Change of cell wall chitin content in amphotericin B resistant Kluyveromyces strains

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Abstract

The culture of two Kluyveromyces species, Kluyveromyces lactis (ATCC 96897) and Kluyveromyces bulgaricus (ATCC 96631), in the presence of subinhibitory doses of amphotericin B leads to the selection of mutants which are resistant to this polyene. The mutants show an alteration of their cell wall composition with the main change corresponding to an increase of chitin. The enzyme activities involved in the metabolism of this polymer, i.e. chitin synthases and chitinase, were measured. The results demonstrate that in both mutants the activity of chitinase was drastically decreased by 99% in comparison with the activity measured in the corresponding wild-type strain while no significant change of the chitin synthase I, II and III activities could be detected.

Keywords: Amphotericin B; Chitin content; Yeast cell wall

1. Introduction

Yeast cell walls are essential for the maintenance of cell shape, prevention of lysis, and regulation of the uptake of substances from the environment. In spite of its apparent rigidity, the yeast cell wall is a dynamic structure that can be modulated in response to different physiological states (e.g., budding, mating, and sporulation) or to morphological changes, such as in Candida albicans during the transition from yeast to hyphal form [1]. Chitin is an essential structural component of the cell wall but is usually present at a very low level [2]. Chitin deposition is spatially and temporally regulated throughout the yeast cell cycle and life cycle [1,3]. Three chitin synthase activities (CSI, CSII, and CSIII) have been described, each having a distinct function. Mutations that affect chitin-synthesis cause osmotic sensitivity [4,5], abnormal morphology, aggregation, and growth arrest with elongated buds [2,6].

CSI is thought to be a repair enzyme, which synthesizes chitin in response to an acid-induced increase in chitinase activity after separation of mother and daughter cells [7]. CSII is localized in the mother–bud junction and synthesizes chitin in the primary septum [2,6,8]. CSIII activity is required for the formation of the chitin ring at the base of the bud; this enzyme is also responsible for the synthesis of more than 90% of the chitin in the cell wall [2,4]. CSIII is also required for chitin deposition in the lateral wall during vegetative growth, as well as for chitin synthesis during mating and sporulation.

Chitinases belong to a group of enzymes capable of degrading chitin directly to low molecular mass products. The enzymatic degradation of chitin appears to occur in two steps, which are similar in prokaryotes and eukaryotes. An endochitinase reduces the polymer to oligomers, which are subsequently degraded to monomers by exochitinase [9].

It is known that the resistance to amphotericin B (Amb) is due to an alteration of the membrane [10]. However, several authors observed that some cell wall constituents
were also involved in the sensitivity or resistance of the cells to AmB [11,12].

Studying the cell wall composition of two AmB resistant Kluyveromyces strains, results indicated that the chitin levels were 6–9-fold higher than that in the respective wild-type strains.

Two types of enzymes are responsible for the level of chitin in the cell wall and a change of activity of one of them may explain the alteration of the chitin content.

In the present study we have investigated these two types of enzyme activities, i.e. CSI, CSII and CSIII, located in the membrane [7], which catalyse the synthesis of this polymer, and chitinase, located in the cell wall [9,13], and involved in the catabolic process.

2. Materials and methods

2.1. Strains and growth conditions

Kluyveromyces bulgaricus (Kb, ATCC 96631) and Kluyveromyces lactis (Kl, ATCC 96897), were isolated from dairy products. K. lactis mutant (Klm, ATCC 96896), an AmB resistant strain, was obtained according to Hakkou et al. [14]. In the present work a similar process was applied in the K. bulgaricus (Kb) strain to obtain an AmB resistant Kbm strain. To induce the chitinase, 2% (w/v) glucose was substituted by 2% (w/v) chitin.

