Yeast ascospore wall assembly requires two chitin deacetylase isozymes

Anna Christodoulidou[a,*], Peter Briza[b], Adi Ellinger[c], Vassilis Bouriotis[a]

[a] *Enzyme Technology Division, Institute of Molecular Biology and Biotechnology and the Division of Applied Biology and Biotechnology, Department of Biology, University of Crete, Heraklion, Greece*
[b] *Institut für Genetik und Allgemeine Biologie, University of Salzburg, Salzburg, Austria*
[c] *Histologisch-Embryologisches Institut, University of Vienna, Vienna, Austria*

Received 13 September 1999

Abstract Chitin deacetylases are required for spore wall rigidity in *Saccharomyces cerevisiae*. Two chitin deacetylase genes (*CDA1* and *CDA2*) have been identified in yeast. In this report we studied the biochemical properties of the chitin deacetylases encoded by *CDA1* and *CDA2* and we show how their elimination directly affects the ascospore wall assembly.

Key words: Chitin deacetylase; Chitosan; Spore wall; Chitin

1. Introduction

It has been shown that the *Saccharomyces cerevisiae* ascospore walls are well ordered structures. Four layers can be distinguished in electron micrographs. Two innermost layers, made primarily of glucan, a 95% chitosan layer and an outermost proteinaceous layer rich in dityrosine form these walls. The two outer layers confer on the spores the characteristic resistance against environmental stress. [1,2]

Chitin deacetylase (CDA) is the enzyme that catalyzes the conversion of chitin to chitosan by deacetylation of *N*-acetyl-D-glucosamine residues. So far, CDA enzymes have been isolated from the fungi *Mucor rouxii* and *Colletotrichum lindemuthianum* [3,4] and genes encoding CDA have been studied in *M. rouxii* and *Saccharomyces cerevisiae* [5,6].

In order to evaluate the role of these enzymes in the formation of the yeast ascospore wall we isolated and studied the corresponding genes. Two genes are responsible for the chitin deacetylase activity in yeast, *CDA1* and *CDA2*. The transcription of these genes is restricted to a distinct time during the process of sporulation. Strains mutated in both genes are viable but fail to emit the natural fluorescence imparted by the dityrosine residues of the outermost wall layer and are sensitive to hydrolytic enzymes, organic solvents and heat shock. Strains mutated on either gene still exhibit fluorescence and chitin deacetylase activity [6].

In this report we distinguish for the first time the enzymatic activity of Cda1p and Cda2p according to their properties and further investigate their role in the formation of the yeast spore wall.

2. Materials and methods

2.1. Strains and growth media

The strains used for the construction of the disruption alleles of *CDA1* and *CDA2* were CEN.PK1 (MATa, *ura3, leu2, his3*), CEN.PK2 (MATα, *ura3, leu2, trp1*), and their diploid derivative CEN.PK2D (MATα/MATa, HIS3/his3 *ura3*/*ura3, trp1*/TRP1, *leu2*/*leu2*). Vegetative cultures were grown on YEPD (1% yeast extract, 2% bactopeptone, 2% glucose), YEPA (1% yeast extract, 2% bactopeptone, 2% potassium acetate), minimal medium (0.7% Difco yeast nitrogen base without amino acids and 2% galactose) supplemented with the appropriate amino acids. Synchronous sporulation was carried out as follows: cells were grown in YEPA to a density of 3×10^7 cells/ml, washed and resuspended in 1% potassium the double density. The cultures were maintained at 30°C under vigorous shaking. The efficiency of sporulation of strain CEN.PK2D was 75% after 36 h.

2.2. Gene disruptions in the yeast genome of strain CEN.PK2

Gene disruptions were performed as described in [6].

2.3. Chitin deacetylase assays

These were performed as described in [3].

2.4. Protein determination

Protein content was determined according to the method of Lowry et al. [7] and equal amounts were used in the radiometric assay described above.

2.5. Dityrosine fluorescence of sporulated colonies

Dityrosine was determined as described by Briza et al. [2].

2.6. Glucosamine determination

Total glucosamine and glucosamine remaining after treating the spores with HNO_2 were determined as described by Wagner et al. [12].

