

## Two Sporulation-specific Chitin Deacetylase-encoding Genes Are Required for the Ascospore Wall Rigidity of *Saccharomyces cerevisiae*\*

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The formation of the ascospore wall of *Saccharomyces cerevisiae* requires the coordinate activity of enzymes involved in the biosynthesis of its components such as chitosan, the deacetylated form of chitin. We have cloned the *CDA1* and *CDA2* genes which together account for the total chitin deacetylase activity of the organism. We have shown that expression of these genes is restricted to a distinct time period during sporulation. The two genes are functionally redundant, each contributing equally to the total chitin deacetylase activity. Diploids disrupted for both genes sporulate as efficiently as wild type cells, and the resulting mutant spores are viable under standard laboratory conditions. However, they fail to emit the natural fluorescence of yeast spores imparted by the dityrosine residues of the outermost ascospore wall layer. Moreover, mutant spores are relatively sensitive to hydrolytic enzymes, ether, and heat shock, a fact that underscores the importance of the *CDA* genes for the proper formation of the ascospore wall.

The cell wall of *Saccharomyces cerevisiae*, in spite of its apparent rigidity, is a highly dynamic structure. Its general biological functions include mechanical protection, determination of cell shape, and modulation of selective uptake of molecules. Moreover, yeast are able to alter the composition and/or the structure of their cell wall throughout the different stages of their life cycle such as budding, mating, and sporulation. Such plasticity requires the coordinated regulation of the expression of genes involved in the formation of the cell wall. Although cell wall assembly and its regulation have been extensively studied during vegetative growth (1–3), there is limited information on the composition and formation of the more complex ascospore cell wall.

Sporulation is the developmental stage that diploid cells enter when starved for nitrogen and a fermentable carbon source. It proceeds with meiosis followed by the encapsulation of the four haploid nuclei within the spore wall. This wall offers increased protection to stress conditions as compared to the wall of vegetative cells (4) and consists of four layers (5, 6). The two inner layers are formed by closely juxtaposed glucans and mannans and appear as a single layer very similar in morphology to the vegetative cell wall (6, 7). The outermost layer

consists of an insoluble macromolecule, probably a protein, which contains a high number of cross-linked tyrosine residues (8–10). Between these layers a chitosan layer has been identified (6). Chitosan, a  $\beta$ -(1→4)-D-glucosamine homopolymer, was initially detected in the cell wall of Zygomycete species (11) and is produced by the deacetylation of nascent chains of chitin, a  $\beta$ -(1→4)-N-acetyl-D-glucosamine homopolymer produced by the action of chitin synthases (12–14). The deacetylation reaction is catalyzed by the enzyme chitin deacetylase (*CDA*)<sup>1</sup> (15, 16).

Chitin deacetylases are involved either in the formation of the cell wall (12, 14, 17) or in the deacetylation of chitin oligosaccharides following the action of an endochitinase on cell walls during autolysis (18). The involvement of this enzyme in plant-pathogen interactions has also been proposed (19, 20). The isolation of the first *CDA*-encoding gene from the fungus *Mucor rouxii* revealed a remarkable sequence similarity to the rhizobial *nodB* proteins, suggesting functional homology of these evolutionary distant proteins (21). Subsequently, it was verified biochemically that *nodB* proteins are chito oligosaccharide deacetylases (22). In this report we describe the identification and molecular characterization of two sporulation-specific genes, *CDA1* and *CDA2*, whose products account for the total chitin deacetylase activity in *S. cerevisiae*. Mutational analysis of the two genes revealed that *CDA* contributes to spore wall resistance.

### EXPERIMENTAL PROCEDURES

**Strains and Growth Media**—The strain used for temporal analysis of *CDA* expression was the DKB96 diploid (*MATa/MAT $\alpha$* , *ura3/ura3*, *leu2/leu2*, *his4/his4*) kindly provided by Dr. D. Bishop. The strains used for the construction of the disruption alleles of *CDA1* and *CDA2* were FY104 (*MATa*, *ura3*, *trp1*, *his3*), the S288C derivative GT44 (*MAT $\alpha$* , *gcn4*, *ura3*, *trp1*) and their diploid derivative GT44D (*MATa/MAT $\alpha$* , *gcn4/GCN4*, *HIS3/his3*, *ura3/ura3*, *trp1/trp1*). Complete, minimal, and sporulation media were as described previously (23). For synchronous sporulation cells were grown in sporulation medium to a density of  $3 \times 10^7$  cells/ml, washed, and resuspended in SPM (0.3% potassium acetate, 0.02% glucose, and appropriate supplements) at the same density. The efficiency of sporulation of DKB96 strain was >95% after 36 h and 15% for all other strains, after 3 days under the same conditions. Ascus formation was monitored by light microscopy using phase-contrast optics.

**Genes and Plasmids**—The *CDA1* and *CDA2* genes were isolated from a YCP50 yeast genomic library (24) using a synthetic DNA oligonucleotide as a probe. Two overlapping clones were chosen, one of which was used for the isolation of a 1.2-kb *XbaI* fragment of *CDA1* and the other for the isolation of a 1.3-kb *EcoRI* fragment of *CDA2*. These were used for disruption experiments. For the expression experiment, two DNA fragments carrying the entire coding region of *CDA1* and *CDA2* were generated by a polymerase chain reaction using specific primers and

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<sup>1</sup> The abbreviations used are: *CDA*, chitin deacetylase; bp, base pair(s); kb, kilobase pair(s).

