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ABSTRACT

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

KEYWORDS

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

ABBREVIATIONS

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

INTRODUCTION

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

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

Marennine is a natural blue pigment and a promising therapeutic alternative against vibriosis in aquaculture [13,14]. It is secreted by *Haslea ostrearia*, a marine microalga responsible for the noticeable greening of oysters cultured in the French Marennes region and harmless for the consumers. Recent works showed that marennine is a water-soluble 10 kDa polyphenol with possibly a glycosidic skeleton, however, the exact structure and nature of this molecule is today still unknown [15]. The probiotic potential of marennine against aquatic Gram(-) bacteria including *Vibrio* species, has already been demonstrated [16-18]. Turcotte *et al.* also showed that in the presence of marennine extracts, the pathogenicity of Gram(-) *V. splendidus* was suppressed [14]. However, the action mechanism of marennine towards marine bacteria has not yet been elucidated. Considering the high molecular weight of marennine and its unlikely penetration in the bacterial membrane, the pigment would favor an interaction with the outer-membrane of Gram(-) bacteria, as suggested by a solid-state nuclear magnetic resonance (SS-NMR) study on *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* ^{2}H 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 phosopholipids, 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* ^{2}H 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 (D_2O) 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 µmol photons·m^{-2}·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-d_{31} (0.3 mM each) micellized in 0.14 mM of Tween-20 detergent to insure fatty acid intake, as described in Bouhlel *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 µg/mL for marennine, and from 1 to 32 µg/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 ^{2}H-labelled bacteria, adjusted to correspond to approximately 10 ± 2 mg (dry weight).

For PxB exposures, ^{2}H-labelled bacteria pellets were resuspended in a 1 mL solution of NaCl (154 mM) in ^{2}H-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, ^{2}H-labelled bacteria pellets were resuspended in a 50 mL solution of of ^{2}H-depleted artificial seawater (ASW) containing NaCl (480 mM), $MgSO_4$ (28 mM), $MgCl_2$ (24 mM), $CaCl_2$ (16 mM) and $NaHCO_3$ (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 ^{2}H-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 CH_2Cl_2/MeOH v/v) and 0.88% KCl solution in a Potter glass

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

Preparation of model bacterial membranes

Model membranes were prepared from lipid mixtures of POPE, POPG and CL in proportions similar to *V. splendidus* membrane composition, deduced from the ^{31}P solution NMR experiments (85% phosphatidylethanolamine (PE), 10% phosphatidylglycerol (PG) and 5% cardiolipin (CL) (in mole %)). For each sample, 30 mg of phospholipid powder mixture was dissolved in CH_2Cl_2/MeOH (3:1) and dried under a stream of N_2 gas for 30 min. Remaining organic residues were further evaporated overnight under vacuum. The dry lipid films were hydrated in 120 µL buffer solution (Tris (100 mM), NaCl (100 mM), EDTA (2 mM) and D_2O; pH=8), resulting in multilamellar vesicles (MLVs) which will be referred to as liposomes. For marennine-containing samples, the appropriate pigment amount was dissolved in the buffer solution before lipid hydration. All preparations were mechanically homogenized and processed through five cycles of liquid N_2 freezing and thawing in a 35°C water bath. The MLV samples were subsequently filled in 4 mm rotors for ^{31}P SS- NMR analysis.

^{2}H solid-state NMR experiments

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

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

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

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

where ω_r is the angular spinning frequency, N the side band number, and A_N the area of each sideband obtained by spectral integration. The integration of the central peak includes the residual HDO peak which leads to a systematic underestimation of the M2 by a maximum of 15%.

^{31}P solid-state NMR experiments

All ^{31}P SS-NMR experiments were carried out using a Bruker Avance III HD Wide Bore 400 MHz NMR spectrometer (Milton, Ontario, Canada) operating at frequencies of 161.9MHz and 400.02 MHz for ^{31}P and ^{1}H, respectively. Samples were analyzed at 25°C, 50°C and 75°C, with a 25 min equilibration step before each temperature. Temperatures were controlled to within ± 0.5°C, and chemical shifts were referenced relative to external H_3PO_4 set to 0 ppm. Static spectra were recorded through a ^{1}H-decoupled Hahn echo pulse sequence with a ^{31}P 90° pulse length of 3µs, a recycle delay of 3s, and 512 scans, in approximately 25 min.