2.7. Electron and fluorescence microscopy

Electron and fluorescence microscopy were carried out according to Briza et al. [8]

3. Results

In a previous study we showed that *CDA1* and *CDA2* are located in the genome of *S. cerevisiae* in a head to tail orientation with an intervening sequence of 1.5 kb between their open reading frames. These two genes are 57% identical and 72% similar throughout their amino acid sequences. Both genes are expressed in a developmentally regulated way as assessed by RNA accumulation analysis and deacetylation activity assays. So far it appears that the function of the two enzymes Cda1p and Cda2p is redundant [6]. In order to further analyze the role of each enzyme and gain some insight into the reason for their conservation, we further studied the biochemical properties of the two enzymes and the effect of their action on the formation of spore walls.

3.1. Time course of chitin deacetylase expression during vegetative growth and sporulation

Since the CDA genes are transcribed at comparable levels under the control of a heterologous promoter during vegetative growth [6,9], we measured the activity of Cda1p and Cda2p at different time points of ectopic expression (Fig. 1)

*Corresponding author. Present address: Laboratoire de Biologie Cellulaire, Institut National de la Recherche Agronomique, Route de Saint-Cyr, 78026 Versailles Cedex, France. Fax: (33) (1) 30833099. E-mail: christod@versailles.inra.fr

PII: S0014-5793(99)01334-4

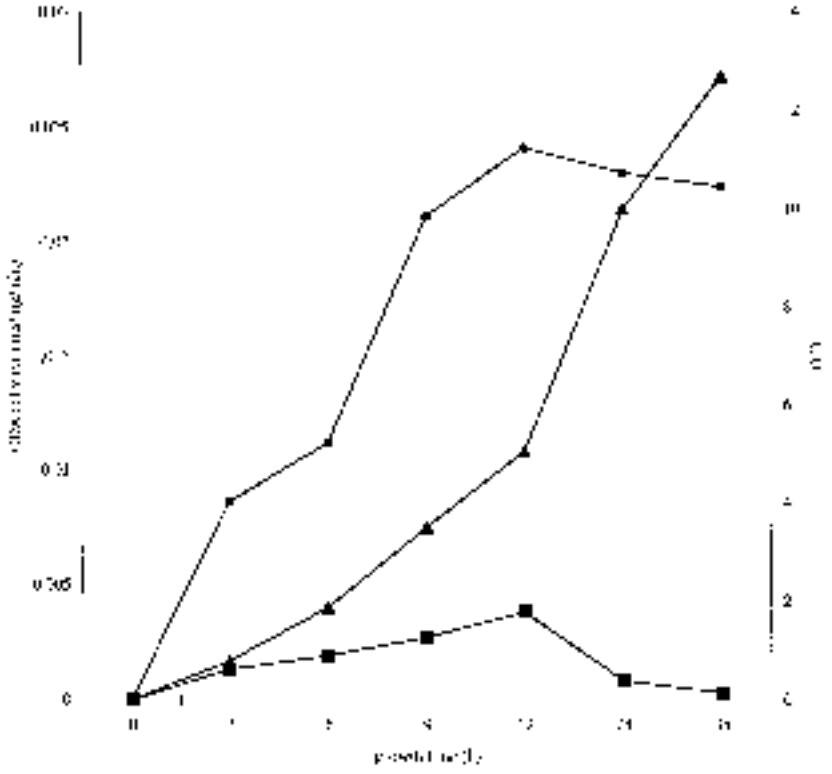

Fig. 1. Enzymatic activity of Cda1p and Cda2p during growth in galactose-containing medium. *CDA1* and *CDA2* were ectopically expressed under the control of the *GAL1* promoter. Cda2p (triangles) and Cda1p1 (squares) relative activity assays. The change of the optical density of the cultures during time is also shown (circles).