ScCDA1	.....CID UDDUTSCFK. .LSQTFDDG PAPA.TEAL. . .KKLAQRT	133
ScCDA2	.....HHCTE HODUYTCSK. .LSQTFDDG PSAS.TTKL. . .DLRKHNS	142
MrCDA	.....KISDI NDDISKCEP ETNGLIYDDG PNC.S.HNAFY DYQEQKLLK	164
BstPDA	.....LNKY GAFYLGDPK KEIYLTFFDDG YENGYTSKIL DVLKKHQUHA	95
R/NodB	NKRPAYMSEV PUNHTSGQEA RCUYLTFFDDG PNPFCPTQIL DVLAEHRUPA	50
ScCDA1	TFVULGINTU NYPDIVYEHIL ERGHLIGTHT ASHEFLPSLS NEEIUAQIE.	182
ScCDA2	TFVNLGINTU QHPDIYQRMQ KEGHLIGSHT LSHUYLPNUS NEKIITQIE.	191
MrCDA	SMFVIGSNVU DLPYGMARGU VDGHLIGASHT LSHPQMTTKT NQEVLAEFY.	203
BstPDA	TFVUTGHYLK TAPDLUKRNU KEGHIVGNHS LSHPDMTTIS ADKIKKELDA	145
R/NodB	TFVATIGSYUK DAPELIARLU REGHDUANHT NTHPDLATCD PKDUKREIDE	100
ScCDA1	NSIWMNATG KHFKPKYRFP VGRIDNRARA TUKQFGLTUV LLDLDTFDWK	232
ScCDA2	NSIWMNATG NHTPKWFRFP VGGIDNRARA ITRQFGLQAV LLDHDTFDWS	241
MrCDA	YTQKAIKLAT GLTPRYWRFP VGDIDNRARW IASQGLGTAV IANLDTFDWS	253
BstPDA	USDKUKELTG QEGTYUWRFP RGIFFSERTLA LSEKYGVYRNI FUSLAFVDWK	195
R/NodB	AHQAIUSACP QALVRHLRFP VGVVTEEDULS ASURAGLGAU HUSADPRDWS	150
ScCDA1	LITNDFFRTE EEILMDINTH KGKRK....G LLEHDGARR TVEVAIKINE	278
ScCDA2	LLLNDSSVITE QEILNQVINU NKSQT....G LLEHDSTEK TUDLAIKINK	287
MrCDA	AGUTTTVEAV EQSYSDVIAM GTNGTFANSG NULVTHEINT TMSLAUENLT	303
BstPDA	UNEQKGRYA YDNIINQI.H. ....PGA IILLH.....T	223
R/NodB	CPGVDU...I UDEULRAAR. ....PGA IULLHDGCPP DEUEQCCLAG	189

FIG. 1. Multiple sequence alignment of CDA1, CDA2, chitin deacetylase from *M. rouxii* (*MrCDA*), nodB protein from *Rhizobium leguminosarum* bv. *visiae* (*R/NodB*), and the deduced amino acid sequence of the presumed deacetylase from *B. stearothersophilus* (*BstPDA*). The black regions indicate residues identical between all five compared sequences and the gray areas those shared only by the three eucaryotic sequences. Amino acid numbers for each protein sequence are given on the right.

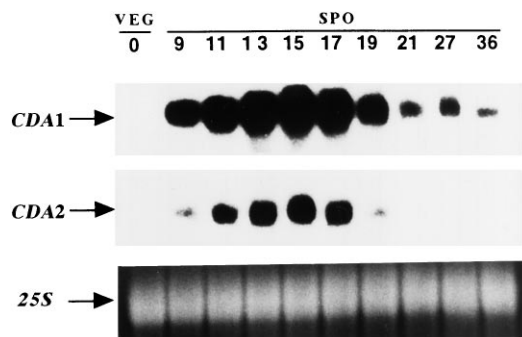


FIG. 2. Sporulation specific transcription of *CDA1* and *CDA2* genes as determined by Northern blot analysis of total RNA samples isolated from vegetatively grown cells (VEG) and at different times after transfer to sporulation medium (SPO). Ethidium bromide stain was used to normalize RNA levels. The 25 S large ribosomal RNA is shown as control.

were cloned into the pYES2 (Invitrogen) expression vector carrying the *GAL1* promoter region.

**Gene Disruptions in the Yeast Genome**—(i) For *CDA1* disruption, a *NcoI-HpaI* 572-bp fragment (−441, +131) was replaced by a 1.1-kb *HindIII URA3* DNA fragment. The resulting 1.8-kb *XbaI* fragment was excised and used to transform *ura3* strains of both mating types. (ii) For *CDA2* disruption, a 346-bp *StyI-NdeI* fragment (+288, +634) was replaced by the 1.1-kb *HindIII URA3* DNA fragment. A 2140-bp *EcoRI* fragment was excised and used to transform *ura3* strains of both mating types. (iii) For *CDA1* and *CDA2* double disruption, the *SmaI-XbaI* 550-bp fragment of the *CDA1* disruption plasmid (the *SmaI* site was created after the insertion of *URA3* gene at its end) was replaced by a *NdeI-EcoRI* 300-bp fragment of the *CDA2* region. The resulting construct had the *URA3* gene inserted between the 5' of the *CDA1* and the 3' of the *CDA2* regions. The resulting 1950-bp *XbaI-EcoRI* was excised and used to transform *ura3* strains of both mating type.

**DNA Blot Hybridization Analysis**—Yeast DNA was prepared as described previously (23), digested with *EcoRI* or *XbaI*, electrophoresed on 1% agarose gel, and transferred to GenScreen membranes (Dupont NEN). Membranes were hybridized with two different  $\alpha$ -<sup>32</sup>P-labeled probes, either a 1.2-kb *XbaI CDA1* fragment or a 1.3-kb *EcoRI CDA2* fragment. No cross-hybridization of the two genes was observed using these probes.

