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# Assessment of membrane labelling mechanisms with exogenous fatty acids and detergents in bacteria☆

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ABSTRACT

Labelling of bacterial membranes using exogenous fatty acids has proven to be a valuable tool to investigate molecular interactions by in-cell solid-state nuclear magnetic resonance (ssNMR) spectroscopy, notably with antimicrobial peptides. However, the mechanism by which this labelling takes place in non-mutated bacteria has not yet been investigated. In this work, we propose a rapid method to assess the fate of the fatty acids during the labelling of bacteria, involving two different methylation schemes and gas chromatography coupled to mass spectrometry. We applied this approach to Gram(+) and Gram(−) bacteria grown with deuterated palmitic acid under different conditions. We assessed the extent of labelling, then the resulting membrane rigidity by $^{2}$H ssNMR. Our results reveal that the labelling mechanism depends on the detergent used to micellize the fatty acids. This labelling can be either *active* or *passive*, whether the fatty acids are metabolized and used in the phospholipids biosynthesis, or remain unmodified in the membrane. We discuss the best labelling protocol for studying peptide-membrane interactions.



## 1. Introduction

While nuclear magnetic resonance (NMR) spectroscopy has been applied to live cells since its early days [1], in-cell solid-state (ss) NMR has experienced a revival in the past decade [2]. First focused on membrane proteins, it is now expanding to the study of cell walls and extracellular matrix organization, as well as lipid membrane fluidity, and the effect of drugs. For this latter application, membrane lipid labelling is a prerequisite, and two main approaches have been suggested, using either wild type strains [3,4] or strains lacking fatty acid oxidation [5,6], both supplemented with deuterated fatty acids during growth.

In the case of wild-type strains, it has been assumed that if the labelled fatty acids were correctly delivered to the bacterial membrane (with the help of micellizing detergents), they contributed to the lipid metabolism, and finally resulted in a membrane containing at least one type of labelled fatty acid chain in *Escherichia coli* [4], *B. subtilis* [7,8] or *Vibrio splendidus* [9]. We will refer to this process as “active labelling”. Upon labelling with exogenous deuterated C16:0, the detection of deuterated C14:0 in *E. coli* and of deuterated C18:0 in *B. subtilis* was a proof that “active” labelling was indeed taking place [10]. In the context of the incorporation of host fatty acids, this mechanism was detailed in a review by Yao and Rock [11], and involves acyl-CoA synthetase in *E. coli*, and fatty acid kinase systems in Gram(+) bacteria, both located on the cytosolic side of the bacterial membrane.

However, it is also possible that a significant part of the exogenous fatty acids remain in the bacterial membrane without further transformation - a process which we term “passive labelling”. Depending on the growth conditions (labelling duration, growth phase, temperature, nature of the detergent, nature and concentration of the exogenous fatty acids etc.), the labelling efficiency is likely to vary, and a combination of “active” and “passive” labelling

*Abbreviations:* DMPC, (dimyristoyl phosphatidylcholine); DPC, (dodecylphosphocholine); DPPC, (dipalmitoyl phosphatidylcholine); DPPC-$d_{62}$, (perdeuterated dipalmitoyl phosphatidylcholine); FAME, (fatty acid methyl ester); FFA, (free fatty acid); GC/MS, (gas chromatography coupled to mass spectrometry); LB, (Lysogeny broth); MA or C14:0, (myristic acid); MAS, (magic-angle spinning); NMR, (nuclear magnetic resonance); PA or C16:0, (palmitic acid); PA-$d_{31}$, (perdeuterated palmitic acid); SA or C18:0, (stearic acid); SA-$d_{35}$, (perdeuterated stearic acid); ssNMR, (solid-state NMR); TotFA, (total fatty acid); Tween 20, (polyoxyethylenesorbitan monolaurate).

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could take place, resulting in differences of membrane rigidity that could in turn affect the interpretation of ssNMR data.

When labelling bacterial membranes, a simple and fast fatty acid profiling is required in order to test a large number of sample preparations and identify the best conditions. To do so, bacterial lipids are extracted using the classical Folch method, and phospholipid classes (headgroups) are identified by $^{31}$P solution NMR [10]. The lipid chain profile is then determined by gas chromatography coupled to mass spectrometry (GC-MS) after the transformation of lipid chains into fatty acid methyl esters (FAMEs). In the one-step approach that simultaneously hydrolyses and methylates the fatty acids [10], both the free fatty acids (FFAs) and esterified fatty acids are totaled. Therefore, to evaluate the extent of passive labelling, we need to methylate the FFAs that were present in the membrane before lipid hydrolysis.

In this work, we propose a simple protocol to evaluate the extent of membrane labelling as well as the percentage of active *vs.* passive labelling. We then apply this protocol to Gram (−) and Gram (+) bacteria grown in different conditions, *i.e. E. coli*, *Bacillus subtilis* and *Staphylococcus aureus*. In each case, we correlate the labelling process ("active" *vs.* "passive") and the resulting membrane fluidity as determined by $^{2}$H ssNMR.

## 2. Materials and methods

### 2.1. Materials

Dipalmitoyl-glycero-phosphocholine (DPPC), dimyristoyl-glycero-phosphocholine (DMPC), deuterated palmitic acid C16:0 (PA-$d_{31}$), deuterated stearic acid C18:0 (SA-$d_{35}$), dodecylphosphocholine (DPC), fatty acid methyl ester mix C4−C24 (FAME mix), formaldehyde solution (37 %), and polyoxyethylenesorbitan monolaurate (Tween 20) were purchased from Avanti/Merck (St-Quentin-en-Yvelines, France).

