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# A $^2$H solid-state NMR study of the effect of antimicrobial agents on intact *Escherichia coli* without mutating

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ABSTRACT

Solid-state nuclear magnetic resonance (NMR) is a useful tool to probe the organization and dynamics of phospholipids in bilayers. The interactions of molecules with membranes are usually studied with model systems; however, the complex composition of biological membranes motivates such investigations on intact cells. We have thus developed a protocol to deuterate membrane phospholipids in *Escherichia coli* without mutating to facilitate $^2$H solid-state NMR studies on intact bacteria. By exploiting the natural lipid biosynthesis pathway and using perdeuterated palmitic acid, our results show that 76% deuteration of the phospholipid fatty acid chains was attained. To verify the responsiveness of these membrane-deuterated *E. coli*, the effect of known antimicrobial agents was studied. $^2$H solid-state NMR spectra combined to spectral moment analysis support the insertion of the antibiotic polymyxin B lipid tail in the bacterial membrane. The use of membrane-deuterated bacteria was shown to be important in cases where antibiotic action of molecules relies on the interaction with lipopolysaccharides. This is the case of fullerenol nanoparticles which showed a different effect on intact cells when compared to dipalmitoylphosphatidylcholine/dipalmitoylphosphatidylglycerol membranes. Our results also suggest that membrane rigidification could play a role in the biocide activity of the detergent cetyltrimethyammonium chloride. Finally, the deuterated *E. coli* were used to verify the potential antibacterial effect of a marennine-like pigment produced by marine microalgae. We were able to detect a different perturbation of the bacteria membranes by intra- and extracellular forms of the pigment, thus providing valuable information on their action mechanism and suggesting structural differences.



## 1. Introduction

New antimicrobial agents are required to act against the emergence of pathogenic bacteria that are resistant to the commonly used antibiotics. Principally designed from less than ten types of molecular scaffolds [1,2], their action mechanism targets few cellular processes such as cell wall and protein biosynthesis as well as DNA replication and repair. Antibiotic resistance is due to their excessive consumption by human patients but could also be related to the widespread routine feeding of antibiotics to livestock [3]. Bacteria inevitably develop resistance to treatments within a time frame of months to years and, unfortunately, resistant strains can transfer their antibiotic resistance genes across pathogen cells from different species [4]. The problem of emerging multidrug resistant strains is compounded with insufficient production of new antibiotics by pharmaceutical companies [5].

Identification and study of antimicrobial peptides (AMPs), biosynthesized by different organisms including plants, insects, anurans and mammals, is currently a dynamic field of research. AMPs are strongly cationic and generally form amphipathic secondary structures such as α-helices and β-sheets [6]. They can selectively interact with bacterial membranes which, contrary to eukaryote membranes, are negatively charged. Indeed, the inner and outer membranes of Gram-negative bacteria are mainly composed of zwitterionic phosphatidylethanolamine (PE), anionic phosphatidylglycerol (PG) and cardiolipin (CL), while lipopolysaccharides (LPS) are only found in the outer membrane [7]. Via electrostatic interaction, AMPs permeabilize the lipid bilayer by creating

*Abbreviations:* ACP, acyl carrier protein; AMP, antimicrobial peptide; CL, cardiolipin; CTAC, cetyltrimethylammonium chloride; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; DMSO, dimethyl sulfoxide; DPC, dodecyl phosphocholine; DOPC, dioleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; ExP, extracellular form of the marennine-like pigment; InP, intracellular form of the marennine-like pigment; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; FA, fatty acid; FAME, fatty acid methyl ester; LPS, lipopolysaccharide; MeOH, methanol; PA-$d_{31}$, perdeuterated palmitic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PxB, polymyxin B; QAC, quaternary ammonium compound; SS-NMR, solid-state nuclear magnetic resonance

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pores, or solubilize the membrane in a detergent-like mechanism [8]. To develop new efficient antibiotics against resistant bacteria, the action mechanism of membrane-disrupting antimicrobial agents should be studied [9].

Deuterium solid-state nuclear magnetic resonance ($^2$H SS-NMR) is an excellent non-invasive technique to probe membrane perturbations because it provides information on the lipid acyl chains' organization and dynamics in the bilayer core. Moreover, the non-perturbing isotopic ($^2$H) labeling does not change the lipid properties contrary to the addition of large reporter molecules used in other methods [10–12]. $^2$H SS-NMR studies have improved our knowledge of the action of antimicrobial agents on biomembranes [13], principally using model membranes as reviewed by Bechinger and coworkers [14]. However, considering the complexity of natural membrane composition, especially the presence of lipopolysaccharides in the outer membrane of Gram-negative bacteria, the interaction of exogenous molecules should ideally be studied *in vivo*, at least as an initial step before refining the interaction mechanism with model membranes.

The lipid metabolism of organisms such as bacteria is known and could be exploited to successfully deuterate membrane lipids of wild-type strains. *Escherichia coli* is a good candidate because of the simplicity of its membrane composition with just three types of phospholipids, i.e. 75% PE, 20% PG and 5% CL, composed of only three different fatty acid chain lengths (C16, C17 and C18) [15,16]. So far in terms of NMR studies of bacteria, membrane lipids have been deuterated in *Acholeplasma laidlawii* [17] and in strains of *E. coli* engineered to uptake exogenous (deuterated) fatty acids, and intact cells were analyzed by $^2$H SS-NMR [10,18,19]. This strategy was carried out on L51 and LA8 auxotroph strains defective in fatty acid oxidation and grown in a solution containing exogenous perdeuterated palmitic acid [10,19]. However, the disadvantage of this method is to rely on the availability of considerably mutated strains.

