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ACEI of *Trichoderma reesei* Is a Repressor of Cellulase and Xylanase Expression

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We characterized the effect of deletion of the *Trichoderma reesei* (*Hypocrea jecorina*) *ace1* gene encoding the novel cellulase regulator ACEI that was isolated based on its ability to bind to and activate in vivo in *Saccharomyces cerevisiae* the promoter of the main cellulase gene, *cbh1*. Deletion of *ace1* resulted in an increase in the expression of all the main cellulase genes and two xylanase genes in sophorose- and cellulose-induced cultures, indicating that ACEI acts as a repressor of cellulase and xylanase expression. Growth of the strain with a deletion of the *ace1* gene on different carbon sources was analyzed. On cellulose-based medium, on which cellulases are needed for growth, the Δ*ace1* strain grew better than the host strain due to the increased cellulase production. On culture media containing sorbitol as the sole carbon source, the growth of the strain with a deletion of the *ace1* gene was severely impaired, suggesting that ACEI regulates expression of other genes in addition to cellulase and xylanase genes. A strain with a deletion of the *ace1* gene and with a deletion of the *ace2* gene coding for the cellulase and xylanase activator ACEII expressed cellulases and xylanases similar to the Δ*ace1* strain, indicating that yet another activator regulating cellulase and xylanase promoters was present.

In filamentous fungi production of the cellulose- and hemicellulose-degrading enzymes, the cellulases and hemicellulases, is controlled at the transcriptional level by the available carbon source. One of the best-studied cellulolytic systems is that of the saprophytic fungus *Trichoderma reesei* (*Hypocrea jecorina*). Cellulase genes of *T. reesei* are repressed in the presence of glucose by the wide-domain carbon catabolite repressor CREI (12, 19, 28, 29), which together with CREA of the aspergilli is the only known repressor of cellulase and hemicellulase genes (for a recent review see reference 4). Cellulase genes are induced in the presence of cellulose or its derivatives or by addition of the disaccharide sophorose to a medium containing a neutral carbon source, such as glycerol (for a review see reference 15). In addition, the monosaccharide L-sorbose was recently reported to induce cellulase genes of *T. reesei* (22). Most of the hemicellulase genes are also controlled by CREI and induced in the presence of the cellulase inducers cellulose and sophorose (20). Xylobiose, arabitol, and different xylans also induce the expression of many of the hemicellulase genes (20).

Even though the carbon source-dependent expression of cellulase genes has been studied in detail, our knowledge about the molecular mechanisms regulating the cellulase genes in filamentous fungi is rather fragmentary. In addition to CREI/CREA, ACEII in *T. reesei* and XlnR in *Aspergillus niger* are known to regulate fungal cellulase promoters. Several genes encoding cellulases and hemicellulases are positively regulated by XlnR, a factor that binds in vitro to a 5′GGCTAA site (8, 35, 36). In *T. reesei* ACEII binds in vitro to a site containing the 5′GGCTAATAA sequence in the *cbh1* promoter and regulates positively all of the main cellulase genes (*cbh1*, *cbh2*, *egl1*, and *egl2*) and the xylanase gene (*xyn2*) in cellulose-induced cultures (1). However, deletion of *ace2* resulted only in decreased cellulase and xylanase expression, not in complete loss of expression, suggesting that additional activators for these genes exist. In addition, the activity of the *cbh2* promoter has been shown to be dependent on the Hap2/3/5 protein complex binding to the CCAAT box, one of the most common elements in eukaryotic promoters (40, 41). Cell-free lysates prepared from *T. reesei* also bind to CCAAT sequences in the promoters of the *xyn1* and *xyn2* xylanase genes, suggesting that the Hap2/3/5 complex regulates xylanase promoters as well (42). Furthermore, the *Aspergillus nidulans* xylanase genes *xlnA* and *xlnB* are differentially controlled with respect to ambient pH by the transcription factor PacC, which activates the expression of alkaline-expressed genes and represses the expression of acid-expressed genes (17).

T. reesei ACEI is a factor that was isolated in a yeast-based screening analysis that selected for factors binding to and activating the *T. reesei cbh1* promoter in yeast (26), a screening analysis that also resulted in cloning of the *ace2* gene (1). ACEI contains three Cys_2His_2-type zinc fingers and was shown to bind in vitro to eight sites containing the core sequence 5′AGGCA scattered along the 1.15-kb *cbh1* promoter. Deletion of the *ace1* gene in *T. reesei* led to reduced colony growth on Solka floc cellulose-containing solid medium, on which cellulases are normally expressed. Growth on glucose plates was not notably affected. ACEI was suggested to be a factor specific for filamentous fungi since no significant sequence similarity to ACEI was found in databases or the *Saccharomyces cerevisiae* genome (26).

In order to expand our understanding of the regulation of cellulase production, we further investigated the role of ACEI in the regulation of *T. reesei* cellulase and xylanase expression. We analyzed the effect of deletion of *ace1* on the expression of all the main cellulase and xylanase genes under different inducing conditions. Our data indicate that ACEI acts in a repressor-like manner rather than as an activator. We also ana-

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lyzed the effect of simultaneous deletion of both the *ace1* and *ace2* genes on cellulase expression.

MATERIALS AND METHODS

Strains. *T. reesei* strains ALKO2221 (A. Mäntylä, unpublished data), VTT-D-01850 (strain ALKO2221 with a deletion of the *ace1* gene) (26), VTT-D-01849 (another transformant with a deletion of the *ace1* gene), and VTT-D-99729 (strain ALKO2221 with a deletion of the *ace2* gene) (1) and two transformants with deletions of both the *ace1* and *ace2* genes (this study) were used.

A *T. reesei* strain with deletions of both the *ace1* and *ace2* genes was made by transforming the VTT-D-01850 strain with the *ace1* deletion (26) and using the *ace2* deletion cassette released from pAS40 (1). In pAS40 1.6 kb of 5′ flanking and 2.2 kb of 3′ flanking sequences of the *ace2* gene are cloned at the 3′ and 5′ ends of the hygromycin resistance cassette derived from the selection plasmid $pRLM_{EX}30$ (18). Transformation was performed as described by Penttilä et al. (23). Southern analysis was used to verify that the transformants contained only one copy of the hygromycin gene and that this copy replaced the coding sequence of the *ace2* gene. Two transformants, VTT-D-01851 and VTT-D-01852 (Δ*ace1* Δ*ace2* strain), were studied.

