Labelling strategy and membrane characterization of marine bacteria *Vibrio splendidus* by *in vivo* ^{2}H NMR

Zeineb Bouhlel[a,b], Alexandre A. Arnold[a], Dror E. Warschawski[a,c], Karine Lemarchand[b], Réjean Tremblay[b], Isabelle Marcotte[a,*]

[a] *Department of Chemistry, Université du Québec à Montréal, P.O. Box 8888, Downtown Station, Montreal H3C 3P8, Canada*
[b] *Institut des Sciences de la Mer de Rimouski, Université du Québec à Rimouski, 310 allée des Ursulines, Rimouski G5L 3A1, Canada*
[c] *UMR 7099, CNRS - Université Paris Diderot, IBPC, 13 rue Pierre et Marie Curie, F-75005 Paris, France*

ABSTRACT

Vibrio splendidus is a marine bacterium often considered as a threat in aquaculture hatcheries where it is responsible for mass mortality events, notably of bivalves' larvae. This bacterium is highly adapted to dynamic salty ecosystems where it has become an opportunistic and resistant species. To characterize their membranes as a first and necessary step toward studying bacterial interactions with diverse molecules, we established a labelling protocol for *in vivo* [2]H solid-state nuclear magnetic resonance (SS-NMR) analysis of *V. splendidus*. ^{2}H SS-NMR is a useful tool to study the organization and dynamics of phospholipids at the molecular level, and its application to intact bacteria is further advantageous as it allows probing acyl chains in their natural environment and study membrane interactions. In this study, we showed that *V. splendidus* can be labelled using deuterated palmitic acid, and demonstrated the importance of surfactant choice in the labelling protocol. Moreover, we assessed the impact of lipid deuteration on the general fitness of the bacteria, as well as the saturated-to-unsaturated fatty acid chains ratio and its impact on the membrane properties. We further characterize the evolution of *V. splendidus* membrane fluidity during different growth stages and relate it to fatty acid chain composition. Our results show larger membrane fluidity during the stationary growth phase compared to the exponential growth phase under labelling conditions - an information to take into account for future *in vivo* SS-NMR studies. Our lipid deuteration protocol optimized for *V. splendidus* is likely applicable other microorganisms for *in vivo* NMR studies.

1. Introduction

Vibrio species are Gram-negative bacteria widely spread in coastal marine and estuarine waters and sediments [1–4]. They might also be found in aquatic animal tissues, causing serious pathologies leading in some cases to economic loss in aquaculture industry [5–8]. To date, most research on marine *Vibrio* species concerned their effects on aquatic organisms acting on immunological process [8–14]. As a matter of fact, a common trait of all members of the *Vibrio* genus is their opportunistic nature, which allows them to benefit from the collapse of the immune system of cultured organisms in specific conditions to

become virulent [14,15]. Investigating the virulence/response of these species requires considering numerous biotic and abiotic factors where the physiological responses can remain elusive. For instance, *V. splendidus* are different from their *Vibrio* congeners by their high genetic variability between strains, where each strain can have a different opportunistic and/or virulence pattern [8,15,16].

Very few studies have so far focused on the membranes of environmental bacteria compared to human pathogens, albeit the importance of the external cell envelopes in biological events. Marine bacteria are of great interest for membrane properties investigation considering their high resistance phenotypes and high adaptability to a

large scale of salinity and temperature, attributable to their permanent exposure to changing ecosystems [1,3,4,11,17–21]. Moreover, marine and coastal environments constitute a dynamic platform for water mixing, thus containing a plethora of molecules such as pollutants, aquatic bioactive components, organic or chemical toxins and industrial antibiotics [22–27]. The cell envelope of indigenous bacteria in such environments represents the first barrier encountered by external molecules which could either cross the membrane and ultimately intracellular sites, or directly affect their structural components [28,29]. Acquiring knowledge on *Vibrio* membrane structure and fluidity would help tackling physiological processes inside the cell and gain a better understanding of the interaction mechanisms of these marine bacteria with molecules in their environment. This should contribute to improve investigations of larval infections and therapeutic treatments in aquaculture.

Historically, *in vivo* nuclear magnetic resonance (NMR) has been associated with the observation of metabolites and metallic ions that were sufficiently abundant so generate an NMR signal [30,31]. Afterwards, thanks to technological progress, new labelling schemes, and genetic manipulations, the term *in-cell* NMR arose, describing the observation of larger molecules inside whole cells [32–34]. For obvious resolution and sensitivity issues, *in cell* solution NMR first focused on small and/or mobile molecules [31,32,34,35]. During the last five years, solid-state (SS) NMR made a significant contribution, to include not just large molecules with limited movement, but also heterogeneous biological systems [36]. Today, *in vivo* NMR generally refers to the study of intact *living* organisms such as bacteria, microalgae, and small invertebrates, as long as their size allows them to fit into the NMR rotor [37–42].

The objective of this work was to establish a protocol to deuterate the lipid acyl chains in *V. splendidus* membranes, to enable the *in vivo* ^{2}H SS-NMR study of this marine bacterium. More specifically we used the indigenous 7SHRW strain isolated from the St. Lawrence Gulf sediments (Canada) which can cause significant mortalities to blue mussels and scallops at larval stages [43,44], and has the advantage to be easily cultured in laboratory conditions. Like other marine bacteria, very few research on membrane phospholipids of *Vibrio* sp. are available [45–49] and studies on *V. splendidus* in this regard are even more sparse. To the best of our knowledge, only one study describes membrane phospholipids of *V. splendidus*, using a strain living in deep anoxic sediments [50].

Deuterium SS-NMR is an excellent tool to investigate the structure and dynamics of membrane lipids at the molecular level [51–53]. Warnet et al. showed that magic-angle spinning (MAS) can be used in ^{2}H in-cell SS-NMR studies to shorten the acquisition time by a factor of 10 while simultaneously maintaining spectral sensitivity, thus favoring the *in vivo* character of the experiment [54]. However, ^{2}H NMR requires isotopic labelling which can be challenging in biological organisms such as *V. splendidus*. Notably, deuteration labelling protocols of bacteria phospholipids require the use of surfactants [55–57] to micellize deuterated fatty acids prior to their uptake by the bacteria and use in the phospholipids biosynthesis. These surfactants can possibly affect the membrane. Moreover, the natural saturated/unsaturated lipid ratio in the bacterial membrane should be preserved. A careful optimization of the deuteration protocol is thus necessary to ensure genuine *in vivo* characterization of these microorganisms.

In this work we propose for the first time a protocol for the deuteration of lipids in a model marine bacterium, *V. splendidus*. To do so, we assessed the effect of different surfactants on the cell growth. We also studied the effect of palmitic and oleic acid on the lipid profile and membrane fluidity by *in vivo* ^{2}H SS-NMR as a function of cell growth stage. The labelling strategy developed here has the potential to be amenable to the *in vivo* NMR investigations of a variety of marine and terrestrial bacteria.

2. Materials and methods

2.1. Materials

Triton X-100 and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS), Brij35, oleic (OA) and deuterated palmitic (d$_{31}$-PA) acids, deuterium-depleted water, as well as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and fatty acid methyl ester (FAME) mix C4-C24 were all purchased from Sigma Aldrich (Oakville, Canada). Dodecylphosphocholine (DPC) and octylglucopyranoside (OG) were obtained from Avanti Polar Lipids (Alabaster, AL). Polyethylene glycol sorbitan monolaurate (Tween® 20) was purchased from BioShop Canada Inc. (Burlington, Canada), whereas LB (Lysogeny broth) Broth Miller was obtained from BioBasic Inc. (Markham, Canada).

2.2. Bacterial growth and ^{2}H labelling protocol

Vibrio splendidus strain 7SHRW were isolated from sediments of Hillsborough River, Prince Edward Island (Gulf of St. Lawrence, Canada) [43]. Cell culture was initiated by adding 100 μL of a frozen cell stock solution (in 40% glycerol at −80 °C in 10 mL LB medium (NaCl 10 g/L, tryptone 10 g/L, yeast extracts 5 g/L)), and incubated at 24.5 °C (± 0.5 °C) on a rotary shaker (INFORS HT Multitron Pro, USA) operating at 100 rpm. After 3 days of growth, bacteria were transferred into 250 mL Erlenmeyer flasks containing 100 mL of LB 1× (initial OD$_{600nm}$ adjusted to ≈0.02) and 1 mL was transferred into a 24-well plate to monitor the growth kinetic with a multiple plate reader (Infinite M200 TECAN, Männedorf, Switzerland). Plates were conditioned to 24.5 °C (± 0.5 °C) and to an agitation of 87 rpm before measuring the absorbance at 600 nm every 30 min during 48 h. For each treatment, 3 to 4 wells were used. For isotopic labelling, *V. splendidus* were grown in the same conditions as described above but in an LB medium enriched with deuterated palmitic acid (d$_{31}$-PA). Prior to each use, the *Vibrio* strain was grown for two days and its purity was verified on LB-agar. The initial bacteria concentration was adjusted to be the same between replicates, *i.e.*, an optical density (OD$_{600nm}$) of 0.02 at 600 nm wavelengths.

