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Biochimica et Biophysica Acta 1512 (2001) 239–250

BIOCHIMICA ET BIOPHYSICA ACTA
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Cytoplasmic membrane of a sensitive yeast is a primary target for *Cryptococcus humicola* mycocidal compound (microcin)[1]

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Received 30 October 2000; received in revised form 30 March 2001; accepted 2 April 2001

Abstract

A basidiomycetous yeast strain, *Cryptococcus humicola* 9-6, secretes a mycocidal compound (microcin) which is lethal for many yeasts. In this study a new protocol for microcin purification has been developed, and TLC-purity product was obtained. Using fluorescein as a pH-sensitive probe it was found that microcin treatment of *Cryptococcus terreus*, a model microcin-sensitive yeast, immediately caused transient alkalization followed by acidification of the cells' cytoplasm. Upon completion of this process, endogenous respiration as well as activity of unspecific esterases were inhibited, and alterations in cell wall and/or capsule started. Microcin was shown to make the cells leaky for intracellular ATP. The mycocidal effect of microcin did not depend on the cell cycle phase of *Cr. terreus*. Based on these observations and on electrical measurements on planar phospholipid bilayers, which indicated a microcin-induced membrane permeabilization, it is suggested that the cytoplasmic membrane of the sensitive yeast is a primary target of microcin action. The conjectured mode of microcin action involves gradual increase of the cytoplasmic membrane's unspecific permeability. Intracellular ion homeostasis changes induced by microcin are considered to be the main cause of enzyme inhibition, alterations in the outer layers of the cell envelope and, finally, division arrest.

Keywords: Mycocidal compound; Cytoplasmic membrane; Permeability; Fluorescence; Planar lipid bilayer; Intracellular pH; *Cryptococcus*

1. Introduction

A basidiomycetous yeast strain, *Cryptococcus humicola* 9-6, was shown to produce a compound which is inhibitory for growth of many yeasts, while the producer is immune to it [1]. Since the main phenomenology resembled the so-called killer phenomenon in yeasts (for review see [2,3]), the compound was considered as killer toxin, yet it noticeably differed from other known yeast killer toxins at least in two ways. Firstly, it had a relatively low molecular mass of about 1 kDa, therefore it was called microcin [1] (we shall refer to this compound

Abbreviations: FDA, fluorescein diacetate; 4-MUA, 4-methylumbelliferyl acetate; 4-MU, 4-methylumbelliferon; FCCP, carbonyl cyanide *p*-(triflouromethoxy)- phenylhydrazone; MIC, minimal inhibitory concentration; TLC, thin-layer chromatography; PS, phosphatidylserine; CPB, citrate–phosphate buffer, pH 4.1

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[1] Part of this work was presented and published in Abstracts of the International Symposium 'Modern Problems of Microbial Biochemistry and Biotechnology', Pushchino, Russia, June 25–30, 2000.

0005-2736/01/$ – see front matter
PII: S0005-2736(01)00325-X

using the same term). Secondly, it exhibited mycocidal activity against a broad spectrum of both ascomycetous and basidiomycetous yeasts, including some human pathogens [1,4].

The mode of the mycocidal effect of microcin upon sensitive yeasts is not known as yet. In our previous studies [4], using the fluorescent dye exclusion methodology, we have demonstrated that microcin rendered the capsule and/or cell wall of a sensitive yeast *Cryptococcus terreus* permeable for some fluorochromes and cetyltrimethylammonium bromide, a cationic detergent. Also, the cells lost intracellular K^+. Although capsule and/or cell wall damage correlated fairly well with cell viability, it was not clear whether this damage was the primary or a secondary effect of microcin action. In other words, the question of the primary target of microcin action is still open.

The present study was undertaken to reveal whether the cytoplasmic membrane of the sensitive yeast might be a primary target for microcin. The work was carried out primarily on a strain of *Cr. terreus* as a model sensitive yeast. Additionally, electrical measurements on artificial planar phospholipid bilayers were performed. In contrast to our previous studies with a crude microcin preparation [4], in this investigation a new microcin purification protocol has been developed and a TLC-purity preparation was used.

The main methodology of this investigation was based on the application of profluorochromes. According to this methodology, hydrophobic nonfluorescent profluorochromes can permeate into the cells, where they are hydrolyzed by unspecific esterases resulting in formation of hydrophilic fluorescent products [5]. In our studies, fluorescein diacetate (FDA) and 4-methylumbelliferyl acetate (4-MUA) were used as profluorochromes giving fluorescent products fluorescein and 4-methylumbelliferon (4-MU), respectively. Using these profluorochromes, the following information can be obtained. (i) The barrier function of the cytoplasmic membrane can be assessed by microscopic or flow cytometric examination of the cells' stainability (the so-called 'dye retention methodology'), since it has been suggested and repeatedly reported for many cell types [5–10] that the cytoplasmic membrane may be impermeable or hardly permeable for fluorescein. (ii) Intracellular pH can be evaluated because fluorescence of both fluorescein [11] and 4-MU [12] is pH dependent. Application of these fluorochromes for intracellular pH evaluation has been demonstrated in yeasts [13] and in mammalian cells [14]. (iii) Unspecific esterase activity of cells can also be determined with the use of these profluorochromes [15]. All these opportunities of the profluorochrome methodology have not been tested on *Cr. terreus* previously.

Voltage-clamp techniques on planar phospholipid bilayers provide information on the possibility of microcin interaction with phospholipid bilayer and the formation of microcin-induced lesions in phospholipid bilayer membranes from an increase in membrane current upon addition of the compound.

2. Materials and methods

2.1. Strains and growth conditions

The microcin-sensitive test strain of *Cryptococcus terreus* VKM Y-2253 was from All-Russian Collection of Microorganisms (VKM). The microcin-producing strain *Cryptococcus humicola* 9-6 was kindly provided by Dr. W.I. Golubev [1]. Strains were maintained on malt agar slants at 4°C.

