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Cédric Laguri, Alba Silipo, Alessandra M Martorana, Paul Schanda, Roberta Marchetti, et al.. Solid State NMR Studies of Intact Lipopolysaccharide Endotoxin. ACS Chemical Biology, 2018, 13 (8), pp.2106 - 2113. 10.1021/acschembio.8b00271 . hal-01875923

HAL Id: hal-01875923

<https://hal.univ-grenoble-alpes.fr/hal-01875923>

Submitted on 22 Oct 2021

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Solid State NMR studies of intact lipopolysaccharide endotoxin

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ABSTRACT

Lipopolysaccharides (LPS) are complex glycolipids forming the outside layer of gram-negative bacteria. Their hydrophobic and heterogeneous nature greatly hampers their structural study in an environment similar to the bacterial surface. We have studied LPS purified from *E. coli* and pathogenic *P. aeruginosa* with long O-antigen polysaccharides assembled in solution as vesicles or elongated micelles. Solid-state NMR with Magic-angle spinning permitted the identification of NMR signals arising from regions with different flexibilities in the LPS, from the lipid components to the O-antigen polysaccharides. Atomic scale data on the LPS enabled to study the interaction of gentamicin antibiotic bound to *P. aeruginosa* LPS for which we could confirm that a specific oligosaccharide is involved in the antibiotic binding. The possibility to study LPS alone and bound to a ligand when it is assembled in membrane like structures opens great prospects for the investigation of proteins and antibiotics that specifically target such an important molecule at the surface of gram-negative bacteria.

INTRODUCTION

Gram negative bacteria lipopolysaccharides (LPSs) are key microbial macromolecules involved in a plethora of functions in prokaryotes and in the elicitation of innate immune response in insects,

animals and plants^{1,2}. Gram negative microbes possess a double system of membranes (Figure 1) in which LPSs are only present on the outer leaflet of the external membrane but in great abundance.

Figure 1 Localization and structure of lipopolysaccharides. LPSs constitute the major component of outer-membrane (OM) external leaflet of gram-negative bacteria (left). LPS is composed of three moieties, the lipid A, core oligosaccharides and O-antigens(right). PG Peptidoglycan, IM Inner-membrane.

LPS is composed of a highly conserved hydrophobic lipid A moiety, a core oligosaccharide (core OS), and in many species a long-chain O-antigenic polysaccharide (Figure 1). Gram negative bacteria are more resistant to antibiotic treatment mainly due to their highly impermeable outer membrane where LPS is considered as the key actor in the membrane integrity³. Indeed, the LPS layer is highly ordered and is stabilized by electrostatic interactions between divalent cations (as Ca^{2+} and Mg^{2+}) and phosphate groups of the lipid A part. This low fluidity of the layer confers to the outer membrane a low permeability to hydrophobic compounds and to higher molecular weight hydrophilic compounds. Only certain antibiotics directed against Gram-negative bacteria, such as polymyxin B or gentamicin, are able to destabilize the outer membrane leading to the disruption of the membrane integrity⁴.

LPS is also a bacterial hallmark, a microbe associated molecular pattern, which is specifically recognized by eukaryotic immunity cell line receptors as a microbial signature^{1,2}. For these organisms, this preliminary step of the innate immunity process, serves to trigger the response in case of pathogenicity or to shut down the response itself in case of beneficial interactions, such as

symbiosis. In animals/humans, LPS is extracellularly detected by its lipid A⁵ at the level of immunity cell lines by a binary protein system (TLR4/MD2) which upon physical binding triggers the downstream reaction cascade¹. Moreover, caspase-4/5 in humans and caspase-11 in mice detect intracellular LPS thus, likely being in direct binding and detection of intracellular pathogens⁶.