Initially, different commonly used surfactants were tested to micellize palmitic acid (0.3 mM) in culture media, and bacterial growths were monitored. Surfactants were used above their critical micelle concentration (CMC): Brij35 (0.1 mM), Tween20 (0.06 mM), Triton-X (0.4 mM), OG (20 mM), CHAPS (6 mM), and DPC (1.5 mM). The lipid deuteration procedure was optimized as follows: LB culture medium was supplemented with a mixture of d$_{31}$-PA (0.3 mM) and Tween-20 (0.14 mM), heated at 85 °C, and the corresponding solution was flash-frozen and heated again several times until the complete dissolution of d$_{31}$-PA crystals. Protonated OA was added in the same proportions (0.3 mM) during bacteria inoculation, to mitigate potential unbalance of the saturated/unsaturated fatty acid (SFA/UFA) ratio within the membrane [54]. Potential effect of the ^{2}H labelling was verified by comparing the growth of bacteria in deuterated and non-deuterated media. The specific growth rate (μ) was estimated from the slope regression of ln (OD$_{600nm}$) as a function of elapsed time [58].

2.3. Fatty acid profile analysis

Fatty acid (FA) profiles were analyzed using gas chromatography combined to mass spectrometry (GC–MS) to obtain the proportion of each FA (deuterated or protonated) in ng·mg^{-1}, and were expressed as relative concentration (weight % relative to total FA contents) as described by Tardy-Laporte et al. [57]. Briefly, starting from three pools of 30 to 60 mg of dry-freezed bacteria, total lipids were extracted using dichloromethane/methanol (2:1 CH$_2$Cl$_2$/MeOH v/v) and 0.88% KCl solution in a Potter glass homogenizer. Neutral and polar lipids were separated by elution through a silica gel column (30 × 5 mm) hydrated

with 6% water. Polar lipids were transesterified using 2 mL of H_2SO_4 (2% in MeOH) and 0.8 mL of toluene. Final extracts were diluted in hexane solution and adjusted to a volume of 0.5 mL before GC–MS analysis (Agilent technology-7890A, Santa Clara, CA, USA). FA analyses were performed in parallel on a FAME mix which was used as a standard.

2.4. Sample preparation for ^2H SS-NMR analysis and viability assays

Freshly collected bacterial cells were centrifuged at 4000 rpm for 10 min to remove the culture medium. Pellets were then suspended in a saline sterile rinsing solution (9‰ NaCl) to remove any residual FAs and detergent molecules, and centrifuged again at 3800 rpm for 5 min. Rinsing was carried out at least 3 times, twice with saline solution prepared with nanopure water, and a final wash with saline solution prepared with deuterium-depleted water. The final pellet was used to fill a 4-mm zirconium oxide rotor, which corresponds to approximately 90 mg of hydrated bacteria. NMR experiments were performed on deuterated bacteria harvested at three different growth times (mid-log, early stationary phase and late stationary phase) and prepared in triplicate.

Following NMR experiments *V. splendidus* viability was determined using MTT reduction assays [59]. Cell suspensions from NMR samples were diluted in 5 replicates to a final OD_{600nm} of 0.1 each, and were mixed with MTT solution (5 mg/ml) to a ratio of MTT/cell suspension of 1:10 (v/v). The mixture was incubated in Eppendorf tubes for 20 min at 25 °C with open caps until the formation of formazan crystals. Preparations were centrifuged ($10.000g \times 1$ min.) and the crystal pellets were dissolved in dimethylsulfoxide and incubated at room temperature for 15 min. Optical density was measured at 550 nm and cell viability of bacteria was expressed in relative percentage compared to freshly harvested bacteria before rinse. MTT assays were performed on triplicates of cultures corresponding to the NMR samples.

2.5. In vivo ^2H SS-NMR experiments and moment analysis

All ^2H solid-state NMR experiments were performed at 25 °C on a Bruker Avance HD III wide Bore 600 MHz spectrometer (Billerica, MA, USA) using a double-resonance magic angle spinning (MAS) probe tuned to 92.1 MHz. Sample spinning frequency was set to 10 kHz. Typically, spectra were acquired using a Hahn Echo pulse sequence (90°-t-180°-t) with the following operating conditions: 5 μs 90° pulses separated by an echo delay of 100 μs and a recycle time of 0.5 s. Each spectrum was obtained in approximately 43 min of acquisition time, corresponding to 4096 scans. A total of 32 k points were acquired for a spectral width of 500 kHz. Spectra were Fourier transformed after application of a 50 Hz exponential line broadening and zero filling to 64 k points.

Spectral moment analysis was performed using MestRenova software V6.0 (Mestrelab Research, Santiago de Compostela, Spain) and a macro developed by Pierre Audet (Université Laval). The second moment (M_2) was calculated as described in Eq. (1) [54]:

$$M_2 = \omega_r^2 \frac{\sum_{N=0}^{\infty} N^2 A_N}{\sum_{N=0}^{\infty} A_N} = \frac{4\pi^2 \nu_Q^2}{5} \langle S_{CD}^2 \rangle \tag{1}$$

where ω_r is the angular spinning frequency, N the side band number, and A_N the area of each sideband obtained by spectral integration, S_{CD} is the mean square order parameter, and ν_Q is the static quadrupolar coupling constant equal to 168 kHz for a C-D bond in acyl chains [60].

3. Results

3.1. Optimized ^2H labelling of V. splendidus for in vivo SS-NMR

The enzymatic machinery of bacteria energetically favors the
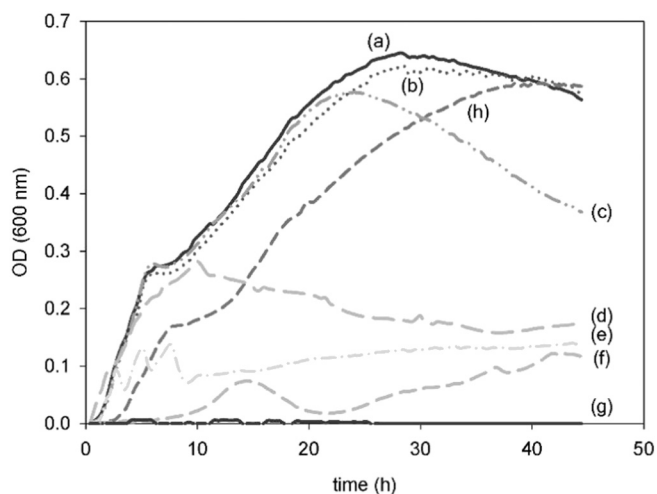

Fig. 1. Effect of different surfactants on *V. splendidus* growth. Control (a) experiment using the growth medium, and was compared to the following detergents at their CMC: Tween-20 (b), Brij-35 (c), DPC (d), Triton-X (e), CHAPS (f) and OG (g). Tween-20 was also tested at 0.25 mM (h).

incorporation of exogenous FAs into phospholipids [61], thus enabling the incorporation of deuterated PA chains exclusively in the membranes. We based our ^2H labelling procedure of *V. splendidus* on a previously published protocol for another Gram($-$) bacterium, *Escherichia coli*, which involved micellization of FAs by DPC in the growth medium to facilitate the FA uptake. However, since the presence of surfactants could be harmful to bacteria, and since another surfactant (Brij-58) had been used by other groups [56,62], we tested a series of detergents to identify the best suited for optimal bacterial lipid deuteration. Therefore, Tween-20, Brij-35, DPC, Triton-X, CHAPS and OG were assessed. To do so, we first monitored bacterial growth in different culture media when mixing protonated palmitic acid (PA) with these non-anionic surfactants above their CMC. Fig. 1 shows that Tween-20 is the less harmful detergent for the lipid deuteration of *V. splendidus*, and that a high concentration of bacteria is measured even at a detergent concentration of 0.25 mM, *i.e.*, well above Tween-20's CMC (0.06 mM). A concentration of 0.14 mM of Tween-20 was thus employed for the rest of the study because it allows complete solubilization of PA (concentration of 0.3 mM) without affecting the bacterial culture.