### 2.2. Synthetic lipid mixture

A lipid mixture composed of DPPC (34 %, in mol%), DMPC (24 %), PA-$d_{31}$ (15 %) and SA-$d_{35}$ (27 %) was prepared in 3:1 $CHCl_3$:MeOH and dried under a nitrogen stream.

### 2.3. Bacterial growth

In this work, *E. coli* K12, *E. coli* BL21, *S. aureus* (ATCC 6538) and *B. subtilis* (CIP 52.65) were studied. They were all grown in 300 ml of LB (Lysogeny Broth) medium at 37 °C (except for *S. aureus* at 24 °C), shaking at 200 rpm until the late log stage is reached. In cases where fatty acids were incorporated into the culture, they were first solubilized with DPC at 2 mM or Tween 20 at 5 mM, in 8 mL of LB medium, analogous to the protocol described in Laydevant et al. 2022. Unless specified, the fatty acids solution was added to the 300 mL culture medium 50 min after starting the bacterial culture (90 min for *S. aureus*). For *E. coli* strains, a final concentration of 100 μM of PA-$d_{31}$ was incorporated, while for all other (Gram+) bacteria, a final concentration of 50 μM PA-$d_{31}$ was added. At such concentrations of exogenous fatty acids and detergents, the bacterial growth was not affected, as compared to the control.

### 2.4. Lipid analysis

Lipids were extracted using the classical Folch method [10]. To establish their fatty acid chain profile, the lipid mixture was then spiked with a (nonmethylated) fatty acid that is not present in the mixture, *i.e.* SA-$d_{35}$. For a dry lipid mixture of approximately 10 mg, we prepared a 3 mM solution of SA-$d_{35}$ in 3:1 $CHCl_3$:MeOH. We then solubilized the dry lipid mixture with 500 μL of this solution, split it in two 250 μL samples for the two methylation protocols, and dried them under a nitrogen stream.

The lipid chain profile was established by GC/MS following their transformation into FAMEs. The one-step approach that hydrolyses and methylates the fatty acids at the same time is derived from Laydevant et al. [10]. It involves solubilizing the dry lipid mixture into 2 mL of $H_2SO_4$ (2 % in MeOH) and 800 μL of toluene, heating for 1h at 100 °C and, after cooling, adding 4 ml of $H_2O$ and 800 μL of cyclohexane, retaining the upper phase. This approach provides the total fatty acid (TotFA) proportions.

Simple methylation was done using our optimized protocol derived from Kail et al. [12]. It involves solubilizing the dry lipid mixture into 2 mL of $H_2SO_4$ (2 % in MeOH) and 800 μL of toluene, heating for 10 min at 50 °C and, after cooling, adding 4 ml of $H_2O$ and 800 μL of cyclohexane, retaining the upper phase. This approach provides the proportion of FFAs. The same initial spiked lipid mixture was used for both protocols to enable the quantitative comparison of the obtained TotFA and FFA proportions.

GC-MS analyses were performed using a GC QP2010 SE chromatograph (Shimadzu, Marne la Vallée, France) equipped with an SH-I-5Sil column of 30 m length, 0.25 mm inner diameter and 0.25 μm film thickness. Electron impact mode was performed at 70 eV. The carrier gas was helium at a constant flow of 0.70 mL/min, and the injector temperature was 250 °C. The column program was as follows: the initial temperature of 140 °C was held for 5 min, then the temperature was ramped at a rate of 4 °C/min up to 300 °C, followed by a second ramp at a rate of 20 °C/min up to 320 °C held for 10 min. The source temperature was kept at 230 °C, and the interface temperature at 280 °C. The total run time was around 1h. Data acquisition and analysis were done with the Shimadzu Insight software package. Peaks were identified by comparing their retention time to that of the standard FAME mix, and by their mass. The area of each peak was converted into percentage by comparing them to those of the FAME mix.

To obtain a quantitative comparison, the FFA percentages were normalized so that the proportion of spiking molecule was equal to the one in the TotFA sample. Therefore, the proportion of esterified fatty acids is the difference between proportions of TotFAs and normalized FFAs. This method provides an important parameter for each fatty acid, *i.e.* the proportion in which they appear as free or as esterified. In the case where the fatty acid was the one used to label the bacteria, these proportions correspond to *passive* or *active* labelling respectively (Equation (1)).

$$\%Passive\ labelling = 100 \times \frac{\%FFA}{\%TotFA} \qquad \text{(Equation 1)}$$

### 2.5. Solid-state NMR analysis

$^{2}$H ssNMR spectra were recorded on a Bruker Avance III 500 wide bore spectrometer (Wissembourg, France), operating at a frequency of 76.8 MHz for $^{2}$H, and equipped with a 4-mm magic-angle spinning (MAS) triple-resonance probe. A 10 kHz MAS frequency was used for all experiments. Spectra were recorded at 30 °C with a Hahn echo sequence using a 100 μs delay (one rotor period) and 0.5 s recycle delay. A typical spectrum was acquired with 6k scans, and a spectral width of 500 kHz, for about 1h. Spectra were processed using a 100 Hz exponential multiplication, and spectral moments were determined as described by Warnet et al. [13], with the MestRenova software V14.2 (Mestrelab Research, Santiago de Compostela, Spain).

