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Role of Four Major Cellulases in Triggering of Cellulase Gene Expression by Cellulose in *Trichoderma reesei*

BERNHARD SEIBOTH,[1] SATU HAKOLA,[2] ROBERT L. MACH,[1] PIRKKO L. SUOMINEN,[2] AND CHRISTIAN P. KUBICEK[1]*

Abteilung für Mikrobielle Biochemie, Institut für Biochemische Technologie und Mikrobiologie, TU Wien, A-1060 Vienna, Austria,[1] and Primalco Ltd., Biotec, Valta-akseli, FIN-05200 Rajamäki, Finland[2]

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The relative contributions of four major cellulases of *Trichoderma reesei* (1,4-β-D-glucan cellobiohydrolase I [CBH I], CBH II, endo-1,4-β-D-glucanase I [EG I], and EG II) to the generation of the cellulase inducer from cellulose were studied with isogenic strains in which the corresponding genes (*cbh1*, *cbh2*, *egl1*, and *egl2*) had been deleted by insertion of the *Aspergillus nidulans amdS* marker gene. During growth on lactose (a soluble carbon source provoking cellulase gene expression), these strains showed no significant alterations in their ability to express the respective other cellulase genes, with the exception of the strain containing Δ*cbh1*, which exhibited an increased steady-state level of *cbh2* mRNA. On crystalline cellulose as the only carbon source, however, significant differences were apparent: strains in which *cbh2* and *egl2*, respectively, had been deleted showed no expression of the other cellulase genes, whereas strains carrying the *cbh1* or *egl1* deletion showed these transcripts. The Δ*cbh1*-containing strain also showed enhanced *cbh2* mRNA levels under these conditions. A strain in which both *cbh1* and *cbh2* had been deleted, however, was unable to initiate growth on cellulose. Addition of 2 mM sophorose, a putative inducer of cellulase gene expression, to such cultures induced the transcription of *egl1* and *egl2* and restored the ability to grow on cellulose. We conclude that CBH II and EG II are of major importance for the efficient formation of the inducer from cellulose in *T. reesei* and that removal of both cellobiohydrolases renders *T. reesei* unable to attack crystalline cellulose.

Cellulose, a linear, essentially insoluble β-1,4-glucosidically linked homopolymer with a size of about 8,000 to 12,000 glucose units, is used as an energy source by numerous and diverse microorganisms, including fungi and bacteria, which produce functionally complete cellulase enzyme systems. Among the best characterized of these systems are the inducible cellulases of the saprophytic fungus *Trichoderma reesei*. They consist of at least two 1,4-β-D-glucan cellobiohydrolases (CBH I and II; EC 3.2.1.91), four endo-1,4-β-D-glucanases [EG I, II, III, and V; 1,3-(1,3;1,4)-β-D-glucan 3(4) glucanohydrolase; EC 3.2.1.4], and two 1,4-β-D-glucosidases (BGL I and II; EC 3.2.1.21), which are formed adaptively in the presence of cellulose to synergistically cooperate in its degradation.

The mechanism by which an insoluble substrate triggers the formation of enzymes for its degradation in the fungus has been a matter of speculation for three decades. We have previously demonstrated the presence of cellulases bound to the conidial surface of *T. reesei* and have shown that they are essential for growth on cellulose as the sole carbon source (10, 17). Evidence for a major role of one of these cellulases, CBH II, to enable the fungus to start growth on cellulose has also been presented (12, 21). We interpreted these data as showing that CBH II releases small amounts of cellobiose, which may, either directly or after further conversion (7, 14), act as inducers of cellulase formation.

Although the deletion of *cbh2* significantly impaired the ability of *T. reesei* to grow on cellulose (21), one may argue that a similar effect would be obtained when any of the other cellulase genes are deleted. Although such strains have been described (23), they have not yet been used to investigate this point. In order to reassess the importance of CBH II in the generation of the cellulase inducer from cellulose, we used a set of isogenic strains (23) in which the major cellulase genes *cbh1*, *cbh2*, *egl1*, and *egl2* had been deleted. Here we report on the ability of a conidial inoculum of these strains to induce the expression of the other cellulase genes by cellulose.