To understand these different biological processes at the molecular level, it is important to have methods to characterize macromolecular organization formed by LPSs in the membrane or in interaction with other proteins and/or ligands. However, because of their glycolipid nature they are generally neither soluble in water nor soluble in any organic solvents hampering the structural and functional characterization of LPSs in their intact state. The determination of the primary structure of a LPS is nowadays carried out separately on lipid A and O-antigen, chemically split in advance, by means of chemical analysis and biophysical methodologies (Gas Liquid Chromatography coupled to Mass spectrometry, GLC-MS; Mass Spectrometry, MS and solution-state Nuclear Magnetic Resonance, NMR)^{7,8}. Intact LPS molecules have been previously investigated, some of them also by NMR but no systematic approach has been adopted so far⁹⁻¹¹. The most exhaustive NMR analysis of intact LPS molecules including 3D structure determination and dynamics were conducted with LPS solubilized in detergent micelles^{12,13}.

While these methods are efficient in attaining chemical structural studies of LPSs, there is a lack of alternative approaches able to study interaction of key molecules (receptors/antibiotics) with membrane surfaces formed by LPS. In fact, it is very important in this perspective to have a thorough view at molecular level at the whole intact molecule, at its overall supramolecular structure, at its delivery and at its atomic epitopes when bound and/or recognized by other ligands.

Atomic scale analysis of cell-surface glycoconjugates is particularly challenging. Magic Angle Spinning (MAS) solid state NMR (ssNMR) has provided in the last years interesting information on complex macromolecules alone or in complex with ligands (ions/proteins)¹⁴⁻¹⁸.

Therefore, we have deemed that ssNMR could provide the missing information on the different supramolecular organizations adopted by these molecules and we have undertaken the study of intact

LPS in an environment close to the bacterial membrane. To this aim, in the following work we studied LPS extracted from different bacterial strains and with the two different molecular arrangements, a rough type LPS (a lipooligosaccharide, R-LPS) from *E. coli* K-12 and a smooth-type LPS (S-LPS) from *P. aeruginosa* PAO1 strains (Figure 1). The analysis of LPSs by ssNMR indeed enabled to observe the main characteristics of both LPS molecules, i.e., comparable to the data obtained when LPS is chemically split and then analyzed by NMR. Moreover, in a proof of principle we also studied PAO1 LPS when bound to gentamicin; this latter approach revealed interesting molecular information confirming the involvement of the O-antigen in the intake of the antibiotic by bacteria.

On this ground, we estimate that ssNMR approach is a very useful and very informative technique to observe the behavior of different LPSs in their intact chemical form and when bound to prokaryotic or eukaryotic ligands.

RESULTS AND DISCUSSION

Characterization of lipid A and core oligosaccharide from rough-type *E. coli* K12 LPS.

E. coli K12 cells were grown in media containing ^{13}C as the sole carbon source and LPS molecules extracted and purified (see Methods). Freeze dried LPS molecules were re-suspended in water and, prior to conducting the NMR analysis, the macromolecular state of LPS was investigated by electron microscopy. Spontaneously *E. coli* R-LPS assembled into vesicles of 100-200 nm diameter with lipid bilayers of 26 ± 4 nm that were protected from staining (Figure 2).

Figure 2 Characterization of *E. coli* R-LPS reconstituted in solution by a) Negative stain Electron

microscopy and b) 1D ^{13}C CP-MAS ssNMR spectrum recorded on a 600 MHz spectrometer and at a spinning rate of 12.5kHz.

The hydrated LPS has been then pelleted into a NMR rotor, of either 1.6 or 3.2 mm diameter and ssNMR experiments were recorded. Figure 2 shows a 1D ^{13}C spectrum of *E. coli* R-LPS that features sharp NMR signals representing the different chemical groups of the LPS. To obtain site-specific information, we collected 2D ^{13}C - ^{13}C correlation experiments, exploiting either the dipolar coupling between the ^{13}C spins (using a Dipolar Assisted Rotational Resonance or DARR experiment) or the scalar coupling (with a heteronuclear Insensitive Nuclei Enhanced by Polarization Transfer INEPT-based experiment), respectively (Figures 3 and Supplementary Figure 1). While the former experiment was able to dissect the rigid moiety of the molecule, the latter was more sensitive to flexible parts of LPS (Supplementary Figure 1). Proton LPS resonances are too broad to be observed at low spinning speed but could be detected at 34kHz MAS rotation in a ^{13}C - ^1H INEPT experiment.

shown in c) in black and green respectively. *P*-EtN and *P*-Gly Phospholipids resonances are also indicated. CH₂x stands for CH₂ at the middle of the lipid chains that are all chemically equivalent.

