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Interactions of the antifungal mycosubtilin with ergosterol-containing interfacial monolayers

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ABSTRACT

Mycosubtilin, an antimicrobial lipopeptide produced by *Bacillus subtilis*, is characterized by strong antifungal activities. The molecular mechanisms of its biological activities on the membranes of the sensitive yeasts or fungi have not yet been clearly elucidated. Our purpose was to mimic the mycosubtilin interactions with these membranes using various Langmuir monolayers. Since the major sterol of yeasts or fungi is ergosterol, the interactions of mycosubtilin with monolayers constituted by ergosterol, DPPC/ergosterol or DPPC/sphingomyelin/ergosterol were examined at different initial surface pressures (ΠI). Plotting the mycosubtilin-induced surface pressure increases versus ΠI allowed to determine that the exclusion pressures of mycosubtilin from these different monolayers is higher than the surface prevailing within the biological membranes. However, this behavior was lost when mycosubtilin was interacting with ergosteryl acetate-containing monolayers. This suggests the involvement of the sterol alcohol group in the mycosubtilin interactions within membranes. Furthermore, the behavior of mycosubtilin with stigmasterol, similar to that observed with ergosterol, differs from that previously observed with cholesterol, suggesting a role of the alkyl side chain of the sterols. The adsorption of mycosubtilin to ergosterol monolayers induced changes in the lipopeptide orientation at the air–water interface as revealed by polarization modulation infrared reflection absorption spectroscopy (PM-IRRAS). Moreover, imaging the air–water interface by Brewster angle microscopy (BAM) indicates that mycosubtilin induced changes in the organization and morphology of monolayers containing pure ergosterol with the appearance of small condensed dots, suggesting again that the target of mycosubtilin might be the ergosterol present in the membranes of the sensitive yeasts or fungi.

1. Introduction

Mycosubtilin has been identified as an iturinic antifungal compound in the culture medium of several strains of *Bacillus subtilis* [1,2]. It is constituted by a heptapeptide, cyclized in a ring with a β-amino fatty acid (Fig. 1) [3]. The β-amino fatty acid chain can be *iso* C_{16}, *n* C_{16}, *iso* C_{17} or *anteiso* C_{17} [4]. Mycosubtilin exhibits biocide activities [5] for some pathogenic strains which are resistant to classical antifungal agents [6]. Among the mycosubtilin-sensitive strains are *Botrytis cinerea*, *Candida albicans*, *Candida tropicalis*, *Candida glabrata*, *Cryptococcus neoformans*, *Fusarium oxysporum*, *Pichia pastoris*, *Saccharomyces cerevisiae* and *Yarrowia lipolytica*. Furthermore, the cell target of the iturinic lipopeptides has been studied using *S. cerevisiae* as model yeast and identified as the cytoplasmic membrane [5,7,8].

In a recent work, the molecular mechanisms of the hemolytic activity of mycosubtilin were analyzed by examining its interactions with monolayers constituted by pure lipids (DPPC, cholesterol or sphingomyelin) or by lipid mixtures (DPPC/cholesterol or DPPC/cholesterol/sphingomyelin) [9]. It has been shown that mycosubtilin induced the most striking changes in the organization and morphology of the monolayers only when they contained the DPPC, cholesterol and sphingomyelin, monolayers which could be considered to mimic the lipid rafts [10]. Furthermore, cholesterol has been identified as the potential cellular target of mycosubtilin [9] but this cannot explain the antifungal activities of mycosubtilin since the cytoplasmic membranes of yeasts or fungi do not contain significant amounts of cholesterol. Indeed the major sterol of their plasma membrane is ergosterol [11,12]. Ergosterol differs from cholesterol by the presence of two additional double bonds,

Abbreviations: BAM, Brewster angle microscopy; Chol, cholesterol; DPPC, dipalmitoylphosphatidylcholine; Erg, ergosterol; GL, gray level; ΠEx, exclusion pressures; Π_i, initial surface pressure; OS, obturation speed; PM-IRRAS, polarization modulation infrared reflection absorption spectroscopy

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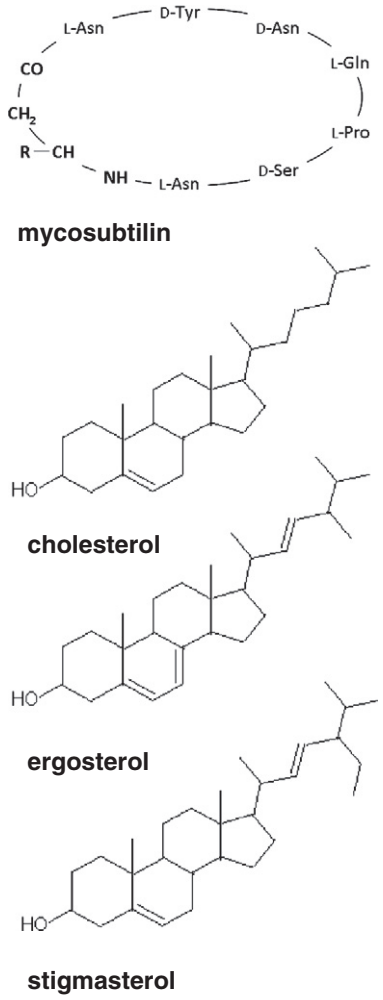

Fig. 1. Schematic structures of mycosubtilin and different sterols.

one in the steroid nucleus and the other one in the alkyl side chain, as well as a by the presence of an extra methyl group in this side chain (Fig. 1).