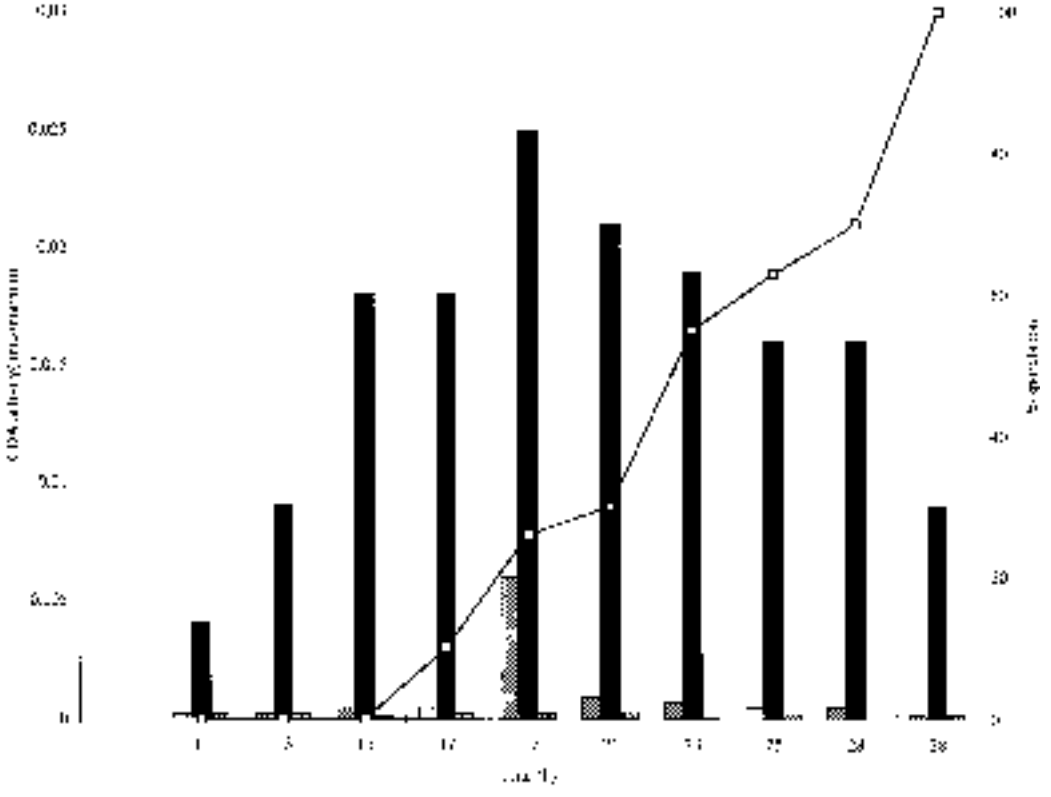

Fig. 2. Relative sporulation-specific expression of *CDA1* (gray bars) and *CDA2* (black bars) as determined by CDA activity assays in the Δ*cda1* and Δ*cda2* deletion strains at various times after transfer to sporulation medium. As control CDA activity of the double disrupted strain Δ*cda1*Δ*cda2* was measured (white bars). The percentage of sporulation was monitored by light microscopy (line).

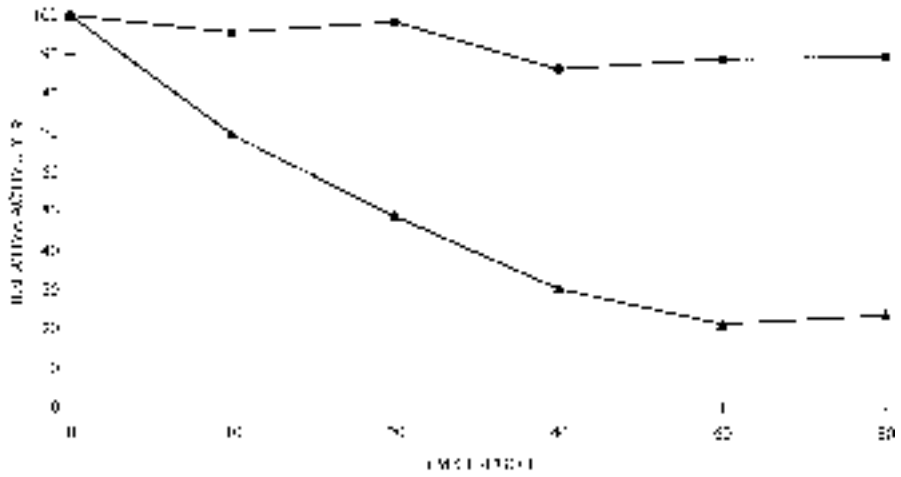

Fig. 3. Effect of acetic acid on Cda1p and Cda2p activity. Relative activity of Cda1p (triangles) and Cda2p (circles) was determined after addition of various concentrations of acetic acid in the reaction mixtures.

The activity of both enzymes increased during the logarithmic phase of growth. At stationary phase Cda1p lost its activity while the activity of Cda2p still increased.

In order to detect any differences in the activity of Cda1p and Cda2p under sporulation conditions, we measured the activity of each enzyme at different time points (Fig. 2). We detected maximum activity of both enzymes around 19 h after transfer to sporulation medium, when the sporulation efficiency was about 26%, which is in good agreement with the time of maximum total CDA activity [6]. However, while the activity of Cda2p appeared as early as at 11 h and remained at high levels even after the completion of sporulation, the activity of Cda1p increased abruptly between 17 and 19 h and almost disappeared at later stages of sporulation.