Resonances from most residues of *E. coli* LPS could be assigned and were in good agreement with those reported by liquid-state NMR (Supplementary Table 1). Signals for the two GlcN (glucosamine) of lipid A, straight assignments of the acyl parts of the molecules and parts of the core region including Kdo (keto-deoxyoctulosonate) and Hep (heptose) could be achieved (Figure 1). Lipid contaminants, probably tightly associated with LPS, were also identified, as cyclopropane modifications, normally present only on phospholipids. Insaturations, potentially occurring either on LPS fatty acids or on phospholipids acyl chains were assigned, as well as the two major phospholipids composing *E. coli* outer membrane inner leaflet, namely phosphatidyl ethanolamine and phosphatidyl glycerol. Peptidoglycan, another major component of the cell envelope, could also be detected, including GlcNAc and MurNAc (N-acetyl muramic acid) backbone sugars of the macromolecule, as well as characteristic Lactoyl signals (highlighted in yellow in figure 3). It is not unusual that peptidoglycan is associated with LPS preparation, presumably because of co-purification with Braun's lipoprotein that anchors PG in the outer membrane¹⁹.

Characterization of core oligosaccharide and O-antigen from *P. aeruginosa* PAO1.

Since rough type K-12 R-LPS from common laboratory strains could be successfully analyzed by ssNMR, we investigated a wild-type S-LPS extracted from the pathogenic *P. aeruginosa* PAO1 strain. This microbe is responsible for severe infections in immunocompromised patients and in particular in cystic fibrosis, and possesses variable LPS O-antigens²⁰. Indeed, *Pseudomonas* LPS are composed of the same lipid A-core oligosaccharide skeleton but to which two different O-antigens are appended termed A and B bands²⁰ (Supplementary Figure 1 and Figure 4 panel a). A-band O-antigen is based on a D-rhamnose repeating unit while B-band is based on a complex trisaccharide unit with two *N*-acylated Mannuronic acids (ManNAc3NAcA or 3 AmA) and a FucNAc (Figure 4 panel a and Figure 5). These two O-antigen polysaccharides are simultaneously present at the surface

of the bacteria.

Figure 4 MAS ssNMR O-antigen from *P. aeruginosa* PAO1. a) Chemical structure of *P. aeruginosa* PAO1 S-LPS. b) EM negative stain micrograph of PAO1 LPS. c) extract of a ^{13}C - ^{13}C Single Quantum J correlation spectrum of *P. aeruginosa* LPS with the assigned resonances showing clearly the O-antigen repeat (labeled Fuc1-Man2-Man3) and several resonances from sugars of the extended core. * Artefacts

^{13}C -labeled PAO1 LPS was resuspended (see Methods) and first analyzed by Electron microscopy. The S-LPS was morphologically different from the *E. coli* R-LPS and formed elongated structures of various length of approximately 8 nm width (Figure 4 panel b). Formation of these elongated structures has been already observed for *N. meningitis* purified LPS²¹. Their shape suggested they assemble into elongated micelles, rather than vesicles, presumably because of their long hydrophilic O-antigen. The presence of the O-antigen might also explain the gel-like behavior of *P. aeruginosa* LPS in solution. Consequently, PAO1 LPS had to be centrifuged at 50000g overnight to be pelleted inside the NMR rotor.

