



HAL
open science

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

Laila Zaatouf, Kiran Kumar, Isabelle Marcotte, Dror E Warschawski

► To cite this version:

Laila Zaatouf, Kiran Kumar, Isabelle Marcotte, Dror E Warschawski. Assessment of membrane labelling mechanisms with exogenous fatty acids and detergents in bacteria. *Biochimie*, 2024, 227 (B), pp.12-18. 10.1016/j.biochi.2024.05.024 . hal-04826831

HAL Id: hal-04826831

<https://hal.science/hal-04826831v1>

Submitted on 9 Dec 2024

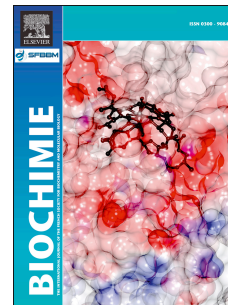
HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Journal Pre-proof

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

Laila Zaatouf, Kiran Kumar, Isabelle Marcotte, Dror E. Warschawski



PII: S0300-9084(24)00134-2

DOI: <https://doi.org/10.1016/j.biochi.2024.05.024>

Reference: BIOCHI 6739

To appear in: *Biochimie*

Received Date: 29 March 2024

Revised Date: 26 May 2024

Accepted Date: 30 May 2024

Please cite this article as: L. Zaatouf, K. Kumar, I. Marcotte, D.E. Warschawski, Assessment of membrane labelling mechanisms with exogenous fatty acids and detergents in bacteria , *Biochimie*, <https://doi.org/10.1016/j.biochi.2024.05.024>.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2024 Published by Elsevier B.V.

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

Laila Zaatouf (1), Kiran Kumar (2), Isabelle Marcotte (2)
and Dror E. Warschawski* (1)

Biochimie special issue following the 2023 GERLI meeting

(1) Laboratoire des Biomolécules, LBM, CNRS UMR 7203, Sorbonne Université,
École normale supérieure, PSL University, 75005 Paris, France

(2) Department of Chemistry, Université du Québec à Montréal, P.O. Box 8888,
Downtown Station, Montreal H3C 3P8, Canada

* Dror E. WARSCHAWSKI, Laboratoire des Biomolécules, Sorbonne Université, 4
place Jussieu, 75005 Paris, France

E-mail address: Dror.Warschawski@sorbonne.universite.fr

Abbreviations

DMPC (dimyristoyl phosphatidylcholine); DPC (dodecylphosphocholine); DPPC
(dipalmitoyl phosphatidylcholine); DPPC-d₆₂ (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-d₃₁ (perdeuterated palmitic acid); SA or
C18:0 (stearic acid); SA-d₃₅ (perdeuterated stearic acid); ssNMR (solid-state NMR);
TotFA (total fatty acid); Tween 20 (polyoxyethylenesorbitan monolaurate).

Abstract

30 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
35 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 ^2H ssNMR. Our results reveal that the labelling
mechanism depends on the detergent used to micellize the fatty acids. This labelling
40 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.

Keywords:

45 Bacterial membranes, labelling, fatty-acids, mass spectrometry, nuclear magnetic
resonance

1. Introduction

50

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.

60 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(+) 70 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 could take place, resulting in

differences of membrane rigidity that could in turn affect the interpretation of ssNMR data.

80

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.

90

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 ^2H ssNMR.

95

100 2. Materials and Methods

2.1. Materials

Dipalmitoyl-glycero-phosphocholine (DPPC), dimyristoyl-glycero-phosphocholine (DMPC), deuterated palmitic acid C16:0 (PA-d₃₁), deuterated stearic acid C18:0 (SA-d₃₅), dodecylphosphocholine (DPC), fatty acid methyl ester mix C4-C24 (FAME

105

mix), formaldehyde solution (37%), and polyoxyethylenesorbitan monolaurate (Tween 20) were purchased from Avanti/Merck (St-Quentin-en-Yvelines, France).