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

^{31}P solution-state NMR experiments

All ^{31}P solution NMR experiments were performed at 25°C using a Bruker Avance III HD 600 MHz NMR spectrometer (Milton, Ontario, Canada) operating at frequencies of 242.84 MHz and 599.95 MHz for ^{31}P and ^{1}H, respectively. For quantitative results, ^{31}P spectra were recorded with a 10 s recycle delay, continuous ^{1}H decoupling during acquisition, without NOE enhancement, with 128 scans in approximately 22 min. Spectra were processed with an exponential line 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 ^{2}H 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 ^{2}H-labelled by growing marine bacteria in a medium enriched with PA-d_{31} [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 (M_2) was used to quantify the membrane lipid acyl chain ordering. The greater the M_2, the higher the lipid chain ordering. As shown by Warnet *et al.*, combination of

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

Figure 1 shows the effect of marennine on *V. splendidus* at low concentration (2 µg/mL) and at the MIC (200 µg/mL), with corresponding M_2 values presented in Table 1. The "low" marennine concentration employed here is 20 times that reported to be effective on *V. splendidus* (7SHRW) in aquaculture conditions [14]. Spectral analysis reveals that marennine has an ordering effect on *V. splendidus* membrane lipid chains at a concentration of 2 µg/mL, as shown by a 35% increase