2.2. Cell wall preparation

A 1-ml sample of overnight culture (approximately $2 \times 10^7$ cells) was transferred to a 1.5-ml Microfuge tube and pelleted by centrifugation. The pellet was suspended in 200 µl of solution A (0.8% NaCl, 0.02% KCl, 0.12% Na2HPO4, 0.02% KH2PO4, containing 0.1% Triton X-100). Glass beads (0.45–0.5 mm diameter) were added to a level of approximately 2 mm below the meniscus and the cells were mixed vigorously for 2 min [9]; this treatment was repeated at least four times. After each mixing the broken cell slurry was recovered with a pipette and the glass beads were washed two times with 0.5 ml of solution A supplemented with Triton X-100. Washings were pooled with the original slurry and the cell walls pelleted. One part of the pelleted cell walls was washed twice with distilled water and lyophilized. This part was used for the chitin quantitation, the other part was used for chitinase activity estimation.

2.3. Cell wall chitin quantitation

The chitin content was estimated after acid and alkali treatment [15,16]. Cell wall acid–alkali insoluble fractions (20 mg) were suspended in 2 ml of 6 N HCl and heated in sealed tubes at 105°C for 12 h. After lyophilization, the hydrolysates were dissolved in distilled water and the liberated glucosamine was estimated [17].

2.4. Chitinase assays

For chitinase estimation in cell walls, the fresh cell wall pellets were washed again two times with 1 ml of solution A supplemented with Triton X-100 and finally suspended in 200 µl of 0.1 M sodium citrate buffer pH 3.0.

A sample of 20 µl of culture medium, cell wall or whole cell suspension was mixed with 80 µl of 250 µmol 4-methylumbelliferyl chitosinose in 0.1 M sodium citrate buffer, pH 3.0 and incubated at 30°C for 1 h. The mixture was then diluted into 2.9 ml of 0.5 M glycine, NaOH buffer, pH 10.4. The residue was removed by centrifugation, and the liberated 4-methylumbelliferone measured using a SPEX Fluorolog II fluorescence spectrophotometer (excitation at 350 nm, emission at 440 nm). Units of chitinase activity are expressed as nanomoles of 4-methylumbelliferone released per min per mg of protein.

2.5. Chitin synthase extraction and assays

The chitin synthases extraction was achieved according to the procedure described previously [18]. The synthase activities of CSI, CSII and CSIII were assayed according to [18] with a slight modification, i.e. UDP [14C]GlcNAc was replaced by 0.15 µg ml⁻¹ 6-O-dansyl-N-acetylglucosamine (DNAG).

2.6. General chemical and analytical procedures

DNAG was synthesized in three steps from N-acetylglucosamine according to the procedure described previously [19]. The anomeric hydroxyl function was first protected by a tert-butyl carbonate group. Condensation of di-tert-butyl carbonate (1.18 g, 5.41 mM) on the N-acetylglucosamine (1 g, 4.52 mM) in the presence of triethylamine (548 g, 5.41 mM) and a catalytic amount of dimethylaminopyridine was achieved in a dioxane–water mixture (1:1 v/v) at 20°C for 4 h. Then, the solvent was evaporated to dryness under reduced pressure. The corresponding carbonate (1.21 g) was isolated in 83% yield after flash chromatography on silica gel. The $^1$H NMR (AMX 250 Brucker) showed the anomeric proton $\mathrm{H}_1$ at 5.9 ppm (d, $J = 3.66$ Hz) typical for the α configuration. Then this compound (1.19 g, 3.7 mM) was dissolved in cold pyridine (0°C) and 5-dimethylaminonaphthalene-1-sulfonyl chloride (dansyl chloride, 0.997 g, 3.7 mM) was added dropwise. After stirring for 12 h at room temperature the solvent was evaporated under reduced pressure, the residue was poured in ether, the precipitate was filtered off and compound 3 was obtained as a yellow solid in 60% yield after flash chromatography on silica gel (eluent ethyl acetate/methanol (AcOEt/MeOH) 95/5). $^1$H and$^{13}$C NMR spectra (performed in deuterated chloroform: CDCl₃) were in...
good agreement with the regiospecific sulfonation at carbon 6. Treatment of compound 3 (1.20 g) with a mixture of ethylene chloride CH$_2$Cl$_2$/trifluoroacetic acid 1/1 for 1 h provided the desired DNAG 4 (α/β anomic mixture 25/75) in quantitative yield. Compound 4 was purified by chromatography on silica gel (eluent AcOEt/MeOH 85/15), then recrystallized in MeOH/ether. 1H and 13C NMR and MS studies were in very good agreement with the proposed structure.

