

ACTION OF ITURIN A, AN ANTIFUNGAL ANTIBIOTIC FROM
BACILLUS SUBTILIS, ON THE YEAST *SACCHAROMYCES*
CEREVISIAE: MODIFICATIONS OF MEMBRANE
PERMEABILITY AND LIPID COMPOSITION

CHANTAL LATOUD, FRANÇOISE PEYPOUX and GEORGES MICHEL

Laboratoire de Biochimie Microbienne, UA (CNRS) 1176, Université Claude Bernard,
Lyon I, 43, Boulevard du 11 Novembre 1918,
F69622, Villeurbanne, Cedex, France

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The action of iturin A on non-growing cells of *Saccharomyces cerevisiae* was tested. This antibiotic gave important modifications in the membrane permeability which permitted nucleotides, proteins, polysaccharides and lipids to escape from cells. The lipid content of cells was strongly disturbed; the level of phospholipids, essentially phosphatidylcholine, decreased while the level of fatty acids increased. A part of these fatty acids were extruded from yeast cells. The role of iturin A in these modifications was discussed.

Iturin A is an antifungal and hemolytic antibiotic produced by some strains of *Bacillus subtilis*; it contains a cyclopeptide moiety with seven L and D α -amino acids and a lipophilic moiety consisting of a long chain β -amino acid¹⁾. Previous studies showed that iturin A lysed protoplasts from *Micrococcus luteus*²⁾ and formed channels in artificial lipid membranes³⁾. Moreover iturin A released intracellular K^+ ions and hemoglobin from erythrocytes⁴⁾. All these results brought evidence that the site of action of iturin A is the cytoplasmic membrane of cells. However, the target material used until now had only a simple natural or artificial membrane. In the case of yeast cells, the problem is complicated by the presence of the cell wall; the antifungal action involves penetration into the cell through the wall barrier and activity on cytoplasmic membrane or on intracellular membranous systems. We here describe modifications in membrane permeability which were observed in cells of *Saccharomyces cerevisiae* by action of iturin A.

Materials and Methods

Chemicals and Antibiotic

Sodium [1-¹⁴C]acetate (55 Ci/mol), [¹⁴C]leucine (54 Ci/mol) and D-[1-¹⁴C]mannose (50 Ci/mol) were purchased from the Commissariat à l'Energie Atomique, Saclay (France). Standard phospholipids, ergosterol, triacylglycerols and fatty acids were purchased from Sigma (U.S.A.), Cytohellicase from IBF (France) and other chemicals from Prolabo (France). Iturin A was prepared as previously described⁵⁾.

Growth Conditions and Antibiotic Activity

Saccharomyces cerevisiae NCYC 366 was grown aerobically at 28°C on a medium containing, per liter, peptone (Bio-Mérieux) 10 g, yeast extract (Bio-Mérieux) 2 g, glucose 20 g, pH 7.0. When the absorbance reached 0.8, antibiotic in 0.15 M NaCl solution was added and the growth was measured by turbidimetry at 600 nm.

Leakage of UV Absorbing Materials

The cells were grown overnight, harvested, washed and resuspended in 0.05 M phosphate - citrate

buffer, pH 6.0 (final absorbance 0.8). Various amounts of iturin A were added to the suspension; a control was made in the same conditions. Samples (2 ml) were taken at various intervals and centrifuged at 4°C, the absorbance of supernatants was measured at 260 and 280 nm. The maximal extractable UV absorbing materials was determined after treatment of cells with 1.2 N perchloric acid at 100°C for 30 minutes, this value was taken as 100% release⁸⁾.

Leakage of [¹⁴C]Leucine and [¹⁴C]Mannose Labeled Compounds

Cells were labeled by growing *S. cerevisiae* overnight in the presence of [¹⁴C]leucine (2 μCi/ml) or of [¹⁴C]mannose (0.2 μCi/ml).

The uptake of radioactive marker by a suspension of yeast cells, after washings (3 times), was measured in presence of Dimilume 30. Soluble radioactive compounds were quantified after the action of Cytohelicase for 2 hours at 37°C on yeast cells and centrifugation at 40,000 × *g*. To determine the leakage of labeled compounds, assays were set up as described above. The radioactivities of supernatants and of trichloroacetic acid (TCA) precipitates were measured. In the latter case one volume of 10% cold TCA was added to each supernatant, the precipitate was collected on a filter (Millipore 0.45 μm) and washed. The filter was placed in a vial containing 10 ml of Dimilume 30 and the radioactivity was measured.

