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The mycocidal, membrane-active complex of *Cryptococcus humicola* is a new type of cellobiose lipid with detergent features

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Abstract

The chemical composition of the mycocidal complex (formerly known as microcin) secreted by *Cryptococcus humicola* was investigated by chemical, mass spectrometric and nuclear magnetic resonance methods. The results indicate that the mycocidal complex is composed of glycolipids with a highly acetylated (up to five acetyl groups) cellobiose backbone [β-D-Glc*p*-(1′→4)-β-D-Glc*p*] linked to the ω-hydroxyl group of α,ω-dihydroxy palmitate [16:0-α,ω-di-OH] with an unsubstituted carboxyl group. The acyl chain forming aglycon can be replaced by [18:0-(α,ω-di-OH)], [18:0-(α,ω-1,ω-tri-OH)], and [18:0-(α,ω-2,ω-tri-OH)]. The complex has a comparatively high surface activity; 0.5 mg/ml of it reduced the surface tension of 0.1 M $NaHCO_3$ from 71 mN/m to 37 mN/m and interfacial tension against *n*-hexadecane from 39 mN/m to 10 mN/m. The critical micelle concentration of the complex at pH 4.0, determined by the fluorometric method with *N*-phenyl-1-naphthylamine as fluorescent probe and by the De Nouy ring method, was 2×10^{-5} M (taking the average molecular mass of the complex to be 750); it did not depend on the presence of 100 mM KCl and was an order of magnitude higher at pH 7.0. By fluorescence resonance energy transfer spectroscopy with *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine as energy donor and *N*-(rhodamine B sulfonyl)-phosphatidylethanolamine as energy acceptor the complex was shown to intercalate into the liposomal lipid matrix. Primary lesions caused by the complex in planar lipid bilayers were revealed as short-living current fluctuations of a broad spectrum of amplitudes. The mycocidal effect of the complex is suggested to be associated with its detergent-like properties.

Keywords: Mycocidal effect; Yeast; Cellobiose lipid; Detergent; Permeability; Lipid membrane; Mass spectrometry; Nuclear magnetic resonance spectroscopy; *Cryptococcus*

Abbreviations: MC, mycocidal complex; PS, phosphatidylserine; Glc, glucose; DOPS, dioleylphosphatidylserine; DPhPC, diphytanoylphosphatidylcholine; ES, ergosterol; SDS, sodium dodecyl sulfate; DTAB, dodecyltrimethylammonium bromide; NPN, *N*-phenyl-1-naphthylamine; CPB, citrate-phosphate buffer; CMC, critical micelle concentration; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine; Rh-PE, *N*-(rhodamine B sulfonyl)-phosphatidylethanolamine; TLC, thin layer chromatography; GLC, gas liquid chromatography; ESI-FT-ICR, electrospray ionization Fourier-transform ion cyclotron resonance; FRET, fluorescence resonance energy transfer; NMR, nuclear magnetic resonance; ^{1}H,^{1}H-COSY, ^{1}H,^{1}H-correlation spectroscopy; HMQC, heteronuclear multiple quantum coherence; HMBC, heteronuclear multiple bond correlation; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; ROESY, rotating frame Overhauser enhancement spectroscopy

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1. Introduction

Cryptococcus humicola, a basidiomycetous yeast, expresses mycocidal activity against a broad spectrum of both basidiomycetous and ascomycetous yeasts. Previously, this activity was assumed to be associated with the secretion of low molecular mass killer toxin, which was called 'microcin', by analogy with low molecular mass bacteriocins [1]. As the term 'microcin' was first introduced and is normally used to refer to low molecular mass bacteriocins [2], in the following we shall use the term 'mycocidal complex' (MC) to avoid confusion.

Isolated and thin layer chromatography (TLC)-purified MC exhibits mycocidal activity [3]. The MC was shown to cause impairment of the cytoplasmic membrane, inhibition of some enzymes, structural alterations in cell wall and/or capsule in a sensitive yeast, and lesions in planar lipid bilayers [3,4]. Disruption of the cytoplasmic membrane selective permeability barrier was suggested to be the primary event of the mycocidal effect [3].

The goal of this study was to elucidate the chemical composition of the MC, to characterize some of its physico-chemical properties, and to investigate its interaction with lipid bilayers. The MC composition was analyzed using chemical methods along with gas liquid chromatography–mass spectrometry (GLC-MS), electrospray ionization Fourier-transform mass spectrometry (ESI-FT-MS) and nuclear magnetic resonance (NMR) spectroscopy [5]. Surface activity of the MC was characterized by ring [6] and fluorometric [7] methods. The MC interaction with lipid bilayers was investigated on liposomes by the fluorescence resonance energy transfer (FRET) technique [8] and on planar lipid bilayers by electrical measurements [9].

