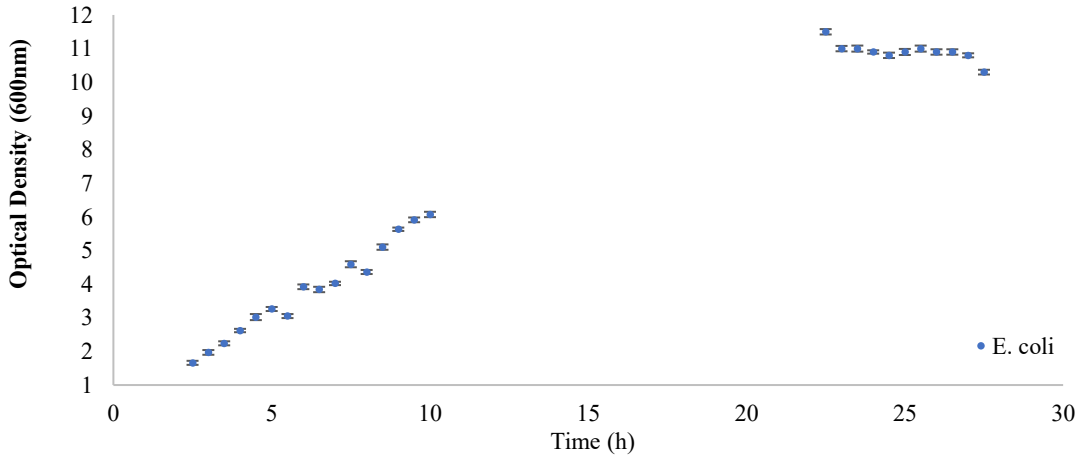


Figure 3-2 Growth curve of *E. coli* at 37 °C measured by spectrophotometer. Tween 20 was used as a detergent to micellize the FAs.

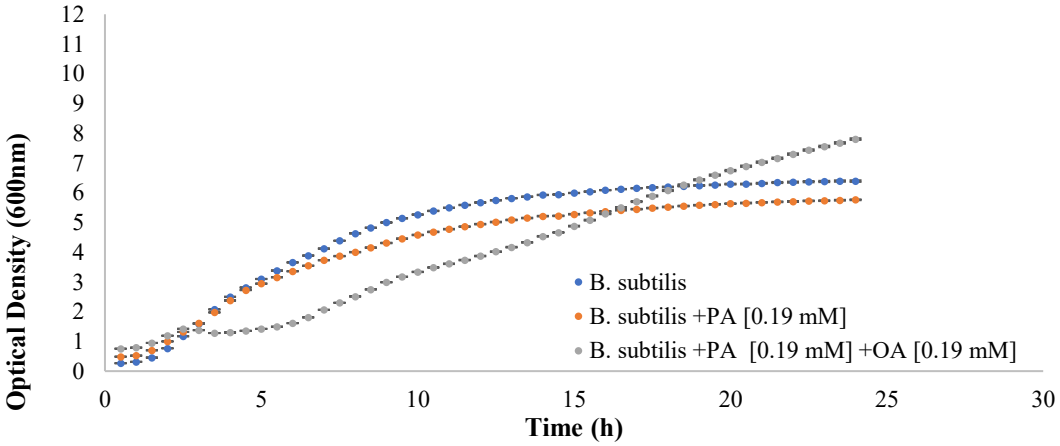


Figure 3-3 Growth curves of *B. subtilis* without (blue), and with PA (orange) and OA (grey) at 37°C with microplate reader. Tween 20 was used as a detergent to micellize the FAs.

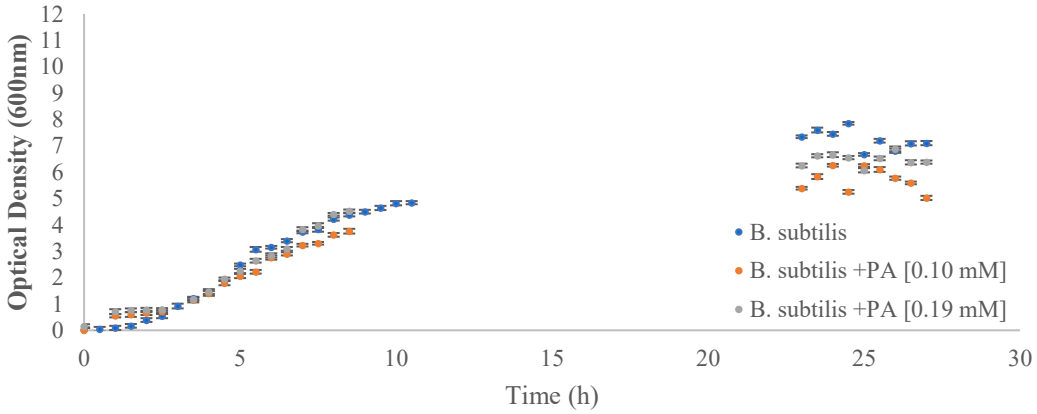


Figure 3-4 Growth curves of *B. subtilis* without (blue), and with PA at a concentration of 0.10 mM. (orange) and 0.19 mM (grey) at 37°C (Measured by spectrophotometer). Tween 20 was used as a detergent to micellize the FAs.

3.2 Escherichia coli

3.2.1 Characterization of the phospholipid profile by ^{31}P NMR Spectroscopy

In the present work, the membrane lipids from the wild-type Gram(-) bacteria *E. coli* strain BL21 have been studied. This bacterium is one of the foremost prokaryotic model organisms. The effect of PA without and with OA on the lipid was studied under different culture conditions in the exponential phase (mid-log) (2.1.2.2). The ^{31}P NMR was conducted in the exponential phase considering the double growth of bacterial cells after each generation. After this phase, the bacterial cells have a constant number (stationary phase) with equal number of growing and dying bacteria. Afterward, it decreases over time until all the cells die (death phase) (Madigan et al., 2014)

To determine the lipid headgroup profile, ^{31}P NMR spectra were recorded from lipid extracts of *E. coli* grown at 37º C. Out of a variety of organic solvent mixtures suggested in the literature, we have selected the one by Estrada et al. 2008 which consists of deuterochloroform ($CDCl_3$), methanol, and *EDTA*. The polar lipid compositions of *E. coli* strains were identified on the basis of the average chemical shift values of the abundant PLs at 0.77, 0.51, and 0.21 ppm corresponding to PG, CL and PE, respectively (Flieger et al., 2000) (Figure 3-5).

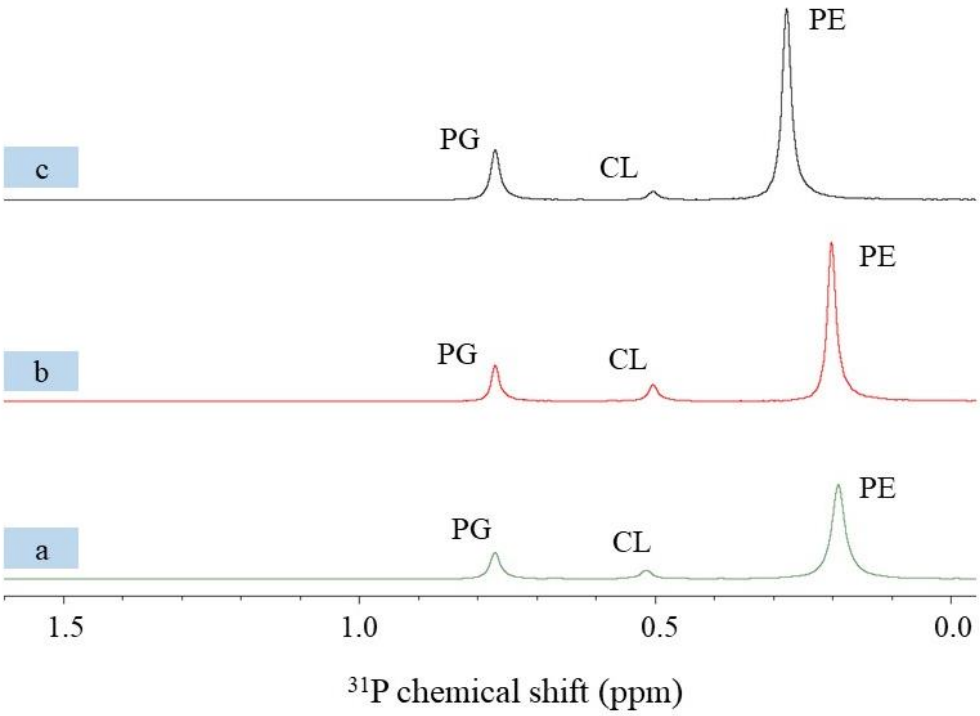


Figure 3-5 ^{31}P NMR spectra of the extracted lipids from a) *E. coli*, b) *E. coli* grown in the presence of PA, and c) *E. coli* grown in the presence of PA and OA. The phospholipid content is identified, and PE is ascribed to phosphatidylethanolamine, PG to phosphatidylglycerol, and CL to cardiolipin.

The lipid profile of the main polar lipids in *E. coli* (control) included PG, CL, and PE. As in Table 3-1 and Figure 3-6, addition of PA and OA in the growth medium have no significant effect on *E. coli's* lipid headgroup profile. The bacterial membranes are composed of about 80% PE, 17% PG and 3% cardiolipin.

Table 3-1 Characterized phospholipids of *E. coli* grown in different conditions, as determined by ^{31}P NMR (Mean and standard deviation of n=3 sets).

Organism \ Headgroups	PE (%)	CL (%)	PG (%)
***E. coli* (Control)**	80.9 (1.0)	2.6 (0.7)	16.5 (1.0)
***E. coli* + PA**	80.2 (0.8)	3.4 (0.8)	16.4 (0.4)
***E. coli* + PA + OA**	79.4 (0.8)	2.4 (0.9)	18.1 (1.7)

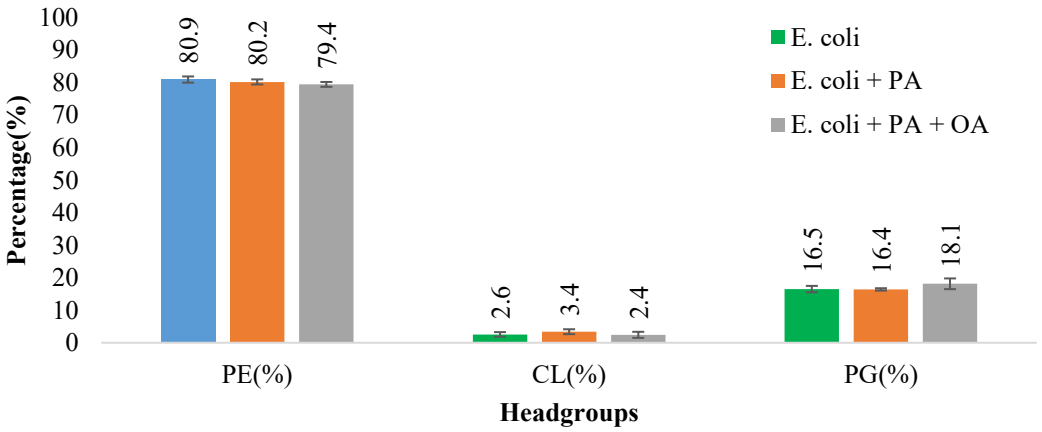
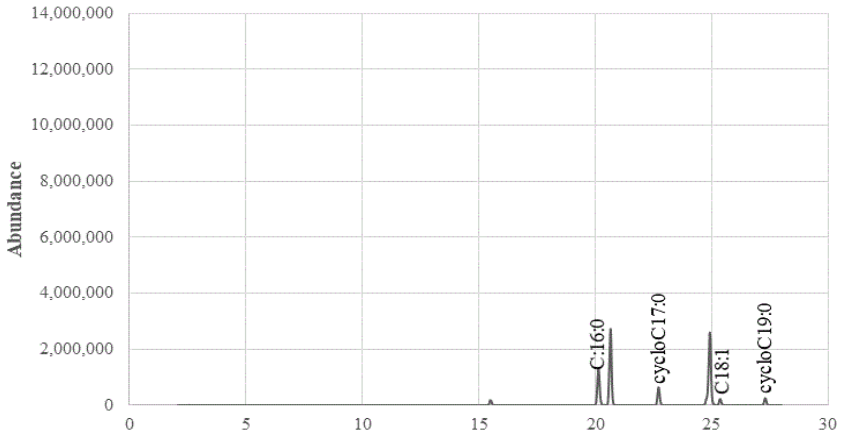