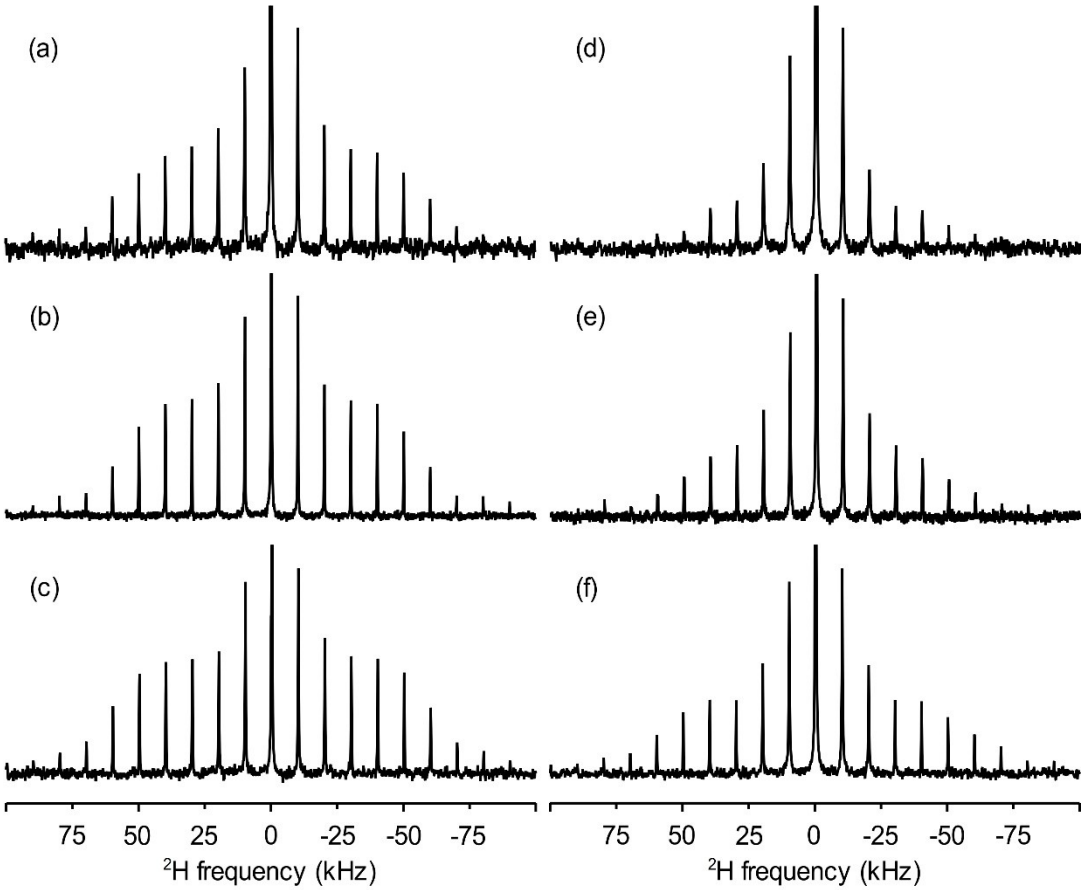

Figure 1: Representative ^{2}H SS-NMR MAS spectra of *V. splendidus* exposed to marennine during the exponential (a-c) and stationary (d-f) stages. Spectra (a) and (d) are control experiments of *V. splendidus* in ASW; (b) and (e) correspond to bacteria exposed to low marennine (2 µg/mL) concentration; (c) and (f) to marennine at MIC (200 µg/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 µg/mL and 8 µg/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 µg/mL	MIC	Control	1 µg/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 µg/mL) and at the MIC (8 µg/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.

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

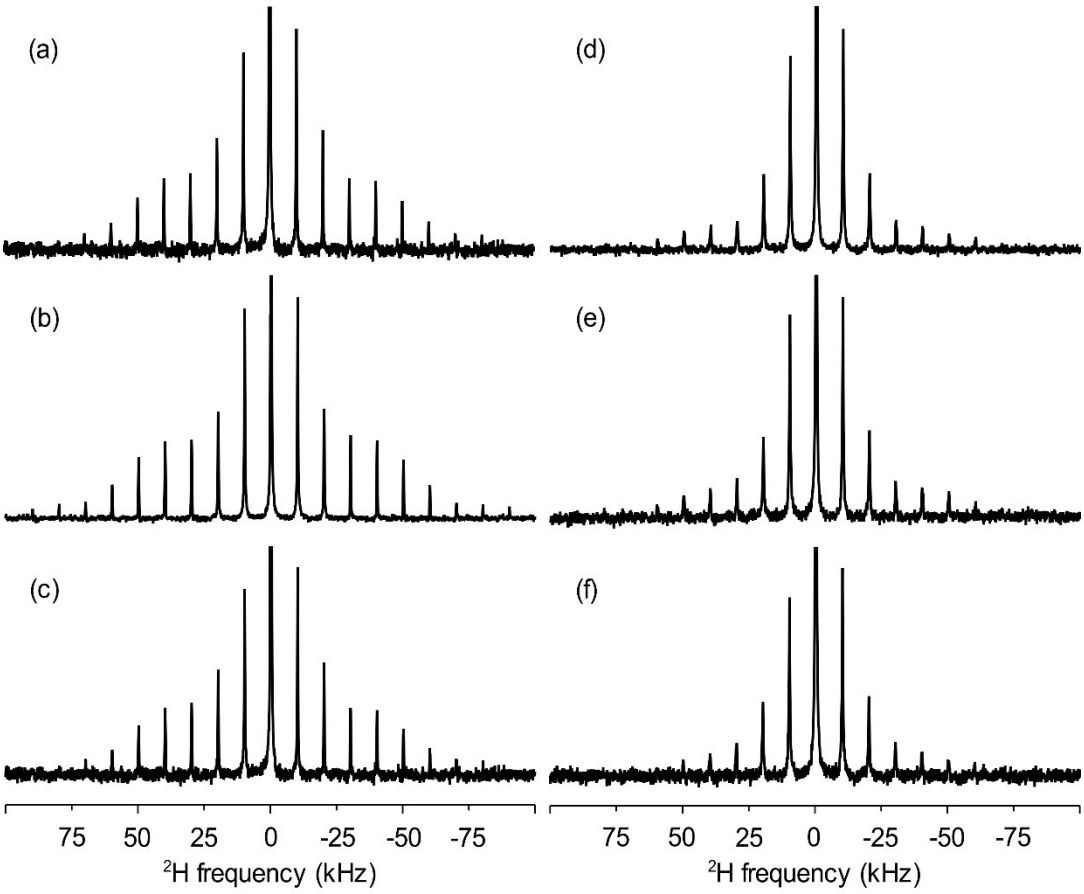

Figure 2: Representative ^{2}H SS-NMR MAS spectra of *V. splendidus* exposed to polymyxin B during exponential (a-c) and stationary (d-f) stages. Spectra (a) and (d) are control experiments, spectra (b) and (e) correspond to bacteria exposed to low PxB (1 µg/mL) concentration; (c) and

