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Polytechnic and Basic Science School**

Department of Chemical Sciences



**Ph.D. in Chemical Sciences**

**Structure, function and properties  
of bacterial cell envelope components**

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## Abstract

Bacterial membranes are highly dynamic structures, exhibiting significant variability in both lipid and protein composition. This flexibility allows the molecules to rearrange, greatly affecting the membrane's fluidity, stability, and permeability. These characteristics are important because they directly influence cellular functions and interactions with the environment. One of the main components, lipopolysaccharides (LPS), plays a crucial role in the outer membrane of Gram-negative bacteria, serving as an essential interface with the environment. Along with capsular polysaccharides (CPS) and exopolysaccharides (EPS), LPS supports bacterial interactions with their hosts and the surrounding microbiota, playing a significant role in adhesion, biofilm development, and immune responses. In addition, hopanoids, structurally and functionally resembling eukaryotic steroids, are essential for stabilizing membranes, ensuring they remain intact and resilient even in tough environmental conditions.

This PhD research investigates the many roles that bacterial glycans play, especially their impact on the immune system and how they interact with immune functions. By carefully characterizing the chemical structures of these glycans, we discovered new insights into how they help shape immune responses in the host and maintain the delicate equilibrium between tolerance and immunity. To achieve this, we isolated and analyzed key components from the bacterial cell envelope, including LPS, CPS and EPS, from specific strains known to interact with both human and plant hosts, such as *Herbaspirillum Root189*, *Methylobacterium extorquens PA1* and *Paenaltcaligenes hominis*. We then analyzed the immune recognition patterns of these glycans to identify the specific structures that trigger immune responses and activate receptors. This approach has enabled us to unveil the molecular pathways through which glycans modulate immune activity and mediate host-microbe interactions.

Glycans play a crucial role in detailed communication between hosts and microbes, influencing health in different ways that can be either beneficial or harmful, depending on the context. In humans and plants, glycans activate the immune system, triggering defense mechanisms against harmful bacteria and enhancing innate immune responses. Conversely, in symbiotic and commensal relationships, glycans promote beneficial interactions, improve the host's tolerance and regulate immune responses, which is essential for maintaining a balanced microbiota that supports health. In plants, microbial glycans have been shown to enhance symbiotic relationships, promoting growth, improving nutrient uptake, and increasing resistance to environmental stresses. Similarly, in human-associated bacteria, glycans can significantly impact on immune responses, emphasizing their important roles in both maintaining health and contributing to disease development.

The main aim of this thesis is to elucidate the complex structure-function relationships of microbial glycans that come from various bacteria, including symbiotic, commensal, and pathogenic strains. This research provides important insights into the molecular mechanisms underlying host-microbe interactions and immune responses. It highlights new structural features and biological roles of bacterial glycans, making an important contribution to the field of glycobiology. By clarifying the complex relationships between microbial life and their hosts, it simplifies aspects that remain poorly understood. These results increase our understanding of how glycans assist communication and recognition between microbes and

hosts. This suggests innovative implications, such as the development of new therapeutic strategies that can modulate the host-microbe interface, refining interventions designed to re-establish a healthy microbial equilibrium, and utilizing beneficial bacteria for applications in agriculture and biomedicine.

Moreover, this work emphasizes the potential of microbial glycans in sustainable and green applications. Understanding these molecular interactions not only advances biomedical research but also sets the stage for environmentally conscious agricultural practices. Promoting beneficial microbial interactions in crops, for example, can naturally enhance growth and resilience, reducing the dependency on chemical fertilizers and pesticides. Similarly, harnessing these mechanisms could lead to greener biotechnological solutions aimed at preserving ecological balance and supporting environmental health. Furthermore, the ability to modulate microbial communities responsibly aligns with global sustainability goals, offering pathways toward less invasive medical treatments and sustainable agricultural practices that reduce environmental impact and support long-term ecosystem health.

**Keywords:** *Gram-negative bacteria, Structure-function relationships, Lipopolysaccharide, Exopolysaccharides, Glycoconjugates, Biological significance.*

## ABBREVIATIONS

|               |                                                   |
|---------------|---------------------------------------------------|
| <b>AA</b>     | Acetylated Alditols                               |
| <b>ABC</b>    | ATP Binding Cassette                              |
| <b>ACC</b>    | 1-aminocyclopropane-1-carboxylate (ACC) deaminase |
| <b>AMG</b>    | Acetylated Methyl Glycosides                      |
| <b>AOG</b>    | Acetylated Octyl- (or Butyl-) Glycosides          |
| <b>ATP</b>    | Adenosine Triphosphate                            |
| <b>ATPase</b> | Adenosine Triphosphatase                          |
| <b>Bam</b>    | $\beta$ -barrel Assembly Machinery                |
| <b>CD14</b>   | Cluster of Differentiation 14                     |
| <b>CFU</b>    | Colony Forming Units                              |
| <b>CID</b>    | Collision-Induced Dissociation                    |
| <b>COSY</b>   | Correlation Spectroscopy                          |
| <b>CPS</b>    | Capsular Polysaccharides                          |
| <b>ConA</b>   | Concanavalin A                                    |
| <b>DLS</b>    | Dynamic Light Scattering                          |
| <b>Dmet</b>   | Methanol self-diffusion coefficient               |
| <b>DMSO</b>   | Dimethyl Sulphoxide                               |
| <b>DNase</b>  | Deoxyribonuclease I From Bovine Pancreas          |
| <b>DPPC</b>   | Dipalmitoylphosphatidylcholine                    |
| <b>EPR</b>    | Electron Paramagnetic Resonance                   |
| <b>EPS</b>    | Exopolysaccharides                                |
| <b>ETI</b>    | Effector-Triggered Immunity                       |
| <b>FMT</b>    | Fecal Microbiota Transplantation                  |
| <b>FT-ICR</b> | Fourier Transform Ion Cyclotron Resonance         |
| <b>GAM</b>    | Gifu Anaerobic Medium                             |

|                  |                                                                                                  |
|------------------|--------------------------------------------------------------------------------------------------|
| <b>GBA</b>       | Gut-Brain Axis                                                                                   |
| <b>GC-MS</b>     | Gas Chromatography–Mass Spectrometry                                                             |
| <b>GM</b>        | Gut Microbiota                                                                                   |
| <b>GNA</b>       | <i>Galanthus nivalis</i> agglutinin                                                              |
| <b>HEK</b>       | Human Embryonic Kidney (e.g., HEK-Blue™ cells)                                                   |
| <b>HHL</b>       | <i>Hippeastrum</i> hybrid lectin                                                                 |
| <b>HMBC</b>      | Heteronuclear Multiple Bond Correlation Spectroscopy                                             |
| <b>HR</b>        | Hypersensitive Response                                                                          |
| <b>HSQC</b>      | Heteronuclear Single-Quantum Correlation Spectroscopy                                            |
| <b>IBD</b>       | Inflammatory Bowel Disease                                                                       |
| <b>IL</b>        | Interleukin                                                                                      |
| <b>IM</b>        | Inner Membrane                                                                                   |
| <b>LBP</b>       | LPS Binding Protein                                                                              |
| <b>LCA</b>       | <i>Lens Culinaris</i> Agglutinin                                                                 |
| <b>LipA</b>      | Lipid A                                                                                          |
| <b>LIR</b>       | Localized Induced Resistance                                                                     |
| <b>Lol</b>       | Localization of lipoproteins                                                                     |
| <b>LolCDE</b>    | ABC transporter for lipoproteins                                                                 |
| <b>LOS</b>       | Lipooligosaccharide                                                                              |
| <b>Lpp</b>       | Braun's Lipoprotein                                                                              |
| <b>LPS</b>       | Lipopolysaccharide                                                                               |
| <b>m-DAP</b>     | meso-Diaminopimelic acid                                                                         |
| <b>MALDI ICR</b> | <b>FT-</b> Matrix-Assisted Laser Desorption/Ionization Fourier Transform Ion Cyclotron Resonance |
| <b>MALDI-TOF</b> | Matrix-Assisted Laser Desorption/Ionization Time-of-Flight                                       |
| <b>MAMP</b>      | Microbe Associated Molecular Pattern                                                             |
| <b>MAPK</b>      | Mitogen-Activate Protein Kinase                                                                  |

|                |                                                                     |
|----------------|---------------------------------------------------------------------|
| <b>MD</b>      | Molecular Dynamics                                                  |
| <b>MD-2</b>    | Myeloid Differentiation Factor 2                                    |
| <b>MeMan</b>   | methyl- $\alpha$ -D-mannopyranoside                                 |
| <b>MeDiMan</b> | methyl-3-O- $\alpha$ -D-mannopyranosyl- $\alpha$ -D-mannopyranoside |
| <b>MetEPS</b>  | Methanol-extracted EPS                                              |
| <b>MM</b>      | Molecular Mechanics                                                 |
| <b>MS</b>      | Mass Spectrometry                                                   |
| <b>MsbA</b>    | ABC transporter for LPS flipping                                    |
| <b>MS/MS</b>   | Tandem Mass Spectrometry                                            |
| <b>Mw</b>      | Molecular Weight                                                    |
| <b>NaBD4</b>   | Sodium borodeuteride                                                |
| <b>NMR</b>     | Nuclear Magnetic Resonance                                          |
| <b>NOE</b>     | Nuclear Overhauser Effect                                           |
| <b>NOESY</b>   | Nuclear Overhauser Spectroscopy                                     |
| <b>NPL</b>     | Narcissus pseudonarcissus lectin                                    |
| <b>OAg</b>     | O-Antigen                                                           |
| <b>OAgAc</b>   | Acetylated O-Antigen                                                |
| <b>OM</b>      | Outer Membrane                                                      |
| <b>OMP</b>     | Outer Membrane Protein                                              |
| <b>OMVs</b>    | Outer Membrane Vesicles                                             |
| <b>OPS</b>     | O-specific Polysaccharide                                           |
| <b>OS</b>      | Oligosaccharide                                                     |
| <b>OSdeAc</b>  | Oligosaccharide Deacetylated                                        |
| <b>PAGE</b>    | Polyacrylamide Gel Electrophoresis                                  |
| <b>PBC</b>     | Periodic Boundary Conditions                                        |
| <b>PBMCs</b>   | Peripheral Blood Mononuclear Cells                                  |

|                                  |                                                  |
|----------------------------------|--------------------------------------------------|
| <b>PCP</b>                       | Petroleum ether/Chloroform/Phenol                |
| <b>PFG NMR</b>                   | Pulsed-Field Gradient Nuclear Magnetic Resonance |
| <b>PGPB</b>                      | Plant Growth-Promoting Bacteria                  |
| <b>PMAA</b>                      | Partially Methylated and Acetylated Alditols     |
| <b>PR</b>                        | Pathogenesis-Related                             |
| <b>PRRs</b>                      | Pattern Recognition Receptors                    |
| <b>PSA</b>                       | Pisum Sativum Agglutinin                         |
| <b>QM</b>                        | Quantum Mechanics                                |
| <b>R-LPS</b>                     | Rough-type Lipopolysaccharide                    |
| <b>RefEPS</b>                    | Reference EPS                                    |
| <b>REGIII<math>\gamma</math></b> | C-type lectin antimicrobial peptide              |
| <b>R<sub>g</sub></b>             | Radius of Gyration                               |
| <b>R<sub>h</sub></b>             | Hydrodynamic Radius                              |
| <b>RNase</b>                     | Ribonuclease A                                   |
| <b>ROESY</b>                     | Rotating Frame Nuclear Overhauser Effect         |
| <b>ROS</b>                       | Reactive Oxygen Species                          |
| <b>S-LPS</b>                     | Smooth-type Lipopolysaccharide                   |
| <b>SANS</b>                      | Small-Angle Neutron Scattering                   |
| <b>SAXS</b>                      | Small-Angle X-ray Scattering                     |
| <b>SCFAs</b>                     | Short-Chain Fatty Acids                          |
| <b>SDS</b>                       | Sodium Dodecyl Sulphate                          |
| <b>SEAP</b>                      | Secreted Embryonic Alkaline Phosphatase          |
| <b>SEC</b>                       | Size-Exclusion Chromatography                    |
| <b>SecA</b>                      | Protein translocation ATPase                     |
| <b>SecYEG</b>                    | Protein-conducting channel complex               |
| <b>Siglec</b>                    | Sialic Acid-Binding Immunoglobuline-Type Lectin  |

|                                |                                                                 |
|--------------------------------|-----------------------------------------------------------------|
| <b>SLS</b>                     | Static Light Scattering                                         |
| <b>SNFG</b>                    | Symbol Nomenclature For Glycans                                 |
| <b>TFA</b>                     | Trifluoroacetic Acid                                            |
| <b>TLR</b>                     | Toll-like Receptor                                              |
| <b>TLR4-MD2</b>                | Toll-like receptor 4 - Myeloid Differentiation factor 2 complex |
| <b>TNF-<math>\alpha</math></b> | Tumour Necrosis Factor $\alpha$                                 |
| <b>TOCSY</b>                   | Total Correlation Spectroscopy                                  |
| <b>VEA</b>                     | Vicia Ervilia Agglutinin                                        |
| <b>VLCFA</b>                   | Very Long Chain Fatty Acid                                      |
| <b>VOCs</b>                    | Volatile Organic Compounds                                      |
| <b>WT</b>                      | Wild Type                                                       |

## SUGAR ABBREVIATIONS

|                |                                            |
|----------------|--------------------------------------------|
| <b>Gal</b>     | Galactose                                  |
| <b>GalA</b>    | Galacturonic acid                          |
| <b>Glc</b>     | Glucose                                    |
| <b>Glc3OMe</b> | 3-O-methyl-D-glucose                       |
| <b>GlcN</b>    | Glucosamine                                |
| <b>GlcNAc</b>  | N-acetyl glucosamine                       |
| <b>Hep</b>     | Heptose                                    |
| <b>Kdo</b>     | 3-deoxy-D-manno-octulosonic acid           |
| <b>Kdo5OMe</b> | 5-O-methyl-Kdo                             |
| <b>Ko</b>      | D-glycero-D-talo-oct-2-ulopyranosonic acid |
| <b>Ko5OMe</b>  | 5-O-methyl-Ko                              |
| <b>L,D-Hep</b> | L-glycero-D-manno-heptose                  |
| <b>Man</b>     | Mannose                                    |
| <b>QuiN</b>    | Quinovosamine                              |
| <b>Rha</b>     | Rhamnose                                   |
| <b>Rha3OMe</b> | 3-O-methyl-D-rhamnose                      |

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*Alla mia piccola Michela.*

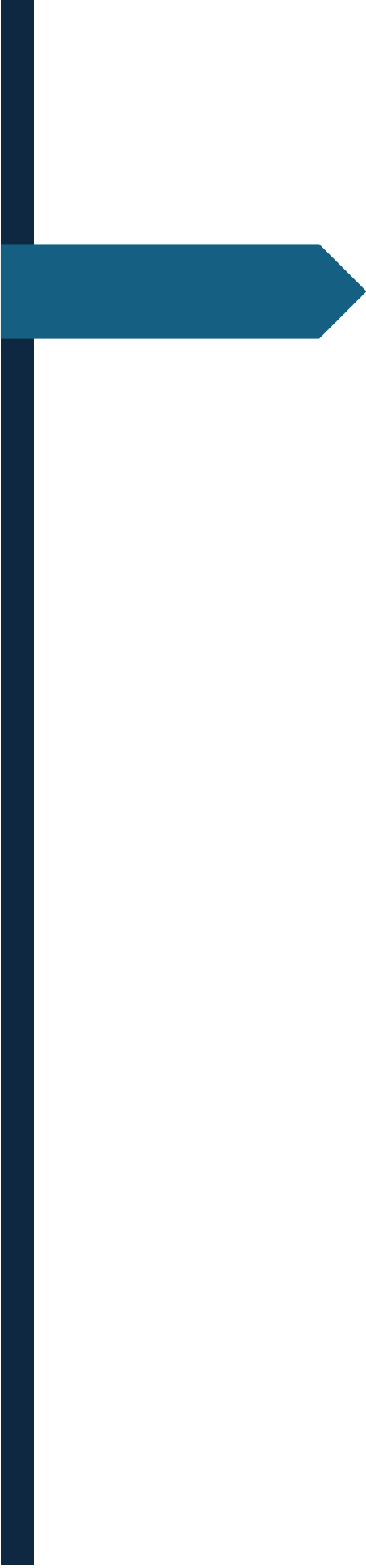
*Questo traguardo è il nostro.*

*Che tu possa sempre credere in te stessa e inseguire i tuoi sogni*

*con la stessa gioia*

*con cui riempi i miei giorni.*



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# **CHAPTER I**

## **Gram-negative bacteria**



## 1.1 The Bacteria cell

Cells are the basic building blocks of life, constantly interacting with their surroundings and with each other. All living organisms are made up of one of two main types of cells: prokaryotic and eukaryotic. Eukaryotic cells are generally larger and more complex, containing membrane-bound organelles like the nucleus, mitochondria, and chloroplasts. These cells make up a wide variety of organisms, from single-celled creatures like algae and protozoa to larger multicellular organisms like plants, fungi, and animals<sup>1</sup>. On the other hand, prokaryotic cells, which include both Bacteria and Archaea, are simpler in structure and lack these internal compartments<sup>2</sup> (Figure 1).

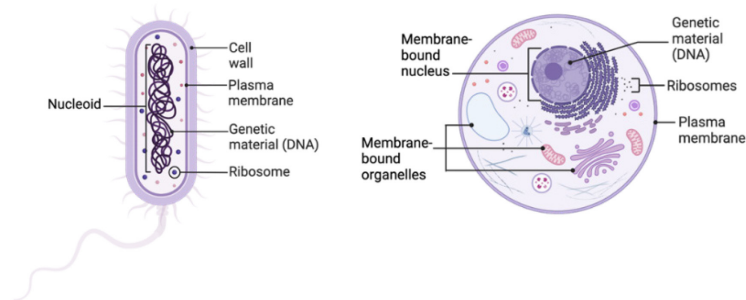


**Figure 1:** Image of prokaryotic and eukaryotic cells, showing the main cellular structures. The eukaryotic cell is depicted with a well-defined nucleus, mitochondria, and chloroplasts (in plant cells), while the prokaryotic cell is shown with its nucleoid region and simpler internal organization.

While Bacteria and Archaea may seem similar at first glance, they actually differ in important ways, especially in their structure, function, and genetic makeup. For example, Archaea are often found in extreme environments, such as places with high temperatures, extreme pH, or intense pressure. Interestingly, Archaea share some molecular traits with eukaryotes, particularly when it comes to processes like transcription and translation<sup>3</sup>.

The way we classify life has changed over time. Today, one of the most widely accepted classification systems is the three-domain model, introduced by Carl Woese in the 1990s. This system groups life into three broad categories: Archaea, Bacteria, and Eukarya<sup>3</sup>. Alternatively, the six-kingdom model divides life into Archaeobacteria, Eubacteria, Protista, Fungi, Plantae, and Animalia. In this system, Archaeobacteria and Eubacteria correspond to the Archaea and Bacteria domains, while the other four kingdoms fall under the Eukarya domain<sup>4</sup>.

Bacteria, as unicellular organisms, show a remarkable variety in terms of size, shape, and behavior, allowing them to thrive in many different environments<sup>5</sup>. Unlike eukaryotic cells, their DNA is not enclosed in a nucleus but is found in a specific region of the cytoplasm called the nucleoid<sup>2</sup>. Bacteria can be classified by shape into several categories: cocci (spherical), bacilli (rod-shaped), spirilla (spiral-shaped), and filamentous forms. After division, some bacteria stick together, forming structures like chains (e.g., *Streptococcus*), clusters (e.g., *Staphylococcus*), or even cubic arrangements (e.g., *Sarcina*)<sup>5</sup>. The diversity in bacterial shapes, including those seen in spirochetes and filamentous bacteria, shows just how adaptable these organisms can be<sup>2</sup>.

## Gram-negative bacteria

Temperature is another key factor in classifying bacteria. Some bacteria, called psychrophiles, thrive in cold temperatures (0–20°C), while mesophiles prefer moderate temperatures (25–45°C). There are also thermophiles that can handle extreme heat, growing best in temperatures as high as 122°C<sup>6</sup>. Prokaryotic cells are typically much smaller than eukaryotic cells, ranging from 0.1–0.2 µm to around 50 µm. This size difference has a big impact on their biology and how they survive in different environments<sup>2</sup>.

One of the key methods used to differentiate bacteria is the Gram stain, developed by Hans Christian Gram in 1884. This technique remains a cornerstone in microbiology, helping to classify bacteria based on the structure of their cell walls. Gram-positive bacteria have a thick peptidoglycan layer that holds onto the crystal violet stain, making them appear purple under a microscope. Gram-negative bacteria, on the other hand, have a thinner peptidoglycan layer and an extra outer membrane (Figure 2). This difference causes them to lose the crystal violet stain during a washing step and instead take on a pink color after counterstaining<sup>7</sup>.

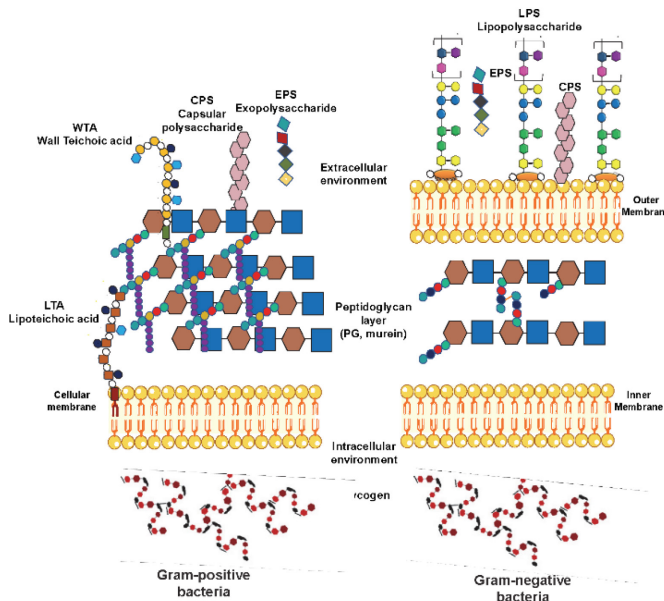


**Figure 2:** Schematic comparison of Gram-positive and Gram-negative bacterial cell walls. Gram-positive bacteria are characterized by a single lipid membrane and a thick peptidoglycan layer, whereas Gram-negative bacteria have both an inner and outer membrane with a thin peptidoglycan layer in the periplasmic space, along with lipopolysaccharides lining the outer membrane. Adapted from ref. 8.

## 1.2 The Gram-negative cell envelope

The cell envelope of Gram-negative bacteria is a complex, multi-layered structure that plays a crucial role in maintaining the integrity of the cell, facilitating interactions with the environment, and regulating the movement of substances across the cell membrane. This envelope consists of three main layers: the inner membrane (IM), the periplasmic space (which

contains a thin peptidoglycan layer), and the outer membrane (OM), the latter of which is a defining feature of Gram-negative bacteria<sup>9</sup>.

The inner membrane (IM) is composed of a glycerophospholipid bilayer that includes integral transmembrane proteins and lipoproteins. These components are key players in several essential processes, such as respiration, lipid biosynthesis, protein export, and nutrient uptake<sup>10</sup>. Unlike eukaryotic cells, which have specialized compartments for these activities, bacterial cells carry out all membrane-associated functions directly within the inner membrane.

The periplasmic space, which lies between the inner and outer membranes, serves as a water-filled compartment housing a variety of enzymes, chaperones, and binding proteins. These molecules are involved in envelope biogenesis, maintaining redox balance, and transporting materials<sup>9</sup>. The backbone of this space is the peptidoglycan layer, which consists of alternating N-acetylglucosamine and N-acetylmuramic acid residues, connected by short peptide chains. In Gram-negative bacteria, the third amino acid in the peptide chain is typically meso-diaminopimelic acid (m-DAP), whereas Gram-positive bacteria often use lysine<sup>11</sup>. Despite the relatively low amount of peptidoglycan in the envelope (just 5-10% of its total mass), it provides vital structural support and protects the cell from osmotic stress.

The outer membrane (OM) is a distinctive feature of Gram-negative bacteria and is made up of an asymmetric lipid bilayer. The inner layer consists of phospholipids, while the outer layer is composed of lipopolysaccharides (LPS). This structure contributes to selective permeability, limiting the entry of harmful substances<sup>12</sup>. The outer membrane is physically connected to the peptidoglycan layer through a covalent bond formed by Braun's lipoprotein (Lpp), which plays a critical role in maintaining structural integrity<sup>13</sup>. Embedded within the outer membrane are two major classes of proteins: lipoproteins and  $\beta$ -barrel outer membrane proteins (OMPs). Although many lipoproteins have unknown functions, OMPs such as porins and efflux pumps are essential for nutrient uptake and waste removal<sup>12</sup> (Figure 3).

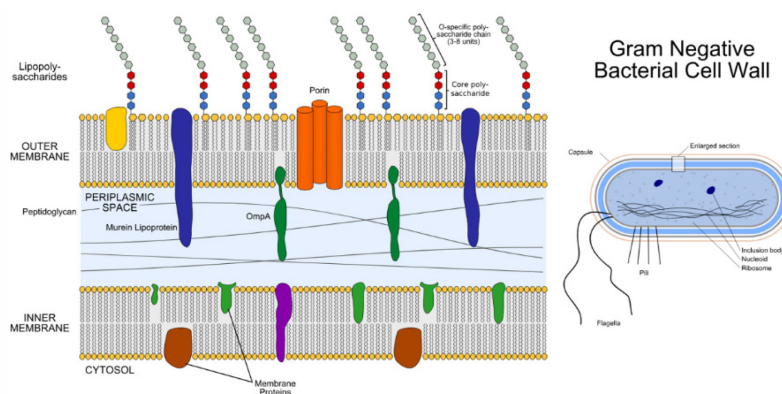


**Figure 3:** Schematic representation of the Gram-negative bacterial cell envelope. This diagram illustrates the three key layers: the inner membrane (IM), the periplasmic space containing a thin peptidoglycan layer, and the outer membrane (OM) composed of lipopolysaccharides (LPS), along with associated proteins involved in transport and structural integrity.

The synthesis of all components of the Gram-negative cell envelope occurs either in the cytoplasm or on the inner surface of the inner membrane. These components must then be translocated from the cytoplasm or flipped through the inner membrane. Proteins destined for the periplasm or outer membrane (OM) are first synthesized as precursors with a signal sequence at their amino terminus. This sequence directs them for translocation. The translocation process is catalyzed by an essential protein complex called SecYEG, with the assistance of the ATPase SecA and the proton motive force<sup>14</sup>. Once in the periplasm, these proteins are stabilized by molecular chaperones to prevent misfolding and aggregation. They are then sent to a site in the outer membrane known as the  $\beta$ -barrel assembly machinery (Bam) complex, where they are integrated into the membrane<sup>15</sup>.

Lipoproteins, after being translocated via the Sec pathway, are processed and sent to the outer membrane through the Lol (localization of lipoproteins) pathway. In this process, lipoproteins are extracted from the inner membrane by the ATP-binding cassette (ABC) transporter, LolCDE, and then carried through the periplasm by the soluble carrier protein, LolA. Finally, they are inserted into the outer membrane by LolB<sup>16</sup>.

LPS is synthesized on the inner leaflet of the inner membrane and flipped to the outer leaflet by the MsbA ABC transporter<sup>17</sup>. The LPS is then transported to the cell surface by a group of highly conserved proteins known as the Lpt (lipopolysaccharide transport) system. This system includes proteins like LptA, LptB, LptC, LptD, LptE, LptF, and LptG, which bridge the periplasmic space and assist in inserting the LPS into the outer membrane<sup>18</sup>.

The assembly and maintenance of the Gram-negative cell envelope is a carefully orchestrated process that requires the involvement of multiple membrane-spanning complexes, enzymes, and chaperone systems. These intricate networks are vital for the cell's function and survival, and they also represent potential targets for new antimicrobial therapies, especially in light of the growing antibiotic resistance in Gram-negative pathogens.

### 1.3 Lipopolysaccharide (LPSs)

Lipopolysaccharides (LPS) are glycoconjugates and represent the major component of the outer membrane of Gram-negative bacteria, significantly contributing to the structural integrity of the bacteria and the protection of the bacterial cell envelope. LPS also increases the negative charge of the cell membrane and helps stabilize the overall membrane structure. LPS constitutes about 10-15% of the total molecules in the outer membrane, covering approximately 75% of the bacterial surface area<sup>19,20</sup>. Due to their external location on the outer bacterial membrane, LPS are responsible for many biological interactions of bacteria with the external environment. In particular, they play a pivotal role in various host-bacteria interaction processes such as adhesion, recognition, pathogenesis, and symbiosis<sup>21</sup>. Furthermore, LPS are also referred to as endotoxins because they are cell-bound, and once released, they play a key role in the pathogenesis of Gram-negative infections due to their ability to trigger the immune system in a wide range of eukaryotic organisms<sup>22</sup>.

To understand the molecular basis of bacterium–host interactions, it is crucial to elucidate the structure of LPS and identify how bacteria modify this structure in response to different environments. It has already been demonstrated that a correct LPS structure is required to

establish disease (pathogens) or to produce beneficial outcomes (symbionts) in host-microbe interactions<sup>23</sup>.

From a chemical perspective, LPS are amphiphilic macromolecules composed of three distinct fractions:

- A lipophilic domain called Lipid A;
- A hetero-oligosaccharide domain, referred to as the Core;
- A hydrophilic polysaccharide fraction known as the O-chain (or O-polysaccharide chain or O-antigen)<sup>20,24</sup> (Figure 4).

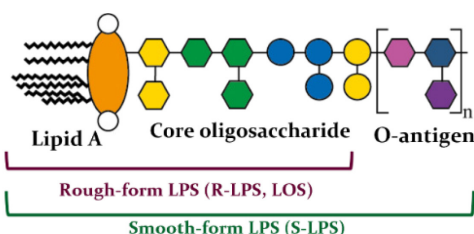


**Figure 4:** Schematic illustration of a lipopolysaccharide (LPS). It is composed of three main domain: lipid A, core oligosaccharide, and specific O-antigen. Adapted from ref. 8.

Bacteria can also synthesize LPS without the O-chain, called Lipooligosaccharides (LOS), or rough-type LPS (R-LPS), which confers a rough morphology to bacterial colonies. Conversely, the presence of an O-chain provides a smooth appearance to bacterial colonies (S-LPS)<sup>120</sup>.

### 1.3.1 Lipid A

Lipid A is a glycolipid and represents the hydrophobic domain of LPS, being responsible for anchoring the entire macromolecule to the outer membrane. It is the region of LPS with the least structural variability. Its general structure consists of a disaccharide backbone formed by a 2-amino-2-deoxy-D-glucopyranose (glucosamine, GlcN) disaccharide, linked by a  $\beta(1,6)$ -glycosidic bond, phosphorylated at positions 1 and 4', and acylated by 3-hydroxy fatty acid residues, called primary fatty acids, linked by ester bonds at positions 3 and 3', and by amide bonds at positions 2 and 2'. These fatty acids can also be further acylated at the hydroxyl position with secondary fatty acids, usually non-hydroxylated and of varying lengths<sup>21</sup> (Figure 5).

## Gram-negative bacteria

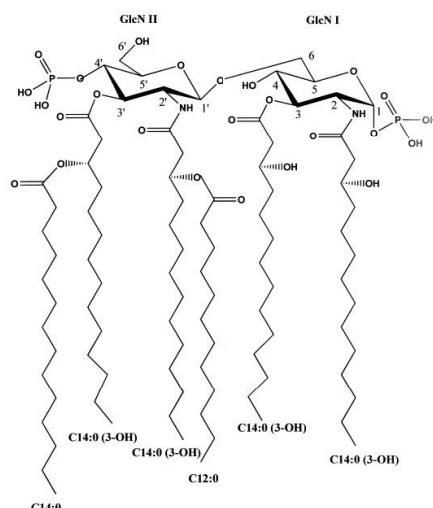


**Figure 5:** Example of Lipid A from LPS of *Escherichia coli*, showing the disaccharide backbone, phosphorylation sites, and acylation pattern.

Lipid A is often composed of a mixture of glycoforms that differ in their acylation and phosphorylation patterns. These subtle chemical differences have been identified as responsible for variations in the virulence of bacteria in response to adaptation to the host environment. In fact, some Lipid A isolated from Rhizobiaceae are mixtures of glycoforms in which one or both GlcNs are replaced by 2,3-diamino-2,3-dideoxy-D-glucopyranose (GlcN3N)<sup>22</sup>. For example, *Rhizobium etili* possesses a Lipid A in which the non-reducing GlcN is  $\alpha$ -1,6-linked to a residue of 2-amino-2-deoxy-D-gluconate, and in position 4, it is substituted by distal galacturonic acid residues. The *Rhizobium* genus is also characterized by the presence of long-chain secondary fatty acids, such as C28:0, C27:0 (OH), C30:0, or C29:0 (OH)<sup>23</sup>.

Lipid A is the endotoxin principle of LPS and is capable of triggering the innate immune system of plants (and mammals). The toxicity of Lipid A is strongly influenced by its primary structure, and changes in this structure significantly affect its ability to interact with and activate immune system receptors<sup>21</sup>.

### 1.3.2 Core

The core domain always contains a complex oligosaccharide component that is directly attached to Lipid A. It consists of approximately fifteen monosaccharide residues, organized into two different regions: the inner core and the outer core. The inner core, which is closest to Lipid A, is structurally less variable and contains peculiar monosaccharides such as heptose (e.g., L-glycero-D-mannoheptose or D-glycero-D-mannoheptose), and Kdo (3-deoxy-D-manno-octulosonic acid), a monosaccharide marker for all Gram-negative bacteria that links the core oligosaccharide to the Lipid A backbone<sup>120</sup>. The composition of the outer core is usually characterized by a few glycosidic residues, including neutral sugars, uronic acids and

aminosugars. Together with Lipid A, the core fraction forms the structural motif common to all LPS, suggesting that these domains are essential for the life of Gram-negative bacteria<sup>20,24</sup>.

### 1.3.3 O-chain

The O-polysaccharide chain, the hydrophilic component of LPS, is the most variable part of the LPS, even among bacteria belonging to the same genus<sup>18</sup>. It is usually composed of a regular polysaccharide, either homopolymeric or heteropolymeric, linear or branched, with subunits consisting of up to eight different sugars, and may have up to 50 identical repeating units. During LPS biosynthesis, the O-chain is formed by polymerization of blocks of different lengths, which are then added to the core. This means that LPS consists of a mixture of species differing in molecular weight, responsible for the characteristic ladder pattern of LPS molecules on SDS electrophoresis gels<sup>20</sup>. The monosaccharides can also exhibit very unique structures. A notable example is a carbocyclic sugar called caryose, found in the O-polysaccharide of *Burkholderia caryophilli*, a phytopathogenic bacterium responsible for the wilting of carnations<sup>24</sup>. The structural variability of the O-chain is attributed to the large number of monosaccharides that can make up the repeating units, as well as to the glycosidic sequence and the presence of non-carbohydrate substituents like phosphate, amino acids, sulfate, acetyl, or formamide groups, often present in non-stoichiometric amounts, which can mask the repeating oligosaccharide unit<sup>20,25</sup>. The significance of these substituents is often unknown, although bacteria frequently modify their LPS to camouflage themselves from the host and avoid detection by the immune system<sup>22</sup>.

The O-chain also plays important roles in plant-microbe interactions, as in the mammalian immune system, where it represents the antigenic moiety of smooth LPS and can be pivotal for bacterial pathogenesis and survival<sup>26</sup>.

## 1.4 Extracellular Polysaccharides (EPS): Functions and Applications

Extracellular Polysaccharides (EPS) are a diverse group of biopolymers produced by both Gram-positive and Gram-negative bacteria. These molecules are secreted outside the bacterial cell wall and play essential roles in bacteria's survival and adaptability to a range of environments. EPS are typically classified into two main categories based on their structure: homopolysaccharides, which consist of a single type of sugar (such as glucose or fructose), and heteropolysaccharides, which are made up of different sugars, like glucose, galactose, and mannose. This diversity in composition allows EPS to perform a wide array of functions for bacteria<sup>27</sup>.

One of the primary roles of EPS is to serve as energy reserves. When bacteria face nutrient scarcity, EPS act as stored carbon sources, providing energy when needed<sup>28</sup>. Additionally, EPS play a significant role in bacterial virulence. Certain pathogenic bacteria, such as *Streptococcus pneumoniae*, produce protective EPS capsules that help them evade the immune system, making infections harder to combat<sup>29</sup>. This protective function extends beyond immune evasion, EPS form a physical barrier that protects bacteria from environmental stresses such as dehydration, temperature fluctuations, and the presence of toxic substances. These properties are particularly important for biofilm formation, where bacterial communities

cluster together and secrete EPS, creating a protective matrix that enhances their resistance to external threats like antibiotics<sup>30</sup>.

EPS also act as a defense mechanism against bacteriophages, viruses that specifically target bacteria. By forming a physical barrier, EPS help prevent these viral infections<sup>31</sup>. Moreover, EPS are crucial in the adhesion process. They mediate interactions between bacteria and their environment, whether by anchoring bacteria to surfaces or facilitating their colonization of plant roots. This ability to adhere is essential for the establishment of symbiotic relationships, such as the one between nitrogen-fixing bacteria like *Rhizobium* and legumes<sup>28</sup>.

Beyond their biological functions, EPS have a wide range of industrial applications. In the food industry, EPS are used as additives to improve the texture and consistency of various products. For instance, curdlan, an EPS derived from *Alcaligenes faecalis*, is widely used in the production of tofu, yogurt, and noodles, where it acts as a stabilizer and thickener<sup>32</sup>. In the pharmaceutical and medical fields, EPS have shown promise in supporting immune health, reducing inflammation, and even fighting cancer. Their biocompatibility and ability to interact with biological systems make them suitable for use in drug delivery systems, where they can carry therapeutic agents to specific parts of the body<sup>33,34</sup>.

EPS are also gaining attention in the field of environmental sustainability. Due to their biodegradable nature, EPS provide an eco-friendly alternative to synthetic polymers, and they hold promise for use in biodegradable packaging and other materials<sup>34</sup>. Furthermore, EPS play a role in bioremediation, as they can help bind and remove toxins from polluted environments. In wastewater treatment, particularly in industries like dairy and brewing, EPS contribute to the management of waste by binding harmful substances and improving the clarity of treated water<sup>36</sup>.

Overall, EPS are far more than just bacterial byproducts; their diverse and multifunctional properties make them invaluable across various industries. From enhancing food products to supporting medical advancements, improving environmental sustainability, and offering alternatives to plastics, EPS are becoming an essential component of modern industrial processes. As research continues, we can expect EPS to play an even greater role in addressing some of the most pressing challenges in health, food security, and environmental protection<sup>37</sup>.

## 1.5 The Plant Microbiota

The plant microbiota is an essential part of the plant holobiont, a community of microorganisms that work together to support the plant's growth and health<sup>38</sup>. By studying how plants interact with their microbiomes and understanding the factors that shape these microbial communities, we gain valuable insights into the role these microorganisms play in plant biology.

The plant microbiota is divided into three key compartments:

**Rhizosphere:** This is the soil surrounding the plant's roots, where microbial communities are heavily influenced by the compounds the plant releases through its roots—such as organic acids and amino acids. These root exudates essentially act like signals, shaping the microbial

populations in this area and helping the plant with nutrient acquisition, protection from pathogens, and even communication with other organisms in the soil.

**Endosphere:** The internal tissues of the plant, both roots and above-ground organs, are home to microorganisms that have either entered the plant through natural openings or made their way inside via direct contact with plant cells. These microbes play essential roles in nutrient cycling, pathogen protection, and overall plant health. They live within the plant, often forming long-term relationships that contribute to the plant's survival.

**Phyllosphere:** The aerial environment of the plant, including its leaves, flowers, seeds, and fruits, hosts a diverse group of microorganisms. These microbes interact with the plant's surface, helping it cope with environmental stresses like high humidity or temperature, while also offering protection against harmful pathogens and pests. The phyllosphere is especially critical for plant defense against airborne diseases.

These three compartments (Figure 6), rhizosphere, endosphere, and phyllosphere, each serve unique roles in the plant's overall biology. While the rhizosphere and endosphere focus primarily on below-ground and internal interactions, the phyllosphere is where the plant meets its environment in the above-ground world<sup>39</sup>.

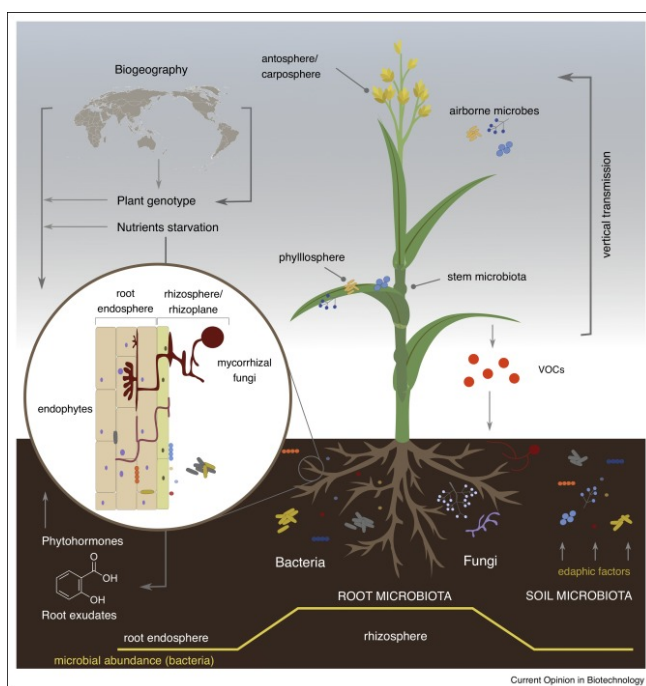


**Figure 6:** Schematic representation of the main factors influencing the composition of plant-associated microbiota across various host compartments. Plant-associated microbes are primarily recruited from the soil, via vertical transmission through seeds, and through airborne microbes. **VOCs:** Volatile Organic Compounds.

### 1.5.1 Functional Roles of the Plant Microbiota

The plant microbiota plays a fundamental role in helping plants grow, stay healthy, and adapt to their environment. Among the many microorganisms that live in and around plants, some are particularly helpful, these are known as **Plant Growth-Promoting Bacteria (PGPB)** (Figure 7). These beneficial microbes support plants in several important ways.

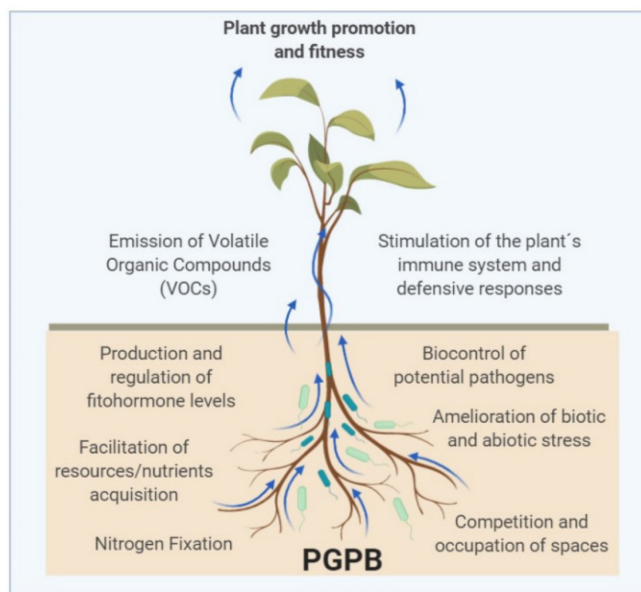


**Figure 7:** Illustration of key functions of Plant Growth-Promoting Bacteria (PGPB) in supporting plant health and development. PGPB enhance plant growth by producing and regulating phytohormones, facilitating nutrient acquisition, and fixing nitrogen. They also protect plants by stimulating immune responses, suppressing pathogens, and mitigating both biotic and abiotic stress. Additional mechanisms include the emission of volatile organic compounds (VOCs) and competition for space, contributing to overall plant fitness and resilience. Adapted from ref. 40.

First, they produce phytohormones like auxins, cytokinins, and gibberellins, which are natural chemicals that regulate how plants grow and develop. They also help plants deal with stress. For example, some bacteria produce an enzyme called ACC deaminase, which lowers levels of ethylene, a hormone that builds up when a plant is stressed, such as during drought or extreme temperatures. By keeping ethylene in check, these microbes help plants cope better with tough conditions.

Another important job of PGPB is improving the plant's ability to take up nutrients. Some microbes can fix nitrogen from the air or make phosphorus in the soil more available, giving the plant better access to these essential elements. Well-known bacteria like *Pseudomonas*, *Arthrobacter*, and *Bacillus* are great examples of microbes that boost plant growth, increase resistance to stress, and promote overall health<sup>41</sup>.

But not all microorganisms are allies. Some, like *Pseudomonas syringae* or *Erwinia amylovora*, are pathogens that produce harmful compounds and cause disease in plants. Fortunately, many beneficial microbes also act as protectors. They produce natural antibiotics, enzymes that break down pathogen cells, and molecules called siderophores that compete for nutrients, effectively starving the bad guys. In doing so, these helpful microbes keep harmful organisms in check, creating a natural defense system that protects the plant.

In this way, the plant microbiota acts like a supportive community, some members help the plant grow, others guard against threats, and together they form a living shield that keeps the plant strong and resilient.

### 1.5.2 Below-ground Microbiota

The microorganisms that live around and inside plant roots are primarily derived from the surrounding soil, but they can also be transmitted from the seeds. The rhizosphere, the soil that is directly influenced by the roots, hosts a rich variety of microbial life. Common microbes here include *Acidobacteria*, *Proteobacteria*, *Planctomycetes*, and *Actinobacteria*. Plant roots release exudates like organic acids and amino acids, which shape the microbial communities around them, a phenomenon known as the rhizosphere effect<sup>42</sup>. For example, maize roots produce secondary metabolites such as benzoxazinoids that influence the types of microbes in the surrounding soil, including those from *Actinobacteria* and *Proteobacteria*<sup>43</sup>. The types and amounts of exudates vary between plant species, and these variations play a significant role in shaping the microbiota.

In addition to surface microbes, plants are also home to endophytes, bacteria that live within plant tissues. These microbes may enter through root cracks or actively colonize plant tissues, depending on the plant's needs and the microbe's abilities. The endophytes present in plant roots vary, with *Proteobacteria*, *Acidobacteria*, and *Actinobacteria* being common inhabitants<sup>43</sup>.

### 1.5.3 Above-ground Microbiota

The microbiota in a plant's aerial parts, such as leaves, stems, flowers, and fruits, includes both endophytes (microbes living inside plant tissues) and epiphytes (microbes that live on the plant surface). The microbiota in these areas differs from those in the roots, as the ecological conditions vary greatly. Endophytes move through the plant via the xylem, which transports water and nutrients. On the other hand, epiphytes tend to colonize the plant surface, adapting to the environmental conditions on the plant's exterior.

For example, in grapevine, the phyllosphere is largely dominated by microbes like *Pseudomonas*, *Bacillus*, and *Erwinia*, while the endophytic community inside the grapevine's berries is enriched with *Ralstonia*, *Burkholderia*, and *Pseudomonas*<sup>39</sup>. Environmental factors such as climate, the presence of pathogens, and farming practices can greatly affect the microbial composition on these parts of the plant.

### 1.5.4 Determinants of Plant Microbiota Composition

The composition of the microbiota in various parts of the plant is shaped by a combination of biotic (living) and abiotic (non-living) factors. For the microbiota in the soil surrounding plant roots, soil properties like pH, moisture, salinity, and organic content are particularly influential. For above-ground plant microbiota, external factors like weather, the presence of pathogens, and human activities (e.g., farming practices) play a significant role in shaping microbial communities.

Moreover, the species and genotype of the plant itself greatly impact which microorganisms are recruited. The plant's root structure, the types of chemicals it releases into the soil, and the compounds it deposits around its roots (known as rhizodeposits) are key factors in microbial community assembly. Interestingly, different plant species can recruit very different microbial communities from the same soil, highlighting the plant's active role in shaping its microbiota<sup>39</sup>.