This radioactivity was compared with that of material obtained by TCA precipitation of a same volume of bacterial suspension (total TCA precipitable radioactivity).

After 5 hours incubation, 150 ml of cell suspension were filtered on a Millipore filter (0.45 μm); the soluble fraction was lyophilized and put on a Sephadex G 50 column (16 × 1.6) in water. The elution profile was established by measuring the radioactivity of the fractions.

Leakage of Lipids

Cells were labeled by growing for 5 hours with sodium [1-¹⁴C]acetate (1 μCi/ml), harvested, washed and resuspended in 0.05 M phosphate - citrate buffer, pH 6.0 (final absorbance 0.8). To the suspension 20 μg/ml of iturin A were added. At intervals, 100 ml were withdrawn and filtered on a Millipore filter (0.45 μm); the cells and filtrates were lyophilized.

Lipids were extracted by stirring lyophilized materials overnight with CHCl₃ - MeOH (1 : 1) and then with CHCl₃ - MeOH (2 : 1) for 8 hours. After filtration on a sintered glass filter and evaporation to dryness, lipids were purified with the solvent of FOLCH *et al.*⁷⁾. The CHCl₃ phase was washed four times with the upper phase and evaporated to dryness; the radioactivity of an aliquot was measured in a toluene based scintillation counting solution. A preliminary fractionation of lipids was carried out on a silicic acid column (Bio Sil HA 325 mesh). The first fraction was eluted with CHCl₃ and the second with MeOH; their radioactivities were determined. Non polar lipids were separated by TLC on Silica gel 60 (Merck). The plates were developed with hexane - diethyl ether - acetic acid (90 : 10 : 1) and the spots detected by iodine vapor were compared with standard triacylglycerols, fatty acids and sterols. Individual phospholipids were separated by bidimensional TLC on Silica gel G with a concentration gradient. The solvents were, first CHCl₃ - MeOH - propan-1-ol - 0.25% KCl - ethyl acetate (25 : 13 : 25 : 9 : 25), then CHCl₃ - EtOH - water - diethylamine (30 : 34 : 8 : 35)⁸⁾. The identification of phospholipids was made by comparison with authentic samples and by using specific reagents. For quantitative analysis each spot was scraped off the plate and the radioactivity was counted in a toluene scintillation fluid.