## 3. Results

### 3.1. Comparison of FAME preparation protocols

To establish the best lipid profiling protocol to quantify FFAs in a membrane, we used a model mixture composed of phospholipids and FFAs, namely DPPC (34 %, in mol%), DMPC (24 %), PA-$d_{31}$ (15 %) and SA-$d_{35}$ (27 %). The resulting actual TotFA and FFA proportions are indicated on Table 1 (columns 2 and 3 respectively). The one-step approach derived from Laydevant et al. [10] with 1h at 100 °C instead of 10 min, and which simultaneously hydrolyses and methylates fatty acids, is very robust. Indeed, it provides the TotFA proportions with a *ca.* 20 % precision (Table 1, column 4). It uses a solution of 2 % $H_2SO_4$ in MeOH for both the hydrolysis and methylation. However, it does not separate free and esterified fatty acids, and therefore does not allow the quantification of FFAs in the sample.

To evaluate the amount of FFAs, a protocol is required that methylates without hydrolyzing. Such protocols have been suggested in the literature, often involving HCl [12,14,15], KOH [16], or $Na_2CO_3$ in methyl iodide and dimethyl sulfoxide [17]. Kail and colleagues [12] used HCl in MeOH, and varied the duration and temperature of the reaction. With low temperatures or short reaction times, only methylation takes place, while at high temperatures and for long reaction times, both hydrolysis and methylation occur.

We adapted Kail's approach to Laydevant's, by using $H_2SO_4$ instead of HCl, and optimized the temperature and duration of the reaction to selectively methylate the FFAs, while leaving the lipids intact. Our improved methylation protocol involves solubilizing the dry lipid mixture into 2 mL of $H_2SO_4$ (2 % in MeOH) and 800 μL of toluene, heating for 10 min at 50 °C and, after cooling, adding 4 ml of $H_2O$ and 800 μL of cyclohexane, retaining the upper phase. This protocol reveals the proportion of FFAs with a *ca.* 20 % precision (See Table 1, column 5). In summary, both our TotFA and FFA profilings make use of $H_2SO_4$ in MeOH, the only difference being the heating of 1h at 100 °C in the first case, and 10 min at 50 °C in the second case.

### 3.2. Application to Gram(−) bacterial membrane

We applied our optimized protocols to investigate the fate of exogenous fatty acids when added to the culture medium for the labelling of bacterial membranes. We first studied the Gram(−) strain *E. coli* K12 grown at 37 °C and harvested at the late log stage. Although the fatty acid chain profile depends on growth temperature and stage, we chose to keep those parameters fixed, in order to evaluate the effects of labelling conditions. Like other *E. coli* strains, PA is the most abundant fatty acid, but the K12 strain has the particularity of having a high proportion of C16:1 [18] in its native membranes, as well as around 5 % of FFAs (see Table 2, columns 2 and 5).

We micellized PA-$d_{31}$ (100 μM) with either DPC or Tween 20, and obtained significantly different results (Table 2). As expected, the most abundant fatty acid is the perdeuterated palmitic acid, which is also the most abundant *free* fatty acid. As shown previously [4,13], the addition of exogenous PA-$d_{31}$ reduces the proportion of natural (non deuterated) PA, but also that of C16:1. However, our results demonstrate for the first time that using Tween 20 as the micellizing agent improves the labelling efficiency compared to DPC (57 % *vs.* 35 %).

By comparing the TotFA and FFA profiles, normalized by the spiked molecule concentration, we evaluated the labelling efficiency (proportion of PA-$d_{31}$ incorporated in the membrane). Moreover, we distinguished between the amount of PA-$d_{31}$ *passively* incorporated into the membrane (using Equation (1)), and that which was *actively* metabolized by the bacteria and incorporated into bacterial lipids. Our results showed that depending on the micellizing agent, the labelling mode is different. Indeed, when using DPC, the majority of the exogenous fatty acids are used in the lipid metabolism through an “active labelling” mechanism. Out of the 35 % PA-$d_{31}$ molecules found in the bacterial membranes, only 7 % were detected as “free PA-$d_{31}$”, as a result of “passive labelling”. In other words, using DPC, only 20 % of the labelling is “passive”. By contrast, all exogenous fatty acids remain as FFAs when Tween 20 is used as a micellizing agent, and none of them are metabolized. The labelling efficiency is high, 57 %, but 100 % “passive”.

Finally, our results reveal that the active and passive labelling mechanisms are respectively favored by the use of DPC and Tween 20, and have an important effect on the resulting $^2$H ssNMR spectra. As shown in Fig. 1, each MAS spectrum is composed of spinning sidebands equally spaced on each side of a central resonance. Either ±2 or ±6 spinning sidebands are seen on the spectra, depending on the labelling pattern. To assess the membrane rigidity (Table 2, bottom line), we measured the second spectral moment, $M_2$, from the spectra (following the procedure described in Ref. [13]), and obtained values between 3 and 26 × $10^9$ $s^{-2}$.

We compared those values to extreme cases, by recording the $^2$H ssNMR spectrum of precipitated PA-$d_{31}$, which is much more rigid, as revealed by the $M_2$ value of 47 × $10^9$ $s^{-2}$ (see Fig. S1). Conversely, labelled bacteria exposed to formaldehyde led to membrane solubilization, with a corresponding low $M_2$ value of 2 × $10^9$ $s^{-2}$ for *E. coli* K12 (see Fig. 2).

When DPC is used, the labelling efficiency is reduced and split into active and passive labelling. The membrane rigidity is small, as determined by the low average $M_2$ value of 3 × $10^9$ $s^{-2}$, similar to a very fluid membrane [13]. Using Tween 20, the labelling is 100 % passive but very efficient, and the corresponding spectra with ±6 spinning sidebands has an average $M_2$ value of 26 × $10^9$ $s^{-2}$ which corresponds to a more rigid membrane.