* Corresponding author. Mailing address: Abteilung für Mikrobielle Biochemie, Institut für Biochemische Technologie und Mikrobiologie, TU Wien, Getreidemarkt 9/1725, A-1060 Vienna, Austria. Phone: 43-1 58801 4707. Fax: 43-1 581 62 66. E-mail: ckubicek@fbch.tuwien.ac.at.

MATERIALS AND METHODS

Plasmids and bacterial and fungal strains. *Escherichia coli* JM 109 and DH5α were used for propagation of the plasmids pMS2 (*egl2* cDNA [19]), pTTc11 (*egl1* cDNA [18]), pCBHI (*cbh1* [22]), pSB1 (*cbh2* [21]), and pACT1 (*act1* [26]).

T. reesei VTT-D-79125 (2) served as the parent strain for construction of all of the strains used in this study. The strains ALKO2862 (*cbh1* deleted), ALKO3067 (*cbh2* deleted), and ALKO3128 (*egl2* deleted) have been described by Suominen et al. (23). ALKO3193 (*egl1* deleted) is one of the transformants with a pALK471 fragment described by Suominen et al. (23), which was confirmed to harbor the expected deletion of 1.35 kb comprising the 5′ and coding regions and 0.3 kb of the 3′ region of the *egl1* locus by the *amdS* marker gene. ALKO3266 (*cbh1* and *cbh2* replaced) was constructed by replacing the *cbh1* locus of ALKO3067 (*cbh2* deleted) with the phleomycin marker (16). An expected deletion of 2.3 kb comprising the 5′ and coding regions and 1.4 kb of the 3′ region of the *cbh1* gene was confirmed by Southern blotting. The deleted area is the same as that in ALKO2862.

Media and culture cultivation. Conidia for preparation of the inoculum were obtained by growing *T. reesei* on potato dextrose agar (Difco) at 30°C for 7 days. Cultivation was carried out in 1-liter Erlenmeyer flasks containing 250 ml of the respective medium incubated at 30°C on a rotary shaker operating at 250 rpm. The liquid cultivation medium contained either 0.5% (wt/vol) Avicel or 2% (wt/vol) lactose or glucose as the carbon source, 0.5% (wt/vol) $(NH_4)_2SO_4$, 1.5% (wt/vol) KH_2PO_4, 5.4 mM $CaCl_2$, 2.4 mM $MgSO_4$, 2 mg of $FeSO_4$ per liter, 0.64 mg of $MnSO_4$ per liter, and 0.56 mg of $ZnSO_4$ per liter and was inoculated with 1.6×10^5 spores/ml.

For induction of cellulase formation in the strains VTT-D-79125 and ALKO3266 (*cbh1* and *cbh2* replaced) by sophorose, conidia were grown on cellulose (Avicel) as the sole carbon source in a 100-ml Erlenmeyer flask containing 25 ml of medium. After 24 or 48 h, respectively, sophorose (final concentration, 2 mM) was added, and cultivation was continued for a further 24 h.

Molecular biology techniques. Standard procedures were used for plasmid isolation, restriction enzyme digestion, and random priming as described in reference 20.

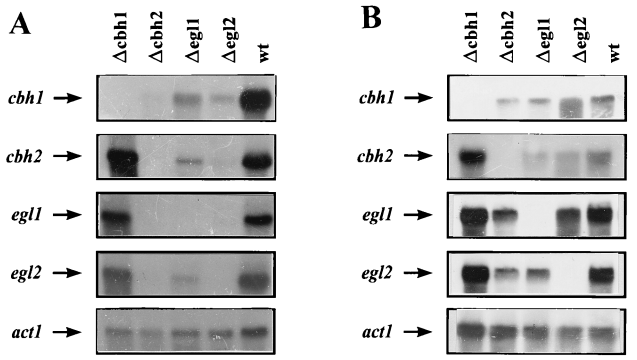

FIG. 1. Northern analysis of cellulase gene transcript formation in *T. reesei* VTT-D-79125 (wild type [wt]) and the cellulase-deleted strains (Δcbh1, Δcbh2, Δcbh1/2, Δegl1, and Δegl2) grown on Avicel cellulose (A) or lactose (B) after 48 h. Hybridizations in a given lane were performed with the cellulase gene indicated on the left. *act1* was used as a loading control. The same blot was used for all hybridizations after stripping of the membrane.