Figure 3-6 Phospholipid composition of *E. coli* grown in different culture conditions, as determined by ^{31}P NMR

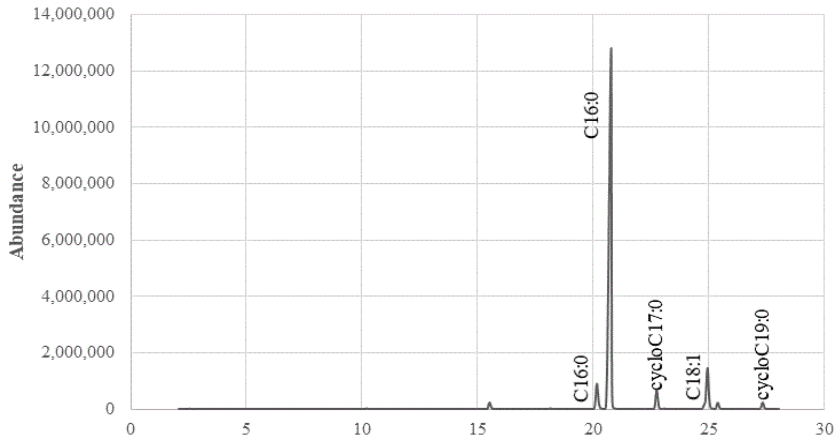
3.2.2 Analysis of fatty acid composition by GC-MS

The profile of FA chains in the membrane of *E. coli* was analyzed by GC-MS under three growth conditions: 1) in absence of PA, 2) in the presence of PA, and 3) in the combined presence of PA and OA. There were mainly seven extracted FAs, including C14:0, C16:0, C16:1, cycloC17:0, C18:0, C18:1, and cycloC19:0 (Figure 3-7), saturated and unsaturated.

a



b



c

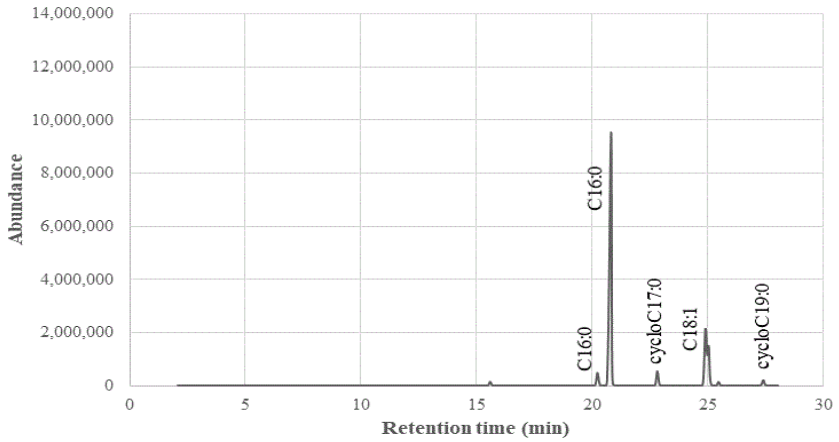


Figure 3-7 GC-MS total ion chromatogram (TIC) of fatty acid methyl esters (FAMEs) from a) *E. coli,* b) *E. coli* grown in the presence of palmitic acid (PA), and c) *E. coli* grown in the presence of palmitic acid (PA) and oleic acid (OA).

As reported in Figure 3-7 and Table 3-2 and summarized in Fig. 3-8, in unlabeled *E. coli*, palmitic acid (16:0) and oleic acid (18:1) are the predominant FAs (respectively 34% and 37%), in good agreement with the results (36%) previously reported by Tardy-Laporte et al., (2013). Li et al. found 35% of C16:0 in the W3110 strain of *E. coli* at 37° C which is also consistent with our finding (Li et al., 2010). Although Morein et al. reported 45% of C16:0 in the K12 strain, their result became 35% when they decreased the growth temperature to 17° C (Morein et al., 1996).

When PA was added to the *E. coli* culture, the percentage of C16:0 increased from 34.0% to 78%. Tardy-Laporte et al. found proportion of C16:0 of 36% in unlabeled membrane of wild type *E. coli*, while C16:0 and C16:0D amounted for 7% and 76%, respectively, in the labeled bacteria (Tardy-Laporte et al., 2013). Adding the combination of PA and OA to the culture intensified the percentage of C18:1 from 12% to 31% while C16:0 decreased from 78% to 56% (Figure 3-8). The same trend was reported by Warnet *et al.* such that C:16:0 was 39% in the unlabeled membrane of *E.coli* and increased to 55%

when the membrane was labeled by PA (Warnet et al., 2016). Labeling the membrane with the combination of PA and OA caused a decrease in C 16:0 from 55% to 45% and increased the percentage of C18:1 from 14% to 23% (Warnet et al., 2016).

Table 3-2 Fatty acid compositions under various growth conditions for *E. coli* (Mean value with standard deviation of n=3 sets). Ratio of saturated and unsaturated fatty acids (S/U) is also reported.

Organism / Fatty acids	*E. coli* (Control) (%)	*E. coli* +PA (%)	*E. coli* +PA + OA (%)
C14:0	2.6 (0.2)	0.8 (0.8)	1.4 (0.3)
C16:0	34.2 (1.1)	77.8 (1.8)	56.4 (7.5)
C16:1	12.0 (0.5)	3.3 (0.7)	3.2 (0.7)
cycloC17:0	8.5 (1.3)	3.5 (1.7)	4.8 (1.3)
C18:0	2.1 (0.6)	1.2 (0.2)	1.0 (0.3)
C18:1	37.3 (1.3)	12.0 (2.8)	30.6 (5.7)
cycloC19:0	3.2 (0.4)	1.2 (0.6)	2.2 (0.5)
Others	Trace	Trace	Trace
Saturated Fatty Acids	38.8	79.8	58.8
Unsaturated Fatty Acids	61.1	20.1	41.1
Ratio (S/U)	0.6	4.0	1.5

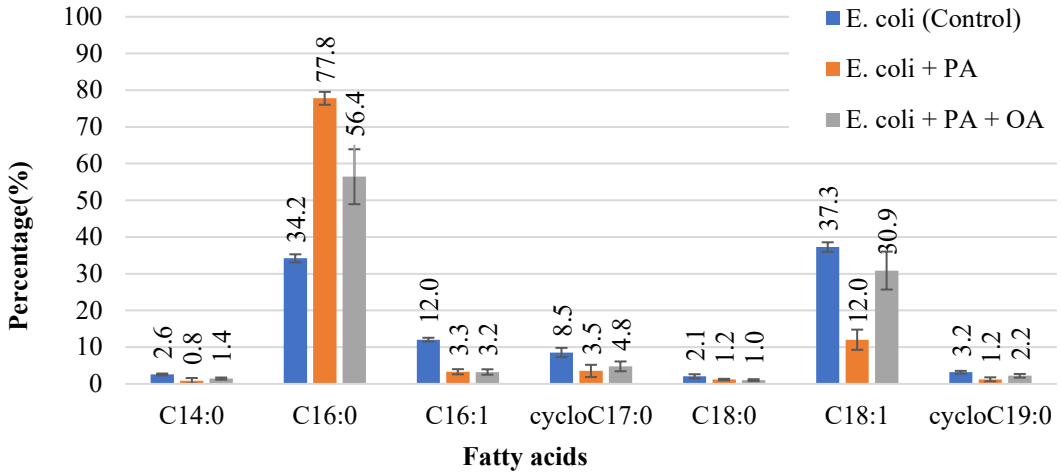


Figure 3-8 Fatty acid composition of the labeled and unlabeled *E. coli* analyzed by using GC-MS.

The ratio of saturated-to-unsaturated fatty acids (S/U) increased from 0.6 to 4.0 when *E. coli* was labeled with PA; however, it decreased from 4.0 to 1.5 by using the combination of PA and OA (Figure 3-9). Therefore, the presence of OA in the culture provided a S/U ratio closer to the "native" unlabeled *E. coli* which is about 0.6.

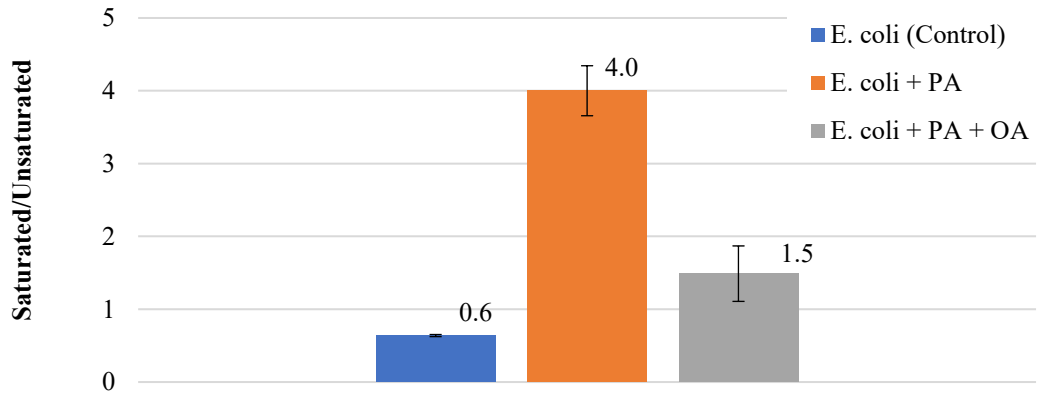
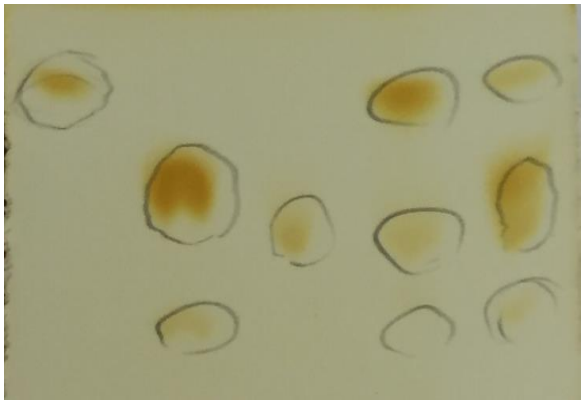


Figure 3-9 The ratio of saturated and unsaturated fatty acids in *E. coli* strain under different conditions.

3.3 Bacillus subtilis

3.3.1 Characterization of phospholipid profile by TLC

The results of thin-layer chromatography (TLC) showed that the membrane of *B. subtilis* contains the same main headgroups as those of tested synthetic phospholipids (Figure 3-10). Also, the mixture of synthetic phospholipids had similar composition as the results of extracted lipids from the membrane of *B. subtilis*. These findings should be verified by ^{31}P NMR to further evaluate the feasibility of this characterization technique.



PE CL PG S M

Figure 3-10 Representative TLC comparing extracted lipids from *B. subtilis* (S), a mixture of synthetic lipids (M) and individual lipids Phosphatidylethanolamine (PE), Cardiolipin (CL), and Phosphatidylglycerol (PG).

3.3.2 Characterization of phospholipid profile of *B. subtilis* by ^{31}P NMR spectroscopy

Although TLC is a fast and low-cost analytical technique, it is associated with some limitations. Exposure of samples to the atmosphere environment may oxidize or hydrolyze the phospholipids. TLC is not as reproducible and sensitive as other characterization techniques are, such as ^{31}P NMR. The last but not the least is that a reference, which should be known beforehand the experiments, for identification of any compound in the sample is required. We, therefore, chose ^{31}P NMR as our main characterization technique to identify the phospholipids of *B. subtilis*.

The extracted lipids from *B. subtilis*, which was grown under various conditions and harvested at mid-log phase, were dissolved in the chloroform-methanol-*EDTA* solvent mixture previously described. To determine the chemical shift of each existing phospholipid in the bacterial membrane of *B. subtilis*, different combinations of PE, PG, and CL as synthetic model membranes (commercial standard) were tested. Three polar lipids, namely PE, PG, and CL, were identified in different *B. subtilis* cultures at the average chemical shift values of 0.21, 0.77, and 0.46 ppm, respectively. It was interesting that a new signal of phospholipid at 0.13-0.16 ppm was recorded, which was absent in *E. coli.* Figure 3-11 represents the ^{1}H-decoupled ^{31}P NMR spectrum, showing four lipids, in the labeled (with 0.19 mM PA) and unlabeled *B. subtilis* membranes.