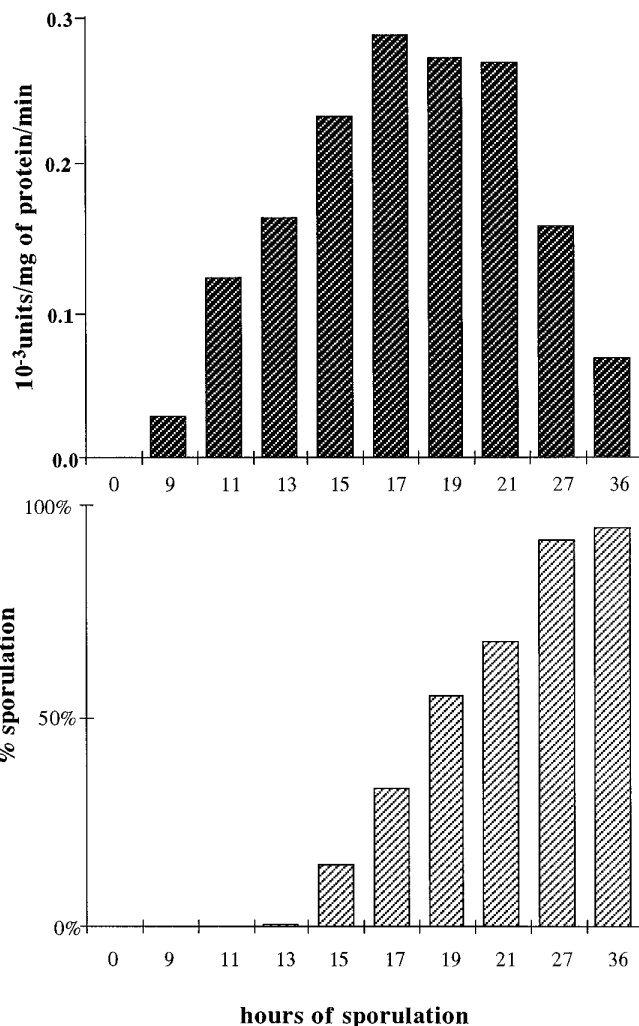


FIG. 3. Sporulation specific synthesis of *CDA1* and *CDA2* enzymes determined by CDA activity assays before and at various hours following transfer to sporulation medium. CDA activity values are given (dark bars). In parallel, the percentage of sporulation (light bars) was also monitored by light microscopy.

**RNA Blot Hybridization Analysis**—Cells (at a concentration of  $5 \times 10^7$ /ml) were collected by centrifugation at 2-h intervals following transfer to sporulation medium and total RNA was isolated by the glass bead/phenol procedure (23). For Northern hybridization analysis RNA was electrophoresed through a 1.5% agarose gel containing 15% formaldehyde and transferred to GenScreen membrane (23). The same membrane filter was sequentially hybridized with  $\alpha$ -<sup>32</sup>P-labeled 1.2-kb *XbaI CDA1* DNA fragment and with  $\alpha$ -<sup>32</sup>P-labeled 1.3-kb *EcoRI CDA2* DNA fragment.

**Cell Extract Isolation**—Cell extracts were isolated, at the same time intervals as for the RNA analysis, disrupted with glass beads in a 50 mM Tris-HCl (pH 8.0) buffer, and centrifuged at 10,000 rpm to remove cell debris.

**Chitin Deacetylase Assays**—For the determination of chitin deacetylase activity we have employed a radiometric assay using as substrate partially *O*-hydroxyethylated chitin (glycol chitin), radiolabeled in *N*-acetyl groups. The substrate was prepared according to Araki and Ito (25). Enzyme assays were performed as described previously (16) in 50 mM Tris-HCl (pH 8.0) buffer at 55 °C for 30 min. One unit of enzymatic activity releases 1.0  $\mu$ mol of [<sup>3</sup>H]acetic acid from glycol chitin per min.

**Dityrosine Fluorescence of Sporulated Colonies**—Wild type and mutant strains were streaked in YPD plates, grown at 30 °C, and replica-plated onto nitrocellulose filters. Filters were grown in YPD plates for 1 day and then transferred to sporulation plates for 3 days. Filters were treated with Glusulase (Sigma) with a mixture of 200  $\mu$ l of H<sub>2</sub>O, 70  $\mu$ l of undiluted Glusulase, and 15  $\mu$ l of  $\beta$ -mercaptoethanol. After 5 h at 30 °C, filters were transferred to 30% NH<sub>3</sub>, and their fluorescence was visualized after exposure to UV light (315 nm) (10).