RNA isolation and analysis. Mycelia of *T. reesei*, grown in liquid culture, were harvested on a sinter funnel and ground to a fine powder in liquid nitrogen. Total RNA was isolated as described by Chomczynski and Sacchi (4). Twenty micrograms of each sample was applied to the gel tracks, and then the samples were separated by electrophoresis, blotted onto nylon membranes (Hybond-N; Amersham), and hybridized for 20 h at 42°C according to the method of Sambrook et al. (20). Washing was performed twice for 5 min each with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.1% sodium dodecyl sulfate at room temperature and for 30 min at 60°C.

Hybridization was performed with a 1.5-kb *Bgl*I fragment of the *cbh1* gene, a 1.3-kb *Hae*II fragment of the *cbh2* gene, the *egl1* cDNA of pTTc11, the *egl2* cDNA of pMS2, or a 1.7-kb *Kpn*I fragment of *act1* of *T. reesei*. The resulting Northern autoradiographs were developed at various times of saturation of the film and quantified by laser densitometry.

RESULTS AND DISCUSSION

In order to investigate the relative importance of the four major, putatively conidium-bound, cellulase components CBH I, CBH II, EG I, and EG II for the triggering of cellulase gene expression in *T. reesei* by formation of inducing oligosaccharides from cellulose, conidia of the strains described in Materials and Methods were inoculated into a medium containing Avicel cellulose as the only carbon source. Peptone and Tween 80, which are usually present in the medium used for cellulase formation (15), were omitted in order to avoid germination of the conidia on carbon sources other than cellulose, because at least for peptone, there is evidence that it is consumed as a carbon source prior to cellulose, thereby already leading to the secretion of low levels of cellulases (11). The expression of four major cellulase genes (*cbh1*, *cbh2*, *egl1*, and *egl2*) in the set of strains described above was then investigated by Northern blot hybridization with *act1* mRNA as a control. Because of the considerably slower growth of all strains in the absence of peptone and Tween 80, samples from 48 h of growth rather than 24 h are used as shown in Fig. 1A, but the results obtained for 24-h samples are consistent with those shown here. All expected cellulase gene transcripts were detected in the control strain and in the strains lacking *cbh1* and *egl1*, suggesting that the deletion of these two genes does not affect the formation of the inducers for the others. Interestingly, the strain harboring the *egl1* deletion showed reduced levels of all transcripts despite secreting practically the same amounts of CBH I and II (quantified by enzyme-linked immunosorbent assay [references 1 and 3 and data not shown]). A comparison of the ratios of intensity of the various cellulase mRNA bands and the actin control by laser densitometry revealed similar ratios (±30%) in the parent strain and the strain with the Δ*cbh1* change, with the exception of the strain lacking *cbh2*, whose transcript level was roughly 2.5-fold increased over the level of the wild type. This high level of expression of all cellulase genes in this strain rather argues against a role for CBH I in inducer generation.

In contrast, the transcripts of the other cellulase genes were almost undetectable in the strains carrying deleted copies of *cbh2* or *egl2*. A strain in which both *cbh1* and *cbh2* had been deleted was completely unable to grow on cellulose as a sole carbon source, and hence no transcripts could be analyzed.

In order to strengthen the interpretation that these differences in cellulase gene expression were due to different effects of individual cellulase deletions on the generation of the inducer from cellulose, a similar set of experiments was also performed with cultures growing on the soluble carbon source lactose, which also provokes cellulase gene expression. The data are given in Fig. 1B. All of the expected transcripts were found. Laser densitometry did not reveal major differences in the intensities of cellulase mRNA bands relative to those of the actin controls. However, in accordance with the findings during growth on cellulose, the *cbh1*-deleted strain again showed a significant increase in the accumulation of *cbh2* mRNA. A doubling of the production of CBH II in this strain has also been described on rich lactose-based medium (23), and the present findings thereby confirm this observation and show that it is due to an enhancement of transcription. Furthermore, preliminary data suggest that the deletion of the promoter and/or terminator regions of *cbh1* is relevant for this effect to be observed (data not shown).