The objective of this work was therefore to establish a protocol to deuterate the phospholipid acyl chains in the membranes of *E. coli* bacteria that are non-mutated. Wild-type *E. coli* are readily available and known to use exogenous fatty acids for their phospholipid biosynthesis, thus enabling acyl chain deuteration [20]. The $^2$H labeling protocol is presented as well as the phospholipid profile of the bacteria membranes compared to non-labeled organisms. Temperature behavior of intact *E. coli* membranes is also shown. Then the responsiveness of these *E. coli* to known antimicrobial agents is assessed *in vivo* by $^2$H SS-NMR using three different types of molecules, namely the antibiotic polymyxin B, fullerenol ($C_{60}(OH)_{24}$) nanoparticles as well as the detergent cetyltrimethylammonium chloride which structures are displayed in Fig. 1. Finally the non-mutated *E. coli* bacteria with deuterated phospholipids are exploited to verify the antibiotic effect and membrane interaction of a marennine-like blue pigment by $^2$H SS-NMR. Marennine is a water-soluble polyphenol which structure is not fully characterized [21]. It is produced by the blue diatom *Haslea ostrearia*, a marine microalga involved in the greening of oysters, and is thus ingested by humans. Marennine exists in an intracellular form which accumulates mainly at the apical regions of the algal cells, and an extracellular one which is released into the aquatic environment [22]. Both forms of marennine differ with regard to their molecular weight and spectroscopic characteristics [21]. Furthermore, they are known for their anticancer and antiviral activities but also for their ability to inhibit the development of pathogenic marine bacteria such as *Vibrio aestuarianus* [23]. In the present study, a marennine-like pigment produced by a new species of blue diatom, *Haslea provincialis* sp. nov. [24], is evaluated.

## 2. Materials and methods

### 2.1. Materials

All solvents (HPLC grade), deuterium depleted water as well as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), sodium chloride, polymyxin B sulfate (PxB) and cetyltrimethylammonium chloride (CTAC) solution (25% w/w) were purchased from Sigma Aldrich (Oakville, ON, Canada). Fullerenol ($C_{60}(OH)_{16-18}(ONa)_{6-8}$) was obtained from M.E.R. Corporation (Tucson, AZ, USA). Dodecyl phosphocholine (DPC) was acquired from Avanti Polar Lipids (Alabaster, AL, USA) while deuterated palmitic acid (PA-$d_{31}$) was obtained from CDN Isotopes (Pointe-Claire, QC, Canada). Bio-tryptone and yeast extract were purchased from Bioshop (Burlington, ON, Canada). The fatty acid methyl ester (FAME) mixture ($C_4$–$C_{24}$) was provided by Supelco (Bellefonte, USA).

### 2.2. Incorporation of deuterated palmitic acid in E. coli membranes

PA-$d_{31}$ (0.25 mM) was sterilely incorporated into DPC (1 mM) micelles by heating the mixture at 95 °C until the fatty acid (FA) crystals had melted. The solution was flash-frozen and warmed to room temperature until a clear solution was obtained, which was then used immediately. One heat/freeze cycle was generally necessary. Ampicillin-resistant *E. coli* pPD117 (kindly provided by Prof. S. Jenna, UQAM) were then grown at 37 °C under moderate shaking in LB medium (10 g of Bio-tryptone, 5 g of yeast extract and 10 g of NaCl per liter of water) supplemented with the DPC/PA-$d_{31}$ micelle solution to ensure an optimal contact with the outer-membrane of bacteria and efficient subsequent uptake by the fadL protein. Cells were harvested in the mid-log growth phase at an $A_{600}$ of 0.4, then pelleted (1500 G for 10 min at room temperature) and rinsed twice with 0.9% (w/w) NaCl solution, followed by a final rinsing step with a saline solution made of $^2$H-depleted water to limit the NMR signal from naturally-occurring HDO. $^2$H SS-NMR experiments were carried out on fresh samples 2 h at most after harvesting.

### 2.3. Exposition of E. coli to antimicrobial agents

PxB, fullerenol and CTAC solutions were prepared prepared with $^2$H-depleted water and NaCl (0.9% w/w). An appropriate amount of antimicrobial agent solution was added to deuterated pelleted cells and the samples were gently mixed. Concentrations of approximately 1%, 5% and 1% (w/w) were respectively determined for PxB, fullerenol and CTAC using the dry weight of the bacteria. These concentrations were used to compare with similar studies carried out on model membranes [25–27]. Finally, 57 μL of the sample was transferred into a 4-mm ZnO rotor and immediately analyzed by $^2$H SS-NMR. The intra- and extracelullar forms of the pigment produced by *H. provincialis* sp. nov. were extracted and purified according to the methods developed for marennine [21]. Saturated pigment solutions were prepared in seawater and bacteria were exposed to approximately 2 mol% of marennine using the dry weight of bacteria and estimating a pigment molecular weight of 10 kDa according to preliminary characterization of marennine [21].

### 2.4. Lipid profile analysis

The lipid profile analysis was performed in triplicate on three different freshly labeled and unlabeled bacteria samples which were flash-frozen, vacuum-dried and stored at −20 °C prior to lipid extraction. Lipid extraction was carried out on approximately 10 mg of lyophilized bacteria sample using $CH_2Cl_2$/MeOH (2:1 v/v) and 0.88% KCl solution in a Potter glass homogenizer [28]. The lipid extract was evaporated to dryness and lipids were recovered by washing three times with 500 μL of $CH_2Cl_2$/MeOH (98:2 v/v) then deposited at the top of a silica gel column (30 × 5 mm i.d. packed with Kieselgel 60, 70–230 mesh, Merck, Darmstadt, Germany) hydrated with 6% water. Neutral lipids (including triglycerides, free FAs and sterols) were eluted with 10 mL of $CH_2Cl_2$/MeOH (98:2 v/v) and polar lipids (phospholipids) were recovered with 20 mL of MeOH [29]. Polar

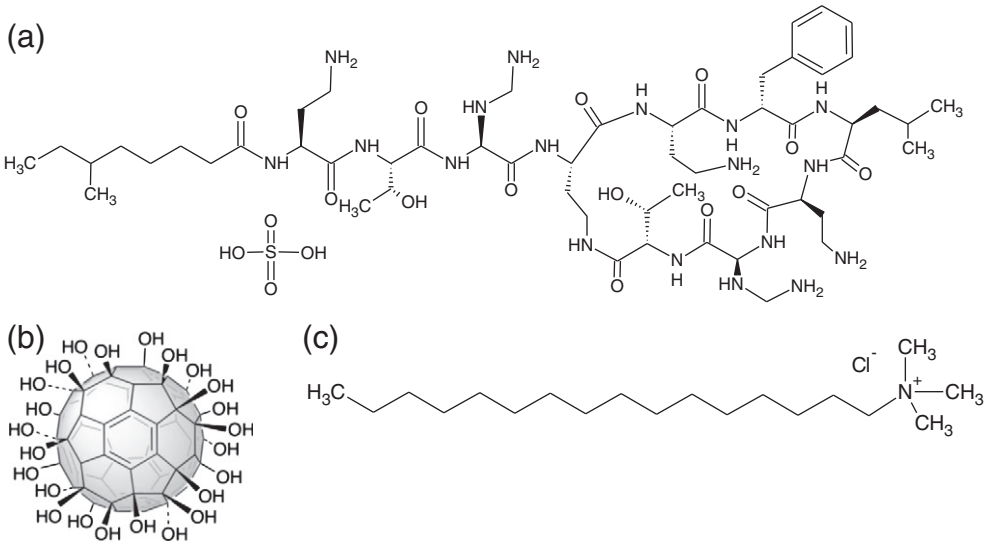


**Fig. 1.** Structures of (a) polymyxin B, (b) fullerenol, and (c) cetyltrimethylammonium chloride.

lipids were evaporated at 40 °C and transesterified in 2 mL of $H_2SO_4$ (2% in MeOH) and 0.8 mL of toluene for 10 min at 100 °C.