(f) to PxB at MIC (8 µg/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* ^{2}H 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

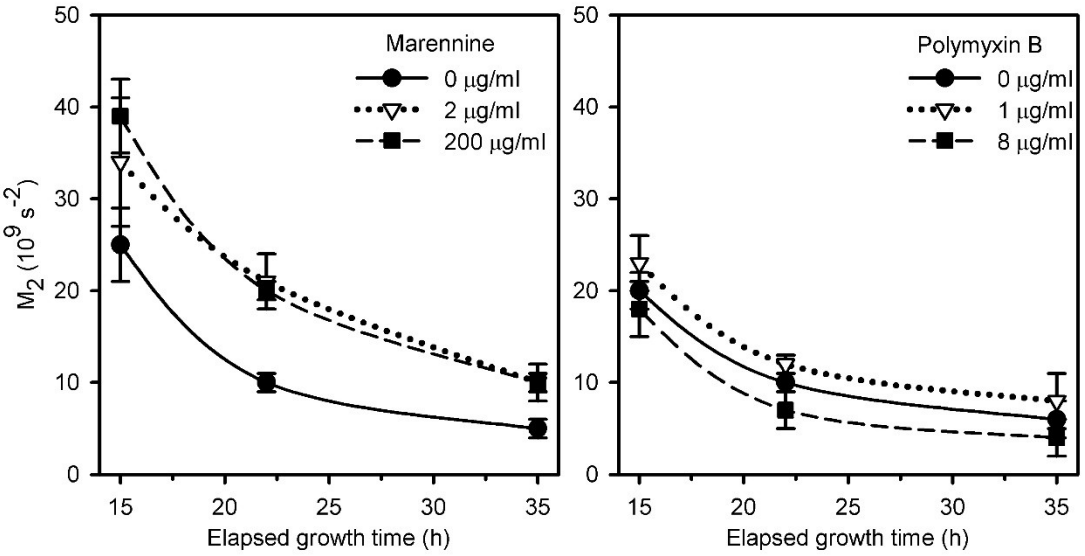

Figure 3: Effect of marennine and PxB on the second spectral moment M_2 of *V. splendidus* after exposure to marennine (left), and PxB (right) at 25°C. Control cells (solid lines) are compared to bacteria exposed to 2 µg/mL of marennine or 1 µg/mL of PxB (dotted lines), and at the respective MICs (dashed 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 ^{2}H 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 ^{31}P 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.

