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**Investigating the action of the microalgal pigment marennine on *Vibrio*
splendidus by *in vivo* ²H and ³¹P solid-state NMR**

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27 ABSTRACT

28 This work investigates the potential probiotic effect of marennine - a natural pigment produced
29 by the diatom *Haslea ostrearia* - on *Vibrio splendidus*. These marine bacteria are often
30 considered a threat for aquaculture, therefore chemical antibiotics can be required to reduce
31 bacterial outbreaks. *In vivo* ^2H solid-state NMR was used to probe the effects of marennine on the
32 bacterial membrane in the exponential and stationary phases. Comparisons were made with
33 polymyxin B (PxB) - an antibiotic used in aquaculture and known to interact with Gram(-)
34 bacteria membranes. We also investigated the effect of marennine using ^{31}P solid-state NMR on
35 model membranes. Our results show that marennine has little effect on phospholipid headgroups
36 dynamics, but reduces the acyl chain fluidity. Our data suggest that the two antimicrobial agents
37 perturb *V. splendidus* membranes through different mechanisms. While PxB would alter the
38 bacterial outer and inner membranes, marennine would act through a membrane stiffening
39 mechanism, without affecting the bilayer integrity. Our study proposes this microalgal pigment,
40 which is harmless for humans, as a potential treatment against vibriosis.

41 KEYWORDS

42 Antimicrobial pigment, polymyxin B, membrane fluidity, model membranes, ^{31}P and ^2H NMR,
43 in-cell NMR.

44 ABBREVIATIONS

45 ASW, artificial sea water; Chloroform-D, deuterated chloroform; CL, cardiolipin; CSA, chemical
46 shift anisotropy; D_2O , deuterium oxide; PA- d_{31} , deuterated palmitic acid; DOPE,
47 dioleoylphosphatidylethanolamine; LB, Lysogeny broth; LPS, lipopolysaccharides; M_2 , second
48 spectral moment; MAS, magic angle spinning; MIC, minimum inhibitory concentration; MLV,
49 multilamellar vesicle; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OA,
50 oleic acid; OD, optical density; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; POPE,
51 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; POPG, 1-palmitoyl-2-oleoyl-sn-
52 glycero-3-phospho-(1'-rac- glycerol); PSU, Practical Salinity Unit; PxB, polymyxin B; SSB,
53 spinning side band; SS-NMR, solid-state nuclear magnetic resonance.

54 INTRODUCTION

55 According to the World Bank, aquaculture is a growing industry and a promising alternative to
56 the fishery crisis [1]. However, like many commercial activities, aquaculture relies on intensive
57 production and in the context of global warming, the stress that cultured animals may undergo
58 can lead to diseases and losses [2,3]. Vibriosis - an infection caused by *Vibrio* bacteria - is
59 amongst the most problematic pathologies in the world for aquatic species [4]. *Vibrio* outbreaks
60 are often associated with important mortality and *Vibrio splendidus* are almost exclusive to
61 bivalve infection especially during the first stages of culture [5,6].

62 For years, aquaculture has counted on antimicrobial agents for prophylactic or therapeutic
63 purposes, and too extensively in some cases such as shrimp and salmon farming [7-9].
64 Nowadays, regulations in aquaculture are stricter since bacterial resistance is a considerable issue
65 [8,10]. Consequently, there is a growing need to develop novel therapeutic approaches that are
66 more environmentally friendly and less harmful for food human consumption than conventional
67 synthetic antibiotics. New strategies include brood-stock conditioning [7], as well as the use of
68 natural biologically active alternatives such as immuno-stimulants, bacteriophages and probiotics
69 [11,12].

70 Marennine is a natural blue pigment and a promising therapeutic alternative against vibriosis
71 in aquaculture [13,14]. It is secreted by *Haslea ostrearia*, a marine microalga responsible for the
72 noticeable greening of oysters cultured in the French Marennes region and harmless for the
73 consumers. Recent works showed that marennine is a water-soluble 10 kDa polyphenol with
74 possibly a glycosidic skeleton, however, the exact structure and nature of this molecule is today
75 still unknown [15]. The probiotic potential of marennine against aquatic Gram(-) bacteria
76 including *Vibrio* species, has already been demonstrated [16-18]. Turcotte *et al.* also showed that
77 in the presence of marennine extracts, the pathogenicity of Gram(-) *V. splendidus* was suppressed
78 [14]. However, the action mechanism of marennine towards marine bacteria has not yet been
79 elucidated. Considering the high molecular weight of marennine and its unlikely penetration in
80 the bacterial membrane, the pigment would favor an interaction with the outer-membrane of
81 Gram(-) bacteria, as suggested by a solid-state nuclear magnetic resonance (SS-NMR) study on
82 *Escherichia coli* [19].