They were then analyzed in a gas phase chromatograph (Trace GC Ultra, ThermoScientific, Asheville, NC, USA) equipped with an autosampler and PTV-type injector (model ITQ900) for FA composition. Separation was done on a Supelco Omegawax 250 capillary column (30-m long, 0.25-mm internal diameter, 0.25-μm film thickness). 1 μL of the sample was injected in splitless mode using an injector temperature of 90 °C. Helium at a flow rate of 1 mL/min was used as carrier gas. The temperature program employed consisted of a 2 min-period at 100 °C, followed by a 1-min period at 140 °C reached after a 10 °C/min ramp. A 5 °C/min ramp was then applied followed by a 2-min final step at 270 °C. The detector and ionization source temperatures were 250 and 225 °C, respectively. Full-scan type detection was employed in positive mode on an m/z interval of 50–650. Peak identification was done with the C4-C24 FAME mix. Quantification was performed using external calibration curves with concentrations ranging from 0.3 to 20 μg/mL. The deuterated palmitic acid methyl ester standard was prepared by transesterification of PA-$d_{31}$ as described above. Data acquisition and analysis were done with Excalibur 2.1 software from Thermo Scientific. C19:0 was used as an internal standard.

### 2.5. Cellular viability assay

An MTT reduction assay was used to determine the cellular viability [30]. Bacteria cell samples were diluted in fresh LB medium to an absorbance of ~0.1 at 600 nm. 20 μL of MTT solution (5 mg/mL) stored at −20 °C then heated at 37 °C was added to 200 μL of cell suspension and incubated at 37 °C for 20 min. Formazen crystals were pelleted (pulse-centrifugation) and dissolved in 1 mL of DMSO at room temperature after the LB medium was removed. Optical absorbance readings at 550 nm were done after 15 min of stabilization. These assays were performed in triplicate.

### 2.6. $^2H$ solid-state NMR experiments and moment analysis

All spectra were recorded on a hybrid solution/solid-state Varian Unity Inova 600 (Agilent, Santa Clara, CA, USA) operating at a frequency of 92.1 MHz for deuterium. $^2H$ SS-NMR spectra were obtained using a solid-echo pulse sequence [31] with a pulse length of 3 μs, an interpulse delay of 20 μs and a repetition delay of 500 ms. Typically 20 k scans were acquired and a line broadening of 150 was applied. Data were analyzed using Matlab® software [32]. The equilibration time in the case of multi-temperature experiments was 15 min.

Spectral moments are used to quantify the intensity distribution on the spectra as a function of quadrupolar splitting and are defined as follows [10,19]:

$$M_n = \frac{\int_0^\infty \omega^n f(\omega) d\omega}{\int_0^\infty f(\omega) d\omega}$$

where ω is the angular frequency with respect to the Larmor angular frequency $\omega_0$ while f(ω) is the intensity at that frequency, thus f(ω) describes the spectrum lineshape. The first spectral moment ($M_1$) reflects the average orientational order parameter of the C–D bonds, i.e., the mean of the distribution of order parameters [33]. The second spectral moment ($M_2$) gives the mean square value of the quadrupolar splittings. $M_1$ and $M_2$ can be used to calculate the relative mean square width ($\Delta_2$) of the distribution of quadrupolar splittings:

$$\Delta_2 = \frac{M_2}{1.35 \times M_1^2} - 1.$$

The description of $^2H$ NMR spectra with the spectral moments is particularly useful when a distribution of quadrupolar splittings is present and specific quadrupolar splittings cannot be measured. Changes of $M_1$ and $M_2$ will be particularly sensitive to changes from gel phase to fluid phase. First and second spectral moments were calculated using dedicated scripts implemented in the MatNMR software.

## 3. Results

### 3.1. Incorporation of deuterated palmitic acid into non-mutated E. coli cell membranes

Results of FA profiles in phospholipids of *E. coli* membranes before and after application of the deuteration protocol show that 76% (w/w) of the phospholipids have deuterated acyl chains with a length corresponding to that of palmitic acid, as displayed in Table 1. No other deuterated FAs were detected, indicating that *E. coli* does not elongate the palmitic acid aliphatic chain. As seen in Table 1, unsaturated FA chains in phospholipids are mostly palmitoleic and oleic acids, with

**Table 1**
Fatty acid composition of the membranes of labeled and unlabeled *E. coli* without mutating.

| *E. coli* sample | % Fatty acid (w/w) | | | | |
|---|---|---|---|---|---|
| | C16:0 | C16:0D | C16:1 | C17:1 | C18:1 |
| Labeled | 7% | 76% | 9% | 1% | 7% |
| Unlabeled | 36% | 0% | 28% | 3% | 33% |

saturated/unsaturated (S/U) FA chain ratio of 36:64 in unlabeled bacteria *vs*. 83:17 after PA-$d_{31}$ enrichment.

To verify if our membrane lipid deuteration procedure affects the viability of the bacteria, cellular viability was determined after harvest, but also before and after $^2$H SS-NMR experiments. This allowed ascertaining if *in vivo* SS-NMR analysis of membrane perturbations was accomplishable with these newly developed membrane-deuterated wild-type *E. coli*. As can be seen in Table 2, cellular viability at harvest is rather similar to that of unlabeled cells. Application of the rinsing and pelleting steps possibly affects the cells as the MTT reduction activity is lowered to 78% of the value at harvest. Nevertheless, the washing procedure allows cells to be viable at the start of the NMR experiment, thus enabling *in vivo* analysis. Some cells die during the static NMR study as the MTT reduction activity diminishes to 42% (Table 2). Still, a significant portion of the bacteria remains alive during the 2-hour analysis. Finally, we have also evaluated the effect of the antimicrobial agents on the bacteria after $^2$H SS-NMR analysis. Table 2 shows that CTAC has a greater impact on cellular viability than PxB, fullerenol and the marennine-like pigment when used at similar concentrations (estimated to 1–5% w/w). Only 4% of the bacteria survive to the addition of the detergent, as opposed to ~24% in the case of the antibiotic, nanoparticles and extracellular marennine-like pigment.