We tested another Gram(−) strain, *i.e. E. coli* BL21 grown with 100 μM of PA-$d_{31}$ in Tween 20, and found similar results (See Table S1), with a 52 % labelling efficiency, 100 % passive labelling, and an $M_2$ of 29 × $10^9$ $s^{-2}$ (see Table 3).

### 3.3. Application to Gram(+) bacterial membrane

We then applied the optimized protocols to determine the membrane labelling mechanism of the Gram(+) *S. aureus* grown at 24 °C and harvested at the late log stage, with or without 50 μM of

**Table 1**
Fatty acid proportions in the model lipid mixture, either actual or experimentally determined. Average values for three replicates are shown with standard deviation. MA for myristic acid (C14:0). PA for palmitic acid (C16:0). SA for stearic acid (C18:0).

| Fatty acid | Actual % TotFA | Actual % FFA | Measured % TotFA | Measured % FFA |
|---|---|---|---|---|
| PA | 43 | 0 | 48 (1) | 0 |
| MA | 30 | 0 | 27 (1) | 0 |
| SA-$d_{35}$ | 17 | 64 | 16 (1) | 69 (5) |
| PA-$d_{31}$ | 10 | 36 | 8 (1) | 31 (5) |

**Table 2**
Fatty acid proportions in *E. coli* K12, grown at 37 °C, with or without 100 μM exogenous PA-$d_{31}$, micellized in DPC or Tween 20. FFAs are proportions of the TotFAs, which is why the total is not 100. Total labelling, as well as active and passive labelling proportions, are indicated. $M_2$ values extracted from $^2$H solid-state NMR spectra of the corresponding samples are also indicated. Average values for three replicates are shown with standard deviation.

| Fatty acid | % TotFA | | | % FFA | | |
|---|---|---|---|---|---|---|
| | Native | With PA-$d_{31}$ micellized in DPC | With PA-$d_{31}$ micellized in Tween 20 | Native | With PA-$d_{31}$ micellized in DPC | With PA-$d_{31}$ micellized in Tween 20 |
| MA | 4 (0.6) | 4 (1) | 3 (1) | 0 | 0 | 0 |
| C15:0 | 4 (2) | 2 (3) | 6 (4) | 0 | <1 | 0 |
| C16:1 | 29 (1) | 4 (2) | <1 | 1 (0.1) | <1 | 0 |
| PA | 47 (2) | 36 (3) | 20 (3) | 4 (0.2) | 11 (3) | 7 (2) |
| PA-$d_{31}$ | X | 35 (14) | 57 (8) | X | 7 (4) | 69 (15) |
| cyC17:0 | 3 (1) | <1 | 8 (5) | 0 | 0 | 0 |
| SA | 1 (0.2) | <1 | <1 | 0 | <1 | 0 |
| C18:1 | 10 (0.1) | 8 (5) | 4 (0.8) | 0 | <1 | 0 |
| cyC19:0 | 0 | 9 (1) | 0 | 0 | <1 | 0 |
| C20:0 | 0 | 2 (2) | 0 | 0 | 0 | 0 |
| Total labelling | X | 35 | 57 | | | |
| Active labelling | X | 80 | 0 | | | |
| Passive labelling | X | 20 | 100 | | | |
| **$M_2$ ($10^9$ $s^{-2}$)** | **X** | **3 (0.3)** | **26 (5)** | | | |

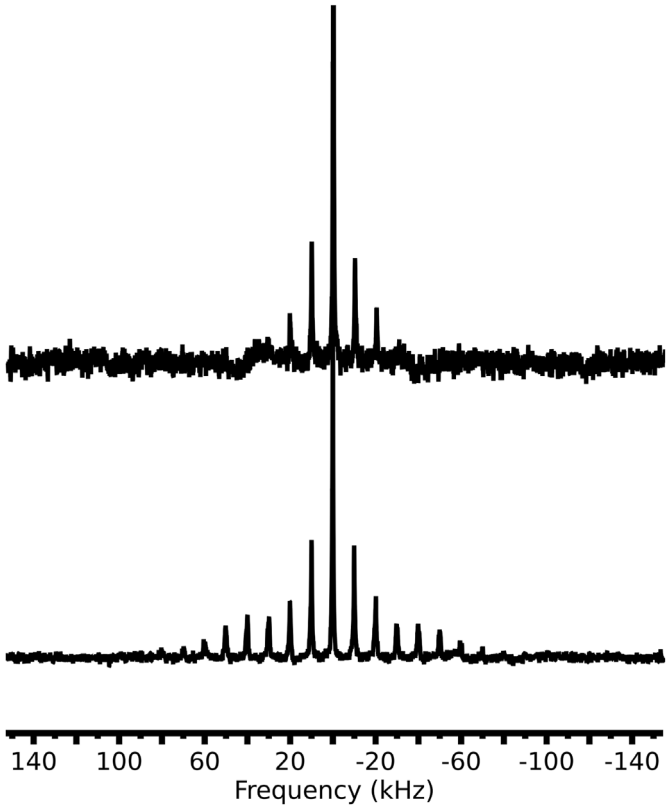


**Fig. 1.** $^2$H ssNMR spectra of *E. coli* K12 recorded at 30 °C with an MAS frequency of 10 kHz. Bacteria were grown with 100 μM of PA-$d_{31}$ micellized in DPC (top) or Tween 20 (bottom), collected at the late log stage, and pelleted. The resulting $M_2$ values are reported in Table 2. Spectra were normalized with respect to the first spinning sideband, and the central peak is thus truncated.