Furthermore the antifungal activities of mycosubtilin have been shown to be inhibited by adding ergosterol to the culture medium of the sensitive cells [6,13], suggesting an interaction between the lipopeptide and ergosterol. The present work is the first attempt to explain the biocide activities of mycosubtilin by looking for the role played by ergosterol in its antifungal power. We also addressed the following question: does mycosubtilin bind preferentially to the lipid rafts of the sensitive cells which are supposed to contain phosphatidylcholine, sphingomyelin and ergosterol? The interactions between mycosubtilin and the cytoplasmic membranes of yeasts or fungi were modeled by using biomimetic Langmuir monolayers at the air–water interface, as it had been done for amphotericin B, another antifungal molecule [14]. It was shown that the nature of the sterols plays a role in the interaction of amphotericin B, we have chosen to analyze the adsorption of mycosubtilin to monolayers constituted by stigmasterol, another sterol.

In the present work, the orientation of the lipopeptide at the air–water interface was analyzed by polarization modulation infrared reflection absorption spectroscopy (PM-IRRAS) which is sensitive to protein or peptide conformations and to the mean orientation of the transition dipole. The morphology of the mycosubtilin/lipid complexes was analyzed by Brewster angle microscopy (BAM), an interfacial technique detecting the presence of condensed domains in monolayers.

2. Materials and methods

2.1. Chemicals

Bovine brain sphingomyelin (SM), dimethylsulfoxide (DMSO), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), ergosterol (Ergo) and stigmasterol were purchased from Sigma Chemical Co. (St. Louis, MO). Ergosteryl acetate (Ergo-Ac) was purchased from Sobioda (Montbonnot St Martin, France). They were used without further purification. All organic solvents were of analytical grade. The ultrapure water, purified with a Millipore filtering system (Bedford, MA), had a resistivity of 18.2 MΩ cm. Stock solutions of the lipids were obtained by dissolving them at 0.545 mM in hexane–ethanol (9/1, v/v). DPPC/Ergo and DPPC/Ergo/SM solutions were obtained by mixing the stock solutions at the desired mole ratio. The purity of mycosubtilin, prepared from cultures of *B. subtilis* strain, was checked by thin-layer chromatography [15], and it was about 98%. Stock solution of mycosubtilin was obtained by dissolving it into DMSO at 0.545 mM [16].

2.2. Adsorption experiments at constant surface area

The film balance was built by R&K (Riegler & Kirstein GmbH, Wiesbaden, Germany) and equipped with a Wilhelmy-type surface-pressure-measuring system. All monolayer experiments were performed at 21 °C. Adsorption experiments were performed on a small Teflon dish (diameter, 4 cm; volume, 10 mL) with an ultrapure water subphase (pH 5.6). The subphase was stirred with a magnetic stirrer spinning at 100 rev. /min. Lipids were spread at the air–water interface to reach the desired final surface pressure. After solvent evaporation and film stabilization [17], mycosubtilin was injected at a final concentration of 0.545 μM into the subphase. The lipopeptide adsorption to the different lipid monolayers was measured as an increase in the surface pressure as described previously [9,18]. To determine whether DMSO was not interfering with the surface chemistry properties, the same volume of pure DMSO was injected under the lipid monolayer as control, and no change in surface pressure was detected as previously shown [9,16].

2.3. PM-IRRAS measurements

PM-IRRAS is an IR spectroscopic method adapted to the air–water interface. It combines Fourier transform mid-IR reflection spectroscopy with rapid polarization of the incident beam between parallel (p) and perpendicular (s) polarization [19–21]. The spectra were recorded on a Nicolet 850 spectrometer equipped with an HgCdTe detector which was cooled down at −196 °C with liquid nitrogen (Thermo Electron, Nicolet Instrument, Madison, WI). In short, the infrared beam was reflected toward the optical bench by a mirror. The reflected beam was then polarized by a ZnSe polarizer and modulated between a parallel (p) and a perpendicular (s) polarization by a photoelastic modulator. The polarized and modulated beam was then directed toward the air–water interface onto a small Langmuir trough (Nima Technology, UK) as described previously [22]. The optimal angle of incidence was 75° to the interface normal. Afterwards, the beam was reflected on the HgCdTe detector. The detected signal allowed obtaining the differential reflectivity spectrum after processing as follows:

$$\Delta R/R = \left[\left(R_p - R_s\right) J_2(\Phi_0)/\left(R_p + R_s\right) + \left(R_p + R_s\right) J_0(\Phi_0)\right],$$

where J_2 and J_0 are the zero- and second-order Bessel functions, while Φ_0 is the phase of the Bessel functions related to variations of the modulation of polarization according to the wavelength and R_p and R_s are the parallel and perpendicular reflectivity,

respectively. In order to remove the liquid water absorption and the contribution of the Bessel function, the monolayer spectrum was divided by that of the pure subphase. For all PM-IRRAS spectra measurements, the resolution was 8 cm^{-1}. Each spectrum was collected at 1024 scans and is at least the average of three spectra measured in an independent manner.