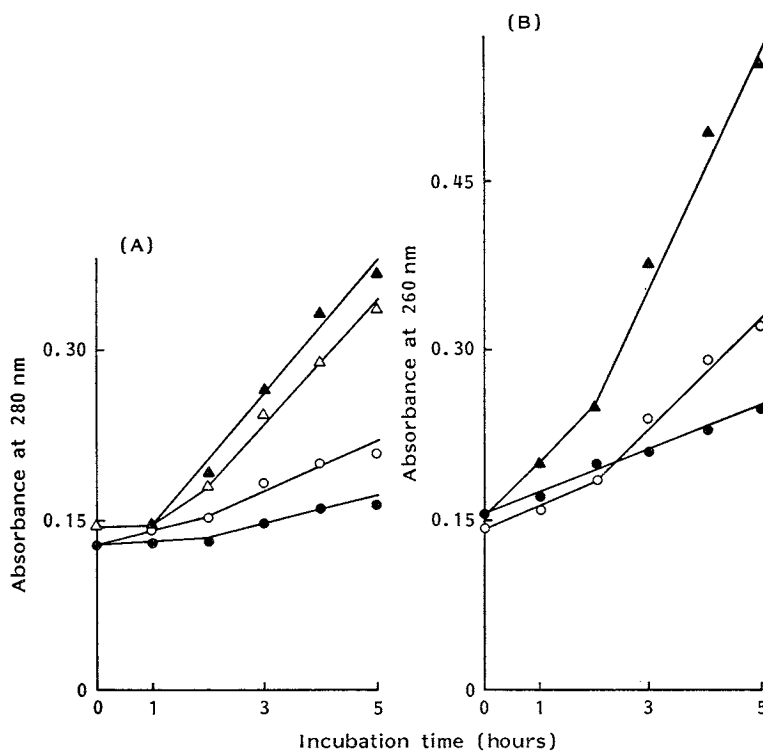
Results

Leakage of UV Absorbing Materials from Yeast Cells

The action of iturin A on the permeability of yeast cell envelopes was tested by measuring the escape of material absorbing at 260 and 280 nm. Various concentrations of antibiotic were added to cell suspensions and samples were examined at several time intervals. The results are indicated in Fig. 1. Compounds absorbing at 260 nm and at 280 nm were released and the amounts increased with concentrations of iturin A up to 20 μg/ml, *i.e.* the minimal inhibitory concentration. This

Fig. 1. Release of UV absorbing materials from resting cells of *Saccharomyces cerevisiae* incubated with various amounts of iturin A.

● Control, ○ 10 $\mu\text{g/ml}$ of iturin A, \triangle 20 $\mu\text{g/ml}$ of iturin A, \blacktriangle 40 $\mu\text{g/ml}$ of iturin A.



This concentration was chosen in the following experiments. After 5 hours incubation the removal of UV absorbing material represented 10% of the total material extractable with perchloric acid.

Leakage of Materials from [^{14}C]Leucine Labeled Cells

Yeast cells were labeled by growing in a culture medium containing [^{14}C]leucine. Then, they were harvested, washed and suspended in a phosphate-citrate buffer as described above. Iturin A was added at a concentration of 20 $\mu\text{g/ml}$, and samples were taken at 1 hour intervals during 5 hours incubation. For each sample the radioactivity of the supernatant before and after TCA precipitation was measured. The results are indicated in Fig. 2: After 2 hours incubation with iturin A, release of radioactive material was observed, the amount increasing up to the end of the experiment. The total radioactivity in the supernatant was about 4-fold higher in the presence of iturin A than in the control. The released radioactivity was 1.5% of the total radioactivity in control cells. This increased to 6.1% when cells were incubated for 5 hours with iturin A.

A part of the radioactive material present in the supernatants was precipitated by TCA. It represented 2.9% of the total TCA precipitable radioactivity when the cells were treated with iturin A and only 0.4% of untreated cells. The released radioactive materials was analyzed by chromatography on a column of Sephadex G 50 (Fig. 3). The elution curves show the presence of high and low molecular weight compounds in much larger amounts in cells treated with iturin A than in control cells.

Fig. 2. Release of [^{14}C]leucine labeled materials in the supernatants of resting cells of *Saccharomyces cerevisiae* suspended in phosphate-citrate buffer pH 7.0 in presence of 20 $\mu\text{g}/\text{ml}$ of iturin A.

○ Radioactivity in the control, □ radioactivity in the iturin A-treated cells, ● radioactivity in the TCA-insoluble fraction from the control, ■ radioactivity in the TCA-insoluble fraction of the iturin A-treated cells.

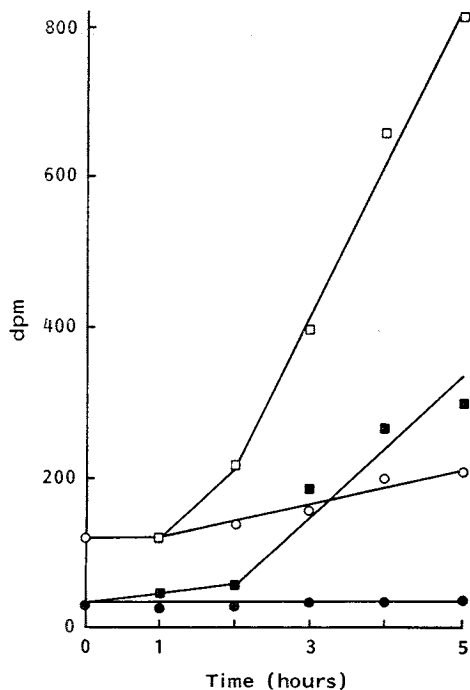
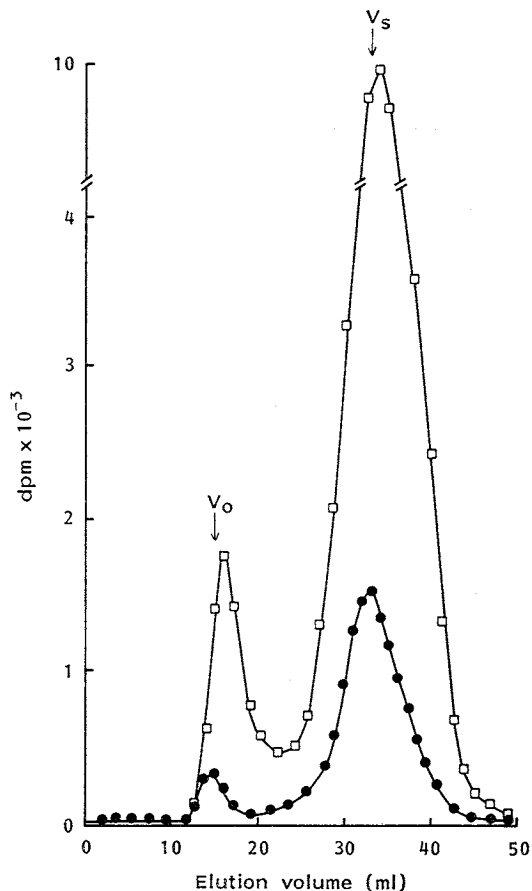