The deuteration of *V. splendidus* membrane lipids was then verified by ^2H SS-NMR. The second spectral moment M_2 was calculated to assess the membrane fluidity. When specific quadrupolar splittings cannot be measured, M_2 values are good reporter of the spectral distribution which reflects the lipid phases - the greater the M_2 value, the greater the lipid ordering. Fig. 2B–C shows that the deuteration protocol used herein was successful as a good signal-to-noise ratio is observed *in vivo* by ^2H SS-NMR when bacteria are sampled in the exponential growth phase. The importance of the surfactant-mediated micellization step can be seen in Fig. 2A which shows that when d_{31}-PA is used without Tween-20, no side bands can be detected (*i.e.*, there is no labelling). Fig. 2C also shows that supplementing the culture medium with oleic acid (OA) in addition to d_{31}-PA leads to a reduction in side bands intensities, indicating an increase in membrane fluidity further demonstrated by the decrease in M_2 value. These findings suggest that both unsaturated (OA) and saturated (d_{31}-PA) FAs were integrated by the cell. The *in vivo* NMR conditions during *V. splendidus* analysis were confirmed by estimating the bacterial viability, which was 95 ± 5%.

3.2. Fatty acid composition as a function of cell growth and labelling conditions

Since the labelling protocol was successful, the membrane FA profile was monitored in order to quantify the assimilation of exogenous
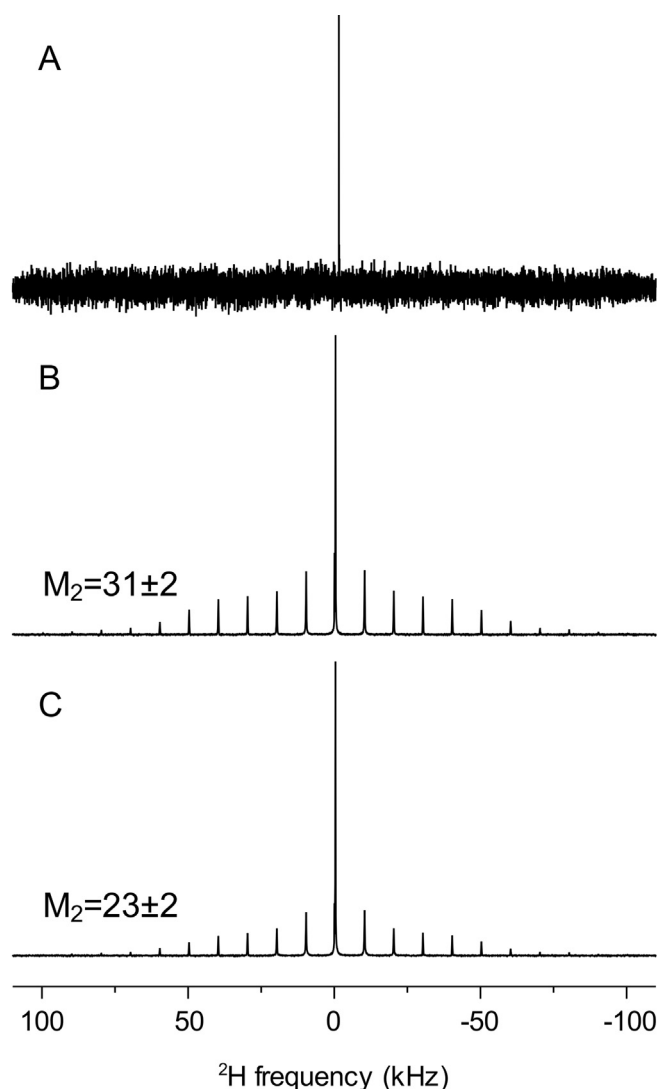
Fig. 2. *In vivo* ^{2}H MAS (10 kHz) SS-NMR spectra of *V. splendidus* harvested in the mid-log phase. Control experiment corresponding to bacteria labelled with d_{31}-PA without detergent (A), bacteria labelled with d_{31}-PA in the presence of Tween-20 (0.14 mM) without (B), and with (C) OA (1:1). Spectra are normalized according to the central peak. Average second spectral moments M_2 are indicated (10^9 s^{-2}).
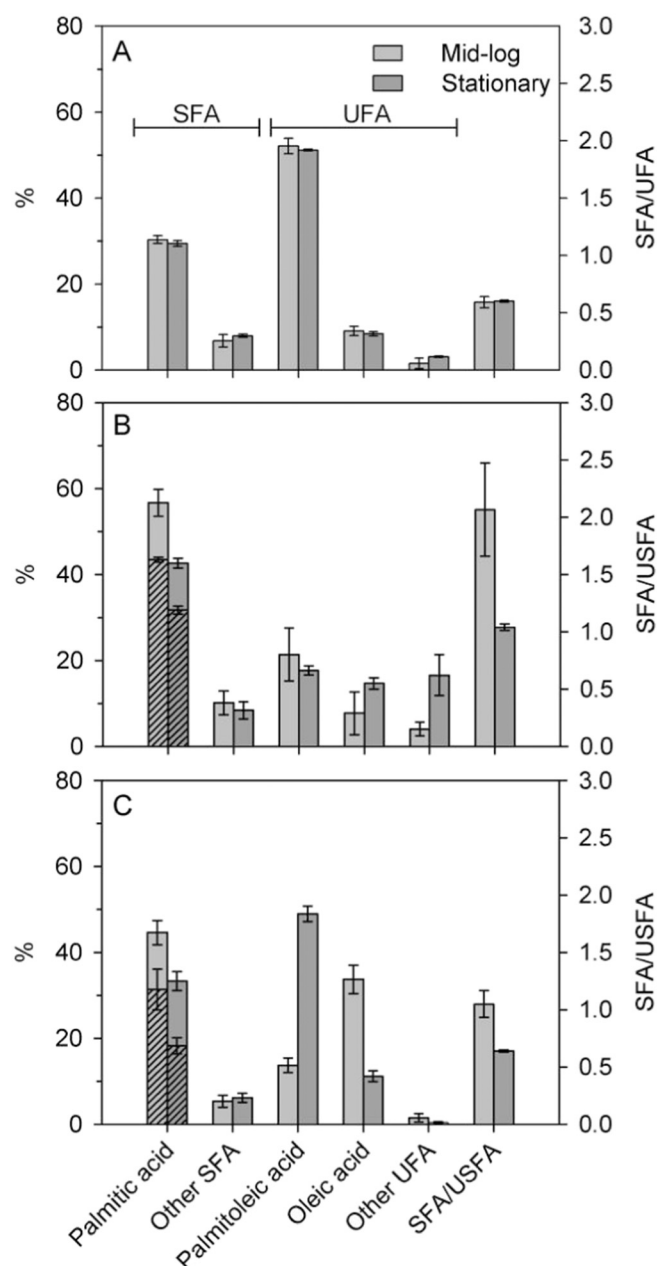

Fig. 3. Fatty acid composition and corresponding SFA/UFA ratio at exponential (mid-log) and early stationary growth phases of *V. splendidus* in the presence of exogenous FAs. (A) Control sample in LB medium, (B) in presence of d_{31}-PA, and (C) in presence of d_{31}-PA and OA (1:1). Other SFAs includes lauric, myristic, pentadecanoic, heptadecanoic, stearic and arachidic acids (see complete assignment in Table SI1). Other UFAs include myristoleic, pentadecenoic, stearidonic, α-linoleic, eicosanoids, cyclopropyl heptadecanoic (cyC17:0), and cyclopropyl nonadecanoic (cyC19:0) acids (see complete assignment in Table SI1). Hatched histograms indicate the relative proportion of d_{31}-PA per total palmitic acid. Saturated/unsaturated fatty acid ratios were calculated from FA content expressed in mol% per total FA (see S.I). Growth temperature was 25 °C for all cultures.

FAs at different growth stages and assess the potential impact of ^{2}H labelling on the membrane. Lipid analyses were thus performed on labelled and non-labelled bacteria and comparisons established between growth media enriched with d_{31}-PA, with and without OA. Cell growth data are presented in the "*Supplementary material*" section. Fig. 3 shows that the incorporation of d_{31}-PA is indeed successful and that 77% of the PA acyl chains in the membrane are deuterated during the mid-log phase for *V. splendidus* grown in the presence of d_{31}-PA (amounting to 44% of all FAs). The deuteration level of PA is maintained as high as 69% (31% of all fatty acids, numerical values are reported in *Supplementary material*, Table SI1) when OA is added in the growth medium, in spite of the high incorporation of OA into the membranes lipids. In the early stationary phase, the level of deuterated PA remains high, *i.e.* 75% of PA (or 32% of all FAs). When OA is added, this deuteration level is reduced to 55% (18% of all FAs), but it is still sufficient to generate a strong ^{2}H NMR signal (see Fig. 4B).