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

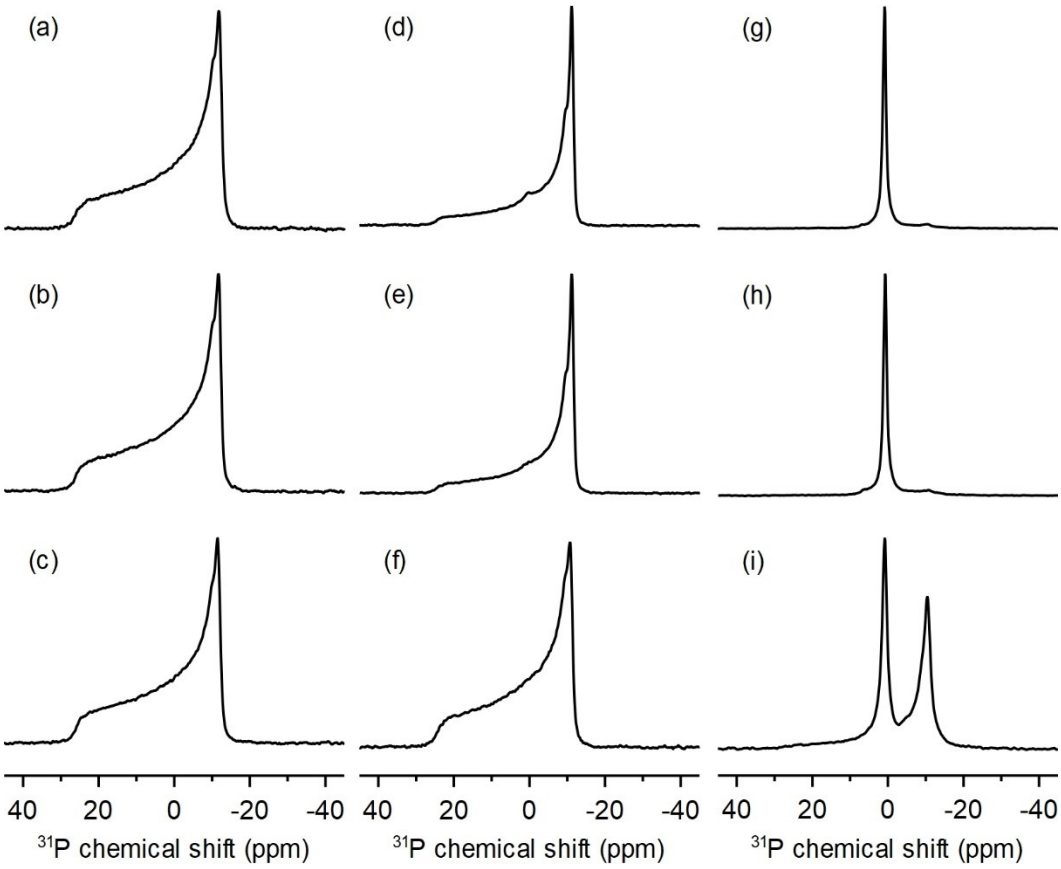

Figure 4: ^{31}P-SS NMR spectra of model *V. splendidus* membranes as a function of temperature: (a-c) T=25°C, (d-f) T=50°C and (g-i) T=75°C. Top three spectra (a,d and g) are controls without marennine, middle spectra (b,e and h) are at a marennine concentration of 2 µg/mL, and bottom spectra (c,f and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