3.2. Effect of acetic acid on chitin deacetylase activity

The activity of only some deacetylases [3,4,10,11] is inhibited by acetic acid. We therefore measured the activity of both CDAs by adding increasing concentrations of acetic acid in the reaction mixture (Fig. 3). We observed only a slight decrease of the activity of Cda2p even at a concentration of 80 mM acetic acid. Cda1p, on the other hand, was already inhibited at a concentration of 10 mM acetic acid and lost 80% of its activity at 80 mM.

3.3. Effects of the CDA mutations on the composition of yeast spore walls

In previous studies [2,6] it was speculated that the deacetylation of the chitin layer by the action of the two chitin deacetylases plays an important role in the subsequent addition of the external dityrosine-rich layer of the walls. In order to investigate this role we compared the levels of dityrosine, total glucosamine and glucosamine corresponding to chitosan in wild type and *CDA* deletion strains. The presence of dityrosine was shown to be dependent on the existence of deacetylated chitin. In the presence of Cda2p, when most of the existing chitin is deacetylated, 75% of the wild type dityrosine was found. In the presence of Cda1p only 20% of the total chitin was deacetylated and 50% of the wild type dityrosine was found. In the double disrupted strain we found only very small amounts of chitosan and dityrosine. Surprisingly, a linear reduction of the total glucosamine was observed in all CDA mutants. The double disruption strain contained only 25% of the total glucosamine of the wild type strain (Fig. 4).

3.4. Phenotypic analysis of CDA mutant strains

Electron microscopy was done in order to show the effects of CDA mutations on the spore wall assembly (Fig. 5). In the wild type strain the chitosan layer was tightly associated with the electron-dense surface layer. These layers appeared very compact and they were hardly distinguishable. In the mutant strain lacking *CDA1*, the chitosan layer was more diffuse and less compact, while the surface layer remained intact. In the *CDA2* disruption strain, the chitosan layer was not detectable (although chemical data indicated that there still was 50% glucosamine, compared to wild type). The surface layer appeared ruptured. In the spore walls of the Δ*cda1*Δ*cda2* strain both outer layers were missing. Instead we observed electron-dense globules accumulating on the surface of the spores. These globules were observed in other sporulation mutants

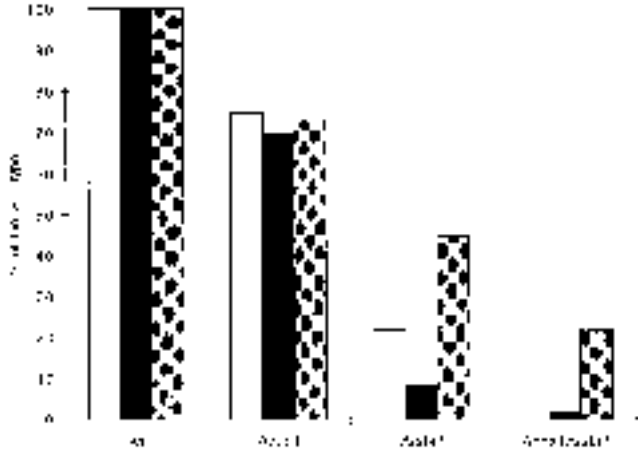

Fig. 4. Dityrosine, glucosamine and total glucosamine of the mutant strains compared to the wild type. Absolute values of the wild type correspond to 1.25, 13.8 and 18 nmol/10^8 cells respectively. Dityrosine (white bars) and total glucosamine (gray bars), corresponding to the sum of chitin and chitosan, were measured on hydrolysates of yeast spores while glucosamine (black bars), corresponding to chitosan, was measured as the HNO_2-soluble fraction of the hydrolysates.

