Effect of Papulacandin B on the Cell Wall and Growth of *Geotrichum lactis*

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Addition of the antifungal antibiotic papulacandin B to an exponential culture of *Geotrichum lactis* inhibited incorporation of glucose into the alkali-insoluble and alkali-soluble glucan fractions of the hyphal wall, although the rate of growth was practically unaltered. Synthesis of other cell wall components (i.e. galactomannan and chitin) was not affected. Papulacandin B also induced the proliferation of branches along the hyphae which continued to branch dichotomously resulting in a 'colonial' pattern of growth. Aculeacin A, another antifungal antibiotic that inhibited β -glucan synthesis also caused morphological alterations similar to those described for papulacandin B. Inhibition of β -glucan synthesis and the altered growth pattern persisted for several hours after removal of the antibiotic. Recovery of β -glucan synthesis and restoration of the normal pattern of growth occurred simultaneously. Growth of *G. lactis* in L-sorbose medium also led to inhibition of β -glucan synthesis and dichotomous branching.

INTRODUCTION

It has been proposed that the antifungal compounds papulacandin B and aculeacin A (Traxler *et al.*, 1977; Mizuno *et al.*, 1977) interfere with cell wall synthesis in yeasts since glucose incorporation into the alkali-insoluble glucan fraction of the cell wall of *Saccharomyces cerevisiae* is inhibited by these antibiotics (Baguley *et al.*, 1979; Mizoguchi *et al.*, 1977). Recently we have established that papulacandin B and aculeacin A specifically inhibit the 1,3- β -glucan synthesis of *Geotrichum lactis* *in vitro* (Pérez *et al.*, 1981).

The experiments reported in this paper describe the *in vivo* effect of papulacandin B, aculeacin A and L-sorbose on the growth of *G. lactis*. The main parameter measured was β -glucan synthesis defined either as glucose incorporation into cell wall glucan by intact hyphae or as 1,3- β -glucan synthase activity levels in cell-free extracts of the fungus.

METHODS

Chemicals. Papulacandin B and aculeacin A were generous gifts of CIBA-Geigy and the Toyo-Jozo Co., Tokyo, Japan, respectively. UDP-[U-¹⁴C]glucose (240 mCi mmol⁻¹), UDP-[U-¹⁴C]N-acetylglucosamine (346 mCi mmol⁻¹), [U-¹⁴C]glucose (295 mCi mmol⁻¹), [2-¹⁴C]uracil (60 mCi mmol⁻¹; where 1 mCi = 37 MBq) and ³H-labelled amino acid mixture were purchased from Amersham. UDP-glucose, UDP-N-acetylglucosamine and GTP were purchased from Sigma. Chitinase from *Streptomyces griseus* (ATCC 23345) was purified by adsorption to chitin as described previously (Molano *et al.*, 1977) and zymolyase-5000 was obtained from Kirin Brewery Co., Tokyo, Japan. L-Sorbose and cellobiose were obtained from Merck.

Culture conditions. *Geotrichum lactis* CECT 1102 (Colección Española de Cultivos Tipo) was grown in YED medium (1%, w/v yeast extract and 1%, w/v dextrose) or sorbose medium (2%, w/v L-sorbose and 1%, w/v yeast extract) as described previously (Pérez *et al.*, 1981). Growth was monitored by measuring the dry weight of mycelium harvested by filtration of cultures through Whatman no. 1 filter paper.

Determination of protein and RNA synthesis. Protein and RNA synthesis as well as glucose incorporation were monitored by measuring the radioactivity in TCA-insoluble material (Santos *et al.*, 1978) from cultures supplemented with [2-¹⁴C]uracil (0.2 μ Ci ml⁻¹), ³H-labelled amino acids (4 μ Ci ml⁻¹) or [U-¹⁴C]glucose (0.2 μ Ci ml⁻¹; where 1 μ Ci = 37 kBq), respectively.