Hexosamine was estimated following the micro-method [17] after hydrolysis of the samples under vacuum with 6 M HCl at 105°C for 12 h.

Protein concentration was estimated by the bicinchoninic acid method [20] using the protein assay kit of Sigma (St. Louis, MO, USA) following Sigma procedure No. TP RO562.

### 3. Results and discussion

#### 3.1. Growth of the yeasts in the presence of AmB and phenotype of the resistant strains

The yeast strains were grown in the presence of AmB MIC (minimal inhibitory concentration), i.e. 10 mg l$^{-1}$ for *K. lactis* and 1 mg l$^{-1}$ for *K. bulgaricus*. For *K. lactis* one generation in the presence of AmB MIC was sufficient to obtain the resistant *K. lactis* mutant strain, which is 10 times more resistant to AmB than the parental strain [14]. The use of concentrations lower than the MIC did not result in the appearance of AmB resistant mutants. In the *K. bulgaricus* strain four successive subcultures in the presence of AmB MIC were necessary to isolate resistant mutants. This result shows a lower level of adaptation of

![Fig. 1. Cell growth and cell wall chitinase activity in Kl, Klm, Kb and Kbm. A: *K. lactis*; B: *K. lactis* AmB resistant mutant; C: *K. bulgaricus*; D: *K. bulgaricus* AmB resistant mutant. △, chitinase activity; □, cell growth (absorbance at 620 nm).](image)

### Table 1

<table>
<thead>
<tr>
<th>Strains</th>
<th>Kb</th>
<th>Kbm</th>
<th>Kl</th>
<th>Klm</th>
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<tbody>
<tr>
<td>CSI</td>
<td>4 (± 0.02)</td>
<td>0.50 (± 0.02)</td>
<td>0.49 (± 0.01)</td>
<td>0.53 (± 0.02)</td>
</tr>
<tr>
<td>CSII</td>
<td>1.04 (± 0.02)</td>
<td>0.42 (± 0.01)</td>
<td>0.41 (± 0.02)</td>
<td>0.36 (± 0.01)</td>
</tr>
<tr>
<td>CSIII</td>
<td>1.06 (± 0.03)</td>
<td>0.62 (± 0.02)</td>
<td>0.54 (± 0.02)</td>
<td>0.20 (± 0.01)</td>
</tr>
</tbody>
</table>

Activity values are expressed as nanomoles of DNAG released per min per mg of protein.

this strain to AmB than that of K. lactis. The resistant K. bulgaricus mutant is 5 times more resistant to AmB than the parental wild-type strain.

In control medium the growth of neither the Kbm nor the Klm mutant strain was similar to that of the wild-type strains. Following the growth curves (Fig. 1), the generation times were 4 h 40 min and 3 h 40 min for K. bulgaricus and K. lactis respectively and 5 h 30 min and 4 h 20 min for Kbm and Klm respectively, with a 15% and 10% reduction of biomass at the end of the growth in Kbm and Klm respectively. During culture, the pH value of the medium decreased from 6.8 to 4.5 for all the strains.

The AmB resistant strains stay resistant to this polyene even after 10 cultures in control medium without AmB. All the strains were conserved at 4°C on Sabouraud solid medium.

3.2. Level of cell wall chitin

The amounts of chitin in the cell walls are reported in Fig. 2. The two mutant strains show a significant increase of chitin in their cell walls. In Kbm and Klm the chitin level is 6- and 9-fold higher than in K. bulgaricus and K. lactis respectively.

In a recent paper we reported a change in the membrane fluidity in an AmB resistant mutant of K. lactis (Klm) [21]. We speculated that this change could be responsible for a modification of the activities of membrane linked enzymes such as the three chitin synthases. We therefore thought to explore the activities of the enzymes involved in the metabolism of this polymer.