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

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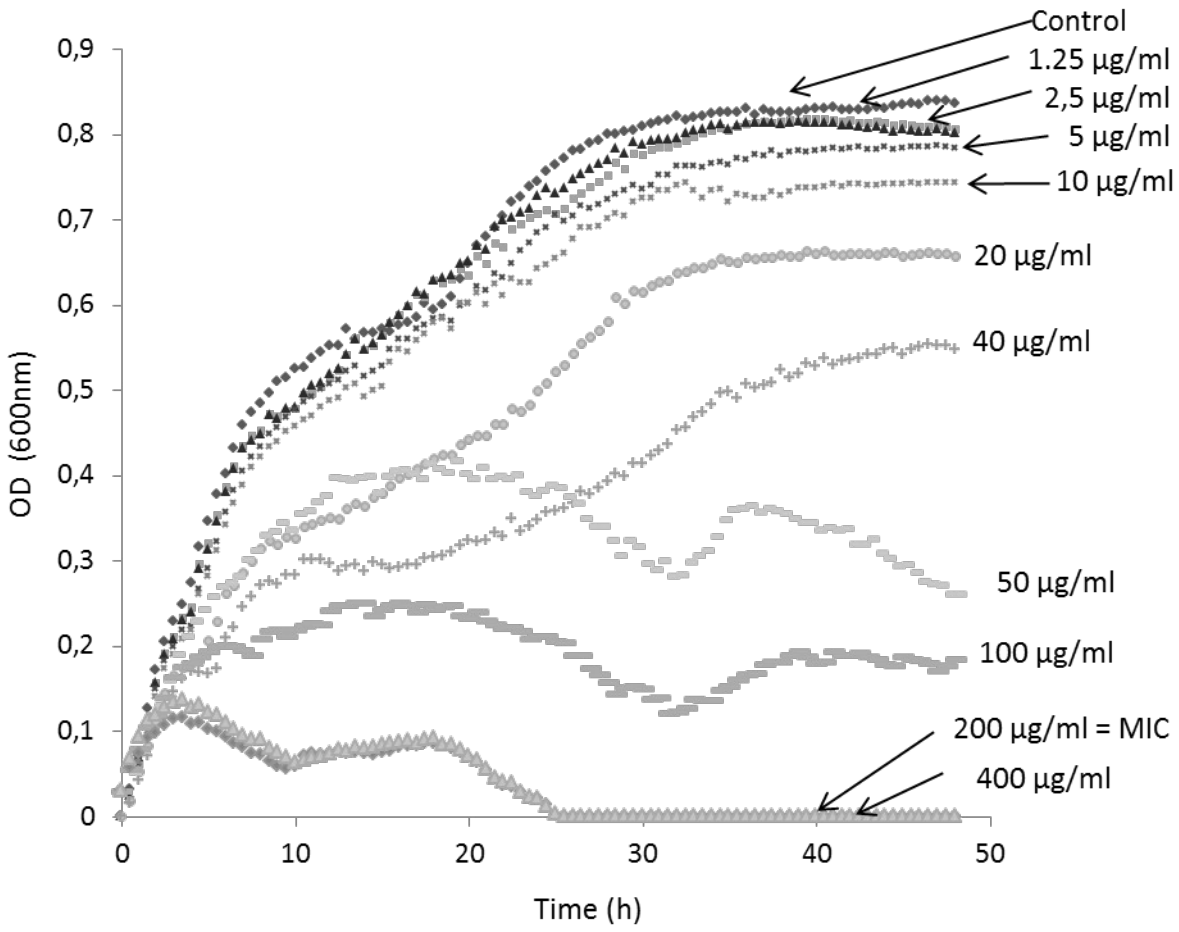

693

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.

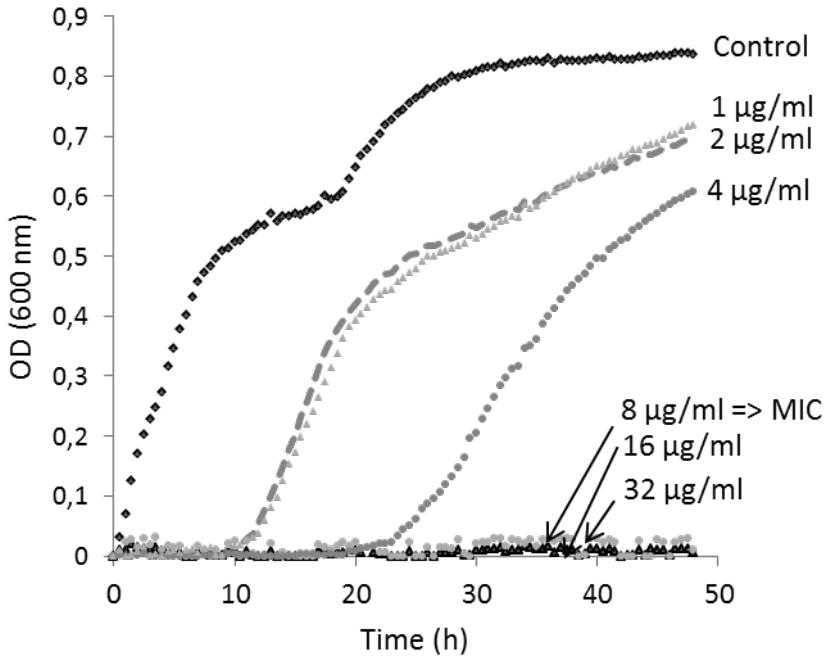

Figure S2: Determination of minimal inhibitory concentration (MIC) for polymyxin B with bacterial growth (OD = 600nm) as a function of time (hours). MIC is indicated.