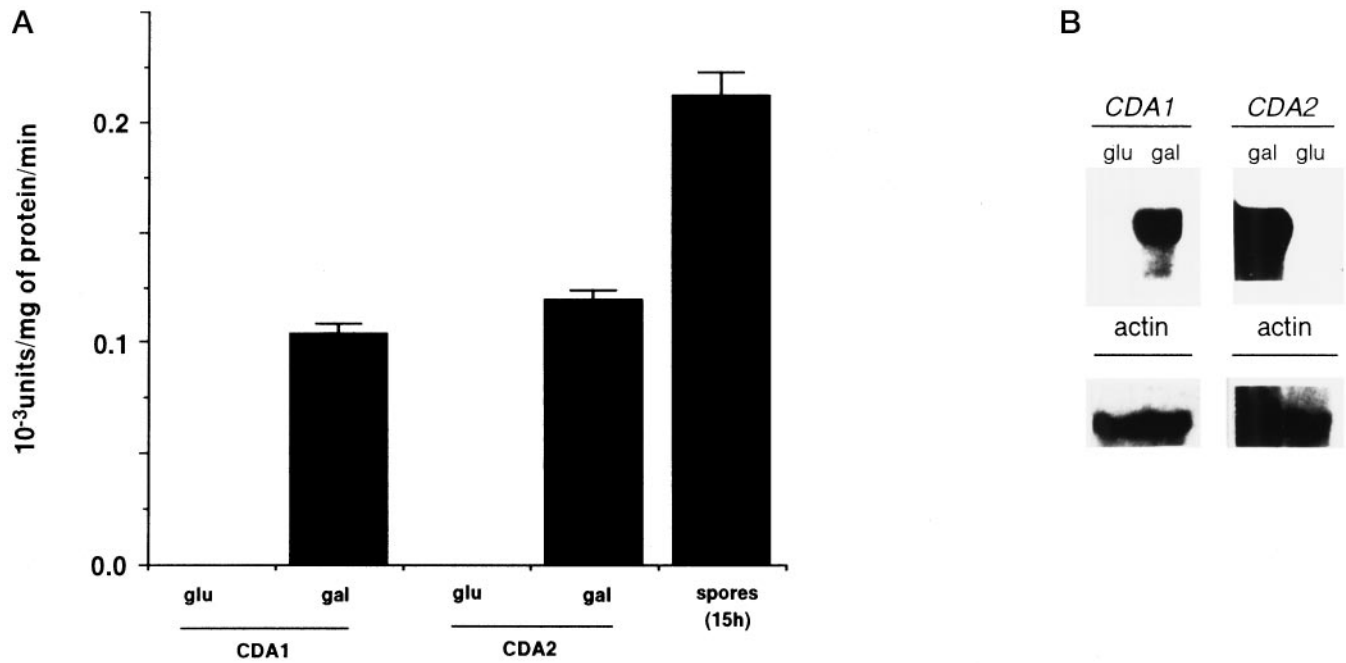


FIG. 4. Expression of *CDA1* and *CDA2* genes under the control of a *GAL1* promoter during growth in glucose containing medium (*glu*) and after transfer in galactose containing medium (*gal*). A, *CDA1* and *CDA2* activity assays. CDA activity of sporulating cells (15 h in sporulation medium) is given as positive control. B, transcription of *CDA1* and *CDA2* genes measured by Northern blot hybridization analysis. *ACT1* was used as control for vegetatively grown cells.

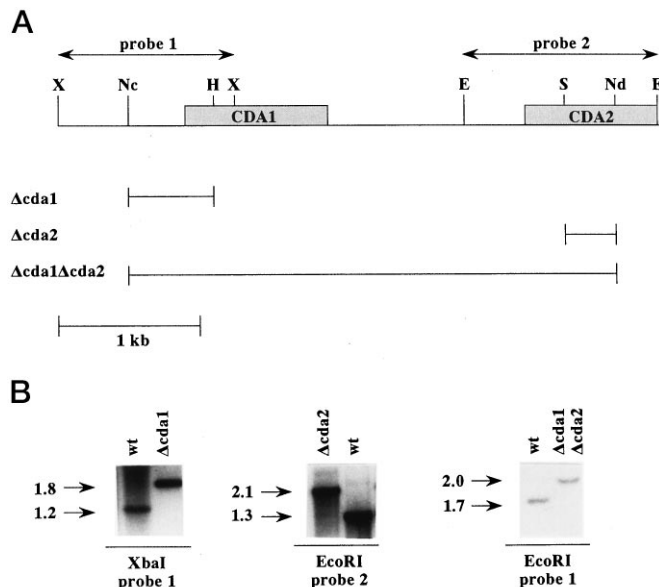


FIG. 5. A, restriction map for selected enzymes of the DNA region containing the *CDA1* and *CDA2* genes. Abbreviations of the different restriction enzyme names are as follows: X, *Xba*I; H, *Hpa*I; Nc, *Nco*I; E, *Eco*RI; S, *Sty*I; Nd, *Nde*I. The coding regions of *CDA1* and *CDA2* are boxed. For Southern blot analysis, *CDA1* and *CDA2* genes were detected using probe 1 and 2, respectively, from regions indicated by two-pointed arrows. Horizontal bars denote regions of the genome replaced by a 1.1-kb *Hind*III fragment containing the *URA3* gene in order to construct the mutant strains  $\Delta cda1$ ,  $\Delta cda2$ , and  $\Delta cda1\Delta cda2$ . B, Southern blot analysis of *CDA1* and *CDA2* gene disrupted strains. Samples of genomic DNA isolated from diploid strains containing a disruption and deletion of *CDA1* ( $\Delta cda1$ ), *CDA2* ( $\Delta cda2$ ), or both ( $\Delta cda1\Delta cda2$ ) as well as the parental wild-type strain were analyzed as described under "Experimental Procedures."

**Glucosylase Treatment, Ether Test, and Heat Shock Resistance**—Sporulated cells from the above filters were resuspended in undiluted Glucosylase at 30 °C in a hemocytometer chamber, and their lysis was observed by light microscopic examination. Resistance of spores to diethyl ether was assessed as described in Law and Segall (26), and the

viability of cells after exposure to 55 °C was determined as described by Briza *et al.* (9)

**DNA and Protein Sequence Analysis**—The *M. rouxii* CDA sequence was compared against the GenBank™/EMBL data base using FastA (27) of the Genetics Computer Group package software (28). Multiple alignment of similar protein sequences was performed using the PILEUP algorithm.

