ACTION OF ANTIFUNGAL PEPTIDOLIPIDS FROM *BACILLUS SUBTILIS* ON THE CELL MEMBRANE OF *SACCHAROMYCES CEREVISIAE*†

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(Received for publication October 13, 1983)

Iturin A and bacillomycin L, antibiotics of the iturin group inhibit the growth of *Saccharomyces cerevisiae* and the lethal doses were respectively 10 and 60 μg/ml. Both antibiotics had an effect on the incorporation of radioactive precursors into macromolecules which decreased with increasing concentrations of antibiotics. However, no specificity was observed on the various macromolecules, proteins, ribonucleic acids and polysaccharides.

The site of action on yeast cells was demonstrated to be the cytoplasmic membrane: both antibiotics of iturin group lysed spheroplasts of *S. cerevisiae*. Moreover, a rapid leakage of potassium ions occurred in the presence of the antibiotics; this leakage was directly associated to the killing effect. These results are consistent with a disruption of the structural integrity of the cytoplasmic membrane correlated to the loss of viability of the yeast cells.

Bacillomycin L[1] and iturin A[2] are peptidolipidic antibiotics of *Bacillus subtilis*[3]. These antibiotics present a narrow antibacterial spectrum and a potent antifungal activity against a large variety of fungi and yeasts[4,5].

Previous work showed that these antibiotics are hemolytic[6] and that iturin A has a lytic action upon protoplasts of *Micrococcus luteus* while bacillomycin L is devoid of this activity[7,8]. These results question the mode of action of the antibiotics of iturin group and especially the ability of bacillomycin L to destroy cytoplasmic membranes.

The purpose of this work was to study the mode of action of the peptidolipidic antibiotics on eukaryotic cells. As these antibiotics are powerful antifungal compounds, a strain of *Saccharomyces cerevisiae* was chosen as test organism. In a first step the possibility of their action on the biosynthesis of macromolecules was examined by measuring the incorporation of labeled precursors into proteins, polysaccharides and RNA. Secondly we studied their effects on yeast spheroplasts and on the permeability of the cytoplasmic membrane. The permeability changes were measured by the leakage of K^+ ions.

Materials and Methods

Antibiotics

Bacillomycin L was a gift of Dr. G. H. WARREN, Wyeth Institute of Applied Biochemistry, Pensylvannia, U. S. A. and iturin A was prepared as described previously[3]. For all experiments, antibiotics were added in methanol solution.

Culture Conditions

S. cerevisiae was grown aerobically at 30°C with shaking in a medium at pH 7.2 containing, per liter, glucose 40 g, peptone (Bio Mérieux) 10 g and yeast extract (Bio Mérieux) 2 g. Growth was estimated

† This paper is dedicated to the memory of ELISABETH BESSON.

by turbidimetry at 600 nm. In assays on the incorporation of labeled glucose the growth medium contained only 0.2% (w/v) glucose.

Viability Determination

S. cerevisiae was grown until A_{600} reached 2.0; cells were collected by centrifugation for 15 minutes at 10,000×*g*, washed twice and resuspended in 0.1 M Na-phosphate buffer, pH 7.6 until $A_{600}=0.750$. Antibiotics were added to the non-growing cell suspension or to the growing cells in the culture medium. Samples were diluted with 1% (w/v) peptone and plated on Sabouraud dextrose agar (Bio Mérieux). After 48 hours incubation at 28°C, the colonies were counted.

Incorporation of Radioactive Precursors

Radioactive precursors were obtained from the Commissariat à l'Energie Atomique, Saclay, France; [^{14}C]uracil (55 mCi/mmol) is labeled in position C_2, L-[^{14}C]isoleucine (240 mCi/mmol) and D-[^{14}C]glucose (288 mCi/mmol), were uniformly labeled. Radioactive uracil, L-isoleucine or D-glucose was added to a log-phase culture of *S. cerevisiae* ($A_{600}=0.5$) at the respective final radioactivity of 0.4, 0.5 and 1.4 μCi/ml. After 10 minutes, the antibiotic was added and, at various time intervals, 1 ml samples of the culture were treated with 1 ml of ice cold 10% (w/v) trichloroacetic acid (TCA). The acid-insoluble fractions were collected on a Millipore filter (0.45 μm) and washed with 5 ml of 5% (w/v) TCA, then with 2 ml of ethanol. The radioactivity was counted with a liquid scintillation spectrometer (Packard Tricarb 2001) in 5 ml of scintillation fluid containing in 1 liter of toluene, 5 g of 2,4-diphenyloxazole and 0.3 g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene. The counting efficiency was 73%.