The objective of this work was to investigate the action mechanism of marennine towards the marine bacterium *V. splendidus*, more specifically the indigenous environmental strain (7SHRW) isolated from the St. Lawrence estuary, which causes significant mortality of blue mussel and scallops' larvae. Marennine has previously been shown to inhibit the virulent action of this strain [14]. To do so, we studied the interaction of marennine with 7SHRW using *in vivo* ^2H SS-NMR, and with model membranes using ^{31}P SS-NMR. Deuterium SS-NMR provides valuable information on acyl chain dynamics and organization in membrane systems made of deuterated phospholipids, while phosphorous NMR is a valuable tool to probe lipid headgroup perturbations. Traditionally used to study model membranes, the deuterium labelling of membrane lipids in intact bacteria enabled *in vivo* ^2H SS-NMR experiments on *E. coli*, *Bacillus subtilis*, and more recently *V. splendidus* [19-22]. It is also a useful tool to examine membrane interactions with exogenous molecules such as detergents, drugs, and peptides, at a molecular-level [23].

In this work, the effect of marennine on *V. splendidus* membranes at two different growth stages (mid-log and stationary phase) is compared to a commercial antibiotic, polymyxin B (PxB), which has a well-documented action mechanism. Polymyxins are amongst the most commonly used antibiotics in aquaculture and are known to specifically act against Gram(-) bacteria by damaging their membranes [24-26]. However, recent regulations have prohibited the use of PxB in aquaculture [24]. By comparing the interaction of marennine and PxB with *V. splendidus* membranes, we propose a possible mechanism involved in the bactericidal action of marennine, and the use of this natural pigment as an alternative to synthetic antibiotics.

MATERIALS AND METHODS

Materials

Oleic acid (OA) and deuterated palmitic acid (PA- d_{31}), as well as deuterium depleted water, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and PxB sulfate salt, were all purchased from Sigma Aldrich (Oakville, ON, Canada). Synthetic lipids 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (POPG), and cardiolipin (CL) were purchased from Avanti Polar Lipids (Alabaster, AL,

USA). Tween-20 (polyethylene glycol sorbitan monolaurate) and tris(hydroxymethyl)-aminomethane were acquired from BioShop (Burlington, ON, Canada), while LB Broth Miller was obtained from BioBasic (Markham ON, Canada), and ethylenediaminetetraacetic acid (EDTA) from Fisher Scientific (Fair Lawn, NJ, USA). Deuterium oxide (D₂O) and deuterated chloroform (chloroform-D) were respectively bought from CDN Isotopes (Pointe-Claire, Quebec, Canada) and Cambridge Isotope Laboratories (Endover, MA, USA). The solution of marennine tested was produced by *H. ostrearia* strain NCC-136-isolated from Bourgneuf Bay, France. Microalgae were cultured with the use of fluorescent growth-light at an intensity corresponding to a photon flux of 180 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and 14/10 hours light/dark cycle in filtered seawater with a salinity of 28 PSU, at 20°C [14]. The supernatant was then extracted and purified according to previously published methods [13].

Bacterial growth and ²H labelling

V. splendidus 7SHRW [27] were grown in LB medium and deuterated using equal proportions of OA and PA-d₃₁ (0.3 mM each) micellized in 0.14 mM of Tween-20 detergent to insure fatty acid intake, as described in Bouhlef *et al.* [22]. The growth kinetics was monitored with a multiple plate reader (Infinite M200 TECAN, Männedorf, Switzerland) using 24-well plates, by measuring absorbance at 600 nm every 30 min. Typically, 3 to 4 wells were used for each treatment. Bacteria were harvested at two different moments, *i.e.*, after the mid-log phase and at the early-stationary phase, centrifuged (2600 g, 10 min), then pellets were washed twice with a sterile NaCl solution (154 mM) prepared with nanopure water. A standard curve relating optical density of the culture and the corresponding dry weight was determined after pellet lyophilization.

Antimicrobial activity measurement

The antimicrobial activity of marennine and PxB towards *V. splendidus* was assessed by measuring the minimum inhibitory concentration (MIC) using a serial dilution technique [17,28]. An LB medium (171 mM NaCl) was used for all dilutions, and final concentrations ranged from 1.25 to 400 $\mu\text{g/mL}$ for marennine, and from 1 to 32 $\mu\text{g/mL}$ for PxB. A total of 200 μL of initial cell suspension was transferred into a 100-well plate to monitor the growth kinetics with a multiple-plate reader at 25°C. The absorbance at 600 nm was recorded every 30 min over a 48h

period. The absorbances were ~0.5 in the mid-log phase and ~0.8 in the stationary phase as shown in the growth curves (Figures S1 and S2).

Bacteria exposure for NMR experiments

Marennine and PxB assays were performed at selected concentrations in triplicate on ²H-labelled bacteria, adjusted to correspond to approximately 10 ± 2 mg (dry weight).