## RESULTS

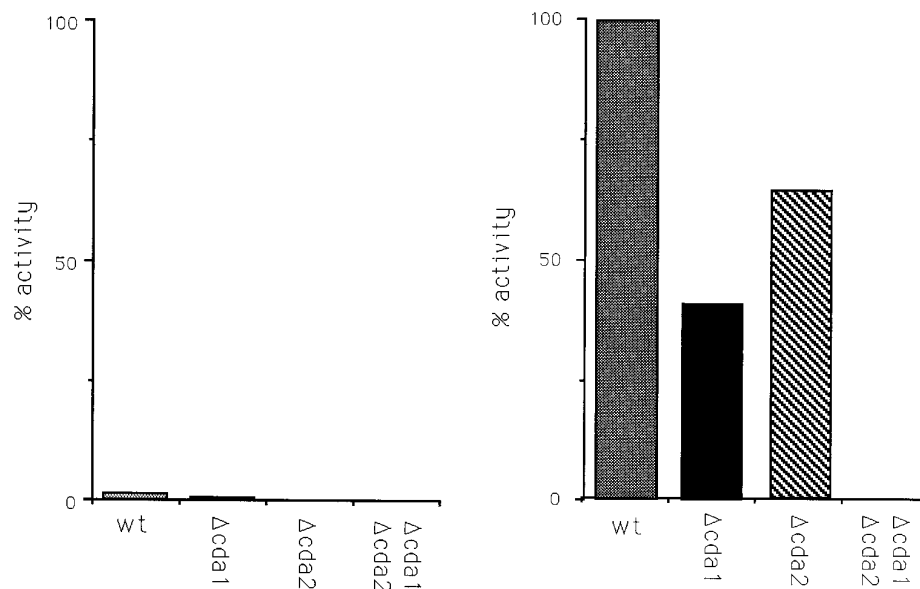
**Identification of Two Yeast Genes Encoding Proteins Similar to Chitin Deacetylases**—Comparison of the deduced amino acid sequence of *M. rouxii* chitin deacetylase (21) with the EMBL data base revealed the existence of two similar and closely linked open reading frames (EMBL accession no. for the region, U17247) on chromosome XII of *S. cerevisiae*. The homology exhibited with *M. rouxii* CDA was 24.5% over 284 amino acids for the first and 27.4% over 274 amino acids for the second open reading frame, *CDA1* and *CDA2*, respectively. The predicted proteins (34.6 and 35.6 kDa, respectively) have an identity of 57% and a similarity of 72% throughout their entire amino acid sequence, with a gap of 10 amino acids starting at position 39 of *CDA1*. The two open reading frames are separated by 1411 nucleotides, and they are transcribed in the same orientation. No other genes were predicted in the intergenic region or in the 800-bp region upstream of *CDA1*.

Multiple sequence alignments between a central region of *M. rouxii* CDA protein (extending from amino acids 121 to 325), *Bacillus stearothermophilus* putative deacetylase and the NodB protein family, which includes chitoooligosaccharide (Nod factor precursors) deacetylases (22), revealed conservation at specific residues (21). We performed a new alignment with the inclusion of the newly identified *ScCDA1* and *ScCDA2* genes, which confirmed the conservation for most of these residues, designating more accurately the invariant ones (Fig. 1). The above sequence comparison studies prompted us to investigate the function of *CDA1* and *CDA2* genes. Toward this goal the two genes were isolated from a yeast genomic plasmid library using an oligonucleotide probe, designed according to the published sequence (for the restriction map, see Fig. 5A).

**Sporulation Specific Expression of *CDA1* and *CDA2***—We



FIG. 6. Relative CDA activities of the wild type and mutant  $\Delta cda1$ ,  $\Delta cda2$ , and  $\Delta cda1\Delta cda2$  strains are shown by differently shadowed vertical bars at the time before CDA expression (0% sporulation) and when maximum expression is measured (30% sporulation).



were not able to detect any *CDA* transcripts in vegetatively grown cells. Since expression of many genes encoding sporulation specific functions is developmentally regulated, we examined the transcription of the putative *CDA* genes during the process of sporulation, when the chitosan layer of spore walls is formed (6). Total RNA from *MATa/MAT $\alpha$*  diploid cells was isolated from vegetatively grown cells and at various time intervals following transfer to sporulation medium. RNA blot hybridization analysis using either probe 1 or 2 (Fig. 5A) revealed that transcripts for both genes began to accumulate 9 h after transfer to sporulation medium (Fig. 2). At that time mature asci had not yet appeared (Fig. 3). Maximal accumulation of *CDA* transcripts occurred at 15 h, and the transcripts almost disappeared after 19 h. The two genes exhibited exactly the same transcriptional pattern and accumulated to comparable levels, a conclusion based on the use of probes of similar specific activity. The appearance of these transcripts was diploid specific since they were not detected in either vegetative or starved haploid cells (not shown).

**CDA Activity in Sporulating Cells**—The samples used for the RNA analysis were also assayed for CDA activity (Fig. 3). No activity was detected during vegetative growth. However, during the process of sporulation CDA activity accumulated in parallel with the transcriptional pattern, but with a 2-h delay. Thus, the progressive increase of CDA activity was initiated at 9 h and reached maximal levels at 17 h. Approximately half of this activity persisted at 27 h, when sporulation was completed and one third of maximal activity remained even after 36 h.

**Expression of *CDA1* and *CDA2* Genes in Vegetatively Grown Cells**—In order to examine the effects of unregulated expression of each *CDA* gene, the coding regions of the *CDA1* and *CDA2* genes were placed downstream of a *GAL1* promoter region. These constructs were introduced into haploid cells which were grown first in glucose and then transferred to galactose minimal medium, to induce *CDA* gene expression. RNA blot hybridization analysis showed that transcripts of the two genes were accumulated in cells grown on galactose (Fig. 4B). Moreover, CDA activity, at levels comparable to that found in sporulating cells, was detected in the transformed cells, indicating that indeed these two genes encode for chitin deacetylases (Fig. 4A). Furthermore, it was concluded that an unregulated expression at these levels had no effect on haploid cell viability.

*CDA1* and *CDA2* Account for the Total CDA Activity of *S.*

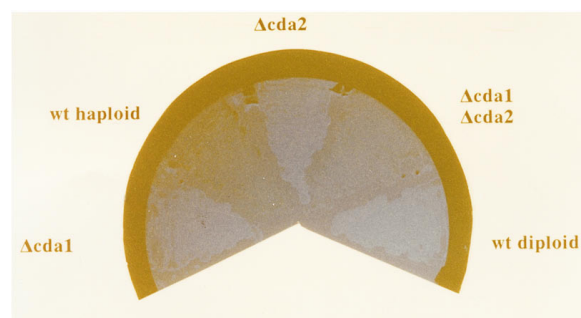


FIG. 7. Natural UV fluorescence, of wild type diploid, wild type haploid, and mutant  $\Delta cda1$ ,  $\Delta cda2$ , and  $\Delta cda1\Delta cda2$  sporulated strains.