Depending on the growth regimes, *V. splendidus* FA profile reveals more than 15 different FA chains with three major and recurring components: palmitoleic acid (C16:1), PA (C16), and OA (C18:1) (Fig. 3). Our results (Fig. 3A) indicate that deuteration with d_{31}-PA

respects the native composition of *V. splendidus*. In addition, exogenous OA was highly incorporated during the exponential phase, at the expense of palmitoleic acid - the most naturally abundant UFA in *V. splendidus* membranes. The choice to enrich the culture with OA as an UFA was to balance as much as possible the incorporation of saturated d_{31}-PA and to allow comparison with previously published deuteration protocols for other bacteria such as *E. coli* [40,54,56]. It also enabled exploring the adaptability of *V. splendidus* to integrate exogenous FAs
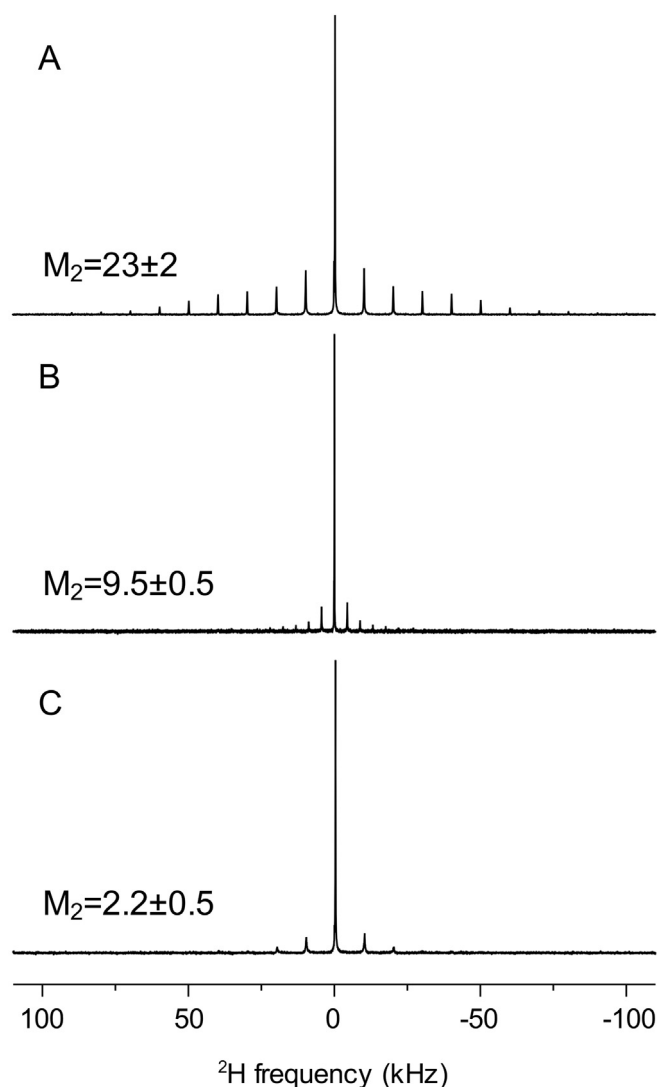
A

$M_2=23\pm2$

B

$M_2=9.5\pm0.5$

C

$M_2=2.2\pm0.5$

| 100 | 50 | 0 | -50 | -100 |

^{2}H frequency (kHz)

Fig. 4. ^{2}H MAS (10 kHz) SS-NMR spectra of intact *V. splendidus* harvested at three different cell growth times: (A) after 15 h in the mid-log phase (OD$_{600nm}$ ≈ 0.3), (B) after 22 h (± 2 h) at the beginning of the stationary stage (OD$_{600nm}$ ≈ 0.5), and (C) after 30 h at advanced stationary phase (OD$_{600nm}$ ≈ 0.5). Bacteria were labelled with d$_{31}$-PA in presence of OA. Average second spectral moments M$_2$ are indicated (10^9 s^{-2}).

and to modulate its FA chain fluidity accordingly.

Indeed bacteria responded to the enrichment with both exogenous FAs by including them in their lipid profile, more strikingly during the exponential phase, without affecting much the proportions of the other FAs (Fig. 3B). Permutational multivariate analysis of variance (Permanova) on Bray-Curtis matrices (Primer 7.0.13) of FAs composition of cell culture from different treatments and growth phases reveal significant interaction between both factors (DF = 2 and 22, Pseudo-F = 4.05, *p* = 0.005). Pair-wise Permanova comparison test indicates more specifically where differences were observed. Interestingly, the FA compositions were similar to that of the native bacteria membranes during the plateau phase (Fig. 3A), suggesting an adaptation of the bacteria to the growth medium (*p* = 0.089). In contrast, growing bacteria with only d$_{31}$-PA leads to a more altered FA profile (*p* = 0.002). For instance, the major FA chains for *V. splendidus*, *i.e.*, palmitoleic acid, was reduced by more than half, and other UFAs such as cyclopropane FAs (cyC17:0 and cyC19:0) were highly synthesized under this regime (see *Supplementary material*).

Under labelling conditions, both with and without OA, the SFA/UFA

ratios were higher in the exponential phase than in the stationary phase. This pattern is due to increased biosynthesis of UFAs such as palmitoleic acid (C16:1) or cyclopropane FAs (cyC17:0 and cyC19:0). It should also be noted that the SFA/UFA ratio of bacteria labelled in the presence of OA and during early stationary phase (SFA/UFA = 0.65 ± 0.01) was close to that of unlabelled cultures which was about 0.59 ± 0.05 and 0.60 ± 0.01 during mid-log and early stationary phases, respectively.

3.3. Membrane fluidity characterization by SS-NMR

The ^{2}H SS-NMR spectra obtained for the labelled bacteria (Fig. 4) show that membrane rigidity varies with cell division. Under the same growth conditions (d$_{31}$-PA with OA) and in a timeframe of 5 h, a reduction was observed in the sideband intensity and number. Accordingly, the M$_2$ values dropped by half going from the mid-log to the stationary phase. These results are consistent with the decreased SFA/UFA ratio which reveal an increase in membrane fluidity as a function of growth time under the same growth conditions.

4. Discussion

4.1. ^{2}H labelling optimization for in vivo study of membranes by ^{2}H SS-NMR

Nuclear magnetic resonance spectroscopy has gained an appreciated reputation among biologists, notably because of its low radiation energy in the radio frequency range, which causes no detrimental effects on biological tissues [35]. This property has allowed NMR to tackle different living systems (cells or tissues) which is referred to as *in vivo* NMR [31,35,36]. Labelling is a common strategy in NMR and deuterium labelling of lipid chains is a useful approach to explore changes occurring in biological membranes using SS-NMR. However, when studying living organisms, the deuteration protocol could be a disturbing factor to physiological pathways, or induce stress. Previously published work has established the feasibility and pertinence of bacterial membrane deuteration on human pathogens *E. coli* and *B. subtilis* [54–57,63]. Here, a marine bacterium, *V. splendidus*, is deuterated for the first time while minimizing disturbances on the microorganism. Although *V. splendidus* and previously characterized *E. coli* [40,54] are both Gram(−) bacteria, differences in ^{2}H SS-NMR spectra between these two species were expected, due perhaps to species-specific biological requirements [64,65].

The capacity of the bacteria to grow under our labelling conditions was first verified. While deuteration of molecules such as FAs does not affect their structure and thermodynamical stability, surfactants used to facilitate the deuterated FA incorporation into the bacteria [66], could have a detrimental effect since detergents are commonly used for biochemical applications such as extraction of membrane proteins. Therefore, the physiological state of the bacteria during labelling procedure was monitored using growth kinetics, and our results showed that Tween-20 had the lowest impact. This result is in agreement with Schuck et al. who compared sensitivities of diverse cells toward a set of detergents and showed that Tween-20 was always the least effective in provoking membrane resistance, and the best at preserving membrane integrity [67]. Tween-20 has a low CMC, does not affect protein activity, and is rarely used for cell lysis or protein extraction, but rather as a gentle washing agent. [67,68]. On the other hand, although harmless for the first 10 h of culture, the zwitterionic detergent DPC used to deuterate *E. coli* and *B. subtilis* lipids showed a hampering action on *V. splendidus* at longer exposure time, which could be explained by its ability to break protein-lipid and lipid-lipid associations [68,69].

De novo synthesis of FAs is the most energetically expensive mechanism in phospholipid synthesis for bacteria membrane [49]. Because they do not have specialized functional cellular compartments, prokaryotic organisms rely on multiple regulation tools to control their

phospholipid composition according to the niche in which they live [47,49,50,70,71]. Overall, bacteria tend to sustain their membrane homeostasis through adaptation mechanisms by maintaining zwitterionic/anionic, protein/lipid [70] or FA chain ratios [72]. The SFA/UFA ratio is one of the most used reporters of membrane fluidity [73–75] as well as bacterial adaptive strategies [73–77]. Our results showed that when PA was added in the growth medium of *V. splendidus* without OA, bacteria adapted by synthesizing more cyclic fatty acids (cyC17:0 and cyC19:0) that restored membrane fluidity. Similarly, when OA was also added and despite the fact that OA is not the major UFA in *V. splendidus*, this FA was highly integrated in the exponential phase, resulting in a FA profile similar to that of unlabelled bacteria. The SFA/UFA ratios determined here confirm the need to enrich the medium with UFAs during labelling with d_{31}-PA in order to avoid extreme shifts from the natural lipid composition (Fig. 3), in agreement with previous work carried out on *E. coli* [40,54,56].

