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Role of Ace2 (Activator of Cellulases 2) within the *xyn2* transcriptosome of *Hypocrea jecorina*

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Abstract

Ace2 (Activator of Cellulases 2)-encoding gene was deleted from and retransformed in the *H. jecorina* QM9414 genome. Comparison of xylanase activity and *xyn2* transcription of the corresponding strains after cultivation on inducing compounds (xylan, xylobiose) revealed a faster initial inducibility in the Δ*ace2*-strain, but final levels of *xyn2* transcript and xylanase activity of the parental strain could not be reached. This suggests a role for Ace2 in the regulation of *xyn2* induction mechanisms, moreover Ace2 is responsible for the basal level of *xyn2* transcription. Furthermore, a palindrome in the *xyn2* promoter consisting of a GGGTAA- and a CCAGCC-element was identified. Both Xyr1 and Ace2 are able to bind the complete motif, the latter also only to one part of it. Phosphorylation as well as dimerization are prerequisites for binding of Ace2 to the *xyn2* promoter. Finally, the impact of Ace2 on *xyr1* transcription could be demonstrated under inducing conditions.

Keywords: *Trichoderma reesei*; *Hypocrea jecorina*; Xylanases; *xyn2* gene expression; Ace2

1. Introduction

The filamentous ascomycete *Hypocrea jecorina* (anamorph *Trichoderma reesei*) is a fungus of noteworthy industrial importance, mainly because it secretes a broad range of hydrolytic enzymes comprising, amongst others, two major specific endo-β-1,4-xylanases, XYNI and XYNII (EC 3.2.1.8) (Törrönen et al., 1994) and two main cellobiohydrolases, CBHI (e.g., Teeri et al., 1983) and CBHII (EC 3.2.1.91) (e.g., Teeri et al., 1983). The whole set of hydrolases works together to completely degrade xylan and cellulose, the predominant bio-polymeric substrates encountered by the fungus. In this brakedown process the enzymes cause the hydrolysis to smaller, soluble oligo- and monosaccharides which are finally either acting directly as low-molecular weight inducer substances (e.g., xylobiose, xylose) (Mach et al., 1995; Zeilinger et al., 1996) or are converted to respective inducers (e.g., sophorose) via transglycosylation (Vaheri et al., 1979).

Whereas in the genus *Aspergillus* the xylanolytic and cellulolytic systems are strictly co-regulated via the inducer xylose (e.g., Gielkens et al., 1999; Hasper et al., 2000), enzymes participating in the respective *H. jecorina* hydrolytic complexes are not. Their differential expression has been reported in several studies (Hrmova et al., 1986; Margolles-Clark et al., 1997; Senior et al., 1989). Only *xyn1* transcription, in contrary to all the other xylanolytic and cellulolytic enzyme-encoding genes in *H. jecorina,* is induced by xylose (Mach et al., 1996). In addition, evidence for a different transcript formation pattern of *xyn1* and *xyn2* with respect to glucose was provided (Mach et al., 1996; Zeilinger et al., 1996). It was demonstrated that *xyn2* transcript arises at a low basal level when the fungus is grown on glucose as sole carbon source. This basal level is elevated by the presence of xylan, xylobiose, or sophorose. Simultaneous presence of glucose and xylan leads to a drop of transcription to the basal level, whereas induction

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of *xyn2* transcription by xylobiose is not affected by glucose (Würleitner et al., 2003).

Recently, we reported the identification of the main activator of hydrolases in *H. jecorina*, Xyr1 (xylanase regulator 1) (Stricker et al., 2006). Xyr1 is a zinc binuclear cluster protein binding to a GGCTAA-motif arranged as an inverted repeat in the *xyn1* promoter (Rauscher et al., 2006) closely resembling the consensus sequence for binding of the XlnR transactivator from *Aspergillus niger* (van Peij et al., 1998b). Xyr1 is both a central regulator protein responsible for the transcriptional activation of at least *xyn1*, *xyn2*, *cbh1*, *cbh2*, *bxl1*, and *bgl1* as well as for the formation of the enzymes involved in degradation of xylan and cellulose. It also contributes to the regulation of the D-xylose metabolism, namely it has strong impact on the formation of D-xylose reductase activity (Gielkens et al., 1999; Hasper et al., 2000; van Peij et al., 1998a).

In addition to Xyr1, the isolation of the two transcription factors Ace1 and Ace2, potentially involved in the regulation of hydrolase formation in *H. jecorina*, has been reported (Aro et al., 2001; Saloheimo et al., 2000). The previously described regulator Ace1 (Aro et al., 2003) was proven to directly antagonize Xyr1 function by competing for one of its binding sites in the *xyn1* promoter, thus acting as a specific repressor of *xyn1* transcription (Rauscher et al., 2006).