### 3.2. Characterization of intact E. coli cells

Characterization of the membrane lipids was carried out by studying the thermotropic behavior of the intact labeled *E. coli* cells by $^2$H SS-NMR. Phospholipids in a bacterial membrane are in a liquid-crystalline phase and undergo a fast rotation along their longitudinal axis. The quadrupolar splitting ($\Delta\nu_Q$) observed on the SS-NMR spectrum for a deuterium nucleus in the lipid acyl chain will depend on its orientation with respect to the magnetic field direction, but also on the location of the C–D bond on the chain which ordering decreases when going from the positions near the headgroup to the terminal $CD_3$. For a C–D bond in a phospholipid bilayer with axial symmetry:

$$\Delta\nu_Q(\delta) = \frac{3}{4}\frac{e^2qQ}{h}\left(3\cos^2\theta - 1\right)S_{CD} \quad (1)$$

where ($e^2qQ/h$) is the quadrupole coupling constant (~167 kHz), θ is the angle between the bilayer normal and the lipid long axis, and $S_{CD}$ is the order parameter of a deuterium bond vector [34,35]. Typically, the positions located in the first half of the phospholipid chain have a similar degree of ordering and form a plateau-like feature on the spectra.

**Table 2**
MTT reduction activities of labeled *E. coli* after exposure to antimicrobial agents with an estimate (%) of live population relative to that after rinsing.

| Analysis | MRU/(mL $OD_{600}$) | |
|---|---|---|
| | Labeled | Unlabeled |
| At harvest | 74.8 ± 29.2 | 68.2 ± 17.3 |
| After rinsing | 58.5 ± 27.1 | – |
| After NMR | | |
| No antimicrobials | 24.3 (42%) | – |
| + PxB | 13.8 (24%) | – |
| + Fullerenol | 14.0 (24%) | – |
| + CTAC | 2.6 (4%) | – |
| + InP | 24.1 (41%) | – |
| + ExP | 14.0 (24%) | – |

InP and ExP respectively refer to the intra- and extracellular forms of the marennine-like blue pigment.

The spectra acquired at 12, 37 and 55 °C are shown in Fig. 2 and the spectral moment analysis is displayed in Table 3. Fig. 2a shows that at 12 °C the spectrum is mostly typical of membrane lipids in mixed gel and liquid-crystal phases. The largest quadrupolar splitting has a value of 125 kHz similar to perdeuterated dipalmitoylphosphatidylcholine (DPPC-$d_{62}$) in the gel phase [10,33]. At about ± 30 kHz, another population of phospholipids in the gel phase is identified. From previous work, it could be ascribed to phospholipids with more chain disorder [10,33]. Evidence for lipids in a fluid phase is revealed by a shoulder at approximately ± 25 kHz. $\Delta\nu_Q$ value of 2 kHz is attributed to the terminal methyl groups. As previously observed on *E. coli* membranes [10], a feature with $\Delta\nu_Q$ of 15 kHz is visible on the spectrum. Called "feature X", this time-dependent feature has been associated with bacteria degradation when the cells are densely packed without fresh nutrients supply. It has been attributed to membrane disruption and possible formation of a hexagonal phase by the phospholipids in the membrane fragments [10]. Feature X is present at all temperatures but is smaller at lower temperatures and when analyses are done on fresh samples.

As the temperature is increased to 37 °C, the contribution of phospholipids in the gel phase ($\Delta\nu_Q$ of ~60 kHz) is very small (Fig. 2b). The signal of phospholipids in the liquid crystalline phase produces a characteristic spectrum presenting the well-defined edges of a plateau which has a quadrupolar splitting of 25 kHz. When heating the bacteria sample to higher temperatures (Fig. 2c), this value slightly decreases due to increased motional averaging, consistent with other work [33]. At 37 °C the quadrupolar splitting of the terminal methyl group (2 kHz) corresponds to previously reported values of *E. coli* biomembranes in a lamellar liquid crystalline phase [10]. At 55 °C, the gel phase is almost absent. Most phospholipids are in a fluid phase but the plateau is barely distinguishable due to the considerable contribution of feature X to the spectrum. Therefore bacteria alteration seems to dominate at high temperatures. Finally, an isotropic signal is present on all spectra and corresponds to traces of deuterated water [10].

Examination of the spectral moments shows that $M_1$ decreases when heating the bacteria samples from 12 to 55 °C consistent with a concomitant increase in phospholipid acyl chain disorder as seen when pure phospholipid bilayers undergo a gel-to-liquid crystalline phase transition [33]. $M_2$ reflects the correspondent smaller spectral width. The increase in $\Delta_2$ value with *E. coli* sample temperature suggests that more lipid phases coexist when increasing the temperature, e.g. a gel and a fluid phase at 37 °C. However the calculation of

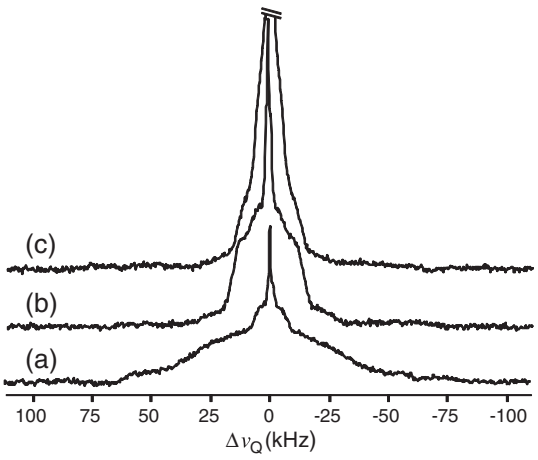


**Fig. 2.** $^2$H SS-NMR spectra of intact *E. coli* without mutating acquired at (a) 12, (b) 37 and (c) 55 °C. The top of the spectrum (c) is truncated.