Incorporation of Radioactive Glucose into Cell Wall Glucan and Mannan

Yeast cells were grown with antibiotic for 20 minutes and then D-[^{14}C]glucose was added to the medium. After 30 minutes the polysaccharides were prepared according to the method of ELORZA and SENTANDREU[9]: the cells were collected by centrifugation and digested with 2 N sodium hydroxide at 100°C for 2 hours, the supernatant obtained by centrifugation was treated with Fehling reagent to form Cu^{++}-mannan complex, and the precipitate was extracted with 0.5 N acetic acid at 100°C for 2 hours to remove glycogen. The radioactivity of the Cu^{++}-mannan complex and of the insoluble glucan residue after acetic acid extraction was counted as described above.

Determination of the Intracellular Potassium Ions from *S. cerevisiae* Cells

Non-growing cells were suspended in 0.1 M Na-phosphate buffer at a concentration of about 5×10^5 cells/ml. The antibiotic in methanol solution was then added. The total amount of solvent never exceeded 1% of the total volume, a concentration which did not modify the cell permeability. The suspension was kept at room temperature with shaking; 3 ml samples were removed at time intervals of 20 minutes and filtered on Millipore filters (0.45 μm). The cells were washed with 3 ml of cold water. The filters were put in 3 ml of distilled water and placed in a boiling water bath for 10 minutes. After centrifugation, K^+ ions were determined by flame spectrometry (Flame photometer Elvi 660). The amount of intracellular K^+ was expressed as a percentage of the intracellular K^+ of control cells.

Preparation of Spheroplasts

Yeast was grown to early stationary phase in liquid medium containing 1% Bacto peptone, 1% yeast extract and 2% glucose. The cells, collected by centrifugation, were washed by distilled water and then by 1.2 M sorbitol. Yeast cells (1 g wet weight) were suspended in 0.05 M phosphate-citrate buffer pH 5.8 (2 ml) containing 1.2 M sorbitol and 0.2 ml of Cytohelicase solution (IBF France) was added. The suspension was incubated at 37°C for 2 hours with gentle shaking. The hydrolysis of yeast cell wall was monitored by measuring A_{600} of 0.01 ml of the suspension diluted in 2 ml of H_2O. After 2 hours incubation, A_{600} was stable. The lysis of the spheroplasts was measured after 1 hour of incubation with antibiotics.

Results

Effects of Antibiotics on *S. cerevisiae*

The inhibition of the growth of *S. cerevisiae* is shown in Fig. 1. Controls and cultures with low

Fig. 1. Effect of antibiotics on the growth of *S. cerevisiae*.

Cells were incubated in the presence (●) or absence (▲) of various concentrations of iturin A (A) or bacillomycin L (B). The arrows indicate the time of addition of antibiotic.

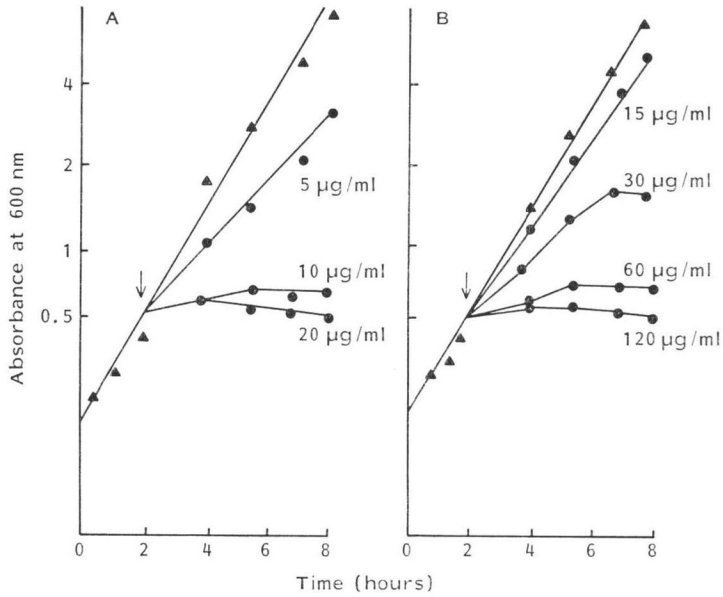
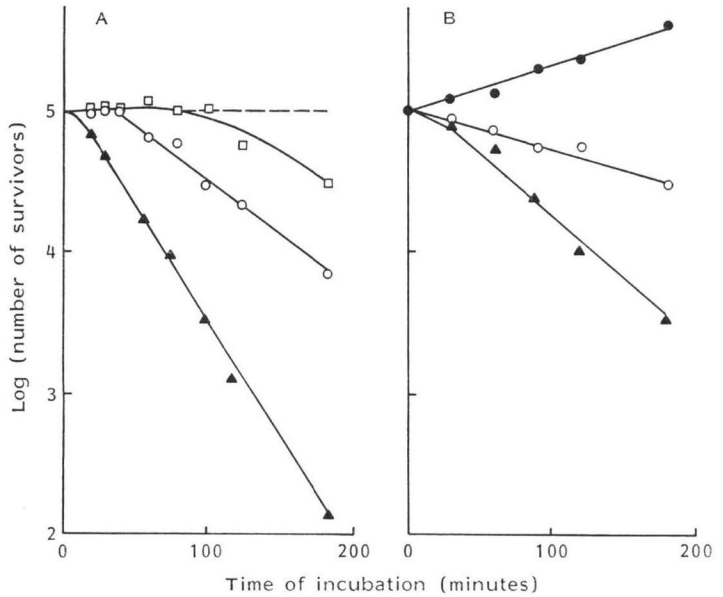