Deletion of *ace2* was demonstrated to clearly reduce the expression levels of the main cellulase genes e.g., *cbh1*, *cbh2,* and *egl1* as well as of *xyn2* on cellulose but did not effect induction on sophorose (Aro et al., 2001). A heterologously expressed binding domain of Ace2 does not bind to the *xyn1* promoter (Rauscher et al., 2006), but contacts the xylanase activating element XAE in the *xyn2* promoter and moreover it does affect the regulation of transcription of the *xyn2* gene (Würleitner et al., 2003). As it was previously stated, the understanding of the function of Ace2 in the regulation of xylanase expression needs more extensive studies (Aro et al., 2001).

2. Materials and methods

2.1. Strains and growth conditions

Hypocrea jecorina (*T. reesei*) QM9414 (ATCC 26921) was used as parental strain throughout this study and as recipient strain for hygromycin B-mediated transformation to delete *ace2* from the genome. It was maintained on malt agar.

For replacement experiments, strains were pre-grown first and mycelia then put into media containing the carbon source of interest. Thus, respective strains were pre-cultured in 1-L-Erlenmeyer flasks on a rotary shaker (250 rpm) at 30 °C for 18 h in 250 mL of Mandels-Andreotti (MA) medium (Mandels, 1985) supplemented with 1% (w/v) glycerol as a carbon source. 10^8 conidia per liter (final concentration) were used as inoculum. Pre-grown mycelia from respective strains were washed and thereafter divided into eight equal amounts of wet weight. One eighth was, respectively, resuspended in 20 mL MA media containing either 1% (w/v) oat spelt xylan (Sigma, Steinheim, Germany), glucose, or glycerol as carbon source. One eighth of mycelium was also replaced on 20 mL MA media without carbon source (control) or on MA media without carbon source but supplemented with 2 mM xylobiose as inducer molecule. Incubation was continued for 8 and 24 h if cultivated on xylan, for 5 h if cultivated on glucose or glycerol, for 3 and 5 h if incubated on xylobiose, and for 3 h if incubated on medium without carbon source.

Cultivation of wild-type, *ace2* deletion and *ace2* retransformation strains in bench top fermenter (Applikon Biotechnology, Schiedam, Netherlands) was carried out using 1 L medium adjusted to pH 4.5 comprising 20 g xylan, 2.8 g $(NH_4)_2SO_4$, 1 g $MgSO_4 \cdot 7\ H_2O$, 4 g KH_2PO_4, 0.5 g NaCl, 0.5 g Tween 80, 0.1 g peptone, 5 mg $FeSO_4 \cdot 7\ H_2O$, 1.7 mg $MnSO_4 \cdot H_2O$, 1.4 mg $ZnSO_4 \cdot 7\ H_2O$, and 2 mg $CaCl_2 \cdot 2\ H_2O$ dissolved/resuspended in distilled water. Some drops glanapon (Becker, Wien, Austria) were added to the medium to avoid excessive foam formation. Glycerol pre-grown mycelia as described above or 10^8 conidia per liter (final concentration), respectively, were used as inoculum. Cultivation was performed at 30 °C water bath temperature, pH 4.5, 0.3 vvm aeration rate and 500 rpm agitation rate until autolysis started. Timing of sample drawing was planned according to the expected time points of *xyn2* transcript formation. Each sample drawing was followed by a microscopic analysis for infection control. Culture supernatant and mycelia were separated by filtration through GF/F glass microfiber filters (Whatman, Brentford, UK).

Computer-aided process control and monitoring was performed using the LIME Process Control software (ATS, Wien, Austria). It shall be noted that all replacement and bench top cultivation experiments described above have been performed at least in biological triplicates.

All strains (parental, *ace2* deletion and *ace2* retransformation strains) showed similar growth on rich media as well as MA medium containing e.g., xylan, glucose, or glycerol.

2.2. Deletion from and retransformation of ace2 in the H. jecorina genome

The deletion strain was constructed as follows. The hygromycin resistance cassette was cloned from $pRLM_{ex}30$ (13) to pBluescript SK+ as a XhoI–HindIII fragment. This new plasmid was named pARO21. The chromosomal copy of *ace2* gene (6.5 kb) had been subcloned into pZerO-1 vector as a HindIII–EcoRI fragment. This plasmid is pAS33. From pAS33, we cloned a 3.3 kb Asp718–SalI fragment containing the 3′ flanking sequence of *ace2* gene into the Asp718–XhoI site of pARO21. pAS33 contained only 0.6 kb of the 5′ sequence of *ace2* gene, which is not enough to result in good gene replacement frequency. Consequently, a 1.6 kb fragment