Media and culture conditions. For RNA isolation and biomass determination all strains were grown in the minimal medium without peptone described by Ilmén et al. (11) supplemented with 2% glycerol, glucose, sorbitol, fructose, or Solka floc cellulose (James River Corp.) as the carbon source; 1 mM α-sophorose (Serva) was added to the glycerol medium to induce cellulase expression. Four hundred milliliters of each growth medium in a 2-liter shake flask was inoculated with 8×10^7 spores of ALKO2221 or a Δ*ace1* or Δ*ace1* Δ*ace2* strain and incubated in a rotary shaker at 250 rpm at 28°C. To analyze the effect of Triton X-100 on the growth of the different strains on Solka floc cellulose, 0.1% Triton X-100 was added to the cellulose media.

To study the growth of the Δ*ace1* and Δ*ace1* Δ*ace2* strains, three parallel shake flasks containing the strains with deletions (VTT-D-01850, VTT-D-01849, VTT-D-01851, VTT-D-01852) and four parallel flasks containing host strain ALKO2221 were inoculated, and the dry weights of the mycelia and pH values of the media were measured for 5 (glucose) or 6 days. The dry weights of the mycelia harvested on filter paper were measured after the preparations were dried at 100°C for 24 h. In Solka floc cellulose medium the growth of a strain was monitored by measuring the amount of the NaOH-extractable protein in the insoluble portion of culture fluid that was washed once with 0.7 NaCl and twice with tap water. NaOH extraction was performed as described by Jayaraman (13), except that the samples were incubated for 24 h at room temperature instead of heated at 90°C. Total protein was measured as described by Lowry et al. (16).

To study the induction of the cellulase genes by cellulose, the Δ*ace1* (VTT-D-01850 and VTT-D-01849), Δ*ace1* Δ*ace2* (VTT-D-01851 and VTT-D-01852), and ALKO2221 strains were grown in shake flasks in triplicate on glycerol medium for 72 h (in the experiment carried out with the Δ*ace1* strain) or for 58 h (in the experiment carried out with the Δ*ace1* Δ*ace2* strain), after which the mycelia from triplicate flasks of each strain were pooled and collected by filtration through Miracloth and transferred into two shake flasks containing cellulose as the sole carbon source and into one shake flask containing glycerol medium as a control. The biomass of the glycerol-grown mycelia was determined to verify that the same amount of mycelia (±6%) from each strain in the experiment was transferred into the cellulose- and glycerol-containing shake flasks. For sophorose induction studies strains were grown on glycerol in two parallel flasks for 72 h, after which 1 mM sophorose was added, and mycelial samples were collected 1, 2, 3, and 6 h after sophorose addition for mRNA analysis.

Northern analysis of cellulase expression. Total RNA was isolated with the Trizol reagent (Life Technologies, Inc.). The probes used for Northern analysis were the entire cDNAs of *cbh1*, *cbh2* (24), *egl1* (25), *egl2* (previously called *egl3*) (27), and *ace1* (26) released from vector sequences. The probes for the β-xylanase genes *xyn1* and *xyn2* were 350-bp fragments prepared by a PCR (20). As an internal loading control the membranes were hybridized with either an actin-encoding (*act1*) or glyceraldehyde-3-phosphate dehydrogenase-encoding (*gpd1*) cDNA fragment. The probes were labeled by using a random primed DNA labeling kit (Roche Molecular Biochemicals) and [α-^{32}P]dCTP (Amersham Pharmacia Biotech). Hybridization signals were detected on phosphor screen autoradiographs by using Phosphorimager SI, were quantified by using the ImageQuant software (Molecular Dynamics), and were normalized for the total amount of mRNA loaded by using *gpd1* or the actin mRNA as a loading control. The signal intensities of the different blots cannot be compared to each other due to the different specific activities of the probes and the different exposure times used for the various blots. The results of Northern analyses shown in Fig. 2A, 3A, and 4A are the results of exposure to film.

Enzyme activity assays. CBHI and EGI activities in the culture supernatants were measured by using 4-methylumbelliferyl-β-D-lactoside (MUL) (Sigma) as the substrate as described by van Tilbeurgh et al. (37); 0.17 mM MUL and 10 min of incubation at pH 5.0 and 50°C were used.

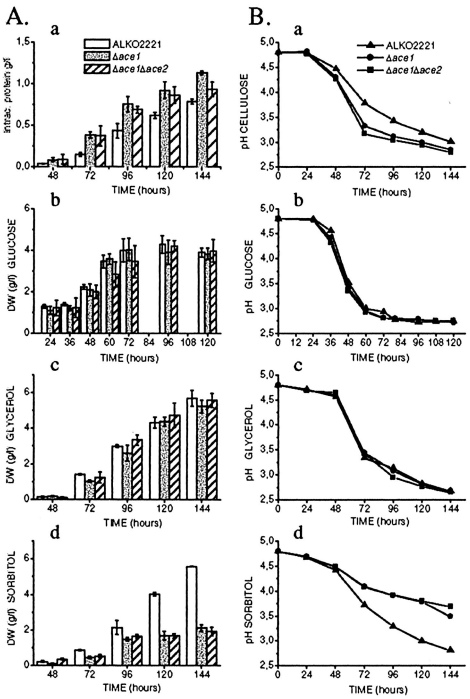
RESULTS

Growth of the Δ*ace1* strain on different carbon sources. Deletion of the *ace1* gene has been shown to result in retardation of the radial growth of colonies on Solka floc and Avicel cellulose-containing plates (26). Repeated plating of the Δ*ace1* strain and host strain ALKO2221 as single-spore colonies on Avicel and Solka floc cellulose plates showed that the diameter of the Δ*ace1* colonies was on average 40% of the diameter of the host strain ALKO2221 colonies (data not shown). A logical explanation for this is that there was reduced cellulase production in the strain with the deletion of the *ace1* gene.

In order to study further the role of ACEI in carbon utilization and cellulase production, the Δ*ace1* strain and the Δ*ace1* Δ*ace2* strain (see below) were cultivated together with host strain ALKO2221 in liquid minimal media containing different carbohydrates, glycerol, sorbitol, glucose, and Solka floc cellulose, as the sole carbon sources. The first two of these compounds are neutral carbon sources with respect to cellulase gene expression. Glucose is a repressing carbon source that causes repression of cellulase promoters by CREI (12, 30). Cellulose is an inducing carbon source on which high levels of cellulases are produced. The growth of the strains was monitored by determining biomass and measuring the pH of the culture medium, a common measure of growth in filamentous fungi. The total-protein amounts extracted from cellulose cultures by 1 M NaOH were measured to determine the rates of biomass accumulation of the different strains. Figure 1 shows that reductions in the pH values of the culture media correlated well with the rates of accumulation of fungal biomass.