### 1.5.5 Plant-associated Bacteria

Bacteria that live in and around plants play a vital role in keeping them healthy and helping them grow. These plant-associated bacteria are part of the larger plant microbiota and can be grouped into three main types based on where they live:

- **Endophytic bacteria:** These microbes live inside the plant's tissues, within roots, stems, or leaves, often without causing any harm. In fact, they usually help the plant by boosting its defense against stress and disease.
- **Phyllospheric bacteria:** These bacteria are found on the outer surfaces of the plant, especially on leaves, stems, and flowers. Though they live externally, they still influence plant health by interacting with the plant's environment.
- **Rhizospheric bacteria:** These reside in the thin layer of soil around plant roots, an area that's rich in nutrients due to the substances (root exudates) the plant releases. This area is a hotspot for microbial activity.

Plant-associated bacteria support growth in many ways. Some do this directly, for example, by fixing atmospheric nitrogen into a form plants can use (a process called biological nitrogen fixation), or by making key nutrients like phosphorus and iron more available through nutrient solubilization. Others produce phytohormones that stimulate plant development. Certain endophytic bacteria called diazotrophs are particularly effective at fixing nitrogen inside the plant, while some rhizobacteria release siderophores, molecules that help plants absorb essential minerals like iron<sup>44,45</sup>.

These bacteria can also help indirectly by outcompeting harmful microbes for resources, or by producing natural protective substances such as antibiotics and antifungal compounds. Additionally, some rhizobacteria and endophytes release organic acids or metal-chelating agents that make minerals in the soil easier for plants to absorb. By strengthening the plant's immune response and increasing its tolerance to stress, these microbes contribute significantly to the plant's resilience and overall vitality<sup>46</sup>.

In short, plant-associated bacteria are not just passengers, they're partners that work side by side with plants to ensure their survival, growth, and productivity.

## 1.6 Roles of LPS in the Plant Immune System

### 1.6.1 Plant Innate Immune System

The innate immune system represents the body's first line of defense against invading pathogens, relying on a set of non-specific mechanisms to quickly respond to potential threats. While this system has been well studied in vertebrates and insects, its role in plants has only recently begun to receive comparable attention. Interestingly, plants, despite lacking an adaptive immune system, have evolved sophisticated ways to detect and respond to microbial invaders. Much like mammals, they recognize specific molecular signatures known as Microbe-Associated Molecular Patterns (MAMPs), which are essential for microbial survival but absent in eukaryotic cells<sup>47</sup>. These patterns are detected by Pattern Recognition Receptors (PRRs) on the plant cell surface, which trigger immune responses when activated. Common examples of such microbial signals include lipopolysaccharides (LPS), peptidoglycan, and flagellin, all of which can strongly stimulate plant immune responses<sup>47,48</sup> (Figure 8).

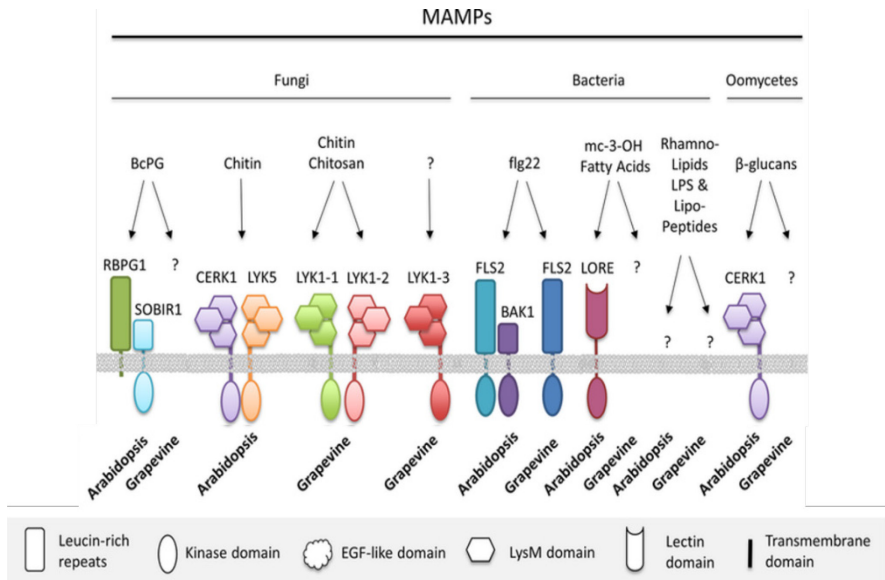


**Figure 8:** Recognition of MAMPs by specific PRRs in Arabidopsis and grapevine. Different microbial patterns from fungi, bacteria, and oomycetes are detected by receptors like FLS2, CERK1, and LORE, triggering innate immune responses. Some PRRs are conserved across species, while others remain unidentified. Adapted from ref. 49.

In mammals, the immune system detects invading pathogens through Toll-like receptors (TLRs), which are proteins found on immune cells like dendritic cells and macrophages. These receptors are made up of two key parts: an extracellular domain that recognizes microbial patterns and an intracellular domain that transmits the signal to the cell. This system is quite similar to the way the human IL-1 receptor works<sup>50,51</sup>. Interestingly, plants have their own version of these receptors. Some of their resistance (R) genes are actually homologous to TLRs, but instead of directly recognizing pathogens, they seem to be more involved in transmitting signals inside the cell<sup>52</sup>.

What makes plants a bit different from animals is that all their pattern recognition receptors (PRRs) are located on the cell surface. Unlike animals, where certain immune receptors are found inside the cell, plants have no known intracellular receptors for pathogen molecules<sup>53</sup>.

Once a plant's PRRs sense the presence of a microbe, they trigger a series of defense responses. This includes the production of reactive oxygen species (ROS) and nitrogen species like nitric oxide, changes to the cell wall, the creation of antimicrobial compounds, and the activation of pathogenesis-related (PR) proteins (Figure 9). Often, this leads to localized cell death, known as the hypersensitive response (HR), which serves as a second layer of defense called effector-triggered immunity (ETI)<sup>47,54</sup>.

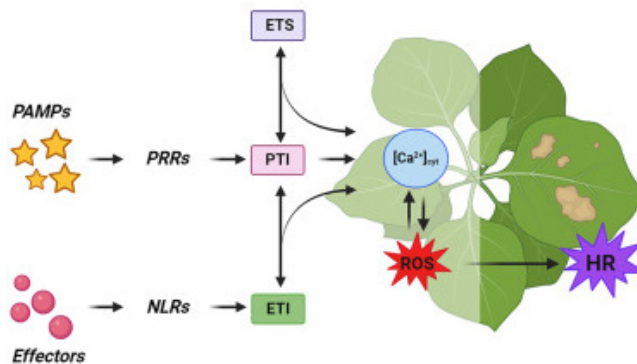


**Figure 9:** Schematic representation of the plant immune system. Pathogen-Associated Molecular Patterns (PAMPs) are recognized by Pattern Recognition Receptors (PRRs), triggering PAMP-Triggered Immunity (PTI). Pathogen effectors can suppress this response, leading to Effector-Triggered Susceptibility (ETS), but are subsequently detected by intracellular Nucleotide-binding Leucine-rich Repeat receptors (NLRs), activating Effector-Triggered Immunity (ETI). Both PTI and ETI result in calcium signaling, reactive oxygen species (ROS) production, and the hypersensitive response (HR), visible as localized leaf necrosis. Adapted from ref. 55.

These defense responses are largely controlled by the plant's R genes, which can detect pathogen effectors. When activated, R genes trigger defense reactions such as callose deposition, lignification, and the production of PR proteins like 1,3-glucanases and chitinases. All these responses work together to stop the pathogen in its tracks, suppressing bacterial growth and causing cell death at the infection site. Interestingly, variations in the structure of

lipopolysaccharides (LPS), one of the most well-known microbe signals, can influence how the plant responds<sup>48</sup>.

Interestingly, LPS can sometimes work in the opposite way and suppress the immune response. For example, if plants are pretreated with heat-killed bacteria or purified LPS, they tend to show fewer disease symptoms and less HR when infected later. This phenomenon is called localized induced resistance (LIR), and it is often linked with an increase in soluble peroxidases, antimicrobial peptides, and other defense molecules like hydrogen peroxide<sup>47</sup>. Changes in the cell wall can be also observed, like thickening or granular deposits forming at the infection site.

Researchers have dug into the structure of LPS to understand what triggers LIR. They've found that the minimal requirement for this immune priming lies in the lipid A-core region of the LPS molecule. Interestingly, while the O-antigen (a part of LPS) isn't necessary for this effect, lipid A by itself doesn't work either because it's too hydrophobic and insoluble in water<sup>56</sup>. That's why the lipid A must be accompanied by some of the core oligosaccharide for it to activate the plant's immune system. It seems that this process helps the plant stay resistant to pathogens without causing excessive damage to its own tissues<sup>57</sup>.

#### 1.6.2 LPS and Pathogenesis

LPS play a crucial role in how many Gram-negative bacteria cause disease in plants. When these bacteria divide or break apart, they release LPS into the plant's environment. This helps the bacteria survive by protecting them from the plant's natural defenses, including antimicrobial compounds and environmental stress<sup>47</sup>. Research has shown that bacteria lacking important parts of the LPS, like the core or lipid A, often cannot infect the plant or survive inside it. This highlights how important LPS is as a protective shield for bacteria<sup>58</sup>.

Interestingly, while the O-antigen (the outer part of the LPS) may not always be necessary for bacteria to cause disease, the inner and outer core parts are essential for the LPS to function properly during infection. When these parts are missing or mutated, bacteria struggle to colonize and become more vulnerable to oxidative stress and chemicals<sup>59</sup>.

There is also growing evidence that LPS might help bacteria dodge the plant's immune system. Some bacteria can tweak their LPS structure to avoid detection or even suppress the plant's defense responses, allowing them to establish a successful infection<sup>60</sup>.

#### 1.6.3 LPS and Symbiosis

LPS aren't just important in the context of pathogens, they also play a key role in mutualistic plant-microbe relationships. For example, in the symbiosis between nitrogen-fixing bacteria and legumes, LPS help with early recognition by the plant, the formation of infection threads, and even suppressing the plant's immune responses<sup>60,54</sup>. The O-antigen is particularly important here, ensuring that the infection thread is initiated and extended correctly, while also masking the bacterial surface to prevent the plant from recognizing it as a threat<sup>38</sup>.

For these interactions to work, the structure of the LPS has to remain intact. Bacteria with truncated or missing O-chains often fail to properly colonize the plant or provoke early defense responses. In this way, the O-antigen acts like a protective shield, keeping more detectable parts of the bacteria, like lipid A and outer membrane proteins, from alerting the plant's immune system<sup>48</sup>.

Interestingly, the lipid A in these symbiotic bacteria is often different from that in pathogenic ones. These differences, whether it's tweaks in the sugar structure or changes in how fatty acids are added, make the bacteria less toxic and help them survive inside the plant without triggering a strong immune reaction<sup>61</sup>. For instance, some mutualistic bacteria have lipid A with galacturonic acid or 2-amino-2-deoxy-gluconic acid, making them less likely to be seen as harmful by the plant<sup>47</sup>.

These variations show how LPS act as both a protective barrier and a communication tool, with their structure carefully adjusted by the bacteria to either help them cause disease or form a beneficial relationship with the plant.

## 1.7 Gut Microbiota

The human gut is home to a remarkably dense and diverse community of microorganisms, collectively known as the gut microbiota. Far from being passive residents, these microbes actively contribute to many aspects of our health, from nutrient metabolism to immune system development and defense against pathogens<sup>62</sup>. Most gut bacteria belong to four dominant phyla: Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria (Figure 10). This microbial composition begins forming early in life and typically stabilizes during childhood, although it remains sensitive to various influences, such as dietary shifts, antibiotic use, and disease states<sup>63</sup>.

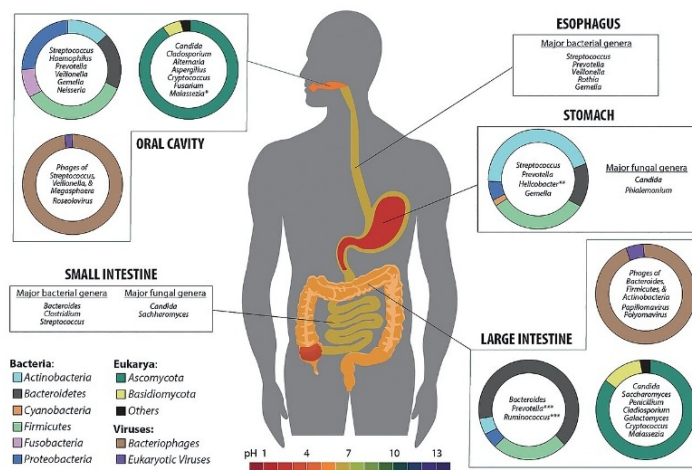


**Figure 10:** Taxonomic composition of the human gut microbiota at the phylum level. The dominant phyla include Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria. These microbial groups collectively play essential roles in host metabolism, immune modulation, and maintenance of gut homeostasis. Adapted from ref. 64.

In a balanced state, the gut microbiota performs essential physiological roles. It ferments complex carbohydrates that humans cannot digest on their own, producing short-chain fatty acids (SCFAs) that nourish colonocytes and support intestinal barrier function<sup>65</sup>. Moreover, gut microbes synthesize important vitamins, such as vitamin K, folate, riboflavin, biotin, and cobalamin, that are indispensable for host metabolic and cellular functions. Beyond its metabolic duties, the microbiota is intricately involved in immune regulation. It shapes both innate and adaptive immune responses, helping the body distinguish between harmful invaders and harmless or beneficial microbes<sup>66</sup>. For instance, certain commensals modulate the production of interleukin-10 (IL-10), a key anti-inflammatory cytokine that regulates immune activation and prevents excessive inflammation. Notably, *Bifidobacterium longum* has been shown to enhance IL-10 production during pulmonary infections with *Klebsiella pneumoniae*, helping to reduce tissue damage and suppress bacterial spread<sup>67</sup>.

A critical function of the microbiota is colonization resistance, the ability of resident microbes to prevent pathogenic species from establishing themselves by outcompeting them for nutrients and producing antimicrobial compounds<sup>68</sup>. However, disturbances to this microbial equilibrium, known as dysbiosis, can impair colonization resistance and create a permissive environment for pathogens. Dysbiosis has been implicated in numerous conditions, including inflammatory bowel disease (IBD), metabolic syndrome, autoimmune diseases, and even neuropsychiatric disorders<sup>69</sup>.

More recently, the gut has been recognized as a central node in systemic communication, particularly through the gut-brain axis (GBA), a bidirectional network connecting the gut and central nervous system via neural, endocrine, and immune pathways<sup>70</sup>. Microbial components, such as lipopolysaccharides (LPS) from Gram-negative bacteria, have gained attention for their potent immunostimulatory effects. LPS can activate toll-like receptor 4 (TLR4) on immune cells, triggering inflammatory responses that may affect both gut and brain health<sup>71</sup>. Understanding how microbial communities interact with pathogens and how elements like LPS contribute to immune modulation offers promising opportunities for novel therapeutic approaches aimed at restoring microbial balance and preventing disease.

### 1.7.1 Interactions Between the Microbiota and Pathogens

The gut microbiota plays a crucial role in defending us against infections, acting as a frontline barrier against harmful pathogens. These beneficial microbes—and the substances they produce, work closely with our immune system to detect and eliminate threats. For instance, *Bacteroides thetaiotaomicron* helps stimulate the production of REGIIIγ, a protein with antimicrobial properties that targets Gram-positive bacteria<sup>72</sup>. Similarly, certain strains of *Bacillus thuringiensis* release bacteriocins that combat dangerous pathogens like *Clostridium difficile*<sup>73</sup> while *Bifidobacterium* species generate organic acids and antimicrobial peptides that suppress the growth of harmful *Escherichia coli* strains<sup>74</sup>.

One of the microbiota's most essential functions is known as *colonization resistance*, the ability of beneficial microbes to prevent pathogens from taking hold (Figure 11).

Gram-negative bacteria

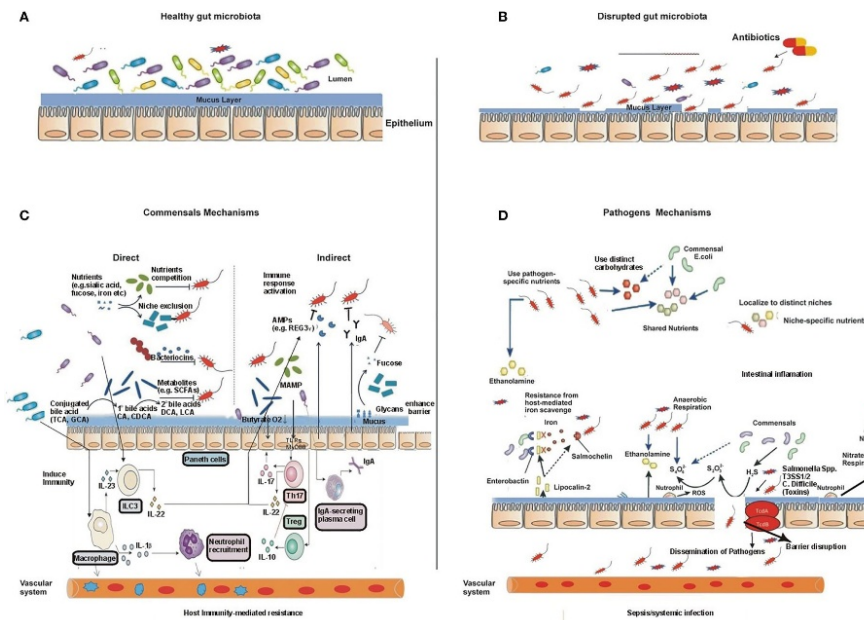


**Figure 11:** (A) In a healthy gut, a diverse and balanced microbial community forms a protective layer above the intestinal epithelium, contributing to homeostasis. (B) Antibiotic treatment disrupts microbial diversity, allowing the expansion of pathogenic bacteria and weakening mucosal protection. (C) Commensal microbes protect the host via direct mechanisms—such as nutrient competition, niche exclusion, and production of bacteriocins and beneficial metabolites (e.g., SCFAs and secondary bile acids)—and indirect mechanisms including immune modulation (e.g., IL-22, REGIIIγ, IgA production, Treg activation), and strengthening of the epithelial barrier. (D) Pathogens exploit specific mechanisms to overcome colonization resistance, including the use of distinct nutrients (e.g., ethanolamine, iron), localization to unique niches, production of virulence factors (e.g., toxins), and anaerobic respiration enhanced by inflammation. These mechanisms can lead to barrier disruption and systemic dissemination, potentially causing sepsis. Adapted from ref. 75.

They achieve this by competing for nutrients, producing antimicrobial substances, and releasing metabolites such as short-chain fatty acids (SCFAs), which not only nourish the gut lining but also boost the immune response and reinforce the intestinal barrier<sup>76,77</sup>. When this balance is disrupted, a state known as dysbiosis, this protective system becomes compromised, leaving us more vulnerable to infections and chronic diseases such as inflammatory bowel disorders, metabolic syndromes, and even neurological conditions<sup>69,78</sup>.

A clear example of this breakdown is *Clostridium difficile*, which often takes advantage of weakened microbiota following antibiotic use. In these imbalanced conditions, *C. difficile* can rapidly multiply and cause serious illness<sup>79</sup>. Thankfully, not all is lost, certain beneficial microbes step in to help. As mentioned, *Bacillus thuringiensis* produces bacteriocins that inhibit *C. difficile*, *Bifidobacterium* counters *E. coli*, and *Bacteroides thetaiotaomicron* promotes the body's own antimicrobial defenses<sup>72,73,74</sup>.

However, there's another emerging concern: the spread of antibiotic resistance genes within the gut microbiota. These genes can linger in the gut environment and be passed on to

harmful bacteria, making infections much more difficult to treat and raising the risk of drug-resistant diseases<sup>80,81</sup>.

In short, maintaining a balanced gut microbiome is vital—not just for digestion and metabolism, but also for protecting against infections and curbing the spread of antibiotic resistance.

## 1.7.2 Novel Therapeutic Strategies Targeting the Microbiota

The gut microbiota plays a vital role in maintaining overall health, and its manipulation has emerged as a promising therapeutic strategy in treating and preventing a variety of diseases<sup>82</sup>. Researchers are increasingly focusing on ways to not only directly combat harmful pathogens but also to restore and support a healthy microbial balance<sup>83</sup>. This growing field aims to harness the power of our microbiota to enhance immune function, prevent infections, and even address conditions that go beyond the gut, such as metabolic disorders and autoimmune diseases.

One of the most talked-about approaches involves probiotics and prebiotics. Probiotics are live microorganisms that can help restore balance in the gut, while prebiotics are substances that help beneficial bacteria grow and thrive<sup>84</sup>. These strategies are being used to prevent conditions like antibiotic-associated diarrhea, and certain probiotic strains, such as *Lactobacillus* and *Bifidobacterium*, have shown promise in regulating immune responses and supporting gut health<sup>85</sup>. By enhancing the balance of beneficial microbes, these treatments aim to bolster the body's defenses against harmful invaders.

Another innovative treatment gaining attention is fecal microbiota transplantation (FMT), which involves transferring stool from a healthy donor to a patient to restore a healthy microbiota. FMT has proven to be highly effective in treating recurrent *Clostridium difficile* infections, which are notoriously difficult to manage with traditional antibiotics<sup>83</sup>. Beyond *C. difficile*, FMT is being studied for its potential in treating other conditions like inflammatory bowel disease (IBD) and even metabolic disorders<sup>79</sup>. However, there are still concerns about the possibility of introducing new pathogens during the process, which highlights the need for continued research and improvements in safety protocols.

Diet, too, plays a critical role in shaping the gut microbiota. Diets rich in fiber promote the growth of beneficial microbes and increase the production of short-chain fatty acids (SCFAs), which support immune regulation and gut health<sup>86</sup>. In contrast, diets high in fat and low in fiber are often linked to an imbalance in microbial communities, contributing to inflammation and chronic diseases<sup>78</sup>. Therefore, dietary changes that promote a healthy microbiota could offer an accessible and effective way to prevent and treat various health issues.

In addition to these established approaches, next-generation biotherapeutics are emerging as potential game-changers. These include genetically engineered bacteria that can deliver therapeutic molecules directly to the gut or modulate the host's gene expression, offering targeted treatments for various conditions<sup>87</sup>. Bacteriophage therapy, which uses viruses to target and kill specific harmful bacteria in the gut, is another exciting area of research<sup>88</sup>. These new therapies could provide more precise methods of controlling harmful pathogens without disrupting the beneficial microbes that are so important to health.

As we continue to learn more about the intricate relationship between our microbiota and overall health, the potential for microbiota-based therapies to revolutionize treatment options becomes more apparent. By supporting and restoring a healthy gut microbiota through probiotics, prebiotics, FMT, dietary changes, and cutting-edge biotherapeutics, we may be able to prevent infections, boost immune function, and even tackle diseases that go beyond the gut. With continued research and development, these therapies could transform how we approach health and disease in the future.

### 1.7.3 The Role of Lipopolysaccharides in Gut Microbiota, Immune Modulation, and Health Implications

In recent years, research into the gut microbiota has moved beyond its traditional role in digestion, revealing its significant influence on our immune system and even mental health. A key player in this intricate relationship is lipopolysaccharides (LPS), molecules found in the outer membrane of Gram-negative bacteria. LPS plays a pivotal role in triggering immune responses, particularly those that can lead to inflammation, which in turn affects overall health<sup>89</sup>. The immunogenic properties of LPS are tightly linked to its chemical structure. Specifically, the Lipid A moiety, which consists of a  $\beta(1\rightarrow6)$  diglucosamine backbone acylated with multiple fatty acids, is responsible for the activation of Toll-like receptor 4 (TLR4). Variations in the acylation pattern, such as the number and length of fatty acid chains, significantly affect its immunostimulatory capacity. For instance, the hexa-acylated form of Lipid A, typically found in *Escherichia coli*, induces a robust immune response, whereas hypo-acylated variants present in *Bacteroides* species trigger milder inflammatory responses, promoting immune tolerance<sup>90</sup>. These molecules vary in structure, particularly in the lipid A component, which determines how strongly they can activate the immune system. For example, the hexa-acylated lipid A from *Escherichia coli* is a potent activator, sparking a strong inflammatory reaction through the activation of the immune receptor TLR4<sup>91</sup>. However, some beneficial gut bacteria, such as *Bacteroides vulgatus* and *Parabacteroides distasonis*, produce a less active form of LPS with fewer fatty acid chains, which induces a milder immune response and helps to maintain balance and tolerance in the gut<sup>92,93</sup>.

When LPS interacts with immune cells, it activates pathways that trigger the release of cytokines and other immune signals to protect the body from infections<sup>94</sup>(Figure 12). However, if the immune system is exposed to too much LPS, whether due to an imbalance in gut bacteria or a weakened intestinal barrier, this can lead to chronic inflammation and contribute to conditions like inflammatory bowel disease (IBD), Crohn's disease, and ulcerative colitis<sup>95</sup>. Moreover, elevated levels of LPS in the bloodstream, a condition known as metabolic endotoxemia, have been linked to metabolic disorders such as obesity, insulin resistance, and type 2 diabetes, particularly when a high-fat diet compromises the gut barrier<sup>96</sup>. Pathogens like *Klebsiella pneumoniae* can even change their LPS structure to avoid detection by the immune system, further complicating the regulation of immune responses<sup>97</sup>.

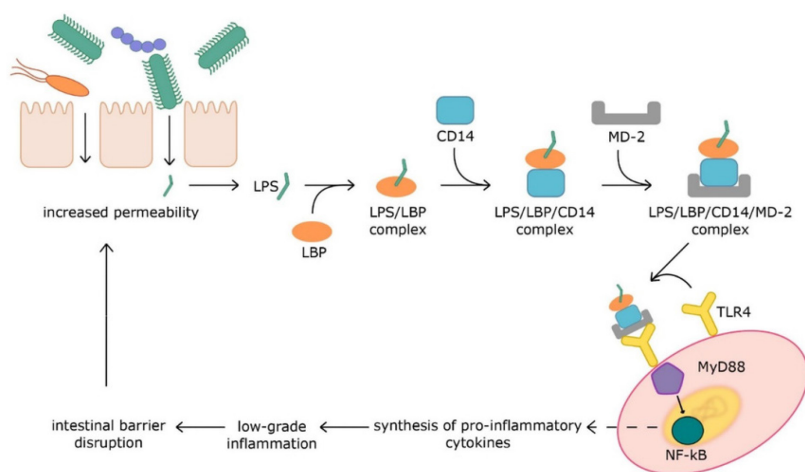


**Figure 12:** Illustration of the immune activation process in the gut via recognition of lipopolysaccharide (LPS). Increased intestinal permeability allows LPS to cross the barrier, forming complexes with LBP and CD14 that activate the TLR4 receptor. This signaling cascade leads to the production of pro-inflammatory cytokines and contributes to intestinal barrier dysfunction and chronic inflammation. Adapted from ref. 98.

Small changes in LPS structure can have significant consequences for health, shaping immune responses in ways that influence disease outcomes. Beyond gut health, LPS can also have systemic effects, with its ability to move from the gut into the bloodstream leading to issues like neuroinflammation, which is linked to conditions such as Alzheimer's disease and depression<sup>99</sup>. This connection underscores the importance of the gut-brain axis (GBA), a bidirectional communication system involving microbial metabolites, immune mediators, and neural signals. Microbial products like short-chain fatty acids (SCFAs) and neurotransmitter precursors such as GABA and serotonin can influence brain function, while changes in the brain can affect gut health<sup>100,101</sup>. Imbalances in the microbiota have been linked to psychiatric conditions such as depression, autism, and Parkinson's disease, suggesting that targeting the microbiota-GBA axis could open new avenues for treating these disorders.

## 1.8 Aims of the thesis

The surface of bacterial cells is composed of complex carbohydrate-based structures that play essential roles in protection, environmental adaptation, and communication with the host. This thesis focuses specifically on the isolation and structural characterization of key surface glycoconjugates, lipopolysaccharide (LPS), lipooligosaccharide (LOS), and exopolysaccharide (EPS), which constitute principal components of the outer membrane in Gram-negative bacteria. These molecules, known as Microbe-Associated Molecular Patterns (MAMPs), serve as critical mediators in the recognition of both pathogenic and commensal bacteria by the host immune system.

Given the close relationship between the chemical structure of these glycans and the immune responses they trigger, elucidating their molecular architecture is fundamental for

distinguishing bacteria perceived as harmful from those considered harmless or beneficial. Consequently, the main objective of this thesis is the comprehensive characterization of bacterial glycans derived from both plant-associated bacteria (*Herbaspirillum* and *Methylobacterium*, Chapters III and IV) and gut-associated bacteria (*Paenalcaligenes*, Chapter V), with particular emphasis on assessing their immunomodulatory properties using in vitro models.

Employing a multidisciplinary approach that integrates structural biology, biophysical analyses, and immunological assays, this work aims to elucidate how the chemical features and supramolecular organization of surface glycoconjugates, including lipooligosaccharides (LOS) and exopolysaccharides (EPS), influence host immune perception. Each chapter delves into the molecular architecture of the glycans produced by these bacteria, evaluating how subtle structural modifications (such as methylation or acetylation of sugar residues) may provoke distinct immune responses.

The ultimate goal is to identify structural patterns that distinguish beneficial bacteria from pathogenic ones, thereby advancing our understanding of the molecular mechanisms underpinning host-microbe interactions and the role of bacterial glycans in immune system modulation.

The thesis is structured as follows:

- **Chapter III** analyzes *Herbaspirillum* Root189, a root-colonizing bacterium associated with *Arabidopsis thaliana*. This chapter investigates how its LPS—rich in modified rhamnose residues—facilitates evasion of the plant immune system and successful colonization of the rhizosphere.

- **Chapter IV** is dedicated to *Methylobacterium extorquens*, a genus of plant-associated bacteria known for its ability to colonize the phyllosphere. This chapter characterizes both the lipooligosaccharides (LOS) and exopolysaccharides (EPS) produced by this bacterium. Special attention is given to the biophysical behavior of EPS in aqueous environments, including diffusion and aggregation properties. The combined analysis of these glycoconjugate classes highlights the structural and functional roles of bacterial surfaces in plant-environment interactions, suggesting adaptation strategies and potential immunological implications, as well as providing insights into how EPS structure correlates with function in ecological contexts.

- **Chapter V** focuses on *Paenalcaligenes hominis*, a bacterium increasingly detected in the gut microbiota of elderly individuals. This chapter explores the structure of its LPS and its potential involvement in low-grade chronic inflammatory states, offering new perspectives on microbiota-host interactions in conditions related to aging and neuroinflammation.

By adopting this multidisciplinary approach, the thesis seeks to bridge the gap between the chemical structures and biological functions of LPS and other bacterial glycans, contributing to a broader understanding of how microbial surfaces influence host interactions across diverse biological kingdoms.

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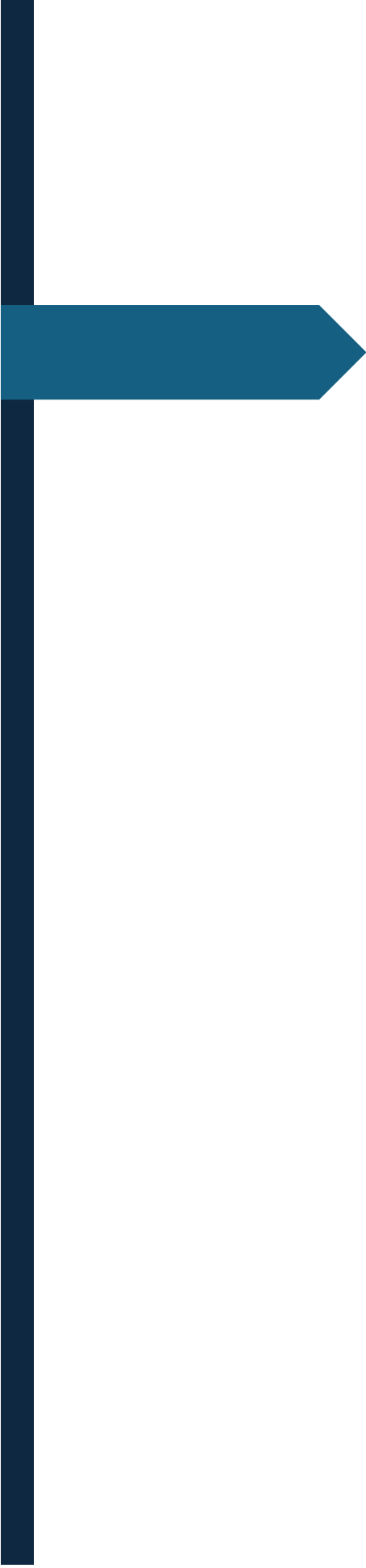
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## **CHAPTER II**

**Extraction, purification and  
structural characterization of  
bacterial glycoconjugates**



## 2.1 Introduction

A deep understanding of the bacterial envelope and its interactions with host receptors is essential for the development of innovative therapeutic and diagnostic strategies. Such knowledge paves the way for the design of new pharmaceuticals capable of targeting biologically significant markers with high precision and effectiveness.

Most of the studies within this PhD thesis focused on the structural characterization of bacterial glycoconjugates, specifically lipopolysaccharides (LPS) and exopolysaccharides (EPS). LPS were extracted from both gut microbiota (GM) and plant-associated bacteria, while EPS were isolated exclusively from plant-associated bacteria. This chapter outlines the main techniques used for their structural determination, describing the methods for extraction, purification, and analysis. Although these methodologies were primarily optimized for LPS characterization, they also proved effective for other GM polysaccharides, such as EPS, even though the corresponding results are not presented in this thesis.

To achieve these structural analyses, a multidisciplinary approach was adopted, combining biophysical, spectroscopic, and computational techniques. In particular, Nuclear Magnetic Resonance (NMR) spectroscopy, Mass Spectrometry (MS), along with various biophysical methods and computational studies, were employed to unravel the intricate architecture of these bacterial cell envelope components and explore their interactions with host receptors.

## 2.2 Extraction of LPS and EPS

The extraction of bacterial glycans, specifically LPS and EPS, represents a crucial step in the structural analysis of these complex molecules. The extraction method largely depends on the type of LPS being isolated, which can be classified as either rough-type (R-LPS or LOS) or smooth-type (S-LPS). These two forms differ in their hydrophobic and hydrophilic properties, primarily due to the presence or absence of the O-antigen polysaccharide<sup>1</sup>.

Two main extraction techniques are commonly employed: the phenol-chloroform-petroleum ether (PCP) method and the hot phenol-water extraction<sup>1,2</sup>.

The PCP method is particularly suited for R-LPS, which lacks the O-polysaccharide antigen and, as a result, is more hydrophobic. In this approach, dried bacterial cells are mixed with petroleum ether, chloroform, and aqueous phenol. Upon the addition of water, the R-LPS selectively precipitates from the phenol phase, facilitating effective separation with minimal contamination<sup>2</sup>. This method yields samples that are generally free from cellular impurities, thereby simplifying subsequent purification steps.

In contrast, the hot phenol-water extraction is the method of choice for S-LPS and EPS, which are more hydrophilic due to the presence of the O-antigen<sup>1,3</sup>. During this process, dried cells are suspended in a 90% phenol/water mixture (1:1 v/v) at 68°C, prompting the LPS to partition primarily into the water phase<sup>1</sup>. In some cases, depending on the specific chemical characteristics of the LPS, the molecule may also partition into the phenol phase, necessitating the collection of both phases to ensure complete recovery<sup>3</sup>.

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Further purification is achieved through dialysis and enzymatic treatments<sup>3</sup>. DNase, RNase, and protease K are employed to degrade nucleic acids and proteins, while amylase and pullulanase remove co-extracted polysaccharides, such as glycogen<sup>3</sup>.

To confirm the nature and purity of the extracted LPS, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is used. Visualization is carried out with silver nitrate staining, which distinctly marks S-LPS with its characteristic ladder-like pattern, reflecting the repeating units of the O-antigen, while R-LPS appears as a sharp band due to its lower molecular weight<sup>4</sup>.

Finally, ultracentrifugation and column chromatography are employed to ensure that LPS and EPS are isolated with minimal contamination and are prepared for detailed structural analysis<sup>3</sup>.

### 2.3 Purification of LPS

Once LPS has been successfully extracted, a series of purification steps are undertaken to eliminate contaminants and isolate the target structures. Commonly employed techniques include enzymatic digestion, ultracentrifugation, and size-exclusion chromatography (SEC). Among these, SEC is particularly effective, as it separates molecules based on their hydrodynamic size, allowing for the high-purity isolation of LPS<sup>5</sup>.

The success of SEC largely depends on the careful selection of resins, which are chosen according to the molecular size of LPS and any potential contaminants. This step is critical to maximizing both yield and purity. Following SEC, further refinement is achieved through ultracentrifugation and gradient separation techniques, which ensure that both LPS and exopolysaccharides (EPS) are obtained in their purest form, ready for downstream structural characterization.

The entire workflow, from extraction to purification and final analysis, lays the groundwork for unravelling the structural complexities of bacterial glycans. These optimized methods enable the high-quality isolation of LPS and EPS, paving the way for in-depth investigation of their composition, structural features, and biological roles<sup>5</sup>.

### 2.4 Structural Analysis of LPS

The chemical analysis of saccharide moieties is fundamental to elucidating the primary structure of poly- and oligosaccharides. Among the most effective techniques for this purpose is Gas Chromatography-Mass Spectrometry (GC-MS)<sup>6</sup>. However, since monosaccharides are inherently non-volatile, they must first be converted into apolar and volatile derivatives to be suitable for GC-MS analysis. This transformation is achieved through specific derivatization methods, each designed to highlight particular structural aspects of the saccharides<sup>6</sup>.

GC-MS is particularly powerful for the structural analysis of Lipopolysaccharides (LPS) and Lipo-oligosaccharides (LOS). Due to their non-volatile nature, both LPS/LOS and their monosaccharide building blocks require derivatization to enable their injection and analysis. This is accomplished by chemically degrading the sugar backbone, followed by GC or GC-

MS analysis of the derivatized monosaccharides. This process allows for the identification of sugar residues based on their characteristic fragmentation patterns and retention times.

To fully harness the capabilities of GC-MS, both sugars and lipids must be rendered volatile through proper derivatization. A combination of methods is often employed to resolve the complex sugar compositions typically found in biological samples such as LPS. Integrating multiple derivatization strategies not only enhances the completeness of monosaccharide composition analysis but also reveals linkage patterns, ring sizes, and absolute configurations. When paired with spectroscopic techniques like Nuclear Magnetic Resonance (NMR), this comprehensive approach enables the construction of a detailed structural map of oligo- or polysaccharides, providing valuable insights into their biological roles and mechanisms of action.

### **2.4.1 Derivatization Techniques for GC-MS Analysis**

The choice of derivatization method is critical to the analysis, as each technique provides unique insights into the structure of the saccharides. The main methods include:

- Acetylated Methyl Glycosides (AMG)
- Partially Methylated and Acetylated Alditols (PMAA)
- Acetylated Alditols (AA)
- Acetylated Octyl- (or Butyl-) Glycosides (AOG)

#### **2.4.1.1 Acetylated Methyl Glycosides (AMG)**

The AMG method is a well-established protocol for GC-MS analysis. It begins with the methanolysis of oligo- and polysaccharides under controlled acidic conditions (MeOH/HCl 0.5–2M at 85°C). This step cleaves the glycosidic bonds, transforming the monosaccharides into O-methyl glycosides. These derivatives are then acetylated with acetic anhydride in pyridine, resulting in per-acetylated O-methyl glycosides (MGA). The MGA derivatives are subsequently injected into the GC-MS, where their retention times and fragmentation patterns enable the identification of individual monosaccharides<sup>3</sup> (Figure 1).

## Extraction, purification and structural characterization of bacterial glycoconjugates

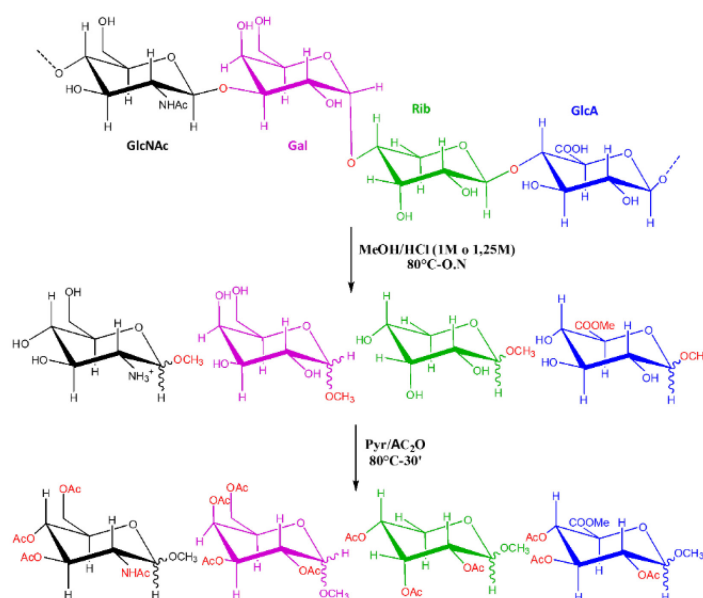


**Figure 1:** Reaction scheme illustrating the transformation of a polysaccharide into its corresponding per-acetylated O-methyl glycosides (MGA) through methanolysis, acetylation, and subsequent analysis via GC-MS. Adapted from ref. 7.

However, one limitation of the AMG method is the generation of isomers, such as pyranose and furanose forms or  $\alpha$  and  $\beta$  anomers, during acidic methanolysis. These isomers manifest as multiple peaks in the chromatogram, complicating quantification. To overcome this issue, the Acetylated Alditols (AA) method is often employed as an alternative, using trifluoroacetic acid (TFA) for hydrolysis and  $\text{NaBH}_4$  for reduction to avoid isomeric complexity.

#### 2.4.1.2 Acetylated Alditols (AA)

The AA method is particularly effective for identifying neutral sugars such as aldoses, ketoses, and aminosugars. The process begins with strong acid hydrolysis using TFA to cleave glycosidic linkages, followed by reduction with  $\text{NaBH}_4$ . The exposed hydroxyl groups are then acetylated, generating volatile alditol derivatives. This derivatization method simplifies GC-MS analysis by producing a single peak for each monosaccharide, enhancing quantitative accuracy<sup>6,8</sup> (Figure 2).

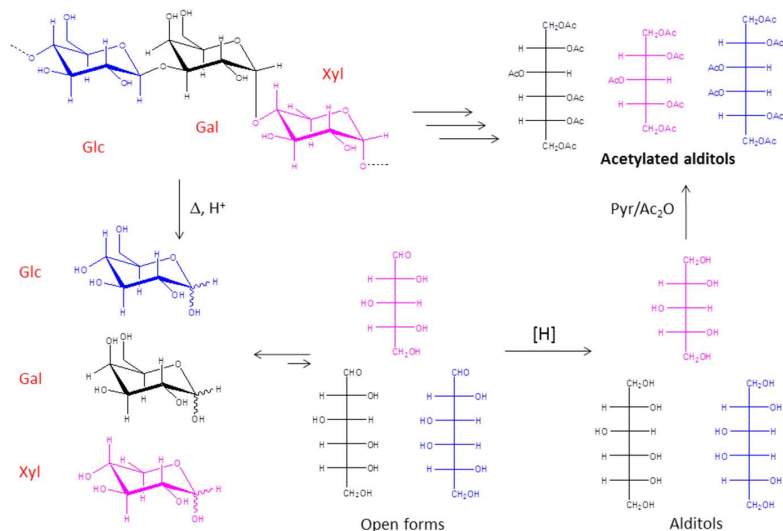


**Figure 2:** Reaction scheme illustrating the transformation of glycans into their corresponding acetylated alditols (AA) via trifluoroacetic acid (TFA) hydrolysis, NaBH<sub>4</sub> reduction, and acetylation. Adapted from ref. 7.

Despite its strengths, the AA method is less effective for acidic residues unless the carboxyl groups are first reduced. Additionally, reactive aldehyde groups formed during reduction may participate in side reactions, potentially affecting the overall yield.

### 2.4.1.3 Partially Methylated and Acetylated Alditols (PMAA)

When it comes to understanding linkage patterns, PMAA derivatization is the method of choice. This process involves four main steps:

1. Methylation of free hydroxyl groups using CH<sub>3</sub>I under strong alkaline conditions.
2. Acid hydrolysis to cleave the glycosidic bonds.
3. Reduction with NaBD<sub>4</sub> to stabilize the sugar alcohols.
4. Acetylation of exposed hydroxyl groups (Figure 3).

## Extraction, purification and structural characterization of bacterial glycoconjugates

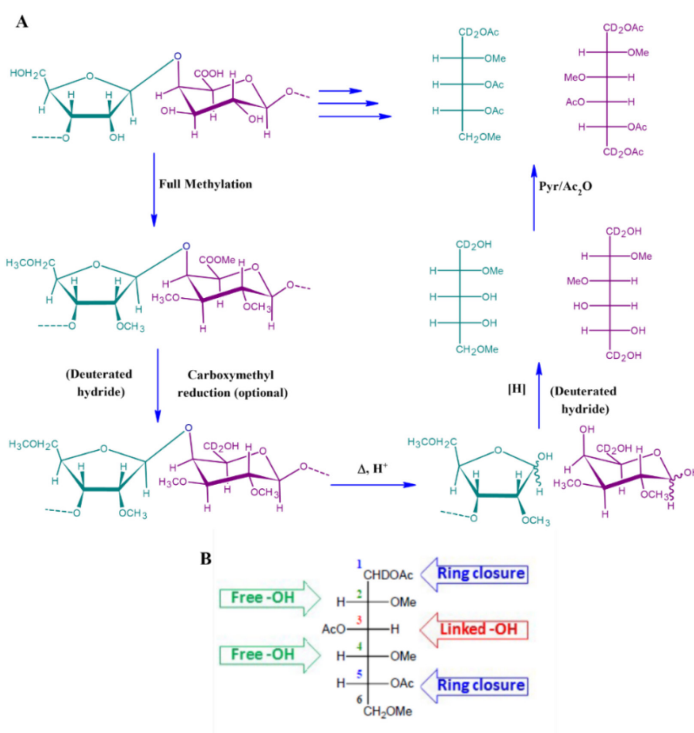


**Figure 3:** (A) Reaction scheme illustrating the transformation of a glycan into its corresponding partially methylated and acetylated alditols (PMAA) through methylation, acid hydrolysis,  $\text{NaBD}_4$  reduction, and acetylation. Adapted from ref. 7. (B) Representation of a 3-linked 6-deoxyhexose as a PMAA derivative. Methyl groups are introduced at the free hydroxyl functions of the original polysaccharide. Acetylation occurs at positions 1 and 5 due to the pyranosidic structure of the residue, while the acetyl group at position 3 indicates its involvement in a glycosidic linkage. Adapted from ref. 7.

The resulting PMAAs are analysed by GC-MS, revealing glycosylation sites and linkage patterns based on the predictable fragmentation of acetyl and methoxyl groups. The use of  $\text{NaBD}_4$  introduces specific mass shifts, aiding in the differentiation of fragments and providing deeper structural insights<sup>9</sup>.

#### 2.4.1.4 Acetylated Octyl- (or Butyl-) Glycosides (AOG)

To determine the absolute configuration of monosaccharides, AOG derivatization is employed. This technique involves solvolysis with enantiomerically pure alcohols such as 2-(+)-octanol or 2-(+)-butanol, followed by acetylation. The process generates a diastereomeric mixture, which is then analysed via GC-MS. By comparing the retention times with known standards, the D- or L-configuration of each sugar can be assigned<sup>10</sup> (Figure 4).