Labelling and fractionation of cell wall polysaccharides. An exponential phase culture of *G. lactis* was harvested and the mycelium resuspended at a concentration of about 1 mg dry wt ml⁻¹ in fresh YED medium supplemented with D-[U-¹⁴C]glucose (0.4 µCi ml⁻¹). Samples were taken at appropriate time intervals and carrier mycelium (1 g wet wt) was added to the radioactive samples and then submitted to mechanical breakage as previously described (Pérez *et al.*, 1981). Cell walls were washed with water by centrifugation at 750 g for 5 min several times and then extracted twice with 5 ml 6% (w/v) NaOH for 90 min at 80 °C. The precipitation of galactomannan from the extract with Fehling reagent was performed as described by Algranati *et al.* (1966). Alkali-soluble glucan and galactomannan were precipitated together by addition of 2 vol. absolute ethanol to the alkali extract. The difference between the counts obtained in the ethanol-precipitated pellet and that produced by the Fehling reagent was taken to represent alkali-soluble glucan. Previous experiments showed that no glycogen was present in any fraction. The residue remaining after NaOH extraction was washed by centrifugation until the pH was neutral and resuspended in 1.5 ml water. Samples of 0.4 ml were incubated for 48 h at 30 °C with 60 milliunits of chitinase (one milliunit is defined as the amount of enzyme that releases 1 nmol *N*-acetylglucosamine equivalents min⁻¹ at 30 °C) or 100 µg zymolyase in 50 mM-phosphate buffer at pH 6.3, in a final volume of 0.5 ml. A sample without enzyme was always included as a control. After incubation the samples were centrifuged, washed and counted. The residues produced after digestion with chitinase or zymolyase were taken to represent alkali-insoluble glucan or chitin, respectively.

Other methods. Radioactivity in insoluble samples was determined with a scintillation mixture containing a thixotropic gel (Keller & Cabib, 1971) using a Beckman LS-8100 scintillation counter. Cell-free extracts were obtained as in Pérez *et al.* (1981). Chitin and glucan synthase activities were assayed and determined essentially as previously described (Durán & Cabib, 1978; Shematek *et al.*, 1980). Protein was measured by the Lowry method. The morphology of the fungus was monitored in a Leitz phase-contrast microscope.

RESULTS

Effect of papulacandin B on fungal growth

Addition of papulacandin B at sub-lethal concentrations (i.e. 6 µg ml⁻¹) to an exponential phase culture of *G. lactis* resulted in a reduction of the extension rate and the appearance of protuberances along the hyphae (Fig. 1*a*) that continued to grow and branch dichotomously at least four to five times (Fig. 1*b*). By contrast, branches in the control cultures were not as frequent and appeared almost exclusively behind septa, never at the tips (Fig. 1*c*; Fiddy & Trinci, 1976). Similar morphological alterations were produced by aculeacin A (Fig. 1*d*), another antifungal antibiotic that inhibits 1,3-β-glucan synthase in *G. lactis* (Pérez *et al.*, 1981). These morphological changes, however, did not alter growth excessively. Thus, after culturing for 6 h in the presence of 6, 10 and 30 µg antibiotic ml⁻¹, yields of 4.3, 4.2 and 3.4 mg dry wt ml⁻¹, respectively, were obtained as compared to 4.5 mg dry wt ml⁻¹ in the control (without antibiotic).

The incorporation of uracil and amino acids from cultures supplemented with 6 µg papulacandin B ml⁻¹ reached values similar to those of the control while incorporation of glucose was slightly inhibited (Fig. 2). Concentrations of antibiotic higher than 60 µg ml⁻¹ resulted in considerable lysis of the mycelium and a substantial decrease in growth (results not shown).

Effect of papulacandin B on synthesis of cell wall polysaccharides

It was important to ascertain whether the action of papulacandin B would specifically affect β-glucan synthesis *in vivo*. Papulacandin B (6 µg ml⁻¹) was added to a growing culture of *G. lactis* followed 30 min later by the addition of [¹⁴C]glucose. Incorporation of label into the galactomannan and chitin fractions of antibiotic-treated mycelium was unaffected, whereas incorporation into the glucan fractions (both alkali-insoluble and alkali-soluble) was reduced by 76% and 47%, respectively (Table 1). Similar results were obtained in two additional independent experiments.