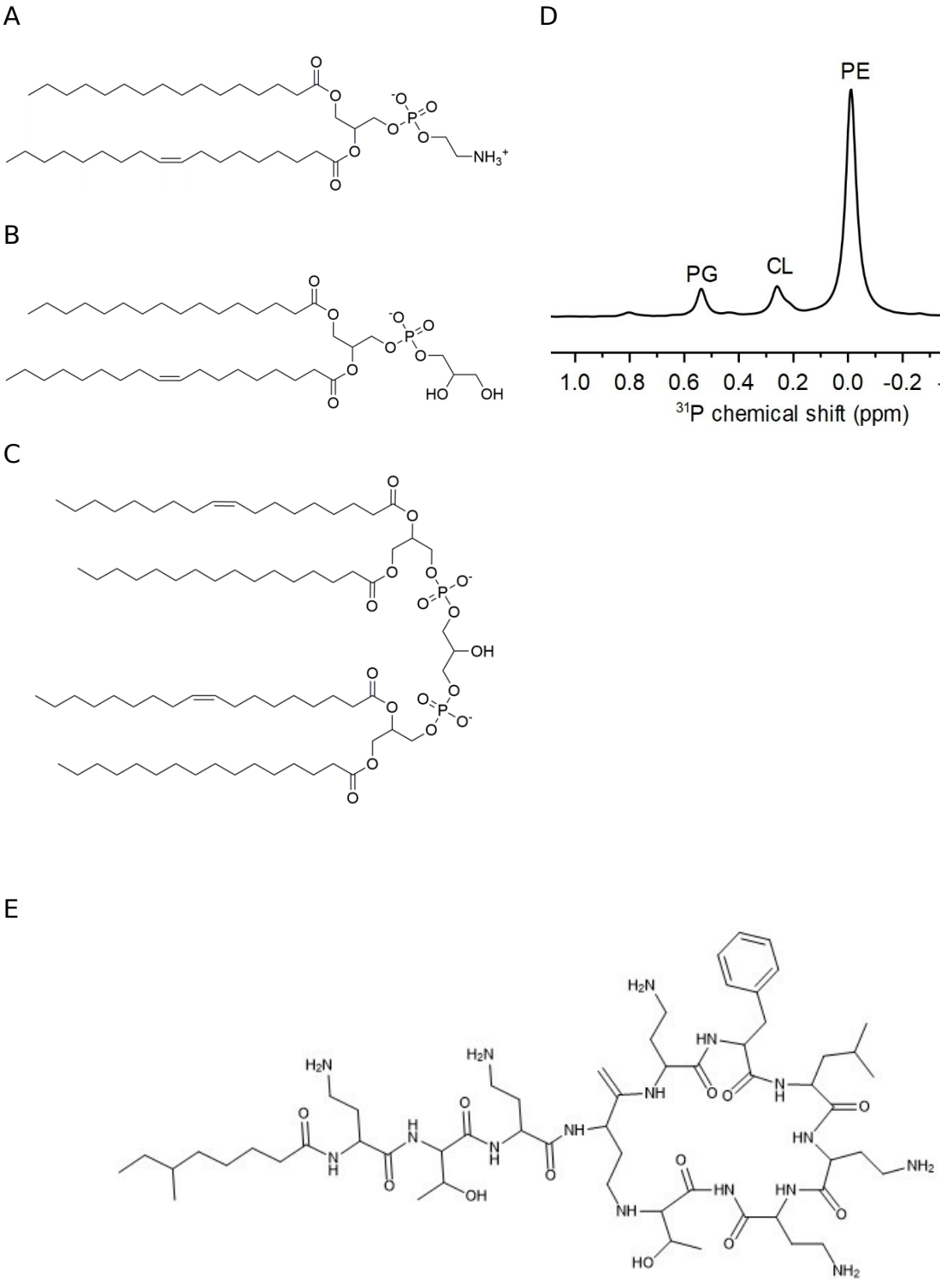

Figure S3: Chemical structures of phospholipids used in model membranes of *V. splendidus*. A: phosphatidylethanolamine (PE), B: phosphatidylglycerol (PG) and C: cardiolipin (CL). D: Characteristic ^{31}P solution NMR spectrum of lipid extracts of *V. splendidus* (7SHRW) grown in