## Extraction, purification and structural characterization of bacterial glycoconjugates

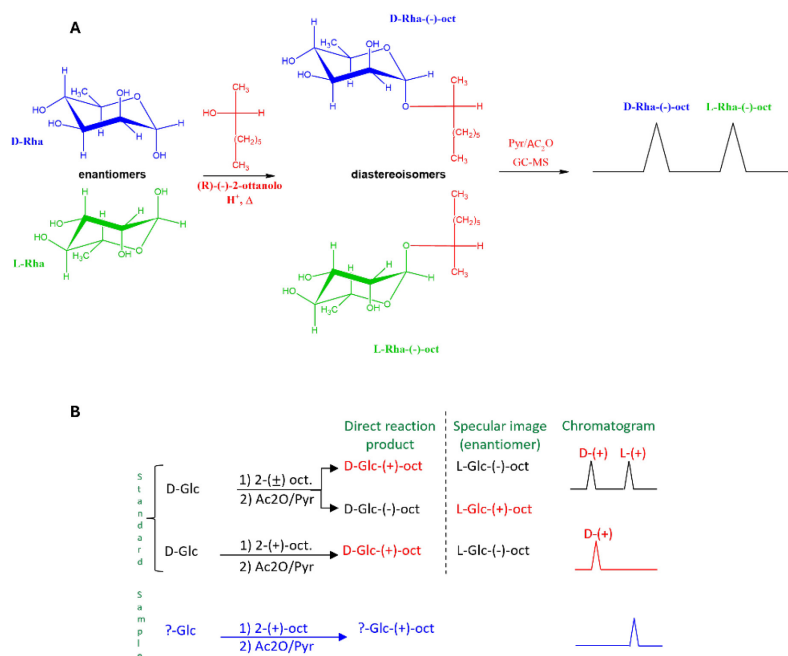


**Figure 4:** Reaction scheme illustrating the solvolysis of an enantiomeric mixture of rhamnose using enantiomerically pure (R)-(-)-2-octanol, followed by acetylation to form Acetylated Octyl Glycosides (AOG). (B) Schematic representation of the preparation and identification of 2-octyl glycosides, highlighting the steps for absolute configuration analysis. Adapted from ref. 7.

This method is particularly effective for distinguishing enantiomers in monosaccharides like rhamnose, fucose, and arabinose.

## 2.4.2 Chemical Degradation Techniques

Due to its amphiphilic nature, Lipopolysaccharides (LPS) tend to form micelles, resulting in low solubility in both aqueous and apolar solvents. This inherent property presents a significant challenge for structural investigation, requiring the development of specific strategies to facilitate analysis<sup>11</sup>. In brief, the carbohydrate portion (O-antigen and core oligosaccharide (OS)) in S-LPS or the core OS in R-LPS can be separated from lipid A using two chemical approaches: one involves a two-step deacylation procedure, and the other involves mild acid hydrolysis.

The first step of the deacylation protocol is O-deacylation using anhydrous hydrazinolysis, which removes the ester-bound acyl chains from lipid A. The second step is N-deacylation through strong alkaline hydrolysis, which cleaves the amide-linked fatty acids<sup>12</sup>. This dual-step method effectively strips away fatty acid groups, enabling the isolation of the saccharide portion of LPS. However, the harsh alkaline conditions employed during N-deacylation can sometimes cause the loss of critical structural information. For example,  $\beta$ -elimination

reactions may occur in 4-substituted uronic acids, leading to the breakdown of the saccharide component at this position. Additionally, if di- and trisaccharide Kdo residues are present, N-deacylation may cause cleavage of these linkages, resulting in the formation of smaller di- and monosaccharide residues. Furthermore, alkaline-labile groups such as phosphodiesteres, pyrophosphates, diphosphodiesteres, acetyl, and carbamoyl groups are also susceptible to cleavage under these conditions<sup>13</sup>.

In contrast, mild acid hydrolysis separates selectively cleaves the acid-labile bond linking the first monosaccharide of the inner core (Kdo) to the non-reducing glucosamine residue of the lipid A disaccharide backbone. This separation step allows for the effective partitioning of the highly hydrophobic lipid A fraction from the water-soluble saccharide moiety<sup>14</sup>. After hydrolysis, the insoluble lipid A is easily separated from the aqueous phase via centrifugation.

Further purification of lipid A can be achieved through the Bligh and Dyer method, which efficiently extracts lipid components from biological samples<sup>15</sup>. Conversely, the saccharide mixture is subjected to size-exclusion chromatography (SEC) to enhance purity and, if necessary, to separate the core OS from the O-antigen. This step is crucial for facilitating the structural investigation of individual LPS components.

One notable drawback of acid hydrolysis is the introduction of microheterogeneity. This arises from the reducing Kdo residue, which can form various  $\alpha$  and  $\beta$  anomers and shift between furanose and pyranose configurations, complicating the analysis of the core OS via NMR spectroscopy<sup>13</sup>. To address this, parallel treatments of O-deacylation and N-deacylation are often recommended, despite the limitations associated with N-deacylation.

To fully decipher the primary structure of the saccharide portions isolated after these chemical treatments, a combination of cutting-edge techniques is employed:

- **Nuclear Magnetic Resonance (NMR) spectroscopy** for high-resolution structural elucidation.
- **Soft-ionization Mass Spectrometry (MS)** to analyse molecular weights and fragmentation patterns.
- **Compositional and linkage analysis** to detail sugar connectivity and branching.

These methodologies collectively enable the detailed structural mapping of LPS and provide insights into its biological roles and interactions<sup>16,17,11</sup>.

### 2.4.3 Structural Characterisation of the Saccharide Moieties Using NMR Spectroscopy

NMR spectroscopy is one of the most powerful and non-destructive techniques for the structural characterization of carbohydrates<sup>18</sup>. Its broad applicability, coupled with the range of experimental setups available, makes it invaluable for analysing oligosaccharides and polysaccharides. However, NMR does come with certain limitations, including the high cost of the equipment, the need for enough pure sample, long experimental times required to obtain all necessary data, and the expertise required for spectrum interpretation.

One of the unique advantages of NMR is its ability to study molecules in their native state in solution, which is especially beneficial for LPS and LOS, as these saccharides are readily soluble in aqueous environments. NMR enables the investigation of carbohydrate structure, conformation, geometry, and dynamics at both atomic and molecular levels<sup>19</sup>. The technique is based on measuring energy absorption between different spin states of nuclei when exposed to a static magnetic field ( $B_0$ ). Only elements with a non-zero quantum nuclear spin ( $I$ ) exhibit a nuclear magnetic moment ( $\mu$ ) and are thus NMR-active. Commonly observed nuclei in glycans include  $^1\text{H}$ ,  $^{13}\text{C}$ , and  $^{31}\text{P}$ , all with  $I = \frac{1}{2}$ , which allows their spins to orient either with or against  $B_0$ <sup>20</sup>.

In liquid-state NMR, a magnetic field is applied to molecules dissolved in a suitable deuterated solvent, which stabilizes the field during experiments. The most used solvent is deuterium oxide ( $\text{D}_2\text{O}$ ), though other solvents like  $\text{CD}_3\text{Cl}$  can be employed when needed. However, the residual water signal can be intense and may obscure nearby signals, making it difficult to capture certain data. This signal appears around 4.7 ppm at 300 K, which coincides with the region where anomeric signals of carbohydrates are located, an essential region for carbohydrate characterization.

#### 2.4.3.1 1D NMR Spectroscopy

The first step in NMR analysis involves acquiring one-dimensional (1D) spectra, specifically  $^1\text{H}$ -NMR (proton) and  $^{13}\text{C}$ -NMR (carbon) spectra, which provide fundamental information about the sample's composition. The spectra are generally divided into four key regions:

- 2.7–1.0 ppm: The aliphatic region, crucial for detecting deoxy sugars. For example, methyl groups of 6-deoxy sugars such as rhamnose or fucose are typically found around 1.1–1.3 ppm.
- 4.4–3.0 ppm: The carbinolic region, which reveals the ring protons of monosaccharide residues and serves as a "fingerprint" for specific glycans. This region is often the most populated part of the spectrum.
- 5.6–4.4 ppm: The anomeric region, which corresponds to the anomeric protons of sugar units. In the pyranose form of sugars, anomeric protons appearing downfield of 4.7 ppm typically indicate an  $\alpha$ -configuration, while those upfield usually represent the  $\beta$ -configuration.
- 8–7 ppm: The aromatic region, which can detect signals from aromatic glycosides or sugar-nucleotides.

The assignment of these regions has been extensively documented in carbohydrate NMR studies<sup>21</sup>, as illustrated in Figure 5.

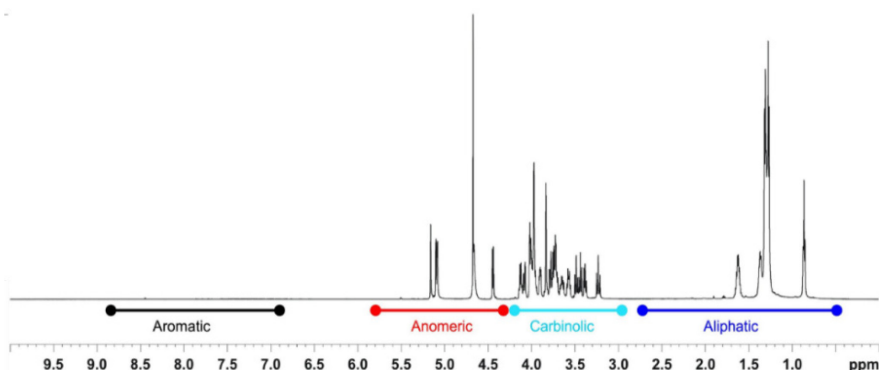


**Figure 5:** <sup>1</sup>H NMR spectrum showing the distinct chemical shift regions: aliphatic (2.7–1.0 ppm), carbinolic (4.4–3.0 ppm), anomeric (5.6–4.4 ppm), and aromatic (8–7 ppm) regions. Adapted from ref 21.

#### 2.4.3.2 2D NMR Spectroscopy

To fully characterize complex oligosaccharides and polysaccharides, two-dimensional (2D) NMR experiments are crucial. Homonuclear experiments, such as Correlation Spectroscopy (COSY) and Total Correlation Spectroscopy (TOCSY), help identify and assign ring protons through scalar couplings. Unlike COSY, TOCSY uncovers correlations between protons within a spin-spin coupled chain, facilitating magnetization propagation through the spin-locking sequence.

Additionally, Nuclear Overhauser Effect Spectroscopy (NOESY) and Rotating Frame Overhauser Enhancement Spectroscopy (ROESY) are used to evaluate spatial proximity between protons. NOESY detects cross-relaxation phenomena and highlights spatial interactions beyond bond numbers, with stronger signals arising from closer nuclei (up to 4 Å). In cases with minimal NOE scenarios, like in small oligosaccharides, ROESY under spin-locked conditions is employed to yield positive signals<sup>20</sup>.

For carbon-proton correlations, Heteronuclear Single Quantum Coherence (HSQC) and Heteronuclear Multiple-Bond Correlation (HMBC) experiments are used. HSQC maps direct <sup>1</sup>H-<sup>13</sup>C interactions, while HMBC reveals long-range couplings, aiding in the determination of glycosidic linkages and cyclic ring structures. HSQC is set to 145 Hz to detect both anomeric (160–170 Hz) and carbinolic (140 Hz) correlations. In contrast, HMBC identifies multiple-bond couplings over two or three bonds, helping establish sugar sequences and inter-residue connectivity (Figure 6).

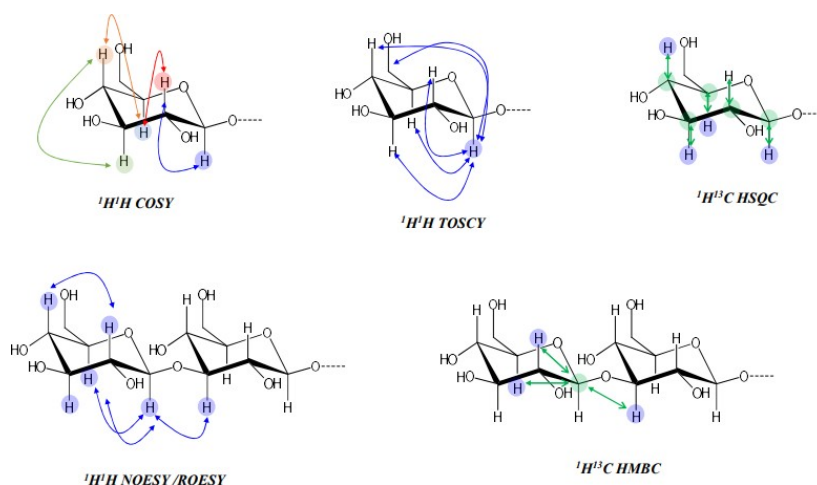


**Figure 6:** Schematic representation of the different correlations obtained via 2D NMR experiments, highlighting COSY, TOCSY, NOESY, ROESY, HSQC, and HMBC interactions.

#### 2.4.4 MALDI-TOF MS and MS/MS Analysis

Recent advances in MS have made it an indispensable technique for studying the structure of oligosaccharides and glycoconjugates, such as lipopolysaccharides (LPS)<sup>22,23</sup>. Improvements in the sensitivity, precision, and speed of modern mass spectrometers have significantly enhanced our ability to perform structural characterization. MS, particularly when combined with soft ionization techniques like electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), has become an essential tool for analysing polysaccharides, oligosaccharides, and intact R-LPS<sup>24</sup>.

MALDI works by focusing a pulsed laser onto a metal target, where the sample is mixed with an organic acidic matrix. The matrix not only aids in ionizing the sample but also absorbs the laser's energy, which protects the sample and ensures the ions remain singly charged, making the analysis easier. When using a MALDI-TOF/TOF system, tandem mass spectrometry (MS/MS) is applied to further break down the sample. This technique isolates specific ions and fragments them, providing detailed information about the oligosaccharide sequence, monosaccharide composition, branching, linkage types, connectivity, and even the size of rings and isomers<sup>25</sup>.

The advantage of MALDI-TOF MS lies in its ability to analyse complex mixtures of oligosaccharides, such as the core oligosaccharides of LPS, without the need for prior purification. This allows the identification of different glycoforms, their components, and even non-saccharide additions. During MS/MS experiments, fragmentation patterns help provide deeper structural insights. A well-established nomenclature system for carbohydrate fragmentations in MS/MS was introduced by Domon and Costello (1988)<sup>26</sup>, which categorizes these fragments into two main groups: those from glycosidic cleavages (B-, C-, Y-, Z-type) and those from cross-ring fragmentations (A- and X-type). The A, B, and C types refer to

fragments containing the terminal sugar (non-reducing end), while the X, Y, and Z types are associated with the reducing end or the aglycon (Figure 7).

MALDI-TOF MS provides a comprehensive profile of the sample, showing ion peaks corresponding to lipid A, the core oligosaccharides, and the entire LPS. For S-LPS, variations in the O-antigen's molecular weight can indicate the size of its repeating unit. Recently, the combination of ion mobility MS (IMS-MS) with tandem MS (IMS-MS/MS) has allowed even higher-resolution analysis of intact R-LPS, offering excellent sensitivity and precision, even for higher molecular weights<sup>27</sup>. Notably, the laser used in MALDI-TOF can also break the acid-labile bond between Kdo and GlcN in lipid A, a bond typically cleaved during mild acid hydrolysis treatments. This ability is clearly reflected in the MALDI-TOF MS spectra, where distinct ion regions can be observed: those from lipid A, the core oligosaccharides, and the complete R-LPS.

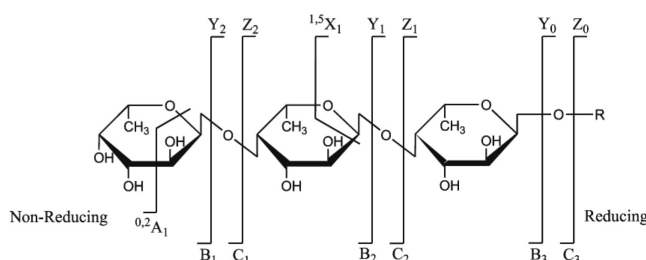


**Figure 7:** Domon and Costello nomenclature for glycoconjugate fragments generated during tandem MS/MS experiments, illustrating the classification of glycosidic (B-, C-, Y-, Z-type) and cross-ring (A- and X-type) cleavages. Adapted from ref. 26.

In summary, MALDI-TOF MS/MS, along with innovations like IMS-MS, has proven to be a powerful tool for the structural analysis of LPS. These techniques provide high-resolution data, enabling the study of oligosaccharide and lipid A structures in detail, even at higher molecular weights, without losing critical structural information.

## 2.5 Immunological Assays for Bacterial Glycoconjugates

The immune system's ability to distinguish between non-infectious self and potentially harmful non-self entities is fundamentally based on the recognition of pathogen-associated molecular patterns (PAMPs) present on bacterial glycoconjugates, such as lipopolysaccharides (LPS) and exopolysaccharides (EPS)<sup>28</sup>. This recognition is primarily mediated by pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), which play a crucial role in pathogen detection and the initiation of inflammatory and immune responses<sup>29,30</sup>. TLRs are strategically positioned on the surface of immune cells and within intracellular compartments to monitor for microbial products. When bacterial glycoconjugates such as LPS interact with TLRs, they activate signaling cascades that result in the expression of pro-inflammatory cytokines, the induction of antimicrobial genes, and the maturation of dendritic cells. This process not only amplifies the local immune response but also enhances the capacity for antigen presentation, effectively linking innate immunity with the adaptive immune response.

To explore these immunological interactions, various experimental approaches are employed. Key among them are cell-based reporter assays and high-throughput microarray technologies, which allow for a detailed evaluation of receptor engagement and cytokine production. These techniques provide a platform for understanding the structural features of bacterial glycoconjugates that drive immune activation and modulation.

Comprehensive immunological evaluation of bacterial glycoconjugates is essential to understand their role in modulating host-pathogen interactions. These insights are crucial for deciphering the mechanisms by which bacterial components influence immune responses and may inform the development of novel therapeutic strategies aimed at modulating these interactions to treat infections and inflammatory diseases.

### 2.5.1 Screening TLR Agonists with HEK-Blue™ Cells

Cell-based reporter assays are valuable methods for evaluating the immune response triggered by bacterial glycoconjugates. These assays involve engineered cell lines, such as HEK-Blue™ cells, which express specific receptors, including Toll-like receptors (TLRs), enabling the detection of microbial component stimulation<sup>31</sup>. Upon activation of these receptors, intracellular signaling cascades are triggered, leading to the activation of transcription factors like NF- $\kappa$ B and the production of inflammatory cytokines.

For example, HEK-Blue™ cells can be engineered to express TLR4, along with its co-receptors MD-2 and CD14, to measure lipopolysaccharide (LPS)-induced activation. The activation is quantified by the secretion of secreted embryonic alkaline phosphatase (SEAP), which serves as a sensitive reporter for immune activation<sup>32,31</sup> (Figure 8). This system allows researchers to evaluate dose-dependent immune responses and to explore the immunogenic potential of specific bacterial glycoconjugates.

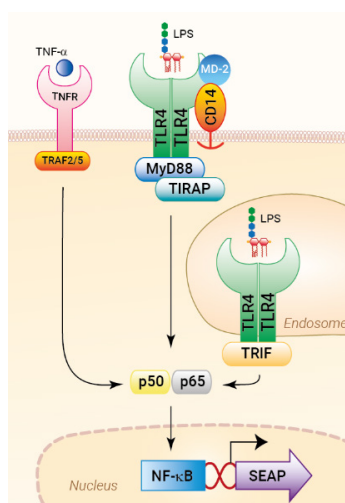


**Figure 8:** Signalling pathways activated in HEK-Blue™ TLR cells upon stimulation, illustrating the cascade leading to SEAP reporter expression. Adapted from ref. 31.

Additionally, HEK-Blue™ cells expressing other TLRs, such as TLR2, provide the ability to assess the activation of alternative immune pathways, facilitating a broader analysis of receptor-specific responses. This dual approach of using different reporter cell lines enables more comprehensive profiling of bacterial component interactions with host immune receptors.

Beyond engineered cell lines, primary human cells, such as peripheral blood mononuclear cells (PBMCs), are also employed in these assays to assess cytokine release and immune modulation in response to bacterial components. This approach offers a more physiologically relevant perspective, capturing the complexity of *in vivo* immune interactions and enhancing the understanding of host-pathogen dynamics<sup>31</sup>.

## 2.5.2 Microarray Studies

Microarray technology is a high-throughput platform widely used to investigate the interactions between glycoconjugates and host immune receptors. This method involves immobilizing a variety of carbohydrates, lectins, or other components on a solid surface, enabling the simultaneous analysis of thousands of binding events. For example, microarray binding assays using various glycoproteins, such as fetuin, asialofetuin, ribonuclease B, and ribonuclease A, allow researchers to identify specific interactions between glycans and immune receptors. These assays involve printing glycoproteins on nitrocellulose-coated glass slides at varying concentrations and using a fluorophore (Cy3) to monitor spot binding via fluorescence scanning<sup>33</sup>.

Lectins and other immune-related proteins are then probed against the microarrays, revealing specific binding interactions that provide insight into the structural determinants governing immune recognition. For instance, in these assays, lectins such as Galectins (1, 3, 4, 7, 8, and 9) and Ficolins (1, 2, and 3) have been tested to study their binding affinity to the immobilized glycans<sup>34</sup>. The binding interactions are subsequently detected by incubating with secondary antibodies conjugated to fluorescent markers, such as AlexaFluor-647, allowing quantification using a GenePix Pro 7 scanner<sup>33</sup> (Figure 9).

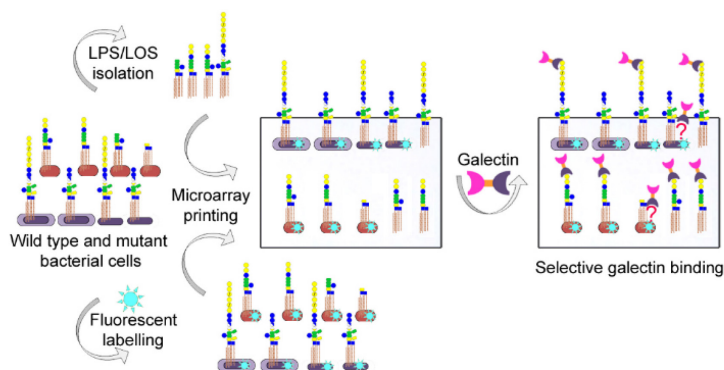


**Figure 9:** Schematic representation of a microbial glycan microarray setup, illustrating the immobilization of glycans on a solid surface and detection of binding interactions via fluorescently labeled probes. Adapted from ref. 33.

More recent studies have extended this approach to explore the ligand profiles of Gram-negative respiratory bacterial pathogens. These investigations have revealed that galectin binding varies significantly with the bacterial surface architecture, emphasizing the role of surface glycan presentation in immune evasion and host interaction<sup>34</sup>.

Microarray technology thus enables detailed mapping of glycan-mediated immune recognition patterns. It has been shown that variations in glycan structure can affect the binding affinity of immune receptors, including lectins and Siglecs, which play a key role in immune response regulation<sup>33</sup>. The ability to perform high-throughput screening of multiple binding partners simultaneously makes this approach invaluable for understanding the immunological roles of glycoconjugates.

By decoding the immunological fingerprint of glycans, microarray studies contribute significantly to the understanding of how glycan structures modulate immune responses, revealing potential targets for therapeutic intervention.

## 2.6 Computational and Molecular Modeling of EPS and LPS

Computational and molecular modelling techniques are essential tools for understanding the structure, dynamics, and interactions of complex biomolecules like exopolysaccharides (EPS) and lipopolysaccharides (LPS). These methods, grounded in quantum and classical physics, enable researchers to simulate the behaviour of molecules under various environmental conditions, including exposure to stressors such as methanol<sup>35,36</sup>. Approaches like molecular dynamics (MD), molecular mechanics (MM), docking simulations, and quantum mechanics/molecular mechanics (QM/MM) allow for the accurate prediction of conformational changes, structural stability, and molecular interactions at the atomic level.

In molecular dynamics simulations, the positions of atoms over time are predicted based on Newton's laws of motion, providing insights into the movement and flexibility of EPS and LPS molecules. The simulation process typically begins with energy minimization to optimize the molecular geometry, followed by solvation with explicit water molecules and the application of periodic boundary conditions (PBC) to minimize surface artifacts. The electrostatic interactions within the system are managed using advanced methods such as particle mesh Ewald (PME) summation. After these initial steps, the system undergoes heating, equilibration, and production phases, during which realistic molecular trajectories are generated. This comprehensive approach not only stabilizes the molecular configurations but also mimics real-world biological environments, where hydration and ionic conditions significantly influence molecular behaviour.

Molecular mechanics methods, employing force fields like Amber/GLYCAM and CHARMM, are crucial for simulating the conformational flexibility of polysaccharides and lipid components<sup>37</sup>. The GLYCAM force field from the Woods Group<sup>38</sup> provides parameters specialized for carbohydrates and glycoconjugates, making it particularly suitable for EPS and LPS simulations. These force fields help to accurately model torsion angles that govern the spatial orientation of glycosidic bonds, such as the  $\Phi$ ,  $\Psi$ , and  $\omega$  angles, which play a significant role in determining the structural behaviour and interactions of EPS and LPS with other biomolecules<sup>39</sup>. These torsion angles, particularly in the case of 1,6 linkages, govern the

flexibility of oligosaccharides, providing valuable information on the stability and adaptability of these molecules in biological systems.

For more precise simulations, QM/MM methods combine quantum calculations for critical regions of the molecule with classical mechanics for the larger molecular environment. This hybrid approach allows for a deeper understanding of electronic properties and reaction mechanisms, which are often difficult to observe experimentally. Moreover, advanced computational techniques, such as adiabatic energy maps, are employed to explore the potential energy surfaces of saccharide dimers, identifying the most energetically favourable conformations.

These *in silico* approaches are often integrated with experimental techniques such as NMR spectroscopy and Small-Angle X-ray Scattering (SAXS), helping to validate the models and provide a more detailed understanding of the conformational dynamics of saccharides. This synergy between computational simulations and experimental data bridges the gap between theoretical predictions and real-world observations, enhancing the reliability and accuracy of the results.

Computational methods are particularly useful for studying how EPS and LPS respond to external stressors, such as methanol exposure, by identifying structural vulnerabilities, adaptive mechanisms, and changes in supramolecular organization. This provides key insights into the resilience of microbial systems and the protective roles of EPS and LPS in fluctuating environmental conditions. These findings not only advance our understanding of microbial survival strategies but also open new possibilities for the development of biotechnological and pharmaceutical applications.

Several software tools facilitate these simulations, including MacroModel from Schrödinger<sup>40</sup> for molecular mechanics calculations, and the widely used AMBER suite<sup>41</sup> was employed to carry out MD simulations, providing a comprehensive environment for system setup, simulation, and analysis. Trajectory processing and analysis are commonly performed using software like PTRAJ and CPPTRAJ, which offer robust tools for evaluating molecular motions and conformational changes<sup>42</sup>.

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## SECTION I

Plant-associated bacteria

# CHAPTER III

Structural characterization  
of *Herbaspirillum* Root189  
LPS and its role in plant  
immune recognition



### 3.1 Introduction

In this chapter, I focus on the characterization of the lipopolysaccharide (LPS) from *Herbaspirillum* Root189, a bacterium found in the root microbiota of *Arabidopsis thaliana*. *Herbaspirillum* species are well-known plant-associated bacteria that often help plants grow better and cope with stress. However, despite their importance, we still know relatively little about how their surface molecules, especially LPS, interact with the plant immune system. Plants provide a multitude of niches for the growth and proliferation of plant microbiota, a complex co-association of microorganisms comprising taxa from diverse phyla, including bacteria, fungi, protists, and nematodes. Such a complex microbial community is persistent and ubiquitous, promotes plant health and growth, productivity and fitness, and mediates beneficial transformation and mobilization of nutrients in the rhizosphere. Furthermore, plant microbiota increases host tolerance to biotic or abiotic stresses and prime immune responses and systemic resistance. The cross-talk between plants and host microbiota represents a yet-to-be-explored research field.

The plant immune system perceives constitutive and conserved microbial epitopes, so-called microbe-associated molecular patterns (MAMPs), to elicit the first line of immune responses. This MAMP-triggered immunity controls the microbial load and, upon non-self-recognition, induces defense responses such as oxidative bursts, nitric oxide generation, extracellular pH increase, cell wall reinforcement, and accumulation of pathogenesis-related proteins to limit pathogen growth. MAMPs include proteins such as bacterial flagellin and elongation factor Tu, as well as cell envelope components like lipopolysaccharides (LPS)<sup>1</sup>, beta-glucans<sup>2</sup>, peptidoglycan<sup>3</sup>, and fungal chitin<sup>4</sup>.

Lipopolysaccharides (LPS) are major components of the outer membrane of Gram-negative bacteria, playing a crucial role in bacterial survival, membrane integrity, and host-microbial interactions. LPS are complex glycoconjugates indispensable for the growth and viability of Gram-negative bacteria, representing the main constituent of the external layer of the outer membrane<sup>5</sup>. LPS is composed of three main regions: lipid A, which anchors the molecule to the bacterial membrane; a core oligosaccharide that connects lipid A to the outer region; and the O-specific polysaccharide (OPS), which is highly variable and determines immunological specificity. The Gram-negative genus *Herbaspirillum* belongs to the Betaproteobacteria class and comprises 14 species to date. *Herbaspirillum* associates with model plants such as *Arabidopsis thaliana*<sup>6</sup> and economically important crops in the family Poaceae and increase growth and productivity<sup>7</sup>. The initial colonization steps are mediated by bacterial envelope components, such as lipopolysaccharide, exopolysaccharide, and adhesins. In fact, *Herbaspirillum* LPS is required for plant colonization, as mutant strains impaired in LPS biosynthesis showed a severe reduction in attachment to the maize root surface and affected internal maize tissue<sup>8</sup>.

Plant-associated bacteria, including *Herbaspirillum*, modulate host immunity largely through the structural variability of their LPS, especially the O-antigen polysaccharide portion. Modifications such as O-methylation and O-acetylation have been reported to influence bacterial survival and immune evasion, enabling successful colonization of the plant root environment<sup>9,10</sup>. Recent investigations revealed that *Herbaspirillum* Root189 synthesizes an O-antigen composed predominantly of an O-methylated and variably O-acetylated D-

rhamnose polysaccharide. *Herbaspirillum* Root189, isolated from the root microbiota of *Arabidopsis thaliana*, produces a unique LPS that is pivotal in mediating host-microbe communication, influencing plant immune responses, and modulating bacterial colonization efficiency. Evasion or suppression of plant immune recognition is therefore not only essential for pathogens to successfully infect plant hosts but must also be critical for commensals to colonize different plant niches or establish successful symbiosis. How the innate immune system discriminates between beneficial and pathogenic microbes, and vice versa, how beneficial microbes evade plant perception remains largely unknown<sup>11</sup>, especially from the molecular point of view. Within this scenario, different studies provided evidence for receptor competition that allows (rice) plants to discriminate microbial cell wall signals and balance immunity and symbiosis signaling<sup>12</sup>. However, it is not clear if the microbe itself can work to adjuvate the process.

Using a combination of chemical, spectroscopic, and computational techniques, our group demonstrated that these O-antigen modifications alter the supramolecular conformation of the LPS, favoring a coiled polymeric structure that affects membrane packing and hydration. Functionally, this structural arrangement diminishes recognition by plant immune receptors, thereby attenuating typical defense responses such as reactive oxygen species (ROS) production and calcium signaling, which are hallmarks of plant immune activation<sup>13</sup>. Given the critical role of LPS chemical structure in shaping host-microbe interactions, this chapter presents an integrated approach to fully characterize the LPS from *Herbaspirillum* Root189. Advanced structural analyses, including NMR spectroscopy, mass spectrometry, light scattering, and neutron reflectometry, were employed to characterize the OPS composition and its conformational dynamics. Furthermore, immunological assays were conducted to elucidate the role of O-acetylation in modulating host immune recognition. These findings offer novel insights into the molecular mechanisms underlying plant-microbe interactions and the strategies employed by beneficial microbes to achieve symbiosis while evading host defences<sup>13</sup>. The results presented in this chapter were previously published in their entirety in: Vanacore, A., Vitiello, G., Wanke, A., Cavasso, D., Clifton, L. A., Mahdi, L., Campanero-Rhodes, M. A., Solís, D., Wuhrer, M., Nicolardi, S., Molinaro, A., Marchetti, R., Zuccaro, A., Paduano, L., & Silipo, A. (2022). Lipopolysaccharide O-antigen molecular and supramolecular modifications of plant root microbiota are pivotal for host recognition. *Carbohydrate Polymers*, 277, 118839.

### 3.2 Structural Characterization of *Herbaspirillum* Root189 LPS

Lipopolysaccharide (LPS) of *Herbaspirillum* Root189 is a crucial bacterial outer membrane component, playing a central role in host and environmental interactions. Its detailed structural characterization is fundamental for understanding the molecular mechanisms underlying its function. The lipopolysaccharide (LPS) of *Herbaspirillum* Root189 is composed of three main regions: Lipid A, a core oligosaccharide, and an O-specific polysaccharide (OPS). The OPS is particularly interesting due to its unique composition of D-rhamnose units enriched with O-methyl and O-acetyl modifications. These modifications play a crucial role in altering the surface properties of the bacterium, enhancing its ability to evade host immune recognition<sup>14,15</sup>. Structural studies employing NMR spectroscopy and mass spectrometry (MS) allowed for the detailed elucidation of the OPS structure. Through NMR

experiments we identified the repetitive tetrasaccharide unit predominantly consisting of  $\alpha$ - and  $\beta$ -rhamnose configurations<sup>16</sup>. The O-methylation and O-acetylation patterns were mapped, revealing their contribution to the hydrophobic character and structural rigidity of the polysaccharide. Mass spectrometry confirmed the molecular weight of the OPS repeating unit, corroborating the findings from NMR analysis<sup>1</sup>.

The structural analysis demonstrated that these modifications contribute significantly to the molecular packing within the LPS layer, affecting both membrane integration and interactions with the host plant's immune receptors<sup>5,15</sup>. This structural adaptation potentially enhances the bacterium's capacity to survive and establish symbiosis within the root environment of *Arabidopsis thaliana*.

### 3.2.1 Purification and Chemical Analyses of LPS from *Herbaspirillum* Root189

LPS from *Herbaspirillum* Root189 was extracted using the classical hot phenol/water method<sup>17</sup>, a well-established procedure for isolating lipopolysaccharides while preserving their structural integrity. Following extraction, enzymatic treatments were applied to remove protein contaminants. The purity and general profile of the LPS were assessed by SDS-PAGE followed by silver nitrate staining<sup>18</sup>, which revealed the typical ladder-like pattern indicative of smooth-type LPS species. Further purification was achieved by size-exclusion chromatography, allowing the separation of high molecular weight fractions and ensuring the isolation of pure LPS for downstream analyses<sup>19</sup>.

Compositional analysis revealed that the LPS predominantly consists of D-rhamnose and a significant amount of 3-O-methyl-D-rhamnose, along with minor core oligosaccharide components including D-glucosamine (GlcN), 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo), L-glycero-D-manno-heptose (L,D-Hep), D-glucose (Glc), and 4-amino-L-arabinose (Ara4N). The relative abundances of these monosaccharides, determined by GC-MS analysis of acetylated methyl glycosides, are summarized in Table 1.

**Table 1:** Monosaccharide Composition of *Herbaspirillum* Root189 LPS. The table reports the relative abundance of the main sugar components, highlighting the predominance of D-rhamnose (Rha) and 3-O-methyl-D-rhamnose (Rha3OMe) in the O-specific polysaccharide (OPS), along with core region constituents.

| Monosaccharide                            | Relative Abundance (%) |
|-------------------------------------------|------------------------|
| 3-O-methyl-D-rhamnose (Rha3OMe)           | 26.17                  |
| D-rhamnose (Rha)                          | 100                    |
| D-glucose (Glc)                           | 7.52                   |
| D-glucosamine (GlcN)                      | 16.91                  |
| L-glycero-D-manno-heptose (L,D-Hep)       | 9.68                   |
| 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) | 3.3                    |

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## CHAPTER III

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### Structural characterization of *Herbaspirillum* Root189 LPS and its role in plant immune recognition

Notably, 3-O-methyl-D-rhamnose (Rha3OMe) is relatively uncommon in bacterial lipopolysaccharides and has been described in only a few environmental or symbiotic species. This unusual substitution likely contributes to the hydrophobicity and conformational rigidity of the polysaccharide chain. By masking hydroxyl groups that could otherwise be recognized by plant pattern recognition receptors, particularly lectin-like domains, O-methylation may act as a molecular shield. Its presence in *Herbaspirillum* Root189 likely represents an evolutionary adaptation for evading immune detection and persisting within the plant rhizosphere.

Methylation analysis was performed using the Ciucanu-Kerek method<sup>20</sup>, a classical technique for identifying linkage positions in polysaccharides. The analysis revealed the presence of 2,3-disubstituted and 3-substituted rhamnose residues, suggesting a branched and complex glycan architecture. These chemical modifications, particularly O-methylation and O-acetylation, play crucial roles not only in conferring structural rigidity and hydrophobicity to the OPS but also in mediating its interactions with the plant immune system. The heterogeneous acetylation patterns, observed across different residues, suggest a fine-tuned regulation of the OPS structure, likely reducing its accessibility to host immune receptors and thereby promoting bacterial survival within the host environment (Table 2).

**Table 2:** Methylation Analysis of *Herbaspirillum* Root189 LPS. Characterization of partially methylated acetylated alditols, indicating a branched OPS structure with various rhamnose linkage patterns.

| Partially Methylated Derivative | Relative Abundance (%) |
|---------------------------------|------------------------|
| 2,3-disubstituted Rhap          | 23.85                  |
| 3-substituted Rhap              | 30.59                  |
| 4-substituted Rhap              | 100                    |

The fatty acid profile of the LPS was also examined, revealing acyl moieties consistent with a typical lipid A structure. Hydroxylated fatty acids such as 10:0 (3-OH) and 12:0 (3-OH), together with saturated fatty acids like 16:0, were identified. These components are known to influence membrane stability, fluidity, and permeability, and may contribute to LPS structural integrity under environmental stress (Table 3). These findings are consistent with previous structural analyses of lipid A acylation patterns and their functional roles in Gram-negative bacteria.

**Table 3:** Fatty Acid Composition of *Herbaspirillum* Root189 LPS. Hydroxylated and saturated fatty acids contribute to membrane stabilization and enhance LPS hydrophobicity.

| Fatty Acid  | Description  |
|-------------|--------------|
| 10:0 (3-OH) | Hydroxylated |
| 12:0 (3-OH) | Hydroxylated |

| Fatty Acid | Description |
|------------|-------------|
| 16:0       | Saturated   |

### 3.2.2 Structural Characterization of the O-specific Polysaccharide (OPS) from *Herbaspirillum* Root189

To dissect the structure of the O-specific polysaccharide (OPS) from *Herbaspirillum* Root189, the purified LPS was subjected to mild acid hydrolysis to cleave the Lipid A moiety from the OPS. This step was crucial for detailed structural analysis and for understanding how O-methylation and O-acetylation impact LPS conformation and interactions with host immune receptors. Following hydrolysis, the isolated OPS was extensively characterized using NMR spectroscopy. A combination of NMR techniques provided comprehensive insights into the chemical environment and connectivity of the sugar residues. The NMR analysis allowed the identification of the anomeric configurations of the monosaccharide units by evaluating coupling constants ( $^3J_{H1,H2}$  and  $^1J_{C1,H1}$ ) and *intra*-residual Nuclear Overhauser Effect (NOE) contacts<sup>21</sup>. Four distinct spin systems, labelled A, B, C, and D were identified. Residues B and C were assigned as  $\alpha$ -rhamnose units, based on scalar correlations observed between ring protons and the methyl signal at position 6 in TOCSY spectra. The manno configuration of these residues was established from low  $^3J_{H-1,H-2}$  and  $^3J_{H-2,H-3}$  values (<2 Hz), indicative of equatorial H-2 orientation, and further supported by diagnostic carbon chemical shifts (>175 Hz) These findings are summarized in Table 4.

The comparison of  $^1H$  NMR spectra for *Herbaspirillum* LPS, OPS, and O-deacylated LPS highlighted key differences in chemical shifts and signal intensity, indicating structural modifications upon hydrolysis and deacetylation (Figure 1).

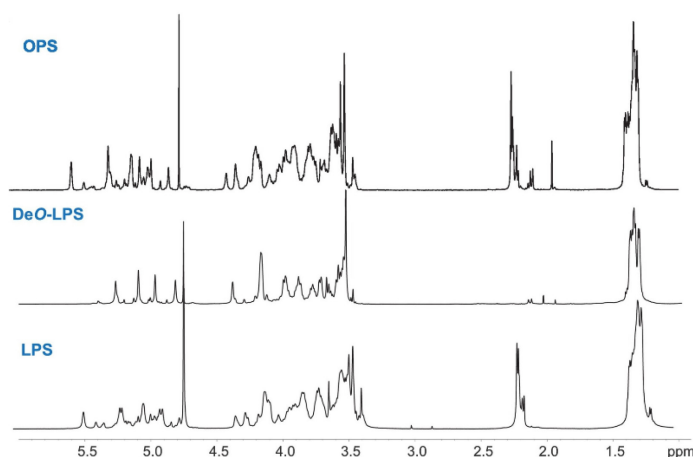


**Figure 1:** Comparison of  $^1H$  NMR spectra of *Herbaspirillum* LPS, OPS, and O-deacylated LPS. The spectra highlight key differences in chemical shifts and signal intensity, indicating structural modifications upon hydrolysis and deacetylation.

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## Structural characterization of *Herbaspirillum* Root189 LPS and its role in plant immune recognition

**Table 4:**  $^1\text{H}$  and  $^{13}\text{C}$  NMR chemical shift values of *Herbaspirillum* OPS and De-acetylated LPS.

| OPS                 |                                        |                                         |                                       |       |       |       |
|---------------------|----------------------------------------|-----------------------------------------|---------------------------------------|-------|-------|-------|
| Unit                | 1                                      | 2                                       | 3                                     | 4     | 5     | 6     |
| A                   | 5.22                                   | 4.28                                    | 3.40                                  | 3.52  | 3.73  | 1.302 |
| 2- $\alpha$ -Rha    | 100.69                                 | 73.05                                   | 79.8                                  | 71.0  | 69.4  | 16.7  |
|                     |                                        |                                         | O-Methyl: 3.467/<br>57.02             |       |       |       |
|                     | 5.22                                   | 4.35                                    | 3.69                                  | 3.56  | 3.87  | 1.30  |
|                     | 100.69                                 | 73.3                                    | 79.7                                  | 71.3  | 67.9  | 16.7  |
|                     |                                        |                                         | O-Methyl: 3.49/<br>57.02              |       |       |       |
| B                   | 5.06                                   | 4.11                                    | 3.90                                  | 3.62  | 3.86  | 1.31  |
| 4- $\alpha$ -Rha    | 101.85                                 | 69.5                                    | 69.2                                  | 83.1  | 67.5  | 16.7  |
|                     | 5.06                                   | 4.14                                    | 3.90                                  | 3.63  | 3.86  | 1.31  |
|                     | 101.8                                  | 69.5                                    | 69.2                                  | 82.7  | 67.5  | 16.7  |
|                     | 5.07                                   | 4.18                                    | 5.168                                 | 3.84  | 3.94  | 1.32  |
|                     | 102.6                                  | 68.26                                   | 72.19                                 | 79.49 |       |       |
|                     | Minor species, <30%, 3-O-Ac            |                                         | Acetyl group:<br>2.18/20.35; --/173.2 |       |       |       |
| C                   | 4.91                                   | 4.09                                    | 3.82                                  | 3.558 | 3.73  | 1.28  |
| 3- $\alpha$ -Rha    | 101.39                                 | 68.8                                    | 77.9                                  | 71.3  | 69.3  | 16.7  |
|                     | 4.94                                   | 4.13                                    | 3.84                                  | 3.558 | 3.73  | 1.28  |
|                     | 101.39                                 | 68.8                                    | 77.9                                  | 71.3  | 69.3  | 16.7  |
| D                   | 5.00                                   | 5.50                                    | 3.89                                  | 3.57  | 3.55  | 1.37  |
| 3- $\beta$ -Rha     | 99.05                                  | 71.0                                    | 77.48                                 | 72.05 | 71.2  | 16.7  |
|                     | Main species,<br>~70%, 2-O-Ac          | Acetyl<br>group: 2.22/20.35<br>--/173.2 |                                       |       |       |       |
|                     | 4.78                                   | 4.129                                   | 3.89                                  | 3.52  | 3.52  | 1.35  |
|                     | 100.2                                  | 70.53                                   | 77.5                                  | 72.7  | 72.7  | 16.7  |
| De-O-acetylated LPS |                                        |                                         |                                       |       |       |       |
| Unit                | 1                                      | 2                                       | 3                                     | 4     | 5     | 6     |
| A                   | 5.26                                   | 4.38                                    | 3.729                                 | 3.62  | 3.906 | 1.35  |
| 2- $\alpha$ -Rha    | 100.73                                 | 73.17                                   | 79.62                                 | 71.22 | 69.3  | 16.7  |
|                     | $^1J_{\text{C,H}} = 175.28 \text{ Hz}$ |                                         | 3.53/ 56.92                           |       |       |       |
| B                   | 5.10                                   | 4.187                                   | 3.99                                  | 3.66  | 3.98  | 1.37  |
| 4- $\alpha$ -Rha    | 101.9                                  | 69.68                                   | 69.09                                 | 82.53 | 67.7  | 16.7  |
|                     | $^1J_{\text{C,H}} = 172.20 \text{ Hz}$ |                                         |                                       |       |       |       |
| C                   | 4.98                                   | 4.170                                   | 3.887                                 | 3.597 | 3.791 | 1.32  |
| 3- $\alpha$ -Rha    | 101.42                                 | 69.87                                   | 78.01                                 | 71.87 | 69.45 | 16.7  |
|                     | $^1J_{\text{C,H}} = 172.98 \text{ Hz}$ |                                         |                                       |       |       |       |
| D                   | 4.82                                   | 4.167                                   | 3.723                                 | 3.56  | 3.55  | 1.386 |
| 3- $\beta$ -Rha     | 100.32                                 | 70.09                                   | 79.79                                 | 71.68 | 71.9  | 16.7  |
|                     | $^1J_{\text{C,H}} = 162.3 \text{ Hz}$  |                                         |                                       |       |       |       |

Spin system A was identified as 3-O-methyl- $\alpha$ -rhamnose, confirmed by the characteristic downfield shift of the C-3 carbon and long-range correlations in the HMBC spectrum, along with NOE contacts between the methoxy proton and H-3. Spin system D was assigned as  $\beta$ -rhamnose, supported by its anomeric  $^1J_{CH}$  coupling constant (162.0 Hz) and intra-residual NOE correlations between H-1 and H-3/H-5 (Table 4). Notably, O-acetylation was detected at the C-2 position of residue D, evidenced by the downfield shift of the H-2 proton resonance and its long-range correlation with an acetyl carbonyl carbon (Figure 2). Variants of the OPS differing in acetylation patterns were also identified, including deacetylated chains and species with additional non-stoichiometric acetylation at O-3 of residue B and O-2 of residue C.

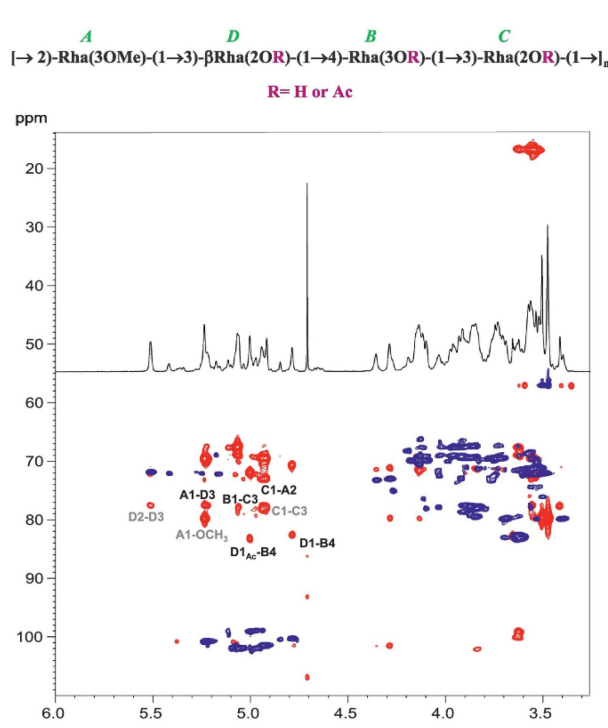


**Figure 2:** HSQC (blue) and HMBC (red) NMR spectra of *Herbaspirillum* OPS. Key inter-residual correlations are indicated, facilitating the assignment of glycosidic linkages and the identification of the spin systems A, B, C, and D. Letters correspond to the assignments in Table 4. Unless specified, Rhamnose residues are  $\alpha$ -configured.