2.4. Brewster angle microscopy measurements

The morphology of lipid and mixed lipid/mycosubtilin monolayers at the air–water interface was observed with a Brewster angle microscope (NFT iElli-2000, Göttingen, Germany) mounted on a Langmuir trough (area, 165 cm^2; volume, 120 mL) (Riegler & Kirstein GmbH, Wiesbaden, Germany) [23]. Lipids, dissolved in hexane–ethanol at 0.545 mM were spread at the air–water interface of the Langmuir trough. After 30 min of waiting for solvent evaporation, the lipid monolayers were compressed to obtain the reference images of the lipid monolayers without mycosubtilin. Then, the lipopeptide was injected into the pure water subphase at a 0 mN/m surface pressure. The molar ratio between spread lipids and injected mycosubtilin was 1/1. A 40 min delay was then required to allow the lipopeptide to reach the air–water interface. BAM images, surface pressures and gray levels (GL) were recorded simultaneously during the compression of the monolayers. Indeed, since the compression of the monolayer induced an increase of its thickness, it is necessary to use different shutter speeds according to the overall increase of the brightness of the film during its compression. The obturation speed (OS) of the camera shutter allows us to select the exposure time adapted to the different illumination levels. The BAM spatial resolution was about 2 μm and the image size was 430 × 320 μm. Each image is representative of at least two series of measurements.

3. Results

3.1. Mycosubtilin adsorption to interfacial ergosterol-containing monolayers

The involvement of ergosterol in the antifungal activities of mycosubtilin was studied using biomimetic interfacial monolayers by increasing step by step the complexity of ergosterol-containing monolayer. Mycosubtilin was injected under different lipid monomolecular film at a constant area. The resulting interaction was measured as an increase in the surface pressure of monolayers constituted by pure ergosterol, by DPPC/Ergo (70/30, mol/mol) or by DPPC/SM/Ergo (30/30/30, mol/mol/mol). The inset of Fig. 2 gives the kinetic curves, obtained for an initial surface pressure (Πi) of about 10 mN/m. The greatest increase in the surface pressure (about 35 mN/m) induced by mycosubtilin was obtained with pure Ergo monolayer, meanwhile similar increases in the surface pressure (about 10 mN/m) were observed with DPPC/Ergo (70/30, mol/mol) and DPPC/SM/Ergo (30/30/30, mol/mol/mol). Besides, Table 1, comparing the initial velocities of the surface pressure increases induced by mycosubtilin for the different lipid monolayers, shows that the velocity observed with pure ergosterol monolayer was at least 1.5 times higher than the DPPC/Ergo or DPPC/Ergo/SM monolayers.

The lipopeptide was then injected under the different lipid monolayers at various Πi. After reaching the equilibrium, the maxima of the mycosubtilin-induced increase in Πi (ΔΠ) were plotted versus Πi (Fig. 2). The ΔΠ vs Πi plot obtained with pure ergosterol monolayer reflects an original behavior of mycosubtilin compared with the plots obtained for DPPC/Ergo (70/30, mol/mol) and for DPPC/SM/Ergo (30/30/30, mol/mol/mol). Indeed, the ΔΠ vs Πi plot of mixed monolayers shows only a decreasing straight line, while the ΔΠ vs Πi plot, obtained with pure ergosterol, shows two parts. For the lowest Πi of pure ergosterol monolayers, the mycosubtilin insertion increases with increasing initial surface pressures, corresponding to an increase

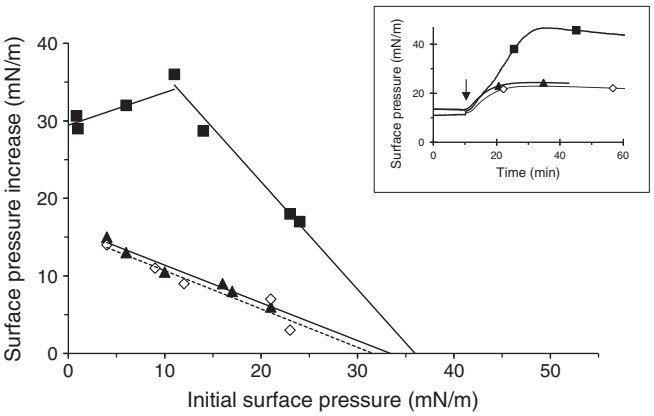

Fig. 2. Interaction of mycosubtilin with interfacial monolayers constituted by pure lipids.Influence of the initial surface pressure (Πi) on the maximal pressure variation (ΔΠ) induced by mycosubtilin adsorption onto monolayers. Each point was obtained from an independent experiment. Squares correspond to pure ergosterol, diamonds to DPPC/Ergo (70/30, mol/mol) and triangles to DPPC/Ergo/SM (30/30/30, mol/mol/mol). Subphase was pure water. Final concentration of mycosubtilin in the subphase was 0.545 μM. The inset gives the kinetics of mycosubtilin adsorption in the presence of the different pure lipids. The arrow indicates the injection of lipopeptide under the lipid monolayer at about 10 mN/m. Each curve is representative of at least two independent assays.

of the number of lipopeptide molecules at the air–water interface. This suggests a specific interaction of the lipopeptide for the sterol. For the highest initial Πi, mycosubtilin insertion decreases with increasing initial surface pressures.