2.2. Synthetic lipid mixture

110 A lipid mixture composed of DPPC (34%, in mol%), DMPC (24%), PA-d₃₁ (15%) and SA-d₃₅ (27%) was prepared in 3:1 CHCl₃:MeOH and dried under a nitrogen stream.

2.3. Bacterial growth

115 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,
120 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-d₃₁ was incorporated, while for all other (Gram+) bacteria, a final concentration of 50 µM PA-d₃₁ was added. At such concentrations of exogenous fatty
125 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
130 acid chain profile, the lipid mixture was then spiked with a (nonmethylated) fatty acid that is not present in the mixture, *i.e.* SA-d₃₅. For a dry lipid mixture of approximately 10 mg, we prepared a 3 mM solution of SA-d₃₅ in 3:1 CHCl₃: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.

135

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 H₂SO₄ (2% in MeOH) and 800 µL of toluene, heating for 1h at 140 100 °C and, after cooling, adding 4 ml of H₂O 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 H₂SO₄ (2% in MeOH) and 800 µL of toluene, heating for 10 min at 50 °C and, after cooling, adding 4 ml of 145 H₂O 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.

150

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 155 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 160 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.

165 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
170 where the fatty acid was the one used to label the bacteria, these proportions
correspond to *passive* or *active* labelling respectively (Equation 1).

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

175 2.5. Solid-state NMR analysis

²H ssNMR spectra were recorded on a Bruker Avance III 500 wide bore spectrometer
(Wissembourg, France), operating at a frequency of 76.8 MHz for ²H, 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
180 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).

185

3. Results

3.1. Comparison of FAME preparation protocols

190

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-d₃₁ (15%) and SA-d₃₅ (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% H₂SO₄ 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.

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)

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 Na₂CO₃ 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.

215 We adapted Kail's approach to Laydevant's, by using H₂SO₄ 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 H₂SO₄ (2% in MeOH) and 800 μ L of
toluene, heating for 10 min at 50 °C and, after cooling, adding 4 ml of H₂O and 800
220 μ L 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 H₂SO₄ 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.

225 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
230 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).

235

We micellized PA-d₃₁ (100 μ M) 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-d₃₁ reduces the proportion of
240 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%).

Table 2. Fatty acid proportions in *E. coli* K12, grown at 37°C, with or without 100 μM exogenous PA-d₃₁, 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. M₂ values extracted from ²H 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			
M₂ (10⁹ s⁻²)	X	3 (0.3)	26 (5)			

250

By comparing the TotFA and FFA profiles, normalized by the spiked molecule concentration, we evaluated the labelling efficiency (proportion of PA-d₃₁

incorporated in the membrane). Moreover, we distinguished between the amount of PA-d₃₁ *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-d₃₁ molecules found in the bacterial membranes, only 7% were detected as “free PA-d₃₁”, 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 ²H ssNMR spectra. As shown in Figure 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, M₂, from the spectra (following the procedure described in [13]), and obtained values between 3 and 26 x 10⁹ s⁻².

We compared those values to extreme cases, by recording the ²H ssNMR spectrum of precipitated PA-d₃₁, which is much more rigid, as revealed by the M₂ value of 47 x 10⁹ s⁻² (see Figure S1). Conversely, labelled bacteria exposed to formaldehyde led to membrane solubilization, with a corresponding low M₂ value of 2 x 10⁹ s⁻² for *E. coli* K12 (see Figure 2).