3.3. Chitin synthases

To assess whether the increase in cell wall chitin content in the AmB resistant strains was due to an increase in the activity of one or other of the three chitin synthases, the separate enzymatic activities of CSI, CSII and CSIII were measured in vitro. Higher activities of the three enzymes were observed in K. bulgaricus by comparison to the other strains (Table 1). CSI exhibited 8 times higher activity than in other strains. CSII was 2.5 times higher and CSIII was 1.7–5 times higher in the other strains. In K. lactis results showed just a slight increase of CSI activity in Klm compared to that of K. lactis while CSII and CSIII activities were 1.1 and 2.7 times lower. Such a small increase of CSI activity may partially explain a higher chitin content, but in general these activities may not be responsible for the very high content of this polymer in the cell walls of this mutant.

3.4. Induction and cellular distribution of chitinase

As chitin synthase activities cannot explain the increase of chitin in the mutant cell walls, it was necessary to check the chitinase activities present in the cell walls, culture media and whole cells. When the four Kluyveromyces strains were grown in control medium containing glucose as carbon source, no chitinase activity could be measured in the cell walls, culture media and whole cells. Chitinase was initially described as an adaptive enzyme, whose activity can be measured only when chitin is present in the culture medium as carbon source. Under such growth conditions the four Kluyveromyces strains expressed chitinase activities as reported in Table 2. Results show that these activities were 3.1–3.5 and 2.1–2.5 times higher in cell walls and in whole cells than in the culture medium for the two K. bulgaricus and K. lactis wild-type strains. However, the chitinase activities were 10-, 75- and

<table>
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<th>Table 2</th>
<th>Chitinase activities in Kb, Kbm, Kl and Klm</th>
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<tr>
<td>Chitinase localization</td>
<td>Strains</td>
</tr>
<tr>
<td>Kb</td>
<td>Kbm</td>
</tr>
<tr>
<td>Culture media&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.25 (± 1)</td>
</tr>
<tr>
<td>Whole cells&lt;sup&gt;b&lt;/sup&gt;</td>
<td>41.2 (± 4)</td>
</tr>
<tr>
<td>Cell walls&lt;sup&gt;c&lt;/sup&gt;</td>
<td>47 (± 4)</td>
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</table>

Activity values are expressed as nanomoles of 4-methylumbelliferone released per min.

<sup>a</sup>Per mg of protein in the cell wall fractions or culture media.

<sup>b</sup>Per 10⁷ whole cells.

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Fig. 2. Chitin content in Kb, Kbm, Kl and Klm cell walls. The levels of chitin are expressed in mg per g of lyophilized yeast cell walls.
100-fold lower respectively in culture medium, whole cells and cell walls in both Kbm and Klm resistant strains than in the corresponding wild strains.

3.5. Chitinase production in relation with yeast cell growth

The rate of growth and time of cell wall chitinase production were determined in the four strains. Fig. 1 shows that for all the strains the maximum of growth in chitin supplemented medium was observed after 9 h of incubation at 25°C. Chitinase production preceded cell growth by 2 h, then the rate of activities was parallel to growth and maximum production occurred when growth reached the stationary phase. These results were found in both K. bulgaricus and K. lactis wild-type strains. In the corresponding mutant strains chitinase activity reminded very low during the whole growth. These results confirmed that in the two mutant strains chitinase activity is drastically decreased.

We can therefore exclude the possibility that the increase in the chitin level in the cell walls results from an induction of chitin synthase enzymes. This notion is supported by previous results [22], which show the non-competitive inhibition of purified chitin synthetase by a high concentration of AmB.

It is relevant to note here that a recent study showed an increase in chitin levels in response to a cell wall stress [23,24]. However, there is less information about the enzymes involved in the response of stress-related chitin synthesis. In this study we have shown that the increase in chitin level in cell walls of the mutant corresponds mainly to a repression or diminution of chitinase activities. However, the precise relationship between AmB resistance and cell wall chitin content remains to be demonstrated.

References