In liquid culture media containing Solka floc cellulose as the sole carbon source the Δ*ace1* strain had a higher growth rate than host strain ALKO2221 (Fig. 1). This was somewhat unexpected since growth of the colonies of the Δ*ace1* strain was reduced on cellulose plates. The media on the cellulose plates and the liquid media used differed only with respect to agar, peptone (which is needed for growth on cellulose plates), and Triton X-100 (a detergent used to restrict the colony size on plates). We repeated the liquid cellulose cultivation experiment with addition of 0.1% Triton X-100, and the growth of all strains was similar to the growth of cultures without Triton X-100 (data not shown), suggesting that peptone might affect the growth of the Δ*ace1* strain on cellulose plates. On glycerol and glucose media the growth of the Δ*ace1* strain was similar to that of the host (Fig. 1, panels b and c). However, in sorbitol medium the growth of the Δ*ace1* strain was clearly retarded. During 6 days of growth on sorbitol, the strain with the deletion accumulated only one-half the biomass accumulated by the host strain (Fig. 1d). Because sorbitol may be converted into fructose by the L-iditol 2-dehydrogenase or by sorbitol dehydrogenase (2), we also cultivated the strains on 2% fructose. Growth of the Δ*ace1* strains on fructose was normal (data not shown).

The amounts of cellulase activity produced by the Δ*ace1* and host strains were measured from culture supernatants obtained



C.

Strain	3 days	4days	5days	6days
Δ*ace1*	3.2 (±0.7)	13.4 (±3.2)	15.9 (±2.2)	28.2 (±4.4)
ALKO2221	2.0 (±0.4)	6.2 (±1.5)	5.6 (±0.9)	15.6 (±2.2)

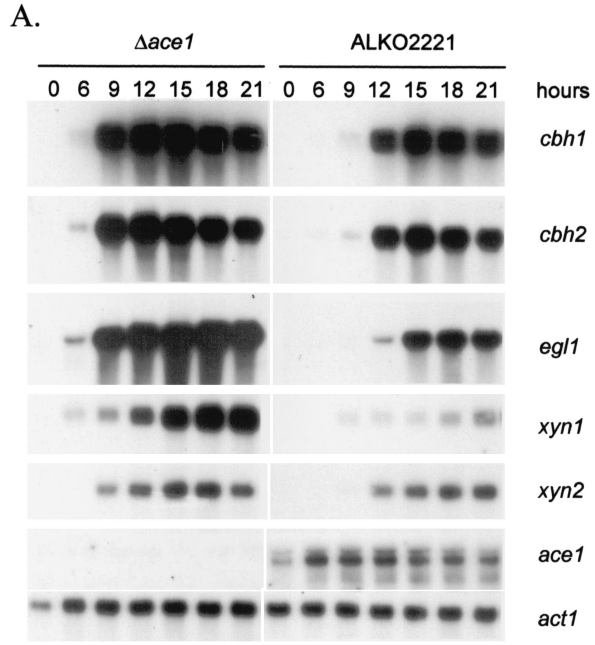
FIG. 1. (A and B) Growth of the ALKO2221, Δ*ace1* (VTT-D-01850 and VTT-D-01849), and Δ*ace1* Δ*ace2* (VTT-D-01851 and VTT-D-01852) strains on cellulose (panels a), glucose (panels b), glycerol (panels c), and sorbitol (panels d). (A) Mycelial biomasses or total amounts of intracellular protein of the ALKO2221, Δ*ace1*, and Δ*ace1* Δ*ace2* strains at different times. The error bars indicate the standard deviations of the mean dry weights (DW) and amounts of total protein. (B) pH values of the culture media of the ALKO2221, Δ*ace1*, and Δ*ace1* Δ*ace2* strains. (C) Cellulase activities (in nanokatals per milliliter; 1 nkat = 1 mmol of methylumbelliferyl released from MUL) in cellulose culture supernatants of Δ*ace1* strains VTT-D-01849 and VTT-D-01850 and host strain ALKO2221 after 3, 4, 5, and 6 days of growth. The values are means based on samples taken from four parallel shake flasks per strain. The values in parentheses are standard deviations.

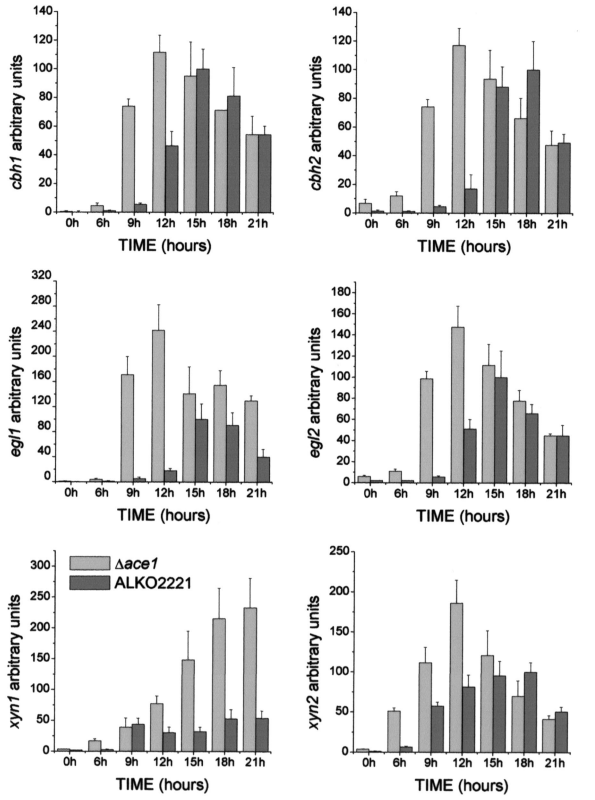
after 3, 4, 5, and 6 days of growth on Solka floc cellulose-containing medium; MUL was used as the substrate (Fig. 1C). The values resulting from MUL activity measurements reflect mainly the amounts of CBHI and EGI produced by the fungus. The strains with a deletion of the *ace1* gene produced more cellobiohydrolase and endoglucanase activities than the host produced. Throughout cultivation the difference in the amounts of MUL activity produced was around twofold, and it was greatest at 5 days. Taken together, the results showed that it is likely that the better growth of the Δ*ace1* strain on cellulose medium resulted primarily from the increased amounts of cellulases produced.