For PxB exposures, ²H-labelled bacteria pellets were resuspended in a 1 mL solution of NaCl (154 mM) in ²H-depleted water, with the appropriate amount of antibiotics (final concentration of 1 µg/mL and the MIC), and vortexed before a 10 min incubation. For marennine experiments, ²H-labelled bacteria pellets were resuspended in a 50 mL solution of ²H-depleted artificial seawater (ASW) containing NaCl (480 mM), MgSO₄ (28 mM), MgCl₂ (24 mM), CaCl₂ (16 mM) and NaHCO₃ (2.4 mM), with a pH adjusted to 8.0 ± 0.1, with the appropriate amount of marennine (final concentration of 2 µg/mL and at the MIC). Exposures were carried out in 250 mL Erlenmeyer flasks placed on a rotary shaker (100 rpm) at a temperature of 22 ± 1 °C for 2h. Note that nutrients were purposely discarded during marennine exposure to avoid continuing bacterial growth, and concomitant changes in membrane fluidity as previously reported [22]. The lack of nutrients and 2h shaking in ASW have a little effect on cell viability (90% ± 5), as estimated with an MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test [20]. Bacteria exposed to ASW only are considered as a control. Both types of sample pellets (control and exposed to marennine) came from the same culture replicates.

Bacteria were collected immediately after exposure to PxB or marennine, and centrifuged at 3200 g for 5 min. Pellets were then washed in a solution of 9‰ NaCl in ²H-depleted water, then centrifuged at 3200 g again for 5 min. The final pellet was used to fill a 4-mm zirconium oxide rotor, which corresponds to approximately 90 mg of hydrated bacteria.

Phospholipid analysis of V. splendidus

Bacteria harvested at the early stationary phase were washed with NaCl solution (154 mM) and freeze-dried. Lipids were analyzed following the protocol of Mahabadi *et al.* (to be submitted). In short, lipids were extracted according to the protocol of Folch [29] using dichloromethane/methanol (2:1 CH₂Cl₂/MeOH v/v) and 0.88% KCl solution in a Potter glass

171 homogenizer. Polar lipids were separated from neutral lipids by elution through a silica gel
172 column (30×5 mm) hydrated to 6% with distilled water [30]. Final polar lipid extracts were
173 dissolved in a mixture composed of 500 µL of deuterated chloroform, 200 µL methanol, 50 µL of
174 an aqueous EDTA (200 mM) solution at pH 6, vortexed, transferred to a solution NMR glass tube
175 for ³¹P NMR analysis, and stabilized until a biphasic solution was obtained.

176 ***Preparation of model bacterial membranes***

177 Model membranes were prepared from lipid mixtures of POPE, POPG and CL in proportions
178 similar to *V. splendidus* membrane composition, deduced from the ³¹P solution NMR experiments
179 (85% phosphatidylethanolamine (PE), 10% phosphatidylglycerol (PG) and 5% cardiolipin (CL)
180 (in mole %)). For each sample, 30 mg of phospholipid powder mixture was dissolved in
181 CH₂Cl₂/MeOH (3:1) and dried under a stream of N₂ gas for 30 min. Remaining organic residues
182 were further evaporated overnight under vacuum. The dry lipid films were hydrated in 120 µL
183 buffer solution (Tris (100 mM), NaCl (100 mM), EDTA (2 mM) and D₂O; pH=8), resulting in
184 multilamellar vesicles (MLVs) which will be referred to as liposomes. For marennine-containing
185 samples, the appropriate pigment amount was dissolved in the buffer solution before lipid
186 hydration. All preparations were mechanically homogenized and processed through five cycles of
187 liquid N₂ freezing and thawing in a 35°C water bath. The MLV samples were subsequently filled
188 in 4 mm rotors for ³¹P SS- NMR analysis.

189 ***²H solid-state NMR experiments***

190 All ²H SS-NMR experiments were carried out using a Bruker Avance III HD Wide Bore 600
191 MHz NMR spectrometer (Milton, Ontario, Canada) and a double-resonance magic angle spinning
192 (MAS) probe tuned to 92.1 MHz. Samples were spun at 10 kHz and at 25°C. Since MAS
193 refocuses the ²H quadrupolar interaction, instead of the classic solid echo used in the static case,
194 the spectra were acquired using a Hahn Echo pulse sequence with 4 µs 90° pulses separated by an
195 echo delay of 96 µs, a recycle time of 0.5 s, 4096 scans, 32k points and a spectral width of 500
196 kHz, for a total of 43 min of acquisition time. Spectra were zero-filled to 64k points, and treated
197 with an exponential line broadening of 40 and 100 Hz for PxB and marennine sample spectra,
198 respectively.