These curves allowed us to estimate the theoretical values of Πi extrapolated for $\Delta\Pi_{max}$ at 0 mN/m, which are usually named exclusion pressures (Πex). Fig. 3 gives the estimated values of Πex for mycosubtilin insertion into the different monolayers which are higher than the surface pressure supposed to prevail within biological membranes [24]. This suggests that the lipopeptide could be inserted in the plasma membranes of the sensitive cells and must be correlated with the *in vivo* data showing strong modifications in the organization of the plasma membrane of the *S. cerevisiae* cells treated by iturin A, another iturinic lipopeptide differing from mycosubtilin only by the replacement of the D-Ser-L-Asn sequence (in mycosubtilin) by the D-Asn-L-Ser sequence (in iturin A) [7]. Moreover, the values of Πex obtained for the different Ergo-containing monolayers, which are all higher than the values of Πex previously determined for pure SM and pure DPPC monolayers [9] (Table 1), as well as the original behavior of mycosubtilin toward Ergo monolayers, suggest that ergosterol may have an important role in the lipopeptide insertion within biological membranes.

Table 1
Comparaison of the initial velocity of the mycosubtilin adsorption to different lipid monolayers at about 10 mN/m.

Lipid monolayer	Initial velocity[a] (mN/m/min)	Exclusion pressure[b] (mN/m)
Ergosterol	1.829 ± 0.012	35.97 ± 5.19
DPPC/Ergo	1.114 ± 0.015	31.91 ± 1.34
DPPC/Ergo/SM	1.167 ± 0.016	33.58 ± 0.53
Cholesterol[c]	1.90	50
DPPC/Chol[c]	0.52	43
DPPC/Chol/SM[c]	1.40	52
DPPC[c]	0.39	28
SM[c]	0.81	27

[a] Initial velocities were determined by linear regression of the linear part of the adsorption curves of Fig. 2 (inset). The error on the initial velocities was calculated using IGOR Pro software.

[b] Exclusion pressures of mycosubtilin from the different monolayers were obtained by linear regression of the curves shown in Fig. 2 and the errors were calculated using IGOR Pro software.

[c] These values have been determined in a previous work [9].

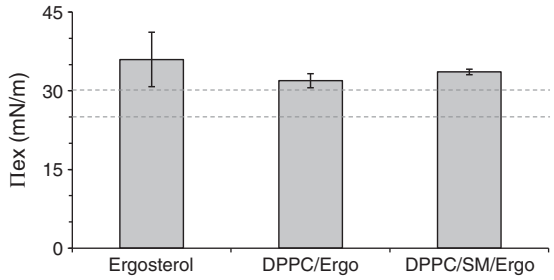

Fig. 3. Influence of the lipids on the exclusion pressures of mycosubtilin from the interfacial monolayers.The exclusion pressures (Πex) were obtained by linear regression and the errors on Πex were calculated using IGOR Pro software. The estimated surface pressure of biological membranes between 25 and 30 mN/m was indicated by dashed lines.

3.2. Conformational analysis of mycosubtilin adsorbed to ergosterol monolayers at the air–water interface

In order to have a reference PM-IRRAS spectrum of pure ergosterol monolayer, Ergo was deposited at the air–water to obtain an initial surface pressure of about 10 mN/m and, after stabilization of the pure Ergo monolayer, its PM-IRRAS spectrum was measured (Fig. 4, dotted line).

As expected, the spectrum did not show any absorbance in the 1750–1500 cm^{-1} region. Then, mycosubtilin was injected under the sterol monolayer and PM-IRRAS spectrum of the interfacial monolayer was measured when the surface was stabilized (i.e. about 45 min after the lipopeptide injection) (Fig. 4, full line). The amide I band shows a maximum at 1654 cm^{-1}, while there was no amide-II band. To determine the influence of ergosterol on the mycosubtilin conformation, this PM-IRRAS spectrum (inset of Fig. 4, full line) was compared with the PM-IRRAS spectrum of a pure mycosubtilin spectrum (inset of Fig. 4, dotted line), which showed a maximum of amide-I band located at 1654 cm^{-1} and a broad amide-II band centered at 1530 cm^{-1}. The absence of amide-II band observed in the PM-IRRAS spectrum measured in the presence of ergosterol and its presence in the spectrum of pure mycosubtilin monolayer suggest ergosterol-induced changes in the orientation and/or organization of mycosubtilin at the air–water interface. This might be due to the modification of the dipole moment of mycosubtilin which can be considered to its peptide cycle. This suggests the involvement of the peptide part of the lipopeptide in its interactions with ergosterol.

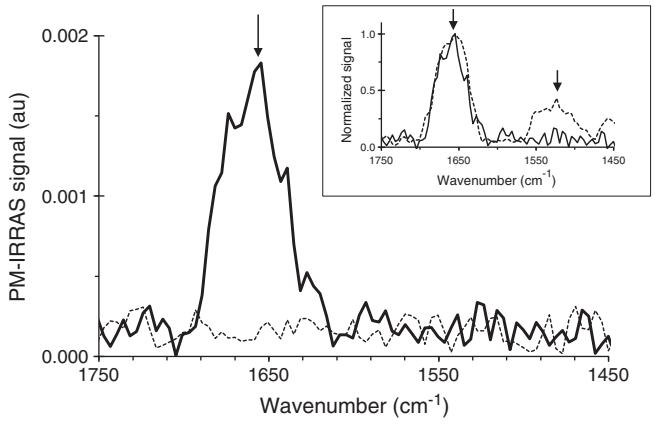

Fig. 4. PM-IRRAS spectra of the ergosterol monolayer before and after injection of mycosubtilin under the monolayer.PM-IRRAS spectrum of the pure ergosterol monolayer (dotted line) was recorded when the surface pressure stabilized at 10 mN/m. The lipopeptide concentration was injected at 0.545 μM and PM-IRRAS spectrum of the mycosubtilin/ergosterol monolayer (full line) was recorded when the surface pressure stabilized at 23 mN/m. The inset compares the normalized PM-IRRAS spectrum of the mycosubtilin/ergosterol monolayer (full line) with the normalized spectrum of a pure mycosubtilin monolayer (dotted line).