The fact that the strain in which both *cbh1* and *cbh2* had been deleted was practically unable to initiate growth on crystalline cellulose of all strains would be consistent with the assumption that this strain is unable to generate the inducer from cellulose. To test this hypothesis, we investigated whether this inability could be overcome by the exogenous addition of a low-molecular-weight cellulase inducer. To this end, we used the putative cellulase inducer sophorose. Addition of small amounts of sophorose (adequate to induce cellulase formation in resting mycelia [cf. reference 21]) to *T. reesei* ALKO3266, cultivated on Avicel for 24 or 48 h, respectively, led to accumulation of *egl1* and *egl2* mRNA (data not given).

In summary, the data presented would be consistent with the hypothesis that the absence of *cbh2* and *egl2* reduces the transcription of the other cellulase genes more severely than the absence of *cbh1* and *egl1*. A lack of effect of *egl1* would be consistent with previous results showing that EG I is apparently absent from *T. reesei* conidia (17), whereas results for *cbh2* are in accordance with earlier findings (21). However, the apparent role of *egl2* has not yet been described. The particular efficacy of CBH II and EG II in the generation of the inducer is puzzling, since the products of their reactions are widely different (5, 9, 13). It is also noteworthy that neither CBH II nor EG II forms transglycosylation products, which have been reported as potent inducers of cellulase formation (23). It is possible that the action of these two enzymes only generates the substrates for transglycosylation by other enzymes. CBH I, on the other hand, has been shown to display transglycosylating activity (8, 25), and the fact that a strain lacking formation of both CBH I and II is practically unable to induce cellulase formation by cellulose, while deletion of CBH I alone has only a minor effect, would render this enzyme a possible candidate for inducer formation. However, we also cannot rule out that one of the other cellulases (e.g., EG III and V, whose genes were not available when this study was carried out) fulfills such a role.