199 Spectral moment analysis was performed using MestRenova software V6.0 (Mestrelab
200 Research, Santiago de Compostela, Spain) and a macro developed by Pierre Audet (Université

201 Laval, Québec, Canada). The second moment (M_2) was calculated using the following equation
202 [21]:

$$M_2 = \omega_r^2 \frac{\sum_{N=0}^{\infty} N^2 A_N}{\sum_{N=0}^{\infty} A_N}$$

203
204 where ω_r is the angular spinning frequency, N the side band number, and A_N the area of each
205 sideband obtained by spectral integration. The integration of the central peak includes the
206 residual HDO peak which leads to a systematic underestimation of the M_2 by a maximum of
207 15%.

208 ***³¹P solid-state NMR experiments***

209 All ³¹P SS-NMR experiments were carried out using a Bruker Avance III HD Wide Bore 400
210 MHz NMR spectrometer (Milton, Ontario, Canada) operating at frequencies of 161.9MHz and
211 400.02 MHz for ³¹P and ¹H, respectively. Samples were analyzed at 25°C, 50°C and 75°C, with a
212 25 min equilibration step before each temperature. Temperatures were controlled to within ±
213 0.5°C, and chemical shifts were referenced relative to external H₃PO₄ set to 0 ppm. Static spectra
214 were recorded through a ¹H-decoupled Hahn echo pulse sequence with a ³¹P 90° pulse length of
215 3μs, a recycle delay of 3s, and 512 scans, in approximately 25 min.

216 Line shapes were fitted using the Bruker SOLA software. Vesicles shapes (spherical vs.
217 ellipsoidal) were determined using dedicated MATLAB scripts based on the analytical model of
218 Dubinnyi *et al.* [31], where the vesicle is described by the ratio ρ between the long and short axis
219 of the ellipsoid ($\rho=1$ if vesicles are spherical).

220 ***³¹P solution-state NMR experiments***

221 All ³¹P solution NMR experiments were performed at 25°C using a Bruker Avance III HD 600
222 MHz NMR spectrometer (Milton, Ontario, Canada) operating at frequencies of 242.84 MHz and
223 599.95 MHz for ³¹P and ¹H, respectively. For quantitative results, ³¹P spectra were recorded with
224 a 10 s recycle delay, continuous ¹H decoupling during acquisition, without NOE enhancement,
225 with 128 scans in approximately 22 min. Spectra were processed with an exponential line
226 broadening of 2 Hz.

RESULTS AND DISCUSSION

Minimum inhibitory concentration

To quantify the antibacterial activity of marennine and PxB on *V. splendidus*, we determined their minimum inhibitory concentration (MIC). The growth curves are displayed in the Supplementary information section (Fig. S1 and S2). Values of 200 µg/mL and 8 µg/mL were found for marennine and PxB, respectively. So far, marennine has been characterized as a potential polyphenol with a glycosidic structure [13,15][13, 15], and its MIC is in the same range as other plant polyphenol extracts acting on Gram(-) bacteria, *i.e.*, from 70 to 500 µg/mL on *Vibrio* species [32,33][32, 33]. As for PxB, its MIC has been reported to range between 1 to 8 µg/mL depending on the bacterial species [34]. Here, we observe that the MIC of PxB for *V. splendidus* is 8 times higher than that reported for *E. coli* [19]. We also notice a different response of *V. splendidus* to marennine as compared to *E. coli*. Indeed, Tardy-Laporte *et al.* reported a change in membrane fluidity when *E. coli* was exposed to 2 µg/mL of marennine in NaCl (171 mM) solution for 15 min [19], while a longer exposure time (2h) was required to observe this effect for *V. splendidus* at the same marennine concentration [19]. However, *V. splendidus* showed membrane collapse after 2 hours of exposure in NaCl (171 mM) solution. We have thus used artificial seawater (ASW) in this work since membrane integrity is preserved for up to 12 hours in ASW (data not shown), and to eliminate any possible effect of the salt and better focus on the antibacterial activity.-

Effect of marennine and polymyxin B on V. splendidus membranes

To better understand the action of marennine and PxB on *V. splendidus*, their effect was studied by ²H SS-NMR at the MIC but also at a lower concentration, during the exponential and stationary phases. In practice, concentrations below the MIC are typically used in aquaculture [8]. As demonstrated in a previous work, membrane lipid chains in *V. splendidus* can be ²H-labelled by growing marine bacteria in a medium enriched with PA-d₃₁ [22]. The presence of OA ensures a lipid profile closer to unlabelled bacteria. A labelling percentage of 69% and 55% of total palmitic acid can be reached in the exponential and in the stationary phases, respectively. The second spectral moment (M₂) was used to quantify the membrane lipid acyl chain ordering. The greater the M₂, the higher the lipid chain ordering. As shown by Warnet *et al.*, combination of

257 MAS and ^2H SS-NMR provides a fast method to ensure the *in vivo* characterization of bacteria
 258 [21]. *In vivo* conditions were confirmed for *V. splendidus* by estimating the bacterial viability
 259 using an MTT reduction assay [21] after 2 h of MAS experiments, which was $95 \pm 5 \%$.