Cr. terreus was grown in liquid medium containing glucose, 1%; peptone, 0.5%; yeast extract, 0.5%; citric acid, 0.68%; Na_2HPO_4, 1.27%; pH 4.0. The starter culture was obtained by inoculating 50 ml of the growth medium with the culture from slants and incubating at 24°C with shaking at 150 rpm for 48 h. Fresh medium (50 ml) was inoculated with 2 ml of the starter culture and incubated under the same conditions. After 24 h the culture reached logarithmic phase of growth. For fluorometric and microscopic experiments as well as for ATP measurements, cells were washed twice and resuspended to approximately 6×10^7 cells/ml in a citrate-phosphate buffer (citric acid, 32 mM and Na_2HPO_4, 36 mM; pH 4.1) (CPB) supplemented with 0.02 mg/ml cycloheximide. Before and during experiments the suspension of cells was kept at room temperature (20–22°C), but not more than for 3 h. For the determination of microcin minimal inhibitory concentration (see below), cells were used without washing in buffer.

2.2. Preparation of microcin

The microcin producing strain *Cr. humicola* 9-6 was grown as described [1]. After 14 days of growth, cells were removed by centrifugation (5000×*g*, 30 min), and the supernatant was filtered through glass microfiber filter GF/A (Whatman, UK). The filtrate 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, after sedimentation by centrifugation, the water-insoluble material was resolved in methanol. The methanol solution was applied to a LH-Sephadex column (40×2 cm) and eluted with methanol (flow rate 20 ml/h). Fractions of 3 ml were collected and assayed for mycocidal activity (see below). Active fractions (approximately 3 ml) were pooled, concentrated by methanol evaporation and subjected to chromatography on LH–Sephadex once again. The fractions with highest activity were pooled, concentrated by methanol evaporation and used for further purification by thin layer chromatography (TLC) on Silufol plates (Kavalier, Czech Republic). Initially, analytical TLC was performed. Development was with solvent system containing chlorophorm/methanol/ammonium hydroxide (25%) (70:30:1.5, v/v/v). The plates were stained in iodine vapor and the main iodine-stainable components of the preparation were revealed (Fig. 1). Then 0.1-ml samples were applied to plates as bands (10×0.3 cm) for preparative TLC. The bands with R_f corresponding to the main iodine-stainable components on the analytical plates were scraped off, extracted by methanol and assayed for mycocidal activity (see below). The mycocidal activity was associated with the fraction F3 with $R_f = 0.8$ (Fig. 1). This fraction was used as a final product (we shall refer further to it as microcin for short). Stock microcin preparations in methanol (6–30 mg/ml) were kept at −12°C. The minimal inhibitory concentration (MIC) of microcin (determined as described below) of different lots was 0.005–0.015 mg/ml.

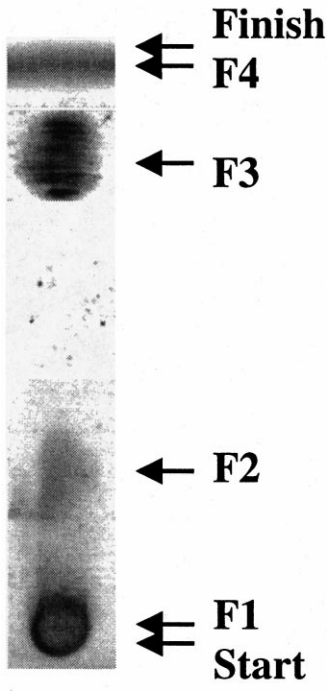

Fig. 1. Analytical thin-layer chromatogram of the microcin preparation after LH–Sephadex column chromatography. Development was with solvent system containing chlorophorm/methanol/ammonium hydroxide (25%) (70:30:1.5; v/v/v). Staining was made in iodine vapor chamber. Arrows indicate iodine-stainable components of the preparation.

2.3. Microcin assay

Microcin activity was estimated using two methods.

2.3.1. Method 1

Sterile 5-mm diameter glass microfiber filter discs GF/C (Whatman, UK) were placed onto the surface of the malt agar in Petri dishes that had been previously inoculated with *Cr. terreus*. 0.005–0.015 ml samples were pipetted onto the discs. The diameter of the growth inhibition zone was measured after 24 h of incubation at 24°C. This method was used for microcin activity assessment in the samples obtained after chromatography on LH-Sephadex and TLC-purification.

2.3.2. Method 2

The MIC of the TLC-purity microcin preparation was estimated as follows. Logarithmic phase cells of *Cr. terreus* were suspended in CPB to 1.5×10^5 cells/ml. Various concentrations of microcin were added to the cells and, upon incubation for 1.5 h at 24°C, cell viability was determined by the slide culture

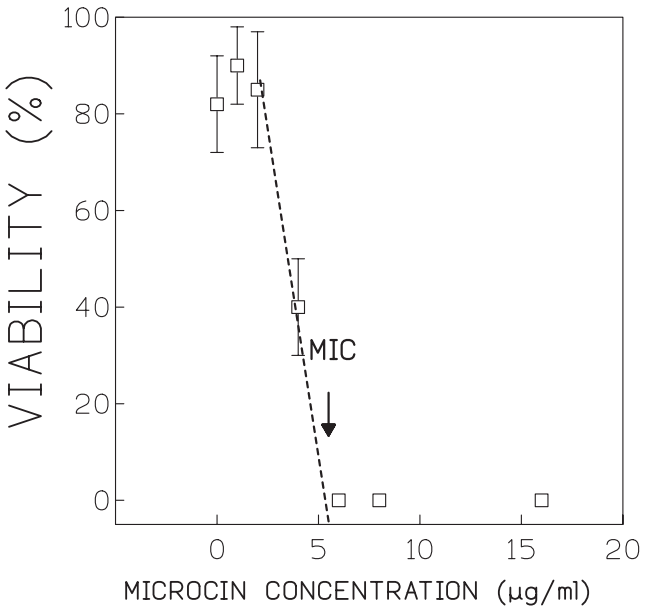

Fig. 2. Determination of the microcin minimal inhibitory concentration (MIC) by slide culture method. Cells of *Cr. terreus* were treated by microcin of varying concentrations in CPB and then viability was determined as described in Section 2 and in [4]. Experimental data in the range of a sharp onset of growth inhibition were approximated by a straight line (dotted) and intercept of this line with *x*-axis gave MIC value (indicated by arrow). Typical results of three experiments with a given lot of microcin preparation are presented.