Effect of disruption of the *ace1* gene on cellulase gene expression. To study whether cellulase expression was indeed elevated in the Δ*ace1* strains and what the role of ACEI in cellulase expression is, the levels of the mRNAs of the individual cellulase genes in the Δ*ace1* strains and the host strain were measured under different inducing conditions. First, the Δ*ace1* strains (transformants VTT-D-01849 and VTT-D-01850) and the control strain were grown on glycerol to obtain biomass. Then cellulase expression was induced by transferring equal amounts of the glycerol-grown mycelia of the Δ*ace1* strains and the control strain into a culture medium containing cellulose as the sole carbon source or into glycerol medium, used as a control. The induction of the cellulase genes was monitored by performing Northern analyses of samples collected 6, 9, 12, 15, 18, and 21 h after the transfer. Expression of the main cellulase genes (*cbh1*, *cbh2*, *egl1*, and *egl2*) was induced earlier and to a higher level in the Δ*ace1* strains than in the host (Fig. 2). Transfer of the mycelia into the glycerol medium did not induce cellulase expression (data not shown). In the Δ*ace1* strains all the cellulases were expressed at a high level as early as 9 h after the transfer into the inducing culture medium, whereas in the host strain high expression was seen only after 15 h. The difference in cellulase transcript levels was generally more profound at the early time points, whereas at the later times the *cbh1*, *cbh2*, and *egl2* mRNA levels were almost the same in the Δ*ace1* and ALKO2221 strains. Nine hours after transfer of the mycelia to the cellulose media, the Δ*ace1* strains expressed on average 13 times more *cbh1* mRNA, 16 times more *cbh2* mRNA, 26 times more *egl1* mRNA, and 16 times more *egll2* mRNA than host strain ALKO2221. Twelve hours after transfer the signals of the main cellulase mRNAs in the Δ*ace1* strains were on average 2.4, 6.8, 11, and 2.8 times higher for the *cbh1*, *cbh2*, *egl1*, and *egl2* mRNAs, respectively (Fig. 2B). Enzyme activities were determined for the culture supernatants taken 15, 18, and 21 h after transfer of the mycelia to the cellulose media. In accordance with the increased expression of the cellulase genes, clearly more cellulase activities were detected in the culture media of the Δ*ace1* strains than in the culture media of the host strain (Fig. 2C).

Since deletion of the cellulase regulator gene *ace2* has previously been found to affect only cellulose-mediated induction of cellulase expression (1), it is possible that different mechanisms function in cellulase gene induction depending on the inducer. Thus, we were interested in determining whether *ace1* also has a role in the induction caused by the known strong and rapid cellulase inducer sophorose. The Δ*ace1* strains (transformants VTT-D-01849 and VTT-D-01850) and host strain ALKO2221 were grown in glycerol medium for 3 days, after which cellulase expression was induced by addition of a small amount of sophorose. The levels of the mRNAs of the three main cellulase genes, *cbh1*, *cbh2*, and *egl1*, were measured 1, 2, 3, 6, and 12 h after sophorose addition. The genes were induced earlier and to higher levels in the strain with a deletion of the *ace1* gene than in host strain ALKO2221 (Fig. 3A; data not shown for *cbh1*). Quantification of the *cbh1*, *cbh2*, and *egl1* mRNAs showed that a Δ*ace1* strain expressed on average two times more *cbh1*, three times more *cbh2*, and two times more *egl1* at the different times analyzed than the ALKO2221 strain expressed (Fig. 3B).

Construction of a strain with deletions of both the *ace1* and *ace2* genes. Transcriptional activity often results from the synergistic action of several factors, and thus the removal of one





C.

Strain	15 hours	18 hours	21 hours
Δ*ace1*	4.3 (0.8)	3.8 (0.5)	4.2 (2.4)
ALKO2221	0.4 (<0.1)	1.6 (0.3)	2.9 (0.4)

factor does not necessarily lead to dramatic changes in the transcriptional activity, as previously seen after deletion of the *ace2* gene (1). Therefore, we wanted to study the effect of simultaneous deletion of the *ace1* and *ace2* genes. A strain with deletions of the two factors was constructed by deleting the *ace2* gene from Δ*ace1* strain VTT-D-01850 (26). In the *ace2* deletion cassette the whole protein-encoding region of the *ace2* gene was removed and replaced with an expression cassette conferring hygromycin resistance. The transformants were screened by Southern hybridization for single copies of the transformed DNA and correct replacement of the *ace2* gene (data not shown). Two transformants, VTT-D-01851 and VTT-D-01852, were used for further studies (referred to below as the Δ*ace1* Δ*ace2* strain).

Growth of the Δ*ace1* Δ*ace2* strain on different carbon sources. A strain with a deletion of only the *ace2* gene has a slightly lower growth rate in liquid cellulose medium than host strain ALKO2221 due to delayed induction and reduced expression of cellulase genes. However, the Δ*ace2* strain grows normally on glucose and glycerol (1). The Δ*ace1* Δ*ace2* strains (transformants VTT-D-01851 and VTT-D-01852) were cultivated together with the Δ*ace1* and ALKO2221 strains on different carbon sources. As Fig. 1 shows, the Δ*ace1* Δ*ace2* strain grew like the strain with a deletion of only the *ace1* gene on all of the carbon sources. Similar growth also occurred in a culture containing fructose as the sole carbon source (data not shown). On sorbitol the poor growth of the Δ*ace1* Δ*ace2* strain resulted from deletion of the *ace1* gene since the strain with a deletion of only the *ace2* gene grew as well as the control strain on sorbitol (data not shown).

Expression of the cellulase genes in the Δ*ace1* Δ*ace2* strain. In order to study the combined effect of the *ace1* and *ace2* deletions on cellulase gene expression, similar cellulose- and sophorose-induced cultures of the Δ*ace1* Δ*ace2* strain (transformants VTT-D-01851 and VTT-D-01852), as were carried out for the Δ*ace1* strain, were grown. The Δ*ace1* Δ*ace2* strains and host strain ALKO2221 were cultivated in glycerol medium, after which the mycelia were collected and transferred into Solka floc cellulose media. The strain with both deletions behaved like the Δ*ace1* strain. *cbh1* and *egl2* were induced earlier and to higher levels in the Δ*ace1* Δ*ace2* strains than in the host

FIG. 2. Effect of disruption of *ace1* on expression of the main cellulases, xylanases, and *ace1* in cellulose-based cultures. (A) Northern blot analysis of *cbh1*, *cbh2*, *egl1*, *xyn1*, *xyn2*, *ace1*, and *act1* (control) mRNAs at different times in Δ*ace1* (VTT-D-01850) and ALKO2221 strains. The probes are indicated on the right. (B) Quantification of the cellulase and xylanase signals normalized with the actin mRNA from two parallel cultures of Δ*ace1* strains VTT-D-01850 and VTT-D-01849 and host strain ALKO2221. The *x* axis indicates the time after the mycelia were transferred to the cellulose medium. The bars indicate the means of the mRNA signals for two parallel shake flasks. The results are expressed relative to the highest value of the ALKO2221 signal, which was defined as 100. The error bars indicate standard deviations. (C) Cellulase activities (in nanokatals per milliliter; 1 nkat = 1 mmol of methylumbelliferyl released from MUL) in cellulose culture supernatants of the Δ*ace1* strains VTT-D-01849 and VTT-D-01850 and host strain ALKO2221 15, 18, and 21 h after transfer into cellulose-containing media. The values in parentheses are standard deviations.