Mapping of the glycosylation sites was achieved by analyzing downfield carbon chemical shifts at O-2 of residue A, O-4 of residue B, O-3 of residue C, and O-3 of residue D (Table 4). Inter-residual NOE contacts, combined with HMBC long-range correlations, established the sequence of the repeating tetrasaccharide unit, as illustrated in figures 2 and 3. Acetylation at residues B and C was found to be nearly stoichiometric (<30%), whereas residue D exhibited acetylation above 70%.

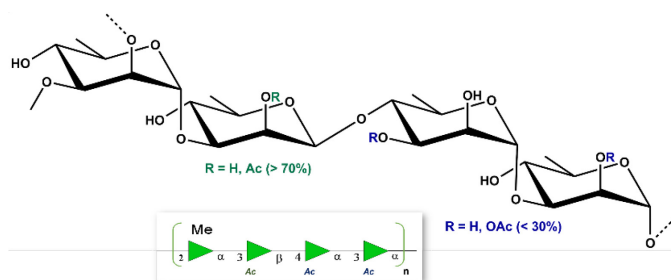


**Figure 3:** *Herbaspirillum* Root189 LPS O-antigen structure. The representation highlights the repeating tetrasaccharide unit with detailed visualization of O-methylation and O-acetylation sites, confirming the structural complexity and linkage positions identified in NMR analysis.

To simplify structural analysis, O-deacetylated OPS (OPS-deO) was prepared. The resulting NMR spectra confirmed that the anomeric configurations and sugar sequence were consistent with the native OPS (Table 4) suggesting strong structural stability even after deacetylation. Validation through HSQC and HMBC experiments demonstrated that the primary saccharide linkages were preserved, maintaining the integrity of the glycan backbone.

To further explore the oligomeric nature of the deacetylated OPS, ultrahigh-resolution MALDI FT-ICR mass spectrometry was performed (Figure 4)<sup>22</sup>. The mass spectrum revealed an oligodisperse mixture with three major species corresponding to 13 to 15 tetrasaccharide repeating units. The observed  $m/z$  difference between the three major ion species matched the theoretical mass of 599.255 Da of the fully deacetylated repeating unit, supporting the structural characterization derived from NMR (Table 4).

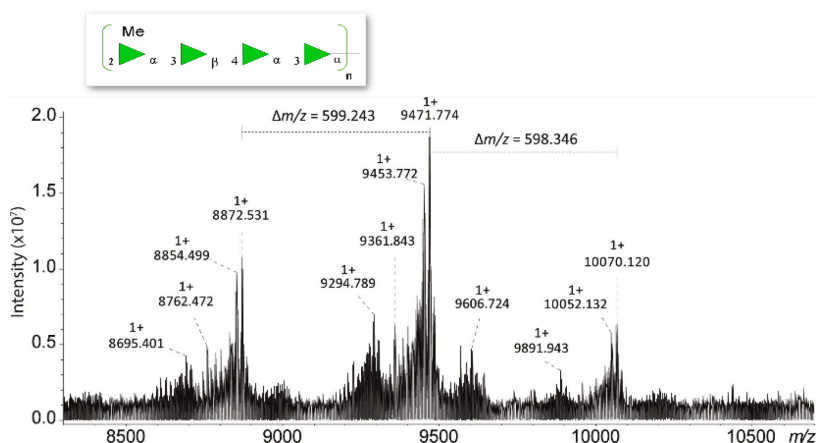


**Figure 4:** MALDI FT-ICR mass spectrum of de-acetylated OPS from *Herbaspirillum* Root189. The spectrum shows the distribution of oligomeric species, with clear peaks corresponding to 13–15 repeating units of the tetrasaccharide.

### 3.2.3 Conformational Analysis and Membrane Properties of *Herbaspirillum* Root189 LPS

The conformational behavior of *Herbaspirillum* Root189 O-specific polysaccharide (OPS) and its de-acetylated derivative (OPS-deO) was investigated through molecular mechanics (MM) calculations and molecular dynamics (MD) simulations. These analyses aimed to explore the structural flexibility, spatial arrangement, and solvent interactions of the OPS under physiological conditions. To evaluate the energetically accessible conformational space, potential energy surfaces of the four disaccharide linkages that constitute the OPS repeating unit were constructed. Adiabatic energy maps were generated for the glycosidic torsions  $\Phi$  (H1-C1-O-CX') and  $\Psi$  (C1-O-CX'-HX') revealing global minima consistent with the exo-anomeric effect (Figure 5A). The energy maps showed moderate flexibility around the  $\Phi$  torsion and higher flexibility around the  $\Psi$  angle, particularly for the Rha $\rightarrow$ 3Rha, Rha(3OMe) $\rightarrow$ 3 $\beta$ Rha, and Rha $\rightarrow$ 2Rha(3OMe) disaccharide units. These units exhibited two global minima separated by a low energy barrier, corresponding to exo- $\Phi$ /syn- $\Psi$  conformations. In contrast, the  $\beta$ Rha $\rightarrow$ 4Rha linkage showed a lowest energy region centered around  $\Phi/\Psi \approx 60^\circ/0^\circ$ , with an additional local minimum in the exo- $\Phi$ /anti- $\Psi$  conformation, suggesting specific torsional preferences (Figure 5A).

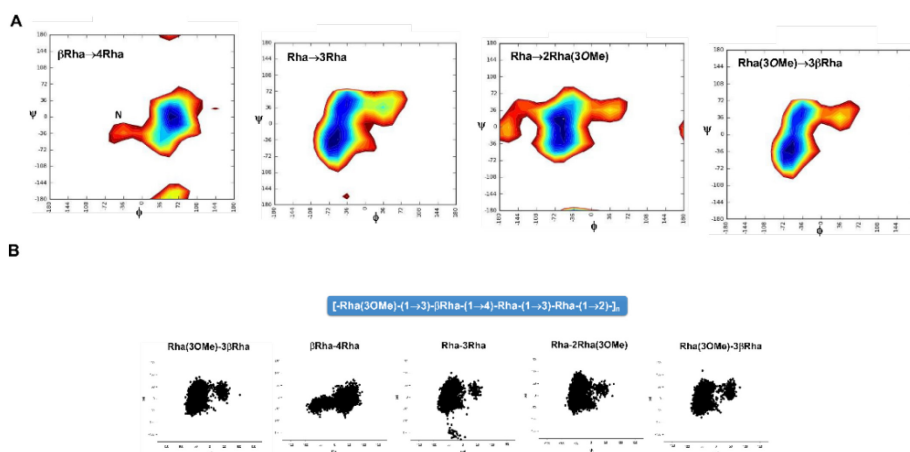


**Figure 5:** (A) Adiabatic energy maps for the disaccharide constituents of the OPS repeating unit. Glycosidic torsions are defined as  $\Phi$  (H1-C1-O-CX') and  $\Psi$  (C1-O-CX'-HX'). A minor local minimum in the non-exo anomeric region is indicated with the letter N in the trisaccharide  $\beta$ Rha $\rightarrow$ 4Rha. (B) Scatter plots of  $\Phi$  vs.  $\Psi$  for the disaccharide units contained in the de-O-acylated OPS of *Herbaspirillum* Root189, highlighting conformational flexibility and preferred orientations during MD simulations.

Based on these energy calculations, saccharide models containing one and two repeating units of OPS-deO were constructed, and their conformational space was further explored using MD simulations. The  $\Phi/\Psi$  scatter plots derived from the MD trajectories confirmed the accessibility of these conformational regions and the strong preference for the exo-anomeric conformation across all glycosidic linkages (Figure 5B). This resulted in limited flexibility and a relatively extended conformation of the glycan chain of *Herbaspirillum* O-deacetylated OPS.

To evaluate the impact of acetylation on OPS structure, MD simulations were performed on hexa- and deca-saccharide models of the acetylated OPS, representing one and two repeating units, respectively (Figure 6A-D).

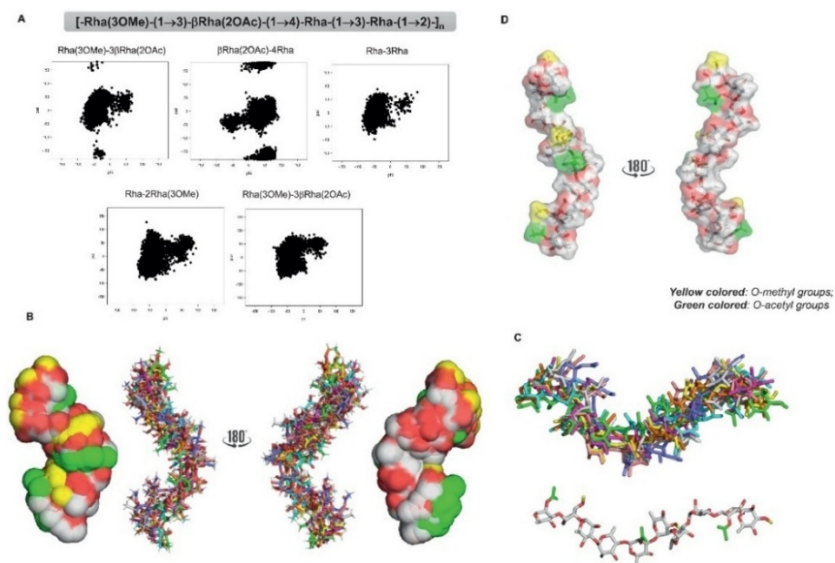


**Figure 6:** (A) Scatter plots of  $\Phi$  vs.  $\Psi$  during the MD simulation for the disaccharide units of *Herbaspirillum* Root189 OPS. (B–C) Conformational behavior of the OPS: representative deca-saccharide conformers and molecular surfaces obtained through NMR and MD simulations. The O-methyl and O-acetyl groups are highlighted with a color code (specified in the figure). Notably, non-sugar substituents are localized on one face of the glycan chain. (D) A representative conformation illustrates the coiled-like structure of the LPS O-antigen, suggesting its potential role in immune evasion.

All structures were energy-minimized and subjected to 100 ns MD simulations in explicit solvent using the AMBER18 force field<sup>23</sup>. Ensemble average inter-proton distances derived from the MD trajectories were converted into NOE contacts using a full-matrix relaxation approach, showing excellent agreement with experimental data (Table 5).

**Table 5:** Experimental (from NOESY experiments) and calculated (from MD calculations) *inter*-proton distances (in Angström) for acetylated and de-acetylated *Herbaspirillum* OPS deca-saccharides.

|       | OPS exp. | OPS Decasaccharide - Calc | OPS-DeO exp. | OPS-DeO - Decasaccharide - Calc |
|-------|----------|---------------------------|--------------|---------------------------------|
| A1-3D | 2.35     | 2.41±0.24                 | 2.42         | 2.42±0.24                       |
| D1-4B | 2.32     | 2.44±0.22                 | 2.35         | 2.37±0.22                       |
| D2-A5 | 2.78     | 2.90±0.49                 | 2.75         | 2.89±0.60                       |
| B1-3C | 2.20     | 2.40±0.34                 | 2.30         | 2.39±0.23                       |
| C1-2A | 2.35     | 2.42±0.22                 | 2.47         | 2.43±0.23                       |

Hydrophobic substituents on the glycan chain, particularly the O-acetyl groups, were found to significantly influence both the shape and hydrophobicity of the OPS. The presence of acetyl groups introduced subtle modifications in the potential energy surface of  $\beta$ Rha $\rightarrow$ 4Rha disaccharide units, notably shifting  $\Psi$  torsion values to around  $180^\circ$ , corresponding to the exo-anti orientation (Figure 6A).

Compared to OPS-deO, the substitution with acetyl groups conferred enhanced flexibility and a reduction in the linear extension of the polysaccharide chain, resulting in a loosely coiled conformation (Figures 6B–C, 7 and 8).

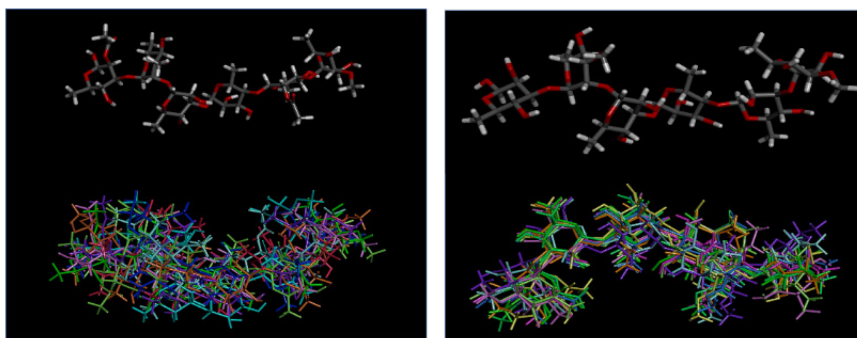


**Figure 7:** Representative hexasaccharide conformers of *Herbaspirillum* OPS and its O-deacetylated form. These models, obtained through NMR analysis and MD simulations. The models highlight structural differences, glycosidic linkages, and solvent-accessible surfaces, illustrating the impact of acetylation on 3D organization.

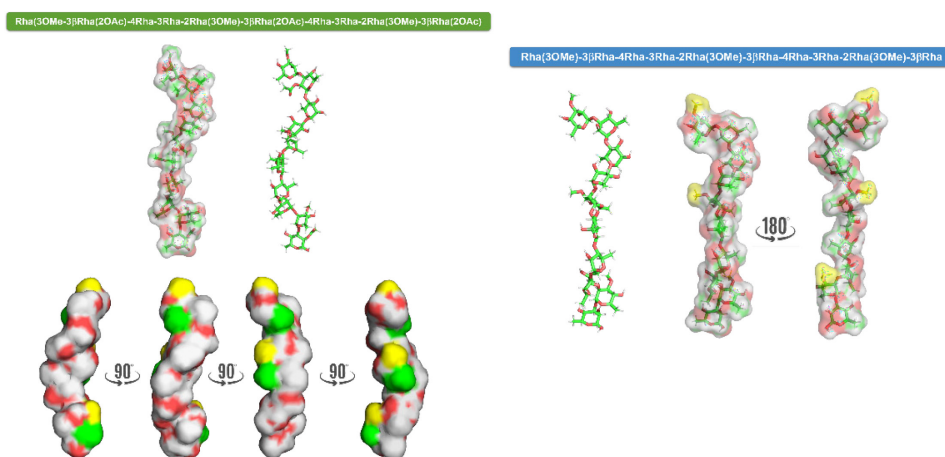


**Figure 8:** Representative deca-saccharide conformers and solvent accessible surfaces of O-deacetylated OPS, determined via NMR analysis and MD simulation. Key conformational features and potential membrane interaction sites are visualized.

The superimposition of representative conformers revealed that acetyl and methyl groups predominantly project from the same face of the polymer backbone, creating an asymmetric hydrophobic distribution along the OPS. This organization is hypothesized to increase membrane affinity and reduce immune visibility by masking specific epitopes (Figures 6B-C).

### 3.2.4 Solvent Affinity, Hydration, and Supramolecular Properties of *Herbaspirillum* Root189 LPS Bilayers

To complement the conformational analysis of the *Herbaspirillum* Root189 O-antigen, the supramolecular behavior and membrane integration properties of the corresponding LPS were investigated using a combination of light scattering techniques and neutron reflectometry (NR). Static and dynamic light scattering (SLS and DLS) analyses<sup>24</sup> revealed a monodisperse population of LPS aggregates with a hydrodynamic radius of approximately  $21.0 \pm 1.0$  nm and low polydispersity, indicative of stable and homogeneous supramolecular assemblies (Figure 9). These aggregates, consisting of approximately 15–35 LPS molecules based on molecular weight estimates, are consistent with micelle-like structures stabilized by hydrophobic interactions among lipid A moieties and dense packing of the O-antigen chains. Importantly, the positive second virial coefficient, determined via Zimm plot analysis<sup>25</sup>, suggested favorable solvent interactions and high hydration of the LPS bilayers, further supporting their thermodynamic stability in water environments (Figure 9).

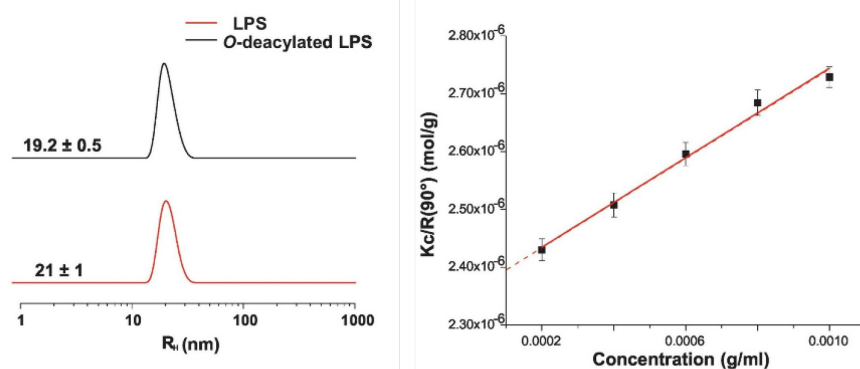


**Figure 9:** Hydrodynamic radius and Zimm plot of *Herbaspirillum* Root189 LPS and its O-deacylated form at a concentration of  $1.0 \text{ mg} \cdot \text{mL}^{-1}$ . Dynamic Light Scattering (DLS) measurements revealed a monodisperse distribution centered at a hydrodynamic radius of  $21.0 \pm 1.0$  nm (shown in red). This well-defined size distribution enabled the estimation of the molecular weight via Static Light Scattering (SLS), yielding a value of  $\sim 420 \pm 20$  kDa. The aggregates are composed of approximately 15 LPS molecules (estimated monomer molecular weight  $\sim 10$  kDa), each with a hydrodynamic radius of  $\sim 21$  nm.

Neutron reflectometry measurements provided high-resolution structural insights into asymmetric lipid bilayers, composed of inner leaflets of perdeuterated DPPC and outer layers of *Herbaspirillum* LPS<sup>26</sup>. Reflectivity profiles, collected under three solution contrasts, revealed a highly stratified bilayer architecture, with a hydrophobic core and a highly hydrated,

extended outer region formed by the O-antigen chains (Figure 10). Model fitting of the NR indicated a  $\sim 25$  Å thick hydrophobic region with limited hydration ( $\sim 15\%$ ), consistent with tight acyl chain packing, and an inner phosphocholine headgroup layer ( $\sim 5$  Å thick) exhibiting  $\sim 85\%$  hydration, likely due to water association with the polar lipid headgroups. In contrast, the outer headgroup region, composed of the LPS carbohydrate moieties, extended approximately 30 Å, while the O-antigen layer protruded an additional  $\sim 100$  Å into the solvent, exhibiting  $\sim 87\%$  hydration. These results demonstrate significant molecular crowding and water penetration, likely mediated by the dense, branched structure and substitutional pattern (O-acetylation and O-methylation) of the OPS chains.

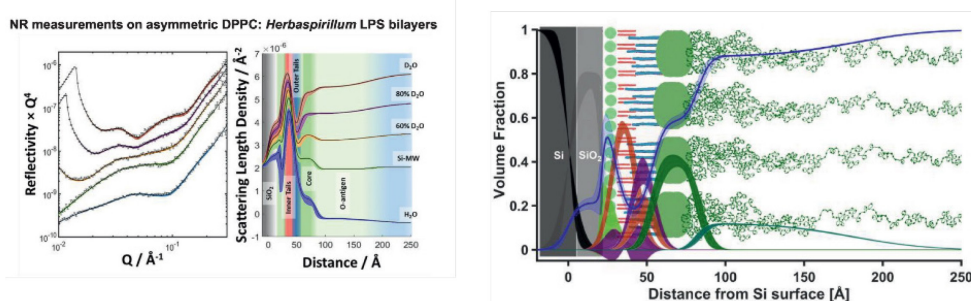


**Figure 10:** Neutron reflectivity profiles (data points) and corresponding model fits (solid lines) for an asymmetric lipid bilayer composed of DPPC (inner leaflet) and *Herbaspirillum* LPS (outer leaflet). The central panel displays the total Scattering Length Density (SLD) profile. Shaded areas indicate 95% confidence intervals, determined using Bayesian error estimation. The volume fraction profiles indicate: silicon (black), silicon dioxide (grey), inner headgroups (green), phosphatidylcholine tails (red), LPS lipid tails (purple), core/outer headgroup region (mid-green), O-antigen chains (dark green), and solvent (blue).

Taking together, these findings highlight the critical role of O-antigen structure in determining bilayer morphology, hydration dynamics and membrane functionality. The broad and rough outer layer ( $\sim 45$  Å roughness) suggests a flexible and irregular extension of the OPS, which may reduce epitope accessibility and thereby enhance immune evasion. Simultaneously, this configuration promotes membrane fluidity and solvent compatibility, contributing to the bacterium's ability to maintain outer membrane integrity under environmental stress.

The integration of molecular modelling, biophysical experiments, and neutron reflectometry presents a coherent picture of *Herbaspirillum* Root189 LPS as a hydrated, supramolecularly stable system, finely tuned for rhizosphere survival and modulation of plant immune responses.

### 3.3 Immunological Studies

The immunomodulatory properties of *Herbaspirillum* Root189 LPS were investigated in *Arabidopsis thaliana* to determine the molecular features that influence host immune recognition. In particular, the impact of O-acetylation on the immunogenic potential of the

LPS O-antigen was evaluated through a combination of early immune response assays and lectin-binding microarray analysis. To this end, both native LPS and its O-deacylated variant (OPS-deO) were tested for their ability to trigger early immune responses in *A. thaliana*, namely apoplastic reactive oxygen species (ROS) production and cytosolic calcium influx. While treatment with native LPS did not elicit a detectable ROS response, removal of O-linked acyl groups on the OPS restored this immune recognition, resulting in a significant oxidative burst in plant tissues (Figure 11A). The deacylated LPS triggered a marked increase in intracellular calcium concentration, while the native molecule remained largely immunologically silent (Figure 11B). These results strongly suggest that O-acetylation functions as a molecular shield, masking immunogenic epitopes on the O-antigen from plant pattern recognition receptors.

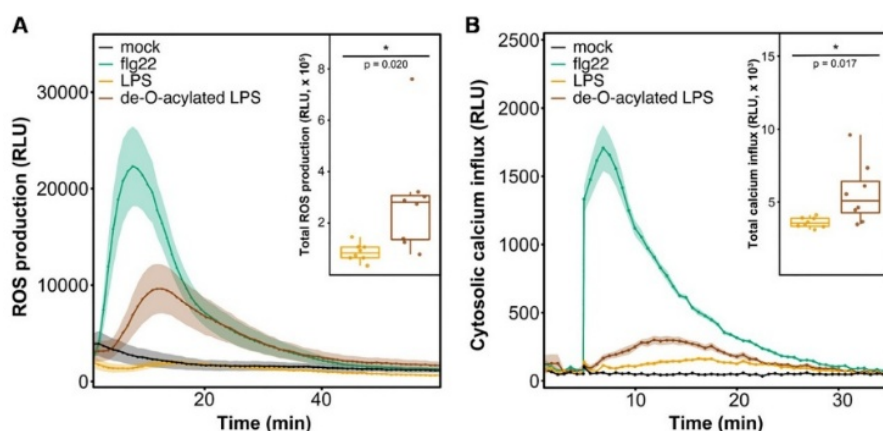


**Figure 11:** (A) Apoplastic ROS production in *A. thaliana* leaf discs treated with sterile Milli-Q water (mock), 250 nM flg22 peptide, 200  $\mu\text{g}\cdot\text{mL}^{-1}$  native *Herbaspirillum* Root189 LPS, or de-O-acylated LPS. Luminescence from luminol-based oxidative burst assays is reported in relative light units (RLU). (B) Cytosolic calcium influx measured using aequorin-expressing seedlings. Values were normalized to maximum calcium discharge following ethanol/ $\text{CaCl}_2$  treatment. Inset panels display cumulative immune responses. Asterisks indicate statistical significance (Welch's t-test,  $p \leq 0.05$ ).

To further characterize the interaction of the bacterial glycome with host recognition systems, a lectin microarray was employed to assess the carbohydrate-binding properties of *Herbaspirillum* Root189 LPS. The LPS was printed on microarray slides in serial dilutions<sup>27</sup>, and a panel of 35 biotin-labelled lectins with defined sugar-binding specificities was screened (Table 5). Notably, specific binding was detected for *Hippeastrum* hybrid lectin (HHL), *Narcissus pseudonarcissus* lectin (NPL), *Galanthus nivalis* agglutinin (GNA), and, to a lesser extent, Concanavalin A (ConA), all of which exhibit affinity for mannose-containing structures. These interactions were dose-dependent and carbohydrate-mediated, as evidenced by successful inhibition upon co-incubation with methyl- $\alpha$ -D-mannopyranoside and methyl- $\alpha$ -(1 $\rightarrow$ 3)-D-mannobioside (MeMan, MeDiMan), which compete for the lectin-binding sites (Figure 12).

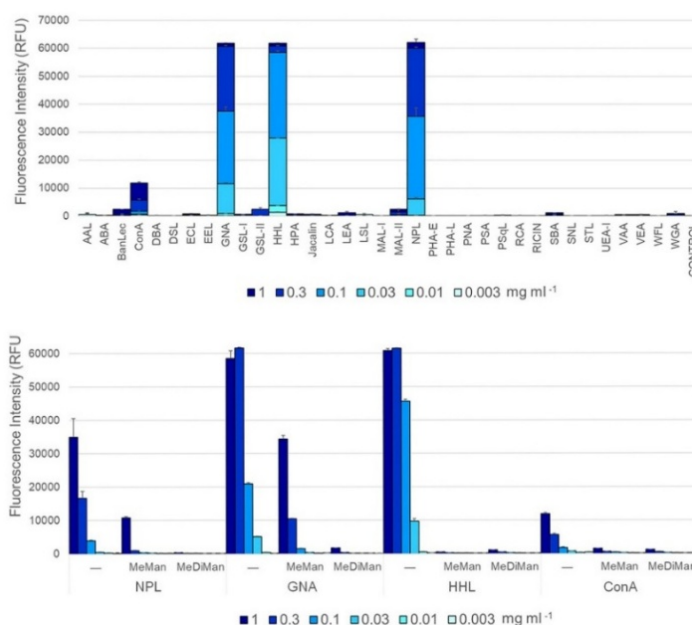


**Figure 12:** Top: Lectin-binding microarray analysis of *Herbaspirillum* Root189 LPS, printed in triplicate at six concentrations (1 to 0.003 mg·mL<sup>-1</sup>), probed with biotinylated lectins and visualized via AF647-streptavidin. Bottom: Competitive inhibition assays of lectin binding in the presence of 50 mM methyl- $\alpha$ -D-mannose (MeMan) or 20 mM methyl-3-O- $\alpha$ -D-mannopyranosyl- $\alpha$ -D-mannopyranoside (MeDiMan).

No detectable binding was observed for lectins recognizing galactose, N-acetyl-galactosamine, fucose, or sialic acid, nor for other mannose-specific lectins such as *Pisum sativum* agglutinin (PSA), *Lens culinaris* agglutinin (LCA), and *Vicia ervilia* agglutinin (VEA). This suggests a unique spatial arrangement of the mannose-like epitopes within the LPS that are selectively recognized only by a subset of mannose-binding lectins. The restricted binding profile observed in the array thus reflects the peculiar structure of the LPS O-antigen, possibly shaped by its rhamnose backbone and site-specific O-substitutions.

Altogether, these findings highlight the dual immunological function of O-acetylation in *Herbaspirillum* Root189 LPS. On one hand, it confers immune evasion by preventing recognition by plant PRRs. On the other, selective deacetylation reveals epitopes that may be involved in lectin-mediated recognition, suggesting a finely tuned glycan presentation that balances concealment and controlled host engagement. This mechanism likely contributes to the adaptation and persistence of *Herbaspirillum* in the rhizosphere, enabling it to modulate immune responses and facilitate beneficial colonization of *A. thaliana* roots.

### 3.4 Discussion and Conclusions

The intricate relationship between plants and their associated microbiota is increasingly recognized as fundamental for plant health, development, and immunity. Just as the human gut

microbiome plays an essential role in modulating host immunity, the plant root microbiome, often referred to as the plant's second genome<sup>28</sup>, actively shapes host physiology and stress responses. However, a fundamental question remains: how do plants tolerate beneficial microbes while maintaining robust immune defenses against pathogens?

This work addresses this question by dissecting the structural and immunological properties of the lipopolysaccharide (LPS) produced by *Herbaspirillum* Root189, a core member of the *Arabidopsis thaliana* root microbiota. Through a multidisciplinary approach combining NMR spectroscopy, mass spectrometry, molecular modeling, light scattering, neutron reflectometry, and immunological assays, we demonstrate that *Herbaspirillum* Root189 LPS possesses a D-rhamnose-based O-antigen that is extensively modified with non-stoichiometric O-acetyl and O-methyl substituents. These modifications are not only structural but functional, playing a pivotal role in modulating host immune recognition<sup>29</sup>.

We show that the native, acetylated form of the LPS is able to evade early plant immune responses, such as apoplastic ROS production and cytosolic calcium influx. Conversely, the deacetylated variant becomes immunogenic, triggering robust immune responses. These findings highlight O-acetylation as a molecular strategy used by commensal bacteria to shield immunogenic epitopes from plant pattern recognition receptors, thereby facilitating successful root colonization.

Molecular dynamics simulations further reveal that acetylation induces a coiled, flexible conformation of the OPS chain, as opposed to the more extended structure of the deacetylated form. This coiling, combined with the asymmetric distribution of hydrophobic substituents, results in an LPS layer with significant hydration but loose packing, as confirmed by neutron reflectometry. The hydrated, rough, and spatially extended structure of the O-antigen likely provides a dynamic shield that both camouflages microbial signatures and fine-tunes interactions with host receptors.

Lectin microarray assays revealed selective binding of Root189 LPS to a subset of mannose-binding lectins (HHL, NPL, GNA, and ConA), suggesting that specific structural motifs, possibly involving D-rhamnose, are recognized by plant lectin-like receptors<sup>30,31</sup>. The binding was inhibited by mannose analogs, underscoring the importance of glycan architecture in determining recognition specificity. Interestingly, several other mannose-specific lectins did not bind, further supporting a model in which the spatial configuration and chemical decoration of the OPS determine its immunological footprint.

Our findings align with recent evidence that plants, like animals, deploy complex immune strategies that can discriminate between pathogenic and non-pathogenic microbes through subtle differences in molecular patterns<sup>29</sup>. The unique O-acetylation pattern of *Herbaspirillum* LPS appears to function as a molecular “pass,” enabling the bacterium to evade immunity while potentially engaging in beneficial interactions. Analogous strategies are used by symbiotic rhizobia<sup>32,33,34</sup> and even some pathogens like *Xylella fastidiosa*<sup>35,36</sup>, indicating that LPS structural modulation is a conserved and versatile strategy in plant-microbe interactions.

From a broader perspective, this thesis contributes to a growing body of work that redefines bacterial LPS not merely as a pathogen-associated molecular pattern (PAMP), but as a context-

dependent signaling molecule capable of shaping immune outcomes. The implications of this paradigm extend beyond plant biology.

Indeed, this chapter builds upon work published in *Carbohydr Polym*<sup>13</sup> that launched our investigation into the structural diversity of LPS molecules in the plant root microbiome and their immunological roles. From this research a new perspective emerged: LPS is not merely a toxic molecule but can serve as a beneficial glycan scaffold capable of modulating immune responses. Looking forward, identifying and characterizing such structure-function relationships will provide vital insights for future biotechnological applications. Decoding the molecular logic by which plants recognize and tolerate their microbial partners opens promising avenues for engineering microbial consortia to enhance crop resilience, nutrient acquisition, and sustainable agriculture. Our long-term vision is to harness the immunomodulatory potential of microbial glycans, such as the LPS studied here, to fine-tune plant immunity and promote beneficial plant-microbe symbioses.

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**SECTION I**

**Plant-associated bacteria**

## **CHAPTER IV**

**Surface glycoconjugates of  
*Methylobacterium extorquens*  
PA1: structural adaptations of  
EPS and LPS to methanol  
tolerance**



## 4.1 Introduction

The complex and multifaceted relationship between plants and their associated microbiota has emerged as a key determinant of plant health, development, and resilience to environmental stressors. Analogous to the human gut microbiome, the plant-associated microbiota, often described as the plant's "second genome", plays an essential role in modulating host physiology and adaptive responses to biotic and abiotic stresses. In addition, the extensively characterized root microbiome, microbial communities colonizing aerial plant surfaces (phyllosphere) and internal tissues (endophytes) also significantly contribute to plant growth and fitness<sup>1</sup>.

Among the most abundant and functionally relevant members of this microbiota are methylotrophic bacteria, particularly those belonging to the genus *Methylobacterium*. These pink-pigmented, facultative Gram-negative organisms, members of the *Rhizobiales* order of *Alphaproteobacteria*, are ubiquitously found in soil, dust, water, sediments, and air<sup>2</sup>. *Methylobacterium* species are metabolically versatile, capable of utilizing reduced single-carbon compounds such as methanol and methylamine. Methanol is naturally released by plants as a metabolic byproduct during pectin demethylation in cell wall biosynthesis<sup>3-4</sup>. This metabolic specialization confers a competitive edge to *Methylobacterium* spp. within plant-associated niches, facilitating their proliferation and interaction with host plants. These interactions are often mutualistic, enhancing plant growth, abiotic stress resistance, and the synthesis of beneficial phytohormones and vitamins<sup>5,6</sup>.

*Methylobacterium extorquens* PA1, a well-established colonizer of the phyllosphere of *Arabidopsis thaliana*, has emerged as a valuable model organism for investigating aerobic methylotrophy. Its relatively simple genomic architecture, rapid growth on methanol, and amenability to genetic manipulation make it ideal for exploring microbe, plant interactions and metabolic plasticity under environmental stress<sup>7</sup>. A key aspect of this bacterium's adaptation strategy lies in the structural modulation of its surface glycoconjugates, which form a dynamic interface with the external environment.

Central to the bacterium's ability to endure methanol-rich environments is the protective role played by its cell envelope, which includes surface glycoconjugates such as exopolysaccharide (EPS) and lipopolysaccharide (LPS), or lipooligosaccharide (LOS) in the case of rough-type strains.

EPSs are high-molecular-weight carbohydrate polymers secreted into the extracellular matrix, where they mediate surface adhesion, biofilm formation, water retention, and protection against abiotic stresses. Meanwhile, the structural adaptability of LPS/LOS molecules, particularly in response to host immune pressure or environmental changes, facilitates bacterial survival and modulates plant colonization, symbiosis, and even virulence. In *M. extorquens* PA1, methanol exposure triggers dynamic changes in the production and composition of these glycoconjugates: under standard conditions, the bacterium synthesizes a predominant EPS rich in rhamnose and O-methyl-glucose, whereas growth on methanol induces secretion of a heterogeneous EPS mixture, with enhanced biophysical properties that confer greater tolerance to solvent stress<sup>8</sup>.

Concurrently, *M. extorquens* PA1 and AM1 produce a unique rough-type LOS characterized by an extensively O-methylated and non-phosphorylated core oligosaccharide enriched with acidic ulosonic residues, including 5-O-methylated Ko or Kdo units. Its lipid A moiety consists of a trisaccharide backbone containing galacturonic acid and very long chain fatty acids (VLCFAs) with unprecedented acyl substitutions, contributing to membrane stabilization and increased order under methanol stress conditions<sup>9</sup>.

Together, these findings highlight the dual biochemical strategies adopted by *M. extorquens* PA1, through structural modulation of both EPS and LOS, to adapt to methanol-rich environments. These surface-level adaptations reflect not only metabolic plasticity but also molecular mechanisms that support ecological fitness under chemical stress. They also offer novel insights into how microbial surface polymers interface with environmental cues, potentially informing future efforts in microbial engineering and sustainable agriculture.

In this context, the present work aims to elucidate the structural and functional adaptations of the two major classes of surface glycoconjugates, EPS and LOS, produced by *M. extorquens* PA1 under methanol-induced stress. By combining advanced chemical characterization with biophysical analyses, including NMR spectroscopy, molecular dynamics simulations, and diffusion-based methods, this chapter explores how these molecules contribute to membrane integrity, solvent tolerance, and microbe–plant interactions.

The data and analyses presented in this chapter have been published in full in the following peer-reviewed articles:

- Vanacore, A., Forgione, M.C., Cavasso, D., et al. (2022). *Role of EPS in mitigation of plant abiotic stress: The case of Methylobacterium extorquens PA1*. *Carbohydr Polym*, 295, 119863.
- Di Lorenzo, F., Nicolardi, S., Marchetti, R., et al. (2023). *Expanding knowledge of methylotrophic capacity: Structure and properties of the rough-type lipopolysaccharide from Methylobacterium extorquens and its role on membrane resistance to methanol*. *JACS Au*, 3, 929–942.

## 4.2 Structural Characterization of *Methylobacterium extorquens* PA1 Exopolysaccharides (EPS)

Exopolysaccharides (EPSs) are key components of the bacterial extracellular matrix, contributing to biofilm formation, cell aggregation, surface adhesion, and protection from environmental stressors<sup>10,5</sup>. In the case of *Methylobacterium extorquens* PA1, a phyllosphere-associated methylotroph capable of thriving in methanol-rich environments, EPS production appears to be tightly linked to its plant-associated lifestyle and stress tolerance<sup>1,11</sup>.

To investigate the molecular features underlying this adaptive strategy, EPS produced by *M. extorquens* PA1 under standard growth conditions (refEPS) and in the presence of 10% (v/v) methanol (metEPS) were extracted, purified, and subjected to detailed structural analyses<sup>12,13,14,15</sup>.

#### 4.2.1 Extraction, Purification, and Compositional Analysis of EPS

Bacterial cells were sequentially extracted using a combination of phenol/chloroform/petroleum ether (PCP) and hot phenol/water methods<sup>16,17</sup>. The crude EPS preparations were enzymatically treated with DNase, RNase, and proteases to remove nucleic acid and protein contaminants, followed by dialysis and gel filtration chromatography to obtain highly purified high-molecular-weight fractions suitable for compositional and spectroscopic analyses.

Monosaccharide composition was assessed by gas chromatography–mass spectrometry (GC-MS) following methanolysis and derivatization into acetylated methyl glycosides<sup>18</sup>. Glycosidic linkages were elucidated by converting the sugars into partially methylated alditol acetates, also analyzed by GC-MS. Additionally, sugars were converted into acetylated octyl glycosides to determine their absolute configuration.

The compositional analysis of refEPS revealed the presence of L-rhamnose (Rha), 3-O-methyl-D-glucose (Glc3OMe), and D-glucose (Glc) in an approximate molar ratio of 1:0.3. Methylation analysis identified a branched polysaccharide architecture with 2-substituted Rhap, 6-substituted Glcp, 2,3-di-substituted Glcp, and 2-substituted Glcp residues. These results are summarized in Table 1.

**Table 1.** Monosaccharide composition and glycosidic linkage analysis of the exopolysaccharide (refEPS) produced by *Methylobacterium extorquens* PA1 under standard growth conditions.

| Monosaccharide                 | Partially Methylated Derivative                               |
|--------------------------------|---------------------------------------------------------------|
| L-rhamnose (Rha)               | 2-substituted Rhap                                            |
| 3-O-methyl-D-glucose (Glc3OMe) | ----                                                          |
| D-glucose (Glc)                | 6-substituted Gcp, 2,3-disubstituted Glcp, 2-substituted Glcp |

In contrast, EPSs produced under methanol stress conditions (metEPS) exhibited a more complex composition. The major components included L-rhamnose (Rha), 3-O-methyl-L-rhamnose (Rha3OMe), 3-O-methyl-D-glucose (Glc3OMe), and D-glucose (Glc). Linkage analysis revealed an extensively branched and methylated architecture comprising 2-substituted, 3-substituted, and 2,3-di-substituted Rhap, as well as 6-substituted Glcp, 2-substituted Glcp, and terminal Glcp residues. A detailed summary of these modifications is reported in Table 2.

**Table 2:** Monosaccharide composition and glycosidic linkage pattern of the exopolysaccharide (metEPS) produced by *Methylobacterium extorquens* PA1 in the presence of 10% methanol.

| Monosaccharide                  | Partially Methylated Derivative                                |
|---------------------------------|----------------------------------------------------------------|
| L-rhamnose (Rha)                | 2-substituted Rhap, 3-substituted Rhap, 2,3-disubstituted Rhap |
| 3-O-methyl-L-rhamnose (Rha3OMe) | ----                                                           |
| 3-O-methyl-D-glucose (Glc3OMe)  | ----                                                           |
| D-glucose (Glc)                 | 6-substituted Glcp, 2-substituted Glcp, terminal Glcp          |

These findings indicate that *M. extorquens* PA1 actively modulates the composition and branching of its EPS in response to elevated methanol levels. The increased complexity and degree of methylation in metEPS likely enhance its biophysical properties, such as water retention and membrane shielding, thereby contributing to solvent stress tolerance and improved survival under environmental constraints.

#### 4.2.2 Structural Characterization by NMR Spectroscopy

Purified exopolysaccharide samples were subjected to extensive NMR spectroscopy to elucidate their carbohydrate polymer structures. The anomeric configurations of the sugar residues were assigned based on  $^3J_{H1,H2}$  and  $^1J_{C1,H1}$  coupling constants and confirmed by intra-residual NOE contacts. Relative configurations were deduced from vicinal  $^3J_{H,H}$  coupling constants and intra-residual NOEs, as previously reported<sup>15</sup>.

For the EPS produced in reference medium (refEPS), three main spin systems were identified, A/A', B, and C (Figure 1A and Table 3). Spin system A was assigned to 3-O-methyl- $\alpha$ -glucose. The methylation site was clearly identified at O-3 by the downfield shift of C-3 and its long-range HMBC correlation with the methoxy protons. The  $\alpha$ -anomeric configuration was confirmed by the  $^1J_{C1,H1}$  coupling constant of 174.3 Hz. A minor variant, spin system A', represented the demethylated form of residue A. Residue B was identified as  $\alpha$ -rhamnose. Its manno configuration was supported by low  $^3J_{H1,H2}$  and  $^3J_{H2,H3}$  values (<2 Hz), indicating an equatorial H-2 orientation. The  $\alpha$ -anomeric configuration was confirmed by the  $^1J_{C1,H1}$  coupling constant of 173.2 Hz. Residue C was assigned as  $\beta$ -glucose, based on a  $^1J_{C1,H1}$  value of 162.1 Hz and intra-residual NOE correlations between H-1, H-3, and H-5. The proposed structure of the repeating trisaccharide unit is shown in Figure 1B.

Surface glycoconjugates of *Methylobacterium extorquens* PA1: structural adaptations of EPS and LPS for methanol tolerance

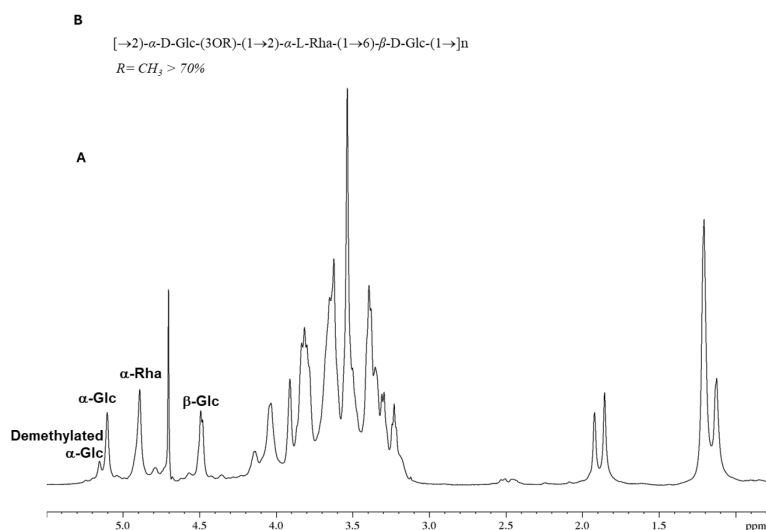


**Figure 1:** (A) <sup>1</sup>H NMR spectrum of the exopolysaccharide (RefEPS) produced by *Methylobacterium extorquens* PA1 under standard growth conditions. (B) Proposed repeating unit structure of RefEPS.

Glycosylation sites were determined from characteristic downfield shifts: O-2 of residues A/A', O-2 of B, and O-6 of C. Inter-residual NOE contacts and long-range HMBC correlations (A1-B2, B1-C6, C1-A2) defined the repeating trisaccharide unit of refEPS. Notably, the α-glucose residue was methylated at O-3 in more than 70% of the units. These correlations are confirmed by the HSQC spectrum shown in Figure 2.

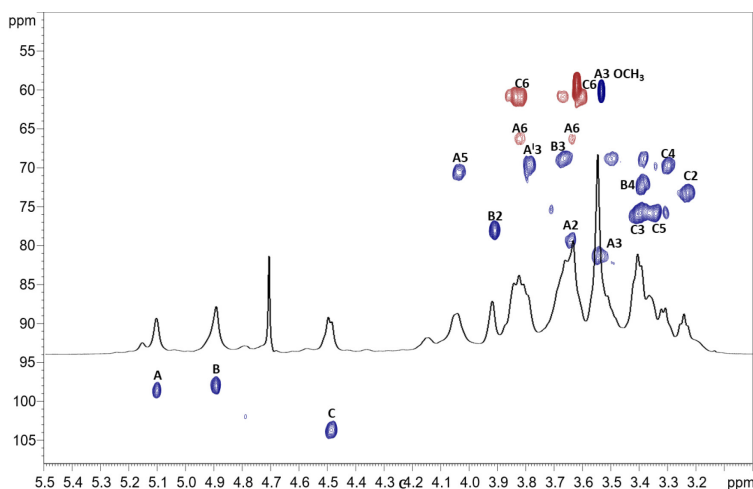


**Figure 2.** HSQC NMR spectrum of RefEPS, showing <sup>1</sup>H–<sup>13</sup>C correlations of anomeric and ring protons/carbons. Peaks confirm the presence of α-Glc, α-Rha, and β-Glc, and 3-O-methylation on α-glucose.

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**Table 3:**  $^1\text{H}$  and  $^{13}\text{C}$  NMR chemical shift values and  $^1J_{\text{C},\text{H}}$  coupling constants of monosaccharide residues in RefEPS.

| <i>refEPS</i>                    |                                    |      |                       |                     |       |            |
|----------------------------------|------------------------------------|------|-----------------------|---------------------|-------|------------|
| Unit                             | 1                                  | 2    | 3                     | 4                   | 5     | 6          |
| <b>A</b>                         | 5.11                               | 3.64 | 3.54                  | 3.50                | 4.04  | 3.84/3.64  |
| <b>2-<math>\alpha</math>-Glc</b> | 98.9                               | 79.4 | 81.4                  | 68.8                | 70.67 | 66.3       |
|                                  | $^1J_{\text{C},\text{H}}=174.3$ Hz |      | O-Methyl: 3.53/ 60.18 | <sup>3</sup><br>.46 |       |            |
| <b>A'</b>                        | 5.15                               | 3.55 | 3.79                  | <sup>6</sup><br>8.7 | 4.03  | 3.84/3.64  |
| <b>2-<math>\alpha</math>-Glc</b> | 98.47                              | 81.5 | 71.18                 |                     | 70.67 | 66.3       |
|                                  | $^1J_{\text{C},\text{H}}=174.3$ Hz |      |                       |                     |       |            |
| <b>B</b>                         | 4.89                               | 3.90 | 3.70                  | 3.39                | 3.66  |            |
| <b>2-<math>\alpha</math>-Rha</b> | 98.17                              | 78.3 | 69.79                 | 74.3                | 69.98 |            |
|                                  | $^1J_{\text{C},\text{H}}=173.2$ Hz |      |                       |                     |       |            |
| <b>C</b>                         | 4.49                               | 3.23 | 3.40                  | 3.29                | 3.34  | 3.83/ 3.61 |
| <b>6-<math>\beta</math>-Glc</b>  | 104.0                              | 73.4 | 76.17                 | 69.6                | 76.01 | 60.8       |
|                                  | $^1J_{\text{C},\text{H}}=162.1$ Hz |      |                       |                     |       |            |

In contrast, the NMR spectra of the exopolysaccharide obtained from cultures grown in methanol-containing medium (metEPS) revealed a significantly more complex mixture of polysaccharides. Representative regions of the  $^1\text{H}$  NMR spectrum are shown in Figure 3. This compositional heterogeneity reflected the presence of at least three distinct polysaccharide structures, each exhibiting microheterogeneity in O-methylation.

