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Yeast Sequencing Report

Cloning and analysis of *CoEXGI*, a secreted 1,3- β -glucanase of the yeast biocontrol agent *Candida oleophila*

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Abstract

Lytic enzymes may have a role in the biological control of fungi. The yeast biocontrol agent, *Candida oleophila*, is an excellent subject to research this matter. In the present study, *CoEXGI*, which encodes for a secreted 1,3- β -glucanase, is the first gene to be cloned from *C. oleophila*. It was isolated from a partial genomic library and analysed. Its open reading frame and putative promoter were expressed in baker's yeast, *Saccharomyces cerevisiae*. The reading frame, expressed under the inducible *GALI* promoter, caused an increased secretion of β -glucanase, and the putative promoter region activated the *lacZ* reporter gene, to which it was fused. Sequencing analysis revealed that *CoEXGI* carries the signature pattern of the 5 glycohydrolases family and has a putative secretion leader, as well as a high degree of identity to yeast 1,3- β -glucanases. The GenBank Accession No. of *CoEXGI* is AF393806. Copyright © 2002 John Wiley & Sons, Ltd.

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Introduction

In recent years, research and development of alternatives for synthetic fungicides, employed in the control of postharvest diseases of fruits, has gained considerable attention, due to mounting concerns of public and health authorities regarding the risks involved in pesticide residues in food. Among the proposed alternatives, the development of antagonistic microorganisms has been the most studied and has made substantial progress (Wilson and Wisniewski, 1994).

Several yeast species have demonstrated biological control to a number of postharvest pathogens in a variety of harvested commodities (Chand-Goyal and Spotts, 1997; El Ghaouth *et al.*, 1998; Roberts, 1990; Stretch, 1989). Yeasts, naturally occurring on fruits and vegetables, have been targeted as potential antagonists of postharvest diseases, since they exhibit a number of traits that confer a greater potential for colonizing fruit surfaces and wound

sites. Such characteristics can aid in the prevention of infection caused by major postharvest pathogens of fruits and vegetables (Sommer, 1982).

The success of some of these microbial antagonists in laboratory and large-scale studies has generated the interest of several agrochemical companies in the development and promotion of postharvest biological products for control of rot in fruits and vegetables. A number of microbial antagonists have been patented and evaluated for commercial use in postharvest treatment. Currently, two yeasts, *Candida oleophila* and *Cryptococcus albidus*, are commercially available under the trade names ASPIRE and YieldPlus, respectively. A few others are at different stages of commercial development and are expected to reach the marketplace within 2–3 years.

In spite of the great body of information and progress in this field, commercial use of these biocontrol products is still very limited and accounts for only a very small fraction of the potential

market. This arises from several limitations that affect their commercial applicability. When used as a stand-alone treatment, none of the biological control agents have been shown clearly to consistently offer an economically sufficient level of disease control that will warrant their acceptance as a viable alternative to synthetic fungicides. The inconsistency and insufficient efficacy under commercial conditions is attributed, in part, to the inability of the antagonists to control previously established infections.

Although the biocontrol activity of antagonistic yeasts has been demonstrated in a variety of commodities, the mode of action of the microbial biocontrol agents has not been fully elucidated. Understanding the mode of action is a prerequisite for the development of successful biocontrol strategies. Several reports have demonstrated that it may involve nutrient competition, site exclusion, direct parasitism, and lytic enzyme action (Wisniewski *et al.*, 1991; Droby and Chalutz, 1994). Growing evidence supports the idea that lytic enzymes play a major role in the mechanism by which yeast antagonists inhibit postharvest disease development. The yeast antagonist *Pichia guilliermondii*, isolate US-7, investigated by Wisniewski *et al.* (1991), appears to produce and secrete high levels of exo-1,3- β -glucanase into the growth medium. The ability to produce high levels of β -glucanase by the yeast is apparently associated with the firm attachment of the yeast cells to fungal hyphae, as well as to pitting, in some areas on fungal mycelium (Wisniewski *et al.*, 1991).