*cerevisiae*—In order to analyze the contribution of the two genes in the total CDA activity of *S. cerevisiae*, we constructed the disrupted mutant strains  $\Delta cda1$ ,  $\Delta cda2$ , and  $\Delta cda1\Delta cda2$  as shown in Fig. 5A. The disruptions were confirmed by DNA blot hybridization analysis (Fig. 5B). Normal growth of the disrupted strains in minimal medium indicated that the two genes were not essential for cell viability.

CDA assays were performed in wild type,  $\Delta cda1$ ,  $\Delta cda2$ , and  $\Delta cda1\Delta cda2$  diploid strains during vegetative growth and at various stages of sporulation. Fig. 6 shows CDA activity detected just prior the formation of asci (0% sporulation) and after it reached a maximum when 30% of the cells were sporulated. The double disrupted  $\Delta cda1\Delta cda2$  strain exhibited no CDA activity at any time during spore formation. Moreover,  $\Delta cda1$  and  $\Delta cda2$  exhibited approximately half of the activity measured in the wild type strain at the same time point. These results demonstrated that the proteins produced by these two genes account for the total *S. cerevisiae* CDA activity and that the two genes contribute essentially equally to the total CDA activity of the cell.

**Double Disrupted Strains Produced Sensitive Spores**—In the  $\Delta cda1\Delta cda2$  strain both the efficiency of spore formation and spore viability, as judged by light microscopy and growth assays, were not affected. Since mutant spores lacking each of the two outermost layers are sensitive to lytic enzymes and stress conditions (9, 29, 30), we examined the sensitivity of the double and single disrupted strains to such treatments. The sensitivity to Glusulase was measured by monitoring either the natural fluorescence of yeast spores imparted by the ditryrosine

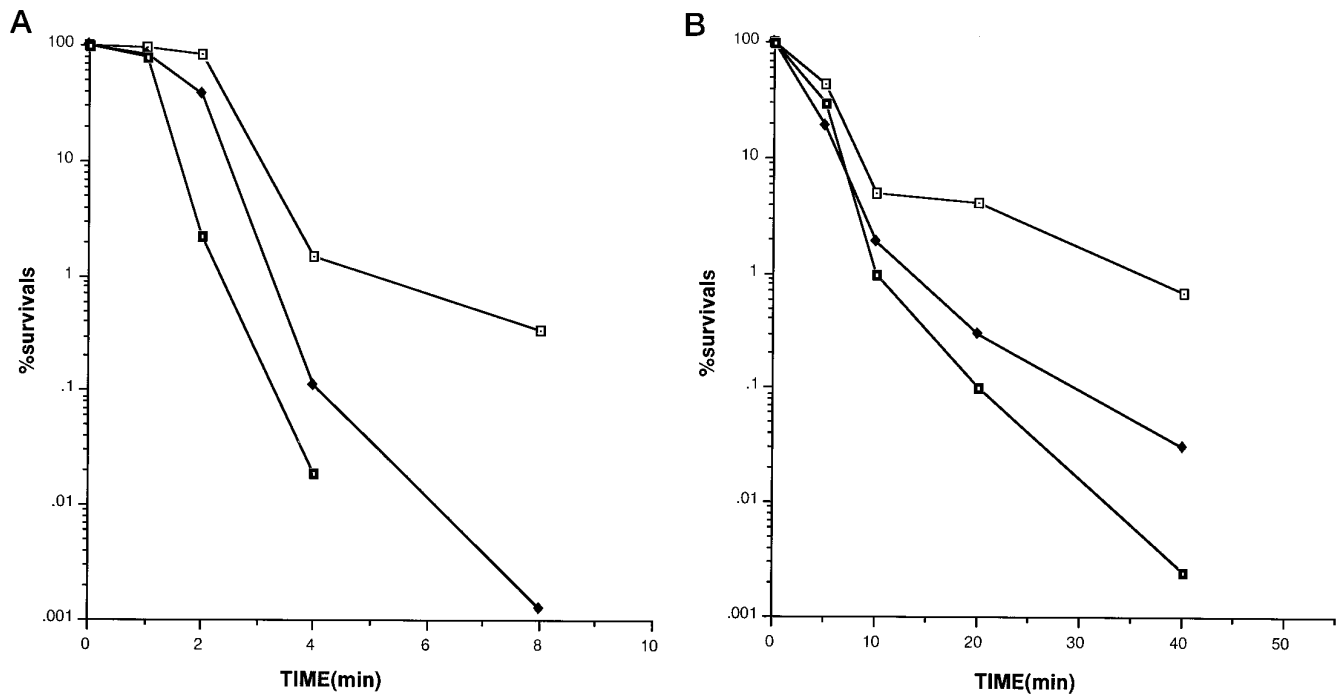


FIG. 8. Sporulated cultures of wild-type (□), double mutant (◆), and unsporulated stationary phase diploid cells (■) were tested for cell viability after exposure to ether (A) and 55 °C (B) for the indicated times. (Vegetative cell viability was below the level of detection after 4-min exposure to ether.)

containing macromolecule of the outermost layer (10), or by observing spore lysis. *CDA* mutants in parallel with wild type strains were allowed to sporulate on filters which were then transferred to undiluted Glusulase. After prolonged treatment with the enzyme (see "Experimental Procedures") double mutant strains failed to emit any fluorescence, while single mutants of both types and the wild type diploid strain exhibited normal fluorescence levels (Fig. 7). Moreover, double mutant spores were lysed by the action of this enzyme whereas single mutant and wild type spores were resistant, as revealed by observation of Glusulase exposed mutant spores under a phase contrast microscope (not shown). The fact that one of the two genes was sufficient to restore normal resistance of the spore wall suggested that the two genes are functionally redundant.