The presence of the 4th peak implies that there is one more unknown phospholipid in the *B. subtilis* membrane at chemical shift of 0.13 to 0.16 ppm and it should be identified. To do so, we followed a procedure to investigate the potential scenario. This peak might be PA, but the comparison with the chemical shift in the literature (Estrada et al., 2008) rejected this assumption. Furthermore, the reported chemical shift of phosphatidic acid (PA) was reported around 5.4 ppm (Cremonini et al., 2004; Culeddu

et al., 1999) which is far enough from the 4th chemical shift we found. The second possibility is to assign this peak to PS.

To test this hypothesis, we performed the analysis with the proposed protocol in the literature (Culeddu et al., 1999) by preparing three organic solvents. The first solvent was made by combining $CDCl_3$, MeOH, and *EDTA* (Flieger et al., 2000); the second solvent, *CUBO*, contained dimethylformamide, triethylamine, and guanidinium (Cremonini, Laghi, & Placucci, 2004; Culeddu, Bosco, Toffanin, & Pollesello, 1999; Furse et al., 2017; Furse, Wienk, Boelens, de Kroon, & Killian, 2015; Murgia, Mele, & Monduzzi, 2003); the third solvent was composed of $CDCl_3$, MeOH, and NaCl (Rehal et al., 2017). The prepared solvents were tested on *B. subtilis* as well the synthetic models including POPG/POPE/CL/POPS, and POPG/CL, then the phospholipids in the membrane and model membrane were identified (Figure 3-11). In *CUBO* solution, however, the resulted peaks were broader and the chemical shifts slightly displaced (PG at 1.25 ppm, CL at around 0.7 ppm, PE around 0.2 ppm, and the fourth lipid around 0.55 ppm (Culeddu et al., 1999; Furse et al., 2017). Comparison of the identified synthetic phospholipids and *B. subtilis* allowed confirming the presence of PE, PG and CL in the bacteria. There is less information about the 4th peak, but putting together, the results of analyses to test our hypotheses showed that chemical shift did not agree with that of PS or PA (Culeddu et al., 1999; Murgia et al., 2003). Thus, our hypothesis is that the 4th peak corresponds to lysilPG (Figure 3-13). In conclusion, similar to the available literature which characterized the *B. subtilis* membrane, we identified four PLs including PE, PG, CL, and lysilPG (Lopez et al., 2006; Lopez, Heras, Ruzal, Sanchez-Rivas, & Rivas, 1998).

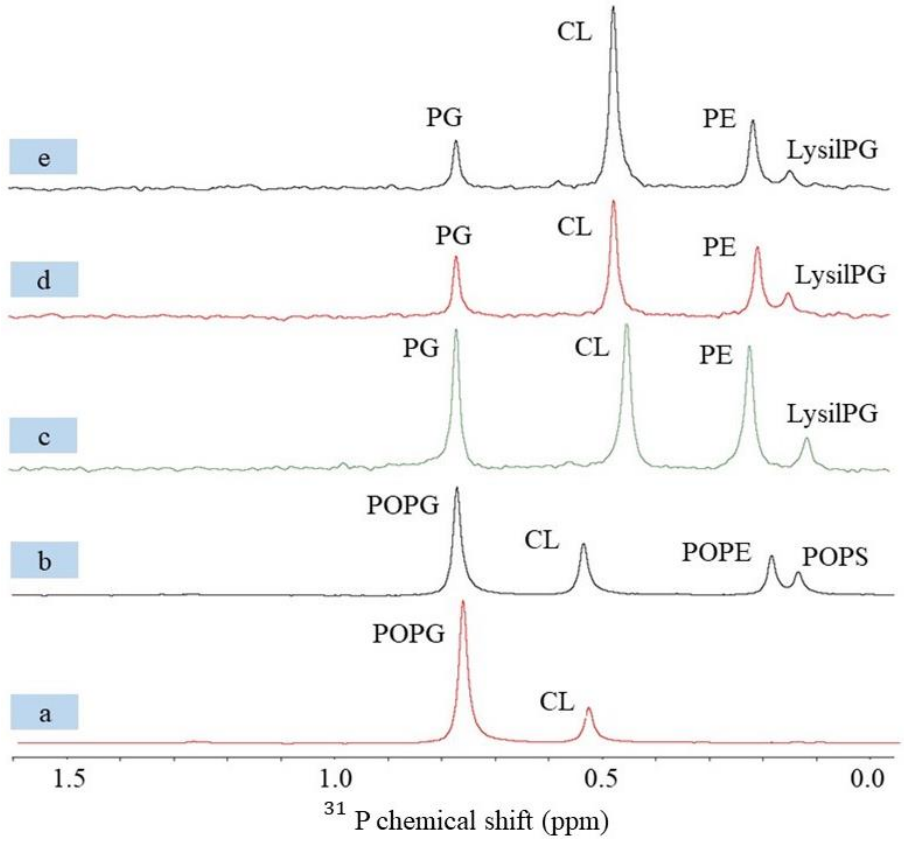


Figure 3-11 ^{31}P NMR spectra, at the mid-log phase, of the a) lipid mixture of 80% POPG, 20% CL, b) lipid mixture of 40% POPG, 30% CL, 20% POPE, and 10% POPS, c) *B. subtilis*, d) *B. subtilis* grown in the presence of palmitic acid, and e) *B. subtilis* grown in the presence of palmitic and oleic acid. Lipids were solubilized in 500 µL of $CDCl_3$, 200 µL of MeOH, and 50 µL of aqueous *EDTA* solution (200 mM at pH6).

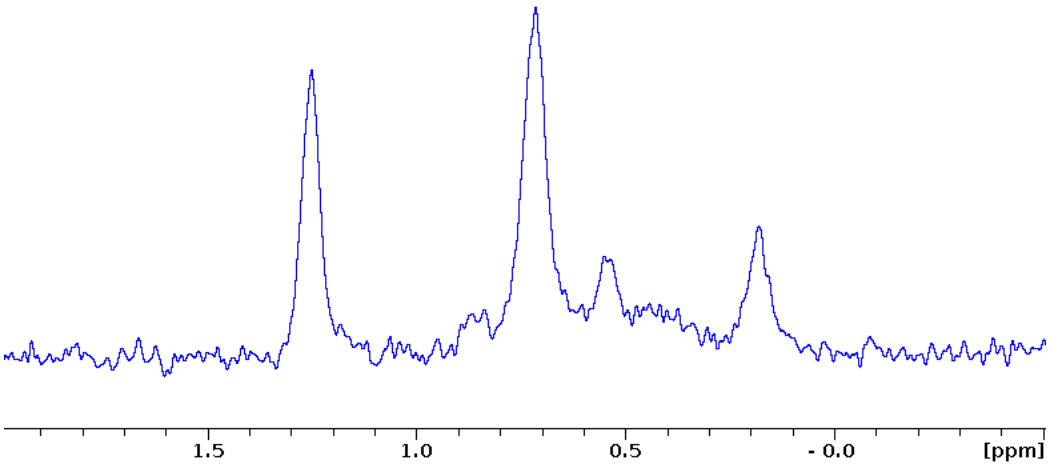


Figure 3-12 ^{31}P NMR spectra of the *B. subtilis* at the mid-log phase in the *CUBO* solvent, made of 500 µl of dimethylformamide, 150 µl of triethylamine, and 50 mg of guanidinium hydrochloride. The spectrum is recorded by 1024 scan at around 3 hours.

Several studies in the available literature have reported the presence of lysilPG in the cell membrane of *B. subtilis*. Mostly, they used TLC method to analyze the headgroups of *B. subtilis* (Atila & Luo, 2016; den Kamp, Redai, & van Deenen, 1969; Minnikin & Abdolrahimzadeh, 1975; Salzberg & Helmann, 2008), but some exploited spectroscopy techniques such as LC-MS (Bernat et al., 2016) and MS-MS (Atila, Katselis, Chumala, & Luo, 2016). This peak also highlights that using TLC for lipid characterization is not precise enough as we previously reported that only three components (PE, PG, and CL) were identified. The content of lysilPG in the membrane of native *B. subtilis* from our experiments (8% at pH of 7.2) was consistent with the results reported previously in the literature (Minnikin & Abdolrahimzadeh, 1975). This study described that the percentage of lysilPG at two different cultures of *B. subtilis* was 7% and 9% at the pH of 7 and respectively increased to 14% and 16% when pH decreased to 5.1.

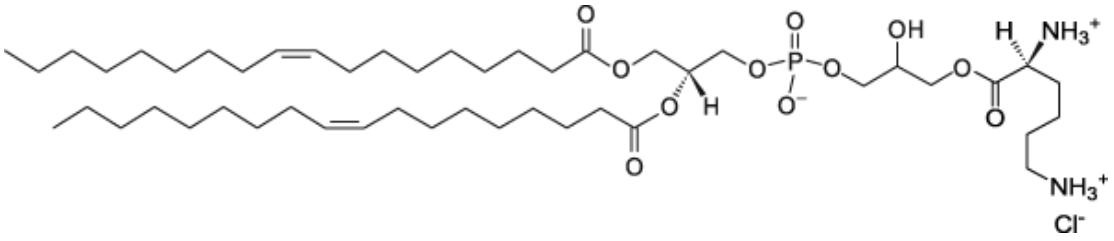


Figure 3-13 Chemical structure of lysilPG

The results of the ^{31}P NMR spectrum show that the phospholipids in the unlabeled *B. subtilis* and the PA-labeled *B. subtilis* membranes are mostly PG (between 30%-35%) and PE (about 30 %). However, the cultivated *B. subtilis* in the presence of PA and OA had CL as the major phospholipid. LysilPG mildly increased from 8.5% to 10.7% and 14.3% when respectively PA and a combination of PA and OA were added to the strain of *B. subtilis* (Table 3-3 and Figure 3-14). Furthermore, it seems that the proportion of anionic phospholipid CL is more synthesized when PA and PA/OA are added to the bacterial growth condition. This, indeed, emphasizes that how CL increases in the case of stress (Lopez et al., 2006; Romantsov, Guan, & Wood, 2009).

Table 3-3 Characterized lipid headgroups of the different cultures of *B. subtilis*, determined by ^{31}P NMR (Mean and standard deviation of n=3 sets).

Headgroups / Organism	PE (%)	CL (%)	PG (%)	LysilPG (%)
***B. subtilis* (Control)**	33.8 (0.7)	21.2 (1.8)	36.5 (2.7)	8.5 (1.5)
***B. subtilis* + PA**	32.2 (2.2)	27.8 (5.0)	29.4 (6.4)	10.7 (2.9)
***B. subtilis* + PA + OA**	25.1 (9.3)	38.6 (1.7)	21.7 (7.6)	14.3 (4.1)

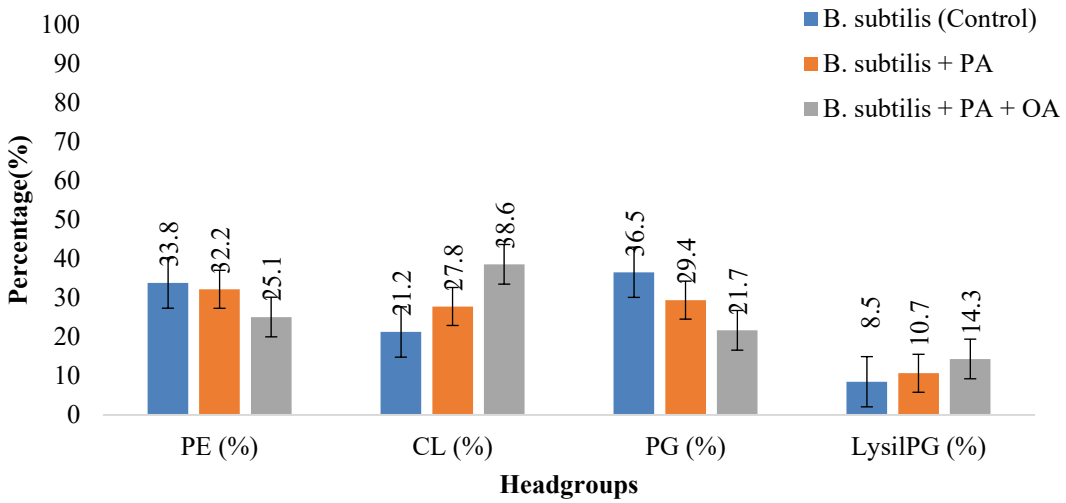


Figure 3-14 Phospholipids headgroup compositions of the extracted lipid of *B. subtilis* grown at the different conditions, determined by ^{31}P NMR.