method as described [4], except that outgrowth of the cells was carried out at 18°C for 28 h. The minimal microcin concentration causing 100% killing effect was determined as it is shown in Fig. 2. This concentration was taken as MIC.

2.4. Measurement of ATP levels

Cr. terreus cells in CPB (6×10^7 cells/ml) were treated by either microcin (0.03 mg/ml) or FCCP (10^{-6} M). At various times, 0.2-ml samples were taken. For intracellular ATP determination, the samples were mixed with 5 ml ice-cold CPB and centrifuged ($5000\times g$, 10 min). The supernatant was removed, the cells were suspended in 0.2 ml of dimethyl sulfoxide and used for ATP determination. For extracellular ATP determination, 0.2-ml samples of the cells were used directly without dimethyl sulfoxide treatment. All samples were diluted with 2 ml of a buffer containing Tris–HCl, 25 mM; $MgSO_4$, 5 mM; EDTA, 0.5 mM; dithiothreitol, 0.5 mM; and albumin, 0.01%; pH 7.8; and after addition of luciferin–luciferase assay, luminescence was measured on a Luminometer 1250 (LKB, Sweden). Calibration was made with standard ATP solutions both in the presence and absence of dimethyl sulfoxide.

2.5. Fluorometry

Fluorescence was measured on a Hitachi-850 spectrofluorometer (Hitachi, Japan) as described [16]. Excitation/emission wavelengths were: 490 nm/520 nm for fluorescein (unless specified otherwise); 360 nm/450 nm for 4-methylumbelliferon; and 500 nm/600 nm for ethidium bromide. All measurements were conducted in 1-ml samples at 24°C. Stock solutions of FDA, 4-MUA and ethidium bromide (2 mM) were prepared in ethanol, dimethyl sulfoxide and water, respectively. In most experiments, the assay mixture contained 3×10^6 cells/ml of *Cr. terreus* in CPB. The final concentration of each dye was 0.02 mM. To avoid sedimentation of the cells, the samples were gently stirred periodically.

2.6. Microscopy

Microscopic observations both in transmission and in fluorescence mode were performed with a LUMAM I2 fluorescence microscope (LOMO, Russia) as described [17] at a magnification of 220×. For fluorescence microscopy, a CC15 blue filter (transmittance maximum at 400 nm, 110 nm half-width) was installed in the excitation path for fluorescein and for 4-methylumbelliferon a UFS6 black filter (transmittance maximum at 360 nm, 60 nm half-width). In the fluorescence path, a GS3 yellow edge filter (transmittance after 450 nm) for both dyes was installed.

2.7. Oxygen and yeast respiration measurements

Oxygen content and yeast respiration were determined as described [16] with a Clark-type electrode and a polarograph PA-2 (Laboratorni Pristroje, Czech Republic) in a 0.7-ml thermostated cell at 20–23°C. Measurements were conducted with the cell suspensions containing approximately 2×10^7 cells/ml in CPB.

2.8. Preparation of planar bilayers and electrical measurements

Planar bilayers were prepared according to the Montal–Mueller technique [29] as described [19,20]. Briefly, phospholipid bilayers were formed by opposing two lipid monolayers prepared on aqueous subphases (bathing solutions) from chloroformic solutions of the lipids at a small aperture (typically 150 μm diameter) in a thin Teflon septum (12.5 μm thickness).

For the formation of bilayer membranes, phosphatidylserine (PS) was dissolved in chloroform (2.5 mg/ml). For electrical measurements, planar membranes were voltage-clamped and the compartment opposite (*trans*-compartment) to the side at which microcin 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 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, 32 mM citric acid, and 36 mM Na_2HPO_4, pH 4.0. Prior to the addition of microcin, membrane stability was tested 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 500 Hz. The sampling frequency was 1500 Hz.

2.9. Chemicals

Fluorescein diacetate, disodium fluorescein, 4-methylumbelliferyl acetate, carbonyl cyanide *p*-(triflouromethoxy)-phenylhydrazone, ethidium bromide and luciferin–luciferase were from Serva Finebiochemica, Heidelberg, Germany; citric acid, ATP, nystatin, bovine serum albumin and cycloheximide were from Sigma, St. Louis, MO, USA; peptone was from Difco Laboratories, Detroit, MI, USA; yeast extract was from Hispanlab, Spain; lipase from wheat germ was from Calbiochem, UK. PS from bovine brain was from Sigma (Deisenhofen, Germany) and was used without further purification. All other reagents were of the highest purity available.

3. Results

3.1. Effects of microcin upon stainability by FDA: microscopic observations

Intact cells of *Cr. terreus* were readily stainable by 0.02 mM FDA. They became yellow-green fluorescent within 2 min after adding the profluorochrome. Upon microcin addition (0.03 mg/ml) to the FDA-stained cells, sharp increase of the cells' glowing lasting for about 5 min was observed. Afterwards there was gradual decrease of fluorescence, and eventually there was complete quenching of the cells fluorescence. If the cells were pretreated by microcin for 5 min or more, they lost the FDA stainability. To explain these observations, FDA hydrolysis by the cells of *Cr. terreus* and fluorescein formation in relation to stainability were studied in more detail by fluorometry.