280

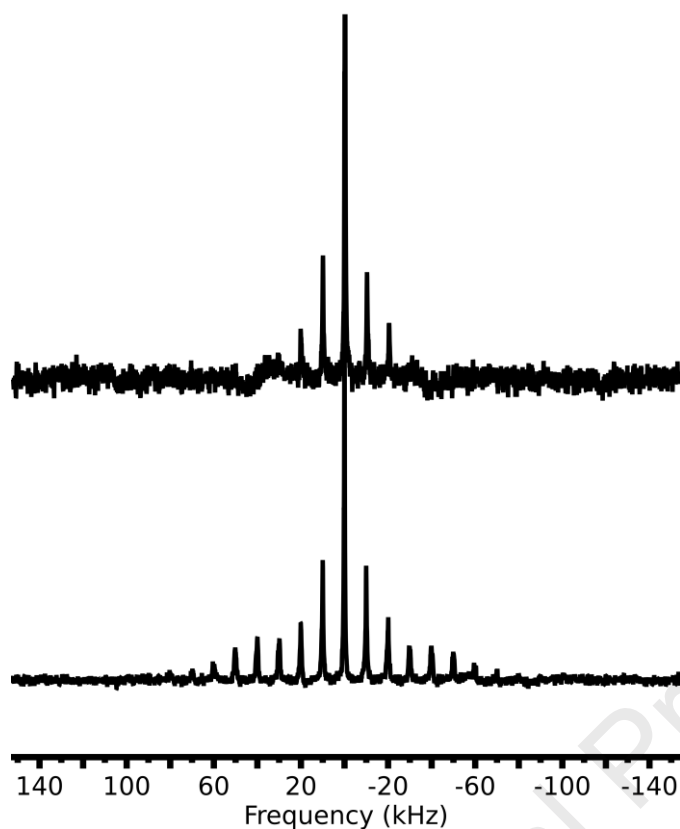


Figure 1. ^2H ssNMR spectra of *E. coli* K12 recorded at 30°C with an MAS frequency of 10 kHz. Bacteria were grown with $100\ \mu\text{M}$ of PA- d_{31} micellized in DPC (top) or Tween 20 (bottom), collected at the late log stage, and pelleted. The resulting M_2 values are reported in Table 2. Spectra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

290

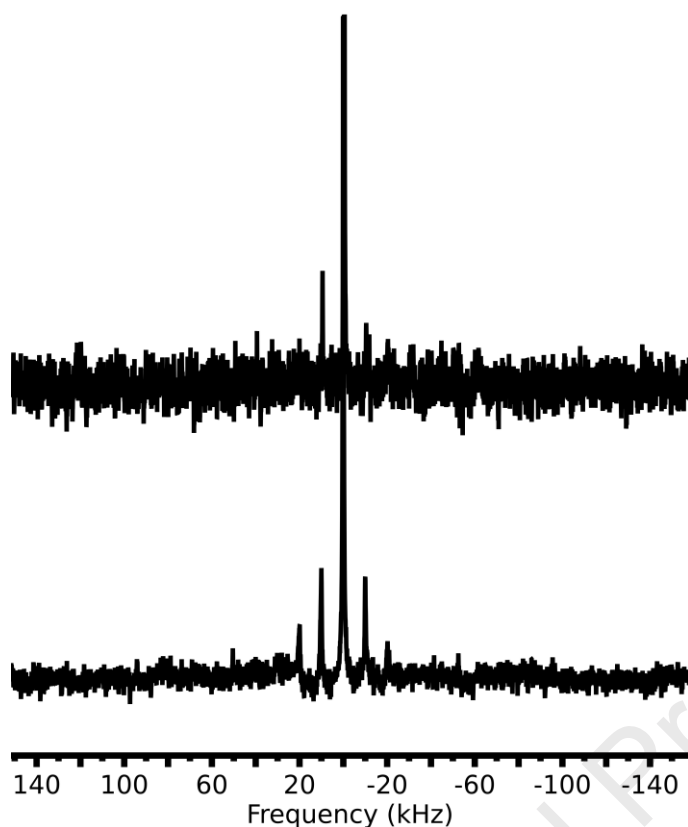


Figure 2. ^2H ssNMR spectra of *S. aureus* (top) or *E. coli* K12 (bottom) recorded at 30°C with an MAS frequency of 10 kHz. Bacteria were grown with 50 μM (*S. aureus*) or 100 μM (*E. coli* K12) of PA-d₃₁ in Tween 20, collected at the late log stage, exposed to formaldehyde (10%) for 20 min, and pelleted. The resulting M_2 is $1 \times 10^9 \text{ s}^{-2}$ (*S. aureus*) or $2 \times 10^9 \text{ s}^{-2}$ (*E. coli* K12). Note that the central peak is truncated to improve the visibility of the remaining spinning sidebands.