Fig. 2. Effect of iturin A on the viability of resting cells and growing cells of *S. cerevisiae*.

Resting cells (A) or growing cells (B) were incubated in the absence (●), or presence of iturin A 5 μg/ml (□), 10 μg/ml (○) or 20 μg/ml (▲).



concentrations of antibiotics, 5 μg/ml for iturin A and 15 μg/ml for bacillomycin L, have an exponential growth. The minimum concentrations giving a total inhibition were 10 μg/ml for iturin A and 60 μg/ml for bacillomycin L.

To distinguish between fungistatic and fungicidal effects, the viability of *S. cerevisiae* was determined after action of various concentrations of antibiotics on growing and non-growing cells. Fig. 2 gives the results of the action of iturin A on non-growing cells at 5, 10 and 20 μg/ml. The number of cells killed increased with increasing concentrations of iturin A, and 99% were killed after 3-hour incubation with the highest concentration. Identical fungicidal effects were obtained with bacillomycin L but with higher concentrations (30 to 120 μg/ml).

In the case of growing cells the apparent effect was lower than with non-growing cells, since about 80% instead 99% of cells were killed after 3-hour incubation with iturin A. In fact, in a culture medium, unkilled cells continued to divide and the true activity of antibiotic was the difference between the growth rate and the death rate. This activity was roughly similar to the activity on non-growing cells.

Effects of Antibiotics on the Biosynthesis of Macromolecules

The *in vivo* biosynthesis of RNA, proteins and polysaccharides in the presence of various concentrations of iturin A or bacillomycin L was studied by measuring the incorporation of radioactive precursors into TCA-insoluble material. The results obtained with iturin A are given in Fig. 3.

The rate of incorporation of radioactive D-glucose, L-isoleucine and uracil was lowered with increasing concentrations of antibiotic but no significant difference was observed in the sensitivity of the biosynthesis of the three macromolecules. Similar results were obtained with bacillomycin L.

The incorporation of [^{14}C]glucose into whole cells and into cell wall polysaccharides, glucan and mannan, was affected by high doses of antibiotics (Table 1). However, there is no significant

Table 1. Percentage of inhibition of [^{14}C]glucose incorporation into cells, mannan and glucan after 30 minutes incubation.

Antibiotic concentration (μg/ml)		Cells	Mannan	Glucan
Iturin A	5	12	17	13
	10	49	52	57
	20	75	81	83
Bacillomycin L	30	10	17	12
	60	23	37	46
	120	85	87	93

Fig. 3. Incorporation of radioactive precursors into the acid-insoluble fraction of *S. cerevisiae* cells. (▲): Incubation without antibiotics, (●) incubation with iturin A, a: 5 μg/ml, b: 10 μg/ml, c: 20 μg/ml. The arrows indicate the time of the addition of antibiotic.

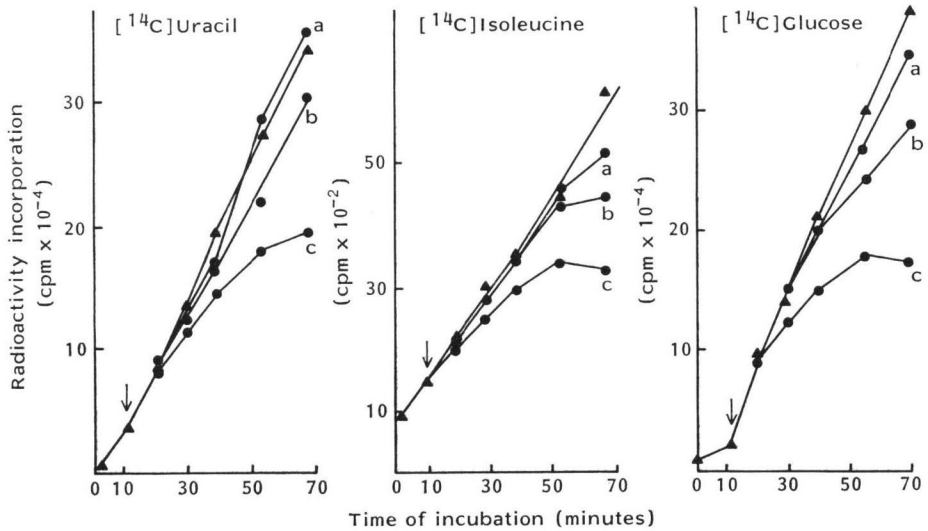


Fig. 4. Lysis of the spheroplasts of *S. cerevisiae* in presence of various concentrations of iturin A (■) or bacillomycin L (▲).