The sensitivity of mutant spores to elevated temperature and ether was also tested. We examined the spore thermotolerance of the wild type and the double disrupted strain after exposure to 55 °C for various periods of time (Fig. 8B). Mutant spore viability was two orders of magnitude lower following a 40-min exposure to elevated temperature, as compared to the wild type spores. We also examined the sensitivity of the above spores on ether exposure. As shown in Fig. 8A double disrupted spores were significantly more sensitive than wild type spores. However, in both tests, mutant strains were more resistant than stationary phase cells. These results indicated that chitin deacetylases contributed to the formation of an ascospore wall, resistant to stress conditions.

#### DISCUSSION

Chitin deacetylase is one of the components of the binary enzyme system leading to chitosan formation in the cell walls of the Zygomycete *M. rouxii*. The other component is chitin synthase, the enzyme that catalyzes the synthesis of nascent chitin chains upon which CDA acts, removing the *N*-linked acetyl groups (12). It has been shown that the second of the four layers forming the ascospore wall in *S. cerevisiae* consists of chitosan (6), and it is reasonable to assume that a similar mode to that of *M. rouxii* operates for its biosynthesis, *i.e.* the coor-

dated involvement of chitin synthase and chitin deacetylase (6, 31). Yeast chitin synthase has been extensively studied (32). Three different enzymes have been detected, each one being nonessential, but lack of all three leads to cell lethality (32). Although none of these genes is strictly sporulation specific, chitin synthase III is the possible candidate for chitin synthesis in spores since the levels of *CSD2* message, the gene presumed to encode for its catalytic subunit (33, 34), increase during sporulation (35) and no chitosan is detected in mutants lacking this gene (30). On the other hand, no study concerning chitin deacetylase has ever been reported.

We have cloned and characterized the *CDA1* and *CDA2* genes encoding two proteins with significant similarity to the *M. rouxii* CDA protein. These genes indeed encode for chitin deacetylase as shown by (a) the lack of CDA activity in  $\Delta cda1$  and  $\Delta cda2$  double disrupted strains and (b) the presence of such an activity upon expression of the cloned genes during vegetative growth. In addition, the fact that the total CDA activity from sporulating cells is abolished when both genes are deleted strongly suggests that no other gene in yeast encodes for chitin deacetylase. Deletion of each gene separately reduces the levels of CDA activity to approximately half indicating that the activities of these enzymes are additive.

The assumption that yeast chitin deacetylase activity has a unique role in sporulation, came from studies on the expression of the *CDA1* and *CDA2* genes. Both are expressed specifically during sporulation, in accordance with the existence of chitosan only in this developmental stage of the *S. cerevisiae* life cycle (6). Substantial accumulation of *CDA1* and *CDA2* mRNA is observed 9 h following transfer to sporulation medium, just prior to the formation of asci. Thus, these two genes probably belong to the mid-late class of sporulation specific genes (10, 36). Two other genes, *DIT1* and *DIT2*, that are involved in the formation of the ascospore wall are also expressed with similar developmental specificity as the CDA genes (10). Although we have not detected any potential common promoter elements among these four genes, we expect that the key regulators of

sporulation specific genes, including *DIT1* and *DIT2*, *IME1* and *IME2*, should play an important role in the expression of the *CDA* genes (36, 37). Within 2 h after the appearance of *CDA* transcripts, chitin deacetylase activity can be detected. This delay might indicate that the enzyme is produced initially as a zymogen and finally is secreted in the periplasmic space, as is the case for the *M. rouxii* enzyme (14).

In order to investigate the importance of the chitin deacetylation process to cell wall assembly, we took advantage of the natural fluorescence that wild type spores emit when excited by ultraviolet light, as well as their resistance to hydrolytic enzymes, ether, and heat shock (8, 10). The fluorescence is due to the bulk of dityrosine residues which are located in the outermost layer of the ascospore wall (8). Wild type cells and single  $\Delta cda$  disruptants exhibit no differences in fluorescence or in Glusulase sensitivity, heat shock and ether, indicating that even one *CDA* gene is sufficient for the proper formation of spore wall. On the contrary, a strain in which both *CDA* genes are deleted, is sensitive to the above treatments and its natural fluorescence is lost after enzymatic hydrolysis.

The unequivocal existence of a chitin deacetylase in *S. cerevisiae* and its importance to cell wall resistance against glucolytic enzymes and stress conditions, reinforces the hypothesis that a mechanism of chitosan biosynthesis similar to that of *M. rouxii* operates and that chitosan is produced by deacetylation of chitin (6, 31). Similar phenotypes to those of the  $\Delta cda$  null mutants are also exhibited by mutations that affect chitin synthesis, such as glucosamine auxotrophies (29), the formation of the chitosan layer, such as *dit101* (30) and the formation of dityrosine bridges, such as *dit1* and *dit2* (9, 10). The lack of chitin deacetylation in a  $\Delta cda\Delta cda2$  strain presumably leaves the second ascospore wall layer untransformed, consisting of pure chitin instead of chitosan. Briza *et al.* (6) proposed that the chitosan layer is somehow associated with the dityrosine-containing macromolecules of the outermost layer and appears to be prerequisite for dityrosine incorporation. Possibly, these interactions are disrupted when the positively charged amino groups are acetylated, leading to a loosely packed structure susceptible to hydrolytic enzymes and stress conditions.