Fig. 3. Elution profile on Sephadex G 50 column of supernatants of cells of *Saccharomyces cerevisiae* labeled with [^{14}C]leucine incubated for 5 hours at 28°C with 20 $\mu\text{g}/\text{ml}$ of iturin A (□) and without antibiotic (●).

V_0 : Void volume, V_s : solvent volume.



Leakage of Materials from [^{14}C]Mannose Labeled Cells

Yeast cells were grown in the presence of [^{14}C]mannose and treated as described above.

The supernatant of the cell suspension treated with iturin A contained 14% of the total radioactivity of the cells while, in the control, it contained only 3.5%. However the TCA-insoluble part was very low, about 1% in the control and 1.3% in the treated sample.

Leakage of Lipids from [^{14}C]Acetate Labeled Cells

The release of lipids was determined with cells grown for 5 hours in the presence of sodium [^{14}C]acetate. Labeled yeast cells were incubated with iturin A, then lipids were extracted from the supernatant and from the cells.

Total cellular lipids and lipids released into the supernatant were fractionated into neutral lipids, including fatty acids, and phospholipids by column chromatography on silicic acid. After 8 hours incubation, in the presence and in the absence of antibiotic only neutral lipids escaped from the yeast

Table 1. Lipid content of cells treated with iturin A.

No. assay	Time of incubation (hours)	Radioactivity ($\text{dpm} \times 10^{-2}$)		Phospholipids/neutral lipids	
		Neutral lipids	Phospholipids	R	R_0/R_t
1	0	8,750	5,400	0.62	
	5	9,650 ^a	5,000 ^a	0.52 ^a	1.2 ^a
		12,730 ^b	2,130 ^b	0.17 ^b	3.6 ^b
2	0	8,208	4,203	0.51	
	8	10,584 ^a	4,090 ^a	0.39 ^a	1.3 ^a
		13,055 ^b	1,950 ^b	0.15 ^b	3.4 ^b

^a Control cells: Yeast cells were kept in resting state in phosphate - citrate buffer.

^b Yeast cells were incubated with 20 $\mu\text{g}/\text{ml}$ iturin A in phosphate - citrate buffer.