2. Materials and methods

2.1. Chemicals

Dioleylphosphatidylserine (DOPS) and diphytanoylphosphatidylcholine (DPhPC) were from Avanti Polar Lipids (Alabaster, AL, USA). Phosphatidylserine (PS) (from bovine brain), 1-stearoyl-2-hydroxy-*sn*-glycero-lysophosphatidylcholine (lyso-PC), ergosterol (ES), asolectin, *N*-phenyl-1-naphthylamine (NPN), sodium dodecyl sulfate (SDS), dodecyltrimethylammonium bromide (DTAB), nonactin, and nystatin were from Sigma (St. Louis, MO, USA). *N*-(7-Nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine (NBD-PE) and *N*-(rhodamine B sulfonyl)-phosphatidylethanolamine (Rh-PE) were from Molecular Probes (Eugene, OR, USA).

2.2. Mycocidal complex preparation

The MC preparation and activity assay protocols were as described in [3]. Briefly, the MC producing strain *C. humicola* 9-6 was grown for 14 days. After separation from cells, the culture medium was lyophilized and suspended in methanol. The methanol extract was separated from the insoluble compounds by filtration. After evaporation, the remaining substance was washed out with deionized water, and the water-insoluble material was resolved in methanol. The methanol solution was applied to LH-Sephadex column and eluted by methanol. After separation, the fractions with mycocidal activity were subjected to chromatography on LH-Sephadex once again. The fractions with highest activity were used for further purification by TLC on Silufol plates (Kavalier, Votice, Czech Republic). Development was with solvent system containing chloroform:methanol:ammonium hydroxide (25%) (70:30:1.5, v:v). The band with $R_f = 0.8$ (see Fig. 1 in [3]), having maximal mycocidal activity, was extracted by methanol. Stock preparation of MC in methanol (6 mg/ml) was kept at −12°C. The minimal inhibitory concentration of the preparation (determined as described in [3]) was 5–10 μg/ml.

2.3. Sugar, fatty acid, and methylation analyses

For sugar analysis, samples of the MC (0.1 mg) were hydrolyzed with aqueous 2 M CF_3CO_2H (100°C, 4 h), reduced with $NaBH_4$ in water, peracetylated with acetanhydride in pyridine (1:1.5, v:v, 85°C, 20 min), and analyzed by GLC [10].

Fatty acid analysis was done with samples (0.1 mg) hydrolyzed by methanolysis (0.5 M HCl/MeOH) at 85°C for 45 min and GLC-MS. For methylation analysis samples (0.2 mg) were methylated with MeI in Me_2SO in the presence of solid NaOH [11].