3.2. Intact cells are leaky for and stainable by fluorescein

Upon FDA addition to a suspension of intact *Cr. terreus* cells, fluorescein formation as a result of FDA hydrolysis was detected fluorometrically (Fig. 3A, curve 1). Curve 1 in Fig. 3A reflects the sum of fluorescence intensities of fluorescein molecules appearing both in the cells (Fig. 3A, curve 2) and in the milieu (Fig. 3A, curve 3). If the cells were preincubated with FDA, sedimented by centrifugation and resuspended in fresh buffer void of FDA, their fluorescence rapidly decreased (Fig. 3B). The remaining fluorescence was shown to be associated with the milieu, and microscopic examination revealed no fluorescing cells.

It should be noted here that fluorescence of fluorescein is pH-dependent mainly due to pH-dependence of the absorption spectrum [11]. This dependence is characterized by the blue shift, isosbestic (pH-independent) point at 435 nm and decreasing absorptivity at 490 nm with decreasing pH [11]. Hence, at an excitation wavelength of 490 nm, the fluorescence increases with the pH value. Intracellular pH in yeast cells is near 6 [13,18], whereas external pH in our system is 4.1.

Taking all this into account, the whole set of data presented in Fig. 3 could best be explained assuming

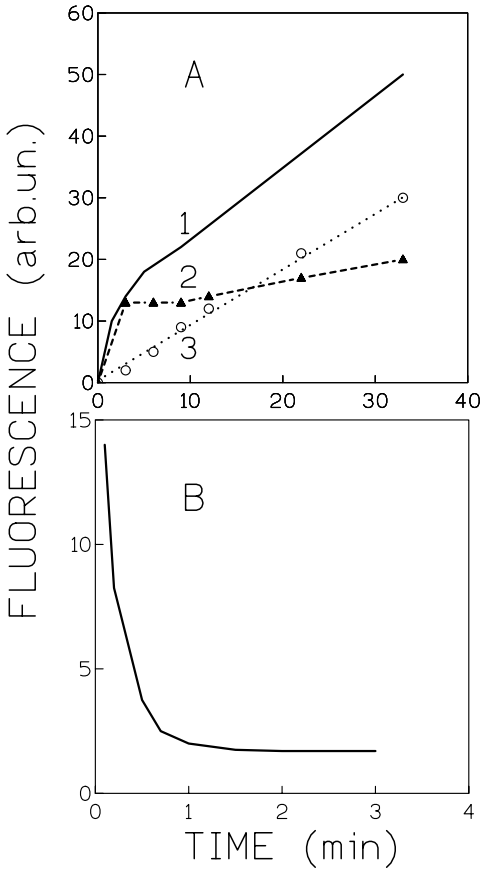

Fig. 3. Fluorometric characterization of the fluorescein formation, intracellular accumulation (A) and outflux (B) as a result of FDA hydrolysis in the cells of *Cr. terreus*. (A) At 0 time 2×10^{-5} M FDA was added to cell suspension in CPB (3×10^{6} cells/ml) and total fluorescein fluorescence was recorded (trace 1) as described in Section 2. At various times, from a parallel sample of the same composition 1.5-ml aliquots were taken, spun down ($6000\times g$, 2 min) and fluorescein fluorescence was measured in supernatants (trace 3). Difference in fluorescence intensities reflected by curves 1 and 3 gave fluorescence associated with the cells (trace 2). (B) The cells were incubated with FDA for 3 min as described above and sedimented ($6000\times g$, 2 min). Supernatant was discarded, the cell pellet was rapidly (no more than for 5 s), resuspended in buffer void of FDA and the fluorescence intensity time course was recorded. The traces in A are offset for the purpose of display. Typical results of two experiments are presented.

that fluorescein is formed inside the cells and then leaks out into the medium. Stainability of the cells, i.e., the cells appear as fluorescing, in microscopic examination, is a consequence of intracellular and extracellular pH difference (more alkaline inside) and steady state of intracellular fluorescein formation and leakage.

If the cytoplasmic membrane of *Cr. terreus* cells was permeable for fluorescein, it might be expected that the cells should be stainable by fluorescein without FDA due to intracellular and extracellular pH difference. Indeed, it was found by microscopy that intact cells of *Cr. terreus* were stainable by 0.02 mM fluorescein.

3.3. Microcin causes intracellular pH changes and inhibition of FDA hydrolysis

Treatment by microcin of the cells hydrolyzing FDA immediately resulted in a splash of fluorescence