In short, our lipid deuteration protocol on *V. splendidus* allowed a similar or better labelling rate as compared to previous work on bacteria. Lipid profile analyses showed that at least 69% deuteration of C16:0 acyl chains was observed in the exponential phase. This deuteration level is slightly higher than what was reported for *E. coli* grown in the presence of d_{31}-PA [40,54]. Except for minor differences in the FA incorporation process [47] and a different culture time lapse, the lipid metabolism of *Vibrio* species is very similar to *E. coli*'s since they are both Gram(−) bacteria with similar transcriptional regulatory genes for FA metabolism and phospholipid composition [47,49,72,76].

4.2. Membrane evolution during bacterial growth

Our results showed that the membrane fluidity varied with bacterial growth during the labelling process. Indeed bacteria experience the fastest growth rate in the exponential phase during which they produce membrane lipids [71]. Once lipids are synthesized, the lipid profile remains the same until the stationary phase is reached, *i.e.*, when nutrients become limited, DNA replication, protein synthesis and respiration are reduced, and cell lysis begins [78]. Previous *in vivo* NMR studies have been carried out on bacteria sampled at the mid-log growth stage [40,54,56,57] which provides excellent NMR signal-to-noise ratio. However, our results revealed that samples obtained at later stages, especially the early stationary stage, provide sufficient labelling for a good NMR signal-to-noise ratio, as well as additional advantages.

Indeed GC–MS analyses showed that exogenous PA and OA were highly integrated in the membrane at mid-log phase during which they become the most abundant FA chains in the membrane (Fig. 3). In subsequent growth stages, PA and OA diminished to reach more "native" proportions (*i.e.*, those measured in the control samples), while the amount of palmitoleic acid increased, through an unknown mechanism that could imply FA conversion or exchange, the addition of a double bond or a CH_2, or the synthesis of new FAs. This is consistent with other investigations on *Vibrio* species, showing that exogenous FAs are incorporated by direct trans-acylation or after incorporation into the biosynthesis pool [47]. Additionally the SFA/UFA ratio, which decreased as a function of growth time, reaching that of unlabelled bacteria (especially when both PA and OA were added to the culture medium), validate the evolution of the FA profile. The ^{2}H SS-NMR spectra also reveal an increase in membrane fluidity as a function of *V. splendidus* growth stages when d_{31}-PA, and to a lesser extent when equal proportions of OA and d_{31}-PA, are added to the growth medium (Fig. 4). In the exponential phase, a more rigid membrane was observed, close to a gel phase (M_2 above $20 \times 10^9 \, s^{-2}$), whereas in later stages the M_2 value was closer to that of a fluid phase membrane (M_2 below $10 \times 10^9 \, s^{-2}$) [54]. Since the FA composition and SFA/UFA ratio were closer to their "native" values in the early stationary phase, the lower M_2 values would thus report with biological membranes in the fluid phase [79].

The contribution of signal from free d_{31}-PA cannot explain the

change in lipid profile during cell growth. Indeed Fig. 2A shows no ^{2}H SS-NMR signal after rinsing. Additionally, free d_{31}-PA is insoluble in water and would lead to a very broad ^{2}H SS-NMR spectrum with M_2 above $70 \times 10^9 \, s^{-2}$ (data not shown), which is incompatible with our results. Moreover, the ^{2}H SS-NMR spectrum of d_{31}-PA crystals would not vary with growth time. In light of these results, the increase in membrane fluidity with cell growth time would be explained by a gradual adaptation of the microorganism metabolism upon isotopic labelling to cope with possible stress and disturbance of metabolic pathways [73–75] due to chemical isotopic labelling. In most cases, stress is known to lead to an increase in membrane rigidity, although the opposite pattern could happen in the case of marine prokaryotic organisms, and specifically for *Vibrio* species [46,75]. The increased fluidity due to increased UFA proportions, and notably cyclopropanes FAs observed when PA was added in the growth medium, seems to prove such environmental stress [75,80]. However, when both PA and OA were present in the medium, cyclopropane FA proportions were inverted (Table SI1), suggesting that stress was reduced [80]. Similarly, the superimposed growth curves (see Fig. SI1) indicate that *V. splendidus* metabolism kinetics under isotopic labelling was not affected by labelling stress. Therefore, the FAs turnover would be fast and all exogenous d_{31}-PA transferred into the membrane lipids at the mid-log phase. Yet, being long and saturated, they would induce large M_2 values. In the early stationary phase, bacteria would have had enough time to regulate their metabolism to the presence of exogenous FAs in the growth medium, generally through adapting their enzymatic machinery [71], and have either converted into or synthesized new UFAs in order to reduce the membrane rigidity, and reach their initial membrane state, as observed by the reduced M_2 values.

5. Conclusion and perspectives

Setting out labelling procedures is an important step toward developing effective and reliable tools for *in vivo* NMR studies. Here, membranes of intact *V. splendidus* were characterized for the first time by SS-NMR, as a model of marine Gram(−) bacteria. This work provides a better understanding of changes in bacterial membrane properties as a function of growth time and conditions that have never been studied so far on any biological system by *in vivo* NMR. Overall, the present study should help in the design of efficient labelling protocols of bacteria, which takes into account not only the technical spectroscopy requirements for analyses (such as signal-to-noise ratio) but also biological considerations. This ^{2}H labelling protocol is useful for ^{2}H SS-NMR, but could also be useful for neutron diffraction, or extended to the labelling of cell membranes by other isotopes such as ^{13}C. Additionally, by characterizing the membrane of *V. splendidus*, a potential virulent bacterium for marine organisms, this work paves the way toward studying the interactions of its membrane with exogenous molecules in the aquatic environment.

Transparency document

The Transparency document associated with this article can be found, in online version.

Competing interests

No competing interests declared.

Author contributions

Z.B. designed and conducted all sampling, data collection and analysis, and wrote the first draft of the manuscript. D.E.W. and A.A. contributed to the data analysis and writing of the manuscript. K.L. provided the initial *Vibrio* strain culture and participated to the revision of the manuscript. R.T. and I.M. designed and supervised the research, contributed to the data analysis and writing of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamem.2019.01.018.

References

[1] H. Urakawa, I.N.G. Rivera, Aquatic environment, The Biology of *Vibrios*, American Society of Microbiology, 2006, pp. 175–189.

[2] F.L. Thompson, T. Iida, J. Swings, Biodiversity of *Vibrios*, Microbiol. Mol. Biol. Rev. 68 (3) (2004) 403–431.

[3] R.A. Cavallo, L. Stabili, Presence of *vibrios* in seawater and *Mytilus galloprovincialis* (Lam.) from the Mar Piccolo of Taranto (Ionian Sea), Water Res. 36 (15) (2002) 3719–3726.

[4] D. McDougald, S. Kjelleberg, Adaptive responses of *Vibrios*, The Biology of *Vibrios*, American Society of Microbiology, 2006, pp. 133–155.

[5] L. DiSalvo, J. Blecka, R. Zebal, *Vibrio anguillarum* and larval mortality in a California coastal shellfish hatchery, Appl. Environ. Microbiol. 35 (1) (1978) 219–221.

[6] R. Crab, A. Lambert, T. Defoirdt, P. Bossier, W. Verstraete, The application of bioflocs technology to protect brine shrimp (*Artemia franciscana*) from pathogenic *Vibrio harveyi*, J. Appl. Microbiol. 109 (5) (2010) 1643–1649.

[7] I. Frans, C.W. Michiels, P. Bossier, K. Willems, B. Lievens, H. Rediers, *Vibrio anguillarum* as a fish pathogen: virulence factors, diagnosis and prevention, J. Fish Dis. 34 (9) (2011) 643–661.

[8] M.-A. Travers, K.B. Miller, A. Roque, C.S. Friedman, Bacterial diseases in marine bivalves, J. Invertebr. Pathol. 131 (2015) 11–31.

[9] M. Velji, L. Albright, T. Evelyn, Immunogenicity of various *Vibrio ordalii* lipopolysaccharide fractions in coho salmon *Oncorhynchus kisutch*, Dis. Aquat. Org. 12 (2) (1992) 97–101.

[10] C. Paillard, F. Le Roux, J.J. Borrego, Bacterial disease in marine bivalves, a review of recent studies: trends and evolution, Aquat. Living Resour. 17 (4) (2004) 477–498.

[11] C. Pruzzo, G. Gallo, L. Canesi, Persistence of *vibrios* in marine bivalves: the role of interactions with haemolymph components, Environ. Microbiol. 7 (6) (2005) 761–772.