**Table 3**
Spectral moment analysis of the $^2$H SS-NMR spectra of labeled *E. coli* obtained at different temperatures, and in the presence of antimicrobial agents.

| Sample | Temp. (°C) | $M_1$ ($10^4$ $s^{-1}$) | $M_2$ ($10^9$ $s^{-2}$) | $\Delta_2$ |
|---|---|---|---|---|
| *E. coli* | 12 | 11.0 | 21.2 | 0.30 |
| *E. coli* | 37 | 6.3 | 9.0 | 0.68 |
| *E. coli* | 55 | 3.8 | 3.3 | 0.69 |
| + PxB | 37 | 7.1 | 11.2 | 0.64 |
| + Fullerenol | 37 | 9.9 | 19.9 | 0.50 |
| + CTAC | 37 | 12.7 | 25.5 | 0.17 |
| + InP | 37 | 5.5 | 5.1 | 0.25 |
| + ExP | 37 | 9.3 | 17.1 | 0.46 |

the $\Delta_2$ value which should be smaller for the *E. coli* membranes in the fluid phase at 55 °C is most likely biased by the large contribution of feature X.

### 3.3. Effect of antimicrobial agents on intact E. coli cell membranes

The response of the membrane-deuterated non-mutated *E. coli* to known antimicrobial agents has been tested using various molecules with different action mechanisms. More specifically, the cationic lipopeptide polymyxin B (PxB) is an antibiotic known to affect the bacterial membrane permeability by causing its breakage [25,36]. Fullerenol is a hydrosoluble nanoparticle that would preferentially interact with negatively-charged phospholipids (PG) and increase the fluidity of the bilayer without leading to membrane rupture, as was observed in model bacterial membranes [27]. Finally, cetyltrimethylammonium chloride (CTAC) is suspected to interact with the polar heads of PGs in *E. coli* membranes similar to CTA bromide which was shown to restrain phospholipid motions [26].

Fig. 3 shows the spectra obtained at 37 °C after exposure of freshly harvested $^2$H-labeled *E. coli* to these different antimicrobial agents. First, the addition of PxB to pelleted cells (~1% w/w of dry bacteria) induces the formation of a gel phase as revealed by the large intensity at $\pm$60 kHz (Fig. 3b). A plateau is also seen on the spectrum with corresponding $\Delta\nu_Q$ value of 25 kHz similar to that of the unexposed cells. This confirms the presence of phospholipids in a fluid phase. Calculation of the first spectral moment in Table 3 is indicative of an overall decrease in the lipid chain motions since $M_1$ increases from 6.3 to $7.1\times10^4$ $s^{-1}$ when PxB is added to the bacteria milieu. The coexistence of a fluid and a gel phase is demonstrated by the spectral shape (higher $M_2$ value) and distribution of quadrupolar splittings with $\Delta_2$ of 0.64 consistent with a "heterogeneous" membrane [33].

Fig. 3c shows that the addition of fullerenol to the bacteria (~5% w/w of dry bacteria) has more effect than PxB on *E. coli*'s $^2$H SS-NMR spectrum lineshape. The gel phase with $\Delta\nu_Q$ of about 120 kHz has a greater contribution to the spectrum while the intensity of both the plateau and feature X is reduced. The quadrupolar splittings of the plateau (25 kHz) and methyl regions (2 kHz) are similar to those of the unexposed cells, suggesting that fullerenol does not affect the acyl chains of the lipids in the fluid portion of the membrane. The increased proportion of lipids in a gel phase induced by the presence of $C_{60}(OH)_{24}$ nanoparticles is better evidenced by the calculation of the spectral moments in Table 3. With fullerenol, both $M_1$ and $M_2$ values are higher than for "pure" *E. coli* (bacteria without antimicrobial), and the smaller $\Delta_2$ value suggests a smaller distribution of chain ordering – i.e. more homogeneity – in the *E. coli* membranes.

Deuterated bacterial cells were also exposed to the detergent CTAC (~1% w/w of dry bacteria) and Fig. 3d reveals significant changes in the *E. coli* $^2$H SS-NMR spectrum. More specifically, an important fraction of the phospholipids are in a gel phase ($\Delta\nu_Q$~120 kHz) with reduced mobility, similar to what was observed in "pure" *E. coli* samples at low temperature. A shoulder with $\Delta\nu_Q$ of about 60 kHz suggests a phospholipid population in a gel phase characterized by more chain disorder. A plateau region is still observable on the spectrum at $\pm$12.5 kHz, though, indicating that some phospholipids are still found in a fluid phase. Table 3 shows that the $M_1$ value calculated for the spectrum of CTAC-exposed bacteria is the highest ($12.7\times10^4$ $s^{-1}$) when compared to the other antimicrobial agents, indicating that the phospholipids' acyl chains are globally more ordered due to the presence of the cationic detergent. The high $M_2$ reflects the dramatic spectral changes and the $\Delta_2$ value of 0.17 illustrates the narrower distribution of quadrupolar splittings consistent with a greater proportion of lipids in a gel phase and less in the fluid phase. Altogether, these results indicate that exposure to CTAC greatly rigidifies *E. coli* membranes. A small isotropic peak is seen on the spectrum and can be ascribed either to traces of deuterated water or to fast-tumbling objects.

Finally, we have exploited the phospholipid deuteration protocol that we have established to verify by $^2$H SS-NMR on intact *E. coli* the potential effect of the blue pigment produced by the Mediterranean diatom *H. provincialis* sp. nov. shown in Fig. 4. This pigment is similar to marennine which is produced by *H. ostrearia* [24]. Marennine, like other similar pigments from blue diatoms, is a natural bioactive molecule which displays antimicrobial activity, but its action mechanism and chemical structure are still unknown [22]. We have more specifically studied the intra- (InP) and extracellular (ExP) forms of the marennine-like blue pigment. The intracellular form is easily seen in Fig. 4 in the apical zones of *H. provincialis* sp. nov.

Fig. 5b shows the $^2$H SS-NMR spectrum of membrane-deuterated *E. coli* obtained in the presence of the pigment's intracellular form.