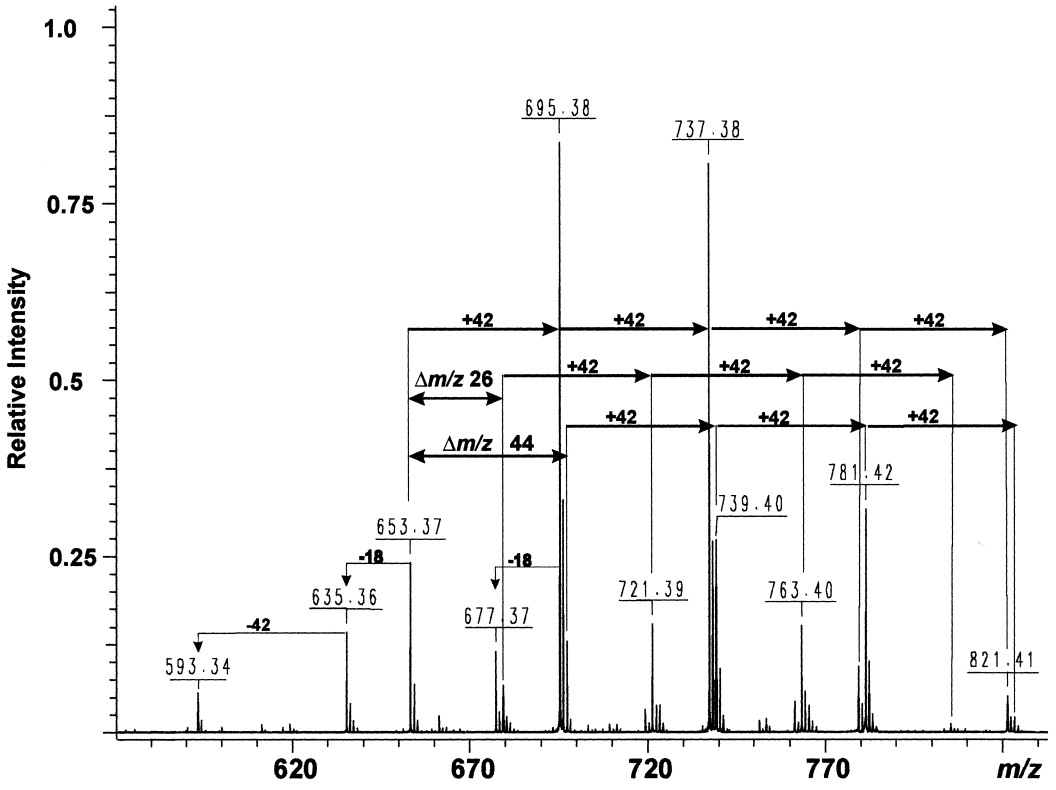

Fig. 1. Negative ion ESI-FT-ICR mass spectrum of the intact MC. Δ*m*/*z* 42 denotes heterogeneity due to an additional acetyl group, Δ*m*/*z* 26 and Δ*m*/*z* 44 replacement of 16:0-di-OH by 18:0(α,ω-di-OH) and 18:0(α,ω-1,ω-tri-OH) or 18:0(α,ω-2,ω-tri-OH), respectively.

Partially methylated sugars were derived by hydrolysis with 4 M CF_3CO_2H (100°C, 2 h), reduced with NaB_2H_4, peracetylated, and analyzed by GLC-MS [12].

2.4. GLC-MS and ESI-MS analysis

For GLC, a Varian 3700 chromatograph (Varian, Germany) equipped with a fused-silica gel SPB-5 column applying a temperature gradient from 150°C for 3 min to 320°C at a heating rate of 5°C/min was used. GLC-MS was performed on an HP 5989A instrument (Hewlett-Packard, Palo Alto, CA, USA) with an HP-5 column under the same chromatographic conditions as in GLC. The intact MC was mass analyzed by electrospray Fourier-transform ion cyclotron resonance mass spectrometry utilizing a Tesla APEX II instrument (Bruker-Daltonics, Germany) in the negative ion mode. The sample was solved in 2-propanol:water:30 mM ammonium acetate (50:50:0.1; v:v:v) and sprayed at a flow rate of 120 μl/h.

2.5. NMR spectroscopy

One-dimensional ^{1}H- and ^{13}C-NMR and two-dimensional (^{1}H,^{1}H-COSY) spectra were obtained with a Bruker DRX-600 AVANCE spectrometer (Bruker-Daltonies, Billerica, MA, USA) at 600 MHz, and ^{13}C-NMR spectra with a Bruker DPX-360 spectrometer at 90.6 MHz. The MC was dissolved in 500 μl methanol-d_4 (D 99.96%, Cambridge Isotope Laboratories, Andover, MA, USA) and transferred to 5 mm ultra high precision NMR sample tubes (Promochem, Wesel, Germany). ^{1}H- and ^{13}C-NMR spectra were recorded at 300 K and chemical shifts were referenced to internal tetramethylsilane (δ_H 0.0, δ_C 0.0). ^{1}H,^{1}H-COSY and heteronuclear correlated (HMQC, HMBC) experiments were performed using standard Bruker software (XWINNMR 2.6).

2.6. Surface activity measurements

Surface and interfacial tensions of the MC were

measured with a Ring Tensiometer K8551 (Krüss, Hamburg, Germany) by the De Nouy ring method [6]. The critical micelle concentration (CMC) of the complex is the concentration at which micelles start to form. It was determined by plotting the surface tension versus MC concentration. The CMC was revealed by the inflection of the curve at a point when surface tension did not change further with increasing MC concentration [6]. Measurements were conducted in citrate-phosphate buffer (CPB), pH 4.0, or in 0.1 M $NaHCO_3$ at 23°C.

2.7. CMC determination by fluorometry

Fluorometric CMC determination was conducted according to [7] utilizing the characteristic of the hydrophobic fluorescent probe NPN to partition into the hydrophobic core of molecular aggregates resulting in an increase of its fluorescence intensity. Various volumes of the MC stock solution in methanol (6 mg/ml) in the presence of NPN (10^{-6} M) were dried under a stream of nitrogen, and were then suspended in CPB of different salt concentrations and pH. The samples were sonicated with a Branson sonicator for 15 min and left to equilibrate for 24 h at 4°C. After 24 h of incubation, fluorescence was measured on a fluorescence spectrometer SPEX F1T11 (SPEX Instruments, Edison, NY, USA). Excitation/emission wavelengths were 360/432 nm. The CMC was determined as the concentration at which NPN fluorescence started to increase with increasing MC concentration.