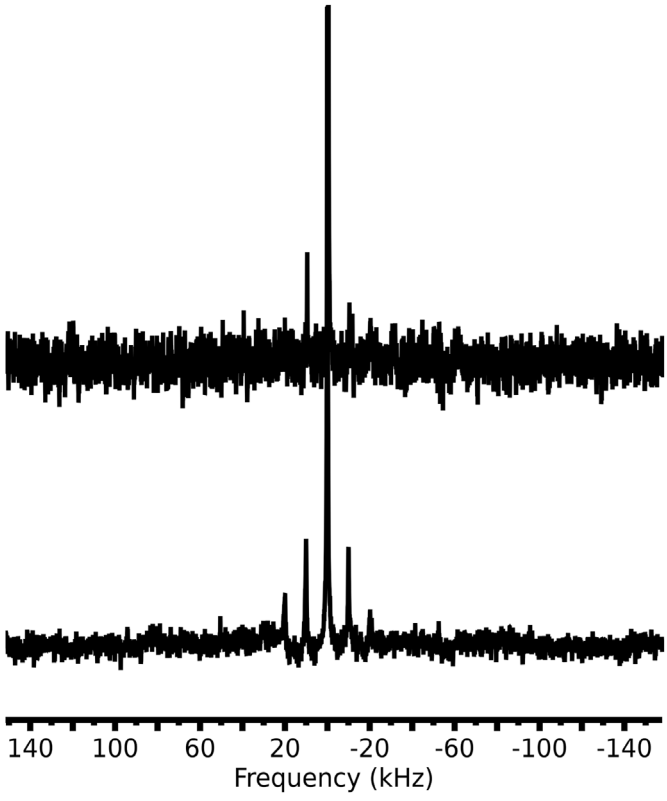


**Fig. 2.** $^2$H ssNMR spectra of *S. aureus* (top) or *E. coli* K12 (bottom) recorded at 30 °C with an MAS frequency of 10 kHz. Bacteria were grown with 50 μM (*S. aureus*) or 100 μM (*E. coli* K12) of PA-$d_{31}$ in Tween 20, collected at the late log stage, exposed to formaldehyde (10 %) for 20 min, and pelleted. The resulting $M_2$ is $1 \times 10^9$ $s^{-2}$ (*S. aureus*) or $2 \times 10^9$ $s^{-2}$ (*E. coli* K12). Note that the central peak is truncated to improve the visibility of the remaining spinning sidebands.

PA-$d_{31}$, micellized with either DPC or Tween 20 detergents (see Table 4). As expected from the literature [19,20], the most abundant esterified fatty acid is the anteiso branched C15. We also found less than 10 % of FFA in native *S. aureus* bacterial membranes.

Upon labelling we observed a decrease in the proportion of branched fatty acids, as reported elsewhere [10,11]. Here again, the labelling efficiency was improved by using Tween 20 over DPC as the micellizing agent (39 % *vs.* 8 %). Table 4 also shows that in *S. aureus*, most of the labelling is passive: 100 % passive in the case of Tween 20, and 75 % passive when using DPC.

Similar to the results obtained with *E. coli*, the $^2$H ssNMR spectra are significantly different with DPC and Tween 20. In the first case, the labelling efficiency is low (8 %), PA-$d_{31}$ being split into active and passive labelling mechanisms, and the membrane thereby remains fluid (average $M_2$ value of $2 \times 10^9$ $s^{-2}$). When Tween 20 is used, a high labelling efficiency is observed (39 %), through a 100 %

**Table 3**
Labelling efficiency, labelling mechanism and resulting rigidity. Bacteria were grown with exogenous PA-$d_{31}$, micellized in DPC or Tween 20.

| Strain | *E. coli* K12 | | *E. coli* BL21 | *S. aureus* | | *B. subtilis* |
|---|---|---|---|---|---|---|
| Detergent used | DPC | Tween 20 | Tween 20 | DPC | Tween 20 | Tween 20 |
| Total labelling (%) | 35 | 57 | 52 | 8 | 39 | 30 |
| Active labelling (%) | 80 | 0 | 0 | 25 | 0 | 7 |
| Passive labelling (%) | 20 | 100 | 100 | 75 | 100 | 93 |
| **$M_2$ ($10^9$ $s^{-2}$)** | **3** | **26** | **29** | **2** | **23** | **32** |

**Table 4**
Fatty acid proportions in *S. aureus*, grown at 24 °C with or without 50 μM exogenous PA-$d_{31}$, micellized in DPC or Tween 20. FFAs are proportions of the TotFAs, which is why the total is not 100. Total labelling, as well as active and passive labelling proportions are indicated. $M_2$ values extracted from $^2$H ssNMR spectra of the corresponding samples are also indicated. Average values for three replicates are shown with standard deviation.