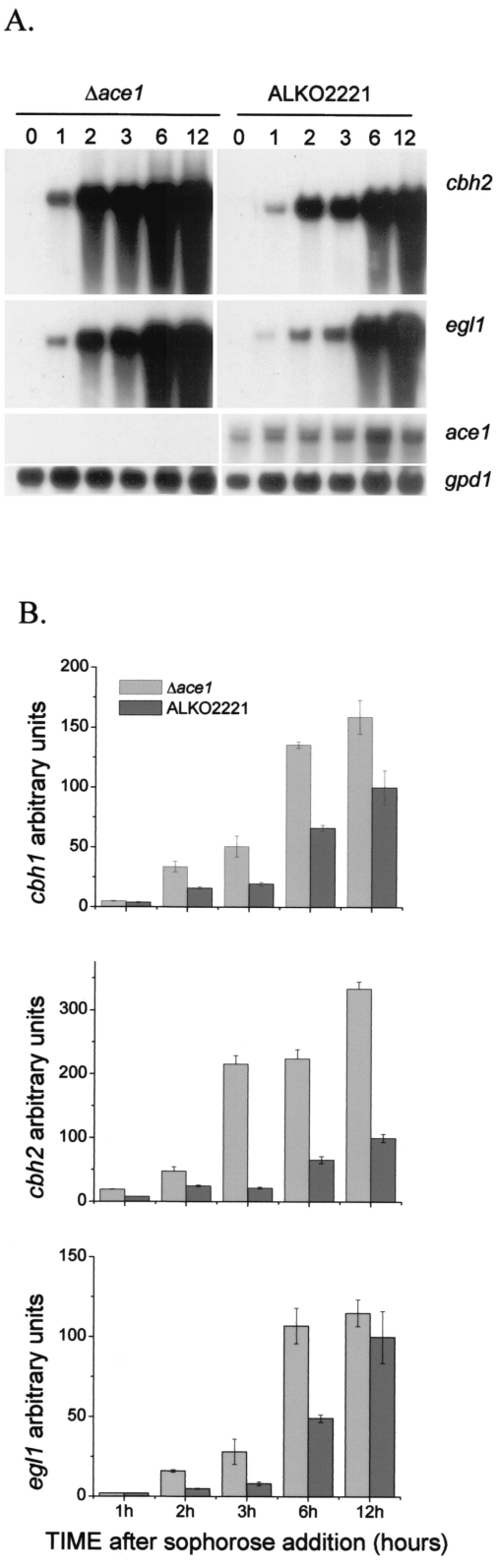


FIG. 3. Effect of *ace1* gene deletion on sophorose induction of cellulase genes. (A) Northern analysis of *cbh2*, *egl1*, *cre1*, *ace1*, and *gpd1* (control) mRNAs at 0, 1, 2, 3, 6, and 12 h after sophorose addition for a Δ*ace1* strain (VTT-D-01850) and strain ALKO2221. The probes are indicated on the right. (B) Quantification of *cbh1*, *cbh2*, and *egl1* signals normalized with the *gpd1* mRNA. The *x* axis indicates the time after sophorose addition. The bars indicate the means for two parallel cultures. The results are expressed relative to the highest value of the ALKO2221 mRNA signal, which was defined as 100. The error bars indicate standard deviations.

strain (Fig. 4). Figure 4B shows the results of quantification of the *cbh1* and *egl2* signals at different times after transfer into the cellulose media. These results show that the *ace2* deletion in the strain with a deletion of the *ace1* gene did not cause a reduction in cellulase expression like that seen in the strain with a deletion of only the *ace2* gene (1); instead, an increase in cellulase expression was seen.

In the sophorose induction experiment, the *cbh1*, *cbh2*, and *egl1* genes were induced to higher levels in the Δ*ace1* Δ*ace2* strain than in host strain ALKO2221 (data not shown). The difference in the amounts of cellulase mRNA in the Δ*ace1* Δ*ace2* strain and the host was similar to the difference observed for the Δ*ace1* strain and the host, as shown in Fig. 3.

Expression of xylanase-encoding genes *xyn1* and *xyn2* in the Δ*ace1* and Δ*ace1* Δ*ace2* strains. It has been shown that glucose repression, mediated by CREI, and the regulatory mechanisms involving *T. reesei* ACEII and *A. niger* XlnR are at least partially shared by cellulase and hemicellulase genes (1, 8, 20, 35). Therefore, we studied whether the *ace1* deletion affects the expression of the *xyn1* and *xyn2* genes encoding the two main endo-β-xylanases. The same filters that were analyzed for cellulase expression in the cellulose transfer assay described above were hybridized with the *xyn1*- and *xyn2*-specific probes. In both the Δ*ace1* and Δ*ace1* Δ*ace2* strains the *xyn1* gene was expressed at a higher level than it was expressed in the ALKO2221 strain at all times analyzed after transfer to cellulose medium (Fig. 2A and 4A). The amount of the *xyn1* mRNA was up to six times higher in the Δ*ace1* strains than in host strain ALKO2221 at all other times except 9 h after the transfer (Fig. 2). The same was true for the strains with both deletions, and the differences were 4- to 19-fold (Fig. 4). Also, the *xyn2* gene was induced to higher levels in the Δ*ace1* and Δ*ace1* Δ*ace2* strains than in host strain ALKO2221. In the Δ*ace1* strains the level of the *xyn2* mRNA was 1.3 to 7.8 times higher than the level in host strain ALKO2221 at the early time points (from 6 to 15 h). At the late time points (18 and 21 h), *xyn2* was expressed similarly in the Δ*ace1* strain and the host strain (Fig. 2B). In the Δ*ace1* Δ*ace2* strain, expression of the *xyn2* gene was 4.2 to 7.3 times greater at the early time points compared to that of the host strain (Fig. 4B).