2.8. FRET measurements

To investigate whether the MC can intercalate into the lipid bilayer, FRET spectroscopy was used as a fluorescent probe dilution assay, as described [8,13]. Briefly, two fluorescent probes, NBD-PE (energy donor) and Rh-PE (energy acceptor), were dissolved in chloroform together with a lipid mixture (DPhPC:PS:ES = 20:2:0.5, w:w:w) in a molar ratio of lipid mixture:NBD-PE:Rh-PE of 100:1:1. The solvent was evaporated under a stream of nitrogen, and the mixture was suspended in CPB, pH 4.0 and sonicated with a Branson sonicator for 1 min. Intercalation could be detected as changes in fluorescence intensities as a function of time (increase of donor signal, decrease of acceptor signal). In the following, the quotient of the donor and the acceptor intensities is denoted as the FRET signal.

Fluorescence was measured on a fluorescence spectrometer SPEX F1T11 (SPEX Instruments) in separate emission channels. The excitation wavelength was 470 nm, and the emission signals were recorded at 531 nm (NBD-PE) and 593 nm (Rh-PE). All measurements were carried out in 1 ml samples at 23°C. The fluorescence intensities of 900 μl of the lipid mixture were recorded for 50 s. Then, 100 μl of the MC was added to a final concentration of 0.1 mM and the fluorescence intensities were recorded for a further 250 s. As a positive control lyso-PC was added (final concentration 0.1 mM) instead of MC. The addition of 100 μl of CPB was used as a negative control.

2.9. Preparation of planar lipid bilayers and electrical measurements

Planar lipid bilayers were prepared according to the Montal–Mueller technique [9] as described before [14,15]. Briefly, phospholipid bilayers were formed by opposing two lipid monolayers prepared on aqueous subphases (bathing solutions) at a small aperture (typically 130 μm diameter) in a thin Teflon septum (12.5 μm thickness).

For the formation of the monolayers, lipids were dissolved in chloroform (2.5 mg/ml) and 6 μl of this solution was applied to each of the subphase surfaces. For electrical measurements, planar membranes were voltage-clamped and the compartment opposite (*trans*-compartment) to the side at which the MC was added (*cis*-compartment) was grounded. Therefore, a positive clamp voltage represents a membrane which is negative on the inner side as observed in the natural system. Current is defined as positive when cation flux is directed towards the grounded compartment.

All measurements were performed at 23°C with bathing solutions consisting of 100 mM KCl in CPB, pH 4.0 (unless specified otherwise). Prior to the addition of the MC, membrane stability was tested by applying clamp voltages of ±50 mV for 1 min each. Only membranes with a basic current of less than ±1 pA at clamp voltages of ±50 mV were used for the experiments. For acquisition of the

data, they were low-pass filtered at 1 kHz. The sampling frequency was 40 Hz.

3. Results

3.1. The mycocidal complex is a mixture of cellobiose lipids

3.1.1. Chemical, GLC-MS, and methylation analysis

Compositional analysis of the MC by GLC and GLC-MS revealed that it was composed of glucose (Glc) and α,ω-dihydroxy palmitic acid [16:0(α,ω-di-OH)] in a molar ratio of ~2:1. Besides [16:0(α,ω-di-OH)] three minor fatty acids could also be identified: α,ω-dihydroxy stearic acid [18:0(α,ω-di-OH)], α,ω-1,ω-trihydroxy stearic acid [18:0(α,ω-1,ω-tri-OH)], and α,ω-2,ω-trihydroxy stearic [18:0(α,ω-2,ω-tri-OH)] acid making up 15–20% of the total fatty acid, thus indicating heterogeneity in the acylation pattern.

Methylation analysis to determine the sugar linkage in the putative disaccharide revealed 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitiol (from terminal Glc) and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-glucitiol (from 4-substituted Glc) in an approximate ratio of 1:1. Therefore, the glycosyl part in the glycolipid was identified as an (1′→4)-interlinked pyranosidic Glc disaccharide.

3.1.2. ESI-MS analysis

These results were further corroborated by electrospray ionization Fourier-transform ion cyclotron resonance (ESI-FT-ICR) mass analysis. The negative ion broad band mass spectrum (Fig. 1) comprises three series of molecular ions $[M_n-H]^-$ resulting from increasing numbers of acetyl groups (Δ*m*/*z* 42).

In accordance with the results of the compositional analysis the most abundant series (*m*/*z* 653.37, 695.38, 737.39, 799.40, and 821.41) consists of a hexose disaccharide to which one dihydroxy palmitate (16:0-di-OH) with an open carboxyl group and up to five acetyl (OAc) groups are linked. The other two series result from the replacement of the fatty acid by either dihydroxy stearic acid [18:0(α,ω-di-OH)] (Δ*m*/*z* 26) or by trihydroxy stearic [18:0(α,ω-1,ω-tri-OH)] and [18:0(α,ω-2,ω-tri-OH)] (Δ*m*/*z* 44), respectively. Furthermore, the spectrum comprises ion peaks of minor intensity (Δ*m*/*z* 18) which are most probably raised due to lactone formation known frequently to occur in such glycolipids having a terminal carboxyl group [5].