Conversely to the *E. coli* R-LPS NMR experiments, only the last 4 carbons of the lipid chains of the PAO1 LPS could be observed (1 CH_3 and 3 CH_2 see Supplementary Table 2) and only few resonances were unambiguously assigned to the inner core oligosaccharides, namely to Kdo and heptose. Residues from the core were particularly visible in dipolar coupling-based experiments, suggesting they belonged to rigid regions of the LPS. Similarly, one rhamnose spin system with chemical shifts matching those of the outer core 3-substituted α -Rha could be identified as well as alanine residues, probably bound to galactose residues (Figure 4)²².

The ssNMR analysis of *P. aeruginosa* LPS showed also the signals of the major B-band O-

antigen trisaccharide α -FucNAc- β -ManNAc3NAcA- β -ManNAc3NAcA repeating unit for which most resonances were unambiguously assigned (Figure 4 and Supplementary Figure 2). The O-antigen was also observed in dipolar-coupling based experiments thus suggesting a significant degree of order in the O-antigen chains, possibly through lateral contacts between O-antigen chains. These last data are in agreement with what observed with gel forming glycan polymers such as maltodextrins that exhibit the same behavior with a degree of order that allows to efficiently transfer the NMR magnetization through dipolar couplings using Cross Polarization (CP) experiments²³.

D-rhamnose A-band repeating unit could not be detected unambiguously at a first glance since rhamnose also composes the outer core oligosaccharide common to both O-antigens. However, one sugar spin system with a lowfield displaced C-2 chemical shift could be unambiguously assigned to a 2-substituted rhamnose residue and cross peaks were assignable up to the methyl group (Figure 4 and Table S2). Several resonances, probably corresponding to 3-substituted rhamnose could be identified in the spectra but only up to the C-2'/H-2 groups, because of severe signals overlapping. Presence of A-Band polysaccharide could be confirmed by the presence of O-methylation (¹³C/¹H resonances at 57.4ppm/3.75ppm) characteristic of O-methyl rhamnose residue found in A-Band O-antigen²⁴. Nevertheless, this latter residue could not be assigned completely due to the lack of long-range distance correlations.

Interaction of *P. aeruginosa* LPS with gentamicin antibiotic.

LPS from PAO1 is a critical factor of virulence, which is particularly harmful in immunocompromised patients as those affected by cystic fibrosis. While A and B band O-antigens are found in patients at early onsets of the infection, only A band polysaccharide can be detected in chronic isolates of *P. aeruginosa*²⁵. Furthermore engineered *P. aeruginosa* A⁺B⁻ bacteria were found to be less sensitive to gentamicin antibiotic, because of a higher affinity of gentamicin for bacteria presenting B-band O-antigen. Nevertheless, the molecular basis of mechanism remains unknown^{26,27}. Based on such previous studies, we decided to investigate the effect of gentamicin on isolated and

intact PAO1 LPS. Gentamicin is a natural antibiotic from the aminoglycosides family produced by *M. echinospora*, a Gram-positive bacterium (Figure 5). *In vitro* gentamicin very efficiently binds to isolated PAO1 LPS and this can be easily observed by solution NMR. Addition of LPS to gentamicin in solution leads to total disappearance of its NMR signals (Supplementary Figure 4), showing that all free gentamicin in solution have bound to LPS. We then used electron microscopy to assess the influence of gentamicin binding on LPS supramolecular organization. Addition of 25mM final concentration of gentamicin to PAO1 LPS did not significantly change the elongated micelles structures of isolated PAO1 LPS in solution (Figure 5 panel a).

Figure 5 *P. aeruginosa* LPS O-antigen is affected by interaction with gentamicin. a) EM pictures with negative staining of *P. aeruginosa* LPS in absence (up) or presence of 25mM Gentamicin (down). b) ^{13}C - ^1H INEPT ssNMR spectrum of LPS in absence or presence (green) of 25mM gentamicin. Spectra are normalized to the intensity of lipid CH_3 peak. 1D slices of Lipid A CH_3 and fucose CH_3 peaks are shown above the spectrum. c) Ratio of peak intensities $I_{\text{gentamicin}}/I_0$ measured in the ^{13}C - ^1H INEPT experiment. d) Chemical structures of B-Band, A-Band trisaccharide repeats and of gentamicin.