Expression of the *ace1* gene. To examine if the transcription of *ace1* is subject to carbon source-dependent control, total RNA was isolated from mycelia grown on glucose, glycerol, and cellulose media and analyzed for *ace1* expression by Northern analysis. Host strain ALKO2221 produced two major *ace1* transcripts (3.2 and 3.0 kb) that were detected under all growth conditions studied at approximately the same relative amounts (data not shown for glucose and sorbitol). The expression of *ace1* differed only slightly at the different times after sophorose addition (Fig. 3A) and increased slightly after transfer from glycerol medium to cellulose medium (Fig. 2A and 4A). We also analyzed the expression of *ace1* in a strain with a deletion of the *ace2* gene (1) in similar experiments, as shown in Fig. 2 and 4, and expression of *ace1* was similar to

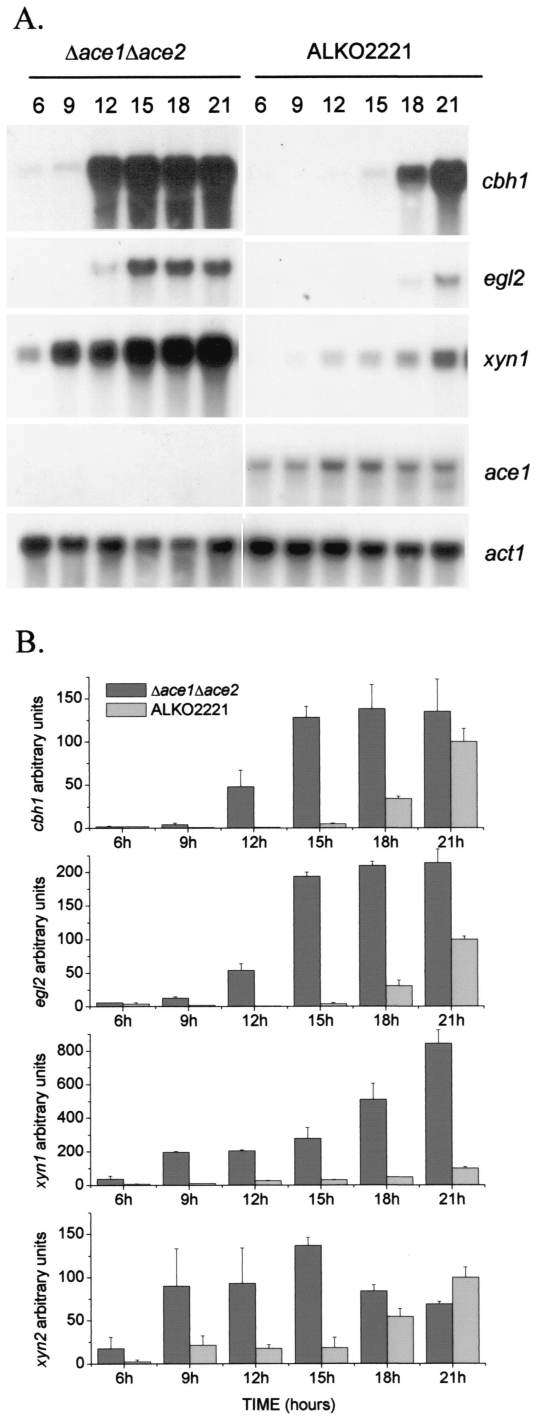


FIG. 4. Induction of cellulase and xylanase genes and expression of *ace1* in the Δ*ace1* Δ*ace2* strain on cellulose. (A) Northern blot analysis of *cbh1*, *egl2*, *xyn1*, *ace1*, and actin (control) mRNAs at 6, 9, 12, 15, 18, and 21 h in the Δ*ace1* Δ*ace2* strain (VTT-D-01851) and host strain ALKO2221. The probes are indicated on the right. (B) Quantification of the *cbh1*, *egl2*, *xyn1*, and *xyn2* signals normalized with the actin mRNA from two parallel cultures of each Δ*ace1* Δ*ace2* strain (VTT-D-01851 and VTT-D-01852) and three parallel cultures of host strain ALKO2221. The *x* axis indicates the time after the mycelia were transferred to the cellulose medium. The bars indicate the means of the mRNA signals. The results are expressed relative to the highest value of the ALKO2221 signal, which was defined as 100. The error bars indicate standard deviations.

expression in the host strain, suggesting that ACEII is not involved in the regulation of *ace1* expression. Furthermore, a 1,000-bp *ace1* promoter contains 11 sites fitting the CREI/CreA binding consensus sequence (5′SYGGRG). We studied whether *ace1* is under the control of CREI by performing a Northern analysis of the *ace1* expression in the Rut-C30 strain expressing a truncated mutant form of the *cre1* gene and the Rut-C30 strain transformed with the full-length *cre1* gene (12). *ace1* mRNA analysis of cultures grown on glucose (1 and 2 days), sorbitol, glycerol, and cellulose (3 and 6 days) showed that *ace1* expression was similar in the two strains under the growth conditions studied, suggesting that expression of *ace1* is not subject to glucose repression and is not affected by CREI (data not shown).

Sequence comparisons of the ACEI protein. We have previously reported the presence of sequences resembling the amino acid sequence of ACEI in the *A. nidulans* and *Neurospora crassa* expressed sequence tag databases (26). More recent sequence similarity searches performed with the BLAST program resulted in identification of a novel *A. nidulans* gene, *stzA* (accession number AF202995), whose product has 58% overall amino acid similarity to the ACEI protein. The database information indicated that *stzA* is a gene that encodes a C_2H_2 zinc finger protein that alleviates sensitivity to salt and DNA-damaging agents. Furthermore, the sequences in the recently released *N. crassa* genome database (2nd release; *Neurospora* Sequencing Project, Whitehead Institute/MIT Center for Genome Research [www-genome.wi.mit.edu]) contain a sequence coding for a polypeptide that has 48% identity and 75% overall similarity with the ACEI protein. The zinc finger regions of the *Neurospora* and *Aspergillus* proteins exhibit 93 and 69% identity with the zinc finger region of ACEI, respectively, suggesting that at least the predicted *Neurospora* protein binds to similar sequences. Comparison of these three proteins allowed us to identify possible meaningful amino acid sequences in addition to the zinc fingers within the ACEI protein. The region encompassing the putative nuclear localization signal, amino acids 387 to 403 in ACEI (Fig. 5), is nearly identical in the three proteins. In addition, the N-terminal parts contain two very similar stretches of amino acids, a region rich in arginine and serine residues and a region where basic and acidic amino acids alternate (Fig. 5). Meaningful sequence similarities of these regions to regions in other proteins are difficult to identify from databases due to the short lengths of the amino acid stretches.