Percentages of lysis were calculated after 60 minutes of incubation with antibiotic. A 100% lysis was obtained when spheroplasts were suspended in water.

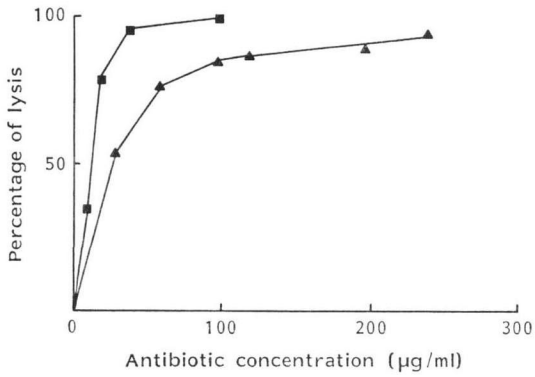
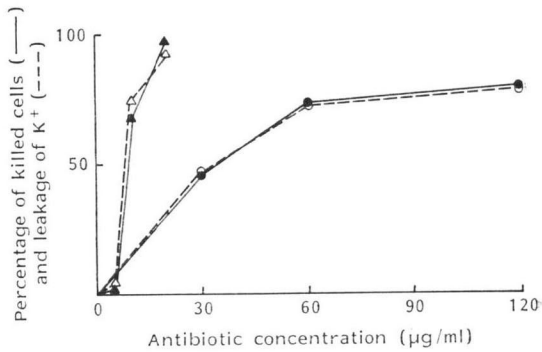


Fig. 5. Effect of iturin A (△, ▲) and bacillomycin L (○, ●) on resting cells of *S. cerevisiae*.



difference between the incorporation of labeled glucose into mannan or glucan.

Effect of Antibiotics on the Spheroplasts of *S. cerevisiae*

Iturin A and bacillomycin L lysed spheroplasts of *S. cerevisiae* (Fig. 4). A 50% lysis was observed with 10 μg/ml iturin A and 30 μg/ml bacillomycin L.

Effect of Antibiotics on the Leakage of Potassium Ions in Resting Cells of *S. cerevisiae*

Antibiotics of iturin group induced leakage of potassium ions in resting cells of *S. cerevisiae*. This effect increased with time and with increasing concentrations of antibiotic. The percentages of leakage of potassium ions and of cell killing were compared after 100 minutes of action for various concentrations of antibiotics (Fig. 5). Both effects were strictly parallel and it is obvious that the loss of potassium ions is a characteristic of the loss of cell viability.

Discussion

The antibiotics of the iturin group are highly active against *S. cerevisiae*. The lethal concentrations are lower than those previously observed against *M. luteus*[7,8] and iturin A has a higher activity than bacillomycin L. These fungicidal activities do not vary with growing or resting state of the cells and are proportional to the concentrations of antibiotics.

The incorporation of radioactive precursors into macromolecules, proteins, RNA and cell wall polysaccharides was reduced when the concentration of antibiotics increased; however this effect was not specific to one macromolecule and is presumed to be a secondary result of other cell damages caused by these antibiotics.

Both iturin A and bacillomycin L lysed the yeast spheroplasts while bacillomycin L was found to have no lytic action on the protoplasts of *M. luteus*[7,8]; thus the composition of the cytoplasmic membranes is of prime importance for the interaction with antibiotics of iturin group.

The fact that the leakage of K^+ from yeast cells is strictly correlated to the loss of cell viability indicates that the action of the antibiotics from the iturin group leads to important and lethal modifications in the cell permeability. The sensitivity of cells or spheroplasts is dependent of the structure of the antibiotics. Both antibiotics are amphiphilic compounds with closely related hydrophobic moieties, they differ by the presence of an anionic group in the peptidic part of bacillomycin L[1]. This structural dif-

ference might explain the difference in the sensibility of cells or spheroplasts to iturin A and bacillomycin L.

Acknowledgments

This work was supported by the Centre National de la Recherche Scientifique (E.R.A. n° 852 and A.T.P. Microbiology 1981). We thank Dr. L. DELCAMBE, Centre National de Production et d'Etude des Substances d'Origine Microbienne, Liège, Belgique, who provided us with iturin.

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