3.3.3 Analysis of fatty acid composition by GC-MS

To assess the FA chain composition of *B. subtilis*, cell pellets of the bacteria were extracted according to the Folch's method; then they were esterified to be analyzed by GC-MS. There are several saturated FAs in *Bacillus* organism, such as C14:0, C15:0, C16:0, C17:0 and C18:0, and unsaturated ones, such as C18:1 in low abundance (Figure 3-15). It is worth noting that C15:0 and C17:0 are two types of FAs that contain iso and anteiso (isomers) in the strain of *B. subtilis*.

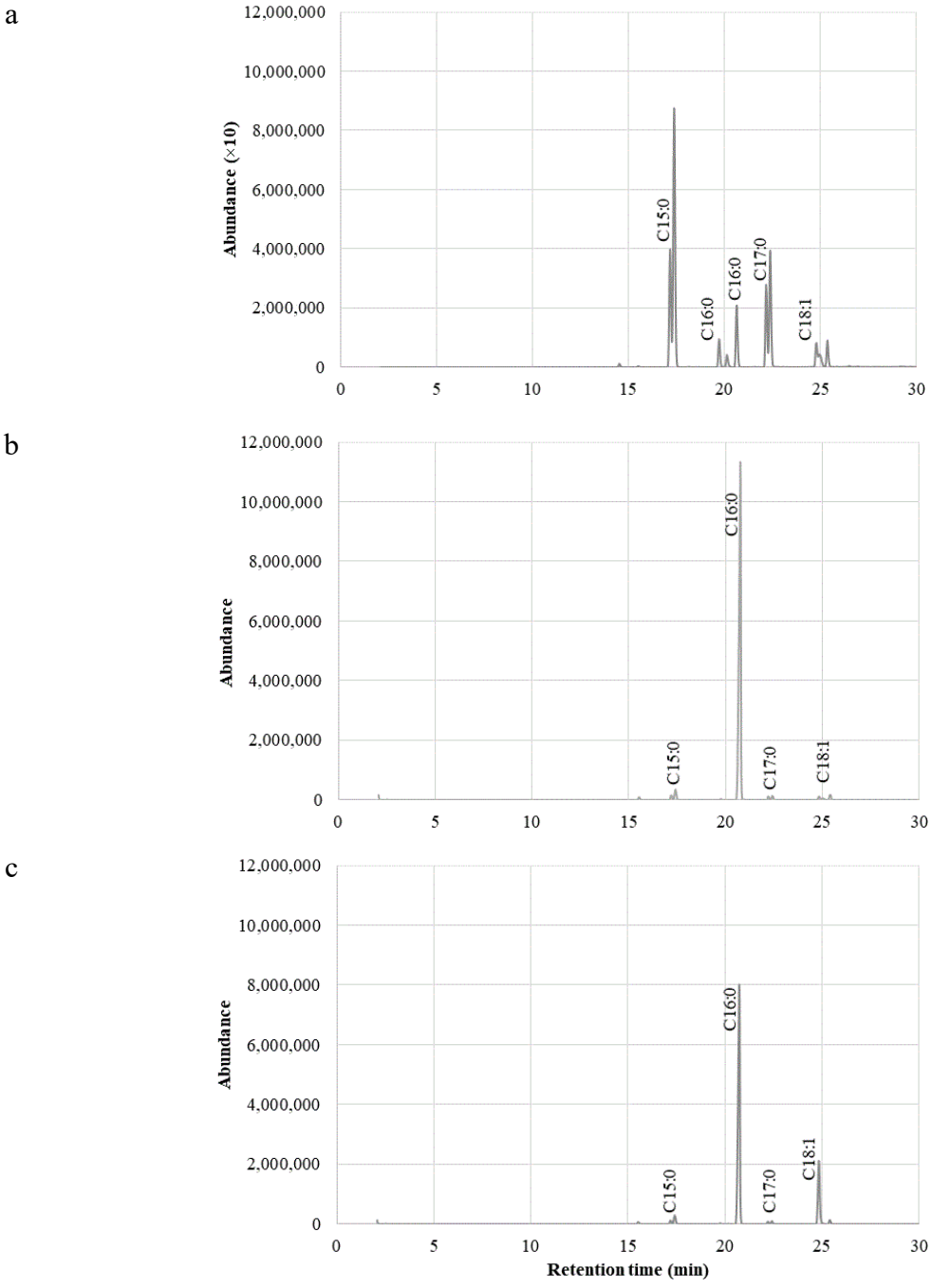


Figure 3-15 Representative GC-MS total ion chromatograms (TIC) of fatty acid methyl esters (FAMEs) from a) *B. subtilis,* b) grown in the presence of palmitic acid (PA), and c) grown in the presence of palmitic acid (PA) and oleic acid (OA).

The results of GC-MS (Table 3-4 and Figure 3-16) indicated that the C15:0 and C17:0 are the major acyl chains in native *B. subtilis* with about 36% and 47% of total C15:0 and C17:0, respectively (iso and anteiso), which is consistent with the reported results by (Bishop, Rutberg, & Samuelsson, 1967). When *B. subtilis* was grown in the presence of PA, the percentage of total C15:0 and C17:0 decreased about 4% for both. By adding the combination of PA and OA, the percentage of total C15:0 and C17:0 dropped to about 3% for both FAs. By adding only PA, the proportion of C16:0 increased from 11% to 89% while it decreased to 70% when also adding OA. Finally, By adding only PA, the proportion of C18:1 decreased from about 5% to 1% and increased to about 22% by adding both PA and OA. It is worth noting that in the presence of only PA or the combination of PA and OA, the proportions of saturated labeled and unlabeled FAs were more than 95%. Our results demonstrate that PA was incorporated into *B. subtilis* membrane. The addition of OA helps restore S/U FA ratio.

Table 3-4 Fatty acid composition of membrane lipid extracts under different growth condition *B. subtilis* strain, determined by GC-MS (Represent the mean and standard deviation of n=3 sets).

Organism / Fatty Acids	***B. subtilis* (Control) (%)**	***B. subtilis* + PA (%)**	***B. subtilis*+PA+ OA (%)**
C14:0	Trace	Trace	Trace
C15:0 (iso)	10.0 (3.3)	1.1 (0.4)	0.9 (0.3)
C15:0 (anteiso)	26.1 (5.9)	3.1 (0.7)	2.3 (0.8)
C16:0	11.3 (1.5)	88.8 (2.2)	70.3 (2.6)
C16:1	Trace	Trace	Trace
C17:0 (iso)	17.3 (3.4)	1.7 (0.2)	1.1 (0.3)
C17:0 (anteiso)	30.0 (7.0)	2.2 (0.3)	1.5 (0.4)
C18:0	3.2 (0.5)	1.1 (0.6)	1.3 (0.4)
C18:1	4.6 (1.3)	1.4 (0.3)	21.9 (2.7)
S/U FA	21.0	70.0	3.5

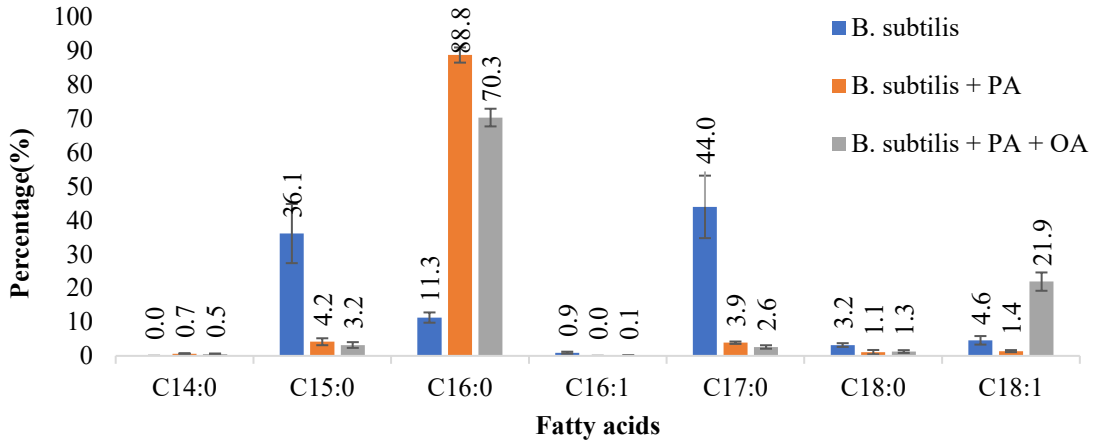


Figure 3-16 FA chain profile in different cultures of *B. subtilis* identified by GC-MS**.**

The values of the ratio of anteiso-to-iso fatty acid of C15:0 in the Bishop's and our study (Table 3-5) were 2.8 and 2.6, respectively, thus showing a remarkable consistency (Bishop et al., 1967). A ratio of 1.6 was reported for C17:0 by Bishop similar to the value of 1.7 that we obtained in our experiment. The anteiso and iso C15:0 and C17:0 content drastically decreased when PA (0.19 mM) and the combination of PA and OA were added to the strain (Figure 3-17), nevertheless, their ratio was almost constant. On the other hand, the ratio of the total anteiso-to-iso FAs substantially diminished from 1.3 to 0.06 by adding PA to the growth culture which represents the decreased fluidity of the cell membrane, but adding OA was not able to restore the fluidity (anteiso/iso=0.05) (Table 3-5).

Table 3-5 The ratio of anteiso to iso compound in *B. subtilis* strain

Organism / Ratio	*B. subtilis* (Control)	*B. subtilis*+PA	*B. subtilis*+PA+OA
Anteiso/Iso (C15:0)	2.6 (0.4)	2.8 (0.5)	2.6 (0.4)
Anteiso/Iso (C17:0)	1.7 (0.2)	1.3 (0.1)	1.4 (0.2)
Anteiso/Iso (Total FAs)	1.3 (0.08)	0.06 (0.01)	0.05 (0.01)

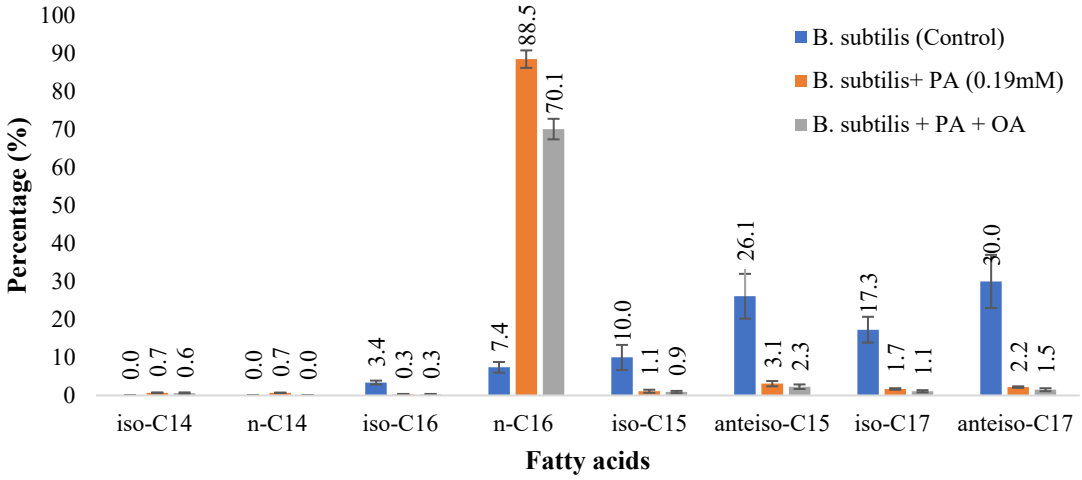


Figure 3-17 Fatty acid composition (n, iso, and anteiso) of *B. subtilis* membrane lipids under different growth conditions.

3.3.4 Characterization of the phospholipid profile as a function of the concentration of PA by ^{31}P NMR spectroscopy

The incorporation of PA and OA affected the lipid profile of bacteria compared to unlabeled ones. To minimize this effect, we have been interested in this project to optimize the concentration of PA used to deuterate the membrane of *B. subtilis*. In the absence of OA, three different concentrations of PA (0.02, 0.05, 0.10 mM) were studied. Figure 3-18 presents the ^{31}P NMR spectra of the extracted lipids from *B. subtilis* cells, which were grown at 37º C in a medium culture supplemented with PA micellized in Tween 20. As previously described, four polar headgroups, namely PE, PG, LysilPG, and CL, were detected.