DISCUSSION

In fungi, the production of cellulolytic enzymes is subject to transcriptional regulation by the available carbon source. So far, the known cellulase regulators include the CREI/CreA carbon catabolite repressors from *T. reesei* and aspergilli and the activators ACEII from *T. reesei* and XlnR from *A. niger*. We have previously reported cloning of the transcription factor ACEI that binds to and activates the promoter of the main cellulase gene (*cbh1*) of *T. reesei* in *S. cerevisiae*. ACEI bound in vitro to eight sites in the *cbh1* promoter, and deletion of the *ace1* gene led to reduced colony growth on cellulose-containing plates (26), suggesting that ACEI regulates cellulase gene expression in *T. reesei*.

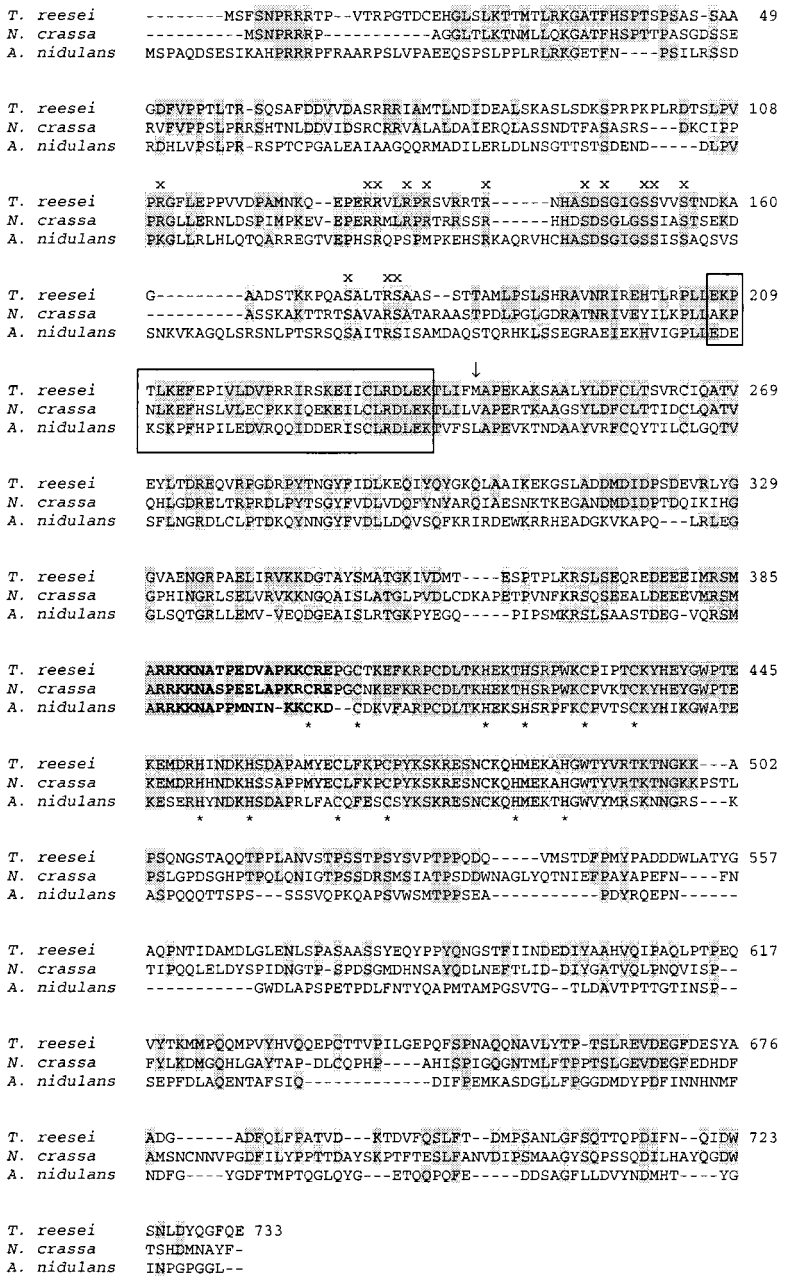


FIG. 5. Alignment of ACEI (*T. reesei*), the protein encoded by the *stzA* gene (StzA) (*A. nidulans*), and the protein deduced from the *N. crassa* genome database (*N. crassa*). Amino acids identical to *T. reesei* ACEI amino acids are indicated by a grey background. The predicted bipartite nuclear targeting signal is indicated by boldface type, and the zinc-coordinating Cys and His residues are indicated by asterisks below the sequence. The first methionine in the original *ace1* clone recovered in the yeast screening analysis and sufficient for activation in yeast (26) is indicated by an arrow. The conserved regions in the three proteins are indicated by x and by boxes. The alignment was constructed by using the ClustalW (1.74) software. Two putative intron sequences that were located at the same positions as the known introns in *ace1* were removed from the *Neurospora* sequence prior to translation on the basis of the general consensus splicing signals suggested for filamentous fungi (32).

Deletion of the *ace1* gene clearly affected the growth of *T. reesei* in cultures containing cellulose as the sole carbon source. The growth of the Δ*ace1* strain was enhanced compared to the growth of the host strain, suggesting that the Δ*ace1* strain was able to degrade cellulose more efficiently than the host strain. This observation was in accordance with the Northern analysis of the cellulase gene expression in cellulose-induced cultures, which showed that transcription of all the major cellulase genes, *cbh1*, *cbh2*, *egl1*, and *egl2*, was induced earlier and to a higher level in the Δ*ace1* strain than in the host strain (Fig. 2). The increases in the amounts of the individual cellulase mRNAs varied from 2- to 30-fold at the early time points (6 to 12 h) after the transfer to cellulose media. Furthermore, the enzyme activity measurements obtained from the culture su-

pernatants of the cellulose cultures showed that more cellulases accumulated in the culture supernatant of the Δ*ace1* strain, both during direct cultivation on cellulose (Fig. 1C) and during the transfer experiments in which cellulase expression was induced by transferring mycelia pregrown on glycerol to cellulose media (Fig. 2C).