3.3. Influence of mycosubtilin on the lipid monolayer morphology

The interactions between mycosubtilin and monolayers constituted by pure ergosterol or DPPC/Ergo/SM were examined by analyzing the morphology of the different films by Brewster angle microscopy imaging.

BAM images of the ergosterol monolayer at different surface pressures were taken before and after mycosubtilin interaction (Fig. 5). At 0–1 mN/m, the BAM images of pure ergosterol monolayers were consistent with the gas–solid phase coexistence, where more compact density states (bright domains) coexist with low molecular ones (dark domains) (Fig. 5, image A). At higher surface pressures, the monolayer of pure ergosterol formed a homogeneous bright zone, which is characteristic of a solid state (Fig. 5, images B and C). These BAM images agree with previous data [25]. In the presence of mycosubtilin and at the lowest surface pressures, the dark domains are greater than in the absence of the lipopeptide (Fig. 5, image A′). Furthermore, the obturation speed decrease, correlated to an increase of the thickness of the film [9], increased in the presence of the lipopeptide. This confirms again the insertion of the lipopeptide into the monolayer. At higher surface pressures, corresponding to a homogenous condensed film for a pure ergosterol monolayer, the mycosubtilin/ergosterol film was heterogeneous and small bright dots appeared (Fig. 5, image B′). At the highest surface pressures, the mycosubtilin/ergosterol film became homogeneous and the small bright dots are

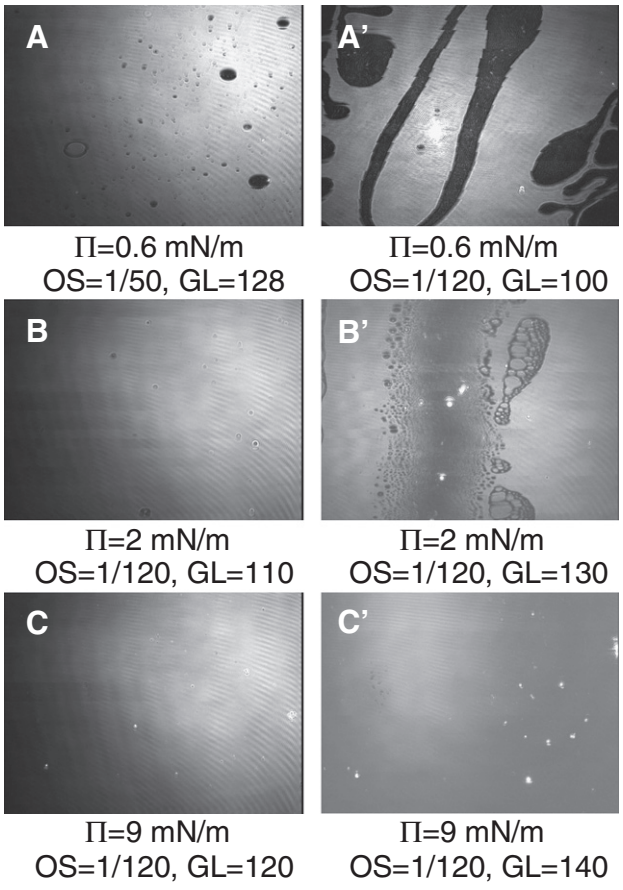

Fig. 5. Influence of mycosubtilin on the organization of monolayers constituted by pure ergosterol.BAM images were taken during the compression of the lipid monolayers before (left column) or 40 min after mycosubtilin injection (right column). Images were recorded at different obturation speeds (OS).

still present (Fig. 5, image C′). These dots might be generated by the presence of the lipopeptide.

We then tested the influence of mycosubtilin on the organization of more complex monolayers constituted by DPPC/Ergo/SM (30/30/30, mol/mol/mol). Fig. 6 (left column) shows the BAM images at different surface pressures of the monolayer before the lipopeptide injection. At 0–1 mN/m (Fig. 6, image A), the BAM images of DPPC/Ergo/SM monolayer show a heterogeneous organization, with the coexistence of bright and dark domains. At higher surface pressures, the DPPC/Ergo/SM monolayer become less and less heterogeneous, leading to a homogenous film (Fig. 6, images B and C). The interaction of mycosubtilin with DPPC/Ergo/SM monolayer does not modify significantly the DPPC/Ergo/SM monolayer organization, suggesting a great miscibility of the four compounds.

Such a behavior was not observed when the monolayers contained the lipopeptide and pure ergosterol. Indeed the BAM images of mycosubtilin/ergosterol monolayer show small bright dots. Dark and bright domains have been also observed in the BAM images of DPPC/Chol (70/30, mol, mol) monolayers at the same surface pressures [9] but the morphology of the domains was different from those observed in the BAM images of the mycosubtilin/ergosterol monolayer (Fig. 5, image A).