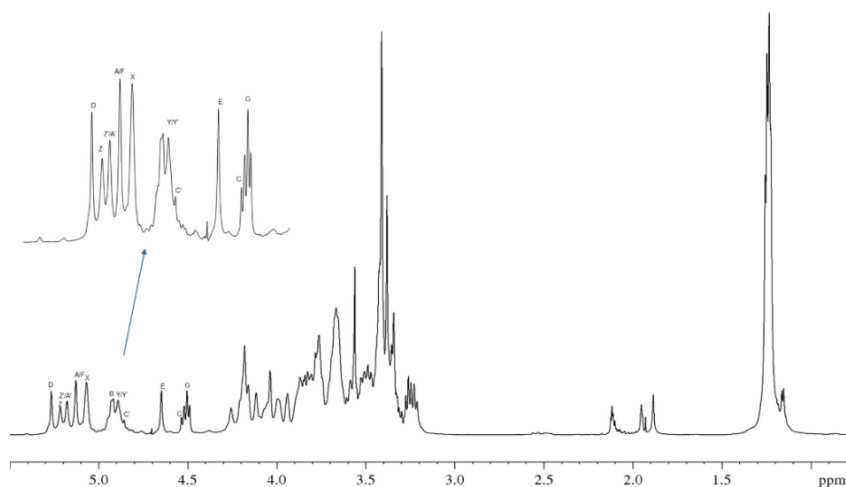


**Figure 3:** Representative  $^1\text{H}$  NMR spectrum of MetEPS, highlighting spectral complexity due to multiple polysaccharide components.

The first component, metEPS\_1, corresponded closely to the refEPS structure but exhibited partial O-methylation (up to 50%) on  $\alpha$ -glucose (residue A), and an additional minor O-methylation on  $\beta$ -glucose (residue C).

The second polysaccharide, metEPS\_2, consisted of residues D (2,3- $\alpha$ -Rha), E (3- $\beta$ -Rha), F (2- $\alpha$ -Rha), and G (terminal  $\beta$ -Glc). Structural elucidation was supported by inter-residual NOE contacts and HMBC correlations, specifically D1–E2, E1–F3, F1–D3, and G1–F2, which established a tetrasaccharide repeating unit. These features are reflected in the HSQC spectrum shown in Figure 4.

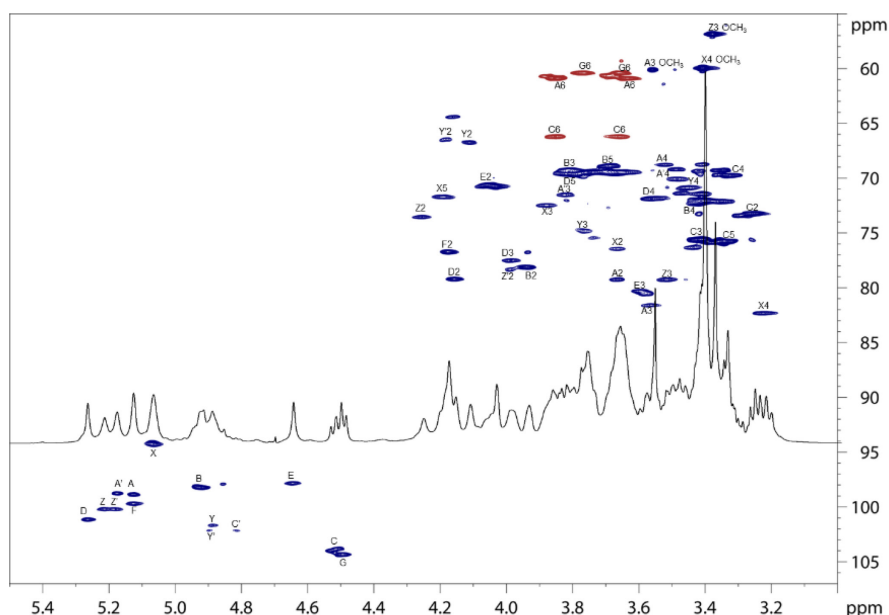


**Figure 4:** HSQC spectrum of MetEPS illustrating the increased branching and methylation complexity relative to RefEPS.

Figure 5 shows an overlay of NOESY and TOCSY spectra, which facilitated the assignment of inter- and intra-residual correlations. Corresponding chemical shift values and coupling constants are summarized in Table 4.

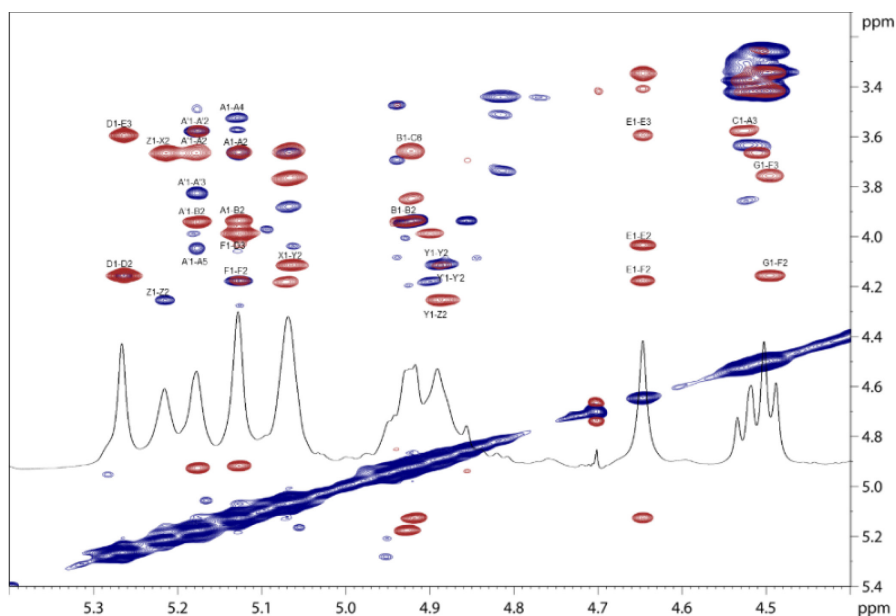


**Figure 5:** Overlay of NOESY (red) and TOCSY (blue) spectra for MetEPS, used for inter-residual and intra-residual assignments.

**Table 4:**  $^1\text{H}$  and  $^{13}\text{C}$  NMR chemical shift values and  $^1J_{\text{C,H}}$  coupling constants for residues in metEPS\_1, metEPS\_2, and metEPS\_3.

| <i>metEPS_1</i>                  |                                  |       |                          |       |       |           |
|----------------------------------|----------------------------------|-------|--------------------------|-------|-------|-----------|
| Unit                             | 1                                | 2     | 3                        | 4     | 5     | 6         |
| <b>A</b>                         | 5.127                            | 3.667 | 3.56                     | 3.51  | 4.06  | 3.85/3.63 |
| <b>2-<math>\alpha</math>-Glc</b> | 98.85                            | 79.26 | 81.6                     | 69.04 | 71.23 | 60.87     |
|                                  | $^1J_{\text{C,H}} = 174.3$<br>Hz |       | O-Methyl: 3.55/<br>60.15 |       |       |           |
| <b>A'</b>                        | 5.18                             | 3.57  | 3.82                     | 3.49  | 4.05  | 3.85/3.63 |
| <b>2-<math>\alpha</math>-Glc</b> | 98.7                             | 80.49 | 71.45                    | 70.1  | 71.23 | 60.87     |
|                                  | $^1J_{\text{C,H}} = 174.5$<br>Hz |       |                          |       |       |           |
| <b>B</b>                         | 4.92                             | 3.93  | 3.82                     | 3.42  | 3.69  | 1.23      |
| <b>2-<math>\alpha</math>-Rha</b> | 98.24                            | 78.16 | 69.15                    | 72.2  | 68.8  | 16.65     |
|                                  | $^1J_{\text{C,H}} = 173.2$<br>Hz |       |                          |       |       |           |

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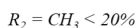
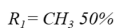
Surface glycoconjugates of *Methylobacterium extorquens* PA1: structural adaptations of EPS and LPS for methanol tolerance

|                  |                                                |                        |                          |                          |       |            |
|------------------|------------------------------------------------|------------------------|--------------------------|--------------------------|-------|------------|
| <b>C</b>         | 4.527/<br>4.510                                | 3.30/ 3.25             | 3.41                     | 3.32                     | 3.34  | 3.66/3.84  |
| <b>6-β-Glc</b>   | 104.28                                         | 73.3                   | 75.58                    | 69.3                     | 75.8  | 66.21      |
|                  | <sup>1</sup> J <sub>C,H</sub> =<br>162.0<br>Hz |                        |                          |                          |       |            |
| <b>C'</b>        | 4.80                                           | 3.51                   | 3.74                     | 3.41                     | 3.43  | 3.72       |
| <b>6-β-Glc</b>   | 102.15                                         | 82.68                  | 75.4                     | 70.8                     | 75.6  | 60.9       |
|                  | <sup>1</sup> J <sub>C,H</sub> =<br>162.1<br>Hz | O-Methyl:<br>3.38/59.9 |                          |                          |       |            |
| <b>metEPS_2</b>  |                                                |                        |                          |                          |       |            |
| <b>D</b>         | 5.26                                           | 4.158                  | 3.98                     | 3.55                     | 3.81  | 1.23       |
| <b>2,3-α-Rha</b> | 101.15                                         | 79.16                  | 77.49                    | 71.9                     | 69.61 | 16.65      |
|                  | <sup>1</sup> J <sub>C,H</sub> =<br>176.0<br>Hz |                        |                          |                          |       |            |
| <b>E</b>         | 4.64                                           | 4.038                  | 3.59                     | 3.41                     | 3.35  | 1.25       |
| <b>3-β-Rha</b>   | 97.9                                           | 70.77                  | 80.36                    | 71.39                    | 75.6  | 16.6       |
|                  | <sup>1</sup> J <sub>C,H</sub> =<br>160.1<br>Hz |                        |                          |                          |       |            |
| <b>F</b>         | 5.128                                          | 4.17                   | 3.74                     | 3.43                     | 3.75  | 1.25       |
| <b>2-α-Rha</b>   | 99.69                                          | 76.65                  | 69.38                    | 69.0                     | 69.52 | 16.6       |
|                  | <sup>1</sup> J <sub>C,H</sub> =<br>172.7<br>Hz |                        |                          |                          |       |            |
| <b>G</b>         | 4.49                                           | 3.26                   | 3.42                     | 3.35                     | 3.43  | 3.66/ 3.76 |
| <b>t-β-Glc</b>   | 104.27                                         | 73.3                   | 75.5                     | 69.7                     | 75.6  | 60.4       |
|                  | <sup>1</sup> J <sub>C,H</sub> =<br>162.7<br>Hz |                        |                          |                          |       |            |
| <b>metEPS_3</b>  |                                                |                        |                          |                          |       |            |
| <b>X</b>         | 5.07                                           | 3.66                   | 3.88                     | 3.22                     | 4.18  | 3.76/3.65  |
| <b>2-α-Glc</b>   | 94.18                                          | 76.4                   | 72.53                    | 82.28                    | 71.6  | 60.4       |
|                  | <sup>1</sup> J <sub>C,H</sub> =<br>172.9<br>Hz |                        |                          | O-Methyl:<br>3.40/ 59.90 |       |            |
| <b>Y</b>         | 4.89/<br>4.90                                  | 4.11 / 4.18            | 3.78                     | 3.48                     | 3.66  | 1.22       |
| <b>3-α-Rha</b>   | 101.64/<br>102.4                               | 66.8                   | 74.8                     | 70.0                     | 69.49 | 16.6       |
|                  | <sup>1</sup> J <sub>C,H</sub> =<br>172.0<br>Hz |                        |                          |                          |       |            |
| <b>Z</b>         | 5.21                                           | 4.25                   | 3.52                     | 3.45                     | 3.67  | 1.23       |
| <b>2-α-Rha</b>   | 100.24                                         | 73.49                  | 79.19                    | 70.86                    | 69.4  | 16.6       |
|                  | <sup>1</sup> J <sub>C,H</sub> =<br>173.3<br>Hz |                        | O-Methyl: 3.37/<br>56.80 |                          |       |            |

|                                  |                         |       |      |      |     |      |
|----------------------------------|-------------------------|-------|------|------|-----|------|
| <b>Z'</b>                        | 5.18                    | 3.99  | 3.80 | 3.44 | 3.6 | 1.23 |
| <b>2-<math>\alpha</math>-Rha</b> | 100.2                   | 78.34 | 69.5 |      |     |      |
|                                  | $^1J_{C,H}=174.1$<br>Hz |       |      |      |     |      |

The third component, metEPS\_3, was composed of residues X (2- $\alpha$ -Glc O-methylated at O-4), Y (3- $\alpha$ -Rha), and Z (2- $\alpha$ -Rha, methylated at O-3 up to 50%). Inter-residual NOE contacts (X1–Y3, Z1–X2, Y1–Z2) and HMBC data defined a trisaccharide repeating unit. The proposed structures of the three polysaccharides identified in MetEPS are illustrated in Figure 6.

#### MetEPS\_1



#### MetEPS\_2



#### MetEPS\_3

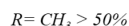


**Figure 6:** Proposed repeating units of the three polysaccharide chains identified in MetEPS.

These findings demonstrate that methanol-rich growth conditions promote significant structural diversification in EPS production by *Methylobacterium extorquens* PA1, reflecting dynamic metabolic adaptations. This structural complexity underscores the essential role of high-resolution NMR techniques in decoding the detailed architecture and biological relevance of microbial polysaccharides.

### 4.2.3 Conformational Analysis and Supramolecular Properties of EPS

To elucidate how structural differences among the exopolysaccharides (EPSs) influence their physical properties and biological functions, we combined molecular mechanics (MM)

calculations, molecular dynamics (MD) simulations, Pulsed-Field Gradient NMR (PFG-NMR), and Dynamic Light Scattering (DLS) analyses<sup>19,20,21,22</sup>.

Five oligosaccharide models were constructed to represent the major EPSs produced by *Methylobacterium extorquens* PA1 under different growth conditions: refEPS, metEPS\_1a/b/c, metEPS\_2 (dodecasaccharide), and metEPS\_3 (nonasaccharide). Each model, containing three repeating units with specific O-methylation patterns, was energy minimized and subjected to MD simulations in explicit water. Cluster analysis of the trajectories revealed that the metEPS\_1a/b/c oligosaccharides share similar random coil conformations, indicating that O-methyl substitutions have only a minor influence on their global shape. In contrast, metEPS\_2 and metEPS\_3 adopted more extended, coiled/random coil-like conformations, reflecting the impact of their unique branching and substitution patterns (Figure 7).

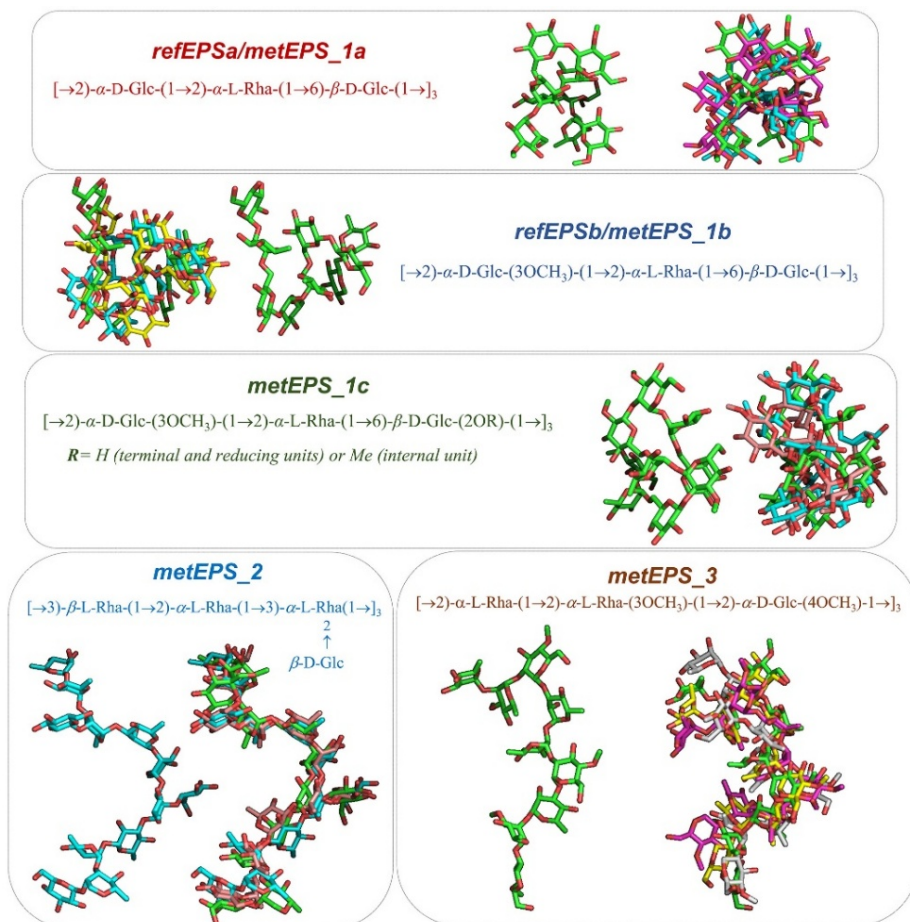


**Figure 7:** Representative conformers of *M. extorquens* PA1 EPSs (refEPS, metEPS\_1, metEPS\_2, metEPS\_3) derived from MD simulations, illustrating conformational diversity influenced by branching and methylation.

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Experimental validation by PFG-NMR showed that for refEPS, the self-diffusion coefficients ( $D_{\text{refEPS}}$ ) decreased with increasing methanol concentration, primarily due to increased solvent viscosity rather than polymer coil expansion. Hydrodynamic radii ( $R_h$ ) calculated via the Stokes–Einstein equation, remained nearly constant across all methanol concentrations tested, indicating that methanol behaves as a good solvent for refEPS, comparable to water. Methanol self-diffusion coefficients ( $D_{\text{met}}$ ) measured in the presence of refEPS were slightly higher than those in its absence ( $D_{\text{met}}^{\circ}$ ), ruling out significant incorporation of methanol into the EPS solvation shell<sup>23,24</sup>(Table 5).

**Table 5:** Self-diffusion coefficients, viscosity, hydrodynamic radius and solvation data for heavy water–methanol–refEPS systems at 25 °C.  $D_{\text{refEPS}}$ : self-diffusion coefficient of *refEPS*;  $D_{\text{met}}$  and  $D_{\text{met}}^{\circ}$ : self-diffusion coefficient of methanol in the presence and in the absence of *refEPS*;  $R_h$ : hydrodynamic radius;  $\eta$ : solvent viscosity.

| Methanol<br>vol% | $D_{\text{refEPS}} \times 10^{11} / \text{m}^2 \text{ s}^{-1\text{a}}$ | $D_{\text{met}} \times 10^{10} / \text{m}^2 \text{ s}^{-1\text{b}}$ | $D_{\text{met}}^{\circ} \times 10^{10} / \text{m}^2 \text{ s}^{-1\text{b}}$ | $\eta \times 10^4 / \text{Pa s}$ | $R_h \times 10^9 / \text{m}^{\text{c}}$ |
|------------------|------------------------------------------------------------------------|---------------------------------------------------------------------|-----------------------------------------------------------------------------|----------------------------------|-----------------------------------------|
| 0                | 3.98                                                                   | -                                                                   | -                                                                           | 9.3                              | 5.8                                     |
| 1                | 3.61                                                                   | 13.8                                                                | 13.9                                                                        | 9.6                              | 6.3                                     |
| 2                | 3.51                                                                   | 15.2                                                                | 13.8                                                                        | 9.8                              | 6.3                                     |
| 3                | 3.41                                                                   | 14.6                                                                | 13.7                                                                        | 10.0                             | 6.4                                     |
| 5                | 3.35                                                                   | 15.4                                                                | 13.7                                                                        | 10.5                             | 6.2                                     |
| 8                | 3.11                                                                   | 15.0                                                                | 13.2                                                                        | 11.1                             | 6.3                                     |

a Experimental uncertainty ~1%.

b Experimental uncertainty <0.5%.

c Uncertainty ~2%.

For metEPS, PFG-NMR revealed a more complex diffusion behavior characterized by multi-exponential decays, consistent with coexistence of distinct diffusing species, likely due to different polysaccharides and/or aggregation states (Table 6). Despite increased uncertainty, reliable diffusion coefficients and hydrodynamic radii were obtained for isolated anomeric signals (Table 7). Notably, metEPS\_2 and metEPS\_3 exhibited slower diffusion and larger hydrodynamic radius compared to metEPS\_1 and refEPS, in line with MD predictions.

**Table 6:** Self-diffusion coefficients and hydrodynamic radii of *metEPS* in heavy water and in heavy water-methanol (8 vol%) at 25 °C.  $D_{\text{metEPS}}$ : self-diffusion coefficient of *metEPS*;  $R_h$ : hydrodynamic radius.

| EPS | $D_{\text{metEPS}} \times 10^{11} / \text{m}^2 \text{ s}^{-1\text{a}}$ | $R_h \times 10^9 / \text{m}^{\text{c}}$ | $D_{\text{metEPS}} \times 10^{11} / \text{m}^2 \text{ s}^{-1\text{a}}$ | $R_h \times 10^9 / \text{m}^{\text{c}}$ | $VDW_{\text{volume}} \text{ \AA}^3$ |
|-----|------------------------------------------------------------------------|-----------------------------------------|------------------------------------------------------------------------|-----------------------------------------|-------------------------------------|
|     | heavy water                                                            |                                         | heavy water -methanol                                                  |                                         |                                     |

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|                                              |     |    |     |    |     |
|----------------------------------------------|-----|----|-----|----|-----|
| <i>metEPS</i> <sub>1</sub><br>(demethylated) | 2.8 | 8  | 2.6 | 7  | 488 |
| <i>metEPS</i> <sub>1</sub>                   | 3.2 | 7  | 3.0 | 6  | 506 |
| <i>metEPS</i> <sub>2</sub>                   | 1.9 | 12 | 1.9 | 10 | 623 |
| <i>metEPS</i> <sub>3</sub>                   | 1.8 | 13 | 1.8 | 11 | 524 |

a Experimental uncertainty ~1%.

b Experimental uncertainty <0.5%.

c Uncertainty ~2%.

**Table 7:** Diffusion coefficients and hydrodynamic radii of *refEPS* and *metEPS* in water and water-methanol (10%) mixtures at 25 °C. “Single chain” indicates individual EPS molecules; “Cluster” refers to larger assemblies detected via DLS.

| Methanol<br>vol% |              | $D_{refEPS} \times 10^{11} / \text{m}^2 \text{ s}^{-1}$ <sup>a</sup> | $D_{metEPS} \times 10^{11} / \text{m}^2 \text{ s}^{-1}$ <sup>a</sup> | $R_h(refEPS) \times 10^9 / \text{m}^b$ | $R_h(metEPS) \times 10^9 / \text{m}^b$ |
|------------------|--------------|----------------------------------------------------------------------|----------------------------------------------------------------------|----------------------------------------|----------------------------------------|
| <b>0</b>         | Single chain | 4.6                                                                  | 4.5                                                                  | 5.8                                    | 5.4                                    |
|                  | Cluster      | 0.2                                                                  | 0.3                                                                  | 160                                    | 87                                     |
| <b>10</b>        | Single chain | 4.1                                                                  | 4.2                                                                  | 5.2                                    | 4.9                                    |
|                  | Cluster      | 0.2                                                                  | 0.2                                                                  | 152                                    | 93                                     |

a Experimental uncertainty ~3%.

b Experimental uncertainty ~5%.

Complementary DLS analyses revealed a bimodal size distribution in both *refEPS* and *metEPS* samples, in water and water/methanol mixtures (Table 7, Figure 8). Two populations were identified: individual EPS chains (~5 nm) and larger polysaccharide clusters (~100 nm or more). Individual chains predominated under all conditions, and methanol addition did not significantly alter diffusion profiles or promote aggregation, further supporting that methanol does not disrupt EPS conformational or supramolecular integrity.

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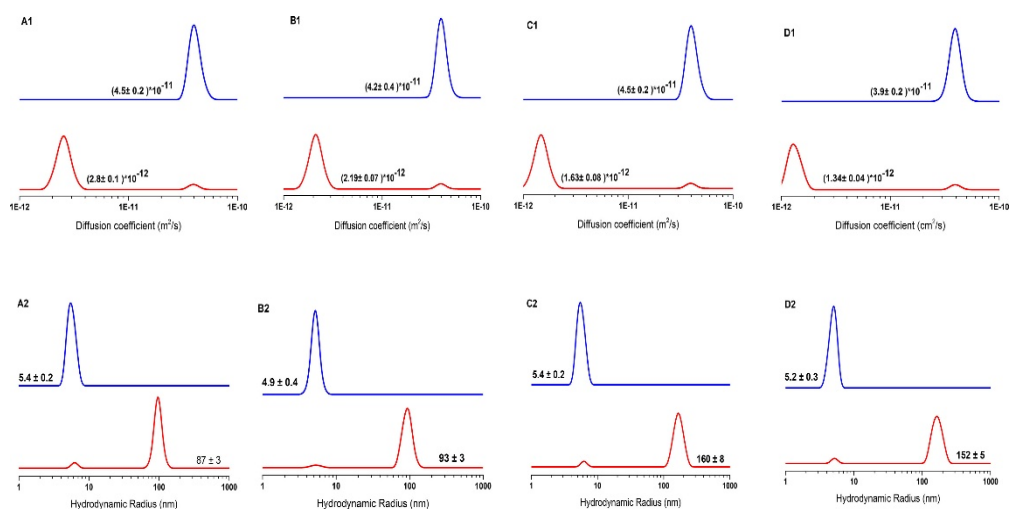


**Figure 8:** Dynamic Light Scattering (DLS) profiles of refEPS and metEPS in water (Panels A and C) and water-methanol (Panels B and D), illustrating bimodal distributions with dominant ~5 nm species and minor large clusters.

Using a self-avoiding random coil model, molecular weights were estimated from the hydrodynamic radius and repeating unit volumes<sup>25</sup>. For refEPS, it was estimated at ~65 kDa (~120 repeating units), while metEPS averaged ~70 kDa (~130 repeating units).

In summary, our combined computational and experimental study demonstrates that methanol-rich environments prompt *M. extorquens* PA1 to produce structurally diverse EPSs with distinct conformational and supramolecular behaviors. These adaptations are likely to contribute to enhanced methanol tolerance, increased cellular stability, and ecological competitiveness.

### 4.3 Structural Characterization of *Methylobacterium extorquens* PA1 Lipopolysaccharide (LPS/LOS)

The outer membrane of *Methylobacterium extorquens* PA1, a prominent colonizer of the *Arabidopsis thaliana* phyllosphere, features a rough-type lipooligosaccharide (LOS), which lacks the extended O-antigen chain found in smooth lipopolysaccharides (LPS)<sup>26,1</sup>. This LOS is distinguished by a non-phosphorylated, extensively O-methylated core oligosaccharide (OS), rich in negatively charged residues such as 3-deoxy-D-manno-octulosonic acid (Kdo) and its rare 5-O-methylated derivative Ko. The lipid A moiety contains a non-phosphorylated trisaccharide backbone and low acylation. It includes three primary fatty acids, one secondary very long-chain fatty acid (VLCFA), and an uncommon 3-O-acetyl-butyrates group<sup>27,28,29</sup>.

These structural features influence OM organization and stability, enhancing resistance to methanol, a central metabolite and stressor for methylotrophic bacteria. Biophysical studies (NMR, ESI-FT-ICR MS, EPR) show that *M. extorquens* LOS promotes increased membrane

ordering under methanol stress, underscoring its role in environmental adaptation and persistence on plant surfaces<sup>3,30</sup>.

### 4.3.1 Isolation and Chemical Analyses of LOS

The LOS from *Methylobacterium extorquens* PA1 was isolated from lyophilized bacterial cells using a modified petroleum ether/chloroform/phenol extraction protocol<sup>16</sup>. To ensure high purity, the crude extract was subjected to enzymatic digestion with DNase, RNase, and proteases, followed by dialysis, ultracentrifugation, and gel-filtration chromatography. The rough nature of the isolated LOS, lacking the O-antigenic polysaccharide chain, was confirmed by SDS-PAGE analysis and silver nitrate staining, yielding a profile consistent with rough-type LPS<sup>31</sup>.

Compositional analysis revealed a complex saccharide profile, including D-glucosamine (GlcN), 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo), D-galacturonic acid (GalA), D-quinovosamine (QuiN), rhamnose (Rha), and D-galactose (Gal). In addition to these common monosaccharides, the LOS also contained rare O-methylated ulosonic acid derivatives such as 5-O-methyl-Kdo (Kdo5OMe) and 5-O-methyl-Ko (Ko5OMe), underscoring a high degree of structural specificity.

Methylation linkage analysis further elucidated the branching and substitution patterns of the core oligosaccharide, identifying residues such as 6- and 3-substituted GlcN, 2-substituted and terminal GalA, and multiple Rha residues with distinct substitution patterns (e.g., 2,4-Rha, 2,3-Rha, 3-Rha, 2-Rha, and terminal Rha) (Table 8). Additional substitutions included 4-substituted Gal and 3-substituted QuiN. Notably, a Ko5OMe-(2→4)-Kdo disaccharide was identified (Figure 9), providing further evidence of unique structural motifs within the LOS core region. This motif may contribute to increased membrane rigidity or resistance to oxidative damage, although further functional studies are warranted.

**Table 8:** Monosaccharide composition and glycosidic linkage analysis of the lipoolysaccharide (LOS) produced by *Methylobacterium extorquens* PA1.

| Monosaccharide                            | Partially Methylated Derivative                                                                  |
|-------------------------------------------|--------------------------------------------------------------------------------------------------|
| D-rhamnose (Rha)                          | 2-substituted Rha, 2,4-disubstituted Rha, 2,3-disubstituted Rha, 3-substituted Rha, terminal Rha |
| D-quinovosamine (QuiN)                    | 3-substituted QuiN                                                                               |
| D-galacturonic acid (GalA)                | 2-substituted GalA, terminal GalA                                                                |
| D-galactose (Gal)                         | 4-substituted Gal                                                                                |
| D-glucosamine (GlcN)                      | 6-substituted GlcN, 3-substituted GlcN                                                           |
| 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) | 4,5-disubstituted Kdo <sub>p</sub>                                                               |
| 5-O-methyl-Kdo (Kdo5OMe)                  | terminal 5-O-methyl-Kdo <sub>p</sub>                                                             |
| 5-O-methyl-Ko (Ko5OMe)                    | terminal 5-O-methyl-Ko <sub>p</sub>                                                              |

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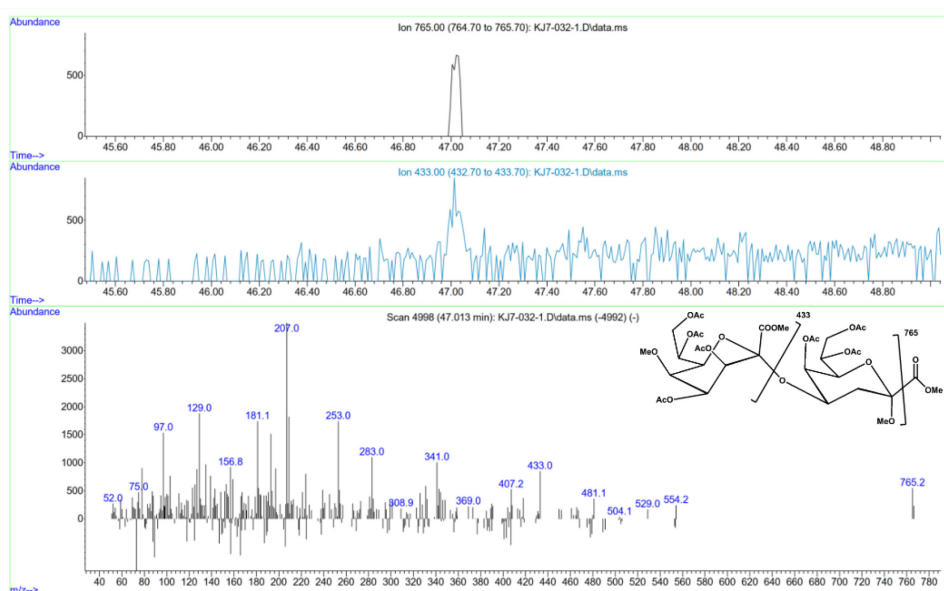


**Figure 9:** GC-MS analysis confirming the structure of the unique Kdo-Ko disaccharide in *Methylobacterium extorquens* PA1 LOS.

Fatty acid analysis revealed the presence of hydroxylated fatty acids such as 14:0(3-OH) and 18:0(3-OH), commonly associated with lipid A architecture. Moreover, very long-chain fatty acids (VLCFAs), including 26:0(25-OH) and 28:0(27-OH), were detected (Table 9). These components are known to influence membrane dynamics, contributing to outer membrane integrity, fluidity, and curvature. Their specific occurrence in *M. extorquens* LOS likely plays a key role in the organism's adaptation to methanol-rich environments.

**Table 9:** Fatty acid composition of *Methylobacterium extorquens* PA1 LOS, showing hydroxylated fatty acids including very long-chain species that influence membrane properties.

| Fatty Acid   | Description  |
|--------------|--------------|
| 14:0 (3-OH)  | Hydroxylated |
| 18:0 (3-OH)  | Hydroxylated |
| 26:0 (25-OH) | Hydroxylated |
| 28:0 (27-OH) | Hydroxylated |

### 4.3.2 Core Oligosaccharide (OS) Structure Determination by NMR and Mass Spectrometry

To elucidate the structure of the core oligosaccharide (OS) from *Methylobacterium extorquens* PA1, the lipooligosaccharide (LOS) was chemically deacylated to obtain the OSdeAc, purified by gel-filtration chromatography. High-resolution NMR spectroscopy enabled full assignment of spin systems and inter-residue connectivity. The OS backbone consists of 14 pyranose units with defined anomeric configurations determined by  $^3J_{\text{H,H}}$  coupling constants and intra-residual NOE contacts<sup>12</sup>. The  $^1\text{H}$  NMR spectrum of OSdeAc (Figure 10) shows the anomeric proton signals labeled according to the assignments in Table 10.

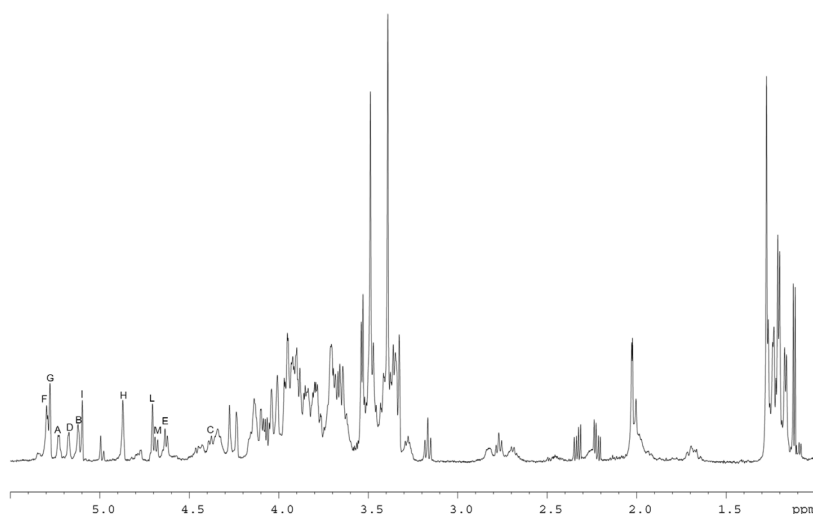


**Figure 10:**  $^1\text{H}$  NMR spectrum of OSdeAc from *M. extorquens* PA1. Anomeric proton signals are labeled as in Table 10.

**Table 10:** Proton ( $^1\text{H}$ ) and carbon ( $^{13}\text{C}$ ) chemical shifts of OS<sub>deAc</sub> saccharide residues from *M. extorquens* PA1.

|                   | 1                         | 2    | 3    | 4    | 5    | 6             | 7 | 8 |
|-------------------|---------------------------|------|------|------|------|---------------|---|---|
| A                 | 5.22<br>$^3J_{12}=3.7$ Hz | 3.89 | 3.94 | 4.23 | 4.27 | ----          |   |   |
| t- $\alpha$ -GalA | 93.8                      | 67.2 | 69.0 | 70.7 | 72.5 | 175.4         |   |   |
| B                 | 5.12                      | 2.84 | 3.64 | 3.51 | 3.94 | 4.14/3.7<br>2 |   |   |
| t- $\alpha$ -GalA | 93.8                      | 54.6 | 73.1 | 69.4 | 71.3 | 68.5          |   |   |
| C                 | 4.35                      | 2.70 | 3.28 | 3.39 | 3.48 | 3.39/3.8<br>3 |   |   |
| $\beta$ -GlcN     | 103.1                     | 56.3 | 75.4 | 70.2 | 74.4 | 63.8          |   |   |

## CHAPTER IV

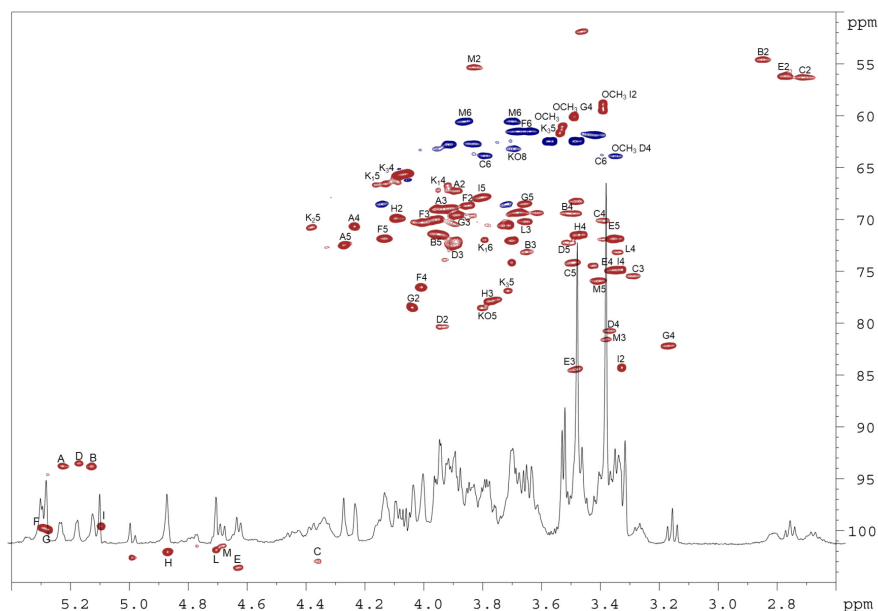
Surface glycoconjugates of *Methylobacterium extorquens* PA1: structural adaptations of EPS and LPS for methanol tolerance

|                   |                                               |                                    |           |                                |                                    |               |      |                            |
|-------------------|-----------------------------------------------|------------------------------------|-----------|--------------------------------|------------------------------------|---------------|------|----------------------------|
| <b>D</b>          | 5.17                                          | 3.94                               | 3.90      | 3.67                           | 3.49                               | ----          |      |                            |
| <b>α-GlcA</b>     | 93.6                                          | 80.4                               | 72.0      | 80.7                           | 72.2                               | 178.1         |      |                            |
|                   |                                               |                                    |           | O-CH <sub>3</sub><br>3.35/63.9 |                                    |               |      |                            |
| <b>E</b>          | 4.62                                          | 2.76                               | 3.48      | 3.35                           | 3.35                               | 1.23          |      |                            |
| <b>β-QuiN</b>     | 103.6                                         | 56.1                               | 84.5      | 74.8/73.2                      | 72.0                               |               |      |                            |
| <b>F</b>          | 5.293<br><sup>3</sup> J <sub>12</sub> =7.6 Hz | 3.84                               | 3.96      | 4.00                           | 4.13                               | 3.68/3.6<br>3 |      |                            |
| <b>α-Gal</b>      | 99.7                                          | 68.7                               | 70.0      | 76.5                           | 71.9                               | 61.6          |      |                            |
| <b>G</b>          | 5.28                                          | 4.03                               | 3.89      | 3.17                           | 3.65                               | 1.27          |      |                            |
| <b>α-Rha</b>      | 99.8                                          | 78.6                               | 69.6      | 82.2                           | 68.6                               | 16.7          |      |                            |
|                   |                                               |                                    |           | O-CH <sub>3</sub><br>3.49/60.3 |                                    |               |      |                            |
| <b>H</b>          | 4.87                                          | 4.09                               | 3.77      | 3.47                           | 3.68                               | 1.20          |      |                            |
| <b>α-Rha</b>      | 102.0                                         | 69.9                               | 77.8      | 71.4                           | 69.3                               |               |      |                            |
| <b>I</b>          | 5.09                                          | 3.33                               | 3.8       | 3.33                           | 3.80                               | 1.205         |      |                            |
| <b>α-Rha</b>      | 99.6                                          | 84.3                               | 78.5      | 74.2                           | 67.98                              |               |      |                            |
|                   |                                               | O-CH <sub>3</sub><br>3.53/6<br>1.1 |           |                                | O-CH <sub>3</sub><br>3.48/60.<br>1 |               |      |                            |
| <b>L</b>          | 4.71                                          | 3.71                               | 3.65      | 3.35                           | 3.91                               | 1.66          |      |                            |
| <b>α-Rha</b>      | 101.9                                         | 70.6                               | 70.3      | 71.8                           | 69.0                               |               |      |                            |
| <b>M</b>          | 4.68                                          | 3.83                               | 3.38      | 3.40                           | 3.49                               | 3.70/3.8<br>6 |      |                            |
| <b>β-GlcN</b>     | 101.5                                         | 52.6                               | 71.6      | 70.1                           | 74.1                               | 60.5          |      |                            |
| <b>K1</b>         | -----                                         | -----                              | 2.00/1.97 | 3.92/3.93                      | 4.13/4.1<br>6                      | 3.77          | 3.84 | 3.69/<br>4.02<br>-<br>3.94 |
| <b>4,5-α-Kdop</b> | 175.0                                         |                                    | 34.2      | 67.0                           | 66.58                              | 72.1          | 70.6 | 63.2                       |
| <b>K2</b>         | -----                                         | -----                              | 1.69/2.24 | 4.33                           | 4.38                               |               |      |                            |
| <b>4,5-α-Kdop</b> | 175.0                                         | 91.2                               | 34.3      | 67.8                           | 71.1                               |               |      |                            |
| <b>K2</b>         | -----                                         | -----                              | 1.69/2.26 | 4.44                           | 4.37                               |               |      |                            |
| <b>4,5-α-Kdop</b> | 175.0                                         | 91.2                               | 34.3      | 67.8                           | 71.8                               |               |      |                            |
| <b>K3</b>         | -----                                         | -----                              | 1.66/1.93 | 4.09                           | 3.71                               |               |      |                            |
| <b>4-α-Kdop</b>   | 175.0                                         | 94.0                               | 35.07     | 65.9                           | 76.8                               |               |      |                            |
|                   |                                               |                                    |           |                                | O-CH <sub>3</sub><br>3.48/60.<br>1 |               |      |                            |
| <b>Ko</b>         | -----                                         | -----                              | 3.68      | 3.89                           | 3.80                               | 3.50          |      | 3.67                       |
| <b>α-Kop</b>      | 175.0                                         | -----                              | 69.3      | 69.1                           | 78.6                               | 72.2          |      | 63.0                       |
|                   |                                               |                                    |           |                                | O-CH <sub>3</sub><br>3.53/61.<br>7 |               |      |                            |

Three galacto-configured residues (spin systems A, B, and F) were identified, with A and D assigned to galacturonic acid (GalA) based on their correlations to carboxyl carbons. Residue E was characterized as β-D-quinovosamine (QuiN), confirmed by its correlation to a

nitrogen-bearing carbon and its methyl substitution at C-6. Glucosamine residues (C and M) were identified through typical  $^{13}\text{C}$  shifts of nitrogen-substituted C-2 atoms, while four  $\alpha$ -rhamnose units (G, H, I, and L) displayed manno-type ring conformations and  $\alpha$ -anomeric configurations.

Residue K was attributed to Kdo based on its distinctive diastereotopic methylene protons in the high-field region of the  $^1\text{H}$  NMR spectrum. Two additional ulosonic acid residues, Kdo and Ko, including 5-O-methyl substituted forms, were also identified. Methoxy groups were confirmed by downfield proton and carbon shifts, supported by diagnostic HMBC and NOE cross-peaks. Particularly notable substitutions occurred at positions D4, G4, I2, I3, K<sub>3</sub>5, and Ko5, highlighting high degree structural variation within the core region. The multiplicity-edited HSQC spectrum (Figure 11) highlights the correlation peaks for sugar residues, with red peaks corresponding to  $-\text{CH}$  and  $-\text{CH}_3$  groups, and blue peaks to  $-\text{CH}_2$  groups, assisting in residue identification.



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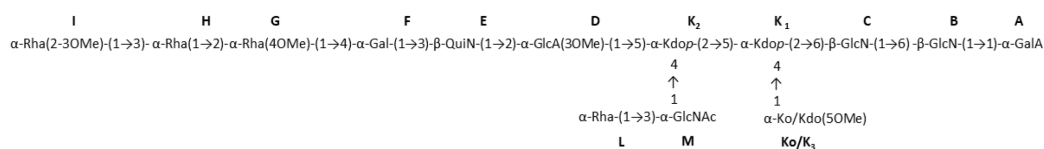


**Figure 12:** Schematic O<sub>SdeAc</sub> structure.

Figure 13 shows the TROESY spectrum, providing further confirmation of spatial proximities and residue connectivity within the OS.

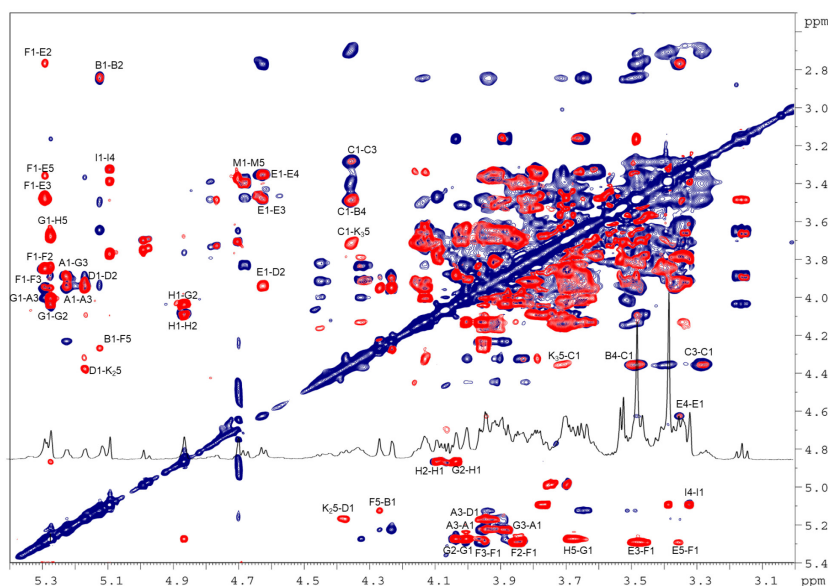


**Figure 13:** TROESY spectrum of OS<sub>dcAc</sub> (400 ms mixing time) showing inter-residue correlations.

Further structural validation was provided by ultrahigh-resolution ESI-FT-ICR mass spectrometry. Analysis in both positive and negative ion modes revealed two predominant OS species (OS<sub>1deAc</sub> and OS<sub>2deAc</sub>), differing by approximately 16 Da, corresponding to the substitution of a methylated Kdo (C<sub>9</sub>H<sub>14</sub>O<sub>7</sub>) with a methylated Ko (C<sub>9</sub>H<sub>14</sub>O<sub>8</sub>) residue (Figure 14). CID spectra of intact and fragmented ions confirmed the presence of an internal trisaccharide composed of Kdo and Ko residues, branching from the non-reducing GlcN, forming the core initiation point<sup>32</sup>.