3.1.3. NMR spectroscopy

^{1}H-NMR analysis of the MC revealed inter alia signals showing coupling constants for two anomeric Glc*p* protons [4.506 ppm, $J_{1,2}$ 8.2 Hz (H-1′) and 4.182 ppm, $J_{1,2}$ 7.9 Hz (H-1)] indicating β-linkage in both Glc-pyranosides. Other signals from 1D and 2D experiments (COSY, TOCSY, data not shown) revealed that it consisted of a highly *O*-acetylated cellobiose disaccharide unit [β-D-Glc*p*-(1′→4)-β-D-Glc*p*]. This finding was also in accordance with ^{13}C-NMR data where the two anomeric carbon signals [104.15 ppm (C-1) and 102.46 ppm (C-1′)] again indicated β-glycosidic linkage. In agreement with the ESI-MS data, the presence of up to five OAc groups [1.99–1.86 ppm (^{1}H) and 21.1–20.7 (^{13}C)] indicated a high degree of acetylation in the disaccharide glycolipid.

The β-glycosidic linkage of the cellobiose disaccharide to the terminal (ω-) hydroxyl group of the 16:0(α,ω-di-OH) was assigned from a HMBC experiment whereby two cross-connectivities could be identified for the anomeric carbon signal (C-1) of Glc with H-ωb and H-ωa (104.15/3.78 ppm) and H-ωb (104.15/3.51 ppm), respectively; a finding which was further corroborated from connectivities between H-1 and H-ωb and H-ωa in the NOESY and ROESY spectra, respectively (data not shown).

In conclusion, the MC produced and secreted by the yeast *C. humicola* was found to be a highly acetylated cellobiose lipid. The chemical structure is depicted in Fig. 2.

3.2. Surface activity of the complex

All known glycolipids are surface-active compounds, i.e. they reduce surface tension of water solutions (for review see [16] and references therein). Cellobiose lipids of the MC have rather high surface activity, too. Table 1 shows that, at the same experimental conditions, surface and interfacial tension reduction caused by cellobiose lipids was higher than that caused by DTAB and comparable to that caused by SDS.

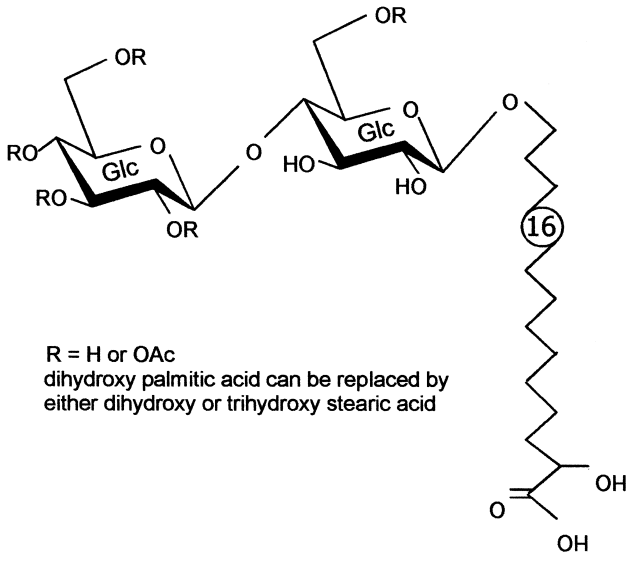

Fig. 2. Chemical structure of the glycolipids comprising the MC.

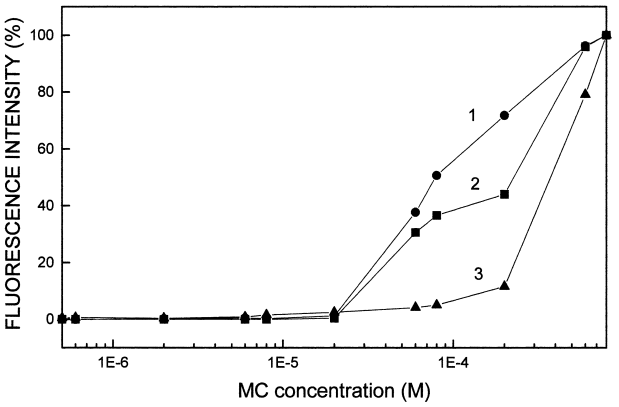

Fig. 3. Relative NPN fluorescence as a function of the MC concentration in (1) CPB, pH 4.0; (2) CPB+100 mM KCl, pH 4.0; (3) CPB, pH 7.0. Sample preparation and NPN fluorescence measurements were as described in Section 2. For convenience, the MC concentration is expressed in M, taking an average molecular mass of the complex of 750 (see Section 3).