In absence of supramolecular reorganization, ssNMR approach was used to investigate the gentamicin interaction with LPS at an atomic resolution. 25mM gentamicin was added to a 40mg/ml PAO1 LPS solution which was ultracentrifuged into the NMR rotor (see Methods). ^{13}C - ^{13}C J-correlation experiments as well as ^{13}C - ^1H INEPT experiments were thus recorded at 34kHz MAS in presence of gentamicin. Gentamicin bound LPS produced a similar spectrum as free PAO1 LPS, showing mostly the O-antigen repeats and the last 4 CH groups of the lipid A part. Small chemical shift variations are observed for the polysaccharides resonances (Supplementary Figure 5), but not for the resonances of the lipid A (Figure 5 panel b). Comparison of the peak intensities before and after addition of gentamicin also reveals that the lipid A resonances are not affected by the gentamicin interaction (Figure 5 panel b). However, polysaccharide resonances of the O-antigen are significantly decreased in intensity in presence of gentamicin. Interestingly, the presence of gentamicin has even a stronger effect on the resonance intensities corresponding to the B-band polysaccharides relative to the saccharides of the A-Band (Figure 5 panels b and c).

This specific decrease in intensity observed for the O-antigen, and more specifically for the saccharide present in the O-antigen B-Band can be interpreted as an interaction of gentamicin with some specific region of the LPS. To analyze in more detail the effect of gentamicin on the local flexibility of the LPS, we have also measured different relaxation parameters. However, only ^{13}C T_1 longitudinal relaxation constant time can be measured with a sufficient sensitivity and in a reasonable experimental time (Supplementary Figure 4). Unfortunately, these longitudinal relaxation rates do not show significant variations and cannot be used to refine the effect of the gentamicin on the O-antigen flexibility.

Though we have information on individual B-band trisaccharide resonances we could not

isolate a unique residue or chemical group that was specifically affected by gentamicin interaction and we can conclude that the whole repeating unit is a determinant of the interaction. In agreement, we can speculate that the B-band possesses two negative charges (the two Mannuronic acids) per repeating unit which should bridge with the free amino groups of gentamicin, i.e., ammonium groups at physiological pH (Figure 5 panel d). Furthermore, additional contacts could be assumed by the several ester and amide carbonyl groups of the O-antigen with the same positively charged groups of gentamicin. Thus, the specific binding effect that we observed between gentamycin and the B-Band O-antigen explains why the PAO1 LPS B band positive strains are sensitive to gentamicin whereas A-band strains are not. Due to avidity phenomena, it can be certainly deemed that this binding process increases local concentration of gentamicin at outer membrane and increases gentamicin lethality on B band possessing strains.

CONCLUSION

The direct observation of LPS endotoxins in their natural environment constitutes an important step in the field of molecular microbiology and, in particular, toward the understanding of interactions of microbes with their eukaryotic hosts, either beneficial or harmful. Many studies have been published so far in which the double role and the action of the endotoxins has been elucidated, i.e., either as barriers to antibiotics or as elicitors/suppressors of innate immunity in eukaryotes^{1,2,28,29}. Beside this, however, very rarely it has been possible to understand the supramolecular behavior and their action as whole molecule, their delivery to the receptors or their action against antibiotics. Nowadays, their study is still confined to a complex operation of bench organic and analytical bio-organic chemistry⁷ with no chances to comprehend how the primary structure affects the overall 3D structure. It is in fact pivotal to study LPS interactions with eukaryotic and prokaryotic lectins, immunity proteins (TLR4/MD2, caspases) and symbiosis proteins and get a perspective at molecular scale of the whole intact molecule when bound and/or recognized by other ligands.

In this work we have employed ssNMR for studying two different and archetypal LPS, with

either long or short carbohydrate chain, in a membrane like structure without any alteration of the molecules, in the absence of detergents and in suitable conditions to study LPS interactions.