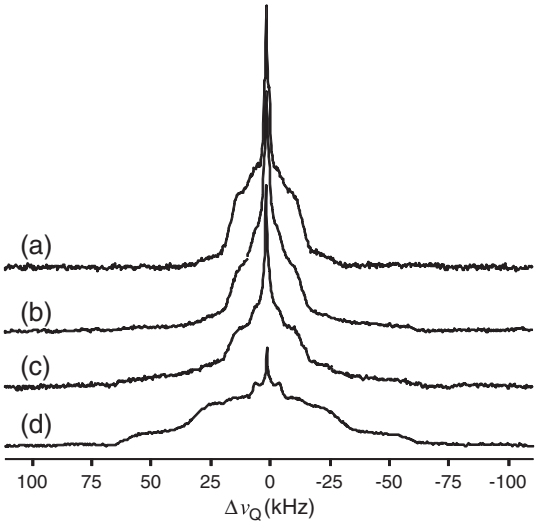


**Fig. 3.** $^2$H SS-NMR spectra of (a) intact *E. coli* without mutating acquired at 37 °C and in the presence of (b) PxB, (c) fullerenol and (d) CTAC.

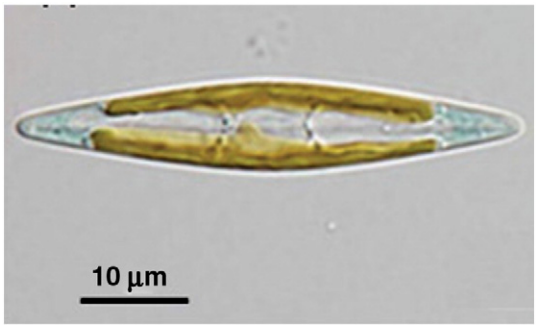


**Fig. 4.** Optical microscopy image of *Haslea provincialis* sp. nov. with blue apices (intracellular form of the marennine-like pigment).

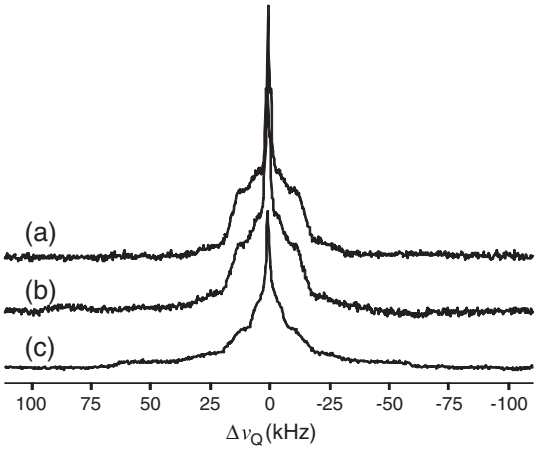


**Fig. 5.** $^2$H SS-NMR spectra of (a) intact *E. coli* without mutating acquired at 37 °C and in the presence of (b) intracellular and (c) extracellular forms of the marennine-like pigment from *H. provincialis* sp. nov.

Although similar to the spectrum of pure bacteria (Fig. 5a), the spectral moment analysis reported in Table 3 reveals a lower $M_1$ value (5.5 compared to 6.3) typical of a slight decrease in phospholipid acyl chain order induced by the interaction of InP with the bacterial membranes, with a lower $M_2$ corresponding to a smaller spectrum width. The decrease in $\Delta_2$ value suggests less phase heterogeneity. InP has little effect on the bacteria viability, as revealed in Table 2 with an MRU similar to cells with no antimicrobial agents that most likely suffer from nutrient privation during the NMR analysis [19].

Interestingly, the effect of the extracellular form of the marennine-like pigment on *E. coli* membranes is very different and resembles that of fullerenol (Fig. 3c). As for the polyhydroxylated fullerene nanoparticles, ExP increases the proportion of phospholipids in a gel phase and the plateau feature indicates that some phospholipids remain in a fluid environment. The similarity between the effect of fullerenol and ExP is better evidenced by comparing the first and second spectral moments in Table 3 which are nearly identical, with $M_1$ and $M_2$ values higher than those of "pure" bacteria. The lower $\Delta_2$ compared to "pure" *E. coli* suggest that the lipid acyl chains are globally more ordered by the presence of ExP. The cell viability is also comparable (24%) for ExP and fullerenol (Table 2).

## 4. Discussion

### 4.1. Successful $^2$H labeling of non-engineered E. coli membranes

We have exploited *E. coli*'s machinery to deuterate the membrane phospholipid chains of *E. coli* without making any use of mutations. This was done by growing bacteria in a medium enriched in deuterated palmitic acid which is the most abundant phospholipid FA chain found in *E. coli*, representing up to 45% of total lipids depending on the growth temperature [37–39]. A 76% deuteration of the phospholipid FA chains was achieved in the bacterial membranes (Table 1). This labeling value is close to what was attained for wild-type *E. coli* (88%) using a similar protocol and radioactive ($^3$H, $^{14}$C) labels [20] but is greater than what was previously reached with the auxotrophic *E. coli* L51 strain (50%) deficient in FA synthesis and oxidation [10]. It compares well with a previous study on the L8 strain [19] assessing that 96% of the PE acyl chains are deuterated. This would correspond to a labeling percentage of 72% considering that PE represents 75% of *E. coli*'s cell membrane phospholipids.

The high energetic cost of FA biosynthesis by the bacteria favors the uptake of exogenous long-chain FAs (12 to 18 carbon atoms) which bind to the fadL protein located within the bilayer of the outer membrane. FAs then travel through the periplasm by protein-mediated transport to reach the cell inner membrane where they are transferred to acyl-CoA in the cytoplasm. There, they can either be degraded via β-oxidation or incorporated into phosphatidic acid by the glycerol-3-phosphate acyltransferase to be later converted into the different phospholipids present in *E. coli* [40–42]. Therefore, the incorporation of exogenous PA-$d_{31}$ is expected to occur in all types of phospholipids equivalently. Only a minor proportion of exogenous FAs can incorporate directly into PE via an acyl carrier protein (ACP) to result in selective deuteration of PE [40]. These pathways, thus, favor the use of exogenous FAs in the phospholipids' synthesis and do not allow accumulation of free FAs inside the bacterial cells [41].