260 Figure 1 shows the effect of marennine on *V. splendidus* at low concentration (2 $\mu\text{g/mL}$) and
 261 at the MIC (200 $\mu\text{g/mL}$), with corresponding M_2 values presented in Table 1. The “low”
 262 marennine concentration employed here is 20 times that reported to be effective on *V. splendidus*
 263 (7SHRW) in aquaculture conditions [14]. Spectral analysis reveals that marennine has an
 264 ordering effect on *V. splendidus* membrane lipid chains at a concentration of 2 $\mu\text{g/mL}$, as shown
 265 by a 35% increase

267 **Figure 1:** Representative ^2H SS-NMR MAS spectra of *V. splendidus* exposed to marennine
 268 during the exponential (a-c) and stationary (d-f) stages. Spectra (a) and (d) are control
 269 experiments of *V. splendidus* in ASW; (b) and (e) correspond to bacteria exposed to low
 270 marennine (2 $\mu\text{g/mL}$) concentration; (c) and (f) to marennine at MIC (200 $\mu\text{g/mL}$). All spectra

were recorded at 25°C and normalized with respect to the first spinning sideband to better highlight the sideband intensity distribution.

in M_2 value (Table 1). This effect is greater for bacteria sampled in the stationary phase, where M_2 is actually doubled. Figure 1 (b,e) indeed shows that spinning side bands (SSBs) span a larger frequency range and have increased intensities. The stiffening effect of marennine on the lipid chains is further observed at the MIC (Table 1). M_2 values increase by 60% compared to the control in the presence of marennine in the exponential phase and are doubled in the stationary phase.

Table 1: Average second spectral moment, M_2 (10^9 s^{-2}) with standard deviation for *V. splendidus* exposed to marennine and polymyxin B at different concentrations. MIC for marennine and PxB are 200 $\mu\text{g/mL}$ and 8 $\mu\text{g/mL}$, respectively. Control for marennine is done in artificial seawater. All spectra were recorded at 25°C. Standard deviations obtained on three replicates are indicated between brackets.

	Marennine			Polymyxin B		
	Control	2 $\mu\text{g/mL}$	MIC	Control	1 $\mu\text{g/mL}$	MIC
Exponential phase	25 (5)	34 (7)	39 (4)	20 (2)	23 (3)	18 (3)
Stationary phase	10 (1)	21 (3)	20 (1)	10 (1)	12 (1)	7 (2)

To explore the marine pigment marennine as a potential antimicrobial agent for aquaculture, its effect was compared to PxB since polymyxins have been widely used for this purpose. Figure 2 shows the effect of PxB on *V. splendidus* at low concentration (1 $\mu\text{g/mL}$) and at the MIC (8 $\mu\text{g/mL}$). Corresponding M_2 values are presented in Table 1. At low PxB concentration (Figures 2b,e), an small increase in SSB intensities and spectral width can be seen, indicating an increased lipid chain ordering. Table 1 shows that this moderate increase in lipid ordering at low concentration of PxB occurs in both the exponential and stationary phase, as revealed by a 15 to 20% increase in M_2 . At the MIC (Figures 2c,f), however, the SSBs intensity is reduced, as well as the spectral width, with a concomitant decrease in M_2 , revealing a disordering effect of PxB on *V. splendidus* inner membrane lipid chains at both growth phases.

296 With ^2H NMR and M_2 determination, we were also able to provide evidence of the effect of
 297 marennine and PxB on *V. splendidus* at different growth times. Control samples were compared
 298 to bacteria exposed to each antibacterial agent after a growth period of 15h (mid-log), 22h (early
 299 stationary) and 35h (late stationary). Figure 3 shows that M_2 values of *V. splendidus* membranes
 300 decrease as a function of the growth stage, in agreement with the increase in membrane fluidity
 301 reported in our previous work [22]. The addition of marennine results in an increase in the lipid
 302 acyl chain ordering at both concentrations, with a more important effect for bacteria sampled at
 303 mid-log phase, compared to the stationary phase (35h of growth). Interestingly, low PxB
 304 concentrations increased the membrane rigidity at all growth stages, while high concentration

306 **Figure 2:** Representative ^2H SS-NMR MAS spectra of *V. splendidus* exposed to polymyxin B
 307 during exponential (a-c) and stationary (d-f) stages. Spectra (a) and (d) are control experiments,
 308 spectra (b) and (e) correspond to bacteria exposed to low PxB (1 $\mu\text{g/mL}$) concentration; (c) and

(f) to PxB at MIC (8 $\mu\text{g/mL}$). All spectra were recorded at 25°C and normalized with respect to the first spinning sideband to better highlight the sideband intensity distribution.