Furthermore, this study further confirmed the capacity of this approach to gain access to parts of the macromolecule that present different flexibilities (Figure 6). MAS ssNMR allowed to either record experiments on the most flexible segments of a molecule through experiments similar as those recorded in liquid-state, or on the most rigid parts by Cross-Polarization experiments. Comparison of the two types of experiments in Figure 6 point out an efficient CP transfer on R-LPS that shows mostly the relatively rigid lipid and core oligosaccharides while CP transfer is less efficient in S-LPS where the spectrum is dominated by flexible O-antigen. The versatility of the method will be particularly efficient in studying LPSs ligand binding, and especially binding to large ligands such as proteins which will significantly rigidify the LPS or when the rigid lipid A part is directly involved in the binding with a receptor (TLR4/MD2, CD14 or LBP protein binding). Further advances in ssNMR methods, in particular higher magic-angle spinning frequencies permit ^1H detection experiments with high resolution and increased sensitivity. High speed spinning (100-111kHz) allows to record 2D and 3D experiments through dipolar or J-couplings similar to liquid state experiments, and on complex $^{13}\text{C}/^{15}\text{N}/(^2\text{H})$ labeled protein systems and on unlabeled molecules with together very low amounts of sample³⁰ and short experimental times. This should prove particularly useful for the study of LPS alone or in interaction with ligands and thus contribute to the elucidation of the fundamental properties of outer membrane permeability.

Figure 6 ssNMR highlights regions of different flexibilities and regions of interactions in LPS.

a) Comparison of 1D ^{13}C Spectra of LPS with direct ^{13}C excitation (red) or through ^{13}C - ^1H Cross polarization experiment (blue) shows that *P. aeruginosa* S-LPS, that assemble into micelles, displays mainly flexible signals (orange) belonging to O-antigens with less efficient CP transfer. These regions were identified as interacting with gentamicin antibiotic.

b) *E. coli* R. LPS in vesicles CP spectrum shows rigid portions of the LPS (blue), mainly core oligosaccharides and lipids.

METHODS

Cell growths and LPS extraction

LPS ^{13}C -labeling was performed by growing *E. coli* BL21(DE3) or *P. aeruginosa* PAO1 in M9 minimal medium³¹ supplemented with 0.1% (w/v) ^{13}C glucose, as the sole carbon source. Cells were harvested from 1 liter of bacterial culture grown to OD₆₀₀ of 0.8 and the bacterial pellet was lyophilized, washed with distilled water, ethanol and acetone followed by ultracentrifugation steps (45,000 rpm, 4 °C, 16 h). LPS were extracted by hot phenol/water extraction³². Both phases were dialyzed and lyophilized, followed by protease and nuclease digestions, then extensive dialysis against water and freeze-drying. Presence of LPS was assessed by SDS-PAGE all along the purification. Gentamicin was obtained from SIGMA(G1264).

Electron microscopy

Samples of LPS at 0.1 to 1mg/ml concentration in water for *E. coli* LPS or 50mM MES pH 6.0 for *P. aeruginosa* PAO1 LPS were used. LPS PAO1 with gentamicin was studied at 0.1 mg/ml in presence of 25mM gentamicin. Samples were absorbed to the clean side of a carbon film on mica, stained with Sodium Silico Tungstate and transferred to a 400-mesh copper grid. The images were taken under low dose conditions ($<10\text{ e}^-/\text{\AA}^2$) at a magnification of 23Kx and 49Kx times with defocus values between 1.2 and 2.5 μm on a Tecnai 12 LaB6 electron microscope at 120 kV accelerating voltage using CCD Camera Gatan Orius 1000.