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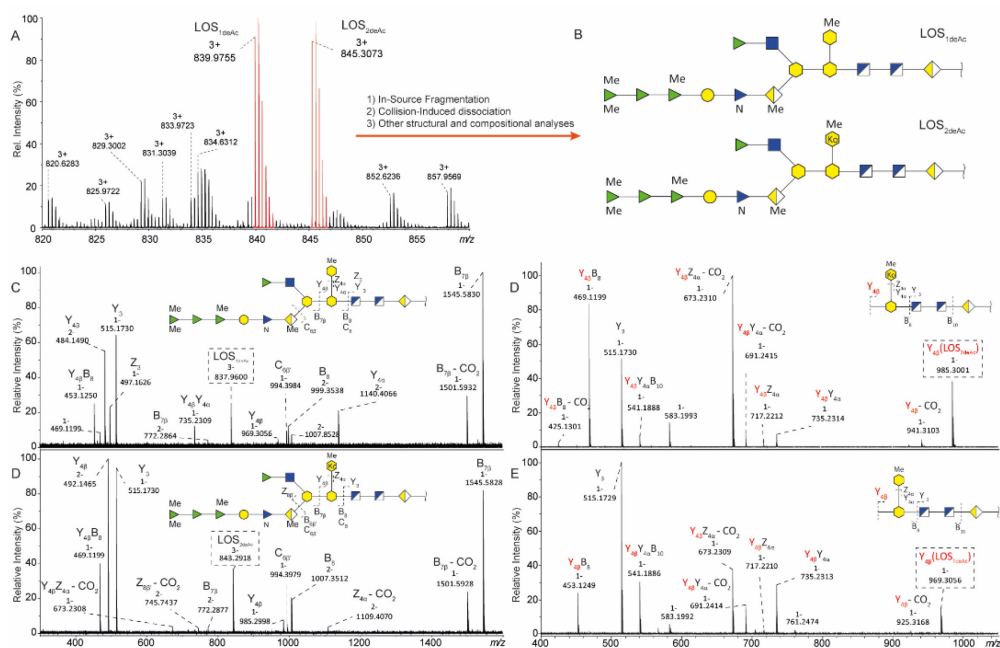


**Figure 14:** (A) Enlarged positive-mode ESI FT-ICR mass spectrum of OSdeAc. (B) Schematic structures of the two main LOS species OS1deAc and OS2deAc. Symbols: green triangle = rhamnose; blue triangle with “N” = quinovosamine; yellow/white diamond = galacturonic acid; blue square = N-acetyl-glucosamine; blue/white square = glucosamine; yellow hexagon = Kdo; yellow hexagon with “Ko” = Ko; Me = methylation. (C-F) Negative mode ESI-CID FT-ICR spectra of OS species and fragments confirming the core structure.

Additionally, mild acid hydrolysis experiments confirmed structural heterogeneity, showing non-stoichiometric 5-O-methylation and sporadic O-acetylation, explaining the microheterogeneous LOS glycoforms.

### 4.3.3 Lipid A Structural Characterization

The lipid A moiety of *Methylobacterium extorquens* PA1 LOS was structurally characterized through a combination of mild acid hydrolysis, chromatographic purification, nuclear magnetic resonance (NMR) spectroscopy, and high-resolution mass spectrometry (MS), including MALDI-TOF and ESI-FT-ICR techniques. NMR analysis (Table 11) revealed a non-phosphorylated trisaccharide backbone: GlcN- $\beta$ -(1 $\rightarrow$ 6)-GlcN- $\alpha$ -(1 $\rightarrow$ 1)-GalA. The acylation pattern was asymmetrical, featuring four fatty acid chains in a 3+1 distribution, located at position 2 of the proximal GlcN and at positions 2 and 3 of the distal GlcN. The GalA unit was  $\alpha$ -linked to the anomeric position of the proximal GlcN.

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**Table 11:** Proton ( $^1\text{H}$ ) and carbon ( $^{13}\text{C}$ ) chemical shifts of the lipid A fraction of LOS from *M. extorquens* PA1 and AM1 (600 MHz, 298 K) obtained after mild acid hydrolysis of the LOS.

|                                                             | 1                   | 2          | 3        | 4                 | 5             | 6         |
|-------------------------------------------------------------|---------------------|------------|----------|-------------------|---------------|-----------|
| <b>A</b>                                                    | 5.06                | 4.01       | 3.74     | 3.36              | 3.93          | 4.11/3.67 |
| $\alpha$ -GlcN                                              | 92.6                | 53.4       | 70.9     | 70.9              | 67.7          | 68.9      |
| <b>B</b>                                                    | 4.56                | 3.87       | 5.02     | 3.58              | 3.39          | 3.75/3.90 |
| $\beta$ -GlcN                                               | 101.2               | 53.7       | 75.6     | 68.4              | 76.1          | 61.2      |
| <b>C</b>                                                    | 5.12                | 3.97       | 3.94     | 4.25              | 4.53          | ----      |
| $\alpha$ -GalA                                              | 93.8                | 71.2       | 71.4     | 70.6              | 71.1          | 170.2     |
|                                                             |                     |            |          |                   |               |           |
| <b>Fatty acid</b>                                           | $\alpha_1/\alpha_2$ | $\beta$    | $\gamma$ | $(\text{CH}_2)_n$ | $\text{CH}_3$ |           |
| (3-OR) fatty acid                                           | 2.38/2.53           | 5.12       | 1.56     | 1.28              | 0.89          |           |
|                                                             | 40.57               | 71.3       | 30.0     | 29.3              | 13.8          |           |
|                                                             |                     |            |          |                   |               |           |
|                                                             | $\alpha_1/\alpha_2$ | $\beta$    | $\gamma$ | $(\text{CH}_2)_n$ | $\text{CH}_3$ |           |
| 3-OH fatty acid                                             | 2.37/2.27           | 3.90       | 1.50     | 1.28              | 0.89          |           |
|                                                             | 43.2                | 68.4       | 33.1     | 29.3              | 13.8          |           |
|                                                             |                     |            |          |                   |               |           |
| 3-OH fatty acid                                             | 2.50/2.44           | 4.02       | 1.48     | 1.28              | 0.89          |           |
|                                                             | 42.0                | 68.2       | 33.0     | 29.3              | 13.8          |           |
|                                                             |                     |            |          |                   |               |           |
| <b>VLCFA</b>                                                | $\omega-2$          | $\omega-1$ | $\omega$ | $(\text{CH}_2)_n$ |               |           |
| <b>1<sup>st</sup> long chain (<math>\omega-1</math>)-OR</b> | 1.50/1.60           | 4.89       | 1.21     | 1.29              |               |           |
|                                                             | 35.6                | 71.4       | 19.5     | 29.3              |               |           |
|                                                             |                     |            |          |                   |               |           |
|                                                             |                     | 3.29       | 3.27     | 1.66              |               |           |
|                                                             |                     | 84.0       | 63.5     | 33                |               |           |

A distinguishing feature of this lipid A structure is the presence of a very long-chain fatty acid (VLCFA), specifically 26:0, esterified at the  $\omega-1$  position by a 3-O-acetyl-butyrate (OAcBu) group. This modification is unprecedented in bacterial lipid A. MALDI-TOF MS in negative-ion mode revealed a heterogeneous set of tetra-acylated lipid A species. The dominant signal at  $m/z$  1829.5 corresponded to a structure composed of two 18:0(3-OH), one 14:0(3-OH), and one 26:0(25-OAcBu) VLCFA, in addition to a GalA substitution. This is illustrated in Figure 15A (Reflectron MALDI-TOF mass spectrum).

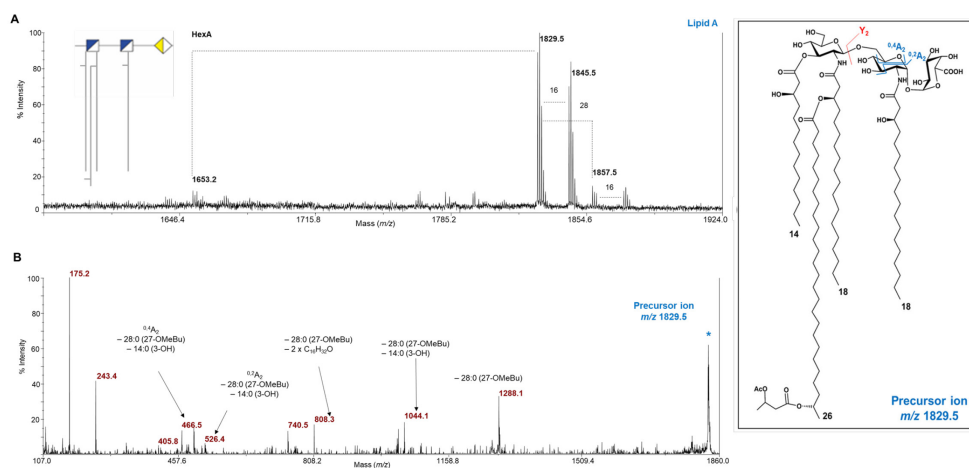
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**Figure 15:** (A) Reflectron MALDI-TOF mass spectrum of lipid A isolated from *M. extorquens*. Differences of 28 amu (a  $-\text{CH}_2\text{CH}_2-$  unit) and 16 amu are also reported in the spectrum. (B) Negative-ion MALDI MS/MS spectrum of a precursor ion at  $m/z$  1829.5 ascribed to a tetra-acylated lipid A species of LOS from *M. extorquens*. Key fragments are assigned, and the proposed structure for the lipid A species is sketched in the inset. Note the loss of  $\text{C}_{16}\text{H}_{32}\text{O}$  (240 mass units), due to a rearrangement on amide-linked 3-OH acyl chains. The asterisk indicates the precursor ion.

MS/MS fragmentation (Figures 15B) provided unambiguous assignment of acyl chain positions. Characteristic fragment ions confirmed the presence of N-linked 18:0(3-OH) and O-linked 14:0(3-OH) on the distal GlcN. The VLCFA (26:0-OAcBu) acts as a secondary acyl chain attached to a primary 18:0(3-OH). The proximal GlcN carried a single N-linked 18:0(3-OH) and was glycosylated at its anomeric carbon by GalA. A key fragment at  $m/z$  466.5, resulting from sugar ring cleavage and loss of VLCFA and 14:0(3-OH), was instrumental in confirming this substitution pattern.

The absence of phosphate groups, along with the unique OAcBu-substituted VLCFA, suggests a specialized lipid A structure with potentially reduced immunostimulatory activity and enhanced membrane-stabilizing capacity. Additional heterogeneity in VLCFA hydroxylation contributes to the overall structural diversity. These features likely reflect an adaptation to methanol-rich environments and interaction with plant hosts in the phyllosphere<sup>27</sup>.

#### 4.3.4 LOS Composition and Core Region Characterization

Negative-ion MALDI-TOF MS analysis of intact LOS from *Methylobacterium extorquens* PA1 and AM1 revealed a highly heterogeneous mixture of molecular species (Figure 16A). The high-mass region ( $m/z$  3400–4300) displayed a series of  $[\text{M} - \text{H}]^-$  ions corresponding to native LOS variants, differing in both acyl chain composition and oligosaccharide structures. In the lower-mass range ( $m/z$  1800–2500), ion fragments derived from cleavage of the acid-labile glycosidic bond between Kdo and lipid A were also observed, providing critical structural information.

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In particular, the core oligosaccharide (core OS) region exhibited significant variability, with five principal core OS species (labeled OSA–OSE) identified in the  $m/z$  1991.7–2061.7 range. These differed primarily in their degree of O-methylation, as illustrated in the inset of Figure 16B. The complex pattern of ions observed reflected a broad structural diversity in both lipid A and core OS regions. The main LOS forms corresponded to combinations of variably acylated lipid A species and distinct core OS structures, underscoring the molecular heterogeneity of *M. extorquens* LOS.

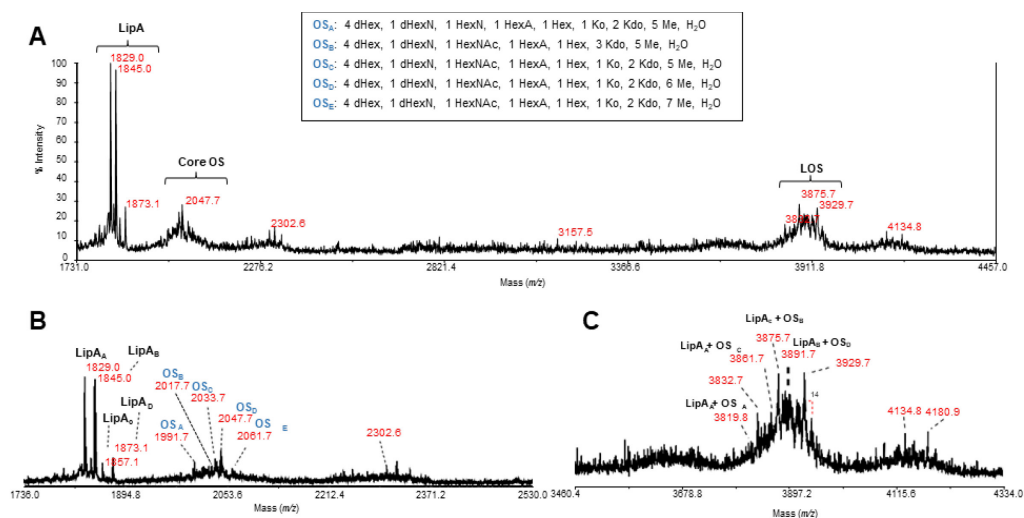


**Figure 16:** (A) Negative-ion MALDI-TOF MS of *M. extorquens* PA1 LOS; (B-C) Zoom of the Lipid A and core OS region. Proposed structural assessment of OS related ions (OSA–E) has been reported in the inset.

#### 4.4 Structural and Membrane Properties of LOS-Containing Lipid Bilayers

The unique chemical structure of *Methylobacterium extorquens* PA1 LOS, particularly the lipid A moiety and the core oligosaccharide (OS), strongly suggests a specialized role in modulating the biophysical properties of the outer membrane (OM) and enhancing bacterial adaptation to environmental pressures. Elucidating the three-dimensional architecture and conformational dynamics of the OS is key to understanding its functional implications.

##### 4.4.1 Conformational Analysis of the Core OS

To focus on the saccharide domain, mild acid hydrolysis was employed to cleave lipid A from the core OS. Negative-ion MALDI-TOF MS analysis revealed a highly heterogeneous OS population. This heterogeneity primarily stems from non-stoichiometric O-methylation of various sugar units and variable acetylation at a glucosamine (GlcN) residue. The major species observed at  $m/z$  2033.7  $[M - H]^-$ , are consistent with the OS2deAc structure (Figure 17). It comprises multiple deoxyhexoses (dHex), deoxyhexosamine (dHexN), N-

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acetylhexosamine (HexNAc), hexuronic acid (HexA), hexose (Hex), one Ko, two Kdo residues, and five methyl groups. Minor peaks at  $m/z$  1991.7, 1859.6, and 1817.6 indicated structural variants lacking acetylation or terminal bis-methylated sugars.

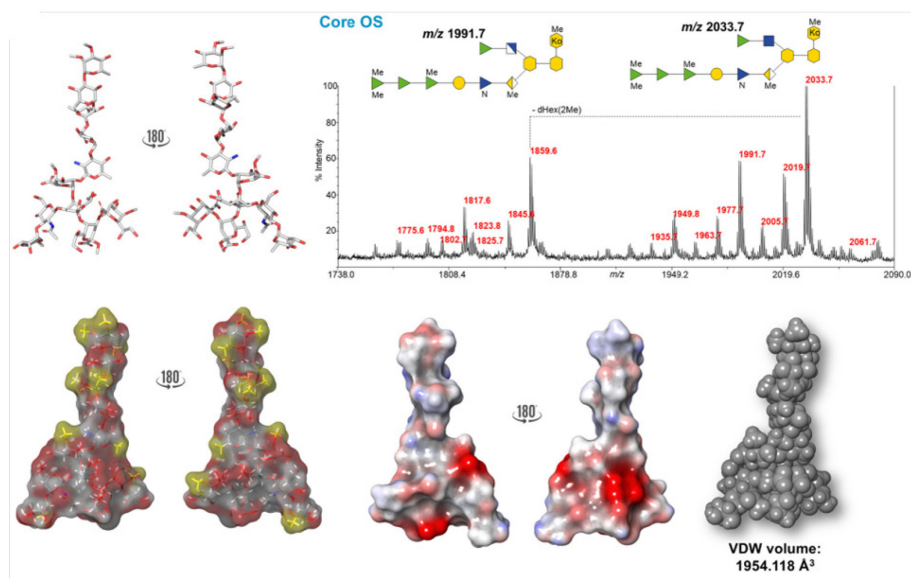


**Figure 17:** Top panel: Negative-ion reflectron MALDI-TOF mass spectrum revealing oligosaccharide species from mild acid hydrolysis of *M. extorquens* LOS. Proposed structures for ions at  $m/z$  1991.7 and  $m/z$  2033.7 are shown using Symbol Nomenclature for Glycans (SNFG). Symbols legend: green triangle: rhamnose; blue triangle with “N”: quinovosamine; yellow/white diamond: galacturonic acid; blue square: *N*-acetyl-glucosamine; blue/white square: glucosamine yellow hexagon: 3-deoxy-*D*-manno-octulosonic acid, Kdo; yellow hexagon with “Ko”: *D*-glycero-*D*-talo-2-octulosonic acid, Ko; Me: methylation. Bottom panel: Different views of the core OS 3D model. Either the Van der Waals (VDW), surface or the molecular surface, with methyl groups depicted in yellow.

#### 4.4.2 Computational Modeling and Functional Implications

To investigate the conformational landscape of the OS, computational methods were applied. Disaccharide fragments were used to generate adiabatic energy maps of the glycosidic torsions ( $\Phi$ ,  $\Psi$ ), and these were assembled into full OS models. Molecular dynamics (MD) simulations in explicit solvent, performed using a customized AMBER parameter set for oligosaccharides, allowed sampling of the accessible conformational space<sup>19</sup>.

Cluster analysis of MD trajectories revealed consistent structural organization: a compact, densely packed region at the reducing end, stabilized by an extensive network of hydrogen bonds and polar interactions, and a more extended terminal region, which likely protrudes into the extracellular space. The widespread O-methylation, combined with the presence of deoxy- and amino-sugars, reduced the hydrophilic character of the OS. Despite the absence of canonical phosphate substituents, the inner core retained a highly negative charge density due to the presence of Kdo, Ko, and galacturonic acid (Gala) residues.

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The final LOS model (Figure 18) integrates these structural elements, showcasing a lipid A core that diverges significantly from classical Gram-negative endotoxins and exemplifies functional adaptation in *M. extorquens*.

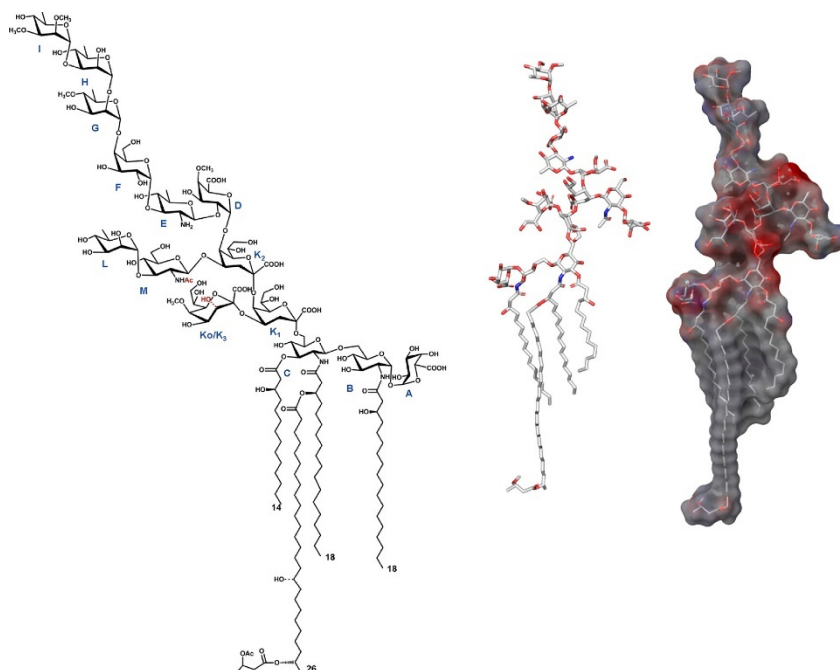


**Figure 18:** Full structure of *M. extorquens* PA1 and AM1 LOS. The 3D model highlights concentrated negative charges in the lipid A inner core and shows the oligosaccharide (OS) conformation with a bent, dense inner region and a more extended terminal end.

These conformational and electrostatic properties likely contribute to the membrane-stabilizing and protective roles of LOS in *M. extorquens*, modulating permeability and host interactions, and facilitating adaptation to methanol-rich or plant-associated environments<sup>33</sup>.

#### 4.4.3 Morphological and Structural Characterization of Lipid Bilayers

The bacterial outer membrane (OM) is composed of both lipooligosaccharide (LOS) and diacylphospholipids, primarily phosphoethanolamine (POPE) and phosphoglycerol (POPG). To understand how *Methylobacterium extorquens* LOS influences membrane architecture and dynamics, simplified lipid bilayers were constructed using POPE and POPG as a model system. This minimalist approach allowed for a precise analysis of LOS-driven effects, which would be difficult to isolate in the native, complex membrane environment. However, it's important to note that extrapolating these findings to physiological conditions requires caution, and further studies using more realistic OM models are necessary<sup>34</sup>.

Small-Angle Neutron Scattering (SANS) analysis showed that the inclusion of *M. extorquens* LOS did not alter the overall scattering profile of POPE/POPG vesicles; both systems exhibited a  $q^{-2}$  decay in intensity, consistent with unilamellar vesicle formation. However, quantitative modeling of the data (Figure 19A) revealed an increase in both vesicle diameter and bilayer thickness upon LOS incorporation (Table 12), indicating that LOS modulates the nanostructure of the membrane.

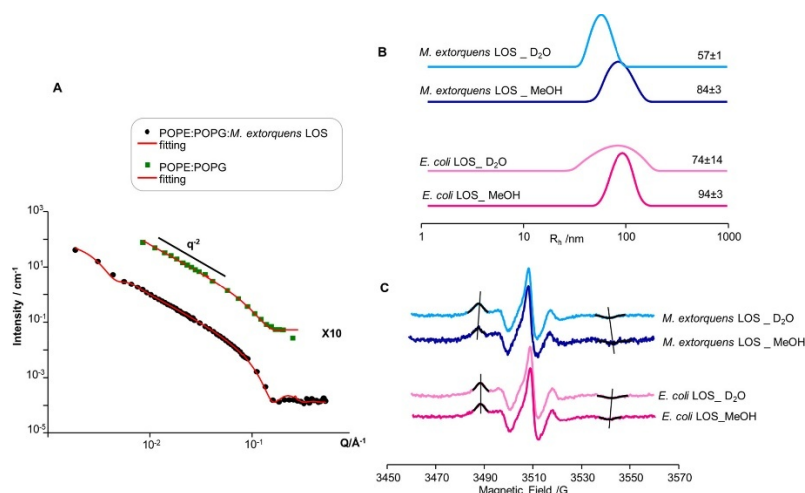


**Figure 19:** (A) SANS profiles and corresponding best-fitting curves of POPE/POPG (green squares) and POPE/POPG/*M. extorquens* LOS (black circles). (B) Hydrodynamic radius distribution and (C) EPR spectra of 5-PCSL in POPE/POPG/*M. extorquens* LOS (49/21/30 molar ratio) and POPE/POPG/*E. coli* LOS (49/21/30 molar ratio) vesicles in D<sub>2</sub>O (light blue and light pink lines, respectively) and in D<sub>2</sub>O/MeOH mixtures (9%vol methanol, blue and pink lines, respectively).

**Table 12:** Structural parameters estimated from the SANS profiles. The solvent was D<sub>2</sub>O in all samples.

| Sample                              | Bilayer composition (mole percent) | Radius /nm | Thickness /nm |
|-------------------------------------|------------------------------------|------------|---------------|
| POPE/POPG                           | 70/30                              | 14.9±0.2   | 3.4±0.1       |
| POPE/POPG/ <i>M. extorquens</i> LOS | 49/21/30                           | 53.3±0.2   | 3.8±0.1       |

To probe membrane responsiveness to solvent perturbation, Dynamic Light Scattering (DLS) and Electron Paramagnetic Resonance (EPR) experiments were conducted in D<sub>2</sub>O and a D<sub>2</sub>O/methanol (9% v/v) mixture (Figure 19B and C). As a control, vesicles containing *Escherichia coli* LOS were analyzed in parallel. DLS profiles indicated a monodisperse vesicle population across all samples, though vesicle size was consistently smaller for *M. extorquens* LOS-containing systems compared to their *E. coli* counterparts. Upon methanol exposure, all vesicles exhibited an increase in hydrodynamic radius ( $R_h$ ), with a more pronounced effect observed in *M. extorquens* LOS vesicles.

EPR spectroscopy using the 5-PCSL spin label provided additional insight into lipid acyl chain dynamics. The outer hyperfine splitting parameter ( $2A_{\max}$ ), a measure of lipid tail ordering, increased upon methanol exposure in *M. extorquens* LOS-containing bilayers (Figure 19C), suggesting enhanced acyl chain packing (Table 13). Conversely, bilayers containing *E. coli* LOS exhibited a decrease in  $2A_{\max}$ , indicating reduced lipid ordering under the same conditions.

**Table 13:** Parameters estimated from the EPR spectra. The solvent was D2O containing the methanol volume percent indicated in the table

| Sample                              | Bilayer composition (mole percent) | Methanol vol. percent | $^2A_{\max}/G$ |
|-------------------------------------|------------------------------------|-----------------------|----------------|
| POPE/POPG/ <i>E. coli</i> LOS       | 49/21/30                           | 9                     | 52.8           |
| POPE/POPG/ <i>E. coli</i> LOS       | 49/21/30                           | -                     | 53.7           |
| POPE/POPG/ <i>M. extorquens</i> LOS | 49/21/30                           | 9                     | 55.1           |
| POPE/POPG/ <i>M. extorquens</i> LOS | 49/21/30                           | -                     | 53.8           |

This differential response highlights the unique ability of *M. extorquens* LOS to promote membrane ordering and structural stabilization in the presence of environmental stressors like methanol<sup>35,36,37,38</sup>.

## 4.5 Discussion and Conclusions

The findings presented in this chapter elucidate the sophisticated strategies employed by *Methylobacterium extorquens* PA1 to adapt and thrive in methanol-rich environments, particularly through the structural modulation of its surface glycoconjugates: exopolysaccharide (EPS) and lipooligosaccharide (LOS). These molecular adaptations are crucial for the bacterium's ecological fitness and its beneficial interactions with plants hosts, especially under conditions of abiotic stress<sup>5,6</sup>.

Our comprehensive structural characterization of EPS revealed that *M. extorquens* PA1 dynamically alters the composition and branching of its EPS in response to methanol exposure. Under standard conditions, the bacterium produces a rhamnose- and O-methyl-glucose-rich polymer (refEPS), while methanol presence induces the secretion of a heterogeneous mixture (metEPS) comprising at least three distinct polysaccharide chains (metEPS<sub>1</sub>, metEPS<sub>2</sub>, metEPS<sub>3</sub>) with increased structural complexity and degrees of O-methylation. This compositional diversification reflects a flexible metabolic response and suggests a functional role in methanol tolerance<sup>10</sup>. Notably, while metEPS<sub>1</sub> shares structural similarity with refEPS, metEPS<sub>2</sub> and metEPS<sub>3</sub> adopt more extended conformations and exhibit slower diffusion rates and larger hydrodynamic radii, indicating increased molecular size and structural complexity.

Biophysical analyses (PFG-NMR, DLS) demonstrated that methanol behaves as a good solvent for both refEPS and metEPS. Despite significant structural variation, the EPS polymers maintain their conformation in water-methanol mixtures, and no direct interaction between

methanol and the EPS solvation shell was detected<sup>37,39</sup>. These observations imply that the protective effect of EPS in methanol environments is mediated through water, rather than direct solvent interaction. The methylation patterns and supramolecular behavior of the EPS likely play a critical role in preserving bacterial cell integrity and facilitating plant colonization under stress conditions<sup>40</sup>.

In parallel, our investigation into the LOS structure of *M. extorquens* PA1 and AM1 revealed a distinctive architecture, divergent from classical Gram-negative endotoxins. The LOS is characterized by a non-phosphorylated, extensively O-methylated core oligosaccharide containing rare sugars like 5-O-methyl-Kdo and 5-O-methyl-Ko, and a high negative charge density due to Kdo, Ko, and GalA residues. The lipid A region further displayed a non-phosphorylated trisaccharide backbone with an asymmetric 3+1 acylation pattern and a unique 3-O-acetyl-butyrate-modified very long-chain fatty acid (VLCFA). This complex and finely tuned structure enables the LOS to contribute to membrane stabilization, particularly in methanol-rich environments.

Biophysical studies using model lipid bilayers (via SANS, DLS, and EPR) confirmed that LOS incorporation increased vesicle diameter and bilayer thickness, it uniquely promoted membrane ordering and structural stabilization in the presence of methanol. This contrasts with *E. coli* LOS-containing bilayers, which showed reduced lipid ordering under similar conditions<sup>41,38</sup>. The low hydrophilicity of the O-methylated saccharide moiety, coupled with solvophobic rhamnose residues, is thought to form a compact, surface-protective outer layer<sup>14</sup>. Additionally, the flexible acyl chains of lipid A, particularly the VLCFAs, contribute to enhanced packing and membrane rigidity, facilitating adaptation to changes in membrane curvature<sup>33</sup>. Overall, the molecular features of *M. extorquens* LOS appear finely tuned to promote asymmetric membrane stabilization and ordering.

Collectively, these findings support a dual-level structural adaptation mechanism in *M. extorquens* PA1, involving both EPS and LOS remodeling. The dynamic changes in EPS composition and branching, alongside the unique and finely tuned structural features of LOS, contribute significantly to the bacterium's ability to maintain cellular stability, resist chemical stress, and successfully colonize plant surface.

Indeed, this chapter builds upon work published in *Carbohydrate Polymers* (2022)<sup>8</sup> and *Journal of the American Chemical Society Au* (2023)<sup>9</sup>, which launched our investigation into the structural diversity of LPS molecules in the plant root microbiome and their immunological roles.

These adaptive strategies may provide a competitive advantage in natural ecosystems and offer valuable insights into the development of methanol-based biotechnologies and plant-microbe interaction models. Furthermore, the unique chemical and functional properties of these glycoconjugates open avenues for applications in agriculture, such as crop resilience enhancement, and in biotechnology sectors requiring microbial resistance to organic solvents.

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**SECTION II**

**Human-associated bacteria**

## **CHAPTER V**

**Structure–Function Analysis of  
the LPS from *Paenalcaldigenes  
hominis*: Implications for Age-  
Associated Neuroinflammation**



## 5.1 Introduction

The intricate interaction between the host immune system and the resident microbiota plays a pivotal role in human health and disease<sup>1,2</sup>. This relationship is especially critical within the gut–brain axis (GBA), a bidirectional communication system that connects the gastrointestinal and central nervous systems via neural, endocrine, metabolic, and immune pathways<sup>3,4</sup>. The gut microbiota, often regarded as a “virtual organ”, influences a wide range of physiological functions, including immune regulation, maintenance of barrier integrity, and cognitive performance<sup>5,6,7</sup>.

Among microbial components, surface molecules such as lipopolysaccharides (LPSs) are key players in host recognition and immune modulation<sup>8,9,10</sup>. *Paenalcaligenes hominis*, a Gram-negative bacterium of the *Alcaligenaceae* family, emerged as a species of interest due to its elevated abundance in the elderly gut microbiota and its association with inflammatory and cognitive disorders. First isolated from the bloodstream of an elderly patient<sup>11</sup>, *P. hominis* has since been linked to age-associated colitis and cognitive decline. These associations are particularly relevant in the context of aging, a physiological condition characterized by microbiota dysbiosis and disruption of gut–brain communication<sup>12,13,14</sup>. Recent studies suggest that fecal microbiota transplants from aged donors enriched in *P. hominis* can worsen colitis and cognition in young mice, possibly via extracellular vesicles reaching the brain through systemic circulation or the vagus nerve<sup>15</sup>.

LPS is a central immunomodulatory molecule of Gram-negative bacteria. It engages host pattern recognition receptors, such as Toll-like receptor 4 (TLR4) triggering innate immune responses<sup>16</sup>. The immunostimulatory activity of LPS varies depending on its molecular architecture, particularly the composition of lipid A, the core oligosaccharide, and the O-antigen<sup>8,16</sup>.

Despite increasing interest in microbiota-derived immune modulators, the structural and functional properties of LPS from non-canonical species such as *P. hominis* remain largely unexplored. In this chapter, we present a multidisciplinary investigation that integrates chemical, spectroscopic, spectrometric, computational, and immunological approaches to comprehensively characterize the LPS of *P. hominis*.

Our findings reveal a structurally atypical LPS: a trisaccharide O-antigen capped with 3-O-methyl-rhamnose, a penta-acylated lipid A, and supramolecular aggregate formation with distinct physical properties. Immunologically, *P. hominis* LPS acts as a weak TLR4 agonist, fails to activate TLR2, induces a modest cytokine response in human PBMCs, and is selectively recognized by lectins such as Ficolin-3 and Galectin-4. Collectively, these features suggest that *P. hominis* LPS functions as a non-canonical immune modulator, potentially contributing to the chronic low-grade inflammation and neuroimmune dysregulation associated with aging.

This chapter is based on the peer-reviewed publication: Nieto-Fabregat, F., et al. (2025). Structure of the lipopolysaccharide from *Paenalcaligenes hominis*: A chemical perspective on immune recognition. *JACS Au*.<sup>17</sup>

## 5.2 Structural Characterization of *Paenaltcaligenes hominis* LPS

To investigate its potential role in gut–brain axis dysfunction and age-associated inflammation, this section provides a detailed structural characterization of *Paenaltcaligenes hominis* LPS through a multidisciplinary approach encompassing its extraction, molecular composition, glycan configuration, and biophysical properties.

Figure 1 illustrates the experimental workflow applied to dissect the structural domains of *P. hominis* LPS.

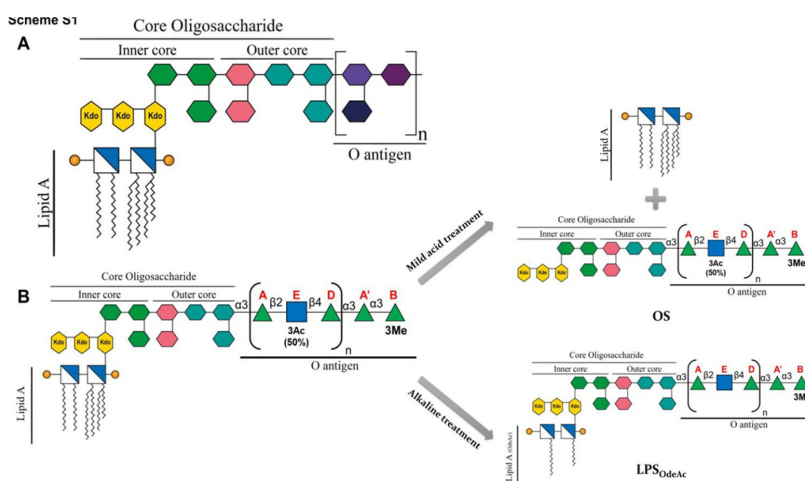


**Figure 1:** (A) Schematic representation of lipopolysaccharide (LPS) architecture, consisting of lipid A, the core oligosaccharide (subdivided into inner and outer regions), and the O-antigen made of repeating sugar units. (B) Isolation strategy for *P. hominis* LPS: mild acid hydrolysis separates lipid A from the core OS–O-antigen; alkaline treatment yields LPS<sub>OAc</sub>. Core and lipid A are schematic; the O-antigen reflects the experimentally defined repeating unit and cap.

### 5.2.1 Extraction, Purification, and Compositional Analysis

LPS was isolated using the classical hot phenol–water extraction<sup>18</sup>, followed by extensive purification: enzymatic digestion (DNase, RNase, protease), dialysis, ultracentrifugation, and gel filtration chromatography. To remove residual lipoproteins capable of activating TLR2, the protocol was supplemented with a chloroform–methanol extraction step<sup>19</sup>, in line with established decontamination protocols<sup>20</sup>. SDS-PAGE analysis with silver nitrate staining revealed a smooth-type LPS, indicated by the characteristic ladder-like pattern corresponding to heterogeneous O-antigen chain lengths. Coomassie staining and bicinchoninic acid (BCA) assays<sup>21</sup>, excluded the presence of protein contaminants, confirming sample purity (Figure 2).

## CHAPTER V

### Structure-Function Analysis of the LPS from *Paenalcaligenes hominis*: Implications for Age-Associated Neuroinflammation

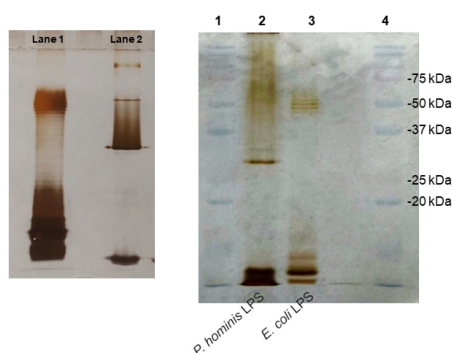


**Figure 2:** *Left:* SDS-PAGE followed by silver staining of *P. hominis* LPS after enzymatic digestion (Lane 2). Smooth LPS (S-LPS) from *Escherichia coli* (Lane 1) was used as a reference. *Right:* Silver-stained SDS-PAGE of *P. hominis* LPS. Lane 2: 8  $\mu$ L of a 1 mg/mL solution of *P. hominis* LPS; Lane 3: 8  $\mu$ L of LPS from *E. coli* O127:B8; Lanes 1 and 4: 2  $\mu$ L of BLUeye Prestained Protein Ladder (reference marker)

Chemical analysis via Gas chromatography–mass spectrometry (GC-MS) identified a predominance of L-rhamnose (L-Rha), D-glucosamine (D-GlcN), and 3-O-methyl-L-rhamnose (Rha3OMe). Additional minor constituents included D-mannose (D-Man), D-galactose (D-Gal), L,D-heptose (L,D-Hep), and 3-deoxy-D-manno-oct-2-ulonic acid (Kdo), common components of the LPS core region in Gram-negative bacteria<sup>16</sup>. Methylation linkage analysis confirmed diverse glycosidic patterns including terminal, 2- and 3-substituted L-Rha; 4- and 3,4-substituted D-GlcN; 3-substituted D-Man and Hep; terminal D-Gal; and both terminal and 5-substituted Kdo. These substitutions suggest a branched core oligosaccharide architecture and a capped O-antigen structure, forming the basis for further structural elucidation via NMR and MS<sup>16,22</sup> (Table 1 and Figure 3).

**Table 1:** Monosaccharide composition and glycosidic linkage analysis of the lipopolysaccharide (LPS) produced by *Paenalcaligenes hominis*.

| Monosaccharide                          | Partially Methylated Derivative                    |
|-----------------------------------------|----------------------------------------------------|
| L-rhamnose (Rha)                        | 2-substituted Rha, 3-substituted Rha, terminal Rha |
| 3-O-methyl-L-rhamnose (Rha3OMe)         | ----                                               |
| D-Mannose (Man)                         | 3-substituted Man                                  |
| D-Galactose (Gal)                       | terminal Gal                                       |
| D-Glucosamine (GlcN)                    | 4-substituted GlcN, 3,4-disubstituted GlcN         |
| Heptose (L,D-Hep)                       | 3-substituted Hep                                  |
| 3-deoxy-D-manno-oct-2-ulonic acid (Kdo) | 5-substituted Kdop                                 |

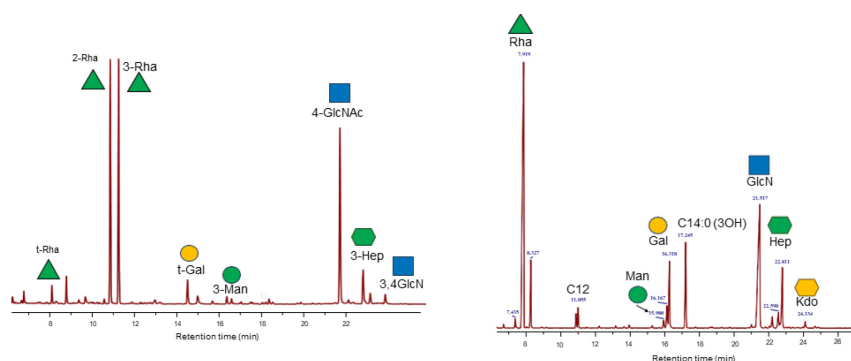
Structure-Function Analysis of the LPS from *Paenaltcaligenes hominis*: Implications for Age-Associated Neuroinflammation

**Figure 3:** GC-MS spectra. **Left:** Partially methylated alditol acetates (PMAAs) derived from the O-antigen repeating unit of *P. hominis* LPS. **Right:** Compositional analysis of monosaccharides. Signals corresponding to the core oligosaccharide region are also highlighted. The chromatograms display retention times and the identification of key monosaccharide derivatives, color-coded according to the Symbol Nomenclature for Glycans (SNFG).

Fatty acid composition analysis, performed via hydrolysis and GC-MS, revealed a penta-acylated lipid A containing dodecanoic acid (12:0), tetradecanoic acid (14:0), (R)-3-hydroxydodecanoic acid [12:0(3-OH)], (R)-3-hydroxytetradecanoic acid [14:0(3-OH)], and hexadecanoic acid (16:0) (Table 2).

**Table 2:** Fatty acid composition of *Paenaltcaligenes hominis* LPS.

| Fatty Acid  | Description  |
|-------------|--------------|
| 12:0        | Saturated    |
| 12:0 (3-OH) | Hydroxylated |
| 14:0        | Saturated    |
| 14:0 (3-OH) | Hydroxylated |
| 16:0        | Saturated    |

These findings are consistent with a non-hexa-acylated lipid A species, previously linked to attenuated TLR4 signaling<sup>8</sup>, and support the hypothesis that *P. hominis* LPS may function as a low-grade immune modulator rather than a classical endotoxin..

### 5.2.2 Structural Characterization by NMR Spectroscopy

A comprehensive structural elucidation of the *Paenaltcaligenes hominis* LPS O-antigen was performed using NMR spectroscopy. Both O-deacylated LPS (LPS<sub>OdeAc</sub>) and isolated O-polysaccharide (O-PS) fractions were analyzed through a suite of NMR experiments, including <sup>1</sup>H, <sup>13</sup>C, COSY, TOCSY, ROESY, NOESY, HSQC, and HMBC. These allowed full

assignment of sugar residues, determination of anomeric configurations, ring forms, linkage positions, and inter-residue connectivity.

The  $^1\text{H}$  NMR spectra of both  $\text{LPS}_{\text{OdeAc}}$  and the O-antigen (OS) are shown in Figure 4. Anomeric signals for each residue were clearly resolved and are attributed in Table 3.

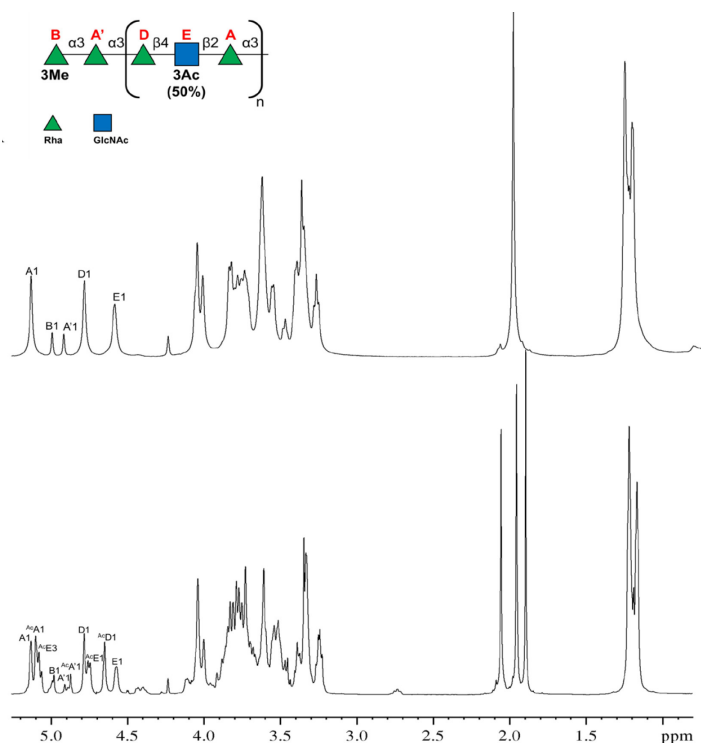


**Figure 4:**  $^1\text{H}$  NMR spectra of O-deacylated LPS ( $\text{LPS}_{\text{OdeAc}}$ , top) and O-antigen (OS, bottom) from *P. hominis* LPS. Anomeric signals are expanded in the inset. Sugar residues are represented using the Symbol Nomenclature for Glycans (SNFG).

The O-antigen comprised five distinct monosaccharide residues, all in the pyranose form. Four of these, designated A, B, A', and D, were identified as L-rhamnose units. Their manno configuration was confirmed by small  $^3J_{\text{H1,H2}}$  and  $^3J_{\text{H2,H3}}$  coupling constants, consistent with equatorial H-2 orientation. Residues A, B, and A' exhibited  $\alpha$ -anomeric configurations, while residue D was assigned to a  $\beta$ -anomeric configuration, based on  $^1J_{\text{CH}}$  coupling constants and intra-residue NOE correlations involving H-1, H-3, and H-5.

Residue E was identified as a  $\beta$ -linked N-acetyl-D-glucosamine (GlcNAc), characterized by ring coupling constants diagnostic of gluco-configuration and a  $^{13}\text{C}$  signal at  $\sim 56.1$  ppm in the HSQC spectrum (Figure 5), indicating a nitrogen-bearing carbon at C-2. Partial O-

acetylation at position 3 of GlcNAc was detected in approximately 50% of the repeating units, introducing structural microheterogeneity within the polysaccharide chain.

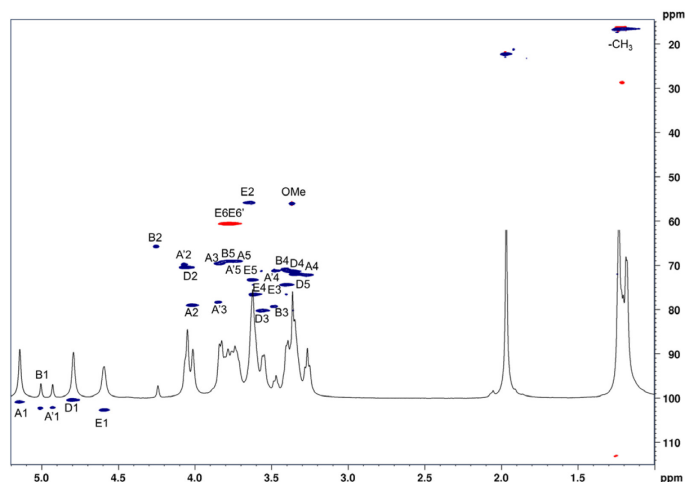


**Figure 5:** Zoom-in of the multiplicity-edited HSQC spectrum (blue and red) of *P. hominis* LPS<sub>OdeAc</sub>, showing  $^1\text{H}$ - $^{13}\text{C}$  correlations and aiding in sugar residue identification.

The glycosidic linkages and sequence were defined through analysis of NOESY, TOCSY, and HMBC spectra. The overlay of NOESY and TOCSY spectra (Figure 6) identified intra- and inter-residue connectivity, crucial for confirming the linear arrangement of the O-antigen.