As presented in Table 1 phospholipids of *E. coli* with or without $^2$H enrichment are constituted mainly from palmitoleic and oleic acid, in accordance with the literature [10,18,20,43–47]. As observed in other studies using mutated strains [10,48], our FAME analysis shows that PA-$d_{31}$ is the only type of deuterated FA chain detected. The elongation of the deuterated palmitic acid chains was not expected because exogenous FAs in *E. coli* have no access to the biosynthetic acyl intermediates provided by the ACP involved in the FA elongation but also in the biosynthesis of lipid A [40,49]. Therefore LPS cannot be deuterated. The S/U FA ratio (Table 1) has considerably increased following the deuteration diet of the bacteria, as already observed in literature [10,18]. The smaller proportion of hydrogenated palmitoleic and oleic acid in the phospholipids in labeled cells indicates that the bacteria have proportionally reduced the *de novo* synthesis of these FAs to compensate for the intake of exogenous PA-$d_{31}$ [50,51]. However, as the MTT reduction activities are similar between labeled and unlabeled bacteria at harvest (Table 2), the changes in the lipid chain profile and characteristics – deuterated lipids that would likely have a higher phase transition temperature – seem not to have an impact on cellular viability at 37 °C. Moreover, the bacterial membranes remain in a liquid crystalline phase at the growth temperature of 37 °C, as demonstrated by the $^2$H SS-NMR spectrum in Fig. 2b. Bacteria, thus, try to maintain the fluidity of their membranes by adjusting their composition which depends on the temperature of growth and exogenous FA used [52]. Indeed an FTIR spectroscopic study performed with *A. laidlawii* grown on myristic-$d_{27}$, pentadecanoic-$d_{29}$ and palmitic-$d_{31}$ acids showed that in all cases the bacteria membranes were in the liquid crystalline phase at the temperature of growth (37 °C) [52]. Nevertheless, introduction of deuterated or protonated oleic acid together with PA-$d_{31}$ in the growth medium could help the organisms maintaining an S/U FA ratio closer to that of "natural" *E. coli*, similar to previous work [10,18,19]. The possibility to reach high phospholipid deuteration levels with non-engineered *E. coli* has important experimental advantages such as easy availability of the bacteria and the near-natural membrane labeling that leads to more native-like *in vivo* studies. Therefore, the use of mutant strains is unnecessary.

### 4.2. $^2$H SS-NMR analysis of intact E. coli membranes and response to antimicrobial agents

Model bacterial membranes are generally employed to investigate the action mechanism of membrane-targeting antimicrobial agents and peptides [12]. $^{31}$P SS-NMR has been used to study the interaction of antimicrobial peptides with *Bacillus cereus* and *Staphylococcus epidermidis* membranes [53]. The very few $^2$H NMR studies carried out on intact *E. coli* cells were performed on mutated strains deficient in total FA biosynthesis [10,19]. The membrane-deuterated non-engineered *E. coli* developed in this work demonstrates high level of cellular viability before (75%) and after (50%) the NMR experiment, confirming the *in vivo* nature of the analyses. Characterization of the intact *E. coli* cells by $^2$H SS-NMR from 12 to 55 °C in Fig. 2 shows the disappearance of a gel phase and a higher proportion of phospholipids in the liquid-crystal phase when increasing the temperature. This membrane behavior presents a lot of concordance with previous $^2$H SS-NMR study of labeled mutated *E. coli* (L51 strain) [10]. Using $^2$H

SS-NMR, Davis et al. [10] showed that the orientational order in the phospholipid acyl chain is greater in the outer membrane of *E. coli* than in the cytoplasmic membrane. The gel-to-liquid crystalline phase transition temperature was shown to be 7 C higher for the outer membrane. When compared to these studies on outer and inner *E. coli* membrane extracts [10], the spectra obtained in our work appear to be the sum of the signals of lipid in both membranes. The increase in feature X proportion with the temperature can be explained by the membrane disruption following bacteria degradation due to lack of nutrients, consistent with the time-dependency reported for this feature. Indeed the spectra were successively recorded at 12, 37 and 55 °C, each spectrum being acquired in 2 h.

We have then evaluated the efficiency of the deuterated non-mutated *E. coli* to examine the action mechanism of antimicrobial agents by $^{2}$H SS-NMR. This was done by exposing labeled bacteria to different molecules with known deleterious effects on bacteria, i.e. an antibiotic, a nanoparticle and a detergent. First, the cationic lipopeptide PxB is known to have a high efficiency against Gram(−) bacteria such as *E. coli* and its action mechanism is well documented [25]. Via electrostatic interactions, polymyxins are believed to bind to LPS in the outermost leaflet of the outer membrane. PxB competes with divalent cations that cross-bridge LPS molecules and this perturbation enables its penetration into the periplasm to reach its target – the cytoplasmic membrane – that can then be permeabilized by PxB's FA chain [25].

The insertion of PxB in *E. coli* membranes is supported using intact bacteria in our study. The $^{2}$H SS-NMR spectrum reveals a higher proportion of phospholipids in a gel phase when bacteria are exposed to PxB. This result is in agreement with previous studies suggesting that at PxB concentration (>2 mol%) the antibiotic reaches the inner membrane and increases its lipid packing due to the insertion of the lipid tail [54]. This is what we observe *in vivo* for PxB which concentration is most likely superior to 3 mol% considering that ~1% antibiotic (w/w) was used. Because the cellular viability falls to 17% in the presence of PxB it is possible that bacteria are lysed by the presence of the antibiotic, in such case contributing to feature X's intensity.

The responsiveness of the non-mutated *E. coli* to antimicrobial agents is further demonstrated with fullerenol. These nanoparticles are reported to have smaller antibacterial activity than non-functionalized fullerene and would inhibit cell growth [55,56]. We observe that *E. coli*'s viability is diminished to 21% by fullerenol interaction at the concentration studied which is superior to that used in a previous work [56]. Comparison of the $^{2}$H SS-NMR spectra for the intact cells with and without fullerenol indicates an increased proportion of lipids in a gel phase. It should be noted that this result differs to what we had previously observed by NMR and infrared spectroscopy on DPPC/DPPG (dipalmitoyl-phosphatidylglycerol) bilayers used to mimic bacterial membranes. Indeed, lipid segregation was seen as well as a disordering effect solely on DPPG acyl chains in the presence of 5 mol% fullerenol [27]. This effect was ascribed to a preferential affinity of polyhydroxylated fullerene for DPPG's hydroxyl group [27]. The outer membrane of *E. coli* comprises LPS that offer several hydroxyl groups for fullerenol to interact, and Aoshima et al. [56] showed that the bactericidal effect of polyhydroxylated fullerene on different bacterial strains was correlated to the number of OH groups on the nanoparticles, suggesting an interaction with the peptidoglycan on Gram(+) bacteria. Therefore, the increased proportion of phospholipids in a gel phase in *E. coli* membranes showed in our study by $^{2}$H SS-NMR can be ascribed to an interaction of the $C_{60}(OH)_{24}$ nanoparticles with LPS at the bacteria surface. This most likely induces a tighter packing of the phospholipids in the outer leaflet of the outer membrane, leaving the more fluid cytoplasmic membrane intact and in a fluid phase [10]. Our results outline the importance of comparing SS-NMR analyses on intact cells with studies on model membranes which are very useful to investigate specific interactions or phenomena.