| Fatty acid | % TotFA | | | % FFA | | |
|---|---|---|---|---|---|---|
| | Native | With PA-$d_{31}$ micellized in DPC | With PA-$d_{31}$ micellized in Tween 20 | Native | With PA-$d_{31}$ micellized in DPC | With PA-$d_{31}$ micellized in Tween 20 |
| Iso-C15:0 | 7 (2) | 7 (0.9) | 1 (2) | 0 | 3 (1) | <1 |
| Anteiso-C15:0 | 57 (2) | 49 (2) | 29 (11) | 5 (0.4) | 14 (8) | 3 (5) |
| PA | 1 (1) | 5 (2) | 6 (1) | 0 | <1 | 8 (4) |
| PA-$d_{31}$ | X | 8 (2) | 38 (23) | X | 6 (0.9) | 41 (17) |
| Iso-C17:0 | 4 (0.3) | 4 (0.4) | <1 | 0 | 1 (1) | 0 |
| Anteiso-C17:0 | 12 (1) | 13 (1) | <1 | 1 (2) | 5 (1) | 0 |
| SA | 5 (3) | 4 (2) | 10 (5) | 3 (4) | <1 | 6 (1) |
| Iso-C19:0 | 4 (3) | 2 (0.3) | 4 (2) | 0 | <1 | 0 |
| Anteiso-C19:0 | 3 (0.9) | 4 (0.5) | 0 | 0 | 1 (0.9) | 0 |
| C20:0 | 6 (4) | 4 (2) | 10 (5) | 0 | <1 | 5 (5) |
| Total labelling | X | 8 | 39 | | | |
| Active labelling | X | 25 | 0 | | | |
| Passive labelling | X | 75 | 100 | | | |
| **$M_2$ ($10^9$ $s^{-2}$)** | **X** | **2 (0.7)** | **23 (5)** | | | |

passive mechanism, leading to high membrane rigidity ($M_2$ ~23 × $10^9$ $s^{-2}$). This difference is directly visible on the $^2$H ssNMR spectra (see Fig. 3). Here again, exposing the labelled bacteria to formaldehyde led to the membrane solubilization, with a resulting decrease in $M_2$ to a very low value of 1 × $10^9$ $s^{-2}$ for *S. aureus* (see Fig. 2).

We also tested the Gram(+) strain *B. subtilis* grown with 50 μM of PA-$d_{31}$ in Tween 20, and found results similar to *S. aureus* (See Table S2), with a 30 % labelling efficiency, 93 % passive labelling, and an average $M_2$ of 32 × $10^9$ $s^{-2}$ (see Table 3).

## 4. Discussion

In this work, we showed that under the labelling efficiency, at least two modes of bacterial membrane labelling can take place, *i.e.* active and passive, when exogenous deuterated fatty acids are used with non-mutated bacterial strains. We also introduced a protocol to determine which mechanism is adopted depending mostly on the nature of the detergent employed to micellize the fatty acids. With two Gram(−) and two Gram(+) bacterial strains, we showed that labelling using Tween 20 as a detergent molecule is very efficient, although 100 % passive and results in a rigid bacterial membrane.

Before proceeding with data interpretation, it is essential to define what is a *rigid* bacterial membrane, and how to assess it using $M_2$ values. Maximal rigidity for PA-$d_{31}$ is observed when precipitated, with an $M_2$ value of 47 × $10^9$ $s^{-2}$. Minimal rigidity occurs when PA-$d_{31}$ is solubilized, resulting in an $M_2$ of 1-2 x $10^9$ $s^{-2}$. For perdeuterated DPPC (DPPC-$d_{62}$) model membranes, $M_2$ values range between ~5 and 27 × $10^9$ $s^{-2}$ when transitioning from the fluid to the gel phase [13]. Therefore, the $M_2$ values between 2 and 32 × $10^9$ $s^{-2}$ obtained in this study indicate the successful incorporation of FFAs into the bacterial membranes.

The $M_2$ values of 23–32 × $10^9$ $s^{-2}$, obtained when Tween 20 was used in the labelling protocol, are indicative of a membrane rigidity comparable to that of DPPC-$d_{62}$ in the gel phase. This is likely due to the large incorporation of saturated fatty acids into the membrane, as well as the presence of membrane proteins, peptidoglycan, lipoteichoic acids [21] and other structural molecules that make a cell membrane more rigid than a DPPC model membrane. For comparison, erythrocyte ghosts passively labelled with PA-$d_{31}$ exhibited $M_2$ values around 12 × $10^9$ $s^{-2}$ [22].

Aiming for *low* labelling efficiency, to maintain membrane rigidity close to the "native" state, involved using DPC as a detergent, which resulted in lower measured $M_2$ values [7,10]. However, labelling efficiency is particularly crucial in $^2$H ssNMR - a relatively insensitive technique considering the low gyromagnetic ratio of the $^2$H nucleus. Therefore, Tween 20 is preferred over DPC to attain the highest deuteration level. Our results confirmed the insertion of the exogenous fatty acids inside the membrane - an obvious prerequisite in order to monitor its rigidity. Our previous studies in which bacteria were deuterated using PA-$d_{31}$ in Tween 20 micelles confirmed that they were alive, even after the NMR experiment, and that their growth curve was not affected by the presence of PA-$d_{31}$ [9,10].

High labelling efficiency is achieved through passive labelling with Tween 20, with the trade-off being the increased rigidity caused by the incorporation of saturated fatty acids. While passive labelling is not ideal, it remains relevant for probing membranes and membrane/peptide interactions. In the case of living cells, such as the bacteria studied here, one may prefer to use actively labelled cells. This can be accomplished by utilizing special strains that favor active labelling, which has proven particularly useful in the case of static $^2$H ss-NMR [6,23,24]. Nevertheless, bacteria whose metabolism is affected by exogenous fatty acids have been argued to be bad antibiotic targets [25,26], and with altered biofilm synthesis behavior [27]. In addition, when such an option is not available (for lack of the adequate strains), passive labelling has been shown to be a very efficient reporter of membrane interactions, both in artificial membranes [28] or in whole cells with a very low metabolism, such