(MIC) showed the opposite pattern. These results are in agreement with an *in vivo* ^2H SS-NMR study of the effect of PxB on the Gram(-) bacterium *E. coli* [19]. They are also compatible with previous work suggesting a two-stage interaction mechanism for this antibiotic with respect to increased concentrations or exposure time [25]. Briefly, PxB first disturbs the bacteria outer membrane by electrostatically binding with the anionic phosphate groups of the lipopolysaccharides (LPS) via its positively charged residues, thus competing with divalent cations such as magnesium and calcium, which play a role in bridging adjacent LPS chains. Then the PxB

Figure 3: Effect of marennine and PxB on the second spectral moment M_2 of *V. splendidus* after exposure to marennine (left), and PxB (right) at 25°C. Control cells (solid lines) are compared to bacteria exposed to 2 $\mu\text{g/mL}$ of marennine or 1 $\mu\text{g/mL}$ of PxB (dotted lines), and at the respective MICs (dashed lines). Values correspond to average means of three replicates with 3-22% error for marennine samples and 8-40% error for PxB samples.

lipid tail can insert itself in the inner membrane, thus disrupting the bilayer, and eventually leading to the loss of membrane integrity and cell leakage [25,26,35]. The importance of LPS is highlighted by the fact that PxB does not significantly affect model membranes made without LPS [36,37].

Altogether, these observations suggest that the action mechanism of marennine is similar to that of PxB at low concentration, but different at high concentration. As opposed to PxB, marennine, even at lethal concentrations, would not disrupt the inner bilayer and interact mostly with the outer layer of *V. splendidus* membrane. Nevertheless, while marennine probably does not disrupt the inner membrane, it can still affect it depending on how much it penetrates the outer membrane. To test this hypothesis, we have decided to study the interaction of marennine with *model* inner bacterial membranes.

Effect of marennine on model membranes

The ²H SS-NMR results obtained *in vivo* suggest a different action of marennine on *V. splendidus*, as compared to PxB, where this natural pigment is shown to reduce the membrane lipid chain dynamics at low concentration and at the MIC, and at both growth stages. Moreover, the action of marennine was observed following a longer incubation time as compared to PxB. To better understand the action mechanism of marennine, we have thus investigated its effect on the lipids using model *V. splendidus* inner membranes, *i.e.*, without LPS. To do so, the phospholipid composition of the bacterium was first determined by ³¹P solution NMR using lipid extracts (a characteristic spectrum is shown in figure S3). Integration of the resonances allowed the quantification of three major phospholipids corresponding to 85% phosphatidylethanolamine (PE), 10% phosphatidylglycerol (PG) and 5% cardiolipin (CL) (in mole %) (phospholipid structures are shown in Figure S3 and the presence of two phosphate headgroups in CL was taken into account in the quantification). These proportions did not change whether samples were harvested at exponential or stationary growth stages. Model membranes (MLVs) of *V. splendidus* were thus prepared using POPE, POPG, and CL, respecting the same proportions as in natural membranes.

355 ³¹P SS-NMR is a useful tool to probe changes in phospholipid headgroup dynamics,
356 orientation and lipid phases [38]. The effect of marennine on the model membranes is presented
357 in Figure 4 at low (2 µg/ml) concentration and at the MIC. Spectra were recorded between 25
358 and 75°C to better evidence any possible changes in membrane morphology and phases. First, the
359 model membranes at 25°C and 50°C are typical of bilayers in liquid-crystalline phase, with a
360 chemical shift anisotropy (CSA, powder spectrum width) of approximately 37.5 and 33 ppm,
361 respectively. The higher intensity of the right edge of the powder spectrum at 50°C can be
362 explained by a change in liposomes from a spherical to an ellipsoidal shape, as confirmed by
363 spectral simulation (Figure S4). Indeed, the ratio ρ between the long and short axis of the
364 liposomes changes from that of a sphere ($\rho=1$) to an ellipsoid ($\rho=1.5$) from 25 to 50°C. This is
365 explainable by the collective orientation of the phospholipids in a fluid membrane in the
366 magnetic field, due to their negative magnetic susceptibility anisotropy [39,40]. At 75°C, the
367 spectrum shows a narrow and symmetric high intensity line and a residual powder pattern.
368 Importantly, when the temperature was reduced, the spectra were identical to those before heating
369 and therefore the isotropic peak does not result from lipid degradation. Bilayers are thus mostly
370 transformed into smaller fast tumbling structures (67%

Figure 4: ^{31}P -SS NMR spectra of model *V. splendidus* membranes as a function of temperature: (a-c) $T=25^\circ\text{C}$, (d-f) $T=50^\circ\text{C}$ and (g-i) $T=75^\circ\text{C}$. Top three spectra (a,d and g) are controls without marennine, middle spectra (b,e and h) are at a marennine concentration of $2\ \mu\text{g/mL}$, and bottom spectra (c,f and i) are at the MIC ($200\ \mu\text{g/mL}$). Note the lineshape difference between spectra d and e which could be fitted with a ratio of long to short ellipsoid axis of $\rho=1.5$ compared to the more spherical cases (a-c, f) with $\rho=1$ as calculated by spectral simulations.