3.4. Mycosubtilin adsorption to ergosteryl acetate-containing monolayers

Previous studies suggested a possible involvement of the secondary alcohol residue of cholesterol [9,13]. Since both sterols (ergosterol and cholesterol) contain a hydroxyl residue, the influence of blocking of this residue was tested by looking for mycosubtilin adsorption toward ergosteryl acetate monolayers. In order to obtain ergosteryl acetate monolayers at different initial surface pressures, different amounts of the lipid were deposited at the air-pure water interface. Whatever the amount deposited, it was impossible to obtain a monolayer stabilized at a well-defined initial surface pressure (data not shown). Ergosteryl acetate was then mixed with DPPC. The addition of 70% of the phospholipid allowed to stabilize the monolayers at different initial surface pressures and then to inject mycosubtilin under the different monolayers. The inset of Fig. 7A compares the kinetic curve obtained with a DPPC/ergosteryl acetate (70/30, mol/mol) monolayer and that previously described for the DPPC/ergosterol (70/30, mol/mol). The mycosubtilin-induced increase in the surface pressure and the initial velocity of the surface pressure increase deceased when mycosubtilin was interacting with a DPPC/ergosteryl acetate monolayer.

The experimental ΔΠ values obtained with monolayers constituted by DPPC/ergosteryl acetate (70/30, mol/mol) at different initial surface pressures were plotted versus Πi (Fig. 7A). The resulting Πex value was significantly lower than the surface pressure supposed to prevail within biological membranes [24] and also lower than the Πex value previously determined for monolayer constituted by DPPC/ergosterol (70/30, mol/mol) (Fig. 7B). This suggests that the secondary alcohol residue of the ergosterol is involved in the interactions between mycosubtilin and this sterol.

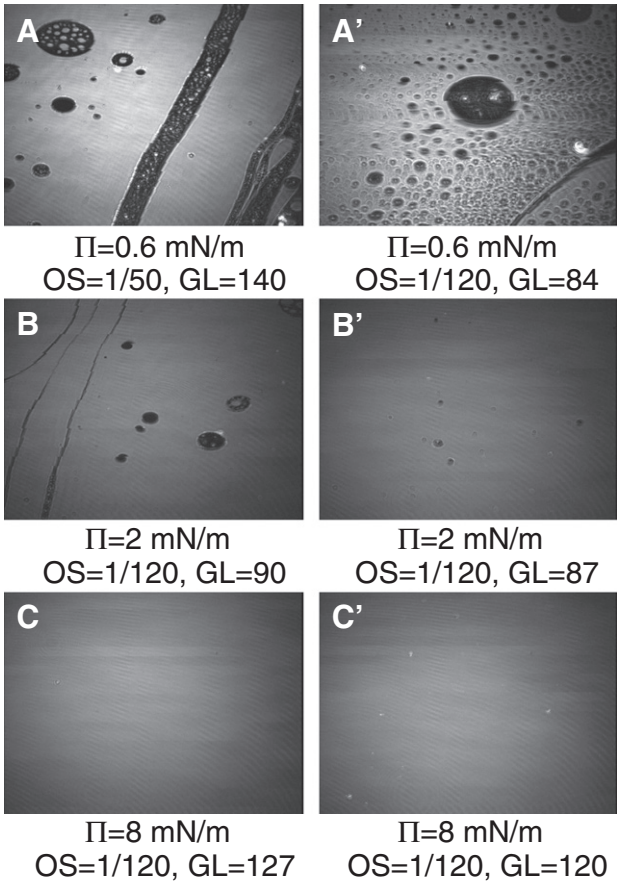

Fig. 6. Influence of mycosubtilin on the organization of monolayers constituted by DPPC/Ergo/SM (30/30/30, mol/mol/mol).BAM images were taken during the compression of the lipid monolayers before (left column) or 40 min after mycosubtilin injection (right column). Images were recorded at different obturation speeds (OS).