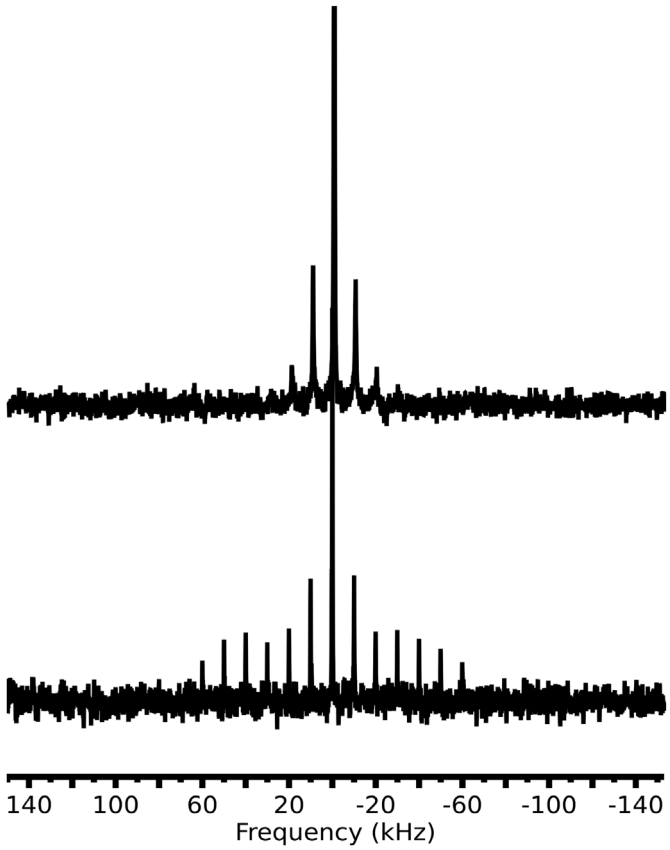


**Fig. 3.** $^{2}$H ssNMR spectra of *S. aureus* recorded at 30 °C with an MAS frequency of 10 kHz. Bacteria were grown with 50 μM of PA-$d_{31}$ in DPC (top) or Tween 20 (bottom), collected at the late log stage, and pelleted. The resulting $M_2$ values are reported in Table 4. Spectra were normalized with respect to the first spinning sideband, and the central peak is thus truncated.

as erythrocyte ghosts [22].

The enhanced rigidity caused by efficient PA-$d_{31}$ labelling, obtained through passive labelling when using Tween 20, can also be an asset. The ensuing larger $M_2$ values allowed us and others to monitor changes in membrane fluidity as a function of antimicrobial peptide concentration, and better understand their interaction mechanism [7,9,10,23,24,29,30]. Previously published data confirm that labelling using Tween 20 results in high $M_2$ values, which we believe results from efficient and passive labelling [9,10]. On the other hand, while labelling using DPC often results in low $M_2$ values [7,10], which most likely results from inefficient and active labelling, some exceptions have been reported [7]. In our hands, it has proven more challenging to observe the effect on bacteria labelled with DPC as the detergent, with starting $M_2$ values of around $3 \times 10^9$ $s^{-2}$ (Zaatouf et al. in preparation).

A question arises as to why the labelling mode and efficiency observed here seems to depend on the nature of the detergent used to solubilize the exogenous fatty acids. It should be stressed that passive labelling only requires the incorporation of fatty acids into the bacterial membrane, while active labelling requires it to cross the membrane towards the cytosolic side where it can be handled by the relevant enzymatic machinery, identified as FadL in the case of *E. coli* [31]. We first note the large difference in the critical micelle concentrations of DPC (1.5 mM) *vs.* Tween 20 (0.06 mM), which may indicate very different partition coefficients and delivery kinetics into the membrane [9,32]. We also note that DPC is zwitterionic, while Tween 20 is nonionic, which could also explain a different behavior in the presence of bacterial membranes. Kumar et al. [22] previously proposed that nonionic surfactants, such as Triton X-100 or Tween 20, underwent slow dissociation, which was favorable for the exchange of fatty acids between micelles and membranes, while residual surfactant molecules would remain in the buffer.

On the other hand, DPC is a "lipid-like" detergent that may partly penetrate the membrane together with the fatty acids, especially in the absence of peptidoglycan, which would explain a slight toxicity for Gram(−) bacteria [9], and the observed reduced $M_2$ values. These different detergent properties may explain why DPC would "carry" the fatty acids within the bacterial membranes to the cytosolic side, while Tween 20 would "drop" them on the extracellular side. Whether DPC enables the passage of the exogeneous fatty acid through a more efficient insertion, or by exerting some form of membrane permeabilization would need to be assessed. Experiments would be required to verify if DPC molecules are indeed inserted in the bacterial membranes, but they exceed the scope of the current work.

Whether the observations made here can be generalized to other cell types is an open question. Nevertheless, it seems important, when labelling a cell membrane, to assess different detergents as transfer molecules, and to verify if the labelled fatty acids are metabolized and esterified as lipids, or if they remain as FFAs. In cases where this was not verified and only TotFAs were assessed, it would be of great interest to determine which labelling mechanism predominates.

Altogether, our work demonstrated the role of the detergent in the deuteration efficacy and mechanism of bacterial membrane labelling with exogenous fatty acids, labelled or not [33]. The protocol described here is valuable to rapidly optimize the labelling conditions, which can be useful for ssNMR but also for other biophysical applications.

## 5. Conclusion

We proposed a fast and efficient protocol to quantify fatty acids in cell membranes, including FFAs. We successfully applied this protocol to evaluate the $^{2}$H-labelling mechanism of Gram(−) and Gram(+) bacteria, and showed that labelling protocols can result in *passive* labelling depending on the detergent used.