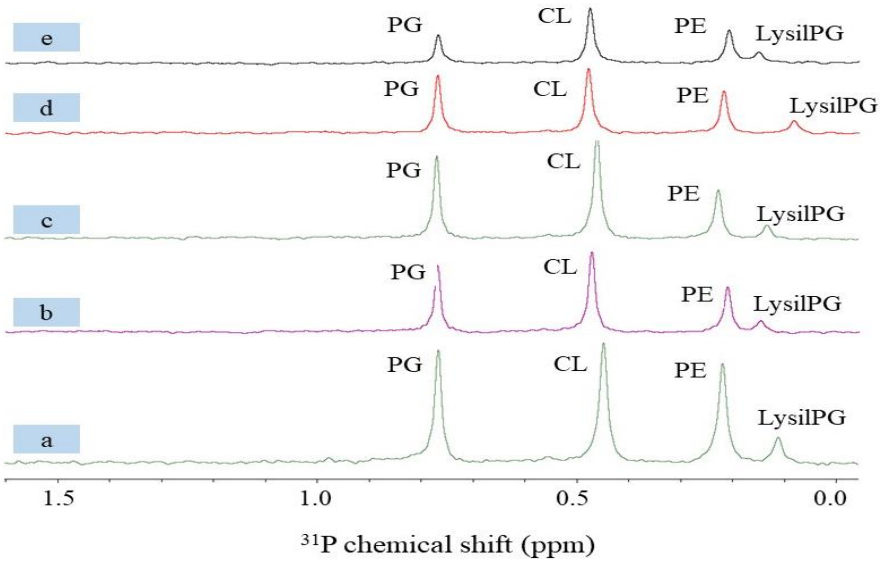


Figure 3-18 ^{31}P NMR spectra of the extracted lipids in a) unlabeled *B. subtilis*, bacteria grown in the presence of different concentrations of PA: b) 0.02 mM. c) 0.05 mM, d) 0.10 mM, and e) 0.19 mM.

The percentage of PE barely changed (from ~30% to ~28%) by increasing the concentration of PA while CL moderately increased (from ~25% to ~29%), except for 0.19 mM. Also, this analysis revealed that the changes observed with PG and LysilPG fall within the error bars (Table 3-6 and Figure 3-19).

Table 3-6 Effect of PA on the phospholipids' classes in *B. subtilis* membranes determined by ^{31}P NMR (Mean and standard deviation of n=3 sets).

Headgroups / Concentration of PA (mM)	PE (%)	CL (%)	PG (%)	LysilPG (%)
0.02	29.5 (0.5)	24.9 (1.7)	36.1 (1.4)	9.5 (0.3)
0.05	28.9 (1.1)	27.2 (2.9)	32.9 (2.2)	11.0 (1.1)
0.10	27.5 (1.0)	29.1 (4.8)	36.1 (3.7)	7.9 (0.8)
0.19	32.2 (2.2)	27.8 (5.0)	29.4 (6.4)	10.7 (2.9)

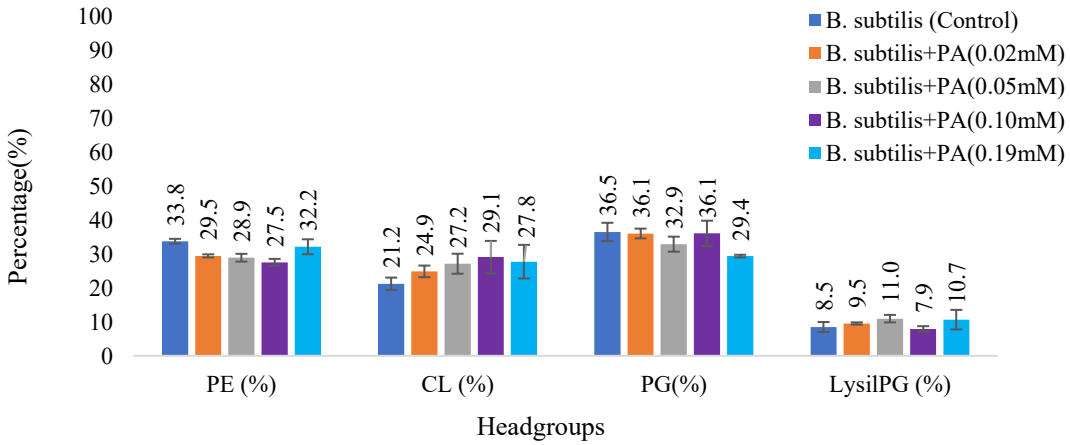
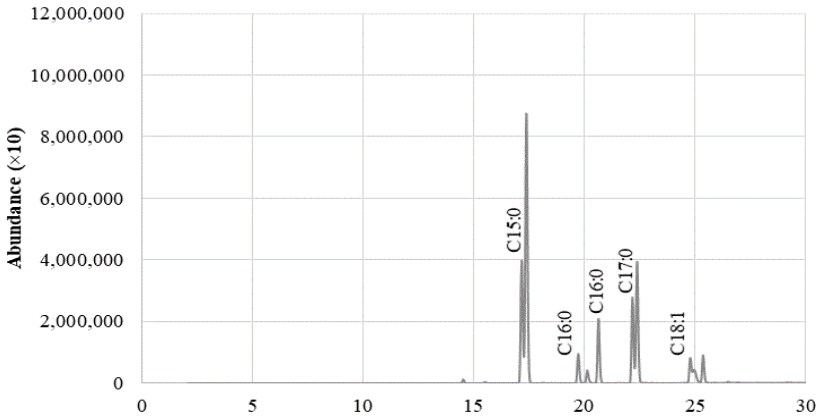


Figure 3-19 Composition of the phospholipid headgroups, detected by ^{31}P NMR, extracted from *B. subtilis* grown in the presence of different concentrations of PA.

3.3.5 Analysis of fatty acid composition at different concentration of PA by GC-MS

To verify if PA affects the acyl chain profile in *B. subtilis* membrane, we studied its profile by GC-MS. *B. subtilis* was grown in the absence and presence of PA at different concentrations (0.02, 0.05, and 0.10, and 0.19 mM); then the cell pellets were collected, and their lipids were extracted, hydrolyzed, methylated and analyzed by GC-MS. Generally, six fatty acid species were observed in the approximate retention times of 17.2, 17.4, 19.7, 20.7, 22.2, 22.4, and 25.4 min, which includes branched-chain saturated (iso and anteiso) and unsaturated fatty acids (Figure 3-20). The most common saturated FAs in the labeled *B. subtilis* are C15:0, C16:0, and C17:0 similarly to normal *B. subtilis,* and the only unsaturated FAwas C18:1 in *B. subtilis* organism.

a



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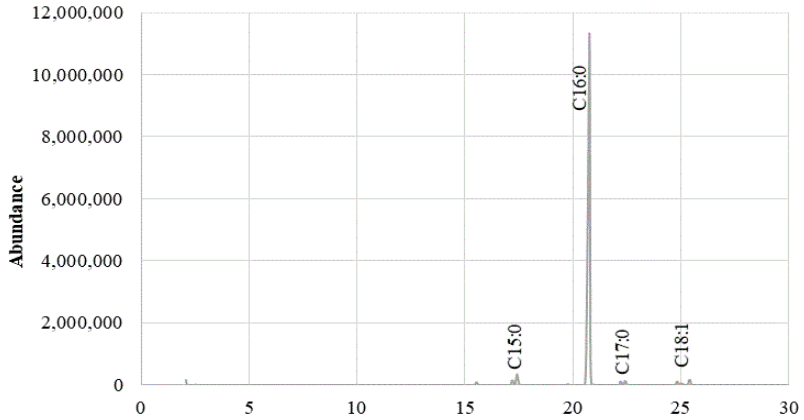

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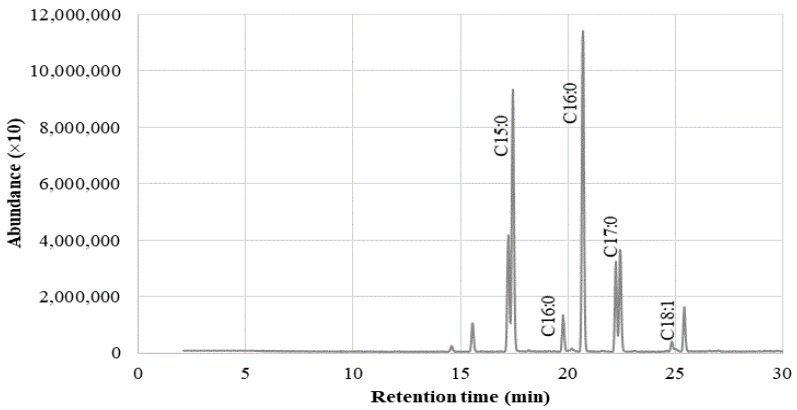

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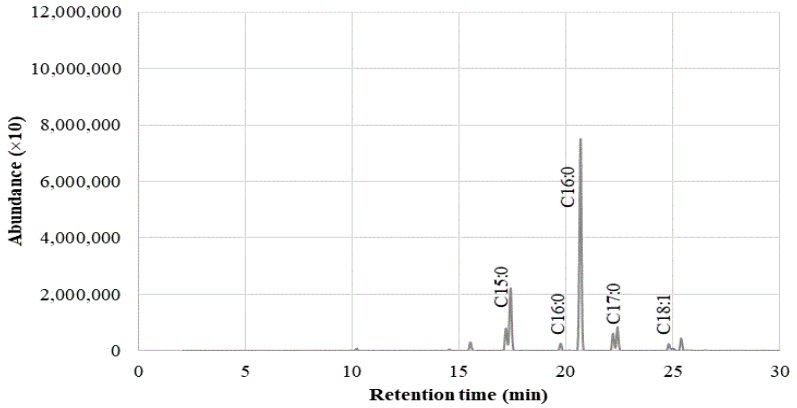


e

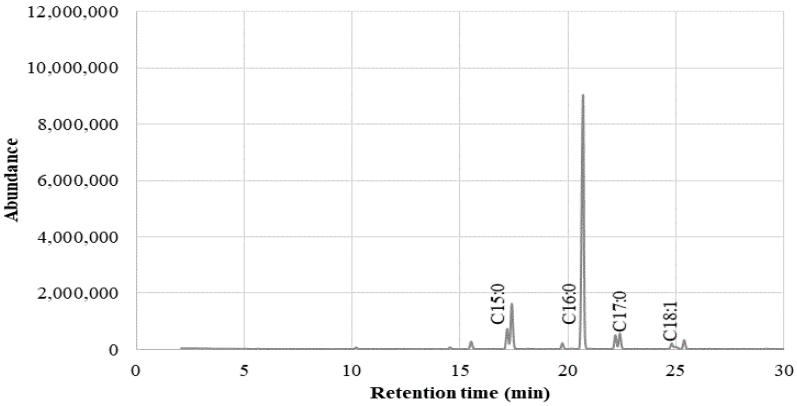


Figure 3-20 Representive GC-MS spectra of fatty acid chain from *B. subtilis* grown in the presence of different concentrations of PA: a) Control b) 0.02 mM, c) 0.05 mM, d) 0.10 mM, and e) 0.19 mM.

The results (Table 3-7 and Figure 3-21) revealed that the composition of FAs is affected by the presence of PA in the growth medium of *B. subtilis*. By increasing the concentration of PA from 0 to 0.19 mM, the percentage of C15:0 and C17:0 drastically decreased from about 36% to 4% and from 44% to 4%, respectively. In addition, the percentage of C18:1 gradually decreased from 5% to 1% when the concentration of PA increased. On the other hand, the fraction of C16:0 sharply raised from 11% to 89% following the increasing concentration of PA.

Table 3-7 Fatty acids chain profile of *B. subtilis* exposed to different concentrations of PA: Control, 0.02 mM, 0.05 mM, 0.10 mM, and 0.19 mM from *B. subtilis* strain, determined by GC-MS.