Cell wall synthase activity levels and growth pattern of mycelium derived from papulacandin B treated arthrospores

Suspensions of arthrospores in YED medium (4 × 10⁶ cells ml⁻¹) were incubated in the presence of papulacandin B (10 µg ml⁻¹) at 30 °C. After 90 min the arthrospores were washed twice and used as inocula for 500 ml flasks containing 125 ml YED medium. Cultures were

Fig. 1. Phase-contrast micrographs of *G. lactis* mycelium grown in the presence of papulacandin B ($6 \mu\text{g ml}^{-1}$) or aculeacin A ($18 \mu\text{g ml}^{-1}$). (a) Protuberances along the hyphae after papulacandin B treatment. (b) Dichotomous branching of mycelium after papulacandin B treatment. (c) Control culture without antibiotic. (d) Mycelium grown in the presence of aculeacin A. The bar markers represent $10 \mu\text{m}$.

harvested after 6, 12 and 18 h and cell wall synthase activities determined. The activities of 1,3- β -glucan synthase in cell-free extracts from 6 and 12 h cultures of papulacandin B-treated arthrospores were 43% and 63% of the control values, respectively, whereas that of 18 h cultures was not inhibited (Table 2). Chitin synthase activity remained essentially unaltered in every case. Mycelium from 6 and 12 h cultures exhibited dichotomous branching at the tips; however, the branching pattern in 18 h mycelium was similar to that observed in the control. Samples from 12 h cultures were resuspended in fresh YED medium supplemented with [^{14}C]glucose and incorporation of label into cell wall fractions was measured after 45 and 90 min of growth. Incorporation into glucan fractions from pre-treated arthrospores was 63% for the alkali-insoluble glucan and 68% for the alkali-soluble glucan as compared to the control, whereas incorporation into galactomannan and chitin was similar in both treated and untreated cultures (data not shown).

Effect of L-sorbose on the 1,3- β -glucan synthase and growth of G. lactis

Although sorbose medium induced 'colonial' growth (as described by de Terra & Tatum, 1961) in *G. lactis*, the addition of L-sorbose (26 mM) to the 1,3- β -glucan synthase assay system only resulted in a slight inhibition of the activity (data not shown). However, cell-free extracts from *G. lactis* grown in sorbose medium showed a reduced level of 1,3- β -glucan synthase activity as compared with that from cultures grown in YED medium; the chitin synthase value was even higher than the one detected in YED medium (Table 3). These values were obtained from exponential cultures with doubling times in YED and sorbose medium of 1.55 and 1.70 h,

Fig. 2. Effect of papulacandin B on (a) glucose incorporation, (b) protein synthesis and (c) RNA synthesis in *G. lactis*. ○, Control; ●, treated with papulacandin B ($6 \mu\text{g ml}^{-1}$).

Table 1. *Effect of papulacandin B on the incorporation of the radioactivity from $[^{14}\text{C}]$ glucose into cell wall polysaccharides*

Papulacandin B was added at a concentration of $6 \mu\text{g ml}^{-1}$. Figures in parentheses are percentages of control values.

Cell wall fraction	$[^{14}\text{C}]$ Glucose incorporated [c.p.m. (mg cell dry wt) $^{-1}$]			
	Control		Plus papulacandin B	
	60 min	120 min	60 min	120 min
Alkali-insoluble glucan	928	1536	161 (17%)	372 (24%)
Alkali-soluble glucan	558	765	400 (72%)	402 (53%)
Chitin	1315	1857	1502	1821
Galactomannan	705	1247	668	1279

respectively. 1,3- β -Glucan synthase activity from sorbose cultures was inhibited *in vitro* by papulacandin B to a similar extent as that in YED medium cultures (Table 3). Cellobiose, another inducer of 'colonial' growth in several fungi (Wilson, 1970), and other L-sugars were neither inhibitors of the synthase nor inducers of morphogenic effects in *G. lactis* (results not shown).