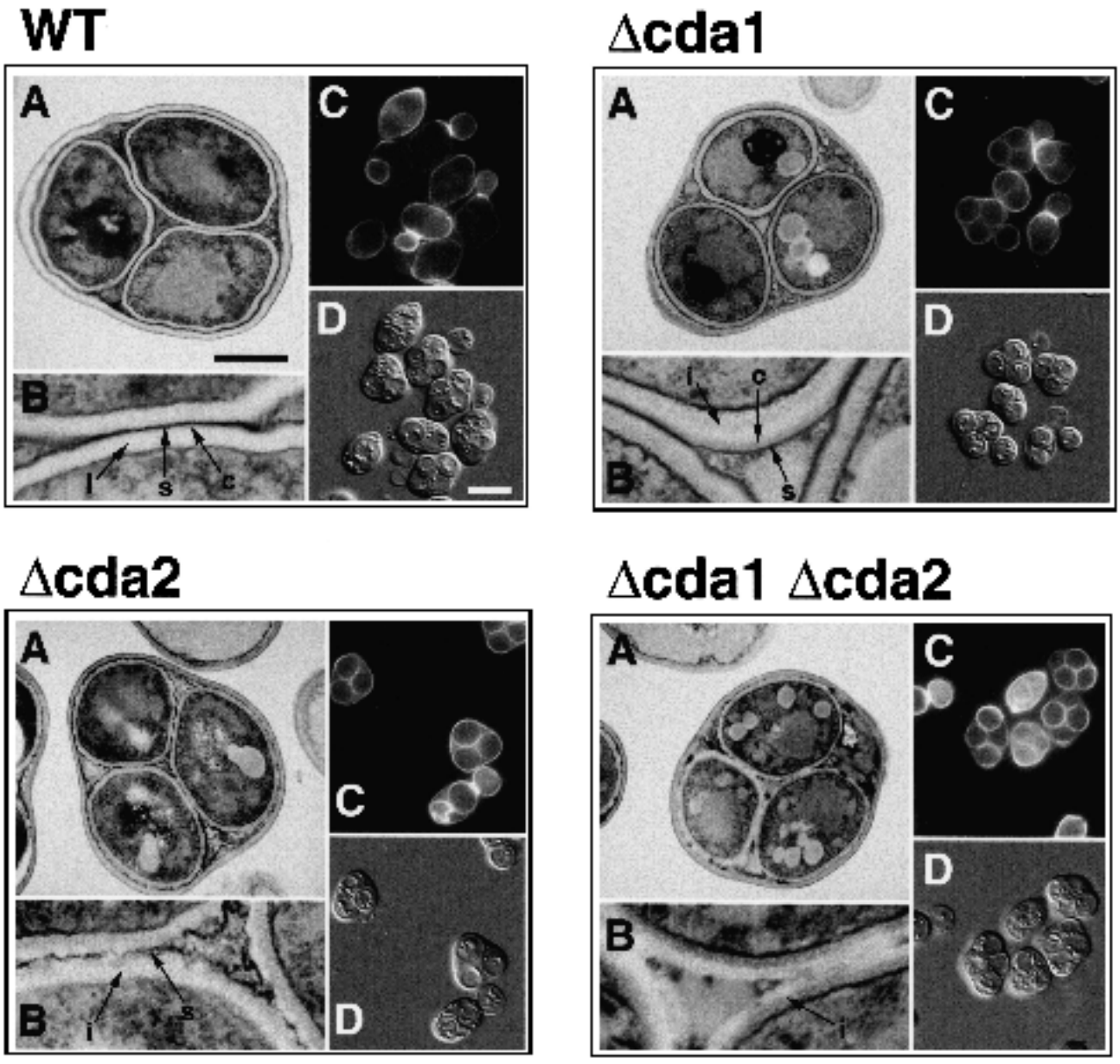

Fig. 5. A: A typical ascus is depicted for the wild type strain CEN.PK2 (wt), the mutant strains Δ*cda1* and Δ*cda2*, and the double mutant Δ*cda1*Δ*cda2*. The black bar corresponds to 1 μm. B: A portion of the spore wall of each ascus is shown at increased magnification relative to A. The outermost thin and very dark layer is denoted with the arrow labeled s. The underlying chitosan-containing layer is denoted with the arrow labeled c. The two inner layers, which often appear as a single layer, are denoted with the arrow labeled i. Fluorescence (C) and differential interference contrast (D) microscopic analyses of sporulated cultures stained with Calcofluor white. Efficient Calcofluor staining of the spore walls occurs only when the spore surface layers are not intact. The white bars corresponds to 5 μm.

as well, and it was speculated that they might represent spore wall material not incorporated into the spore wall (spore surface) due to defects in wall maturation [12]. Calcofluor staining further demonstrated the aberrant spore wall assembly pattern in the mutant strains. The staining properties of a spore by Calcofluor white indicate if the spore wall surface layer is intact or if it is damaged (or missing), and therefore has become permeable for small molecules. As can be seen in Fig. 5, there was a continuous increase of Calcofluor fluorescence from the wild type strain (no fluorescence of the spore walls) to the double disruption strain (bright fluorescence of the spore walls). These data are in excellent agreement with the results of electron microscopy.