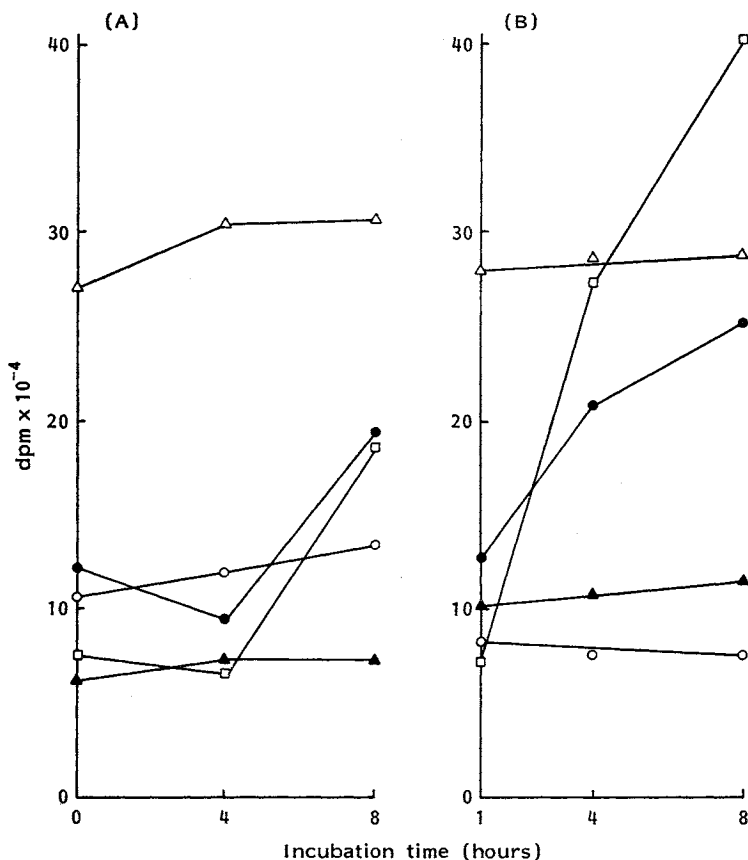
R_0 : Ratio phospholipids/neutral lipids at initial time.

R_t : Ratio phospholipids/neutral lipids after 5 or 8 hours of incubation.

Fig. 4. Effect of iturin A on neutral lipid composition of resting cells of *Saccharomyces cerevisiae*.

(A) Control cells, (B) cells treated with 20 $\mu\text{g}/\text{ml}$ of iturin A. Δ Sterol esters, \bullet triacylglycerols,

\square free fatty acids, \blacktriangle sterols, \circ unidentified.

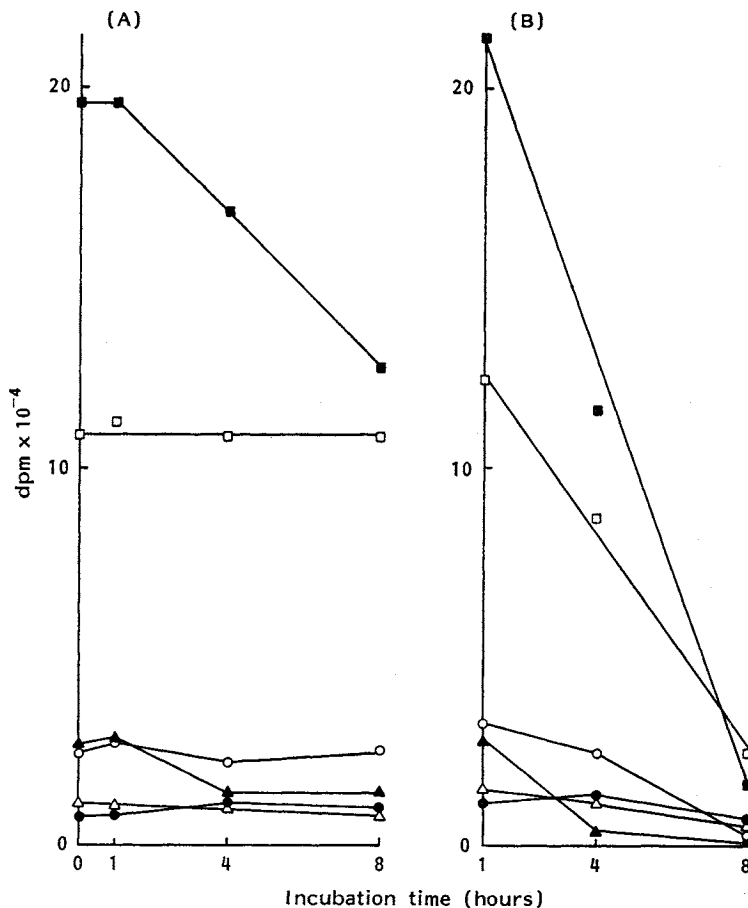


cells. When cells were treated with iturin A, 2.9% of the total cell lipids were found in the supernatant and a much smaller amount, 0.2%, escaped from control cells in the absence of antibiotic. These neutral lipids were analyzed by TLC: Fatty acids were the sole components of released lipids.