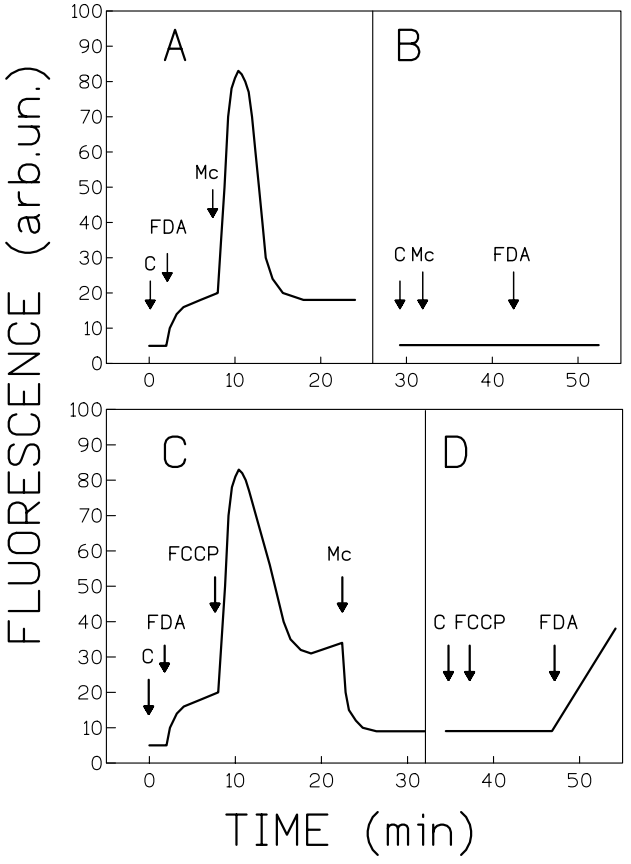

Fig. 4. Effects of microcin (A,B) and a protonophore FCCP (C,D) upon fluorescence dynamics in the course of the FDA hydrolysis by *Cr. terreus* cells. Arrows indicate the moments of the following additions to the fluorescence cuvette containing CPB: C (3×10^{6} cells/ml); FDA, fluorescein diacetate (2×10^{-5} M); Mc, microcin (0.03 mg/ml); FCCP, carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone (10^{-6} M). Measurements were conducted as described in Section 2. Typical results of 12 experiments with three different lots of microcin preparations are presented.

in the system. Then fluorescence decreased reaching the level below that before treatment and did not change further (Fig. 4A). In a cell-free supernatant of the cells hydrolyzing FDA, there were no changes of fluorescence upon microcin addition (data not shown). Microcin-pretreated cells lost the ability to hydrolyze FDA (Fig. 4B). However, at concentrations of up to 0.06 mg/ml, microcin did not affect FDA-hydrolyzing activity of lipase from wheat germ (data not shown).

The amplitude of the fluorescence splash did not depend on microcin concentration, but duration of the fluorescence splash was a function of the microcin concentration. At microcin concentrations of 0.015, 0.03 and 0.06 mg/ml, the time between microcin addition and apparent FDA hydrolysis cessation was 30, 12 and 7 min, respectively.

Similar, but not identical, fluorescent response was found upon treatment by a protonophore FCCP (Fig. 4C). One noticeable difference was that after FCCP treatment the cells did not lose the ability to hydrolyze FDA (Fig. 4D). It was observed also that

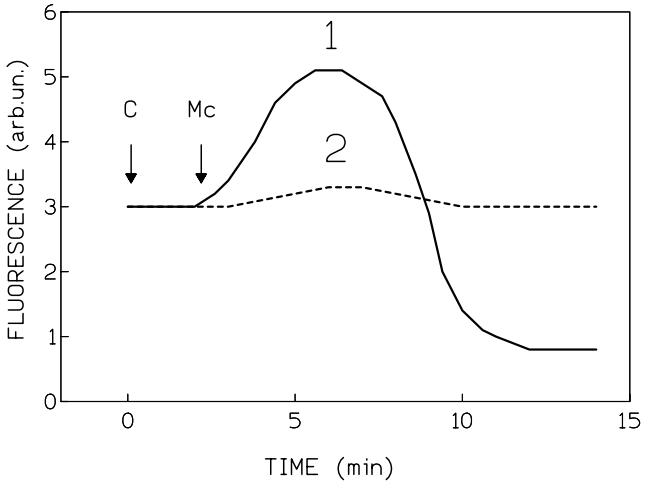

Fig. 5. Effects of microcin upon fluorescence of the cells of *Cr. terreus* loaded by sodium fluorescein. Cells at a concentration of 6×10^7 cells/ml twice washed in CPB, supplemented with 2×10^{-6} M disodium fluorescein and then resuspended at the same concentration in the same solution. Upon incubation for 15 min a sample of this suspension was pipetted into the fluorescence cuvette containing the buffered fluorescein solution (arrow C, final cell concentration 3×10^6 cells/ml). Afterwards microcin (0.03 mg/ml) was added at a moment indicated by arrow (Mc). Trace 1, excitation/emission wavelengths 490/520 nm; trace 2, excitation/emission wavelengths 435/520 nm. The traces 1 and 2 are offset for the purpose of display. Typical results of three experiments are presented.

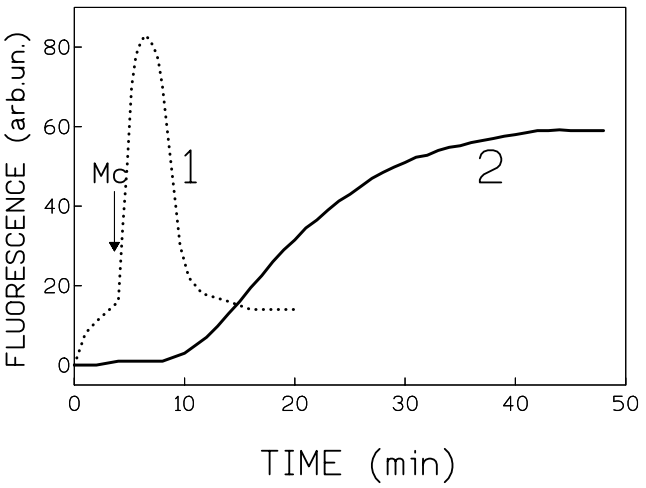

Fig. 6. Comparison of the time courses of the microcin effects upon intracellular pH (1) and alterations in the cell envelope detectable by ethidium fluorescence (2) in the cells of *Cr. terreus*. At time 0, to the cells in the buffer either 2×10^{-5} M FDA (trace 1) or 2×10^{-5} M ethidium bromide were added and fluorescence was recorded as described in Section 2. Upon 4 min of incubation, microcin (0.03 mg/ml) was added (indicated by arrow). The traces 1 and 2 are offset for the purpose of display. Typical results of three experiments are presented.

addition of microcin to FCCP-treated cells after fluorescence splash cessation resulted in further fluorescence decline and inhibition of FDA hydrolysis (Fig. 4C).