[12] R. Beaz-Hidalgo, S. Balboa, J.L. Romalde, M.J. Figueras, Diversity and pathogenecity of *Vibrio* species in cultured bivalve molluscs, Environ. Microbiol. Rep. 2 (1) (2010) 34–43.

[13] S. De Decker, D. Saulnier, Vibriosis induced by experimental cohabitation in *Crassostrea gigas*: evidence of early infection and down-expression of immune-related genes, Fish Shellfish Immunol. 30 (2) (2011) 691–699.

[14] X. Liu, C. Ji, J. Zhao, H. Wu, Differential metabolic responses of clam *Ruditapes philippinarum* to *Vibrio anguillarum* and *Vibrio splendidus* challenges, Fish Shellfish Immunol. 35 (6) (2013) 2001–2007.

[15] M. Gay, T. Renault, A.-M. Pons, F. Le Roux, Two *Vibrio splendidus* related strains collaborate to kill *Crassostrea gigas*: taxonomy and host alterations, Dis. Aquat. Org. 62 (1–2) (2004) 65–74.

[16] R. Liu, L. Qiu, Z. Yu, J. Zi, F. Yue, L. Wang, H. Zhang, W. Teng, X. Liu, L. Song, Identification and characterisation of pathogenic *Vibrio splendidus* from Yesso scallop (*Patinopecten yessoensis*) cultured in a low temperature environment, J. Invertebr. Pathol. 114 (2) (2013) 144–150.

[17] M. Kogut, N.J. Russell, The growth and phospholipid composition of a moderately halophilic bacterium during adaptation to changes in salinity, Curr. Microbiol. 10 (2) (1984) 95–98.

[18] M. Eguchi, E. Fujiwara, N. Miyamoto, Survival of *Vibrio anguillarum* in freshwater environments: adaptation or debilitation? J. Infect. Chemother. 6 (2) (2000) 126–129.

[19] S. Manjusha, G.B. Sarita, K.K. Elyas, M. Chandrasekaran, Multiple antibiotic resistances of *Vibrio* isolates from coastal and brackish water areas, Am. J. Biochem. Biotechnol. 1 (4) (2005) 201–206.

[20] L. Masini, G. De Grandis, F. Principi, C. Mengarelli, D. Ottaviani, Research and characterization of pathogenic *vibrios* from bathing water along the Conero Riviera (Central Italy), Water Res. 41 (18) (2007) 4031–4040.

[21] W. Soto, J. Gutierrez, M. Remmenga, M. Nishiguchi, Salinity and temperature effects on physiological responses of *Vibrio fischeri* from diverse ecological niches, Microb. Ecol. 57 (1) (2009) 140–150.

[22] D.J. Phillips, The use of biological indicator organisms to monitor trace metal pollution in marine and estuarine environments—a review, Environ. Pollut. (1970) 13 (4) (1977) 281–317.

[23] C.F. Olsen, N. Cutshall, I. Larsen, Pollutant—particle associations and dynamics in coastal marine environments: a review, Mar. Chem. 11 (6) (1982) 501–533.

[24] S.W. Nixon, Coastal marine eutrophication: a definition, social causes, and future concerns, Ophelia 41 (1) (1995) 199–219.

[25] F. Baquero, J.-L. Martínez, R. Cantón, Antibiotics and antibiotic resistance in water environments, Curr. Opin. Biotechnol. 19 (3) (2008) 260–265.

[26] K. Kümmerer, Antibiotics in the aquatic environment–a review–part I, Chemosphere 75 (4) (2009) 417–434.

[27] T. Riedel, M. Zark, A.V. Vähätalo, J. Niggemann, R.G. Spencer, P.J. Hernes, T. Dittmar, Molecular signatures of biogeochemical transformations in dissolved organic matter from ten world rivers, Front. Earth Sci. 4 (2016) 85.

[28] R.E. Hancock, The bacterial outer membrane as a drug barrier, Trends Microbiol. 5 (1) (1997) 37–42.

[29] Y. Shai, Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by α-helical antimicrobial and cell non-selective membrane-lytic peptides, Biochim. Biophys. Acta Biomembr. 1462 (1) (1999) 55–70.

[30] R.G. Ratcliffe, *In vivo* NMR studies of higher plants and algae, Adv. Bot. Res. 20 (1994) 43–123.

[31] Z. Serber, L. Corsini, F. Durst, V. Dötsch, In-cell NMR spectroscopy, Methods Enzymol. 394 (2005) 17–41.

[32] P. Selenko, G. Wagner, Looking into live cells with in-cell NMR spectroscopy, J. Struct. Biol. 158 (2) (2007) 244–253.

[33] Z. Serber, A.T. Keatinge-Clay, R. Ledwidge, A.E. Kelly, S.M. Miller, V. Dötsch, High-resolution macromolecular NMR spectroscopy inside living cells, J. Am. Chem. Soc. 123 (10) (2001) 2446–2447.

[34] A.Y. Maldonado, D.S. Burz, A. Shekhtman, In-cell NMR spectroscopy, Prog. Nucl. Magn. Reson. Spectrosc. 59 (3) (2011) 197.

[35] S. Reckel, F. Löhr, V. Dötsch, In-cell NMR spectroscopy, Chembiochem 6 (9) (2005) 1601–1606.

[36] X.L. Warnet, A.A. Arnold, I. Marcotte, D.E. Warschawski, In-cell solid-state NMR: an emerging technique for the study of biological membranes, Biophys. J. 109 (12) (2015) 2461–2466.

[37] A.A. Arnold, B. Genard, F. Zito, R. Tremblay, D.E. Warschawski, I. Marcotte, Identification of lipid and saccharide constituents of whole microalgal cells by ^{13}C solid-state NMR, Biochim. Biophys. Acta Biomembr. 1848 (1) (2015) 369–377.

[38] M.S. Chauton, O.I. Optun, T.F. Bathen, Z. Volent, I.S. Gribbestad, G. Johnsen, HR MAS ^{1}H NMR spectroscopy analysis of marine microalgal whole cells, Mar. Ecol. Prog. Ser. 256 (2003) 57–62.

[39] A.J. Simpson, Y. Liaghati, B. Fortier-McGill, R. Soong, M. Akhter, Perspective: *in vivo* NMR—a potentially powerful tool for environmental research, Magn. Reson. Chem. 53 (2015) 686–690.

[40] M. Laadhari, A.A. Arnold, A.E. Gravel, F. Separovic, I. Marcotte, Interaction of the antimicrobial peptides caerin 1.1 and aurein 1.2 with intact bacteria by ^{2}H solid-state NMR, Biochim. Biophys. Acta Biomembr. 1858 (12) (2016) 2959–2964.

[41] V. Booth, D.E. Warschawski, N.P. Santisteban, M. Laadhari, I. Marcotte, Recent progress on the application of ^{2}H solid-state NMR to probe the interaction of antimicrobial peptides with intact bacteria, Biochim. Biophys. Acta Protein Proteomics 1865 (11 Pt B) (2017) 1500–1511.

[42] A. Poulhazan, A. Arnold, D. Warschawski, I. Marcotte, Unambiguous *ex situ* and *in cell* 2D ^{13}C solid-state NMR characterization of starch and its constituents, Int. J. Mol. Sci. 19 (12) (2018) 3817.

[43] D.R. Mateo, A. Siah, M.T. Araya, F.C. Berthe, G.R. Johnson, S.J. Greenwood, Differential *in vivo* response of soft-shell clam hemocytes against two strains of *Vibrio splendidus*: changes in cell structure, numbers and adherence, J. Invertebr. Pathol. 102 (1) (2009) 50–56.

[44] F. Turcotte, J.-L. Mouget, B. Genard, K. Lemarchand, J.-S. Deschênes, R. Tremblay, Prophylactic effect of *Haslea ostrearia* culture supernatant containing the pigment marennine to stabilize bivalve hatchery production, Aquat. Living Resour. 29 (4) (2016) 401.

[45] J.D. Oliver, R.R. Colwell, Extractable lipids of gram-negative marine bacteria: phospholipid composition, J. Bacteriol. 114 (3) (1973) 897–908.

[46] J.D. Oliver, W.F. Stringer, Lipid composition of a psychrophilic marine *Vibrio sp.* during starvation-induced morphogenesis, Appl. Environ. Microbiol. 47 (3) (1984) 461–466.

[47] D.M. Byers, Elongation of exogenous fatty acids by the bioluminescent bacterium *Vibrio harveyi*, J. Bacteriol. 171 (1) (1989) 59–64.

[48] R.N. Brown, P.A. Gulig, Regulation of fatty acid metabolism by FadR is essential for *Vibrio vulnificus* to cause infection of mice, J. Bacteriol. 190 (23) (2008) 7633–7644.

[49] Y.-M. Zhang, C.O. Rock, Transcriptional regulation in bacterial membrane lipid synthesis, J. Lipid Res. 50 (2009) S115–S119 (Suppl.).