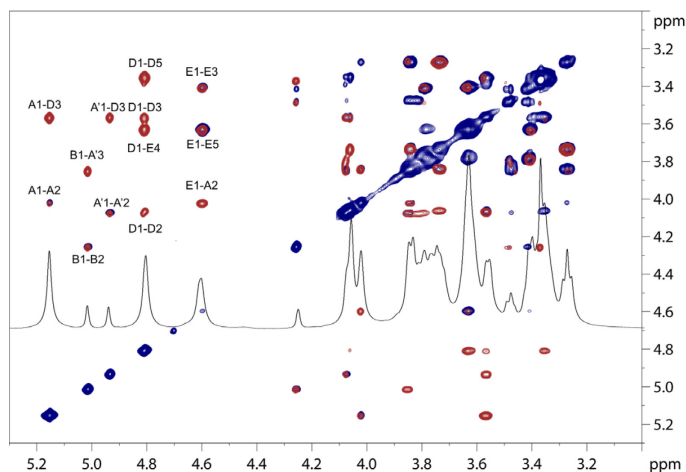


**Figure 6:** Overlay of NOESY (red) and TOCSY (blue) spectra of *P. hominis* LPS<sub>OdeAc</sub>. Key cross-peaks confirm inter-residue linkages and ring connectivity.

Further confirmation was obtained via HMBC data, shown in Figure 7, which revealed long-range heteronuclear couplings supporting the proposed sugar sequence.

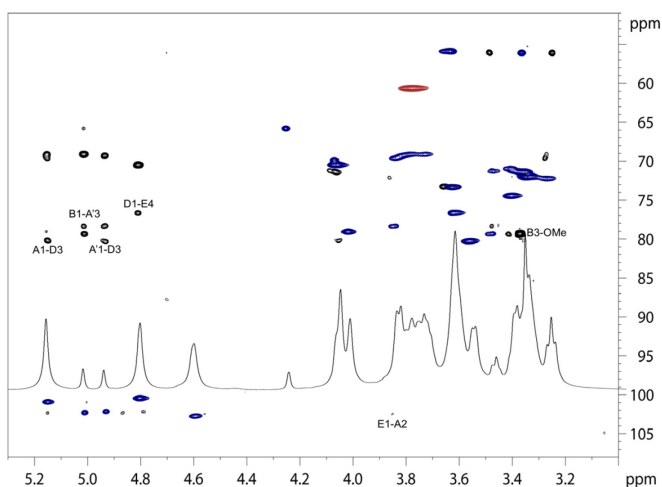


**Figure 7:** Superimposition of HSQC (blue/red) and HMBC (black) spectra of *P. hominis* LPS<sub>OAc</sub>. Cross-peaks confirm glycosidic linkage positions and anomeric configurations.

The repeating unit of the O-antigen (Figure 8) was identified as a linear trisaccharide backbone:



This core is capped at the nonreducing end by a terminal  $\alpha$ -3-O-methyl-L-rhamnose (residue B), yielding the following complete sequence:

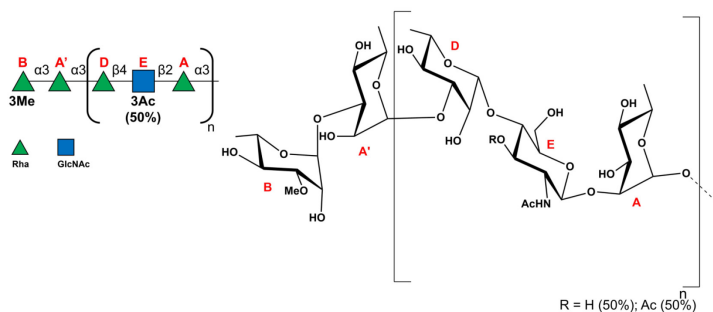
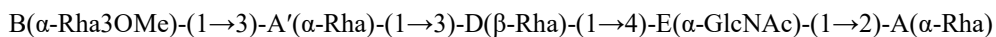


**Figure 8:** Structure of *P. hominis* LPS O-antigen.

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Approximately 50% of the GlcNAc residues were found to be O-acetylated at position 3, introducing structural microheterogeneity.

Mild acid hydrolysis selectively cleaved the Kdo–lipid A linkage, allowing isolation of the saccharide moiety for further analysis without disrupting the glycan backbone. The detailed chemical shifts and coupling constants supporting all assignments are provided in Table 3.

**Table 3:** NMR assignment of *P. hominis* LPS O-antigen. Reported values include  $^1\text{H}$  and  $^{13}\text{C}$  chemical shifts for each sugar residue, and  $^1J_{\text{CH}}$  coupling constants used to define anomeric configurations.

| LPS <sub>OdeAc</sub>   |                                                               |                                                               |           |      |      |           |
|------------------------|---------------------------------------------------------------|---------------------------------------------------------------|-----------|------|------|-----------|
|                        | 1                                                             | 2                                                             | 3         | 4    | 5    | 6         |
| A                      | 5.15                                                          | 4.02                                                          | 3.84      | 3.27 | 3.73 | 1.16      |
| 2- $\alpha$ -Rha       | 100.9                                                         | 79.1                                                          | 69.7      | 72.2 | 69   | 16.9      |
|                        | $^1J_{\text{CH}} = 172.2$<br>Hz                               |                                                               |           |      |      |           |
| B                      | 5.01                                                          | 4.26                                                          | 3.49      | 3.41 | 3.79 | 1.23      |
| t- $\alpha$ -Rha(3OMe) | 102.5                                                         | 65.8                                                          | 79.3      | 70.9 | 69   | 16.9      |
|                        | $^1J_{\text{CH}} = 171.2$<br>Hz                               | $^1\text{H}$ and $^{13}\text{C}$ resonances of O-Methyl group |           |      |      |           |
|                        |                                                               |                                                               | 3.36/56.0 |      |      |           |
| C                      | 102.3                                                         | 69.7                                                          | 78.4      | 71.3 | 69   | 16.9      |
| 3- $\alpha$ -Rha       | 4.93                                                          | 4.08                                                          | 3.85      | 3.48 | 3.8  | 1.23      |
|                        | $^1J_{\text{CH}} = 173.4$<br>Hz                               |                                                               |           |      |      |           |
| D                      | 4.81                                                          | 4.06                                                          | 3.56      | 3.36 | 3.35 | 1.23      |
| 3- $\beta$ -Rha        | 100.5                                                         | 70.5                                                          | 80.4      | 71.3 | 72.3 | 16.9      |
|                        | $^1J_{\text{CH}} = 163.0$<br>Hz                               |                                                               |           |      |      |           |
| E                      | 4.6                                                           | 3.65                                                          | 3.41      | 3.62 | 3.63 | 3.81 3.75 |
| 4- $\beta$ -GlcNAc     | 102.7                                                         | 55.9                                                          | 74.4      | 76.7 | 73.5 | 60.6      |
|                        | $^1J_{\text{CH}} = 166.1$<br>Hz                               |                                                               |           |      |      |           |
|                        | $^1\text{H}$ and $^{13}\text{C}$ resonances of N-Acetyl group |                                                               |           |      |      |           |

|                  |                                                                               |                |            |       |      |           |
|------------------|-------------------------------------------------------------------------------|----------------|------------|-------|------|-----------|
|                  |                                                                               | 1.97/22.2      |            |       |      |           |
| <b>O-antigen</b> |                                                                               |                |            |       |      |           |
|                  |                                                                               |                |            |       |      |           |
| <b>E</b>         | 4.76                                                                          | 3.79           | 5.08       | 3.85  | 3.49 | 3.75/3.69 |
| 4-β-GlcNAc(3OAc) | 101.9                                                                         | 54             | 74.6       | 75.4  | 75.5 | 61        |
|                  | <sup>1</sup> J <sub>CH</sub> = 165.6<br>Hz                                    |                |            |       |      |           |
|                  | <sup>1</sup> H and <sup>13</sup> C resonances of N-Acetyl and O-Methyl groups |                |            |       |      |           |
|                  |                                                                               | 1.89/21.9<br>8 | 2.05/20.29 |       |      |           |
| <b>E'</b>        | 4.57                                                                          | 3.63           | 3.38       | 3.59  | 3.62 | 3.77      |
| 4-β-GlcNAc       | 102.73                                                                        | 55.8           | 74.4       | 76.58 | 73.3 | 60.44     |
|                  | <sup>1</sup> J <sub>CH</sub> = 166.0<br>Hz                                    |                |            |       |      |           |
|                  | <sup>1</sup> H and <sup>13</sup> C resonances of N-Acetyl group               |                |            |       |      |           |
|                  |                                                                               | 1.95/22.2<br>1 |            |       |      |           |

### 5.2.3 Structural Characterization by Mass Spectrometry

Mass spectrometry (MS) analyses were instrumental in complementing NMR data and elucidating the microheterogeneity and domain organization of *Paenaltcaligenes hominis* lipopolysaccharide (LPS). Negative-ion mode matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS and electrospray ionization (ESI-MS) were employed to investigate the structural details of lipid A, the core oligosaccharide (core OS), and the O-antigen domains<sup>23</sup>.

Negative-ion MALDI-TOF MS analysis of purified lipid A (Figure 9) revealed a series of ion peaks in the m/z 1261.5–1585.6 range, corresponding to lipid A species differing in acylation and phosphorylation.

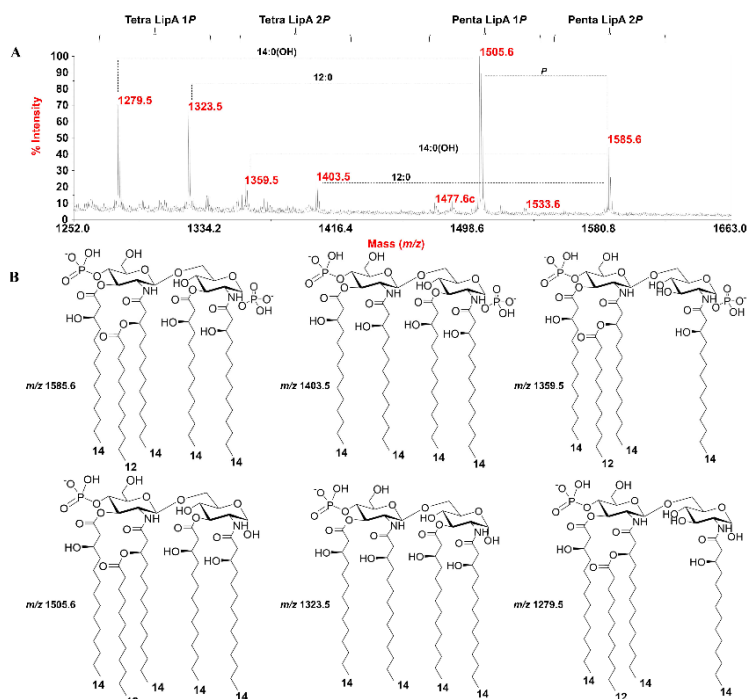
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**Figure 9:** (A) Negative-ion MALDI-TOF MS spectrum of the lipid A fraction from *P. hominis* LPS, recorded in reflectron mode. (B) Proposed structures of the predominant mono- and bis-phosphorylated lipid A species.

The most intense peak at  $m/z$  1505.6 was attributed to a monophosphorylated, penta-acylated lipid A comprising four 14:0(3-OH) primary acyl chains and one secondary 12:0 chain. Additional peaks at  $m/z$  1279.5, 1323.5, and 1585.6 corresponded to tetra-acylated and bis-phosphorylated species. MS/MS fragmentation of selected precursors enabled unambiguous localization of phosphate groups and secondary acyl chains. In particular, the absence of a fragment corresponding to the combined loss of 14:0(3-OH) and 12:0 provided evidence for the amide-linked position of the 12:0 chain.

To further dissect the saccharide architecture, an aliquot of LPS underwent selective O-deacylation via mild ammonium hydroxide treatment. The resulting O-deacylated LPS (LPS<sub>deOAc</sub>) was analyzed by negative-ion ESI-MS (Figure 10), revealing a complex profile of multiply charged species, including intact LPS<sub>deOAc</sub> ions in the (M-4H)<sup>4-</sup> and (M-5H)<sup>5-</sup> states, along with diagnostic Y-type (lipid A-derived) and B-type (sugar-derived) fragments. The minimal core OS, detected at  $m/z$  1230.42, was assigned to a heptasaccharide containing one anhydro-Kdo, one heptose, one N-acetylhexosamine (HexNAc), two deoxyhexoses (dHex), and two hexoses (Hex), consistent with previously reported core OS structures in related bacteria<sup>24</sup>. An acetylated variant at  $m/z$  1272.43 featured an additional acetyl group on the HexNAc residue.

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In the high-mass range, intense peaks at  $m/z$  1622.88 and 1745.18 matched to  $LPS_{deOAc}$  species containing mono- and bis-phosphorylated lipid A, respectively, linked to a defined core OS, multiple O-antigen repeating units (up to 10), and a capping trisaccharide comprising two dHex and one O-methylated dHex. Repeating units were shown to be partially O-acetylated, as inferred from regular mass differences of 42 Da, in agreement with NMR findings and consistent with O-antigen modifications reported in other Gram-negative bacteria<sup>25</sup>. The main ions and their proposed structures are summarized in Table 4, providing further support to the assignments discussed above.

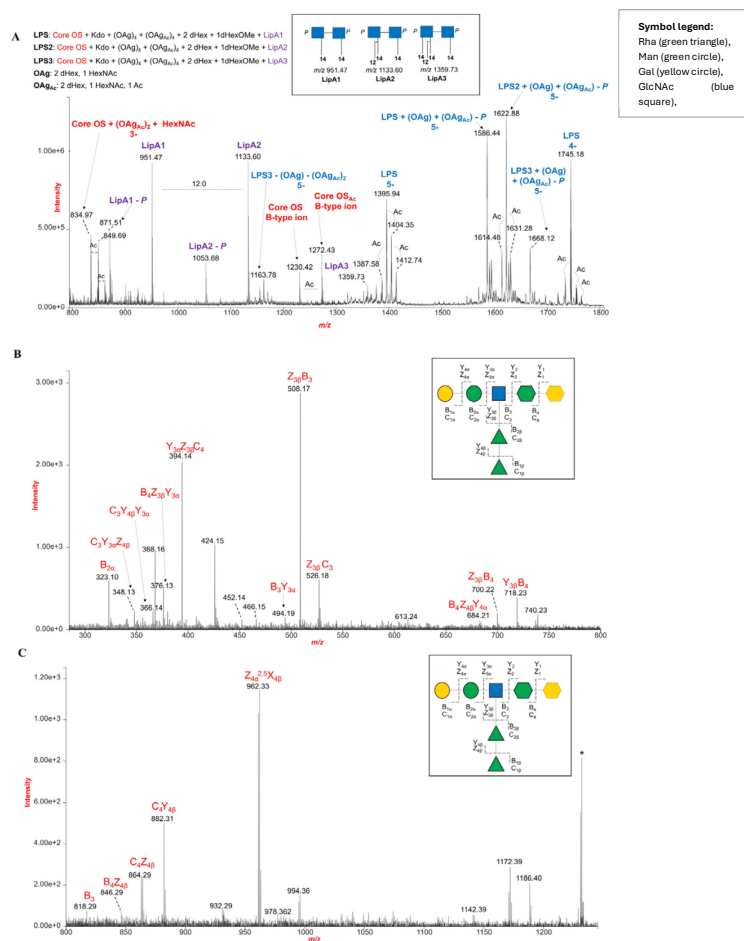


**Figure 10:** (A) Negative-ion ESI mass spectrum of *P. hominis* O-deacylated LPS ( $LPS^{OdeAc}$ ). Major LPS species appear as 4+ and 5+ ions; lipid A and core OS as 1+ and 3+ ions. Key ions and proposed structures are listed in Table 4. OAgAc and OAg indicate O-antigen units with or without an extra acetyl group. LipA variants represent bis-phosphorylated lipid A with increasing acylation. Inset shows deacylation sites. (B, C) Enlarged MS/MS of the core OS ion ( $m/z$  1230.42) with main glycosidic fragments labeled; additional peaks likely derive from cross-ring cleavages. Proposed OS structures are shown in the insets.

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**Table 4:** Observed Ions and related assignment derived from ESI-MS of *O*-deacylated *P. hominis* LPS

| Observed ion peaks ( <i>m/z</i> ) | Proposed structure                                   | Proposed composition                                                                                                                                                                   | Interpretation of the Ion |
|-----------------------------------|------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------|
| 834.94                            | Core OS + (OAg <sub>Ac</sub> ) <sub>2</sub> + HexNAc | (Hex) <sub>2</sub> , Hep, Kdo, (dHex) <sub>6</sub> , (HexNAc) <sub>4</sub> , (Ac) <sub>2</sub>                                                                                         | [M-3H] <sup>3-</sup>      |
| 871.51                            | LipA1 - P                                            | (HexN) <sub>2</sub> , [14:0(3-OH)] <sub>2</sub>                                                                                                                                        | [M-H] <sup>-</sup>        |
| 951.47                            | LipA1                                                | (HexN) <sub>2</sub> , [14:0(3-OH)] <sub>2</sub> , P                                                                                                                                    | [M-H] <sup>-</sup>        |
| 1053.68                           | LipA2 - P                                            | (HexN) <sub>2</sub> , [14:0(3-OH)] <sub>2</sub> , (12:0)                                                                                                                               | [M-H] <sup>-</sup>        |
| 1133.60                           | LipA2                                                | (HexN) <sub>2</sub> , [14:0(3-OH)] <sub>2</sub> , (12:0), P                                                                                                                            | [M-H] <sup>-</sup>        |
| 1163.78                           | LPS3 – (OAg) – (OAg <sub>Ac</sub> ) <sub>2</sub>     | (Hex) <sub>2</sub> , Hep, (Kdo) <sub>2</sub> , (dHex) <sub>14</sub> , (HexNAc) <sub>6</sub> , (Ac) <sub>2</sub> , dHexOMe, (HexN) <sub>2</sub> , [14:0(3-OH)] <sub>3</sub> , (12:0), P | [M-5H] <sup>5-</sup>      |
| 1230.42                           | Core OS (B-type ion)                                 | (Hex) <sub>2</sub> , HexNAc, (dHex) <sub>2</sub> , Hep, Kdo                                                                                                                            | [M-H] <sup>-</sup>        |
| 1272.43                           | Core OS <sub>Ac</sub> (B-type ion)                   | (Hex) <sub>2</sub> , HexNAc, (dHex) <sub>2</sub> , Hep, Kdo, Ac                                                                                                                        | [M-H] <sup>-</sup>        |
| 1359.73                           | LipA3                                                | (HexN) <sub>2</sub> , [14:0(3-OH)] <sub>3</sub> , (12:0), P                                                                                                                            | [M-H] <sup>-</sup>        |
| 1387.58                           | LPS - Ac                                             | (Hex) <sub>2</sub> , Hep, (Kdo) <sub>2</sub> , (dHex) <sub>20</sub> , (HexNAc) <sub>9</sub> , (Ac) <sub>3</sub> , dHexOMe, (HexN) <sub>2</sub> , [14:0(3-OH)] <sub>2</sub> , P         | [M-5H] <sup>5-</sup>      |
| 1395.94                           | LPS                                                  | (Hex) <sub>2</sub> , Hep, (Kdo) <sub>2</sub> , (dHex) <sub>20</sub> , (HexNAc) <sub>9</sub> , (Ac) <sub>4</sub> , dHexOMe, (HexN) <sub>2</sub> , [14:0(3-OH)] <sub>2</sub> , P         | [M-5H] <sup>5-</sup>      |
| 1586.44                           | LPS + (OAg) + (OAg <sub>Ac</sub> ) - P               | (Hex) <sub>2</sub> , Hep, (Kdo) <sub>2</sub> , (dHex) <sub>4</sub> , (HexNAc) <sub>11</sub> , (Ac) <sub>5</sub> , dHexOMe, (HexN) <sub>2</sub> , [14:0(3-OH)] <sub>2</sub>             | [M-5H] <sup>5-</sup>      |
| 1622.88                           | LPS2 + (OAg) + (OAg <sub>Ac</sub> ) - P              | (Hex) <sub>2</sub> , Hep, (Kdo) <sub>2</sub> , (dHex) <sub>24</sub> , (HexNAc) <sub>11</sub> , (Ac) <sub>5</sub> , dHexOMe, (HexN) <sub>2</sub> , [14:0(3-OH)] <sub>2</sub> , (12:0)   | [M-5H] <sup>5-</sup>      |
| 1668.12                           | LPS3 + (OAg) + (OAg <sub>Ac</sub> ) - P              | (Hex) <sub>2</sub> , Hep, (Kdo) <sub>2</sub> , (dHex) <sub>24</sub> , (HexNAc) <sub>11</sub> , (Ac) <sub>5</sub> , dHexOMe, (HexN) <sub>2</sub> , [14:0(3-OH)] <sub>3</sub> , (12:0)   | [M-5H] <sup>5-</sup>      |
| 1745.18                           | LPS                                                  | (Hex) <sub>2</sub> , Hep, (Kdo) <sub>2</sub> , (dHex) <sub>20</sub> , (HexNAc) <sub>9</sub> , (Ac) <sub>4</sub> , dHexOMe, (HexN) <sub>2</sub> , [14:0(3-OH)] <sub>2</sub> , P         | [M-4H] <sup>4-</sup>      |

Complementary MALDI-TOF MS analysis of intact, native LPS revealed a distribution of [M-H]<sup>-</sup> ions in the *m/z* 5817–7720 range (Figure 11). The spectrum displayed a ladder-like

pattern with mass differences of 42, 486, and 537 Da, consistent with incremental O-antigen repeating units and variable O-acetylation.

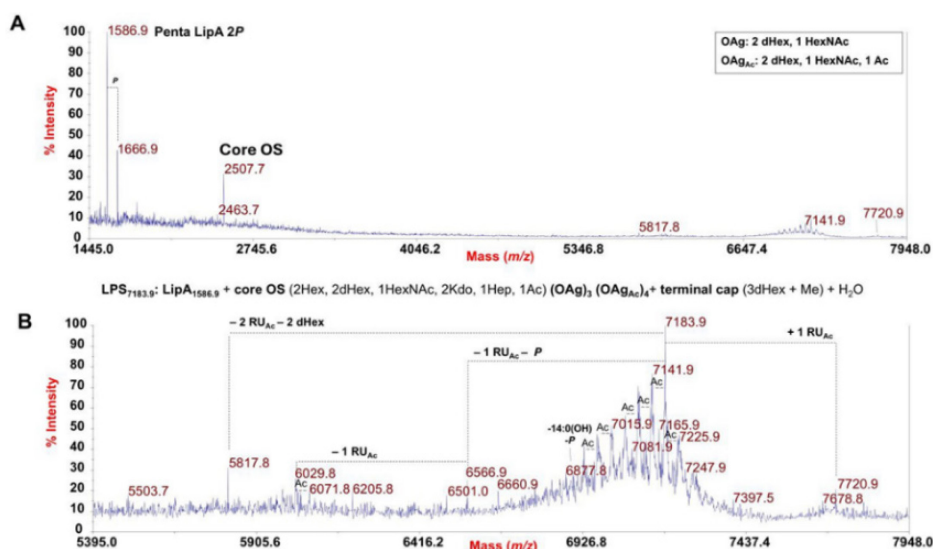


**Figure 11:** (A) Negative-ion MALDI-TOF MS spectrum of *P. hominis* LPS, recorded in reflectron mode. (B) Enlarged view of the  $m/z$  5395–7948 range, showing peaks corresponding to LPS species. OAg and OAg<sub>Ac</sub> indicate O-antigen repeating units with or without an additional acetyl group, respectively.

Fragment ions in the lower  $m/z$  range (1500–2510) were diagnostic of glycosidic bond cleavage between Kdo and lipid A, providing further confirmation of the LPS architecture<sup>23,24</sup>. Notably, a previously undetected tris-phosphorylated lipid A species was observed at  $m/z$  1666.9. The intact core OS was also observed at  $m/z$  2507.7, with a decarboxylated form at  $m/z$  2463.7 (–44 Da), consistent with CO<sub>2</sub> loss from the reducing Kdo residue.

Collectively, the integrated MALDI-TOF and ESI-MS/MS analyses defined a complex and microheterogeneous LPS structure in *P. hominis*, featuring a conserved core OS and structurally diverse lipid A and O-antigen domains<sup>24,26</sup>. The variability in acylation, phosphorylation, O-acetylation, and polysaccharide chain length underscores the non-canonical architecture of this endotoxin, with potential implications for its immunomodulatory properties.

## 5.2.4 Computational Modeling

To better understand the three-dimensional architecture and dynamic behavior of the *Paenalcigenes hominis* O-antigen, we employed a computational strategy combining molecular mechanics (MM) and molecular dynamics (MD) simulations. This approach complemented experimental data from NMR and mass spectrometry, providing mechanistic

insights into conformational plasticity, supramolecular organization, and potential immunological implications<sup>27-32</sup>.

Initial adiabatic energy scans were performed on disaccharide fragments representative of the O-antigen repeating units. Glycosidic torsion angles ( $\Phi = \text{H1-C1-O-C'X}$ ;  $\Psi = \text{C1-O-C'X-H'X}$ ) were systematically varied to produce adiabatic energy maps defining the low-energy conformational space for each glycosidic linkage. These maps (Figure 12) served as a reference for constructing larger O-antigen models, including octasaccharides and dodecasaccharides composed of a terminal tetrasaccharide (B-A'-D-E) and one to three repeating trisaccharide units  $[-2-\alpha\text{-Rha}-3-\beta\text{-Rha}-4-\alpha\text{-GlcNAc}]_n$ .

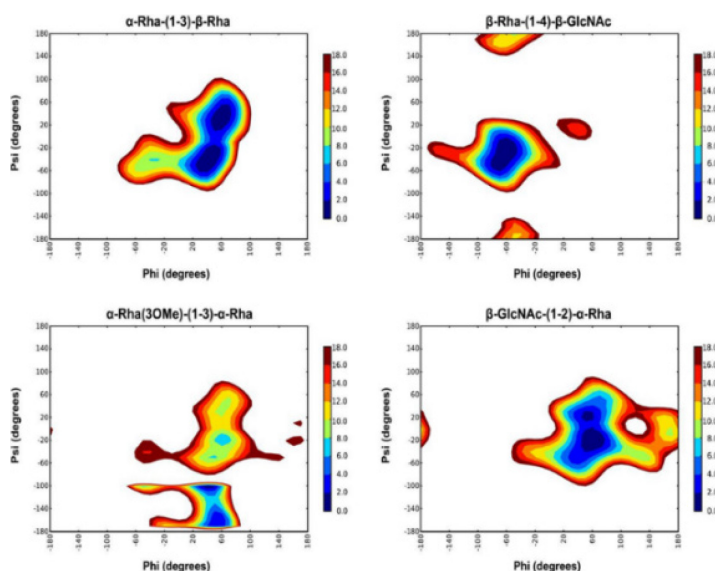


**Figure 12:** Adiabatic maps of  $\Phi$  and  $\Psi$  torsion angles for the glycosidic linkages in *P. hominis* LPS, calculated using molecular mechanics. The color gradient represents the corresponding energy landscape.

Subsequently, MD simulations were conducted in explicit solvent using the AMBER force field<sup>28</sup> to investigate conformational dynamics. Both fully O-acetylated and non-acetylated variants were analyzed to assess the influence of 3-O-acetylation on the terminal GlcNAc residue (E unit). Over 200 ns simulation, torsion angles were monitored for each glycosidic bond, and their distributions were plotted as 2D density maps (Figure 13).

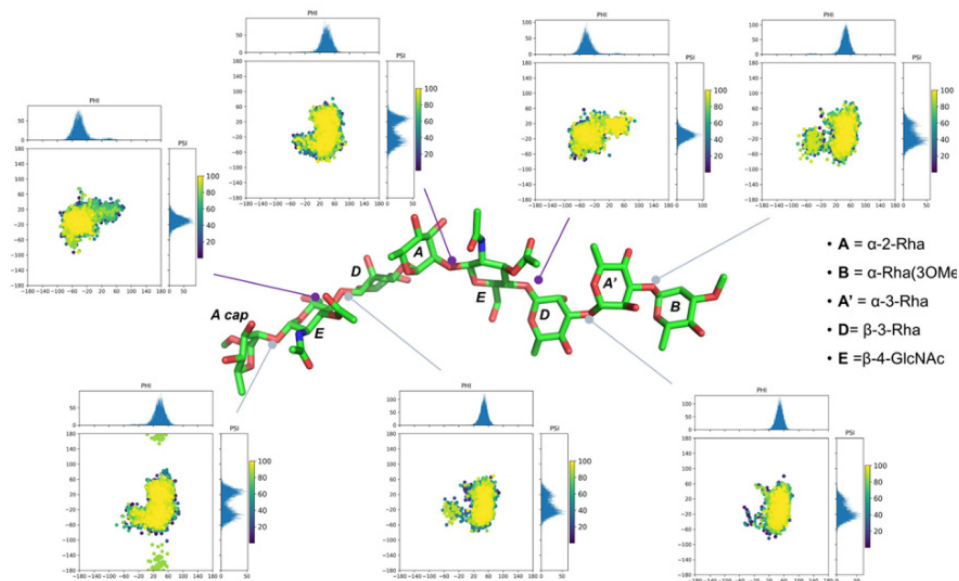
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**Figure 13:** Torsion angle distributions around the glycosidic linkages during the MD simulation of the *P. hominis* LPS<sub>deOAc</sub> octasaccharide. The  $\Phi$  and  $\Psi$  torsion angles were monitored for each glycosidic bond throughout the simulation. The two-dimensional plots show the distribution of these angles, with the color gradient indicating the frequency of occurrence. The LPS<sub>deOAc</sub> octasaccharide is depicted in its most representative conformation, with each sugar residue labeled as in Table 3.

The MD results confirmed a loosely coiled topology, consistent with the MM-derived low-energy conformers.  $\Phi$  angles predominantly adopted exo-syn conformations, while  $\Psi$  angles fluctuated within a narrow range ( $-20^\circ$  to  $+20^\circ$ ). Structural overlays of representative conformations (Figure 14) revealed minimal differences between O-acetylated and non-acetylated forms, suggesting limited influence of acetylation on overall geometry.

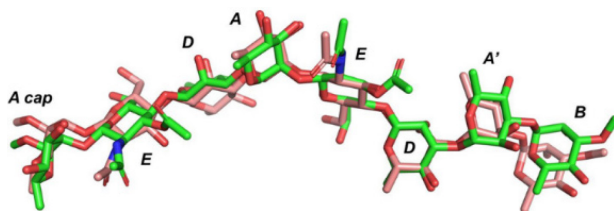


**Figure 14:** Superposition of the most representative conformers from the MD simulations of the OS (green) and LPS<sub>deOAc</sub> (salmon) octasaccharides. The near-identical conformations reflect limited structural impact from GlcNAc acetylation.

To explore the effect of chain length, MD simulations were extended to tetradecasaccharide models comprising a terminal tetrasaccharide and three repeating trisaccharide units [(A–D–E)<sub>3</sub>]. Both O-acetylated and non-acetylated forms exhibited conformational features like those of the octasaccharides, though the non-acetylated species appeared slightly more compact.

This was visualized through representative structures and overlays (Figure 15).

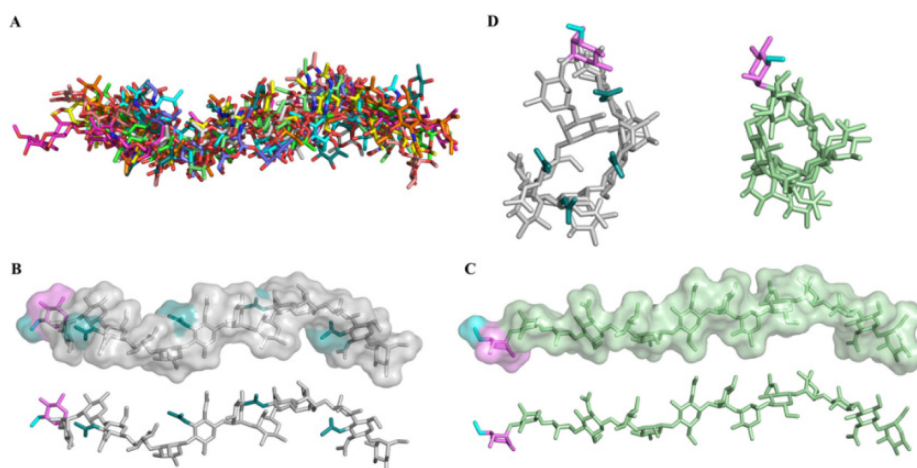


**Figure 15:** Computational modeling of the MD simulation of *P. hominis* O-antigen and LPS<sub>deOAc</sub> tetradecasaccharide. (A) Overlay of all conformations sampled during the MD simulation of the O-antigen tetradecasaccharide. (B) Most representative conformation of the O-antigen (white), showing acetyl groups (teal), terminal sugar (B residue, pink), and its O-methyl group (cyan), with and without surface rendering. (C) Representative structure of LPS<sub>deOAc</sub> tetradecasaccharide (green), with the same highlights as in panel B. (D) Front view comparison of O-antigen (white) and LPS<sub>deOAc</sub> (green) conformers, revealing their shared helical geometry.

Angle distributions for each glycosidic bond in the tetradecasaccharide system were also monitored throughout the simulation (Figure 16), confirming the persistence of *exo-syn*  $\Phi$  angles and restricted  $\Psi$  variability.

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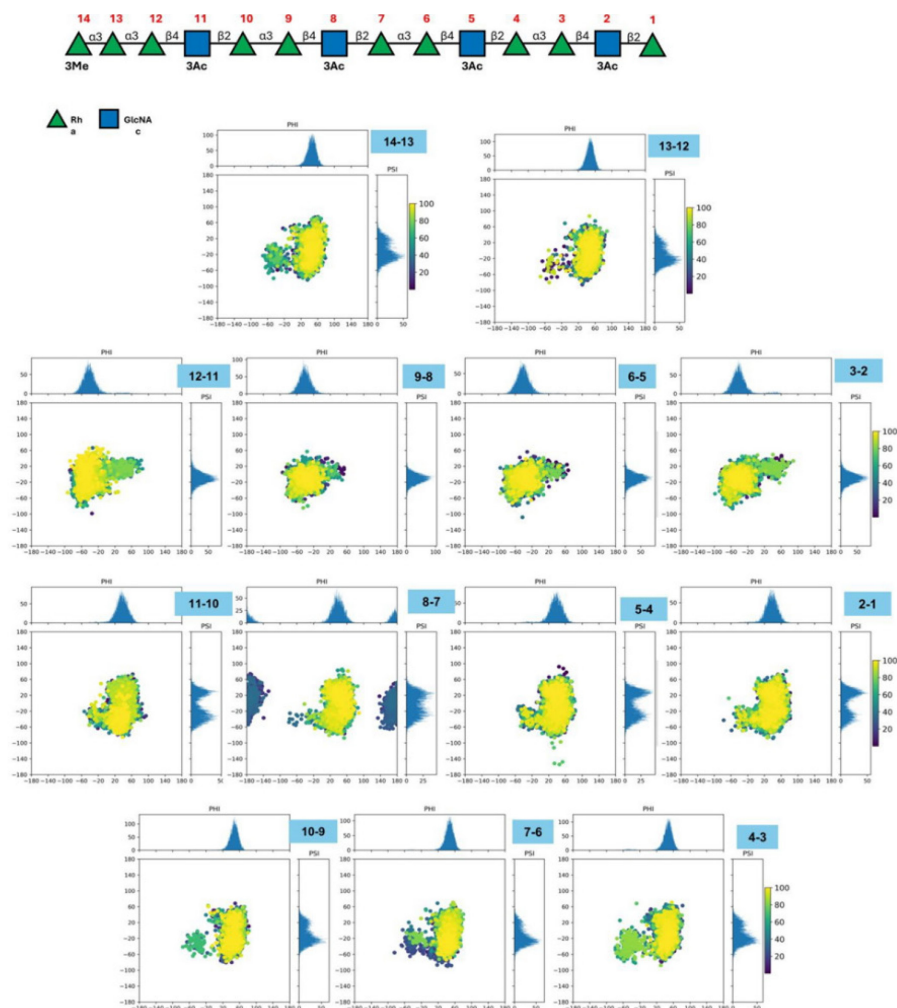


**Figure 16:** Distributions of  $\Phi$  and  $\Psi$  torsion angles across each glycosidic linkage during the MD simulation of the *P. hominis* OS tetradecasaccharide. Data confirms consistent torsional preferences and local flexibility.

These findings are consistent with general properties of bacterial exopolysaccharides<sup>25,33</sup> and suggest that acetylation modulates surface hydrophobicity and molecular recognition more than it alters the oligosaccharide's backbone topology. Notably, the simulations revealed dynamic exposure of terminal and internal residues, providing a structural rationale for selective recognition by host pattern recognition receptors (PRRs), such as galectins and Ficolin-3<sup>34,35</sup>.

In summary, computational modeling supports a model in which the *P. hominis* O-antigen exhibits significant structural adaptability and dynamic epitope accessibility. These features reinforce its potential role as a non-inflammatory or context-dependent immune modulator.

### 5.2.5 Characterization of LPS Aggregation and of Asymmetric LPS-Containing Bilayers Using DLS, SLS, and Neutron Reflectometry

To elucidate the supramolecular organization of *Paenaltcaligenes hominis* lipopolysaccharide (LPS) in aqueous solution and its structural integration within biomimetic membranes, we employed a combination of dynamic light scattering (DLS), static light scattering (SLS), and neutron reflectometry (NR)<sup>36-39</sup>. These complementary biophysical techniques enabled a detailed characterization of LPS aggregation behavior and membrane insertion under physiologically relevant conditions.

DLS analysis revealed that *P. hominis* LPS forms a monodisperse population of supramolecular aggregates with a hydrodynamic radius ( $R_h$ ) of  $30 \pm 2$  nm, indicative of compact, micelle-like assemblies (Figure 17A). This aggregation profile consists of smooth-type LPS and contrasts with larger, polydisperse vesicular systems<sup>36,37</sup>. Complementary SLS measurements estimated an average molecular weight of approximately  $430 \pm 10$  kDa per aggregate (Figure 17B). Considering the monomeric LPS molecular mass of  $\sim 7$  kDa (Figure 11), each aggregate comprises about 61 LPS units per micelle. This stoichiometry aligns with aggregation patterns observed in hypo-acylated LPS species, which often exhibit attenuated TLR4 activation and modified immunogenic profiles.

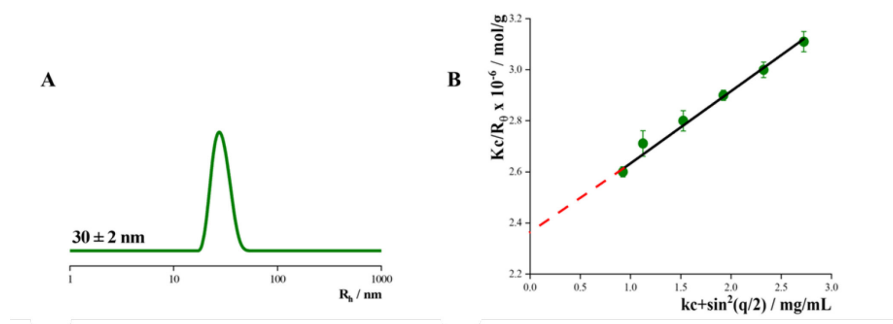


**Figure 17:** (A) Hydrodynamic radius distribution of *P. hominis* LPS. (B) Zimm plot of *P. hominis* LPS aggregates from static light scattering.

Functionally, such defined micelle-like structures may critically influence membrane interactions, receptor clustering, and immune recognition. Their uniformity and size stability support a model wherein aggregation state serves as an important determinant of downstream immune signaling.

To investigate membrane incorporation, NR studies were performed on asymmetric supported bilayers prepared via Langmuir–Blodgett and Langmuir–Schaefer techniques.

These biomimetic membranes featured a deuterated DPPC (d-DPPC) inner leaflet, and an outer leaflet composed of *P. hominis* LPS, thus recapitulating key aspects of the bacterial outer membrane architecture<sup>37,40</sup>. Neutron reflectivity profiles (Figure 18A) confirmed successful formation of highly asymmetric bilayers, supported by the corresponding scattering length density (SLD) profile (Figure 18B). The inner d-DPPC headgroup region measured  $6 \pm 1$  Å thickness with approximately 30% hydration, while the lipid core, comprising both d-DPPC and lipid A chains, was  $\sim 20.3$  Å thick, exhibiting low hydration ( $\sim 16\%$ ) and minimal roughness ( $\sim 4$  Å), indicative of tight lipid packing<sup>37,33</sup>.

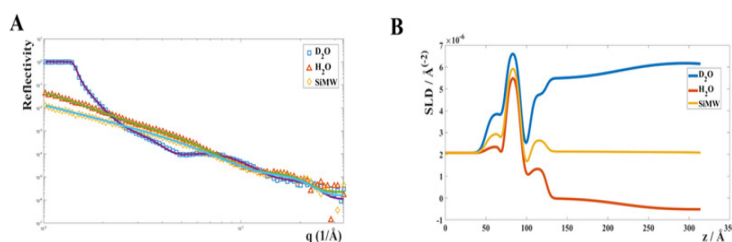


**Figure 18:** (A) NR reflectivity data (points) and model fits (lines); (B) Scattering length density (SLD) profiles.

Above this, the oligosaccharide core extended to  $\sim 20$  Å with  $\sim 26\%$  hydration. The O-antigen domain projected  $\sim 90$  Å into the aqueous phase and was highly hydrated ( $\sim 81\%$ ), consistent with a flexible, brush-like polysaccharide layer, as shown in the volume fraction occupancy profile (Figure 19). This configuration is comparable to that observed in other bacterial glycan-rich surfaces known to regulate immune accessibility<sup>25</sup>.

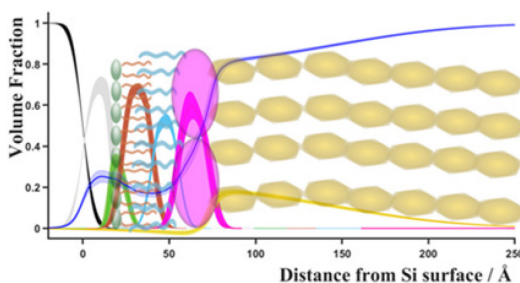


**Figure 19:** Volume fraction occupancy of bilayer components: silicon (black), silicon dioxide (gray), inner headgroups (light green), PC tails (orange), LPS tails (purple), core region (mid-green), and O-antigen region (dark green).

The ability of *P. hominis* LPS to incorporate into asymmetric bilayers without compromising membrane integrity, coupled with its extended, hydrated O-antigen, supports a

role as a modulatory interface facilitating immune evasion or selective host receptor engagement<sup>25</sup>.

Collectively, the DLS, SLS, and NR data describe *P. hominis* LPS as a supramolecularly organized, biophysically compatible, and immunologically strategic molecule. These features are particularly pertinent in contexts such as the aging gut, where altered immune surveillance and chronic low-grade inflammation intersect with microbiome-host dynamics.

### 5.3 Immunological Studies

To evaluate the immunomodulatory potential of *Paenalcaligenes hominis* LPS and its role in shaping innate immune responses, a comprehensive set of assays was conducted focusing on Toll-like receptor (TLR) activation, cytokine profiling, and lectin-mediated recognition<sup>41</sup>.

Initial stimulation experiments using HEK-Blue™ cells expressing human TLR4, MD-2, and CD14 revealed that *P. hominis* LPS is a weak TLR4 agonist. NF-κB activation was significantly lower compared to that induced by *E. coli* O111:B4 LPS at equivalent concentrations (1, 10, and 100 ng/mL), with  $p < 0.001$  at 1 and 10 ng/mL, and  $p < 0.01$  at 100 ng/mL (Figure 20A). This attenuated response correlates with the penta-acylated structure of *P. hominis* lipid A, which lacks the sixth acyl chain typically required for high-affinity TLR4/MD-2 engagement<sup>42,41</sup>. Although the acyl chains in *P. hominis* LPS are similar in length to those of *E. coli* (C12 and C14), the reduced number of chains is a key determinant of its dampened immunostimulatory profile<sup>41</sup>.

In contrast, no activation was observed in HEK-Blue cells transfected with TLR2, confirming the absence of TLR2 agonists or lipoprotein contaminants (Figure 20B).

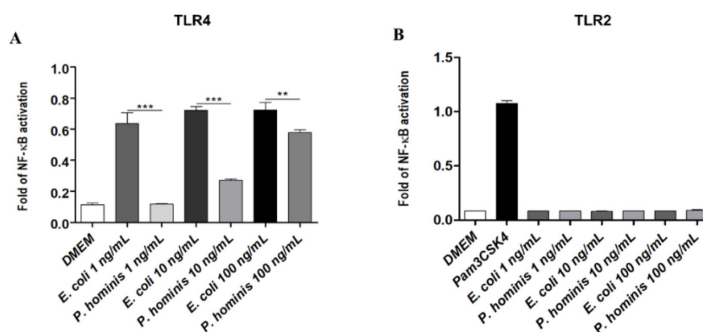


**Figure 20:** TLR activation by *P. hominis* LPS. (A) Stimulation of HEK-Blue hTLR4 and (B) HEK-Blue hTLR2 cells. Secreted embryonic alkaline phosphatase (SEAP) levels (OD) upon stimulation with *E. coli* and *P. hominis* LPS at 1, 10, and 100 ng/mL. *E. coli* O111:B4 LPS was used as a positive control in (A), and Pam3CSK4 in (B). Significance levels: \*\*\* $p < 0.001$ , \*\* $p < 0.01$ . Data represent mean  $\pm$  SD from three independent experiments.

Further characterization in peripheral blood mononuclear cells (PBMCs) from six healthy donors confirmed the non-canonical immune profile of *P. hominis* LPS. A moderate increase

## CHAPTER V

### Structure-Function Analysis of the LPS from *Paenalcigenes hominis*: Implications for Age-Associated Neuroinflammation

in TNF- $\alpha$  and IL-10 was observed, while IL-12p70 and IL-17A were significantly downregulated compared to unstimulated controls (Figure 21).

This cytokine pattern suggests reduced Th1/Th17 polarization and points toward an immunoregulatory rather than pro-inflammatory phenotype. The induction of IL-10, a hallmark cytokine of immune tolerance, supports a potential role in immune homeostasis and low-grade inflammation, rather than acute immune activation.

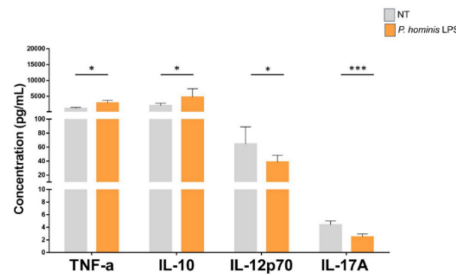


**Figure 21:** Secreted cytokine evaluation from PBMCs. Concentrations (pg/mL) of TNF- $\alpha$ , IL-10, IL-12p70, and IL-17A secreted by PBMCs from six donors after stimulation with *P. hominis* LPS (orange bars) vs unstimulated controls (gray bars). \* $p < 0.05$ , \*\* $p < 0.001$ ; paired t-test.

In parallel, glycan microarray studies using two distinct LPS preparations, Mix1 (containing both long and short O-antigen forms) and Mix2 (enriched in short O-antigen and core oligosaccharide), revealed selective binding to innate immune lectins (Figure 22). Notably, galectin-4 exhibited strong binding to Mix2, likely due to greater exposure of galactose residues in the core OS<sup>43,44</sup>.

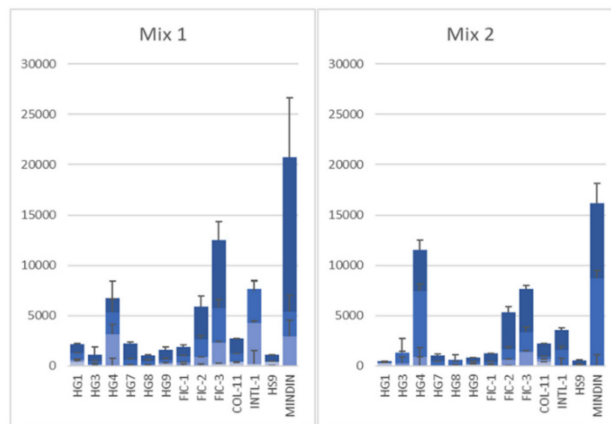


**Figure 22:** Microarray analysis of *P. hominis* LPS recognition by lectins and immune-related proteins. LPS preparations Mix1 (long + short O-antigen) and Mix2 (short O-antigen + core OS) were printed at different concentrations. Fluorescence intensity reflects binding strength. HG = human galectin, FIC = ficolin, COL = collectin, INTL = intelectin, HS = human Siglec.