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

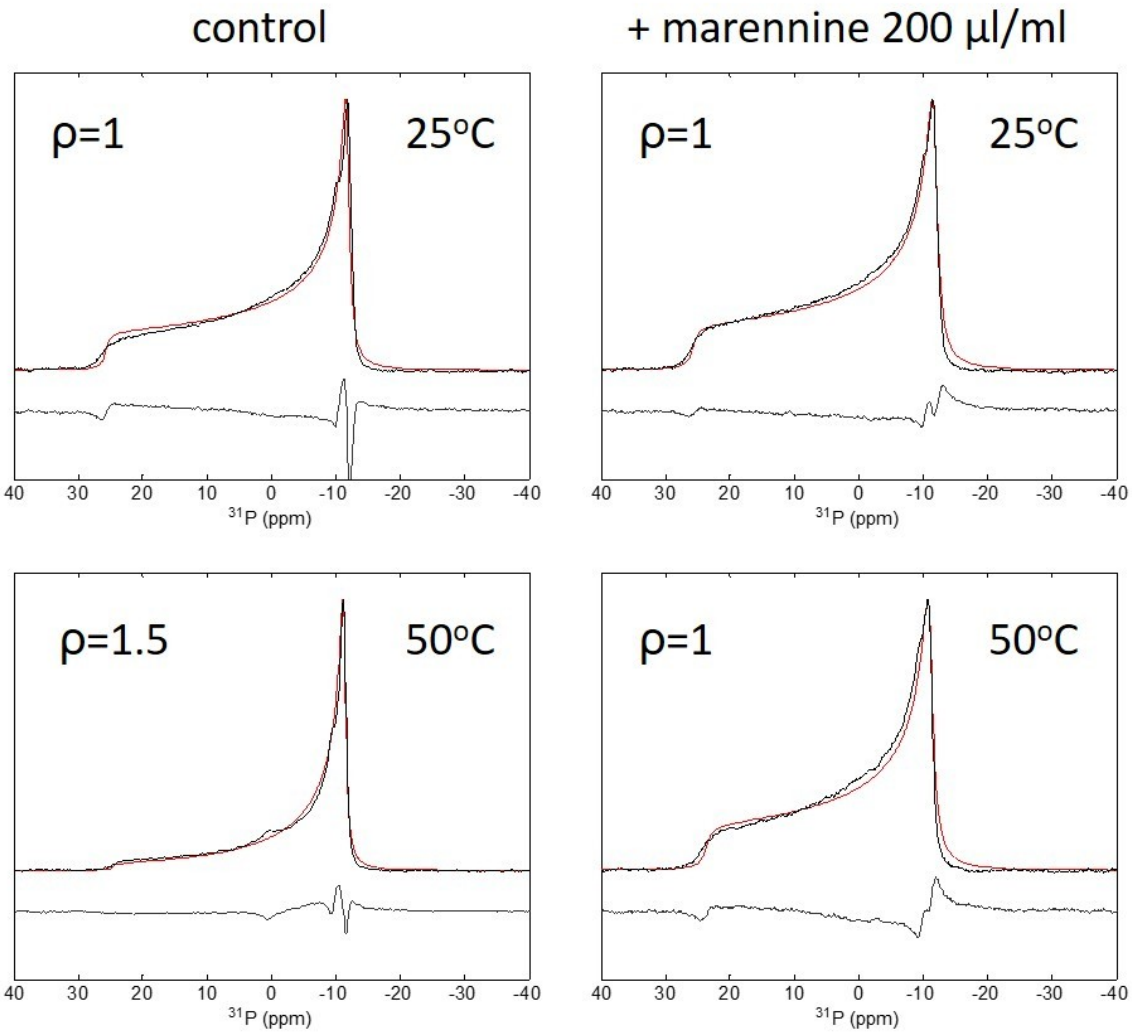

Figure S4: Experimental (black) and simulated spectra (red) of *vibrio splendidus* model membranes without marennine and in the presence of 200 µg/mL marennine at 25 and 50 °C. The ellipsoid long-to-short axis ratio ρ is indicated.