A further feature of surface-active glycolipids is their aggregation in aqueous environments [16]. This feature is characterized by a minimal concentration of the compound, called CMC, at which aggregation starts. The CMC of the MC in CPB at pH 4.0 as determined by the fluorometric method (Fig. 3, curve 1) and by the De Nouy ring method (data not shown) was 2×10^{-5} M. The CMC did not depend on the presence of 100 mM KCl (Fig. 3, curve 2) and was markedly higher at pH 7.0 (Fig. 3, curve 3).

3.3. Interaction of the complex with liposomes

Surface-active compounds like lysolipids are known to intercalate into the lipid matrix of liposomes. Therefore, we have compared the capability of the MC and lyso-PC to intercalate into liposomes made from a lipid mixture (DPhPC:PS:ES = 20:2:0.5, w:w) using FRET spectroscopy (Fig. 4). We observed a significant increase in the FRET signal with respect to the control (100 μl CPB) after addition of 0.1 mM MC or lyso-PC. The FRET signals for the MC and lyso-PC did not differ significantly. These data indicated intercalation of the MC into lipid matrix of the liposomes.

Table 1
Surface activity of the MC in comparison to known detergents

Sample	Surface tension (mN/m)	Interfacial tension (mN/m)
0.1 M $NaHCO_3$	71	39
MC	37	10
SDS	32	6
DTAB	51	21

Surface and interfacial tensions were measured by the ring method as described in Section 2. 0.5 mg/ml solutions of the compounds in 0.1 M Na HCO_3 were used. Interfacial tension was measured against *n*-hexadecane.

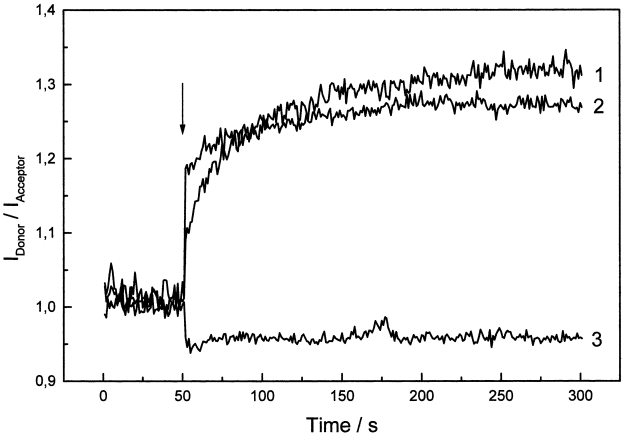

Fig. 4. Changes of the FRET signal vs. time after addition (arrow) of either 100 μM lyso-PC (1), 100 μM MC (taking an average molecular mass of 750, see Section 3) (2) or CPB (3) to 10 μM suspensions of liposomes (DPhPC:PS:ES = 20:2:0.5, w:w:w) double-labeled with NBD-PE and RH-PE. Buffer: citrate-phosphate at pH 4 and 23°C.

3.4. Effects of the complex on planar lipid bilayers

Effects of the MC upon the permeability of planar lipid bilayers of various composition were investigated. It was found that membranes formed of DPhPC, DOPS, asolectin, and DPhPC:ES mixture (4:1, w:w) were very vulnerable to the treatment and ruptured within 0.5 min after addition of the complex to the subphase at a concentration of 0.001 mM (at least 10 membranes of each composition were examined) under otherwise identical experimental conditions. This effect was observed at different potentials applied (0, ±20, 50 mV).

A lipid composition (DPhPC:PS:ES = 20:2:0.5, w:w:w) was used, which resembled that of the yeast cytoplasmic membrane to some extent [17,18] and which yielded artificial membranes resisting MC concentrations in the subphase up to 5×10^{-6} M for some minutes before rupturing. Using membranes of this composition, primary effects of the MC on bilayer permeability could be observed (Fig. 5B). As can be seen from Fig. 5B, the MC induced in lipid bilayers current fluctuations of various amplitudes and durations.

The effect of the MC was compared to the effects of nonactin (K^+ carrier [19]), nystatin (channel former [20]), and SDS (an anion detergent) under the same experimental conditions. Nonactin, at a concentration of 6×10^{-9} M, caused a membrane current increase from 1 to 35 pA, and there were no further current changes after doubling the concentration of the drug (data not shown). Membrane treatment by nystatin (10^{-5} M) resulted in current fluctuations (Fig. 5C). These fluctuations differed significantly from those caused by the MC. Amplitudes of the nystatin-induced current fluctuations were multiples of 0.4 pA, and each current state was markedly longer than those induced by the MC. Primary effects of SDS were observed at a concentration of 2×10^{-5} M and were similar to those of the MC, i.e. mostly short-living current fluctuations of a broad spectrum of amplitudes appeared (data not shown).