The effect of the antimicrobial agent CTAC is successfully evidenced with intact non-mutated *E. coli*. CTAC is a cationic antimicrobial detergent which action mechanism is most likely to disrupt the bacterial membrane and induce cell leakage [57]. The action mechanism of CTAC and CTA bromide on Gram(−) bacteria is not fully known but would most likely be concentration-dependent [58]. A study on Gram(−) *Proteus mirabilis* revealed that membrane fluidity is increased by the action of surfactants but that membrane fluidity could not be correlated to biocidal activity [59]. Electrostatic interactions are believed to be responsible for the effect of quaternary ammonium compounds (QACs) on membrane lipids [60]. $^{31}$P and $^{2}$H SS-NMR investigations on DPPC vesicles showed that CTAB restricted the lipid headgroup movement [26]; however, a thermodynamics study showed that hydrophobic effect is important in the interaction of CTAC with dioleyl-PC (DOPC) bilayers and that long-chain QACs are more efficient to decrease the membrane permeability [60]. The phase diagram for CTAB in dimyristoyl-PC (DMPC) shows that the formation of mixed micelles occurs only at high (1.5:1) detergent/lipid molar ratio [61]. Similarly, membrane disruption by CTAC is observed a detergent/lipid ratio superior to 1 for DOPC vesicles [60].

With the CTAC concentration of 1% w/w used in this work, the *E. coli* $^{2}$H SS-NMR spectrum and moment analysis are strongly suggestive of membrane rigidification with phospholipids predominantly in a gel phase. Most of the cells died due to exposure to CTAC since cellular viability falls to about 5%. The spectrum indicates little or no formation of fast-tumbling structures such as mixed micelles; therefore, membrane disruption is not observed at the CTAC concentration studied. We believe, thus, that cell death is ascribed to the lack of membrane fluidity and subsequent loss of important biochemical functions, for example those performed by membrane proteins. Considering the cationic nature of CTAC, it is very likely that the detergent molecules interact electrostatically with the negatively-charged PG found in the bacteria's outer and inner membranes. CTAC hydrophobic chain would be inserted into the lipid bilayer, thus restraining the lipid acyl chain movements and inducing the formation of a gel phase. The possibility of a phase separation between PE and the negatively-charged phospholipids cannot be ruled out as observed with AMPs [9,62], and such segregation could lead to a PE-enriched phase which is more ordered [63]. Spectroscopy measurements have actually suggested that PE and PG are naturally segregated in *E. coli* membranes [64]. It is also possible that toroidal pores are formed by the detergent in the bacterial membrane as was proposed for fallaxidin 4.1a [63]. Both electrostatic interaction and lipid acyl chain ordering were suggested from $^{31}$P and $^{2}$H SS-NMR spectra when this AMP interacted with model DMPC/DMPG membranes. Our result contrasts to the increased fluidity of *P. mirabilis* membranes in the presence of amine oxide [59], but it can be explained by the absence of an apolar chain on this small amphiphilic surfactant. Our results agree with Marcotte et al. [60] who proposed that hydrophobic interactions would be involved in the action mechanism of QACs, especially with long aliphatic chains such as CTAC.

Finally, the deuteration protocol for the membrane lipids of non-mutated *E. coli* allowed investigating the potential biocidal effect of the intra- and extracellular forms of the marennine-like pigment produced by the marine diatom *H. provincialis* sp. nov. According to the $^{2}$H SS-NMR spectra (Fig. 5) InP has a slight disordering effect on bacterial membranes while the effect of ExP is quasi identical to that of fullerenol. More specifically, the bacteria spectrum is characterized by a greater contribution of lipids in the gel phase as compared to the fluid phase. According to preliminary characterization [21], marennine is a 10 kDa non-hydrolysable polyphenolic molecule, and the pigment produced by *H. provincials* sp. nov. is similar to marennine, as evidenced by *in vivo* UV–visible spectrophotometry and Raman spectroscopy [24]. Therefore as for fullerenol, ExP would exert its antibiotic action by interacting with LPS on *E. coli* surface where this location would restrain the phospholipids motion. Although both forms of marennine are not strong antibiotics against terrestrial pathogens [23], our results demonstrate that the marennine-like pigment produced by *H. provincialis* sp.

nov. does interact with *E. coli* membranes and that the extracellular form is more deleterious than the intracellular one. To a larger extent, these results also provide valuable information on their interaction with Gram(−) bacteria. They also suggest that the structure of the intracellular form is significantly different to that of the extracellular form to present another action mechanism on *E. coli* membranes.

## 5. Conclusion

A protocol to deuterate membrane phospholipids in non-engineered *E. coli* was successfully established by exploiting their natural lipid biosynthesis pathway and using perdeuterated exogenous palmitic acid. These bacteria which do not require genetic modification have the advantage of being readily available and showed a deuteration level comparable to that of fatty-acid auxotrophs. The signal-to-noise ratio of the $^{2}$H SS-NMR spectra allowed studying the interactions of known (PxB, fullerenol and CTAC) and potential (marennine-like pigments) antimicrobial agents on intact bacteria in native-like conditions and bringing valuable information on their action mechanism. The lipid tail insertion of PxB into *E. coli* membranes was supported experimentally. We were also able to propose that membrane rigidification could play a role in the biocide activity of the detergent CTAC. The use of membrane-deuterated bacteria was shown to be important in cases where the antibiotic action of molecules relies on the interaction with lipopolysaccharides that are not used in model membranes. This was demonstrated with polyhydroxylated molecules, i.e. fullerenol nanoparticles and a bioactive pigment produced by a blue diatom from the genus *Haslea*. A difference was indeed observed between the interaction of fullerenol with intact *E. coli* and model DPPC/DPPG membranes by $^{2}$H SS-NMR. Finally, we were able to detect a different perturbation of the bacteria membranes by the intra- and extracellular forms of a marennine-like microalgal blue pigment, thus providing an insight on their action mechanism and suggesting structural differences between these molecules.

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