Galectin-4, which is abundantly expressed in the gut and contains two carbohydrate-recognition domains, was the only galectin to show significant interaction. This binding was specifically inhibited by galactose-3-sulfate, confirming its epitope specificity<sup>44</sup>. In contrast, ficolin-3, intelectin-1, and mindin preferentially recognized Mix1, indicating that longer O-antigen chains or additional surface glycans enhance lectin affinity<sup>45-49</sup>. Ficolin-3, an activator of the lectin complement pathway, likely recognizes acetylated sugar moieties such as GlcNAc<sup>45</sup>. Intelectin-1, known for its role in microbial glycan recognition, showed strong interaction with Mix1<sup>46,47</sup>. Interestingly, mindin, a matrix-associated pattern recognition receptor not previously reported to bind *P. hominis* LPS, exhibited robust interaction, suggesting the existence of non-canonical recognition mechanisms<sup>48,49</sup>.

This phenotype may facilitate microbial persistence in the aging host and contribute to age-associated conditions such as colitis and cognitive decline. While *P. hominis* LPS is a heterogeneous mixture, the observed effects likely result from the combined influence of multiple coexisting LPS species. Future studies dissecting individual LPS forms will be essential to fully elucidate host-microbe immune crosstalk.

## 5.4 Discussion and Conclusions

The complex interplay between the human host and its gut microbiota is increasingly recognized as crucial for maintaining health, especially in the context of the gut-brain axis and age-related disorders. Analogous to how plant root microbiomes influence host stress responses and development, the human gut microbiome profoundly shapes immune function, barrier integrity, and neurophysiology. A key question remains: how does the host discriminate between pathogenic and commensal microbes while preserving immune tolerance?<sup>8,41</sup>

This study provides insight by characterizing the structural and immunological features of lipopolysaccharide (LPS) from *Paenaltcaligenes hominis*, an emerging gut bacterium linked to cognitive decline and colitis in elderly individuals. Using an integrated suite of methods, including NMR spectroscopy, mass spectrometry, molecular modeling, light scattering, neutron reflectometry, and immunological assays, we reveal that *P. hominis* produces a structurally and functionally non-canonical LPS, markedly distinct from classical endotoxins such as *E. coli* LPS.

The *P. hominis* O-antigen consists of an uncommon trisaccharide repeating unit of L-rhamnose and D-glucosamine, capped by a terminal 3-O-methyl-L-rhamnose, with partial, non-stoichiometric O-acetylation adding complexity. The lipid A moiety is hypo-acylated, predominantly penta-acylated with minor tetra-acylated species, and the core oligosaccharide is truncated and relatively short. These structural features profoundly influence the molecule's interaction with host receptors, aggregation behavior, and membrane organization.

Functionally, *P. hominis* LPS exhibits markedly reduced TLR4 activation compared to canonical *E. coli* LPS, a difference attributable to its lower acylation pattern. It does not activate TLR2 and induces only moderate cytokine production in human peripheral blood mononuclear cells (PBMCs), with modest increases in TNF- $\alpha$  and IL-10, alongside decreased IL-12p70 and IL-17A. This cytokine profile suggests an immunomodulatory rather than pro-

inflammatory role, which may favor bacterial persistence within the aging gut, a milieu characterized by both immune tolerance and dysregulation<sup>8,41</sup>.

Molecular dynamics simulations indicate that the O-antigen adopts a flexible, loosely coiled conformation, largely unaffected by acetylation, supporting a dynamic shielding mechanism for underlying epitopes. Neutron reflectometry confirms that *P. hominis* LPS incorporates into asymmetric model membranes, generating highly hydrated, extended O-antigen layers (~90 Å) with loose packing, features compatible with lectin binding and potential immune evasion. Light scattering data reveals stable supramolecular aggregates comprising about 61 LPS units, which may facilitate effective molecular display at the host interface.

Lectin microarray analyses uncover selective interactions with innate immune lectins such as Ficolin-3, Galectin-4, and Mindin. Ficolin-3 preferentially binds longer O-antigen chains, while Galectin-4 targets shorter forms likely exposing core galactosyl residues. Mindin broadens the range of host immune sensors engaged by *P. hominis* LPS. These findings highlight a lectin-mediated recognition pathway distinct from classical TLR engagement, potentially underpinning the nuanced immune modulation observed in elderly hosts.

Taken together, these data position *P. hominis* LPS as a functionally specialized molecular pattern: structurally adapted to evade potent pro-inflammatory responses while allowing selective recognition by innate immune components. This balance may contribute to chronic low-grade inflammation in the elderly, influencing conditions such as colitis, cognitive decline, and gut-brain axis dysregulation<sup>3,13,14</sup>.

By dissecting the fine structural determinants underlying immune recognition of a non-canonical gut LPS, this work challenges traditional binary classifications of LPS as purely pathogenic or commensal. It underscores how subtle chemical modifications, O-methylation, hypo-acylation, partial acetylation, can profoundly reshape host-microbe interactions. As the gut-brain axis gains prominence in aging and neurodegeneration, these microbial signals open promising avenues for precision microbiome-based therapies, including the rational design of immunomodulatory glycoconjugates and microbiota-targeted interventions for age-associated diseases<sup>3,8</sup>.

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### General Conclusions

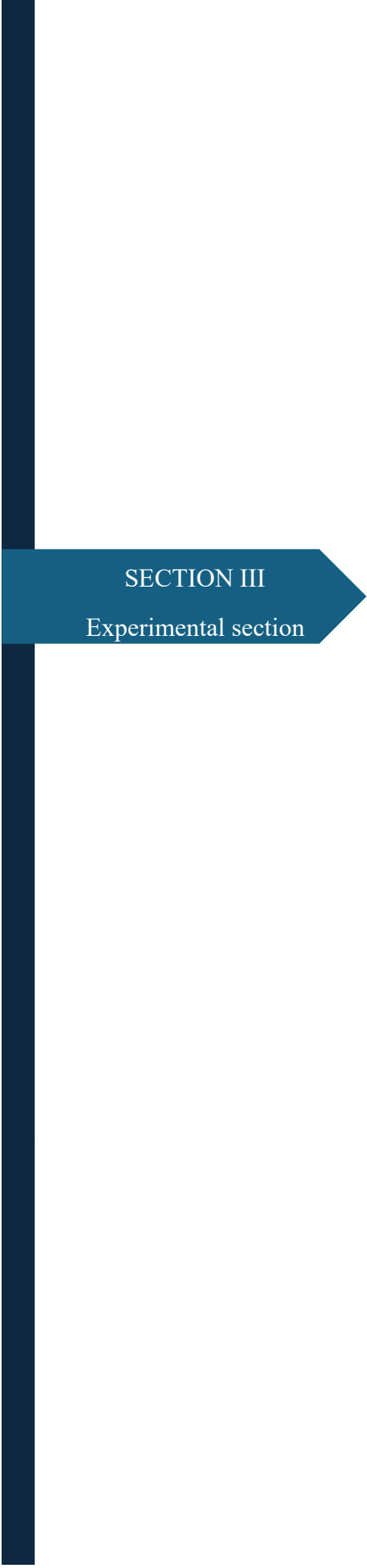
This Ph.D. thesis provides a comprehensive and multi-faceted characterization of bacterial surface glycoconjugates (LPS, LOS, and EPS) sourced from both plant-associated (*Herbaspirillum* Root189 and *Methylobacterium extorquens* PA1) and gut-associated (*Paenalcaldigenes hominis*) bacteria. By successfully integrating structural analysis (NMR, MS), biophysical characterization, and immunological assays, the work decisively demonstrates that subtle chemical modifications, such as methylation or acetylation, are critical modulators of host recognition and microbial fate.

Specifically, the studies yielded three high-impact contributions:

- ***Herbaspirillum* Root189 LPS:** Elucidating the molecular mechanisms of plant immune evasion, providing crucial structural blueprints for the design of next generation biofertilizers and sustainable agriculture strategies.
- ***Methylobacterium extorquens* PA1 EPS and LOS:** Identifying specific structural features linked directly to methanol tolerance, essential for optimizing green biotechnological applications and industrial processes based on C1-substrates.
- ***Paenalcaldigenes hominis* LPS:** Offering novel evidence that connects glycan structure to age-associated neuroinflammation, highlighting the potential of these molecules as therapeutic targets within glycomedicine and microbiome research.

Collectively, these findings significantly advance the fundamental field of glycobiology and microbial surface architecture, effectively bridging the gap between chemical structure and biological function across diverse ecological systems. This thesis establishes that bacterial glycans are central modulators of microbial fate and host response, providing both deep mechanistic insights and actionable strategies for leveraging the microbiome in agriculture, biotechnology, and medicine.

These conclusions confirm that the original aims of the thesis, linking bacterial glycan structure to host-microbe interactions, have been successfully achieved, demonstrating the relevance and impact of this work across multiple biological and applied contexts.



SECTION III

Experimental section

# CHAPTER VI

## Materials and Methods



## 6.1 Introduction

This chapter describes the materials, bacterial strains, and experimental methodologies used for the isolation, structural characterization, and functional analysis of surface glycoconjugates, lipopolysaccharides (LPS), lipooligosaccharides (LOS), and exopolysaccharides (EPS) from three bacterial strains of environmental and immunological interest: *Herbaspirillum Root189*<sup>1</sup>, *Methylobacterium extorquens*<sup>2,3</sup>, and *Paenalcaligenes hominis*<sup>4</sup>.

The techniques employed ranged from chemical-physical characterization (e.g., GC–MS, NMR, SLS/DLS), to the study of supramolecular properties (e.g., SANS, EPR), molecular dynamics simulations (MD), interactions with model membranes (e.g., NR), and immunological response assays in both plant and human cell models. The rationale for including these species lies in their biological relevance and the structural diversity of their respective polysaccharidic components. Methods were adapted to suit the specific structural features of each sample.

The methods described in this chapter were applied, unless otherwise specified, to all three bacterial species investigated in this study: *Herbaspirillum Root189* (Chapter III), *Methylobacterium extorquens* (Chapter IV), and *Paenalcaligenes hominis* (Chapter V). Common methodologies are reported in the main sections, while species-specific variations or procedures are clearly indicated within the relevant paragraphs.

## 6.2 Bacterial Strains and Growth Conditions

All bacterial strains used in this study were obtained from microbial culture collections or collaborating laboratories.

*Herbaspirillum Root189* (Chapter III) was cultured in nutrient broth under aerobic conditions at 30 °C with shaking at 180 rpm. Cells were harvested during the exponential growth phase for downstream LPS extraction.

For immunological assays, *Arabidopsis thaliana* Col-0 plants expressing cytoplasmic apoaequorin (Col-0AEQ) were employed as model hosts<sup>5</sup> Seeds were surface sterilized, dried, and sown on half-strength Murashige and Skoog (½ MS) medium (pH 5.7) supplemented with 0.5% sucrose and 0.4% Gelrite (Duchefa, Haarlem, Netherlands). After stratification for 3 days at 4 °C, seedlings were germinated under controlled climate conditions (8 h light / 16 h dark cycle, 22/18 °C, light intensity of 110  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) for 7 days.

For oxidative burst assays, 7-day-old seedlings were transferred into soil and grown for an additional three weeks. Leaf discs (3 mm) from 28-day-old plants were placed in white 96-well plates with 200  $\mu\text{L}$  of MilliQ water and incubated overnight. The next day, water was replaced with 100  $\mu\text{L}$  of fresh MilliQ water containing 20  $\mu\text{g/mL}$  horseradish peroxidase (Sigma-Aldrich, Germany) and 20  $\mu\text{M}$  L-012 (Wako Chemicals, Germany), followed by a 20-minute dark incubation. 100  $\mu\text{L}$  of double-concentrated LPS solutions (200  $\mu\text{g/mL}$ ) or 250 nM flg22 were then added, and chemiluminescence was measured immediately using a TECAN SPARK 10M microplate reader (450 ms integration time).

For calcium influx assays, 12-day-old seedlings were transferred to white 96-well plates containing 200  $\mu$ L of MilliQ water. Prior to overnight dark incubation, water was replaced with 150  $\mu$ L of 10  $\mu$ M coelenterazine (Roth, Germany). The following day, the plate was placed in a TECAN SPARK 10M reader. After a 5-minute baseline measurement, 50  $\mu$ L of fourfold-concentrated LPS or flg22 solutions were added, and luminescence was recorded for 30 minutes. Residual aequorin was discharged using 100  $\mu$ L of ethanol/CaCl<sub>2</sub> buffer, and the data were normalized to total detected chemiluminescence.

LPS preparations were solubilized at 1 mg/mL and stirred for 4 hours at room temperature before using in the assays. Final elicitor concentrations were standardized to 200  $\mu$ g/mL for LPS and 250 nM for flg22.

*Methylobacterium extorquens* AM1 and PA1 (Chapter IV) were cultivated in 10 L of succinate–methanol medium (30 mM succinate, 150 mM methanol, 30 mM NH<sub>4</sub>Cl, 0.81 mM MgSO<sub>4</sub>, 40  $\mu$ M Na<sub>2</sub>-EDTA, 11  $\mu$ M FeSO<sub>4</sub>, 16  $\mu$ M ZnSO<sub>4</sub>, 13  $\mu$ M CoCl<sub>2</sub>, 5  $\mu$ M MnCl<sub>2</sub>, 16  $\mu$ M H<sub>3</sub>BO<sub>3</sub>, 1.65  $\mu$ M Na<sub>2</sub>MoO<sub>4</sub>, 1.2  $\mu$ M CuSO<sub>4</sub>, and 20.4  $\mu$ M CaCl<sub>2</sub>, buffered with 10 mM phosphate at pH 6.7). Cultures were incubated at 28 °C with shaking at 150 rpm and harvested after four days at the late exponential phase. Cell pellets were obtained by centrifugation at 4,000  $\times$  g for 30 minutes and freeze-dried, yielding approximately 8 g of dried biomass.

*Paenalcogenes hominis* (Chapter V), initially stored in semisolid Gifu Anaerobic Medium (GAM; Nissui Pharmaceutical Co., Tokyo, Japan), was grown in 5 L of liquid GAM broth under strict anaerobic conditions at 37 °C for 18 hours until reaching stationary phase. Bacterial cells were collected by centrifugation at 5,000  $\times$  g for 20 minutes, washed twice with sterile ultrapure water, and lyophilized for further analysis.

All procedures involving bacterial isolates and plant materials were carried out in accordance with national and international biosafety and ethical guidelines. Ethical approvals were obtained from the appropriate institutional review boards where required.

### 6.3 Extraction and Purification of LPS, LOS and EPS

Two main procedures were employed for LPS extraction in this study. The first involved the preparation of a Phenol/Chloroform/Petroleum Ether (PCP) solution (2:5:8 v/v/v)<sup>6</sup>. Freeze-dried bacterial pellets were suspended in this solution at room temperature and stirred for 90 minutes. Afterward, the mixture was centrifuged at 8,000  $\times$  g for 30 minutes. The supernatant was transferred into a round bottom flask, and the extraction was repeated two additional times.

To isolate the phenol-soluble components, the combined PCP extracts were subjected to rotavapor step, during which chloroform and petroleum were removed. The remaining phenol solution was transferred into falcon tubes, a few drops of ultrapure water were added, and samples were centrifuged again (8,000  $\times$  g, 60 min, room temperature). The resulting precipitate, enriched in R-LPS (LOS), was collected for further purification.

The second method applied was the classic hot phenol/water extraction<sup>7</sup>, performed either after PCP treatment or directly on freeze-dried bacterial pellet. Samples were suspended in warm distilled water and mixed with an equal volume of phenol preheated to 70 °C. The mixture was stirred vigorously at 70 °C for 120 minutes and then centrifuged (8,000  $\times$  g, 30

min, 4 °C). The supernatant, formed by the water phase, was collected and the extraction was repeated two times more. Water and phenol phases were dialyzed separately against ultrapure water using 12–14 kDa cut-off dialysis tubing over 7 days and then freeze-dried.

Both fractions (water and phenol) were then subjected to enzymatic digestion with DNase I (Sigma-Aldrich, Darmstadt, Germany), RNase A (Sigma-Aldrich), and subsequently with proteinase K (Sigma-Aldrich) at 310 K and 329 K, respectively, to remove nucleic acid and protein contaminants<sup>8</sup>.

To assess the nature and purity of the extracted material, 13.5% SDS-PAGE gels were run and stained using silver nitrate<sup>9</sup>. Protein contamination was assessed with Coomassie Brilliant Blue G-250 staining (0.5% w/v in 40% methanol, 5% acetic acid)<sup>10</sup>.

In the case of *Paenaltcaligenes hominis* (Chapter V), additional purification steps were employed to ensure the complete removal of potential TLR2-stimulating contaminants and trace lipoproteins<sup>11</sup>. Following the standard extraction, nucleic acid and protein digestion, dialysis, ultracentrifugation, and chromatography, the LPS was washed extensively with chloroform–methanol (1:2, v/v) and chloroform–methanol–water (3:2:0.25, v/v/v) to eliminate residual phospholipids. After complete removal of the organic solvents, the sample underwent a repurification step according to Hirschfeld et al. (2000), designed to remove residual lipoproteins and abrogate unwanted TLR2-mediated signaling.

Further specific purification protocols were carried out for each project and are detailed in the following sections.

#### 6.4 Isolation of O-antigen, Core Oligosaccharide, and Lipid A

Isolation procedures were performed to separate and recover the core oligosaccharide (OS), O-antigen polysaccharide (OPS), and lipid A components from LPS or LOS preparations derived from *Herbaspirillum* Root189 (Chapter III), *Methylobacterium extorquens* (Chapter IV), and *Paenaltcaligenes hominis* (Chapter V).

For *Herbaspirillum* Root189 (Chapter III), the separation of lipid A and O-antigen polysaccharide was achieved via mild acid hydrolysis using acetate buffer (pH 4.4) at 100 °C for 5 hours. The resulting mixture was subjected to Bligh and Dyer extraction<sup>12</sup> to separate the hydrophilic carbohydrate moiety from the hydrophobic glycolipid (lipid A).

For *Methylobacterium extorquens* PA1 and AM1 (Chapter IV), an aliquot of LOS (approximately 40 mg) was subjected to O-deacylation by incubation with anhydrous hydrazine (2 mL, 37 °C, 90 min). The reaction mixture was cooled, precipitated with ice-cold acetone, centrifuged (5,000 × g, 30 min), and the pellet was washed with additional cold acetone, dried, dissolved in ultrapure water, and freeze-dried. The resulting O-deacylated sample was then N-deacylated using 4 M KOH at 120 °C for 16 hours. To eliminate salts and low-molecular-weight contaminants, gel filtration was performed on a Sephadex G-10 column (50 × 1.5 cm; Pharmacia), and the high-molecular-weight fractions containing the OS<sub>deAc</sub> material were collected and freeze-dried for structural characterization<sup>13</sup>.

For both *Methylobacterium extorquens* (Chapter IV) and *Paenaltcaligenes hominis* (Chapter V), lipid A and carbohydrate portions were separated through mild acid hydrolysis.

An aliquot (5 mg for *Methylobacterium*, variable for *Paenaltcaligenes*) was treated with acetic acid (100 °C, 4 h), followed by extraction with a CHCl<sub>3</sub>/MeOH/H<sub>2</sub>O mixture (100:30:30, v/v/v). The organic phases containing lipid A were collected, washed with distilled water or a Bligh-Dyer mixture (CHCl<sub>3</sub>/CH<sub>3</sub>OH/H<sub>2</sub>O, 2:2:1.8, v/v/v), and freeze-dried. The water phases containing O-antigen or core OS were freeze-dried and further purified when necessary.

For *Paenaltcaligenes hominis* (Chapter V), O-deacylation of LPS was performed by incubation with 1 M aqueous ammonia (400 µL, stirred overnight at 37 °C). After drying under a gentle air stream and freeze-drying, the LPS<sub>OdeAc</sub> fraction was obtained for downstream structural analyses.

These fractionation protocols enabled the selective isolation and purification of structurally distinct LPS/LOS components for in-depth chemical and functional studies.

## 6.5 Chemical analysis

### 6.5.1 Acetylated O-methyl glycosides (AMG)

An aliquot (~0.5 mg) of each purified sample (LPS, OS, or O-antigen) was subjected to methanolysis with 300 µL of 1.25 M HCl/CH<sub>3</sub>OH at 80 °C for 16 hours. The reaction mixture was neutralized by successive methanol washes. Subsequently, the released monosaccharides were acetylated by adding 25 µL of acetic anhydride and 25 µL of pyridine, incubated at 80 °C for 30 minutes. The acetylated O-methyl glycosides were extracted with chloroform and water, and the organic phase was separated and dried under a gentle stream of nitrogen.

The purified acetylated sugars were dissolved in 100 µL of acetone and analyzed by GC-MS to determine the monosaccharide composition<sup>14</sup>.

### 6.5.2 Partially methylated alditol acetates (PMAA)

For linkage analysis, approximately 1 mg of each sample was suspended in DMSO. Methylation was initiated by the addition of powdered NaOH and stirring at room temperature for 1.5 h, followed by methyl iodide (CH<sub>3</sub>I) addition to complete permethylation. After hydrolysis with 4 M trifluoroacetic acid (TFA) at 100 °C for 4 h, the sample was dried under airflow and neutralizing with methanol washes. Carbonyl groups were reduced using NaBD<sub>4</sub> in ethanol for 16 hours at room temperature. Reduction was stopped by adding a few drops of 2 M HCl and neutralized again with methanol. Acetylation was performed with pyridine (25 µL) and acetic anhydride (25 µL) at 80 °C for 30 minutes, and PMAA derivatives were analyzed by GC-MS for the determination of glycosidic linkage positions<sup>15</sup>.

### 6.5.3 Acetylated alditols (AA)

To determine neutral sugar composition an aliquot (~0.5 mg) of sample was hydrolyzed with 2 M TFA at 120 °C for 2 hours, then dried under airflow. Monosaccharides were reduced to alditols using NaBH<sub>4</sub> in ethanol. Alditols were acetylated with 25 µL of acetic anhydride and 25 µL of pyridine at 80 °C for 30 minutes. After drying, acetylated alditols were dissolved in 100 µL acetone and analyzed by GC-MS<sup>16</sup>.

### 6.5.4 Octyl-glycosides

Absolute configurations of monosaccharides were determined by derivatization to octyl glycosides. Aliquots (~1 mg) of the hydrolyzed samples were treated with 100  $\mu$ L of enantiopure 2-(+ or -)-octanol, while a parallel aliquot was treated with 100  $\mu$ L of racemic 2-( $\pm$ )-octanol. In both samples, 15  $\mu$ L acetyl chloride was added, and incubated at 60  $^{\circ}$ C for 16 hours. Samples were dried, acetylated with acetic anhydride in pyridine at 80  $^{\circ}$ C for 30 minutes, then analyzed by GC-MS and compared to authentic standards to assign absolute configurations<sup>17</sup>.

### 6.5.5 Gas Chromatography-Mass Spectrometry (GC-MS)

All chemical analyses were conducted using an Agilent Technologies 6850A gas-chromatograph equipped with a 5973N mass selective detector and a Zebron ZB-5 capillary column (30 m  $\times$  0.25 mm i.d., 0.25  $\mu$ m film thickness). Helium was used as the carrier gas at 1 mL/min. The temperature program for fatty acid (lipid) analysis was as follows: initial temperature of 140  $^{\circ}$ C (5 min), ramp to 280  $^{\circ}$ C at 10  $^{\circ}$ C/min, final hold at 280  $^{\circ}$ C for 10 min. For sugar derivatives, the program was: 150  $^{\circ}$ C (5 min), ramp to 280  $^{\circ}$ C at 3  $^{\circ}$ C/min, and hold at 280  $^{\circ}$ C for 5 min.

## 6.6 Nuclear Magnetic Resonance (NMR) methods

NMR spectroscopy was employed for the structural elucidation of isolated polysaccharide fractions (OPS, OS, or LPS) from *Herbaspirillum* Root189 (Chapter III), *Methylobacterium extorquens* (Chapter IV), and *Paenaltcaligenes hominis* (Chapter V).

All spectra were acquired in D<sub>2</sub>O at 298 K (pD ~7) with a cryoprobe-equipped Bruker 600 AVANCE NEO.

Total correlation spectroscopy (TOCSY) experiments were conducted with spinlock times of 100 ms using data sets ( $t_1 \times t_2$ ) of 4096  $\times$  512 points. Rotating-frame Overhauser enhancement spectroscopy (ROESY) and nuclear Overhauser enhancement spectroscopy (NOESY) experiments were performed using data sets ( $t_1 \times t_2$ ) of 4096  $\times$  512 points with mixing times ranging from 100 to 400 ms. Double-quantum-filtered phase-sensitive correlation spectroscopy (DQF-COSY) experiments were carried out using data sets of 4096  $\times$  912 points. The data matrix in all homonuclear experiments underwent zero-filling in both dimensions to obtain a matrix of 4 K  $\times$  2 K points. Subsequently, resolution enhancement was applied in both dimensions using a cosine-bell function prior to Fourier transformation. Coupling constants were determined using 2D phase-sensitive DQF-COSY measurements.

Heteronuclear single-quantum coherence (HSQC) and heteronuclear multiple-bond correlation (HMBC) experiments were performed in the <sup>1</sup>H-detection mode using single-quantum coherence with proton decoupling in the <sup>13</sup>C domain, employing data sets of 2048  $\times$  400 points. For HSQC, sensitivity improvement and phase-sensitive mode were used, together with echo/antiecho gradient selection and multiplicity editing during the selection step. In HMBC experiments, optimization was carried out for long-range coupling constants, incorporating a low-pass J filter to suppress one-bond correlations, and applying gradient pulses for selection. A 60 ms delay was applied for the evolution of long-range correlations, and HMBC spectra were optimized for coupling constants in the range of 6–15 Hz. To enhance

resolution, the data matrix in all heteronuclear experiments was extended to 2048×1024 points by means of forward linear prediction extrapolation<sup>18,19</sup>.

## 6.7 Pulsed-Field Gradient (PFG) NMR: *Methylobacterium extorquens* (Chapter IV)

Self-diffusion coefficient measurements<sup>20</sup> were performed exclusively for the EPS produced by *Methylobacterium extorquens* using Pulsed-Field Gradient (PFG) NMR. EPS samples were dissolved in pre-formed D<sub>2</sub>O/methanol mixtures with increasing methanol content (0%, 1%, 2%, 3%, 5%, and 8% v/v). The experiments were conducted using a cryoprobe-equipped Bruker 600 AVANCE NEO spectrometer at 298 K. The echo delay ( $\Delta$ ) was set to 150 ms for EPS and 80 ms for methanol, while the gradient pulse duration ( $\delta$ ) was fixed at 4 ms for EPS and 2 ms for methanol. A linear gradient ramp, incremented in 16 steps from 2% to 95% of the maximum gradient amplitude (5.35 G cm<sup>-1</sup>), was applied.

The resulting data were acquired as 2D NMR experiments, with baseline correction applied to the 1D FT <sup>1</sup>H spectra ( $F_2$  dimension). The diffusion dimension ( $F_1$ ) was subsequently processed using TopSpin software (version 4.1). Self-diffusion coefficients ( $D$ ) were calculated from the exponential decay of signal intensities, specifically at 1.24 ppm for EPS (methyl group at position 6 of rhamnose) and 3.26 ppm for methanol, according to the Stejskal–Tanner equation:

$$I = I_0 \cdot \exp [ - D \cdot (G \gamma \delta)^2 \cdot (\Delta - \delta/3 - \tau/2) ]$$

where  $I$  and  $I_0$  represent the signal intensities in the presence and absence of the magnetic field gradient, respectively,  $\gamma$  is the proton magnetogyric ratio, and  $\tau$  the gradient recovery delay.

## 6.8 MALDI-TOF Mass Spectrometry and MS

Mass spectrometry analyses were performed using different platforms depending on the bacterial species. For all experiments, spectra were recorded in both negative and positive ion modes, in reflectron or ultrahigh-resolution mode where appropriate. Each MS spectrum was acquired by accumulating 2000 laser shots, while MS/MS data were generated from 5000–7000 shots. All experiments were performed in technical replicates.

For *Herbaspirillum* Root189 (Chapter III), MALDI FT-ICR MS analysis was performed on the same 15 T solariX XR system. De-acetylated OPS was spotted onto a “ground steel” MALDI plate with 1  $\mu$ L of 1 mM NaCl and 1  $\mu$ L of super-DHB (9:1 DHB:2-hydroxy-5-methoxybenzoic acid in 50:50 ACN\water)<sup>21</sup>. Mass spectra were acquired from  $m/z$  3493–30,000 using 512 K data points (transient time 3.985 s), with laser power at 30% and 200 laser shots per spectrum.

For *Methylobacterium extorquens* (Chapter IV), OSdeAc samples were analyzed by direct infusion FT-ICR MS on a 15 T solariX XR (Bruker Daltonics) with a Combi-Source and ParaCell. Samples were diluted in 50:50 ACN\water and infused at 2  $\mu$ L/min (capillary voltage 4500 V). Mass spectra were acquired in the  $m/z$  range 92–5000 with 2 M data points. In-source CID was applied by ramping the skimmer voltage (15–100 V), and MS/MS analysis

was performed in the collision hexapole with optimized parameters. MALDI-CID FT-ICR MS was also conducted using DHB matrix, analyzing OSdeAc in positive ion mode in the  $m/z$  range 298.8–5000 with 1 M data points.

For *Paenaltcaligenes hominis* (Chapter V), intact LPS was suspended in 5 mM EDTA, vortexed, and briefly sonicated. The sample was desalted using Dowex 50X (NH<sub>4</sub><sup>+</sup> form) cation exchange beads and mixed with an equal volume of 2,5-dihydroxybenzoic acid (DHB) dissolved in 0.1% citric acid. A 1  $\mu$ L aliquot of the LPS/DHB mixture was deposited on a stainless-steel MALDI plate and air-dried. Lipid A was dissolved in CHCl<sub>3</sub>/CH<sub>3</sub>OH (1:1, v/v), and analyzed using 2,4,6-trihydroxyacetophenone (THAP) matrix (75 mg/mL in CH<sub>3</sub>OH/0.1% TFA/CH<sub>3</sub>CN, 7:2:1, v/v)<sup>22</sup>. OSdeAc fractions were mixed 1:1 with DHB matrix in 0.1% TFA/CH<sub>3</sub>CN (80:20), and spotted onto the MALDI plate. All spectra were acquired on an ABSCIEX TOF/TOF 5800 mass spectrometer equipped with an Nd:YAG laser ( $\lambda$  = 349 nm, 3 ns pulse, up to 1000 Hz).

Spectra were visualized using DataAnalysis 5.0 SR1 (Bruker), and theoretical  $m/z$  values of glycan fragments were calculated using GlycoWorkbench software.

## 6.9 Biophysical Characterization

A comprehensive biophysical characterization of the lipopolysaccharides (LPS) from *Herbaspirillum Root189* (Chapter III), *Methylobacterium extorquens* (Chapter IV), and *Paenaltcaligenes hominis* (Chapter V) was conducted using a combination of static and dynamic light scattering (SLS and DLS), neutron reflectometry (NR), small-angle neutron scattering (SANS) and electron paramagnetic resonance (EPR) spectroscopy. These techniques provided complementary insights into the molecular size, hydrodynamic properties, supramolecular organization, and membrane interaction dynamics of LPS samples

### 6.9.1 Static and Dynamic Light Scattering (SLS/DLS)

Static (SLS) and dynamic light scattering (DLS) measurements<sup>23</sup> were carried out to evaluate the hydrodynamic behavior and molecular parameters of the lipopolysaccharide (LPS) or lipooligosaccharide (LOS) aggregates. All experiments were conducted at 25.0  $\pm$  0.1  $^{\circ}$ C and at a fixed scattering angle of  $\theta$  = 90 $^{\circ}$ , using aqueous solutions filtered through 0.2  $\mu$ m membranes. Hydrodynamic radii (Rh) were determined from the translational diffusion coefficients (D) via the Stokes–Einstein equation. At least five independent measurements per sample were performed, and data were analyzed with the “Precision Deconvolve” software using a regularization-based fitting procedure<sup>24</sup>.

For *Herbaspirillum Root189* (Chapter III), SLS and DLS experiments were performed using a custom-built instrument equipped with a Photocor compact goniometer, a SMD 6000 Laser Quantum 50 mW laser (532.5 nm), a PMT-120-OP/B photomultiplier, and a Flex02-01D correlator (Correlator.com). The size of the LPS aggregates ( $\ll \lambda/10$ ) limited angular analysis; thus, SLS measurements focused solely on concentration dependence. The mass-average molecular weight (Mw) and second virial coefficient (B) were derived using Zimm plot analysis<sup>25</sup>. DLS correlation functions were evaluated with a modified version of CONTIN.

For *Methylobacterium extorquens* (Chapter IV), DLS analysis was conducted at 1.0 mg/mL under highly diluted aqueous conditions. The intensity autocorrelation function  $g^2(t)$  was related to the electric field autocorrelation function  $g^1(t)$  through the Siegert relation. A variation of the CONTIN algorithm was used to perform Laplace inversion, followed by regularization. Assuming  $\eta \approx \eta_\infty$  (infinite dilution approximation), Rh values were estimated with the Stokes–Einstein relation.

For *Paenaltcaligenes hominis* (Chapter V), measurements were carried out using an LSI spectrometer equipped with a fiber-coupled 638 nm laser source. DLS was performed at 1.0 mg/mL, and SLS over a concentration range of 0.1–1.0 mg/mL. The average Mw, radius of gyration (Rg), and second virial coefficient (A<sub>2</sub>) were obtained from Zimm plot fitting. DLS data were processed using the CORENN algorithm to calculate diffusion coefficients and corresponding Rh values under dilute condition assumptions.

### 6.9.2 Asymmetric Supported Lipid Bilayers and Neutron Reflectometry: *Herbaspirillum* Root189 (Chapter III) and *Paenaltcaligenes hominis* (Chapter V)

Asymmetric supported lipid bilayers incorporating lipopolysaccharides (LPS) from *Herbaspirillum* Root189 (Chapter III) and *Paenaltcaligenes hominis* (Chapter V) were constructed on polished single-crystal silicon substrates using a sequential Langmuir–Blodgett (LB) and Langmuir–Schaeffer (LS) deposition protocol. In both cases, the LB technique was employed to transfer the inner monolayer onto the hydrophilic silicon surface by vertically lifting the substrate through a compressed lipid or LPS film at the air–water interface. The LS method was subsequently used to deposit the outer leaflet by horizontal contact of the bilayer with a second monolayer at the interface.

For *Herbaspirillum* Root189 (Chapter III), both bilayer leaflets were composed of LPS<sup>24</sup>. The LB deposition transferred the LPS monolayer directly from the subphase onto the silicon surface, while the LS step completed the bilayer with a second LPS monolayer on the outer leaflet. Specific details on buffer conditions and monolayer preparation are provided in the Supporting Information.

In the case of *P. hominis* (Chapter V), an asymmetric architecture was used in which the inner leaflet consisted of fully deuterated dipalmitoylphosphatidylcholine (d-DPPC), deposited from a chloroform solution onto a pure water subphase containing 5 mM CaCl<sub>2</sub> at 10 °C and compressed to a surface pressure of 35 mN·m<sup>-1</sup>. The silicon substrate was lifted vertically at a constant speed of 4 mm/min. The outer leaflet was subsequently formed via LS deposition by transferring a monolayer of *P. hominis* LPS, prepared from a phenol:chloroform:petroleum ether solution (2:5:8, v/v), under identical surface pressure conditions.

Specular neutron reflectometry (NR) experiments for both bilayer systems were carried out using the INTER reflectometer at the ISIS Neutron and Muon Source (Didcot, UK). A broad-spectrum white neutron beam with wavelengths from 1 to 16 Å enabled reflectivity data collection over a wide momentum transfer range (Qz) by measuring at two glancing angles, 0.7° and 2.3°.

For *P. hominis* (Chapter V), isotopic contrast variation was introduced to enhance structural resolution of the bilayer components. This was achieved by sequentially perfusing the solid–liquid interface with buffers containing H<sub>2</sub>O, D<sub>2</sub>O, or silicon-matched water (SMW, 38% D<sub>2</sub>O/62% H<sub>2</sub>O) using a Knauer Smartline 1000 liquid chromatography pump at a flow rate of 1.5 mL·min<sup>-1</sup>. A total of ~15 mL of each contrast buffer (corresponding to five times the internal volume of the flow cell) was used to ensure complete exchange.

NR data from both systems were analyzed using RasCal (version 2019, A. Hughes, ISIS), which applies the optical matrix formalism derived from the Abeles model. Each interfacial architecture was modeled as a sequence of discrete slabs characterized by their scattering length density (SLD), thickness, and interfacial roughness. Fitting was performed via least-squares minimization by comparing the calculated reflectivity profiles to experimental data.

Uncertainty estimation was conducted using RasCal's built-in Bayesian framework based on a Delayed Rejection Adaptive Metropolis (DRAM) algorithm. Posterior distributions were generated for each fitted parameter, and best-fit values were reported as the maxima of the marginalized distributions with associated 95% confidence intervals.

### 6.9.3 Small-Angle Neutron Scattering (SANS): *Methylobacterium extorquens* (Chapter IV)

Small-Angle Neutron Scattering (SANS) was employed to characterize the supramolecular architecture of *Methylobacterium extorquens* (Chapter IV) LPS aggregates in aqueous environments. Experiments were performed on the D11 instrument at the Institut Laue-Langevin (ILL, Grenoble, France), using neutrons with a wavelength spread  $\Delta\lambda/\lambda \leq 0.09$ . Data were collected using three Wavelength/Collimation/Detector (W/C/D) configurations, covering a scattering vector range ( $q = 4\pi/\lambda \sin(\theta/2)$ ) between 0.008 and 0.5 Å<sup>-1</sup>. Samples were loaded into sealed quartz cuvettes to ensure stability and prevent evaporation.

Scattering profiles  $I(q)$  were corrected for background, empty cell, and incoherent contributions. Data analysis was carried out using SasView (version 5.0), employing a vesicle model suitable for bilayered unilamellar structures. The model decomposed  $I(q)$  as:  $I(q) = \phi_p V_p P(q) S(q) + \text{bkg}$

where  $\phi_p$  is the particle volume fraction,  $V_p$  the particle volume,  $P(q)$  the form factor describing vesicle shape and bilayer structure, and  $S(q)$  the structure factor accounting for interparticle interactions. The background (bkg) reflects inelastic scattering primarily due to hydrogen atoms.

The vesicle model featured two concentric shells: an inner core (radius  $R_{\text{core}}$ ) and a surrounding bilayer shell ( $R_{\text{tot}}$ ), from which the membrane thickness ( $R_{\text{tot}} - R_{\text{core}}$ ) was derived. Form factors included spherical Bessel functions and SLD contrasts among solvent (D<sub>2</sub>O), lipid bilayer, and LPS core domains.

LPS-containing vesicles were generated by co-dissolving POPE and POPG lipids (Avanti Polar Lipids, purity >99%) in chloroform at a 70:30 molar ratio and combining them with *Methylobacterium extorquens* LOS, also dissolved in chloroform, to achieve an overall phospholipid-to-LOS molar ratio of 70:30. The resulting mixtures were dried under a gentle

nitrogen stream followed by vacuum desiccation to form thin lipid films. These were subsequently rehydrated in D<sub>2</sub>O, vigorously vortexed to obtain multilamellar vesicle suspensions, and then extruded 25 times through 100 nm polycarbonate membranes to yield unilamellar vesicles. Comparative structural analysis with control POPE/POPG vesicles (previously characterized on the KWS-2 instrument at the Heinz Maier-Leibnitz Zentrum, Garching) enabled assessment of the impact of LOS incorporation on bilayer curvature, thickness, and morphology.

#### 6.9.4 Electron Paramagnetic Resonance (EPR): *Methylobacterium extorquens* (Chapter IV)

For EPR spectroscopy<sup>27</sup>, 2% by weight of spin-labeled phosphatidylcholine (5-PCSL, Sigma-Aldrich) was added to lipid mixtures containing POPE, POPG, and *Methylobacterium extorquens* LPS (Chapter IV). The components were co-dissolved in chloroform, and the solvent was evaporated under a stream of nitrogen, followed by vacuum desiccation to form a thin lipid film. The dried films were rehydrated with either D<sub>2</sub>O or a D<sub>2</sub>O/methanol mixture (9% vol MeOH) to a final lipid concentration of 1 mg/mL and vortexed to produce multilamellar vesicle suspensions. These labeled vesicles were analyzed using a Bruker Elexys E-500 X-band spectrometer (9 GHz) at 25 °C. EPR spectral analysis enabled the assessment of local lipid order and membrane fluidity, providing detailed insights into the dynamic organization and biophysical behavior of LPS within biomimetic membrane environments.

This integrated biophysical approach supports the structural and functional characterization of bacterial LPS aggregates, enhancing our understanding of their supramolecular assembly in aqueous environments and their interaction within lipid bilayers.

#### 6.10 Microarray Binding Assays: *Herbaspirillum* Root189 (Chapter III) and *Paenaltcaligenes hominis* (Chapter V)

Microarray binding assays were conducted to investigate the interaction profiles of *Herbaspirillum* Root189 (Chapter III) and *Paenaltcaligenes hominis* (Chapter V) lipopolysaccharides (LPS) with a diverse set of lectins and innate immune receptors. For both species, LPS samples were arrayed on 16-pad nitrocellulose-coated glass slides (ONCYTE NOVA, Grace Biolabs) using a non-contact microarrayer (Sprint, Arrayjet Ltd)<sup>28</sup>. *Herbaspirillum* Root189 LPS was spotted in triplicate at six concentrations ranging from 1 to 0.003 mg/mL, while *P. hominis* LPS preparations (Mix1 and Mix2) were printed in duplicate at four concentrations (1 to 0.03 mg/mL). A selection of control glycoproteins, fetuin, asialofetuin, ribonuclease B, and ribonuclease A, was also included on all arrays<sup>29</sup>. All LPS and protein solutions were supplemented with Cy3 fluorophore (1 µg/mL, GE Healthcare) to enable quality control through fluorescence signal scanning at 532 nm using a GenePix 200-AL scanner (Axon, Molecular Devices).

Slides were blocked for 1 hour using either PBS- or TBS-based buffers containing 0.25% (v/v) Tween-20, followed by incubation with lectins. *Herbaspirillum* arrays were incubated with a panel of 35 biotin-labeled plant and microbial lectins (10 µg/mL), while *P. hominis*

arrays were probed with recombinant human innate immune lectins, including Galectins-1, -3, -4, -7, -8, and -9, Ficolins-1, -2, and -3, Collectin-11, Mindin, Intelectin-1, and Siglec-9.

For the *P. hominis* (Chapter V) assays, His-tagged lectins were applied at 20 µg/mL in PBS containing 0.1% Tween-20, with 4 mM β-mercaptoethanol added for galectins. Non-His-tagged Galectin-1 was detected using a biotinylated rabbit anti-human galectin-1 antibody. After incubation and washing, bound lectins were visualized with AlexaFluor-647 (AF647)-labeled streptavidin (1 µg/mL, Invitrogen), and arrays were scanned for fluorescence at 635 nm (red laser). Detection of lectin binding was further validated using goat anti-lipid A antibodies (Abcam), revealing comparable reactivity between the two *P. hominis* LPS mixes. Negative controls included full antibody cascades applied to uncoated spots, which produced no detectable signal, confirming the specificity of the assay. Data analysis and quantification of fluorescence intensity were performed using GenePix Pro 7 software.

These assays provided valuable insights into the glycan-binding properties of the studied LPS structures and their potential recognition by host immune receptors.

### 6.11 Immunological Assays with *Paenalcaligenes hominis* LPS (Chapter V)

To evaluate the immunomodulatory potential of *P. hominis* lipopolysaccharides (LPS), both primary human immune cells and engineered cell lines were employed. Peripheral blood mononuclear cells (PBMCs) were isolated from six healthy adult donors (three males and three females, median age 32), recruited under a protocol approved by the IRCCS Pascale Institutional Ethical Review Board (Protocol no. 4/21, 2021). PBMCs ( $5 \times 10^5$  cells per well) were incubated overnight at 37 °C in complete DMEM medium with 200 ng/mL of *P. hominis* O-antigen. Following stimulation, supernatants were collected and cytokine levels were quantified using the LEGENDplex™ Human Inflammation Panel (13-plex, BioLegend), according to the manufacturer's instructions.

Additionally, functional stimulation assays were carried out using HEK-Blue™ cell lines expressing either human Toll-like receptor 4 (hTLR4) or TLR2. Cells were seeded into 96-well plates at a density of  $3 \times 10^4$  cells per well and allowed to adhere overnight. Subsequently, cells were treated for 18 hours with either *P. hominis* LPS or *E. coli* O111:B4 LPS (positive control for TLR4 activation). In parallel, HEK-Blue hTLR2 cells were stimulated with 500 ng/mL PAM3CSK4, a known TLR2 agonist. Activation of NF-κB signaling was assessed by measuring secreted embryonic alkaline phosphatase (SEAP) in cell supernatants using the Quanti-Blue™ detection assay (InvivoGen), following the manufacturer's instructions. SEAP levels served as a proxy for TLR-mediated NF-κB activation in response to LPS stimulation.

### 6.12 Molecular Modelling and Molecular Dynamics Simulations

To explore the conformational preferences and dynamic behavior of lipopolysaccharide (LPS) and extracellular polysaccharide (EPS) fragments from *Herbaspirillum Root189* (Chapter III), *Methylobacterium extorquens* (Chapter IV), and *Paenalcaligenes hominis* (Chapter V), a combined molecular mechanics (MM) and molecular dynamics (MD) approach was employed. MM calculations were carried out using the MM3\* force field implemented in MacroModel 8.0 (Schrödinger Inc.), applying a dielectric constant of 80 to simulate aqueous

conditions. For each representative disaccharide, the  $\Phi$  (phi) and  $\Psi$  (psi) glycosidic torsion angles were scanned in  $18^\circ$  increments across the full range, and each point on the resulting grid was energy minimized using 2000 steps of Polak–Ribiere conjugate gradient optimization. This allowed the generation of adiabatic energy maps for each glycosidic linkage, providing insight into the preferred conformational spaces of the oligosaccharides.

MD simulations were performed using the AMBER18 software suite<sup>30</sup>, with carbohydrate parameters derived from the GLYCAM-06j-1 force field. Each glycan structure was solvated in an explicit solvent box using TIP3P water molecules, with a buffer of 10–15 Å from solute atoms to the box edges. To ensure electroneutrality, counterions were added using the Leap module. For simulations involving EPS from *Methylobacterium*, a mixed solvent environment (water:methanol 9:1) was used, generated via the Packmol-Memgen protocol. After solvation, all systems underwent energy minimization in multiple stages, beginning with heavy solute restraints ( $100 \text{ kcal/mol} \cdot \text{Å}^2$ ) that were progressively reduced to zero.

Thermal equilibration was conducted by gradually heating the systems from 0 to 300 K: initially under constant volume conditions (0–100 K), followed by constant pressure conditions (100–300 K). The SHAKE algorithm was applied to constrain all bonds involving hydrogen atoms, enabling a 2 fs integration time step (1 fs for *Paenaltcaligenes*). Simulations were carried out under periodic boundary conditions with long-range electrostatics treated via particle mesh Ewald summation. Equilibrated systems were simulated under isothermal–isobaric conditions ( $T = 300 \text{ K}$ ,  $P = 1 \text{ atm}$ ), employing the Langevin thermostat with a collision frequency of  $1.0 \text{ ps}^{-1}$ .

For each system, trajectory frames were collected every picosecond, yielding 10,000 structures per production run. Trajectories were analyzed using the ptraj or cpptraj modules in AMBER18<sup>31</sup>, assessing structural parameters such as RMSD, hydrogen bonding patterns, radius of gyration, and torsional fluctuations. Clustering analysis based on glycosidic RMSD was applied to identify dominant conformational ensembles. Visualization of molecular structures and dynamic trajectories was performed with VMD<sup>32</sup>, 2.3 Software (<https://pymol.org/2/>), and Discovery Studio<sup>33</sup>. Energy profiles and conformational distributions were plotted using SigmaPlot (Systat Software, San Jose, CA).

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