4. Discussion

Many microorganisms have been shown to secrete diverse types of glycolipids (for review see [16,21] and references therein). The only known secreted glycolipids with cellobiose as a sugar moiety are ustilagic acids A and B. They are produced by the smut fungus *Ustilago zeae* (= *U. maydis*) [22] as a mixture of glycolipids in which β-cellobiose (acylated with acetic, hexanoic, 3-hydroxyhexanoic or 3-hydroxyoctanoic acid) is esterified with hydroxy acids, ustilic acid A (15D,16-dihydroxyhexadecanoic acid) or ustilic acid B (2D,15D,16-trihydroxyhexadecanoic acid) as aglycon [23–25].

One more example of cellobiose lipids was demonstrated to be produced by resting cells of *Arthrobacter* sp., but, strictly speaking, secretion of the compounds was not investigated [26]. In these glycolipids, known as corynomycolates, the cellobiose moiety is mono- or diesterified with high molecular mass (C_{32}–C_{46}) β-hydroxy fatty acids (the corynomycolic acids).

Cellobiose lipids of the MC secreted by *C. humicola* (Fig. 2) significantly differ from both known glycolipids, derivatives of cellobiose. The peculiarities of *C. humicola* cellobiose lipids are their high degree of acetylation (up to five acetyl groups) and the β-glycosidic linkage of the cellobiose disaccharide to the terminal (ω-) hydroxyl group of one of the hydroxy fatty acids, [16:0-α,ω-di-OH], [18:0-(α,ω-di-OH)], [18:0-(α,ω-1,ω-tri-OH)], and [18:0-(α,ω-2,ω-tri-OH)], with open carboxyl group.

Cellobiose lipids secreted by *C. humicola* have mycocidal activity against a broad spectrum of both basidiomycetous and ascomycetous yeasts [1]. Ustilagic acids were shown to have antibiotic activity against some bacteria and fungi [27]. Unfortunately, at present, activities of these two glycolipids cannot be compared, since different microorganisms and assay systems were used in these two studies. We can compare activities of the MC and the well known fungicidal drug, nystatin. Minimal inhibitory concentrations for the MC and nystatin assessed at the same experimental conditions [3] were 5–10 μg/ml and 1–5 μg/ml, respectively. These data indicate a relatively high mycocidal activity of the *C. humicola* cellobiose lipids.

For a better understanding of biological functions and potential applications, surface activity of microbial glycolipids has been characterized [16,21]. Surface activity parameters of the *C. humicola* cellobiose lipids, surface and interfacial tensions (Table 1), and