4. Discussion

We observed significant differences in the activity patterns of Cda1p and Cda2p in sporulating and in vegetative cells. In contrast to Cda2p activity, Cda1p activity was greatly reduced in stationary phase cells when the corresponding genes were expressed under control of the *GAL1* promoter. This result may suggest that Cda1p is unstable in vegetative cells. In sporulating cells the maximum activity of Cda1p was only 25% of the Cda2p activity, although no differences at the RNA expression levels of the two genes were observed [6,9]. We speculate that the *CDA1* protein deacetylates a specific substrate produced at a certain time during sporulation and

is important for the maturation of the spore wall outer layers. Cda2p, on the other hand, is responsible for the formation of the chitosan-containing spore wall layer. We conclude this from the fact that the strain with Cda2p activity (Δ*cda1*) formed spores with nearly intact outer layers, while the strain expressing only *CDA1* (Δ*cda2*) produced spores with an aberrant surface. In vitro experiments suggested different specificity of chitin deacetylases on chitin oligosaccharides [13,14]. CDA from the fungus *M. rouxii* [14], which is inhibited by acetic acid [3], is able to deacetylate chitin oligosaccharides with a degree of polymerization higher than 3 and it fully deacetylates only $(GlnNAc)_4$ and $(GlnNAc)_5$. *C. lindemuthianum* CDA, on the other hand, which is not inhibited by acetic acid [4], is able to deacetylate $(GlnNAc)_2$ and fully deacetylates $(GlnNAc)_3$ to $(GlnNAc)_5$ [13]. Such differences in specificity might contribute to the formation of a set of partially deacetylated chitin oligomers important in a number of cases [15–17]. For example, Vander et al. [15] found that partially acetylated chitosans of certain deacetylation degrees are much more active as elicitors of defense reactions in higher plants than purified GlcAc and GlcN oligomers. As a consequence, they suggested that some of the fragments resulting from enzymatic degradation of partially acetylated chitosans may be highly elicitor active and that studies of the biological activity of purified partially acetylated oligomers are needed to obtain a more detailed understanding of the resistance reactions. The purification of the two expressed proteins which is currently being done in our laboratory will contribute to such findings.

In order to demonstrate the role of chitin deacetylases in the formation of the spore walls we compared the dityrosine and glucosamine contents of spore walls from a wild type strain, *CDA1* and *CDA2* disruption strains and a *CDA1,CDA2* double disruption strain. It was previously shown that the outermost layer of the yeast spore wall consists of a dityrosine-rich polymer closely associated with the underlying chitosan layer. It was speculated that chitosan is a prerequisite for the addition of the dityrosine layer [2]. In accordance with this assumption we detected only very low levels of dityrosine when most of the chitin was acetylated. Surprisingly, the decrease of deacetylation also seemed to affect the total quantity of the chitin polymer produced. Especially in the absence of Cda2p it appeared that the remaining chitin could not form a distinct polymer, although it still supported the addition of a small portion of the external electron-dense layer. Elimination of all chitin deacetylase activity prevented any addition of this layer. Consequently, it appears that not only chitosan but also chitin production is dependent on chitin deacetylation. It was shown that later events of spore wall assembly such as the formation of the outer spore wall layers are regulated by previous events such as changes of SMK1 mitogen-activated protein kinase activity [12]. Our data suggest that other events, such as deacetylation levels, can influence the very same steps. According to the model of Bartnicki-Garcia for the biosynthesis of chitin and chitosan, chitin chains that are not modified by enzymes such as chitinases or chitin deacetylases tend to form crystallized complexes that prevent further elongation of the chains [18]. Another explanation for the low levels of glucosamine in spore walls of Δ*cda1*Δ*cda2* strains could be that the newly synthesized chitin is rapidly degraded by the chitinase Cts1p. This enzyme is expressed in sporulating cells, apparently in the lumen of the prospore where the spore wall material is delivered [19]. Other models that include a more complex interaction between chitin synthase III, which produces spore chitin, and chitin deacetylase might be considered.

The results reported here indicate that two CDA enzymes, Cda2p which is more active and performs most of the deacetylation task, and Cda1p which probably contributes to a fine tuning process, are indispensable for proper ascospore wall assembly.

Acknowledgements: We thank M. Tzanodaskalaki for technical assistance. We also thank E. Georgatsou and D. Kafetzopoulos for helpful discussions. This work was supported by the 'Jubiläumsfonds der Österreichischen Nationalbank', Project 6868 (to P.B.).

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