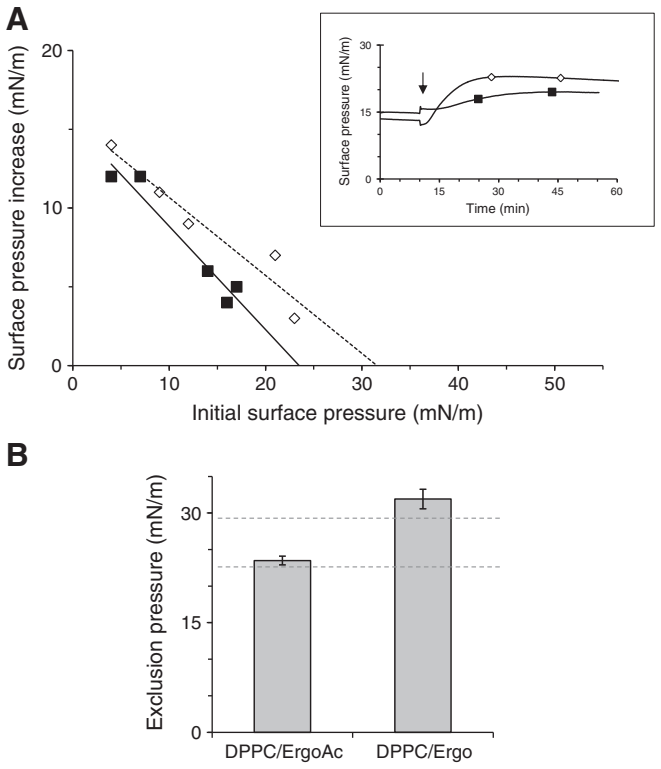

Fig. 7. Role of the acetylation of the secondary alcohol residue of ergosterol on the mycosubtilin adsorption to interfacial monolayers.(A), Influence of the initial surface pressure on the maximal pressure variation induced by mycosubtilin adsorption onto monolayers constituted by DPPC/ergosteryl acetate (70/30, mol/mol) (black squares). Each point was obtained from an independent experiment. Subphase was pure water. Final concentration of mycosubtilin in the subphase was 0.545 μM. The data obtained with DPPC/ergosterol (70/30, mol/mol) (open diamonds), shown in Fig. 2, were given to help in the comparison. The inset compares the kinetics of mycosubtilin adsorption to the DPPC/ergosteryl acetate (black squares) and DPPC/ergosterol (open diamonds). The arrow indicates the injection of lipopeptide under the lipid monolayer. Each curve is representative of at least two independent assays.(B), Comparison of the exclusion pressure of mycosubtilin from the same monolayers. The exclusion pressure (Πex) of mycosubtilin from DPPC/ergosteryl acetate monolayer was obtained by linear regression and the error on Πex was calculated using IGOR Pro software.

3.5. Mycosubtilin adsorption to stigmasterol monolayers

Since previous studies on the interactions between various sterol monolayers showed that the nature of the sterols has an influence on the organization of amphotericin B, another antifungal compound, at the air–water interface [14], we have chosen to test the interactions of mycosubtilin with stigmasterol monolayers. Stigmasterol differs from cholesterol only in the structure of its aliphatic chain (Fig. 1).

Thus, the adsorption kinetics of mycosubtilin toward stigmasterol monolayers at different initial surface pressures was measured (data not shown). The resulting experimental ΔΠ values were plotted versus Πi. Fig. 8 shows that the ΔΠ vs Πi plot, obtained with pure stigmasterol, has two parts, as it was previously observed with pure ergosterol monolayers. Furthermore, the exclusion surface pressure of mycosubtilin was very similar to the Πex value previously determined for pure ergosterol monolayer (Fig. 8, inset) but was significantly lower than that obtained with cholesterol [9]. Since both ergosterol and stigmasterol contain a double bond in their alkyl side chain (Fig. 1), suggesting that the alkyl side chain of the sterols plays a role in the interactions between mycosubtilin and the different sterols.

4. Discussion

Mycosubtilin is a lipopeptide characterized by its strong antifungal activities. Even thought the cellular target of this lipopeptide has been identified to be the cytoplasmic membrane of the sensitive cells [5], there are only a few works conducted to understand the molecular mechanism of mycosubtilin. In this study, our objective was to get information on the mycosubtilin interactions with ergosterol-containing membranes, at the molecular level, using Langmuir monolayers as model membrane.

4.1. Presence of ergosterol within membranes as a driving force for the antifungal activity of mycosubtilin

Previous studies showed that the biocide activity of mycosubtilin could be inhibited by adding free sterol molecules in the culture medium, suggesting specific interactions between the lipopeptide and sterols [6,13]. Since sterols are not alone within membranes, it was necessary to analyze the role of sterols in a phospholipid environment mimicking the membrane of the lipopeptide-sensitive cells. Meanwhile mycosubtilin was discovered on account of its antifungal activities [1], there was no study dedicated to the interactions between the lipopeptide and ergosterol which is the major sterol of the fungi membranes. We thus modeled the interactions of the lipopeptide with biological membranes using different Langmuir monolayers which contain ergosterol as sterol. This membrane biomimetic system was chosen because, unlike liposomes or multilayer vesicles, which cannot be prepared from a pure sterol, it is possible to prepare pure ergosterol monolayers and then compare the behavior of mycosubtilin for different lipids.

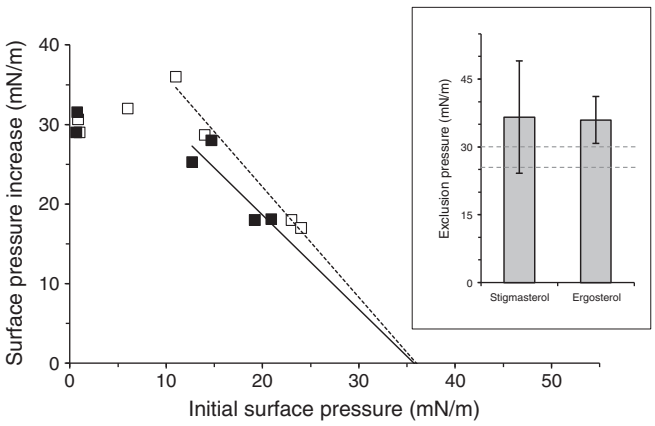

Fig. 8. Role of the nature of the sterol on the mycosubtilin adsorption to interfacial monolayers.Influence of the initial surface pressure on the maximal pressure variation induced by mycosubtilin adsorption onto monolayers constituted by pure stigmasterol (black squares). Each point was obtained from an independent experiment. Subphase was pure water. Final concentration of mycosubtilin in the subphase was 0.545 μM. The data obtained with pure ergosterol (open squares), shown in Fig. 2, were given to help in the comparison.The inset compares the exclusion pressure of mycosubtilin from the same phospholipids. The exclusion pressure (Πex) of mycosubtilin from ergosterol monolayer was obtained by linear regression and the error on Πex was calculated using IGOR Pro software.