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 $3 \times 10^9 \text{ s}^{-2}$, 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 $26 \times 10^9 \text{ s}^{-2}$ which corresponds to a more rigid membrane.

310 We tested another Gram(-) strain, *i.e.* *E. coli* BL21 grown with $100 \mu\text{M}$ of PA-d₃₁ in Tween 20, and found similar results (See Table S1), with a 52% labelling efficiency, 100% passive labelling, and an M_2 of $29 \times 10^9 \text{ s}^{-2}$ (see Table 3).

315 **Table 3.** Labelling efficiency, labelling mechanism and resulting rigidity. Bacteria were grown with exogenous PA-d₃₁, micellized in DPC or Tween 20.

Strain	<i>E. coli</i> K12		<i>E. coli</i> BL21	<i>S. aureus</i>		<i>B. subtilis</i>
	DPC	Tween 20	Tween 20	DPC	Tween 20	Tween 20
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
$M_2 (10^9 \text{ s}^{-2})$	3	26	29	2	23	32

3.3. Application to Gram(+) bacterial membrane

320

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 $50 \mu\text{M}$ of PA-d₃₁, 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
 325 in native *S. aureus* bacterial membranes.

Table 4. Fatty acid proportions in *S. aureus*, grown at 24°C with or without 50 µM exogenous PA-d₃₁, 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. M₂ values extracted from ²H 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			
M₂ (10⁹ s⁻²)	X	2 (0.7)	23 (5)			

335

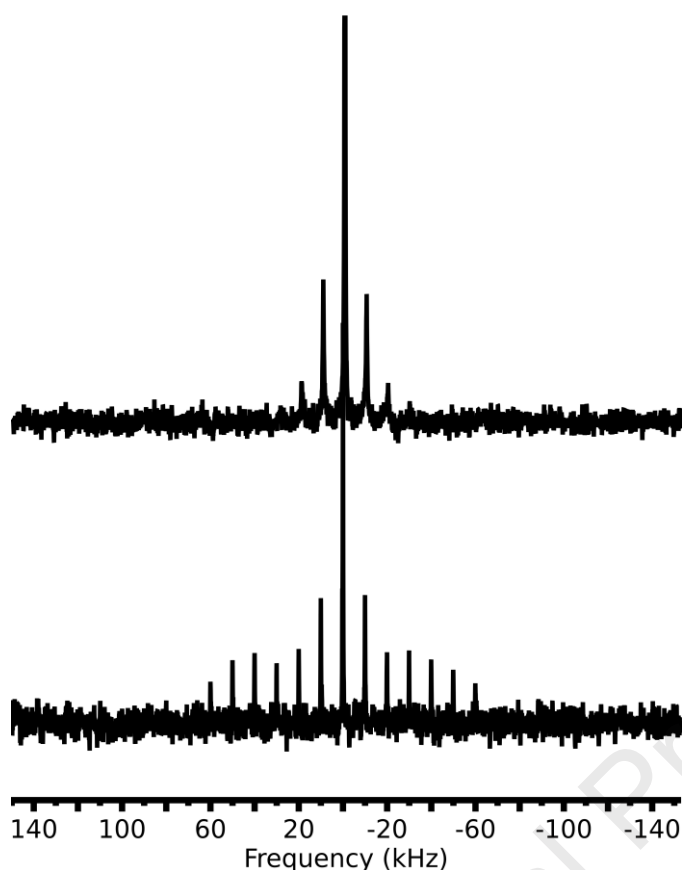


Figure 3. ^2H ssNMR spectra of *S. aureus* recorded at 30°C with an MAS frequency of 10 kHz. Bacteria were grown with 50 μM of PA-d₃₁ in DPC (top) or Tween 20 (bottom), collected at the late log stage, and pelleted. The resulting M₂ values are reported in Table 4. Spectra were normalized with respect to the first spinning sideband, and the central peak is thus truncated.