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REFERENCES

1. **Aho, S., V. Olkkonen, T. Jalava, M. Paloheimo, R. Bühler, M.-L. Niku-Paavola, D. H. Bamford, and M. Korhola.** 1991. Monoclonal antibodies against core and cellulose-binding domains of *Trichoderma reesei* cellobiohydrolases I and II and endoglucanase I. Eur. J. Biochem. **200:**643–649.
2. **Bailey, M. J., and K. M. H. Nevalainen.** 1981. Induction, isolation and testing of stable *Trichoderma reesei* mutants with improved production of solubilizing cellulase. Enzyme Microb. Technol. **3:**153–157.
3. **Bühler, R.** 1991. Double-antibody sandwich enzyme-linked immunosorbent assay for quantitation of endoglucanase I of *Trichoderma reesei*. Appl. Environ. Microbiol. **57:**3317–3321.
4. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
5. **Claeyssens, M., H. van Tilbeurgh, J. P. Kamerling, J. Berg, M. Vrsanska, and P. Biely.** 1990. Studies of the cellulolytic system of the filamentous fungus *Trichoderma reesei* QM 9414. Substrate specificity and transfer activity of endoglucanase I. Biochem. J. **270:**251–256.
6. **Fowler, T., M. Gritzali, and R. D. Brown, Jr.** 1993. Regulation of the cellulase genes of *Trichoderma reesei*, p. 199–210. *In* P. Suominen and T. Reinikainen (ed.), Proceedings of the Second TRICEL Symposium on *Trichoderma reesei* Cellulases and Other Hydrolases, Espoo, Finland, 1993, vol. 8. Foundation for Biotechnical and Industrial Fermentation Research, Helsinki, Finland.
7. **Fritscher, C., R. Messner, and C. P. Kubicek.** 1990. Cellobiose metabolism and cellobiohydrolase I biosynthesis in *Trichoderma reesei*. Exp. Mycol. **14:**451–461.
8. **Gusakov, A. V., O. V. Protas, V. M. Chernoglazov, A. P. Sinitsyn, G. V. Kovalysheva, O. V. Shpanchenko, and O. V. Ermolova.** 1991. Transglycosylation activity of cellobiohydrolase I from *Trichoderma longibrachiatum* on synthetic and natural substrates. Biochim. Biophys. Acta **1073:**481–485.
9. **Knowles, J. K. C., P. Lehtovaara, M. Murray, and M. L. Sinnott.** 1988. Stereochemical course of the action of the cellobioside hydrolases I and II of *Trichoderma reesei*. J. Chem. Soc. Chem. Commun. **1988:**1401–1402.
10. **Kubicek, C. P., G. Mühlbauer, M. Grotz, E. John, and E. M. Kubicek-Pranz.** 1988. Properties of the conidial-bound cellulase system of *Trichoderma reesei*. J. Gen. Microbiol. **134:**1215–1222.
11. **Kubicek, C. P.** 1993. From cellulose to cellulase induction: facts and fiction, p. 181–188. *In* P. Suominen and T. Reinikainen (ed.), Proceedings of the Second TRICEL Symposium on *Trichoderma reesei* Cellulases and Other Hydrolases, Espoo, Finland, 1993, vol. 8. Foundation for Biotechnical and Industrial Fermentation Research, Helsinki, Finland.
12. **Kubicek-Pranz, E. M., F. Gruber, and C. P. Kubicek.** 1991. Transformation of *Trichoderma reesei* with the cellobiohydrolase II gene as a means for obtaining strains with improved cellulase productivity and specific activity. J. Biotechnol. **20:**83–94.
13. **Macarron, R., C. Acebal, M. P. Castillon, J. M. Dominguez, I. de la Mata, G. Petterson, P. Tomme, and M. Clayessens.** 1993. Mode of action of endoglucanase III from *Trichoderma reesei*. Biochem. J. **289:**867–873.
14. **Mach, R. L., B. Seiboth, A. Myasnikov, R. Gonzalez, J. Strauss, A. M. Harkki, and C. P. Kubicek.** 1995. The *bgl1* gene of *Trichoderma reesei* QM 9414 encodes an extracellular, cellulose-inducible β-glucosidase involved in cellulase induction by sophorose. Mol. Microbiol. **16:**687–697.
15. **Mandels, M., and R. E. Andreotti.** 1978. The cellulose to cellulase fermentation. Proc. Biochem. **13:**6–13.
16. **Mattern, J. E., P. J. Punt, and C. A. M. J. J. van den Hondel.** 1988. A vector of *Aspergillus* transformation carrying phleomycin resistance. Fungal Genet. Newsl. **35:**25.
17. **Messner, R., E. M. Kubicek-Pranz, A. Gsur, and C. P. Kubicek.** 1991. Cellobiohydrolase II is the main conidial-bound cellulase in *Trichoderma reesei* and other *Trichoderma* strains. Arch. Microbiol. **155:**601–606.
18. **Penttilä, M., L. André, M. Saloheimo, P. Lehtovaara, and J. K. C. Knowles.** 1987. A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. Gene **45:**253–263.
19. **Saloheimo, M., P. Lehtovaara, M. Penttilä, T. T. Teeri, J. Stahlberg, G. Johansson, G. Pettersson, M. Claeyssens, P. Tomme, and J. K. C. Knowles.** 1988. EG III, a new endoglucanase from *Trichoderma reesei*: the characterization of both gene and enzyme. Gene **63:**11–21.
20. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
21. **Seiboth, B., R. Messner, F. Gruber, and C. P. Kubicek.** 1992. Disruption of the *Trichoderma reesei cbh2* gene coding for cellobiohydrolase II leads to a delay in the triggering of cellulase formation by cellulose. J. Gen. Microbiol. **138:**1259–1264.
22. **Shoemaker, S., V. Schweickart, M. Ladner, D. Gelfand, S. Kwok, K. Myambo, and M. Innis.** 1983. Molecular cloning of exo-cellobiohydrolase from *Trichoderma reesei* strain L27. Bio/Technology **1:**691–696.
23. **Suominen, P. L., A. L. Mäntylä, T. Karhunen, S. Hakola, and K. M. H. Nevalainen.** 1993. High frequency one-step gene deletion in *Trichoderma reesei*. II. Effects of deletions of individual cellulase genes. Mol. Gen. Genet. **241:**523–530.
24. **Vaheri, M. P., M. Leisola, and V. Kaupinnen.** 1979. Transglycosylation products of the cellulase system of *Trichoderma reesei*. Biotechnol. Lett. **1:**41–46.
25. **Vrsanaska, M., and P. Biely.** 1992. The cellobiohydrolase I from *Trichoderma reesei* QM 9414: action on cello-oligosaccharides. Carbohydr. Res. **227:**19–27.
26. **Zeilinger, S.** 1996. Ph.D. thesis. University of Vienna, Vienna, Austria.