[50] E. Freese, H. Rütters, J. Köster, J. Rullkötter, H. Sass, Gammaproteobacteria as a possible source of eicosapentaenoic acid in anoxic intertidal sediments, Microb. Ecol. 57 (3) (2009) 444–454.

[51] J.H. Davis, The description of membrane lipid conformation, order and dynamics by ^{2}H-NMR, Biochim. Biophys. Acta Rev. Biomembr. 737 (1) (1983) 117–171.

[52] J. Li, T. Vosegaard, Z. Guo, Applications of nuclear magnetic resonance in lipid analyses: an emerging powerful tool for lipidomics studies, Prog. Lipid Res. 68 (2017) 37–56.

[53] J. Seelig, Deuterium magnetic resonance: theory and application to lipid membranes, Q. Rev. Biophys. 10 (3) (1977) 353–418.

[54] X.L. Warnet, M. Laadhari, A.A. Arnold, I. Marcotte, D.E. Warschawski, A ^{2}H magic-angle spinning solid-state NMR characterisation of lipid membranes in intact bacteria, Biochim. Biophys. Acta Biomembr. 1858 (1) (2016) 146–152.

[55] N.P. Santisteban, M.R. Morrow, V. Booth, Protocols for studying the interaction of

MSI-78 with the membranes of whole Gram-positive and Gram-negative bacteria by NMR, Methods in Molecular Biology, 2017, pp. 217–230.

[56] J. Pius, M.R. Morrow, V. Booth, ^{2}H solid-state nuclear magnetic resonance investigation of whole *Escherichia coli* interacting with antimicrobial peptide MSI-78, Biochemistry 51 (1) (2011) 118–125.

[57] C. Tardy-Laporte, A.A. Arnold, B. Genard, R. Gastineau, M. Morançais, J.-L. Mouget, R. Tremblay, I. Marcotte, A ^{2}H solid-state NMR study of the effect of antimicrobial agents on intact *Escherichia coli* without mutating, Biochim. Biophys. Acta Biomembr. 1828 (2) (2013) 614–622.

[58] D. Nedwell, M. Rutter, Influence of temperature on growth rate and competition between two psychrotolerant Antarctic bacteria: low temperature diminishes affinity for substrate uptake, Appl. Environ. Microbiol. 60 (6) (1994) 1984–1992.

[59] H. Wang, H. Cheng, F. Wang, D. Wei, X. Wang, An improved 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) reduction assay for evaluating the viability of Escherichia coli cells, J. Microbiol. Methods 82 (3) (2010) 330–333.

[60] L. Burnett, B. Muller, Deuteron quadrupole coupling constants in three solid deuterated paraffin hydrocarbons: C2D6, C4D10, C6D14, J. Chem. Phys. 55 (12) (1971) 5829–5831.

[61] I. Marcotte, V. Booth, ^{2}H solid-state NMR study of peptide–membrane interactions in intact bacteria, Advances in Biological Solid-State NMR, 2014, pp. 459–475.

[62] J.H. Davis, C.P. Nichol, G. Weeks, M. Bloom, Study of the cytoplasmic and outer membranes of *Escherichia coli* by deuterium magnetic resonance, Biochemistry 18 (10) (1979) 2103–2112.

[63] J.-H. Davis, Deuterium magnetic resonance study of the gel and liquid crystalline phases of dipalmitoyl phosphatidylcholine, Biophys. J. 27 (3) (1979) 339–358.

[64] J. Farmer, The family Vibrionaceae, The Prokaryotes, Springer, 2006, pp. 495–507.

[65] S. Gauthier-Clerc, I. Boily, M. Fournier, K. Lemarchand, *In vivo* exposure of *Mytilus edulis* to living enteric bacteria: a threat for immune competency? Environ. Sci. Pollut. Res. 20 (2) (2013) 612–620.

[66] M. Dal Molin, G. Gasparini, P. Scrimin, F. Rastrelli, L.J. Prins, ^{13}C-isotope labelling for the facilitated NMR analysis of a complex dynamic chemical system, Chem. Commun. 47 (46) (2011) 12476–12478.

[67] S. Schuck, M. Honsho, K. Ekroos, A. Shevchenko, K. Simons, Resistance of cell membranes to different detergents, Proc. Natl. Acad. Sci. 100 (10) (2003) 5795–5800.

[68] M. Johnson, Detergents: Triton X-100, tween-20, and more, Mater. Methods 3 (1) (2013) 163.

[69] M. le Maire, P. Champeil, J.V. Møller, Interaction of membrane proteins and lipids with solubilizing detergents, Biochim. Biophys. Acta Biomembr. 1508 (1) (2000) 86–111.

[70] I. Shibuya, Metabolic regulation and biological functions of phospholipids in *Escherichia coli*, Prog. Lipid Res. 31 (3) (1992) 245–299.

[71] L.L. Barton, Cellular growth and reproduction, Structural and Functional Relationships in Prokaryotes, Springer, Editor, New York, 2005, pp. 292–346.

[72] D.F. Silbert, F. Ruch, P.R. Vagelos, Fatty acid replacements in a fatty acid auxotroph of *Escherichia coli*, J. Bacteriol. 95 (5) (1968) 1658–1665.

[73] L. Beney, P. Gervais, Influence of the fluidity of the membrane on the response of microorganisms to environmental stresses, Appl. Microbiol. Biotechnol. 57 (1) (2001) 34–42.

[74] N. Mykytczuk, J. Trevors, L. Leduc, G. Ferroni, Fluorescence polarization in studies of bacterial cytoplasmic membrane fluidity under environmental stress, Prog. Biophys. Mol. Biol. 95 (1) (2007) 60–82.

[75] Y. Yoon, H. Lee, S. Lee, S. Kim, K.-H. Choi, Membrane fluidity-related adaptive response mechanisms of foodborne bacterial pathogens under environmental stresses, Food Res. Int. 72 (2015) 25–36.

[76] C. Rock, S. Jackowski, Pathways for the incorporation of exogenous fatty acids into phosphatidylethanolamine in *Escherichia coli*, J. Biol. Chem. 260 (23) (1985) 12720–12724.

[77] T. Denich, L. Beaudette, H. Lee, J. Trevors, Effect of selected environmental and physico-chemical factors on bacterial cytoplasmic membranes, J. Microbiol. Methods 52 (2) (2003) 149–182.

[78] C. Carty, L. Ingram, Lipid synthesis during the *Escherichia coli* cell cycle, J. Bacteriol. 145 (1) (1981) 472–478.

[79] S. Morein, A.-S. Andersson, L. Rilfors, G. Lindblom, Wild-type *Escherichia coli* cells regulate the membrane lipid composition in a window between gel and non-lamellar structures, J. Biol. Chem. 271 (12) (1996) 6801–6809.

[80] W.M. O'Leary, The fatty acids of bacteria, Bacteriol. Rev. 26 (4) (1962) 421.

Supplementary Material for

Labelling strategy and membrane characterization of marine bacteria *Vibrio splendidus* by *in vivo* ^{2}H solid-state NMR

Zeineb Bouhlel[a,b], Alexandre A. Arnold[a], Dror E. Warschawski[a,c], Karine Lemarchand[b,] Réjean Tremblay[b] and Isabelle Marcotte[a]*

[a]Department of Chemistry, Université du Québec à Montréal, P.O. Box 8888, Downtown Station, Montreal, Canada, H3C 3P8

[b]Institut des Sciences de la Mer de Rimouski, Université du Québec à Rimouski, 310 allée des Ursulines, Rimouski, Canada G5L 3A1

[c]UMR 7099, CNRS - Université Paris Diderot, IBPC, 13 rue Pierre et Marie Curie, F-75005 Paris, France