Cellulose induction and sophorose induction of cellulase genes could be mediated by the same mechanism since cellobiose, a disaccharide released from cellulose, can be converted to sophorose by a β-glucosidase (33). However, our recent results indicated that deletion of the *ace2* gene encoding the activator ACEII affected the induction mediated by cellulose but not the induction mediated by sophorose, thus suggesting that at least partially different mechanisms operate in cellulose-mediated induction and sophorose-mediated induction (1). ACEI, however, seems to function in both. The transcription of the cellulase genes was enhanced in the Δ*ace1* strain both in the cellulose-based cultures and when the genes were induced by sophorose.

Our current knowledge concerning xylanase gene expression suggests that it involves mechanisms that are partially similar to mechanisms involved in cellulase gene induction, while there is also evidence of a separate mechanism (20, 42). Expression of the two xylanase genes (*xyn1* and *xyn2*) increased in the strain with a deletion of the *ace1* gene, indicating that ACEI also regulates the xylanase genes (Fig. 2). In this respect ACEI behaves like all the other (hemi)cellulase regulatory factors studied so far, including CREI, ACEII, and XlnR. The data presented in this paper clearly show that although originally isolated based on its ability to activate transcription, ACEI in fact negatively regulates, either directly or indirectly, cellulase and xylanase expression in *Trichoderma*.

Knowledge concerning the DNA binding repressors of filamentous fungi is limited to a few isolated factors, including the glucose repressor CREI/CreA from various different fungi (5, 6, 12, 28, 30, 31, 38) and NREB involved in nitrogen control in *Penicillium chrysogenum* (9). Repressors for fungal extracellular enzyme-encoding genes other than CREI/CreA have not been reported previously. Promoter regions reported to mediate repression under inducing conditions have been identified for the promoter of the *cgxA* gene encoding a xylanase of *Chaetomium gracile* and for a cutinase gene of *Fusarium solani* (14, 21). In *A. nidulans*, *creA* mutation increased arabinase production during growth on arabitol, suggesting that CreA down regulates arabinase genes also under inducing conditions (34).

The amino acid sequence of ACEI shows similarity to an amino acid sequence (a putative ACEI equivalent) deduced from the *N. crassa* genome database and to the product of the *A. nidulans stzA* gene (accession number AF202995) that has been deposited in a database as a C_2H_2 zinc finger protein that alleviates sensitivity to salt and DNA-damaging agents. The sequence comparison in Fig. 5 shows that in addition to the putative nuclear targeting signal, the N-terminal halves of the proteins contain at least two other highly conserved regions. Based on the similarity of these regions, it can be assumed that they are important for function. ACEI was originally cloned based on its activator function in yeast (26). The cDNA clone recovered, however, was truncated and lacked most of these N-terminal regions (Fig. 5), suggesting that the transcription-regulating function of the truncated form of ACEI might differ from that of the full-length ACEI. It is also possible that ACEI functions in a context-dependent manner and that it may act either as a repressor or as an activator, perhaps depending on the interactions with another factor(s) that influences its function.

Cellulase promoters, especially that of *cbh1*, are among the strongest eukaryotic promoters known. Certain strains have been reported to produce up to 35 g of extracellular protein per liter, and the major part of this consists of CBHI encoded by a single gene (7). Therefore, it is possible that that there are mechanisms that down regulate the cellulase and xylanase promoters also under inducing conditions in order to maintain the balance between the amount of mRNA transcribed and the protein translation and/or secretion rate. This type of regulation could be mediated by ACEI. Furthermore, it is possible that the strong induction of the cellulase genes is somehow controlled by the amount of glucose (or disaccharides) released from cellulose, although such carbon catabolite repression is likely to be mediated by CREI. No significant amounts of glucose, however, accumulated in the supernatants of the cellulose cultures of the Δ*ace1* and ALKO2221 strains (data not shown).

It is still not known why the *ace1* deletion reduced growth on cellulose plates but on the other hand improved growth in liquid cultures containing cellulose as the sole carbon source. Furthermore, our observation that on sorbitol the growth of the Δ*ace1* strain was impaired was unexpected. This suggests that *ace1* has a role in the regulation of expression of some other genes in addition to those encoding cellulases and hemicellulases. The activator of the cellulase and xylanase genes, XlnR of *A. niger*, also regulates the *xyrA* gene encoding D-xylose reductase, an intracellular enzyme involved in the utilization of xylose as a carbon source (10). So far, the genes involved in sorbitol utilization in filamentous fungi have not been characterized. Sorbitol utilization may be mediated by nonspecific dehydrogenases/reductases, one of which is the xylitol dehydrogenase which in *A. niger* also uses sorbitol as a substrate (39). It is also possible that deletion of *ace1* leads to the accumulation of a harmful metabolite during growth on sorbitol or somehow sensitizes the mycelia to sorbitol, which at high concentrations has been shown to cause an osmotic shock to the cells (3). It remains to be determined at what level disruption of the *ace1* gene affects the growth on sorbitol.

In accordance with a more general regulator role, the *ace1* gene was found to be transcribed under all the conditions studied in this work, as is the case with the glucose repressor CREI (12). Similarly, expression of *ace1* appeared to be similar in a strain expressing a mutant glucose repressor CREI and in a strain with a deletion of the *ace2* gene encoding the cellulase activator ACEII (data not shown), suggesting that expression of *ace1* is not regulated by these factors. Expression of *cre1* encoding the glucose repressor CREI appeared to be similar in the strain with the deletion and the host strain in experiments whose results are shown in Fig. 2 and 3 (data not shown), suggesting that ACEI does not regulate *cre1* expression under the conditions studied.

In eukaryotic organisms, appropriate transcriptional regulation often requires the combinatorial and synergistic action of different repressors and activators bound at multiple sites in

the promoter. The transcriptional response to these factors can be either graded or binary, only modulating the level of expression or completely turning expression off or on. We analyzed the effect of simultaneous deletion of *ace1* and *ace2* on the induction of the cellulase and xylanase genes in order to see whether the activator ACEII mediates the increased cellulase and xylanase expression seen in the strain with a deletion of the *ace1* gene. Deletion of the *ace2* gene in the Δ*ace1* strain allowed expression of cellulases at high levels that were comparable to those seen in the Δ*ace1* strain, indicating that the repressive function of ACEI is dominant and/or that the increase in cellulase and xylanase expression seen in the Δ*ace1* strain is mediated not by ACEII but by another factor or factors. According to the data now available on the regulation of cellulase and xylanase expression in *T. reesei*, it can be concluded that the level of cellulase and xylanase gene expression is determined by the balance of both positively and negatively acting regulators, including ACEII, ACEI, and CREI. The effect of these factors is graded, so that they are individually or together only partially responsible for complete regulation of the cellulase and hemicellulase genes.

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