In principle, the microcin-induced splash of the intracellular fluorescence might be a consequence of changes of (i) intracellular pH, (ii) the cytoplasmic membrane permeability for FDA and/or fluorescein and (iii) the activity of unspecific esterases. To investigate what accounts for this splash, the cells were loaded with fluorescein by preincubation with the dye, when no FDA hydrolysis was involved. Fig. 5 shows that, in this case, the microcin-induced splash was observed if fluorescence was excited at 490 nm, the wavelength at which fluorescein absorption is pH-dependent. There were almost no detectable microcin-induced fluorescence changes upon excitation at 435 nm, where absorption and hence fluorescence are pH-independent. These data indicate that the microcin-induced fluorescence splash is a result of intracellular pH changes, namely, transient alkalization followed by acidification. The FCCP-induced splash (Fig. 4C) can be interpreted the same way.

As we have shown earlier, crude microcin preparations caused alterations in the cell wall and/or cap-

sule that resulted in interaction of ethidium with the cytoplasmic membrane [4]. TLC-purity microcin caused the same effect, and kinetics of ethidium interaction with the cells had the distinguishing S-shape too (Fig. 6, trace 2). It is worth noting that, under the same conditions, intracellular pH changes were found to take place immediately upon microcin treatment (Fig. 6, trace 1), while ethidium detectable alterations in the cells developed only after a delay and were much slower.

3.4. Microcin inhibits 4-MUA hydrolysis and endogenous respiration

It was observed microscopically that intact cells of *Cr. terreus* were not stainable by 4-MUA at concentrations of up to 0.2 mM. The cells, however, hydrolyzed 4-MUA (Fig. 7). In contrast to fluorescein formed from FDA, all 4-MU, a fluorescent product of the 4-MUA hydrolysis, was found in milieu (data not shown). Hence, either 4-MU was formed extracellularly, or it was formed intracellularly, but the rate of its efflux was much higher than the rate of 4-MUA permeation and hydrolysis.

Fig. 7 also shows the inhibitory effect of microcin upon 4-MUA-hydrolyzing activity. No splash of fluorescence preceding inhibition of the hydrolysis, as it was the case for FDA (Fig. 4A), was observed.

Microcin was found to completely inhibit endogenous respiration of the cells within 20 min. At the same time, KCN at a concentration of 2 mM did not cause any change in FDA-hydrolyzing activity or intracellular pH of the cells, although it effectively inhibited respiration (data not shown). This means that inhibition of respiration was a consequence rather than a cause of intracellular pH changes.

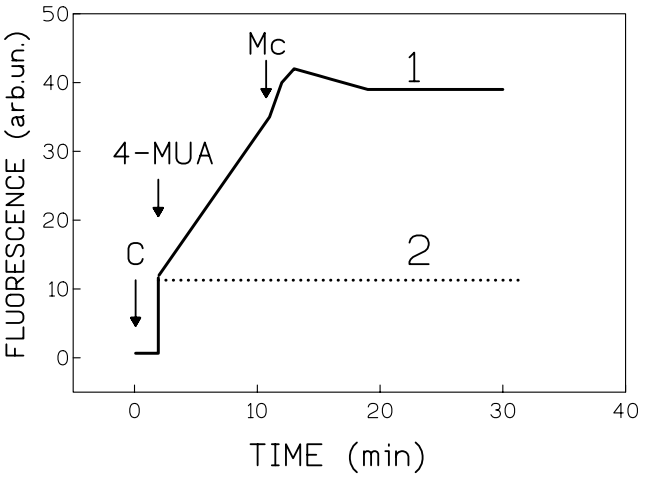

Fig. 7. Effects of microcin upon 4-MUA hydrolysis in the cells of *Cr. terreus*. Arrows indicate the moments of the following additions to the fluorescence cuvette containing CPB: C (3×10^6 cells/ml); 4-MUA, 4-methylumbelliferyl acetate (2×10^{-5} M); Mc, microcin (0.03 mg/ml). Trace 1, intact cells; trace 2, the cells after preincubation with microcin for 6 min. Measurements were conducted as described in Section 2. Typical results of three experiments are presented.

3.5. ATP leaks out from the microcin-treated cells

The data presented in Table 1 indicate that, upon microcin treatment, the intracellular ATP content significantly decreased with time, and ATP appeared in the milieu. Similar to microcin, FCCP halved the intracellular ATP content within 5 min, but in contrast to the effect of microcin, the ATP content stabilized at this level. Another noticeable difference in the effect of FCCP was that there was no detectable extracellular ATP.

Table 1
Effects of microcin and FCCP upon ATP content in *Cr. terreus* cells and in the medium

Time (min)	Intracellular ATP (nmol/cell$\times10^7$)			Extracellular ATP (nmol/cell$\times10^7$)		
	Control	Microcin	FCCP	Control	Microcin	FCCP
0	2.00	2.05	2.08	0	0	0
5	2.06	1.16	1.13	0	0.51	0
10	2.07	0.50	1.09	0	1.00	0
15	1.84	0.28	1.13	0	1.26	0

Cr. terreus cells in CPB (6×10^7 cells/ml) were treated by either microcin (0.03 mg/ml) or FCCP (10^{-6} M). At various times, 0.2-ml samples were taken and intracellular and extracellular ATP were determined as described in Section 2. Typical results of three experiments are presented. Values are given as the mean of three measurements. Standard deviations did not exceed 12%.