345

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.

350

Similar to the results obtained with *E. coli*, the ^2H ssNMR spectra are significantly different with DPC and Tween 20. In the first case, the labelling efficiency is low (8%), PA-d₃₁ being split into active and passive labelling mechanisms, and the membrane thereby remains fluid (average M_2 value of $2 \times 10^9 \text{ s}^{-2}$). When Tween 20 is used, a high labelling efficiency is observed (39%), through a 100% passive mechanism, leading to high membrane rigidity ($M_2 \sim 23 \times 10^9 \text{ s}^{-2}$). This difference is directly visible on the ^2H ssNMR spectra (see Figure 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 $1 \times 10^9 \text{ s}^{-2}$ for *S. aureus* (see Figure 2).

We also tested the Gram(+) strain *B. subtilis* grown with 50 μM of PA-d₃₁ 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 $32 \times 10^9 \text{ s}^{-2}$ (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-d₃₁ is observed when precipitated, with an M_2 value of $47 \times 10^9 \text{ s}^{-2}$. Minimal rigidity

occurs when PA-d₃₁ is solubilized, resulting in an M₂ of 1-2 x 10⁹ s⁻². For perdeuterated DPPC (DPPC-d₆₂) model membranes, M₂ values range between ~5 to 27 x 10⁹ s⁻² when transitioning from the fluid to the gel phase [13]. Therefore, the M₂ values between 2 and 32 x 10⁹ s⁻² obtained in this study indicate the incorporation of
385 FFAs into the bacterial membranes.

The M₂ values of 23 to 32 x 10⁹ s⁻², obtained when Tween 20 was used in the labelling protocol, are indicative of a membrane rigidity comparable to that of DPPC-d₆₂ in the gel phase. This is likely due to the large incorporation of saturated fatty
390 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-d₃₁ exhibited M₂ values around 12 x 10⁹ s⁻² [22].

395 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 M₂ values [7,10]. However, labelling efficiency is particularly crucial in ²H ssNMR - a relatively insensitive technique considering the low gyromagnetic ratio of the ²H nucleus. Therefore, Tween 20 is preferred over DPC to attain the highest deuteration
400 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-d₃₁ 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-d₃₁ [9,10].

405

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

410 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 ^2H 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), 415 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 as erythrocyte ghosts [22].

420 The enhanced rigidity caused by efficient PA-d₃₁ labelling, obtained through passive labelling when using Tween 20, can 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 425 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_2 430 values of around $3 \times 10^9 \text{ s}^{-2}$ (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 435 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
440 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
445 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
450 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 exogenous fatty acid through a more efficient insertion, or by exerting
some form of membrane permeabilization would need to be assessed. Experiments
455 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
460 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.

465 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.

470

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 ^2H -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 ^2H 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.

485

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.

490

Acknowledgments

The authors would like to thank Omar Khaled for technical training and assistance, and Alexandre Arnold for insightful discussions and critical reading of the manuscript.

495

Funding sources

LZ was supported by a Ph.D. grant from Sorbonne Université. KK would like to thank the Quebec Network for Research on Protein Function, Engineering, and Applications (PROTEO) – Strategic Cluster of the Fonds de recherche du Québec – Nature et Technologies (FRQNT) for the award of a scholarship. This work was supported by the Centre National de la Recherche Scientifique (UMR7203 and IEA PamGram+ to DEW), the Agence Nationale de la Recherche (ANTARBioTic to DEW) and the Natural Sciences and Engineering Research Council of Canada (RGPIN-2018–06200 to IM).

Declarations of interest

None.