Concentration of PA (mM) / Fatty acids	Control (%)	0.02 (%)	0.05 (%)	0.10 (%)	0.19 (%)
C14:0	0.0	3.9 (0.5)	3.3 (0.6)	1.8 (0.6)	0.7 (0.2)
C15:0 (iso)	10.0 (3.3)	10.9 (1.0)	9.2(1.9)	4.4 (1.6)	1.1 (0.4)
C15:0 (anteiso)	26.1(5.9)	27.0 (0.6)	23.9 (3.6)	10.5 (3.5)	3.1 (0.7)
C16:0	11.3 (1.5)	26.0 (2.3)	37.5 (7.1)	70.3 (7.3)	88.8 (2.2)
C16:1	Trace	Trace	Trace	Trace	Trace
C17:0 (iso)	17.3(3.4)	10.4 (1.2)	8.7 (1.9)	4.1 (0.6)	1.7 (0.2)
C17:0 (anteiso)	30.0 (7.0)	13.1 (2.1)	10.6 (1.8)	4.8 (0.5)	2.2 (0.2)
C18:0	3.2 (0.5)	4.0 (0.2)	3.8 (0.7)	1.9 (1.1)	1.1 (0.6)
C18:1	4.6 (1.3)	4.6 (3.0)	3.6 (1.3)	2.1 (1.0)	1.4 (0.3)
S/U FA	17	20	26	45	70

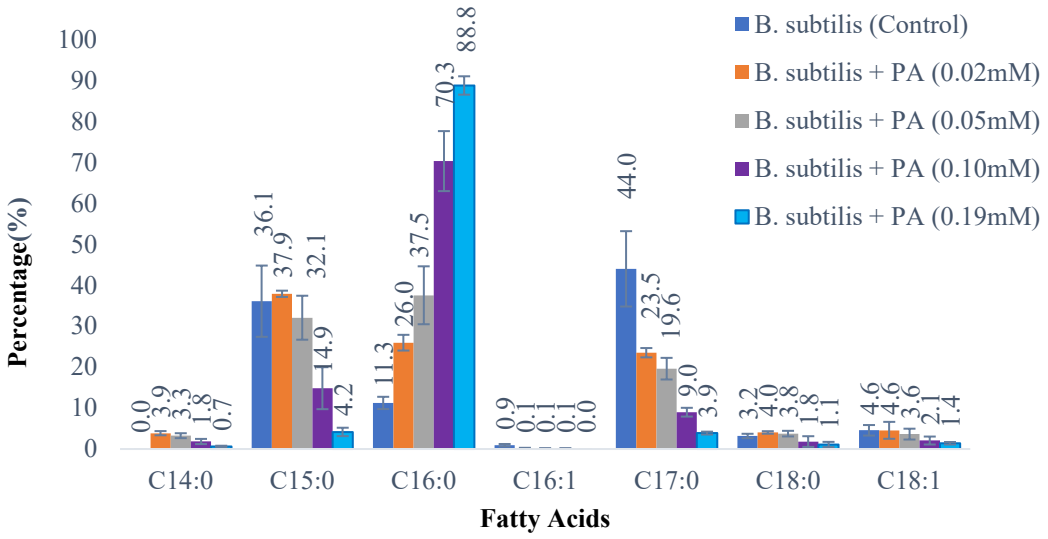


Figure 3-21 The GC-MS analysis of the fatty acids chain profile of *B. subtilis* as a function of different PA concentrations.

The iso and anteiso fatty acid compositions of C15:0 and C17:0 were drastically decreased when PA was added to the strain (Figure 3-22); however, the ratio of anteiso-to-iso compositions was identical for both C15:0 and C17:0 (Table 3-8).

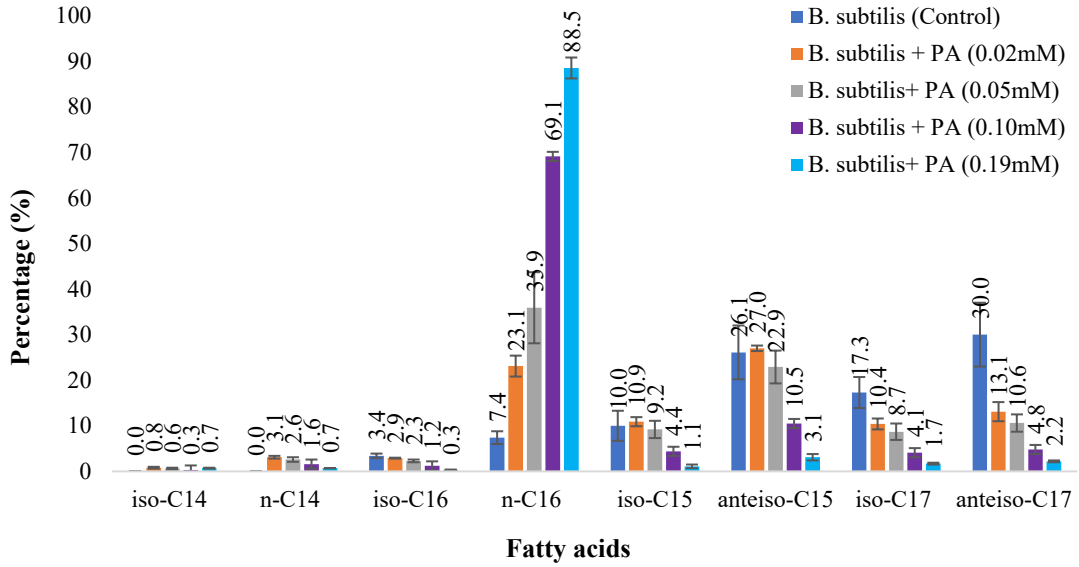


Figure 3-22 Anteiso and iso fatty acid compositions of *B. subtilis* grown in the presence of different concentrations of PA.

Table 3-8 Anteiso/iso fatty acid compositions of the *B. subtilis* grown in the presence of different concentrations of PA.

	Control	0.02 mM	0.05 mM	0.10 mM	0.19 mM
Anteiso/Iso (C15:0)	2.6 (0.4)	2.5 (0.2)	2.5 (0.2)	2.4 (0.04)	2.8 (0.5)
Anteiso/Iso (C17:0)	1.7 (0.2)	1.3 (0.1)	1.2 (0.1)	1.2 (0.02)	1.3 (0.1)

The percentage of the branched and unbranched FAs in the membrane of *B. subtilis* showed a remarkable dependency on the concentration of PA in which the strain was grown. The percentage of branched FAs decreased from 89% (control) to 8.4% (0.19 mM of PA) while the fraction of unbranched FAs increased from 11% (control) to 90% (0.19 mM of PA) (Figure 3-23).

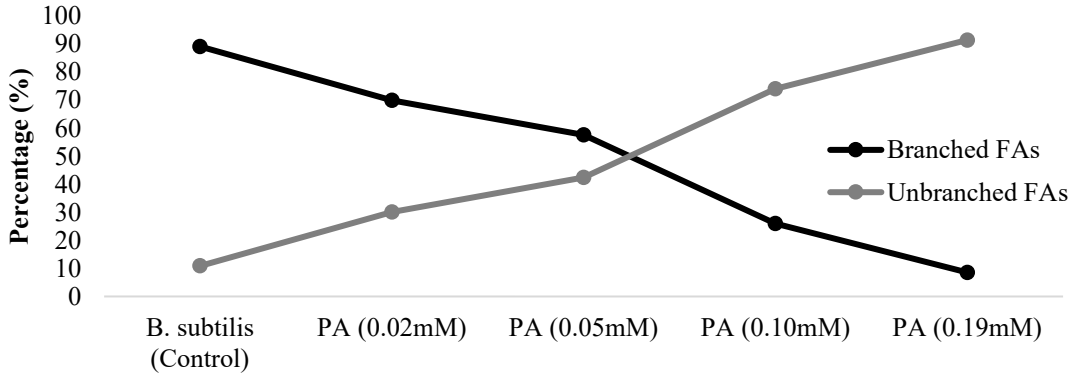


Figure 3-23 Composition of branched-unbranched chains of fatty acids from cultures of *B. subtilis* grown in different concentrations of PA.

Altogether, our results show that at mid-log phase, the bacteria did not change their headgroups profile or produced more unsaturated lipids to compensate the increase in saturated PA chains. Our result demonstrated that the percentage of phospholipid headgroups were quite similar in different concentration of PA, but the proportion of lipids with 0.02 mM had more similar value to the control sample (natural *B. subtilis*). It implies that the proper concentration of PA to label *B. subtilis* is 0.02 mM, which did not affect the nature of the bacterial cell membrane. However, the percentage of deuteration would need to be determined to verify if the signal would be sufficient for the acquisition of *in vivo* ^{2}H SS-NMR spectra.

3.3.6 Experiments with the *B. subtilis* grown in the late-log phase

We verified if the response of the bacteria to the presence of PA in the growth medium changed as a function of the growth phase. We thus carried out the same experiment as a function of PA concentration but harvested the bacteria at the late-log phase, i.e. after 6 hours of growth. We have first looked at the headgroup profile by ^{31}P NMR (Figure 3-24).

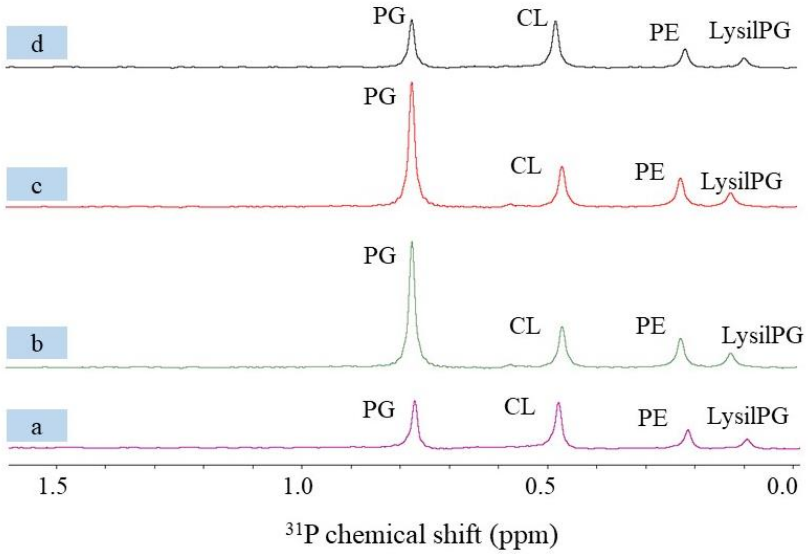


Figure 3-24 ^{31}P NMR spectra of the extracted lipids in *B. subtilis* grown in the presence of different concentrations of PA: a) Control, b) 0.02 mM, c) 0.10 mM, and d) 0.20 mM.

The percentage of PE was around 19% in the control, and exposed to 0.02 mM PA, and 0.10 mM PA, but slightly decreased to 16% when the concentration of PA increased to 0.2 mM. The percentage of CL gradually increased from 16% to 41% when the concentration of PA increased. , while PG decreased from 55% to 34 %. There was not a prevailing trend in the fractions of LysilPG with regard to the increasing concentration of PA (Figure 3-25).

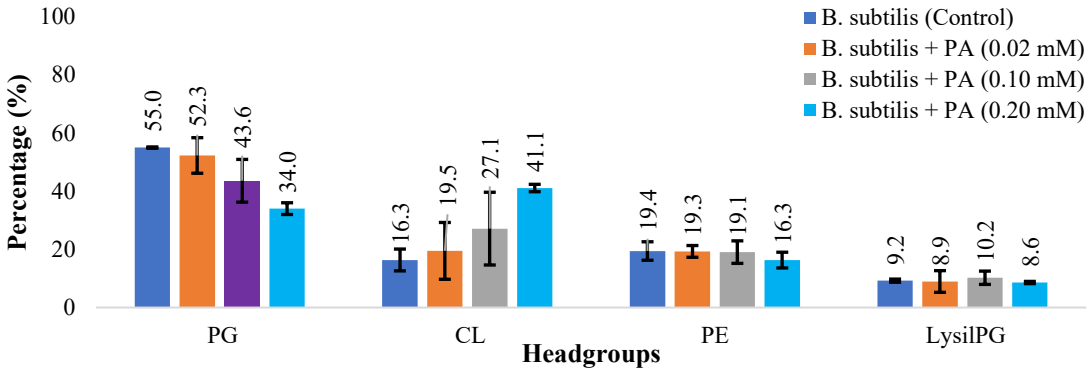


Figure 3-25 Effect of PA concentration on the lipid headgroup profile of *B. subtilis* in the late-log phase (n=3).