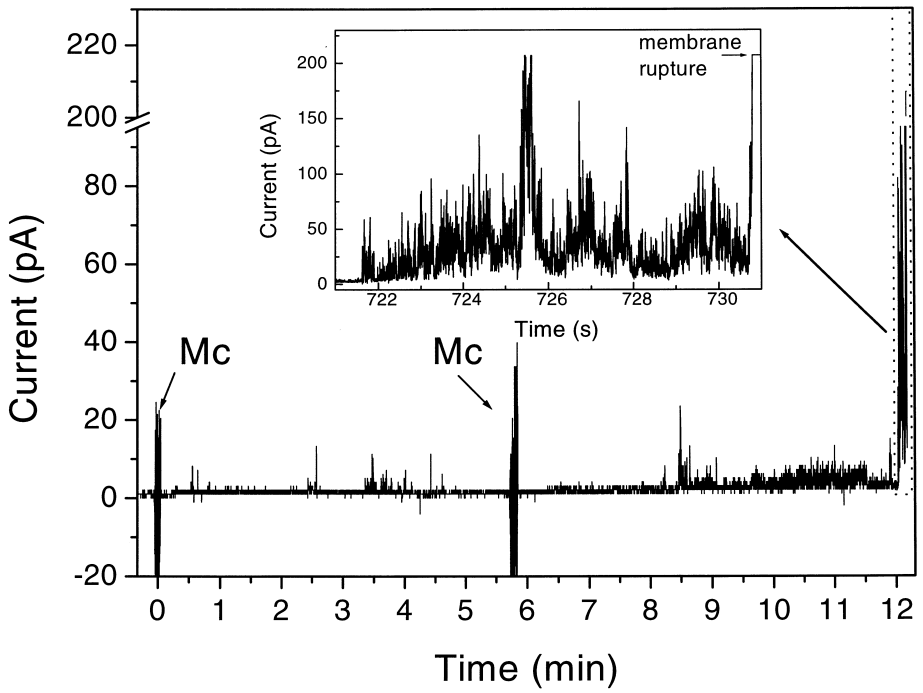

Fig. 8. Influence of microcin on the electrical conductance of planar phosphatidylserine membranes. Time course of membrane current after the addition (arrows) of microcin (Mc) at a final concentration of 0.6 μg/ml to the *cis* side of the membrane. The inset shows the increasing current fluctuations directly before membrane rupture (sudden current increase at the end of the trace). The clamp voltage was 50 mV. Bathing solution: 100 mM KCl, 32 mM citric acid, 36 mM Na_2HPO_4; pH 4, temperature 23°C.

3.6. *Microcin induces lesions in planar phospholipid bilayers*

The addition of microcin to the cis side of symmetric PS bilayers led to transient current fluctuations. Fig. 8 presents a sample of 20 current traces recorded, where these fluctuations were most pronounced. No changes in membrane current were observed when the same amount of methanol was added to the membrane. In this case, a sudden membrane rupture without any preceding current fluctuations occurred at the end of the lifetime of the membrane (data not shown).

It was also noticed that after spontaneous rupture of the 'intact' membrane it can be repeatedly restored from the preformed monolayers. After microcin treatment (addition to the subphase) and membrane rupture, the new membrane could not be formed. So the obtained data present evidence of the principal possibility of the microcin interaction with phospholipids that can cause lesions in phospholipid bilayers.

3.7. *Mycocidal effect of microcin does not depend on the cell cycle phase*

It was shown that killer toxin of *Kluyveromyces lactis* causes sensitive *Saccharomyces cerevisiae* cells to arrest irreversibly in the unbudded (G1) phase of the cell cycle [21]. We have studied whether the microcin mycocidal effect is dependent on the phase of the *Cr. terreus* cell cycle. Fig. 9 shows that the division arrest caused by microcin did not result in detectable changes in fractions of single, budding, and dividing cells. These results indicated that division arrest caused by microcin is independent of the cell cycle of the sensitive yeast.

4. Discussion

After Rotman and Papermaster [5], staining by FDA of numerous cell types was widely used for cytoplasmic membrane integrity assessment upon

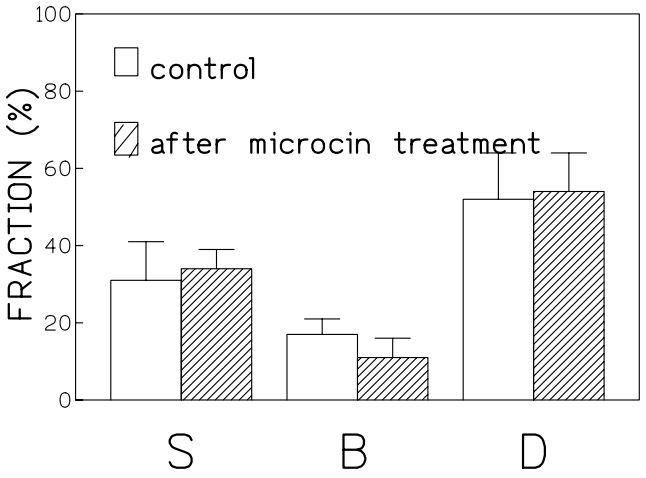

Fig. 9. Fraction relationship of single (S), budding (B) and dividing (D) cells in the populations of *Cr. terreus* before (open bars) and after (diagonal bars) microcin treatment. Suspension of the cells from the logarithmic phase of growth was diluted to 1.5×10^5 cells/ml and divided into two portions. A sample (0.005 ml) from the first portion was pipetted onto malt agar mount on a microscopic slide and examined microscopically as described in Section 2 and in [4] for proportions of single, budding and dividing cells (total >300). The second portion, after incubation with microcin at a concentration of 0.03 mg/ml for 1.5 h, was assayed for growth inhibitory effect as described for MIC determination in Section 2, and again proportions of single, budding and dividing cells (total >300) were estimated. Typical results of three experiments are presented. Values are given as the mean ± S.D. for three experiments performed with different batches.

various treatments [6–10]. Loss of stainability was considered to be a consequence of fluorescein leakage from the cells with compromised membranes. However, when examined more closely, the phenomenon of stainability by FDA appeared to be more complex. It was shown with *Saccharomyces cerevisiae* that the fluorescence staining depends on several interdependent parameters such as uptake of profluorochrome and efflux of fluorescent product, esterase activity and intracellular pH, temperature and fluorescence quenching [22]. We too have observed that, although membranes of intact *Cr. terreus* are leaky for fluorescein (Fig. 3), the cells are stainable by FDA and even by fluorescein. Most probably the later fact is mainly due to a more alkaline cytoplasm as compared to the milieu, otherwise there was no difference in fluorescence intensities of the cells and background upon microscopic examination.