Our results showed that mycosubtilin interacts preferentially with ergosterol compared to the other classes of lipids and that the presence of ergosterol makes easier the insertion of the lipopeptide into the monolayers. Since the membranes of fungi or yeasts contain significant amount of ergosterol, our data suggest that the interactions of mycosubtilin with ergosterol found within membranes could constitute the driving force of its antifungal activity. Furthermore the PM-IRRAS data suggest ergosterol-induced changes in the orientation and/or the organization of mycosubtilin at the air–water interface, suggesting that the peptide part of the lipopeptide could be involved in its interactions with ergosterol. Considering the interaction of mycosubtilin at the cell level, we can suggest that, in a first step, the lipopeptide interacts with the ergosterol molecules present in the external leaflet of the plasma membrane via its peptide part and that, in a second step, an interaction between the lipid part of mycosubtilin and the aliphatic chain of ergosterol occurs. Such interactions were supported by the BAM images showing that, meanwhile the pure ergosterol monolayer was a homogenous condensed film, the mycosubtilin/ergosterol film was heterogeneous with the presence of small bright dots (even at the highest surface pressures) which might be generated by the presence of the lipopeptide.

4.2. Does mycosubtilin interact in the same way with cholesterol and ergosterol?

Our data suggest a stronger interaction of mycosubtilin with cholesterol than with ergosterol (Πex value from pure cholesterol monolayer was higher than Πex value from ergosterol). This difference is corroborated by previous results showing that cholesterol is a stronger inhibitor of the antifungal activity of mycosubtilin than ergosterol [13]. Moreover, it has been shown that a mutant strain of *S. cerevisiae*, containing cholesterol instead of ergosterol, was more sensitive to iturin A (another iturinic lipopeptide differing from mycosubtilin only by the inversion of two aminoacids) than the wild-type yeast [26].

The chemical structure of ergosterol presents a double bond between 22nd and 23rd carbons. This double bond hindering the rotation of the molecule seems to induce more packed molecular arrangement for the ergosterol molecules compared to the cholesterol molecules in the monolayer [27,28]. This means that the ergosterol monolayer, which should be more rigid than the cholesterol one, should be less accessible for the penetration of the lipopeptide. The weak insertion of mycosubtilin into ergosterol monolayers could be explained in terms of restricted accessibility of the ergosterol monolayers for the lipopeptide [27].

Besides, using complex monolayers containing ergosterol, sphingomyelin and saturated phospholipids in a raft-mimicking molar ratio did not show the synergic effect of sphingomyelin previously observed with cholesterol [9]. This could suggest that mycosubtilin has not a preferential interaction with ergosterol-enriched lipid rafts within the yeast plasma membranes but it must be remembered that ergosterol have be demonstrated to be less miscible in DPPC bilayers than cholesterol for a DPPC/sterol (70/30, mole/mole) [29,30]. In order to connect these in vitro data with in vivo data, it should be noticed that sterol-free lipid rafts within yeast

membranes have been recently discovered [31] and, as further investigation, it would be interesting to test the influence of mycosubtilin on the distribution of the lipid rafts in yeast cells.

4.3. The key group of the interactions between mycosubtilin and sterols

In a previous study, we have demonstrated that the hydroxyl group of cholesterol was crucial for the strength interactions of mycosubtilin with this sterol [9]. Our present results confirm this involvement of the hydroxyl group of sterol in the case of ergosterol. Since the hydroxyl group constitutes the hydrophilic part of sterols, we can suggest an interaction of this group with the hydrophilic part of mycosubtilin constituted by the peptide part. A possible interaction with the phenolic group of tyrosyl residue and the hydroxyl group of sterols was suggested in the case of bacillomycin Lc, another lipopeptide belonging to iturin family [32]. Moreover, the authors have speculated on a hydrogen bond formation between these two chemical groups. This hypothesis was supported by the absence of antifungal activities of mycosubtilin when the phenolic group of its tyrosyl residue was methylated or acetylated [13]. In the light of that, we can suggest that the insertion of mycosubtilin into ergosterol monolayers is dependant, in a large extent, of the presence of the sterol hydroxyl group.

4.4. Mycosubtilin and phytosanitary field

Our analysis of the interactions of mycosubtilin with stigmasterol shows that the exclusion surface pressure of the lipopeptide from pure stigmasterol was very similar to that from pure ergosterol monolayer. These results obtained with stigmasterol monolayers confirm the role played by the double bond between 22nd and 23rd carbons of the sterols. In fact our data are in agreement with a stronger interaction of mycosubtilin with cholesterol than with stigmasterol. This could be related with its more rigid monolayers restricting the accessibility of the lipid for the lipopeptide.

Furthermore, the strong antifungal activities of mycosubtilin had been planned to be applied in the biocontrol of plant diseases [33]. Thus, in conclusion, the present work could contribute for phytosanitary applications of the lipopeptide to fight against phytopathogenic fungi, as *B. cinerea* [34].

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