Table 2. Cell wall synthase activity of *G. lactis* mycelium grown from arthrospores pretreated with papulacandin B

Geotrichum lactis arthrospores were treated with papulacandin B ($10 \mu\text{g ml}^{-1}$). After 90 min the arthrospores were washed and used as inoculum. Samples of mycelium were harvested at appropriate times and the synthase activities measured in the corresponding particulate fraction. The values presented are means from two experiments.

Enzyme	Treatment	Specific activity [milliunits (mg protein) $^{-1}$]*		
		6 h	12 h	18 h
1,3- β -Glucan synthase	Control	2.1	2.7	3.3
	Papulacandin B	0.9	1.7	3.3
Chitin synthase	Control	10.2	8.0	7.1
	Papulacandin B	8.7	8.1	7.3

* One milliunit is defined as the amount of enzyme that incorporates $1 \text{ nmol substrate min}^{-1}$ into product.

Table 3. Cell wall synthase activity of *G. lactis* grown in glucose or L-sorbose medium, and its inhibition by papulacandin B

Growth medium contained 1% (w/v) yeast extract and 2% (w/v) of the corresponding sugar. The final concentration of papulacandin in the *in vitro* assay was $18 \mu\text{M}$. The values presented are the means and standard deviations calculated from four independent experiments.

Growth medium	1,3- β -Glucan synthase activity [milliunits (mg protein) $^{-1}$]*		Chitin synthase activity [milliunits (mg protein) $^{-1}$]*
	Control	Plus Papulacandin B	
Glucose	3.4 ± 0.29	1.0 ± 0.10	6.8 ± 1.0
L-Sorbose	2.0 ± 0.21	0.6 ± 0.07	8.5 ± 1.1

* For definition of milliunits see footnote to Table 2.

DISCUSSION

Analysis of cell wall components of *G. lactis* grown in the presence of papulacandin B clearly indicates that synthesis of β -glucan is partially inhibited. Under these conditions RNA and protein synthesis is slightly greater than in control cultures while growth yields, after 6 h of growth and even longer, are almost identical (results not shown). Therefore it seems that growth is not significantly altered.

Parallel to the inhibition of β -glucan synthesis, papulacandin B induces branch proliferation and 'colonial' growth. Aculeacin A, another antifungal antibiotic that specifically inhibits 1,3- β -glucan synthase in *G. lactis* (Pérez *et al.*, 1981) also induces morphological alterations similar to the ones described for papulacandin B. Likewise, L-sorbose, a known inducer of morphological effects in *Neurospora crassa* (Mishra & Tatum, 1972), reduces the 1,3- β -glucan synthase activity levels of *G. lactis in vivo* and induces 'colonial' growth. On the other hand the results in Table 2 indicate that inhibition of β -glucan synthesis still persists for up to several hours after removal of the antibiotic. Recovery of β -glucan synthesis and restoration of a morphologically normal pattern of growth seems to occur simultaneously. This recovery is not explained by the development of resistance to the antibiotic as the fungus remains sensitive to further additions of papulacandin B. The data presented suggest that inhibition of β -glucan synthesis and 'colonial' growth in *G. lactis* may be correlated. A rationale for this correlation is suggested. Trinci (1979) has proposed a hypothesis to account for the regulation of branch initiation in fungi. According to this, apical branching as well as lateral branching in the absence of septation may occur when the rate of supply of components required for primary wall growth (i.e. vesicles, wall precursors

and enzymes which synthesize or lyse wall polymers) exceeds the rate at which they can be incorporated into the tip wall. Papulacandin B, by inhibiting 1,3- β -glucan synthesis, would reduce the rate of incorporation of this material into the wall (β -glucan is quantitatively and qualitatively an important structural component). This unbalanced situation with a relative excess of hydrolytic enzymes (i.e. β -glucanases) would weaken the wall and consequently branching would occur mainly at the tip and also along the hypha. The reason why branches are initiated at precise locations has yet to be understood.

It may be relevant to mention that growth of *Schizosaccharomyces pombe* in the presence of aculeacin A induces swelling of the cells at the growing pole (Miyata *et al.*, 1980) and that papulacandin B induces both inhibition of 1,3- β -glucan synthesis *in vivo*, and an identical morphological effect (R. Varona & A. Durán, unpublished results).

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