In the cells, modification in the lipid composition was observed after incubation with iturin A

Fig. 5. Effect of iturin A on phospholipid content of resting cells of *Saccharomyces cerevisiae*.

(A) Control cells, (B) cells treated with 20 $\mu\text{g/ml}$ of iturin A. ■ Phosphatidylcholine, □ phosphatidylethanolamine, ○ phosphatidylserine, ▲ phosphatidic acid, ● phosphatidylinositol, △ diphosphatidylglycerol.



(Table 1). At the initial time the ratio of phospholipids/neutral lipids was 0.56 ± 0.06 . This ratio was not significantly modified in resting cells after 5 or 8 hours without antibiotic (0.46 ± 0.07). When yeast cells were incubated with 20 $\mu\text{g/ml}$ iturin A the ratio decreased to 0.16 ± 0.01 . This decrease was due to both a diminution of the radioactive phospholipids and an increase of the amount of neutral lipids.

Neutral lipids were separated by TLC and each component was counted. The results are indicated in Fig. 4. In resting cells without antibiotic there was a slight increase of the level of fatty acids and triacylglycerols, the ratio R of the amounts at 8 hours to the amounts at the initial time was $R_{FA} = 2.3$ and $R_{TG} = 1.6$, respectively. This increase probably resulted from a residual synthesis of neutral lipids in resting cells and from a slight hydrolysis of phospholipids as shown later. When the cells were incubated with iturin A the ratio increased strongly for fatty acids ($R_{FA} = 5.8$) and moderately for triacylglycerols ($R_{TG} = 2$).

Phospholipids were analyzed by TLC and counted; the results are shown in Fig. 5. When cells were kept in a resting state for 8 hours, no significant variation of phospholipid components was ob-

served except for phosphatidylcholine, which decreased from 17.7 to 11.1% of the total cellular lipids. In the presence of iturin A, the levels of major phospholipids (phosphatidylcholine and phosphatidylethanolamine) and of minor phospholipids (phosphatidylserine and phosphatidic acid) decreased. The most abundant phospholipid, phosphatidylcholine, represented 17.7% of total lipids initially and only 5% after 8 hours incubation with iturin A.

Discussion

Iturin A is known to have a potent fungicidal action towards growing cells of *S. cerevisiae*. We demonstrate that non-growing cells are also sensitive to this antibiotic. Cellular components that escape from the cell in the presence of iturin A at the minimal inhibitory concentration apparently include nucleotides and polynucleotides absorbing at 260 nm, proteins absorbing at 280 nm, polysaccharides and lipids.

The case of lipids is interesting: Only fatty acids escaped from the cells incubated with iturin A but severe modifications were observed in the lipid composition of cells. The level of phospholipids, notably phosphatidylcholine, was drastically decreased by the action of iturin A and the level of fatty acids concomitantly increased. The ratio of phospholipids/neutral lipids was about 3-fold smaller after incubation with iturin A than in resting cells without antibiotic. Such a modification in the lipid composition must strongly disturb the permeability of the cell membrane and accounts for the release of cellular components. The hydrolysis of phospholipids did not give lysophospholipids, thus both ester linkages were split by phospholipases.

Two hypotheses could be put forward for the mechanism of action of iturin A:

1) Iturin A stimulates phospholipase activities in the yeast cells as does polymyxin B in bacterial cells. In *Pseudomonas aeruginosa*, polymyxin B induced the degradation of phospholipids by *in vivo* activation of phospholipase A which liberated fatty acids, especially from phosphatidylethanolamine and phosphatidylglycerol⁹. More recently phospholipase C was also found to be activated by polymyxin B¹⁰. The mechanism of activation is unknown but it does not consist in an enhancement of the biosynthesis of these enzymes as both phospholipases exist in latent forms in bacteria.

2) Another explanation could be proposed. Previous results have shown that iturin A interacts with cytoplasmic membranes of erythrocytes⁴ and with artificial membranes inducing the fusion of phospholipidic liposomes¹¹ and the formation of pores in bilayer membranes⁹. The presence of iturin A must disturb the distribution of lipid components in the cytoplasmic membranes of yeast and phospholipids might become accessible to phospholipases. The confirmation of these hypotheses needs further investigations which are currently in progress.

Acknowledgments

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