We then monitored the FA chain profile as a function of PA concentration. Our GC-MS results (Figure 3-26) revealed that the composition of FAs is affected by the percentage of PA in the growth medium of *B. subtilis* in the late-log phase. By increasing the concentration of PA from 0 to 0.2 mM, the percentage of C15:0 and C17:0 drastically decreased from ~42% to ~9% and from ~52% to 10%, respectively. The percentages of C14:0 and C18:1 were fairly constant (~1%).

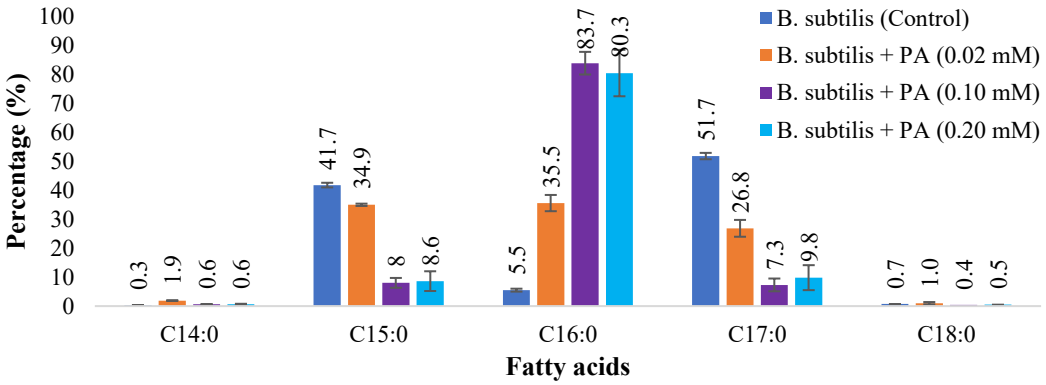


Figure 3-26 Effect of PA concentration on the FA profile of *B. subtilis* in the late-log phase (n=3).

The ratio of the anteiso-to-iso FA chain in the control strain was 2.5 and 1.5 for C15:0 and C17:0 (Figure 3-27), respectively, which was similar to those of our tested *B. subtilis* strains in mid-log phase. i.e. 2.6 and 1.7, respectively. These results also are consistent with Bishop's measurements which respectively were 2.8 and 1.6 (Bishop et al., 1967). The iso and anteiso FA compositions of C15:0 and C17:0 were drastically decreased when PA was added to the strain, but their ratio was identical for both C15:0 (2.5 *vs.* 2.9) and C17:0 (1.5 *vs.* 1.4).

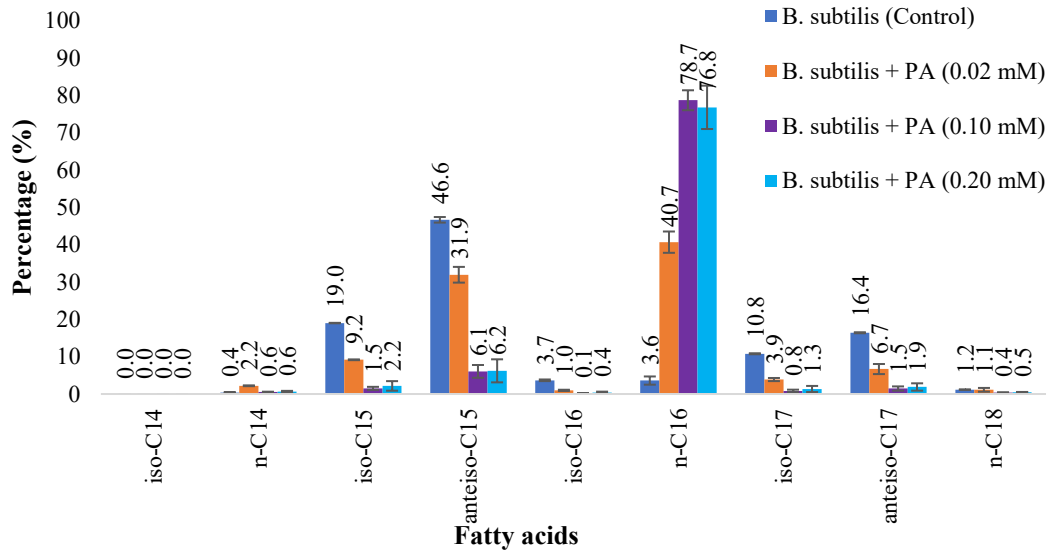


Figure 3-27 Anteiso and iso fatty acid compositions of the *B. subtilis* grown in the presence of different concentrations of PA.

Altogether, our results show some degree of adaptation of *B. subtilis* in the late-log phase. Indeed, there is an increase in CL which can be attributed to the stress response (Lopez et al., 2006; Romantsov et al., 2009). Although in mid-log phase the bacteria did not change the headgroup profile or did not produce more unsaturated lipids, there

was only saturated lipids in the late-log phase. This results suggest to harvest bacteria at the mid-log phase for the future *in vivo* SS-NMR in order to preserve the lipid profile.

Our results demonstrated that the percentage of phospholipid headgroups were quite similar in different concentrations of PA, but the proportion of lipids with 0.02 mM had more similar value to the control sample (natural *B. subtilis*). It implies that the proper concentration of PA to label *B. subtilis* is less than 0.02 mM, which does not change the nature of the bacterial cell membrane.

CONCLUSION

AMPs have garnered tremendous attention because of their role in the innate immune system and their considerable potential to be used as antimicrobial agents (Booth et al., 2017), which may be the solution to antibiotic resistance. Indeed, AMPs, unlike the conventional antibiotics that are target-based, disrupt several biological functions which this offers many therapeautic abilities. Selection of the proper AMP depends on its mechanism of action on the cell membrane, which is determined by several parameters such as existing lipids in the membrane, thickness and curvature of the cell wall, and membrane fluidity (Warschawski et al., 2011). At this view point, it is crucial to distinguish and quantify the existing PLs and FAs in the cell membrane of each bacteria in order to better understand the mechanism of actions of AMPs to disrupt the cell membrane.

In this project, we used a fast and simple protocol involving ^{31}P solution NMR followed by GC-MS to characterize the lipid and FA chain profile of the Gram(-) *E. coli* and Gram(+) *B. subtilis* bacteria in different growth conditions. We started this project by developing a protocol to label the strains of *E. coli* and *B. subtilis* when they were grown in the presence of exogenous palmitic acid (PA) and oleic acid (OA). As SS-NMR is in the early stage of application to bacteria, the concentration of exogenous labeled FAs and also the growth conditions need to be adjusted. Thus, we optimized the bacterial diet by changing the concentration of PA and OA and monitoring the culture at different growth times.

Previously, in the Marcotte's lab, Warnet et al. (2016) and Laadhari et al. (2016) established a protocol in which DPC was used to micellize deuterated PA to label the phospholipid acyl chains and characterize the bacterial cell membrane by ^{2}H SS-NMR. In this project, to label the phospholipid acyl chains in the strains of *E. coli* and *B. subtilis*, we used Tween 20, which is a detergent with nonionic emulsification ability, in the growth medium in the presence of PA and OA. The proposed protocol for lipid extraction by Folch was modified and showed a promising outcome and reproducibility. Three different solvents in NMR analysis, namely MeOH+$CDCl_3$+EDTA, MeOH+$CDCl_3$+NaCl, dimethylformamide+triethylamine+guanidinium, were tested to obtain the lipid content of *E. coli* and *B. subtilis* strains. Using the first solvent in the NMR experiments resulted in four distinct peaks while they overlaid with the use of the other two solvents. The validity of our procedure was achieved by testing similar synthetic model membranes, which revealed identical spectra.

The extracted phospholipids from the late-log phase of unlabeled and labeled *B. subtilis* revealed that the same four major phospholipids observed in the mid-log phase (PG, CL, PE, and LysilPG). When the exogenous FA was incorporated, the percentage of PG approximately decreased by 40% while the content of CL increased by up to 2.5 times. On the contrary, the contents of PE and LysilPG were identical in both diets. The two major FA chains in the late-log phase of *B. subtilis* cell membranes were C15:0 and C17:0 and their content decreased below 10% in the presence of PA. The majority of the characterized FAs were mostly saturated (approximately 100%), in good agreement with the literature (Clejan, Krulwich, Mondrus, & Seto-Young, 1986; Lopez et al., 1998), and the ratio of anteiso-to-iso FAs were almost similar. Also, C15 and C17 were dominant FAs, and the proportion of branched C17 *vs.* C15 were more affected by the growth condition. The level of FAs was remarkedably altered when bacteria were collected at the late-log compared to the mid-log phase (Clejan et al., 1986; Kaneda, 1977).

To study the lipid profile, literature propose using several solvent mixtures (Culeddu et al., 1999; Estrada et al., 2008; Flieger et al., 2000; Furse et al., 2017; Meneses & Glonek, 1988; Rehal et al., 2017) or aqueous detergent solutions (Singh, MacKenzie, Girnun, & Del Poeta, 2017) for ^{31}P NMR. In this study, we forestalled using the molecules which might be confused with FAs. After some replications, we adapted the test conditions presented in the literature (Flieger et al., 2000), including the solubilizede dry lipid pellets in in 500 µL of $CDCl_3$, 200 µL of MeOH, and 50 µL of aqueous *EDTA* solution (200 mM at pH6). We also solubilized the dried lipid pellets in the *CUBO* solutions as it is suggested by Culeddu and collaborators (Culeddu et al., 1999). In this condition, the signals of ^{31}P NMR were broader with less resolution and sensitivity and their corresponding shifts were displaced; however, based on the comparison of the extracted spectrum from *B. subtilis* with the available literature (Furse et al., 2017), we were abled to rejected that the fourth peak was PA or PS and we confirmed that this represents lysilPG.

The corresponding linewidths and chemical shifts of the lipids might change with the temperature and the lipids-to-*EDTA* ratio. For example, we increased the amount of *EDTA* once it was necessary and it resulted to a slight narrowing and shifiting of resonances. Since the chemical shift of PG is relatively independent of temperature, we assigned the chemical shift of 0.77 ppm to PG (Flieger et al., 2000). Regarding to this reference, the chemical shifts of CL and PE were around 0.5 ppm and at 0.2 ppm, respectively. This internally developed reference for the chemical shifts provided us with a reproduceable basis to precisely identify and quantify the lipid profiles by ^{31}P NMR.

The extracted phospholipids from the cultures of *E. coli* and *B. subtilis* in different diets, namely control, labeled with PA, without and with the presence of OA, were compared to determine the lipid profile of each bacterium at the mid-log phase. Three major phospholipids in three diets of *E. coli* were PE, PG, and CL, and their contents were almost independent of the diet, consistent with the available literature (Morein et al., 1996; Pluschke et al., 1978). In *B. subtilis* cultures, four major phospholipids were observed. The contents of the phospholipids were fundamentally changed as a function of the diet in which *B. subtilis* was grown. For example, the percentage of CL considerably increased up to 80% when the diet was PA and OA compared to that of control, while PG decreased up to 40% under the same condition.

Two major fatty FAs in *E. coli* cell membranes were C16:0 and C18:1, which was consistent with the reported results by Warnet et al. (2016) and Tardy Laporte et al. (2013). Similarly, when PA was added to the bacterial diet, the percentage of C16:0 increased while C18:1 decreased. This resulted in a significant rise in the ratio of saturated-to-unsaturated fatty acids which represents an impaired fluidity of the bacterial membrane. To preserve the fluidity of the cell membrane of *E. coli*, a mixture of PA and OA was added to the bacterial growth diet (Warnet et al., 2016). It was expected to achieve a ratio of saturated-to-unsaturated FAs close to that of the control, but there was a difference between them. However, the decreasing ratio by adding the mixture of PA and OA could significantly recover the compromised fluidity.

Two major FA chains in *B. subtilis* cell membrane were C15:0 and C17:0 which was consistent with the literature (Bishop et al., 1967; Kaneda, 1977). The growth of bacteria in the presence of PA increased the percentage of C16:0 about eight times while the percentage of C15:0 and C17:0 significantly decreased. The majority of the characterized FAs were saturated (more than 94%), and also the ratio of anteiso-to-iso FAs were almost similar. Since the percentage of C16:0 experienced a major increase

when PA was in the growth medium, we added a mixture of PA and OA aiming to decrease this value, but the variation of C16:0 was decidedly lower than what we expected (88% decreased to 70 %). In addition, the ratio of S/U FAs decreased by about 15%. Thus, testing different concentrations of PA was considered so as to mitigate this percentage.