of the spectrum area, Table 2). It should be noted that the isotropic signal may also result from lipid lateral diffusion in a highly curved bilayer and non-bilayer lipid structures [41]. DOPE, the most abundant lipid (85%) in our model membranes, is known to adopt a hexagonal phase at high temperatures. However, such phase could not be observed on the spectra at all temperatures most likely due to the presence of PG and CL. Indeed, Warschawski *et al.* [42] reported that only 20% of PG in a PG/PE mixture can govern the phase behaviour of the membranes at 75°C . Under our

Table 2: Estimated phase contributions expressed in % from area integration of *V. splendidus* model membranes at 75°C, in the absence and presence of marennine at two concentrations. Standard deviations obtained on three replicates are indicated between brackets.

	Marennine		
	Control	2 µg/mL	MIC
Isotropic phase	87% (8)	79% (6)	33% (8)
Lamellar phase	13% (8)	21% (6)	67% (8)

conditions, with only 10% PG in the mixture, CL seems to contribute to maintaining a lamellar phase, as it is known to exert a major stabilizing effect on zwitterionic lipids [42,43].

The effect of marennine on the phospholipid headgroups of *V. splendidus* model membranes seems to depend on the concentration and temperature. At 25°C, Figure 4 shows no effect at both pigment concentrations. However, at 50°C, high concentrations of marennine re-establish the initial spherical shape of the MLVs, with κ going from 1.5 to 1 in the presence of the pigment. These observations suggest that marennine increases the rigidity of the membrane and lipid liposomes therefore become harder to deform. This result is consistent with the increase in ^2H M2 observed on the bacteria. Previous studies have reported that compounds such as antimicrobial peptides [44-46] or phenolic molecules [47] can have a similar action, *i.e.*, altering the membranes without necessarily changing the CSA value. The interaction of marennine would thus impede the temperature-induced fast lateral diffusion and motion of the lipid polar headgroups, as was, for example, reported for proteins interacting with DMPC (1,2-dipalmitoylphosphatidylcholine) membranes [48]. An alternative explanation could be that marennine, with an anisotropy of diamagnetic susceptibility of opposing sign to the one of the lipids, would counter the field-induced deformation of the membrane. However, since the exact structure of marennine is unknown, this is for the moment a simple hypothesis. At 75°C and in the presence of 200 µg/mL, lipids are mostly found in a lamellar phase (67%) as shown in Table 2, as compared to about 10% in the control sample. Altogether, our results suggest that marennine protects the lamellar phase.

The weak effects of marennine on the model bacterial membranes are comparable to those of negatively charged molecules observed on membrane systems [45,49]. Reported effects of

anionic molecules on zwitterionic membranes include alterations of lipid packing and gelation [50], vesicle shrinkage [50], or aggregation, as a result of hydration or condensation of the acyl chains [51]. The model *V. splendidus* membranes used in our work are composed of zwitterionic PE and anionic PG and CL (Figure S3). It is possible that the negative charges of marennine interact with PE's positive amine group, while the hydroxyl groups of the pigment form H-bonds with PG and CL, similarly to polyhydroxylated fullerene (fullerenol) nanoparticles which were shown to selectively interact with PG in model membranes, and remain at the lipid/water interface [52].

Interaction mechanism of marennine with V. splendidus

The outer membrane of *Vibrio* species is extremely selective, blocking the permeation of molecules greater than 600-700 Da [53]. A 10-kDa molecule such as marennine is therefore unlikely to traverse the outer membrane. The great diversity in responses to marennine exposure observed with different species and strains of *Vibrio*, ranging from growth stimulation to complete inhibition, could thus originate from molecule specificity and diversity at the bacterial membrane level [17]. Our model membrane study reveals that, if marennine penetrated the outer membrane and reached the inner membrane, it would only weakly perturb the ^{31}P atom vicinity, consistent with the absence of change in CSA. It would also slightly affect the membrane elastic properties, consistent with the stiffening effect observed by ^2H SS-NMR. The results obtained by ^{31}P SS-NMR using MLVs highlight the importance of marennine's charged and hydroxylated groups upon molecular interactions, and help understanding the stiffening effect of marennine observed by ^2H SS-NMR *in vivo* with *V. splendidus*.

Altogether, our results confirm our initial hypothesis that, as opposed to PxB, and even at concentrations at which marennine inhibits bacterial growth, marennine does not disrupt bacterial membranes. Marennine most likely interacts with the LPS on the bacterium outer membrane, as was suspected with *E. coli* [19]. Similarly, Tardy-Laporte *et al.* studied the effect of fullerenol nanoparticles on *E. coli*, and hypothesized that the membrane stiffening effect of this polyhydroxylated molecule was due to an interaction with the polysaccharide core of the LPS, thus inducing a tighter packing of the phospholipids in the outer leaflet of the outer membrane [19]. A similar effect to fullerenol is thus suspected for marennine towards *V. splendidus*.