References

[1] E. Odeblad and G. Lindstrom "Some Preliminary Observations on the Proton Magnetic Resonance in Biologic Samples" *Acta Radiol.*, 43:469-476 (1955)

<http://dx.doi.org/10.3109/00016925509172514>

[2] X. L. Warnet, A. A. Arnold, I. Marcotte and D. E. Warschawski "In-Cell Solid-State NMR: An Emerging Technique for the Study of Biological Membranes" *Biophys. J.* 109:2461–2466 (2015)

<https://doi.org/10.1016/j.bpj.2015.10.041>

[3] E. Oldfield, D. Chapman and W. Derbyshire "Lipid mobility in acholeplasma membranes using deuterium magnetic resonance" *Chem. Phys. Lipids* 9:69-81 (1972)

[https://doi.org/10.1016/0009-3084\(72\)90034-5](https://doi.org/10.1016/0009-3084(72)90034-5)

- 525 [4] C. Tardy-Laporte, A. A. Arnold, B. Genard, R. Gastineau, M. Morançais, J. L. Mouget, R. Tremblay and I. Marcotte "A H-2 solid-state NMR study of the effect of antimicrobial agents on intact *Escherichia coli* without mutating" *Biochim. Biophys. Acta* 1828:614–622 (2013)
<https://doi.org/10.1016/j.bbamem.2012.09.011>
- 530 [5] J. H. Davis, C. P. Nichol, G. Weeks and M. Bloom "Study of the cytoplasmic and outer membranes of *Escherichia coli* by deuterium magnetic resonance" *Biochemistry* 18:2103–2112 (1979)
<https://doi.org/10.1021/bi00577a041>
- 535 [6] J. Pius, M. R. Morrow and V. Booth "2H solid-state NMR investigation of whole *Escherichia coli* interacting with antimicrobial peptide MSI-78" *Biochemistry* 51:118–125 (2012)
<https://doi.org/10.1021/bi201569t>
- 540 [7] M. Laadhari, A. A. Arnold, A. E. Gravel, F. Separovic and I. Marcotte "Interaction of the antimicrobial peptides caerin 1.1 and aurein 1.2 with intact bacteria by 2H solid-state NMR" *Biochim. Biophys. Acta* 1858:2959–2964 (2016)
<http://dx.doi.org/10.1016/j.bbamem.2016.09.009>
- 545 [8] N. P. Santisteban, M. R. Morrow and V. Booth "Effect of AMPs MSI-78 and BP100 on the lipid acyl chains of 2H-labeled intact Gram positive bacteria" *Biochim. Biophys. Acta* 1862:183199 (2020)
<https://doi.org/10.1016/j.bbamem.2020.183199>
- 550 [9] Z. Bouhlel, A. A. Arnold, D. E. Warschawski, K. Lemarchand, R. Tremblay and I. Marcotte "Labelling strategy and membrane characterization of marine bacteria

- Vibrio splendidus by in vivo ^2H NMR" *Biochim. Biophys. Acta* 1861:871-878 (2019)
- 555 <https://doi.org/10.1016/j.bbamem.2019.01.018>
- [10] F. Laydevant, M. Mahabadi, P. Llido, J.-P. Bourgoïn, L. Caron, A. A. Arnold, I. Marcotte and D. E. Warschawski "Growth-phase dependence of bacterial membrane lipid profile and labeling for in-cell solid-state NMR applications" *Biochim. Biophys. Acta* 1864:183819 (2022)
- 560 <https://doi.org/10.1016/j.bbamem.2021.183819>
- [11] J. Yao and C. O. Rock "Exogenous fatty acid metabolism in bacteria" *Biochimie* 141:30-39 (2017)
- 565 <https://doi.org/10.1016/j.biochi.2017.06.015>
- [12] B. W. Kail, D. D. Link and B. D. Morreale "Determination of Free Fatty Acids and Triglycerides by Gas Chromatography Using Selective Esterification Reactions" *J. Chromatogr. Sci.* 50:934–939 (2012)
- 570 <https://doi.org/10.1093/chromsci/bms093>
- [13] X. L. Warnet, M. Laadhari, A. A. Arnold, I. Marcotte and D. E. Warschawski "A ^2H magic-angle spinning solid-state NMR characterisation of lipid membranes in intact bacteria" *Biochim. Biophys. Acta.* 1858:146-152 (2016)
- 575 <https://doi.org/10.1016/j.bbamem.2015.10.020>
- [14] M. I. Avelano, M. VanRollins and L. A. Horrocks "Separation and quantitation of free fatty acids and fatty acid methyl esters by reverse phase high pressure liquid chromatography" *J. Lipid Res.* 24:83-93 (1983)
- 580 [https://doi.org/10.1016/S0022-2275\(20\)38027-5](https://doi.org/10.1016/S0022-2275(20)38027-5)