*Corresponding author

Tel: 1-514-987-3000 #5015
Fax: 1-514-987-4054
E-mail: marcotte.isabelle@uqam.ca

1

(i) <u>*Effect of labelling on bacterial growth*</u>

Growth curves were acquired by periodic measurements of optical density (OD) at 600 nm for cultures enriched with exogenous fatty acids. Figure SI1 shows that supplementing the culture medium with either d_{31}-PA or the combination of d_{31}-PA and OA in the presence of Tween-20 did not induce major shifts in the delimitations of the growth phases. The transition between exponential and stationary phases was smooth, making delimitations hard to define. Typically, we define the mid-log phase occurring 15h to 16h after inoculation, if we consider stationary phase beginning around 25h and fully established after 30h of growth. Specific growth rates (μ) (see Materials & Methods) were calculated and showed no significant differences in the various labelling regimes of the cell growth (Fig. SI1). Similarly, whilst maximum optical densities in the enriched medium were slightly below those of the control, log phases durations were exactly the same (about 3 hours), indicating a good bacterial adaptation and no particular stress or toxic effect on growing bacteria in the ^{2}H enriched medium.
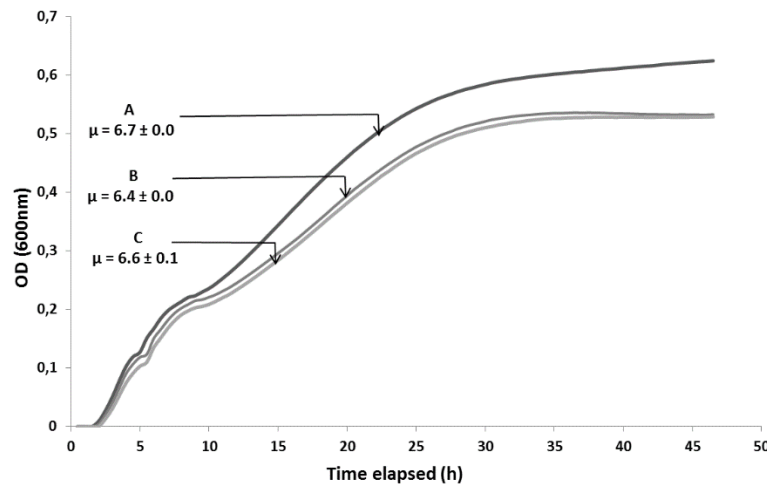

Figure SI1: Representative growth patterns of *V. splendidus* in culture medium (A), culture medium enriched with d_{31}-PA (B), with d_{31}-PA and OA (C) in presence of Tween-20. Cells were inoculated from cultures that had previously grown for 2 days. Specific growth rate (μ) was deduced from the first 25 hours of culture.

(ii) *Fatty acids contents*

Table SI1: FAs content in *V. splendidus* expressed in molar % per total FA content, deuterium fatty acid expressed in % per total palmitic acid and SFA/UFA ratio with cell growth phases under different labelling regimes: *V. splendidus* grown in contol LB-medium, in medium enriched with d_{31}-palmitic acid, and in medium enriched with d_{31}-palmitic acid and oleic acid. Bacteria were sampled during the exponential (Mid-log) and early stationary phase. Growth temperature was 25°C for all cultures.

Fatty Acid	Control		medium + d^{31}-PA		medium + d^{31}-PA + OA	
	Mid-log	Stationary	Mid-log	Stationary	Mid-log	Stationary
^{2}H-Palmitic acid ^{2}H-C16:0	0.09 (0.13)	n.d.	43.5 (0.6)	31.7 (0.9)	31.4 (4.7)	18.4 (1.8)
^{1}H-Palmitic acid ^{1}H-C16 :0	30.2 (0.9)	29.4 (0.7)	13.2 (3.7)	10.9 (1.2)	13.2 (1.9)	15.1 (0.4)
isotopic labeling %	n.a.	n.a.	77%	75%	69%	55%
Total Palmitic acid C16:0	30.3 (1.0)	29.4 (0.7)	56.7 (3.1)	42.6 (1.1)	44.6 (2.8)	33.4 (2.2)
Palmitoleic acid C16:1	52.11 (1.78)	51.2 (0.2)	21.38 (6.12)	17.7 (1.1)	13.7 (1.7)	49.1 (1.8)
Oleic acid C18:1	9.1 (1.0)	8.4 (0.5)	7.7 (4.9)	14.7 (1.1)	33.7 (3.3)	11.2 (1.2)
Lauric acid C12:0	1.35 (0.18)	1.17 (0.02)	3.14 (1.37)	2.12 (1.08)	0.96 (0.37)	0.83 (0.22)
Myristic acid C14:0	3.72 (0.65)	3.87 0.07)	4.57(0.74)	5.58 (0.42)	2.89 (0.79)	3.34 (1.33)
Pentadecanoic acid C 15:0	0.23 (0.18)	0.14 (0.10)	0.19 (0.07)	0.03 (0.04)	0.29 (0.22)	0.15 (0.14)
Heptadecanoic acid C17:0	0.26 (0.2)	0.13 (0.19)	0.21 (0.06)	n.d.	0.13 (0.11)	0.33 (0.36)
Stearic acid C18:0	1.22 (0.32)	2.65 (0.10)	1.92 (1.15)	0.67 (0.43)	0.97 (0.20)	1.18 (0.08)
Arachidic acid C20:0	0.05 (0.10)	n.d.	0.08 (0.12)	n.d.	0.12 (0.15)	n.d.
Myristoleic acid C14 :1	0.16 (0.24)	0.09 (0.13)	0.29 (0.11)	0.07 (0.10)	n.d.	0.16 (0.12)
cis-10-pentadecanoic acid C15:1	0.012 (0.027)	n.d.	0.11 (0.15)	n.d.	n.d.	n.d.
Cyclopropaneoctanoic acid-2-hydroxyl acid C17:1(cyC17:0)	0.19 (0.18)	n.d.	0.97 (0.52)	11.63 (0.48)	0.53 (0.45)	0.30 (0.40)
Stearidonic acid C18:4n	n.d.	n.d.	0.92 (0.86)	n.d.	0.24 (0.23)	n.d.
Alpha-linoleic acid C18:3n3	0.03 (0.08)	n.d.	0.96 (0.92)	n.d.	0.25 (0.21)	n.d.
Cyclopropaneoctanoic acid, 2-octyl acid C19:1(cyC19:0)	0.11 (0.16)	n.d.	0.32 (0.31)	4.95 (0.87)	0.52 (0.40)	n.d.
Eicosenoid FA C20:1n9 + C20:2 + C204n6 +C20:5n3	1.03 (1.18)	2.94 (0.06)	0.48 (0.48)	n.d.	n.d.	n.d.
Total SFA	37.16 (1.96)	37.37 (0.43)	66.86 (4.03)	50.06 (0.73)	51.06 (2.87)	39.24 (0.51)
Total UFA	62.73 (1.97)	62.63 (0.43)	33.15 (4.03)	49.94 (0.73)	48.94 (2.87)	60.76 (0.44)
SFA/UFA	**0.59 (0.05)**	**0.60 (0.01)**	**2.07(0.40)**	**1.04 (0.03)**	**1.05 (0.12)**	**0.65 (0.01)**

Values corresponds to means and respective standard deviations
"n.d."indicates that these measurements could not be detected
"n.a." indicates that these measurements are not applicable (unlabelled medium)

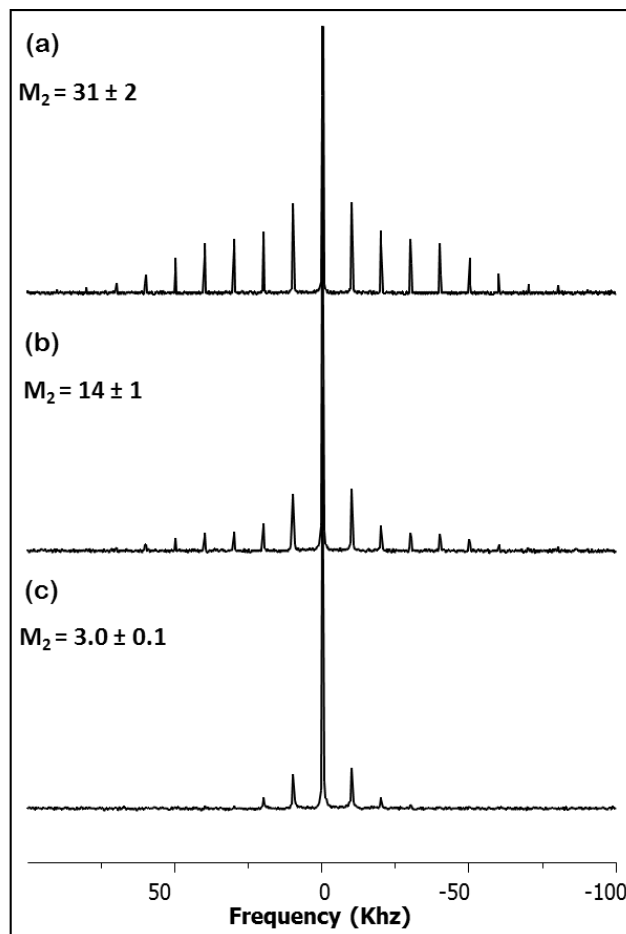*NMR spectra of bacteria enriched with d₃₁-PA in presence of Tween-20*

Figure SI2: ^{2}H MAS (10 kHz) SS-NMR spectra of intact *V. splendidus* harvested at three different cell growth times: (a) after 15 h in the mid-log phase (OD$_{600nm} \approx 0.3$), (b) after 22 h (±2h) at the beginning of the stationary stage (OD$_{600nm} \approx 0.5$), and (c) after 30 h at advanced stationary phase (OD$_{600nm} \approx 0.5$). Average second spectral moments M$_2$ are indicated (10^9 s^{-2}).