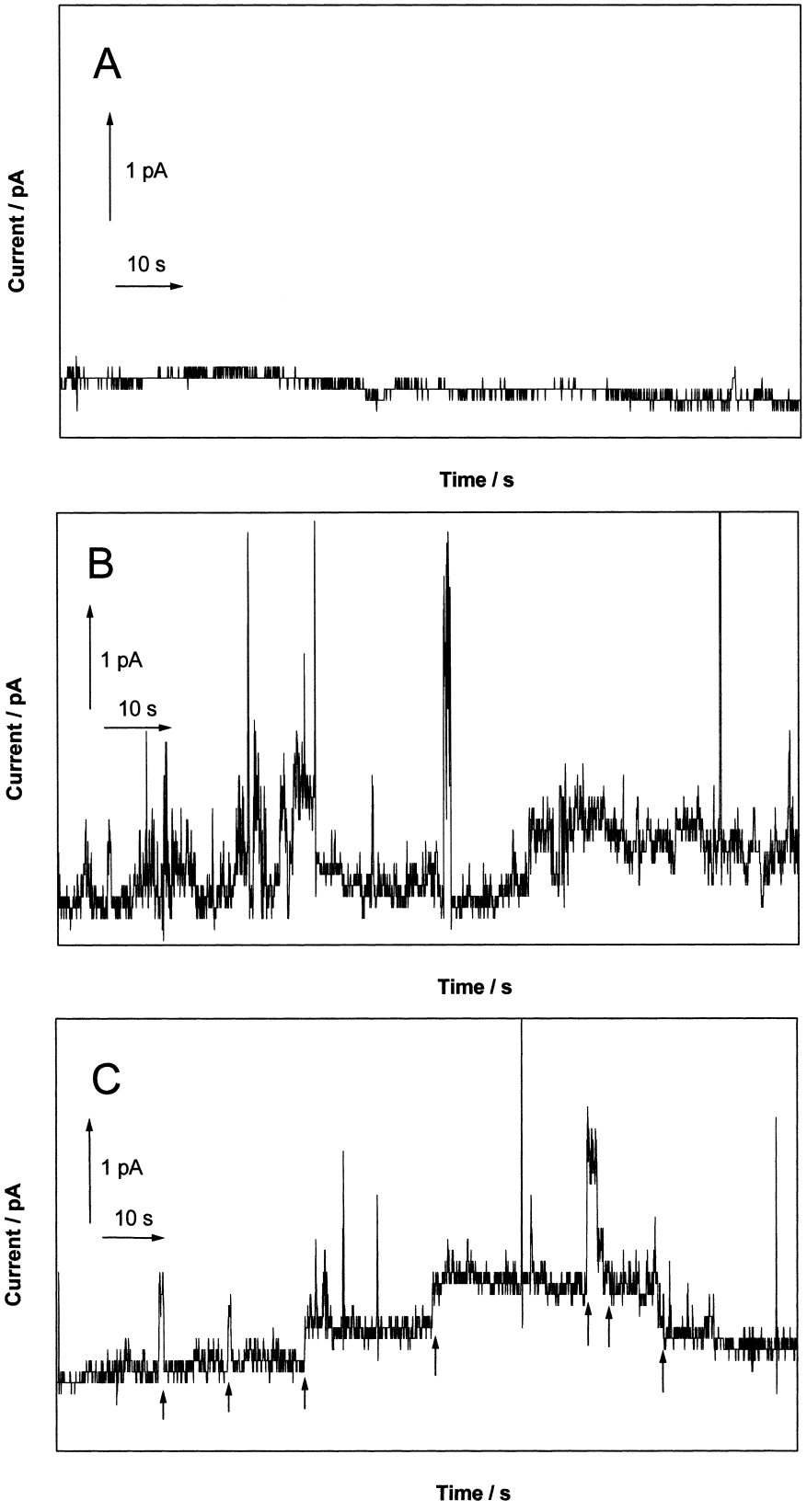
A
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Fig. 5. Time courses of membrane current before treatment (A) and after the addition of either the MC (B) or nystatin (C). Membranes were formed, and electric measurements were performed as described in Section 2. The lipid composition of the membranes was DPhPC:PS:ES = 20:2:0.5, w:w:w. The clamp voltage was 50 mV. Bathing solution: 100 mM KCl, CPB, pH 4, temperature 23°C. MC and nystatin concentrations were 8×10^{-7} M (on the basis of an average molecular mass of 750, see Section 3) and 10^{-5} M, respectively. Arrows in B indicate current increments, which are multiples of 0.4 pA. In each case, one typical result is presented. ←

the CMC (Fig. 3) are comparable not only to some known detergents (Table 1), but also to many microbial surfactants [16,21]. It is worth noting here that the CMC of the MC is markedly higher at pH 7 than at pH 4 (Fig. 3). This may be due to some changes in glycolipid structure and may be somehow related to the fact that the MC has no mycocidal activity at pH > 6 [1].

In a previous publication [3], evidence was presented that unspecific permeability increase of the cytoplasmic membrane in a sensitive yeast is a primary event of the MC mycocidal effect. The data presented here provide some insight into the mechanism of this permeabilization. FRET experiments on liposomes (Fig. 4) indicate that cellobiose lipids of the MC can intercalate into the lipid matrix. This intercalation results in permeabilization of the lipid bilayer that strongly differs from the effects of the ion carrier nonactin or the channel former nystatin (Fig. 5). We hypothesize that permeability changes caused by the MC are probably due to the disturbances in the bilayer structure, which, at small intramembrane MC concentrations (at the initial steps of interaction), appear as short-living channels of various size. With increasing MC concentration gross permeability increase occurs [3].

There are two main natural roles that were suggested for surface-active compounds (including glycolipids) secreted by microorganisms, increasing availability of hydrophobic substrates and regulating attachment–detachment to and from surfaces [16]. High mycocidal activity of the MC secreted by *C. humicola* makes it possible to suggest one more role for the producers of this type of compounds. This may be a defense (competition) strategy in natural habitats. To date, the only defense strategy based on secretion of compounds which are toxic for other species or strains in yeasts was associated with killer toxin production [28].

Potential applications of the compounds with high surface activity (including glycolipids) produced by microorganisms are mainly associated with their emulsifying ability. Their application is considered in the oil and petroleum industries, cleaning and pharmaceutical industries, food production, bioremediation, cosmetics, etc. [16]. *C. humicola* glycolipids have one more important feature, high mycocidal activity. This makes them candidates for some medical and/or veterinary applications.

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