[15] K. Ichihara and Y. Fukubayashi "Preparation of fatty acid methyl esters for gas-liquid chromatography" *J. Lipid Res.* 51:635–640 (2010)

<https://doi.org/10.1194/jlr.D001065>

585

[16] E. Koch, M. Wiebel, C. Hopmann, N. Kampschulte and N. H. Schebb "Rapid quantification of fatty acids in plant oils and biological samples by LC-MS" *Anal. Bioanal. Chem.* 413:5439–5451 (2021)

<https://doi.org/10.1007/s00216-021-03525-y>

590

[17] C. I. Ciucanu, D. C. Vlad, I. Ciucanu, V. Dumitraşcu "Selective and fast methylation of free fatty acids directly in plasma for their individual analysis by gas chromatography- mass spectrometry" *J. Chromatogr. A* 1624:461259 (2020)

<https://doi.org/10.1016/j.chroma.2020.461259>

595

[18] W. Bai, W. E. Anthony, C. J. Hartline, S. Wang, B. Wang, J. Ning, F.-F. Hsu, G. Dantas and F. Zhang "Engineering diverse fatty acid compositions of phospholipids in *Escherichia coli*" *Metab. Eng.* 74:11-23 (2022)

<https://doi.org/10.1016/j.ymben.2022.08.011>

600

[19] Z. DeMars, V. K. Singh and J. L. Bose "Exogenous fatty acids remodel *Staphylococcus aureus* lipid composition through fatty acid kinase" *J. Bacteriol.* 202:e00128-20 (2020)

<https://doi.org/10.1128/JB.00128-20>

605

[20] T.-H. Lee, P. Charchar, F. Separovic, G. E. Reid, I. Yarovsky and M.-I. Aguilar "The intricate link between membrane lipid structure and composition and membrane structural properties in bacterial membranes" *Chem. Sci.* 15:3408-3427 (2024)

<https://doi.org/10.1039/d3sc04523d>

610

- [21] T. Gutberlet, J. Frank, H. Bradaczek and W. Fischer "Effect of lipoteichoic acid on thermotropic membrane properties" *J. Bacteriol.* 179:2879–2883 (1997)
<https://doi.org/10.1128/jb.179.9.2879-2883.1997>
- 615 [22] K. Kumar, M. Sebastiao, A. A. Arnold, S. Bourgault, D. E. Warschawski and I. Marcotte "In situ solid-state NMR study of antimicrobial peptide interactions with erythrocyte membranes" *Biophys. J.* 121:1512-1524 (2022)
<https://doi.org/10.1016/j.bpj.2022.03.009>
- 620 [23] V. Booth "Deuterium Solid State NMR Studies of Intact Bacteria Treated With Antimicrobial Peptides" *Front. Med. Technol.* 2:621572 (2021)
<https://doi.org/10.3389/fmedt.2020.621572>
- [24] S. Kumari and V. Booth "Antimicrobial Peptide Mechanisms Studied by Whole-
625 Cell Deuterium NMR" *Int. J. Mol. Sci.* 23:2740 (2022)
<https://doi.org/10.3390/ijms23052740>
- [25] S. Brinster, G. Lamberet, B. Staels, P. Trieu-Cuot, A. Gruss and C. Poyart "Type
630 II fatty acid synthesis is not a suitable antibiotic target for Gram-positive pathogens"
Nature 458:83-86 (2009)
<https://10.1038/nature07772>
- [26] C. Morvan, D. Halpern, G. Kénanian, A. Pathania, J. Anba-Mondoloni, G. Lamberet, A. Gruss, K. Gloux "The *Staphylococcus aureus* FASII bypass escape
635 route from FASII inhibitors" *Biochimie* 141:40-46 (2017)
<http://dx.doi.org/10.1016/j.biochi.2017.07.004>
- [27] J. S. Sabirova, J.-P. Hernalsteens, S. De Backer, B. B. Xavier, P. Moons, A. Turlej-Rogacka, H. De Greve, H. Goossens, S. Malhotra-Kumar "Fatty acid kinase A