While a direct interaction of marennine can explain this membrane stiffening, the implication of other biochemical mechanisms cannot be ruled out. Under stress situations such as the presence of toxic organic compounds or nutrient deprivation, *Vibrio* species can react by rigidifying their outer membrane, for example via a *cis-trans* conversion process within the lipid unsaturated fatty acids [54,55]. These isomerizations have been reported to increase with increasing phenol concentrations [56], aligning with the increased effect of marennine at the MIC, which may suggest increased phospholipid recruitment for isomerization. If marennine can hardly traverse *V. splendidus* outer membrane, it could still interfere with bacterial outer membrane proteins and induce such rearrangement at the membrane level. Indeed, it has been observed *in vitro* that marennine can interact with and precipitate proteins, a similar effect to that of polyphenols (Mouget, unpublished results). Stiffening could also be induced by cell shrinking due to lower water permeability under stress conditions [57].

Functional consequences of membrane fluidity alteration by marennine are enough to affect bacterial viability, as it may involve the perturbation of lipid-protein domains, and affect processes such as biofilm formation, cytokinesis or enzymatic pathways [58,59]. More specifically, for virulent bacteria under stress, the release of chemical molecules during quorum sensing process could be directly affected by the alteration of membrane fluidity [60].

CONCLUSION

In this work, the action of marennine towards the marine bacterium *V. splendidus* was studied for the first time, at the molecular level, by *in vivo* ^2H SS-NMR, at different growth phases. The antibiotic PxB was used as a reference antibiotic acting against Gram(-) bacteria. While PxB was proved efficient against infection threats in aquaculture, nowadays' regulations as well as increased bacteria resistance prompt more natural alternatives. Our study demonstrates that marennine affects *V. splendidus* membranes at low concentrations, below the MIC. This microalgal pigment, which is not harmful for humans, could thus constitute a potential treatment against vibriosis. Our results also showed that marennine and PxB act against this bacterium via different mechanisms. As proposed in other studies [25,26,35], PxB would disturb the bacteria outer membrane before accessing and disrupting the inner membrane. However, marennine would act in the vicinity of *V. splendidus* outer membrane through a stiffening mechanism. Marennine could interact with the LPS in the outer membrane, and/or trigger the isomerization of

unsaturated fatty acids, as a stress-resistance reaction. More investigations would be required to test these hypotheses.

Finally, our results indicate that both marennine and PxB had a stronger effect in the stationary phase than in the exponential phase. Beney and Gervais [61] have reported that the membrane capacity to resist a source of stress by increasing the lipid ordering is highly related to the initial membrane fluidity state during cell growth. As shown in our previous study [22], cell membranes are more fluid in the stationary phase as compared to the mid-log phase, which would explain the more dramatic stiffening effect of the antimicrobial agents observed in the stationary phase. In all cases, results showed herein indicate that the membrane stiffening induced by marennine occurs even in balanced healthy stage cultures of *V. splendidus* (exponential phase), suggesting that this natural pigment is a potential candidate for antibacterial treatment in aquaculture.

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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. J-S.D. monitored microalgal culture, performed marennine production and purification, and participated in the revision of the manuscript. J-L.M. provided the initial *Haslea*

strain culture and participated in the revision of the manuscript. A.A.A. and D.E.W assisted with NMR operation and analysis, and participated in 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.

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671 Supplementary Materials for

672

673 **Investigating the action of the microalgal pigment marennine on *Vibrio***
674 ***splendidus* by *in vivo* ^2H and ^{31}P solid-state NMR**

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694 **Figure S1:** Determination of minimal inhibitory concentration (MIC) for marennine with
 695 bacterial growth (OD = 600nm) as a function of time (hours). MIC is indicated.

696

697 **Figure S2:** Determination of minimal inhibitory concentration (MIC) for polymyxin B with
 698 bacterial growth (OD = 600nm) as a function of time (hours). MIC is indicated.

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702 **Figure S3:** Chemical structures of phospholipids used in model membranes of *V. splendidus*. A:
 703 phosphatidylethanolamine (PE), B: phosphatidylglycerol (PG) and C: cardiolipin (CL). D:
 704 Characteristic ^{31}P solution NMR spectrum of lipid extracts of *V. splendidus* (7SHRW) grown in

705 LB medium at 25°C. Lipids were solubilized in 500 μL of CDCl_3 , 200 μL methanol, and 50 μL
706 aqueous EDTA solution (200 mM at pH 6), **E: structure of polymyxin B.**
707

709 **Figure S4:** Experimental (black) and simulated spectra (red) of *vibrio splendidus* model
710 membranes without marennine and in the presence of 200 $\mu\text{g}/\text{mL}$ marennine at 25 and 50 °C. The
711 ellipsoid long-to-short axis ratio ρ is indicated.

712