The percentage of C16:0 had a direct correlation with the concentration of PA, so that, increasing concentration of PA resulted in a remarkable rise in the percentage of this FA. Increasing concentration of PA not only multiplied the percentage of C16:0, but also shifted the proportion of anteiso and iso composition of other FAs, including C15:0 and C17:0. However, the ratio of anteiso-to-iso composition of those FAs was almost similar when the phospholipid acyl chains were labeled by PA. The composition of branched and unbranched chains of FAs also respectively decreased and increased when PA was added to the bacterial growth medium. Furthermore, the percentage of phospholipid headgroups were quite similar in different concentration of PA, but the lowest concentration (0.02 mM) had closer value to the control sample. It may infer that 0.02 mM could be a proper concentration for labeling the cell membrane of *B. subtilis* as this diet led to a lower change in the nature of the bacterial membrane.

In this project, we developed and replicated a protocol to characterize the phospholipid headgroups and FAs in the cell membranes of *E. coli* and *B. subtilis* when the bacteria were grown in the different diets. This protocol can be used in the future for the *in vivo* study of the bacteria, harvested at the mid-log or late-log phase, by ^{2}H SS-NMR. Indeed, it was the first time that this type of characterization by using ^{31}P NMR and GC-MS was conducted. The phospholipids extracted from the biological membranes have been usually identified by other spectroscopy techniques such TLC (Bishop et al., 1967; den Kamp et al., 1969; Minnikin & Abdolrahimzadeh, 1975), HPLC followed by fluorescence detection, spectrophotometric (UV-Visible) or MS (Bernat et al., 2016;

Bierhanzl et al., 2016). These techniques need calibration on the basis of internal standards. In contrast, ^{31}P NMR spectroscopy is a technique of choice because of being high-resolution, non-destructive, rapid, and with no need of any chemical derivatization. Furthermore, TLC may damage the structure of existing phospholipids in the membrane of bacteria (Pulfer & Murphy, 2003) and is a low-resolution approach, as we have shown.

The phospholipid headgroups and FAs of the cell membrane of *B. subtilis* are characterized for the control sample and they were compared when PA and a mixture of PA and OA were added to the diet. The concentration of PA only varied in the growth medium of *B. subtilis*, but we expect that the lipid profile of the cell membrane of *E. coli* might be subjected to a similar variation. To further optimize the proposed protocol for lipid profiling of both labeled bacteria, it would be interesting to replicate the experiments with concentrations lower than 0.02 mM. It is also of interest to extend this study to the stationary phase when different concentrations of OA in addition to PA are added to the diet of bacteria. It would bring more insight into our knowledge of variable fluidity of bacterial cell membranes, which is beneficial in the context of the development of new antimicrobial agents.

Since matrix, the components in the solvent rather than analyte, can affect the recorded signal in GC-MS. It is recommended to use an internal standard to increase the test precision. Literature has proposed the use of odd chain FAs for quantification of bacterial FAs (Politz, Lennen, & Pfleger, 2013) and C19:0 was used as the internal standard in ^{2}H SS-NMR study of *E. coli* membrane (Tardy-Laporte et al., 2013). The use of an internal standard and creation of a calibration curve for *B. suntilis*, as the future of this study, might correct the loss of FAs during sample preparation or sample injection.

The presented protocol in this study is optimized for ^{31}P NMR to be used in different labeling strategy of the *B. subtilis* bacteria with exogenous PA and OA. Verification of the labeling of cell membrane is crucial to assure that the exogenous FAs are not just adhered to the cell surface rather than incubation. Thus, we suggest to perform ^{2}H SS-NMR and to assess the membrane fluidity by comparing the second spectral moment (M_2), as it is previously used for *V. splendidus* (Bouhlel et al., 2019). Then, it is also worth to evaluate the deuteration level of PA with respect to the incubation time at different concentration of FAs.

APPENDIX A

PREPARATION OF PRECULTURE

Preparation of preculture of *E. coli / B. subtilis*

- Add 100 mL of sterile LB medium, 100µl *E. coli / B. subtilis*
- Incubate the precultures up to 16 hours to obtain viable bacteria (At 37 ° C, 220 rpm).

Measurement Optical Density:

- Calibrate the spectrophotometer at 600 nm with 1ml LB medium. For bacteria Optical Density, take:
  1- 900 µl LB Medium, 100µl preculture of *E. coli*.
  2- 900 µl LB Medium, 100µl preculture of *B. subtilis*.
- Measure the absorbance at 600 nm of cultures. Verify that the absorbance corresponds to the expected absorbance at this step of growth.
- Calculate all the initial volume for 300 ml LB to growth bacterial culture.
- Centrifuge tubes at 3400 rpm for 10 minutes.
- Throw out the supernatant.

Growth of bacteria in LB medium

- Transfer each bacterial suspension (*B. subtilis* without label) in 300 ml LB medium.
- Put erlenmeyers in incubator (At 37 ° C and 220 rpm).
- Verify that the absorbance corresponds to the expected absorbance at this step of growth every 30 min (OD=0.8).
- Divide the volume (300 mL) into 6 falcons of 50 ml.
- Centrifuge falcons at 3400 rpm for 10 minutes.
- Remove the supernatant

Preparation of solution of palmitic acid, oleic acid and Tween 20

For 300 ml LB:

- In a falcon tube (50 ml), weigh Tween 20 (0.15 mM) and palmitic acid (0.19 mM) for 300 ml of LB.
- In another tube, weigh Tween 20 (0.15 mM), 14, palmitic acid (0.19 mM), and oleic acid (0.19 mM) for 300 ml of LB.
- Fill each tube with 15 mL of sterile LB medium.
- Place the tubes in a beaker filled with hot water.
- Place a thermometer in the beaker.
- Heat the water in the beaker to 90° C until dissolution of the Tween 20 and the palmitic acid in the LB medium.
- Thaw the tubes by immersing them in liquid nitrogen for 2 or 3 minutes.
- Mix by vortex for 20-30 seconds.
- Repeat the same cycle again until the Tween 20 and palmitic acid are completely dissolved.

Growth of bacteria in LB medium in the presence of palmitic acid and palmitic acid/oleic acid

a) *E. coli*

- Follow the procedure as described in the section named "preparation of preculture of *E. coli / B. subtilis."*
- Take the erlenmyers of 285ml LB medium for each culture then transfer *E. coli* bacteria suspension. At the end, add prepared 15ml of (palmitic acid + oleic acid + Tween 20+ LB) and another one 15 ml (Palmitic acid+Tween 20+LB) into erlenmyers.
- Put the erlenmeyers in incubator (At 37° C and 220 rpm).
- Verify that the absorbance corresponds to the expected absorbance at this step of growth every 30 min (OD=0.8).
- Divide the volume (300 mL) into 6 falcons of 50 ml.
- Centrifuge falcons at 3400 rpm for 10 minutes.
- Remove the supernatant.

B. subtilis

- Follow the procedure as described in the section named " Preparation of preculture of *E. coli / B. subtilis"*.
- Take the erlenmyers of 285ml LB medium for each culture then transfer *B. subtilis* bacteria suspension. At the end, add prepared 15ml of (palmitic acid + oleic acid + Tween 20 + LB) and another one 15 ml (Palmitic acid+Tween 20+LB) into erlenmyers
- Put erlenmeyers in the incubator (At 37 ° C and 220 rpm).
- Verify that the absorbance corresponds to the expected absorbance at this step of growth every 30 min (OD=0.8).
- Divide the volume (300 mL) into 6 falcons of 50 ml.
- Centrifuge falcons at 3400 rpm for 10 minutes.
- Remove the supernatant.

- Repeat this prodecure for 0.02 mM, 0.05 mM, and 0.10 mM concentration of palmitic acid.

Washing of bacterial pellet

- Rinse with nanopure water.
- Centrifuge flacons at 3400 rpm for 10 minutes.
- Remove the supernatant.
- Place the tubes in lyophilizer (overnight).
- Weigh the dry mass of the samples.
- Collect up in freezer at -20° C.

APPENDIX B

LIPID EXTRACTION

Materials

- KCl 0.88% solution: 0.88g KCl in 100mL of nanopure water.
- Dichloromethane-methanol solution (2:1): 100 mL of dichlorométhane and 50 mL of methanol.

Method

- Transfer the pellets into a 50 mL vial.
- In a graduated cylinder, measure 100 mL of the dichloromethane-methanol (2:1) solution.
- Add about 30 mL of solvent to the pellet and vortex to dissolve it.
- Sonicate the dry pellet of bacteria in the solvent for 10 minutes at 8 W (sonicate for 1 minute and put in an ice bath for 1 minute. Repeat this process 5 times).
- Transfer the sample to the separating funnel and add the rest of 100 mL solvent to the funnel.
- Shake vigorously for 2 minutes and degas to relieve pressure.
- Let stand for 5 minutes.
- Add 27 mL of the KCl solution to the separating funnel

- Shake vigorously for 2 minutes and degas to relieve pressure.
- Let stand 15-20 minutes until phase separation is complete.
- If the organic phase (the lower one) is unclear, filter this phase. If it remains unclear, add a few mL of the dichloromethane-methanol solution.
- Weigh the 5 mL vial in which the lipids will be stored.
- Evaporate the organic phase using the rotary evaporator.
- Transfer the lipids to the vial that has been weighed.
- Evaporate the MeOH at 40° C under a nitrogen flow rate.
- Once well dried, put the vial in the freeze-dryer (30 to 45 minutes).
- Weigh the dry mass of lipids and store them in the refrigerator at -20° C.

NMR experiment

- Add 500 µl of Chloroform-D, 200 µl of MeOH and 50 µl of *EDTA* 0.2 M (PH=6) to the lipid vial.
- Mix and vortex (20 seconds).
- Transfer the sample to the NMR tube.

APPENDIX C

METHYLATION AND GAS CHROMATOGRAPHY-MASS SPECTROSCOPY

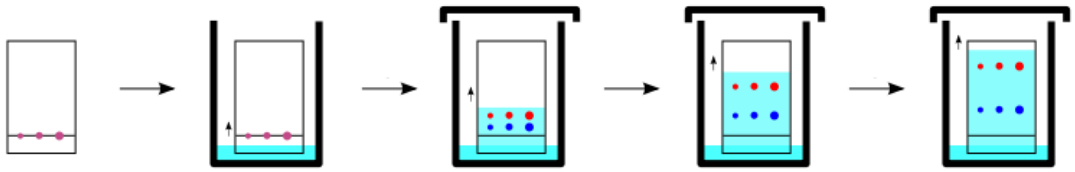
- After NMR, add 2 ml of H_2SO_4 (2% in MeOH) and 0.8 ml of toluene to extracted lipids.
- Keep it sealed and heat them for 10 min at 100° C (do not exceed 105° C) and vortex 2 or 3 times.
- Let cool to room temperature (~ 10 min)
- Add 4 ml of distilled water and 0.8 ml of hexane, then shake and wait for phase separation. Centrifugal at 2000 rpm for 2 minutes.
- Transfer the upper phase to a 2 ml vial.
- Evaporate to dry and weigh the dry mass.
- Add the desired volume of hexane (0.5 ml of hexane for GC-MS) and store it in the freezer (at -20° C).

MeOH-H_2SO_4 must always be renewed because it is a reversible solution.

APPENDIX D

THIN-LAYER CHROMATOGRAPHY (TLC) EXPERIMENT

- Prepare 0.6 mg of Phosphatidylethanolamine (PE), Phosphatidylglycerol (PG), and 1.8 mg of Cardiolipin (CL) as standards.
- Dissolve the extracted lipids in 1ml Dichloromethane: Methanol (2:1).
- Prepare mixture solvent of Chloroform-Methanol-Water (65:25:4).
- Place a small amount of mobile phase in the container. The solvent level must be below the starting line of the TLC, otherwise the spots will dissolve away.
- Touch the plate briefly at the start line. Spot a TLC plate, allow the solvent to evaporate and spot at the same place again.
- Put the TLC plate into the container and wait the solvent slowly travels up the plate.
- Allow the solvent to rise until it almost reaches the top of the plate.
- Allow the chromatogram to dry and then place it in enclosed iodine crystals.



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