Recent information about chitin deacetylases from different species revealed that although these enzymes have the same catalytic activity and stringent specificity for chitinous substrates, they are involved in many different biological processes depending on the individual organism. Thus in fungi *M. rouxii* and *Absidia coerulea*, CDAs are constitutively expressed enzymes (16, 17), localized in the periplasmic space (14) in order to perform their task in cell wall construction, whereas in the plant pathogen *Colletotrichum lindemuthianum* and in *Aspergillus nidulans* CDAs are secreted enzymes postulated to act on chitin oligomers released by fungal cell walls, in order to promote plant invasion (20) or cell wall degradation (18). In prokaryotes, the NodB family of genes encodes CDA which participates in the biosynthesis of the Nod factors that promote plant nodulation (21, 22), while putative CDA homologs are found in nonendosymbiotic bacteria (21). Given this diversity in the

biological functions of CDA, the availability of the *S. cerevisiae*  $\Delta cda$  mutant phenotype as well as the easily measurable CDA activity offer important tools not only toward understanding of cell wall formation but also toward the elucidation of the structure-function relationships of chitin deacetylases.

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#### REFERENCES

- Cid, J. V., Duran, A., del Rey, F., Snyder, P. M., Nombela, C., and Sanchez, M. (1995) *Microbiol. Rev.* **59**, 345–384
- Klis, M. F. (1994) *Yeast* **10**, 851–869
- Stratford, M. (1994) *Yeast* **10**, 1741–1752
- Miller, J. J. (1989) in *The Yeasts* (Rose, A. H., and Harrison J. S., eds), pp. 3/489–3/550, Academic Press, New York
- Kreger-van Rij, N. J. W. (1978) *Arch. Microbiol.* **117**, 73–77
- Briza, P., Ellinger, A., Winkler, G., and Breitenbach, M. (1988) *J. Biol. Chem.* **263**, 11569–11574
- Kathoda, S., Konno, K., Sasaki, Y., and Sakamoto, S. (1984) *Agr. Biol. Chem.* **48**, 895–901
- Briza, P., Winkler, G., Kalchauer, H., and Breitenbach, M. (1986) *J. Biol. Chem.* **261**, 4288–4294
- Briza, P., Winkler, G., Ellinger, A., and Breitenbach, M. (1990) *J. Biol. Chem.* **265**, 15118–15123
- Briza, P., Breitenbach, M., Ellinger, A., and Segall, J. (1990) *Genes Dev.* **4**, 1775–1789
- Bartnicki-Garcia, S., and Nickerson, W. J. (1962) *Biochem. Biophys. Acta* **58**, 102–119
- Davis, L. L., and Bartnicki Garcia, S. (1983) *Biochemistry* **23**, 1065–1073
- Davis, L. L., and Bartnicki Garcia, S. (1984) *J. Gen. Microbiol.* **130**, 2095–2102
- Bartnicki-Garcia, S. (1989) in *Chitin and Chitosan: Sources, Chemistry, Biochemistry, Physical Properties and Applications* (Skjark-Braek, G., Anthonen, T., and Sandford, P., eds) pp. 23–35, Elsevier, Essex
- Araki, Y., and Ito, E. (1975) *Eur. J. Biochem.* **55**, 71–78
- Kafetzopoulos, D., Martinou, A., and Bouriotis, V. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 2564–2568
- Gao, X., Katsumoto, T., and Onodera, K. (1995) *J. Biochem. (Tokyo)* **117**, 257–263
- Alfonso, C., Nuero, M. O., Santamaria, F., and Reyes, F. (1995) *Curr. Microbiol.* **30**, 49–54
- Kauss, H., Jeblick, W., and Young, H. D. (1983) *Plant Sci. Lett.* **28**, 231–236
- Tsigos, I., and Bouriotis, V. (1995) *J. Biol. Chem.* **270**, 26286–26291
- Kafetzopoulos, D., Thireos, G., Vournakis, N. J., and Bouriotis, V. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 8005–8008
- John, M., Rohring, H., Schmidt, J., Wieneke, U., and Shell, J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 2564–2568
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, A. J., and Struhl, K. (1987) *Current Protocols in Molecular Biology*, Wiley Interscience, New York
- Driscoll-Penn, M., Thireos, G., and Greer, H. (1984) *Mol. Cell. Biol.* **4**, 520–525
- Araki, Y., and Ito, E. (1988) *Methods Enzymol.* **161**, 510–512
- Law, D. T. S., and Segall, J. (1988) *Mol. Cell. Biol.* **8**, 912–922
- Pearson, V. R., and Lipman, J. D. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 2444–2448
- Devereux, J., Haberli, P., and Smithies, O. (1984) *Nucleic Acids Res.* **12**, 9509–9524
- Ballou, C. E., Maitra, K. S., Walker, W. J., and Whelan, L. W. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 4351–4355
- Pammer, M., Briza, P., Ellinger, A., Schuster, T., Stucka, R., Feldmann, H., and Breitenbach, M. (1992) *Yeast* **8**, 1089–1099
- Bulawa, C. E. (1993) *Annu. Rev. Microbiol.* **47**, 505–534
- Shaw, J. A., Mol, P. C., Bowers, B., Silverman, S. J., Valdivieso, M. H., Duran, A., and Cabib, E. (1991) *J. Cell Biol.* **114**, 111–123
- Bulawa, C. E. (1992) *Mol. Cell. Biol.* **12**, 1764–1776
- Valdivieso, M. H., Mol, P. C., Shaw, J. A., Cabib, E., and Duran, A. (1991) *J. Cell Biol.* **114**, 101–109
- Choi, W., Santos, B., Duran, A., and Cabib, E. (1994) *Mol. Cell. Biol.* **14**, 7685–7694
- Mitchel, P. A. (1994) *Microbiol. Rev.* **58**, 56–70
- Hepworth, R. S., Ebisuzaki, K. L., and Segall, J. (1995) *Mol. Cell. Biol.* **15**, 3934–3944