- 640 is an important determinant of biofilm formation in *Staphylococcus aureus* USA300"
BMC Genomics 16:861 (2015)
<https://10.1186/s12864-015-1956-8>
- [28] N. Tsapis, R. Ober, A. Chaffotte, D. E. Warschawski, J. Everett, J. Kauffman, P.
645 Kahn, M. Waks and W. Urbach "Modification of the elastic constants of a peptide-
decorated lamellar phase", *Langmuir* 18:4384-4392 (2002)
<https://doi.org/10.1021/la015745c>
- [29] V. Booth, D. E. Warschawski, N. P. Santisteban, M. Laadhari and I. Marcotte
650 "Recent progress on the application of 2H solid-state NMR to probe the interaction of
antimicrobial peptides with intact bacteria". *Biochim. Biophys. Acta* 1865:1500-1511
(2017)
<https://doi.org/10.1016/j.bbapap.2017.07.018>
- [30] M.-A. Sani, S. Rajput, D. W. Keizer and F. Separovic "NMR techniques for
655 investigating antimicrobial peptides in model membranes and bacterial cells"
Methods 224:10-20 (2024)
<https://doi.org/10.1016/j.ymeth.2024.01.012>
- [31] J. M. Salvador López and I. N. A. Van Bogaert "Microbial fatty acid transport
660 proteins and their biotechnological potential" *Biotechnol. Bioeng.* 118:2184-2201
(2021)
<https://doi.org/10.1002/bit.27735>
- [32] H. Heerklotz and J. Seelig "Correlation of membrane/water partition coefficients
665 of detergents with the critical micelle concentration" *Biophys. J.* 78:2435-2440
(2000)
[https://doi: 10.1016/S0006-3495\(00\)76787-7](https://doi:10.1016/S0006-3495(00)76787-7)

- 670 [33] K. Kumar, A. A. Arnold, R. Gauthier, M. Mamone, J.-F. Paquin, D. E.
Warschawski and I Marcotte "19F solid-state NMR approaches to probe
antimicrobial peptide interactions with membranes in whole cells" *Biochim. Biophys.*
Acta Biomembr. 1866:184269 (2024)
<https://doi.org/10.1016/j.bbamem.2023.184269>

675

Journal Pre-proof

**Assessment of membrane labelling mechanisms
with exogenous fatty acids in bacteria**

Laila Zaatouf (1), Kiran Kumar (2), Isabelle Marcotte (2)

and Dror E. Warschawski* (1)

Biochimie special issue following the 2023 GERLI meeting

(1) Laboratoire des Biomolécules, LBM, CNRS UMR 7203, Sorbonne Université, École normale supérieure, PSL University, 75005 Paris, France

(2) Department of Chemistry, Université du Québec à Montréal, P.O. Box 8888, Downtown Station, Montreal H3C 3P8, Canada

* Dror E. WARSCHAWSKI, Laboratoire des Biomolécules, Sorbonne Université, 4 place Jussieu, 75005 Paris, France

E-mail address: Dror.Warschawski@sorbonne.universite.fr

Highlights

- Membrane labelling using fatty acids can occur through two different mechanisms
- GC-MS analysis allows the estimation of labelling efficiency and mechanism
- Detergent used to micellize the fatty acids influence the labelling mechanism
- Passive labelling is more efficient and results in a stiffer bacterial membrane
- The best labelling scheme for solid-state NMR studies uses Tween 20 as a detergent