By comparing two membrane deuteration approaches, we identified that the most useful one for studying peptide-bacterial membrane interactions by $^{2}$H ssNMR makes use of Tween 20 as the detergent to solubilize the deuterated fatty acid. The labelling is efficient, 100 % passive, and the resulting bacterial membrane rigidity is comparable to that of labelled erythrocytes. These are ideal conditions for following the alteration of membrane rigidity by peptides, and will be used in our future projects.

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## Author's contributions

DEW and IM acquired funding. LZ and DEW carried out the experiments. All authors conceived the project, wrote the paper, and approved the final version.

## CRediT authorship contribution statement

**Laila Zaatouf:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Kiran Kumar:** Writing – review & editing, Methodology, Conceptualization. **Isabelle Marcotte:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Dror E. Warschawski:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

## Declaration of competing interest

None.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biochi.2024.05.024.

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**Supplementary information for:**

# Assessment of membrane labelling mechanisms with exogenous fatty acids and detergents in bacteria

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**Table S1.** Fatty acid proportions in *E. coli* BL21, grown at 37°C, with 100 μM exogenous PA-$d_{31}$ micellized in Tween 20. FFAs are proportions of the TotFAs, which is why the total is not 100. Total labelling, as well as active and passive labelling proportions are indicated. $M_2$ values extracted from $^2$H solid-state NMR spectra of the corresponding samples are also indicated. Average values for three replicates are shown with standard deviation.

| **Fatty acid** | With PA-$d_{31}$ micellized in Tween | |
|---|---|---|
| | **% TotFA** | **% FFA** |
| MA | 1 (2) | 0 |
| C15:0 | 6 (2) | 0 |
| PA | 24 (6) | 7 (3) |
| PA-$d_{31}$ | 52 (5) | 52 (10) |
| cyC17:0 | 2 (0.3) | 0 |
| C18:1 | 14 (0.6) | 2 (0.7) |
| C20:0 | < 1 | 0 |
| Total labelling | 52 | |
| Active labelling | 0 | |
| Passive labelling | 100 | |
| **$M_2$ ($10^9$ $s^{-2}$)** | **29 (8)** | |

**Table S2.** Fatty acid proportions in *B. subtilis*, grown at 37°C, with 50 μM exogenous PA-$d_{31}$ micellized in Tween 20. FFAs are proportions of the TotFAs, which is why the total is not 100. Total labelling, as well as active and passive labelling proportions are indicated. $M_2$ values extracted from $^2$H solid-state NMR spectra of the corresponding samples are also indicated. Average values for two replicates are shown with standard deviation.

| | With PA-$d_{31}$ micellized in Tween | |
|---|---|---|
| | **%TFA** | **%FFA** |
| MA | 3 (0.2) | 0 |
| Iso-C15:0 | 14 (0.3) | 2 (2) |
| Anteiso-C15:0 | 24 (4) | 3 (3) |
| PA | 13 (5) | 7 (5) |
| PA-$d_{31}$ | 30 (0.2) | 28 (0.2) |
| Iso-C17:0 | 8 (1) | 0 |
| Anteiso-C17:0 | 5 (2) | 0 |
| SA | 3 (1) | 0 |
| Total labelling | 30 | |
| Active labelling | 7 | |
| Passive labelling | 93 | |
| **$M_2$ ($10^9$ $s^{-2}$)** | **32 (7)** | |

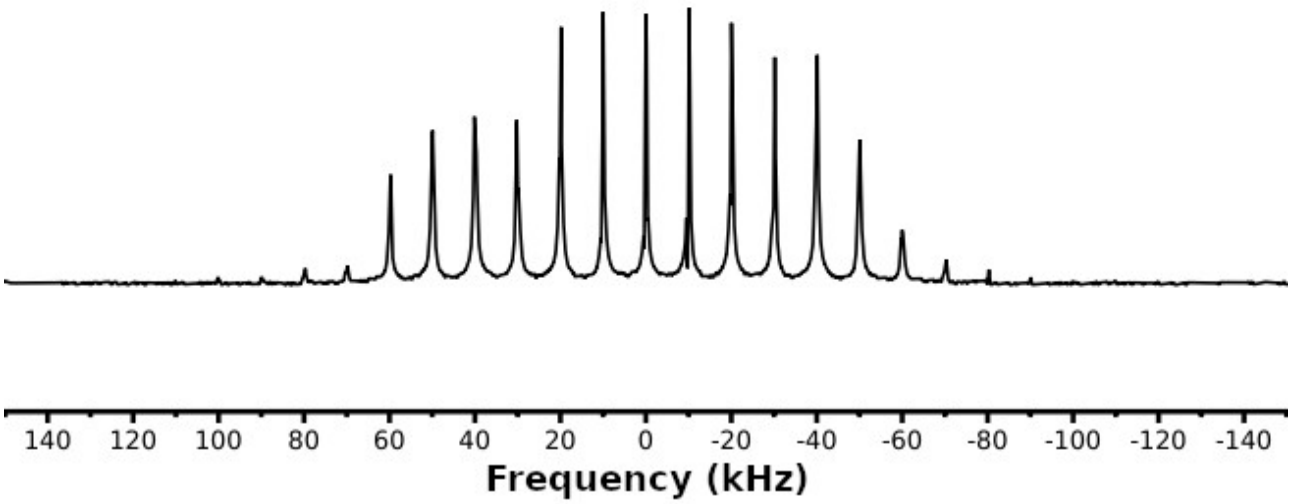


**Figure S1.** $^{2}H$ ssNMR spectrum of precipitated PA-$d_{31}$ recorded at 30°C with an MAS frequency of 10 kHz. The resulting $M_2$ is 47 x $10^9$ $s^{-2}$.