The cells of *Cr. terreus* effectively hydrolyzed 4-MUA with fluorescent 4-MU formation (Fig. 6). However, in contrast to mammalian cells [15,23], they were not stainable by this profluorochrome upon microscopic examination and no cell-associated fluorescence was found fluorometrically. Two possible reasons (not necessarily excluding one another) could result in these phenomena. First, the rate of efflux of intracellularly formed 4-MU is too high for to allow detection of the fluorescent product inside the cells by the methods used. Second, 4-MUA hydrolysis is catalyzed by esterases located at the outer side of the cell envelope, so that all product is formed outside the cell. Multiple forms of esterases hydrolyzing various substrates with different effectiveness and being located both inside the cells and in their envelopes were demonstrated in baker's yeast [24].

To explain the effects of microcin upon the cells of *Cr. terreus* as revealed in this work, the following hypothesis is put forward. The cytoplasmic membrane of the cells is the primary target for microcin. Interaction of microcin with the membrane results in the development of the disruption of the selective permeability barrier with time. This effect of microcin can directly be seen from the current trace after microcin addition to the phospholipid bilayer membrane (Fig. 8). The observed transient current fluctuations are indicative of the transient membrane lesions' formation. Similar traces can be observed for the interaction of detergents with lipid bilayer membranes [25]. The detergent-like effect of microcin was also observed on bilayer membranes formed from brain lipids (V.P. Topaly, personal communication). In the initial phase of the microcin interaction with the cytoplasmic membrane, a small increase of the permeability, similar to that induced by the uncoupler FCCP, is caused. This leads to dissipation of the membrane potential and, as a consequence, to activation of membrane H^+-ATPase, which is known to be activated upon membrane potential reduction [26]. Since H^+-ATPase is the main intracellular pH regulator [27], its activation results in enhanced proton extrusion and transient alkalization of the cytoplasm (fluorescence increase in Figs. 4 and 5). In the next step, cytoplasm alkalization phase is followed by acidification (fluorescence decrease in Figs. 4 and 5). Upon treatment by both microcin and FCCP, acidification starts after reduction of the in-

tracellular ATP level (Table 1). As one can see from Table 1, ATP level reduction caused by microcin is more severe and is a result mainly of ATP leakage, while FCCP-induced intracellular ATP reduction seems to be a consequence of activated ATPase. Also, addition of microcin after FCCP treatment results in further intracellular fluorescein quenching (Fig. 4C), possibly due to additional acidification and/or enhanced leakage of the dye. Thus, in the case of FCCP treatment, the acidification stage may be a consequence of reduction or inhibition of ATPase activity. Finally, microcin induces more severe impairment of the permeability barrier. This would not only lead to equilibration of intracellular and extracellular pH, but also to influx and outflux of molecules of at least ATP size. Experiments on planar bilayer membranes show (Fig. 8) that lesions caused by microcin are most probably due to its direct effect on phospholipid bilayer.

Selective permeability barrier disruption by microcin must cause gross alterations in intracellular homeostasis. We further suggest that these alterations are the main reason of intracellular enzymes' inactivation. In this work we have observed the inhibition of unspecific esterases (Figs. 4B and 7) and endogenous respiration (data not shown). As regards unspecific esterases, one indirect argument in favor of this suggestion is the fact that microcin did not affect activity of wheat germ lipase, catalyzing FDA hydrolysis (data not shown). It was shown that yeast FDA-hydrolyzing [22] and ATPase [28] activities are dramatically reduced at pH below 5. Presumably, intracellular pH changes induced by FCCP were not large enough for FDA hydrolysis inactivation (Fig. 4C,D). The fact that the mycocidal effect of microcin does not depend on the cell cycle (Fig. 9) can, too, be interpreted as a result of unspecific and generalized perturbations in the cells such as alterations in cytoplasm homeostasis. Finally, microcin-induced alterations in the cell wall and/or capsule [4] most probably are the consequence of the cytoplasmic membrane permeability barrier disruption. This conclusion is drawn from the fact that membrane impairment preceded alterations in the cell wall and/or capsule (Fig. 6).

In conclusion, the results of this work show that the cytoplasmic membrane of the sensitive yeast *Cr. terreus* is a primary target for microcin of *Cr. humicola* 9-6. Microcin causes a gradually increasing unspecific permeability of the membrane. This results in a change of intracellular homeostasis and as a consequence inactivation of enzymes, cell wall/capsule alteration and, eventually, cell death.

Acknowledgements

We are grateful to Dr. W.I. Golubev for initiating these studies, providing us with the microcin producing strain *Cr. humicola* 9-6 and advice on zymology. Thanks are due to Professor M.N. Preobrazhenskaya, Drs. A.Y. Pavlov and O.V. Miroshnikova for invaluable help in the development of the protocol for microcin purification. We are grateful to N.V. Cheretayeva and E.V. Kulakovskaya for accurate and devoted technical assistance. This research was supported in part by Grant N 00-04-48082 from the Russian Foundation for Basic Research (E.O.P., T.V.K.) and by the Deutsche Forschungsgemeinschaft SFB 470, project B5 (A.W., U.S.). E.O.P. expresses his gratitude to the Directorate of the Research Center, Borstel for providing him with a guest scientist grant.

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