Nuclear Magnetic Resonance

Lyophilised LPS sample was resuspended at ~50mg/ml final concentration in Milli Q water for *E. coli* at LOS or 50mM MES pH 6.0 for *P. aeruginosa* PAO1 LPS. *E. coli* LOS was sedimented into rotors by centrifugation (16-50.10³g) for 5 minutes. *P. aeruginosa* LPS was sedimented into rotors at 50000g for 16 hours. NMR experiments were recorded on Agilent 600MHz VNMRs spectrometer equipped with MAS 3.2 mm or 1.6 mm HCN probes. MAS frequencies used were 12.5 kHz when employing 3.2 mm probe and 34 kHz with the 1.6 mm probe. The sample temperature was set to ca. 25°C. For 2D ¹³C-¹³C correlation experiments acquisition times were typically 14 -20ms for direct dimension and 5-7 ms in the indirect dimensions, interscan delay was set to 2-4 seconds. ¹H SPINAL-64 decoupling with a radio-frequency (rf) of 100-110 kHz was applied. In CP experiments, the ¹H radiofrequency field was between 55 and 60 kHz, and the ¹³C rf field adjusted to match the Hartmann-Hahn condition with n=1, and the CP time was set between 0.5 and 1.5ms. 1D ¹³C experiments with direct ¹³C excitation or ¹H-¹³C CP-MAS were recorded with 12-19ms acquisition time and an interscan delay of 2-3 seconds. Typically, ¹³C-¹³C DARR experiment on *E. coli* LPS in 3.2 mm rotor was acquired in 41 hours with a 100ms mixing time and ¹³C-¹³C Single Quantum J experiment in 16 hours. ¹³C-¹H INEPT experiments (T_{inept}=1ms) and 2D ¹³C-¹³C Single Quantum J experiment of PAO1 LPS in 1.6mm rotor were recorded in 6 and 24 hours respectively. A PAO1 LPS sample at 40mg/ml in 50mM MES pH 6.0 was split in two and gentamicin at 25mM final concentration was added to one of the sample and they were ultracentrifuged into NMR rotor as described above. ¹³C T₁ longitudinal relaxation time constants were obtained with series of ¹³C-¹H INEPT experiments at 34kHz with inversion recovery periods and delays from 1ms to 1.3s in absence and presence of gentamicin. T₁ values were fitted with CcpNmr software and presented with the fitting error provided by the software. NMR experiments were processed with NMRPipe³³ or Topspin 3.5 and analysed with CcpNmr 2.42 software³⁴. Comparison of 1D ¹³C Spectra of LPS with direct ¹³C excitation or through ¹³C-¹H Cross polarization experiment Spectra were recorded at 600 MHz and at a spinning

rate of 12.5kHz with identical numbers of scans for each sample (PAO1-LPS 128, K12 LPS 64), and baseline was shifted for improved clarity.

ACKNOWLEDGEMENTS

This work was supported by the Train2Target project granted from the European Union's Horizon 2020 framework program for research and innovation (Project #721484) and partly supported by the French National Research Agency in the framework of the " Investissements d'avenir" program Glyco@Alps (ANR-15-IDEX-02). This work used the platforms of the Grenoble Instruct centre (ISBG; UMS 3518 CNRS-CEA-UJF-EMBL) with support from FRISBI (ANR-10-INSB-05-02) and GRAL (ANR-10-LABX-49-01) within the Grenoble Partnership for Structural Biology (PSB). The electron microscope facility is supported by the Rhône-Alpes Region, the Fondation Recherche Medicale (FRM), the fonds FEDER, the Centre National de la Recherche Scientifique (CNRS), the CEA, the University of Grenoble, EMBL, and the GIS-Infrastructures en Biologie Sante et Agronomie (IBISA). We thank D. Fenel and G. Schoehn, from the Electron Microscopy platform of the Integrated Structural Biology of Grenoble (ISBG, UMI3265). We gratefully thank M. Richerd for great assistance in spectra analysis and C. Bougault and B. Bersch for stimulating discussions.

ASSOCIATED CONTENT

Supporting information is available including NMR chemical shifts table and additional NMR spectra: This material is available free of charge via the Internet at <http://pubs.acs.org>.

The authors declare no competing financial interest.

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