Grevesse *et al.* (1997) have suggested the involvement of exo-1,3- β -glucanase in the biocontrol activity of the yeast *Pichia anomala* against *Botrytis cinerea* in apples. An exo-1,3- β -glucanase, purified from the yeast culture filtrates, has exhibited *in vitro* inhibitory effects on conidia germination and germ tube growth of *B. cinerea*. The addition of *B. cinerea* cell walls, to a suspension of *P. anomala*, stimulated *in situ* exo-1,3- β -glucanase activity. Increased activity was also associated with the greater efficacy of the biocontrol agent.

Enhancing of the biocontrol agents' activity could contribute to the success in their control of fruit diseases and to the ultimate acceptance of biological control in commercial disease management. Despite extensive research in the field of biological control of postharvest pathogens, superior antagonists have not yet been obtained through

conventional screening of the naturally occurring microflora. In an effort to identify the genetic traits of the yeast, *C. oleophila* and determine its potential in enhancing biocontrol activity, we have cloned and expressed the *C. oleophila* exo-1,3- β -glucanase gene.

Materials and methods

Strains and culture conditions

The *Escherichia coli* strain DH5 α (Hanahan, 1983) was used as the host for the maintenance and propagation of plasmids. The *Candida oleophila* strain, I-182 [registered as Aspire™ (US-EPA Registration No. 55638-29)], served in the analysis of the secretion of endogenous exoglucanase. Part of its genome was cloned into a plasmid and served as a DNA library. The *Saccharomyces cerevisiae* strain: YPA84 (*MATa*, *ade2*, *ura3*, *his3*, *leu2*, *trp1*, Δ *exg1*, *exg2::ADE2*) has a particularly low endogenous exoglucanase activity (Esteban *et al.*, 1999a) and therefore served in analysing the expression of the *C. oleophila* exoglucanase. A303-1A is identical to YPA84, except that it is not mutated in the *EXG1* and *EXG2* genes (Esteban *et al.*, 1999a). Both of the strains were kindly provided by F. del Rey (University of Salamanca, Spain). yGO535 (*MATa*, *ura3-52*, *his3- Δ 200*, *leu2-3*, *112*, *ade2-101*) was another *S. cerevisiae* strain which served for *lacZ* activity identification.

Bacterial cultures were grown in LB (Sambrook *et al.*, 1989) and when required the medium was supplemented with ampicillin (100 μ g/ml).

Yeast was grown in YEPD rich medium (1% yeast extract, 2% peptone and 2% glucose) and transformants were selected on synthetic complete medium without uracil or leucine, as required (Sherman *et al.*, 1986). For the induction of the *GALI* promoter, 2% galactose was added to the medium instead of glucose.

Plasmids

pES213 carried an internal fragment of the *CoEXG1* gene cloned in pGEM-T (Promega). The fragment was amplified from the genome of *C. oleophila* by the polymerase chain reaction (PCR) with the degenerate primers 5'-ATT T/AC/AAGTTGCT/CTGT/CAAC/ATGGGG-3' (*CaEXG5-1*) and 5'-C/TC/GA/GCATTGGTTT/

AGGG/ATATTG/TC/TC-3' (CaEXG3-1) corresponded to the putative polypeptides of the *C. albicans XOG* gene, encoded by the nucleotides 1164–1187 and 1546–1567, respectively, sequence Accession No. X56556. This fragment served as a probe in the colony hybridization screening performed on the *C. oleophila* genomic library (described in the results). pES214 was isolated from the genomic library of *C. oleophila*. It carried the *CoEXG1* gene on a 5 kb segment in the multicopy yeast vector, pRS426 (Christianson *et al.*, 1992). Two subclones were generated from pES214 by PCR. The open reading frame was amplified with the primers 5'-**CGTCGACTTGCTTACATTGCTCC**-3' (CoORF5) and 5'-**AGATCTGTAGGTTGCGCAACCC**-TCG-3' (CoORF3) corresponding to the 5' end without the first six nucleotides, and the 3' end of the *CoEXG1* open reading frame, both ends flanked by *SalI* and *BglII*, respectively (shown in bold). The 1.5 kb PCR product obtained was ligated into the *SalI* and *BglII* sites of YcpIF2 (Foreman and Davis, 1994), resulting in pTA255, which carried the *CoEXG1* open reading frame under the control of the strong inducible *S. cerevisiae GAL1* promoter. The promoter was amplified as a 1.7 kb segment, from a fragment adjacent and upstream to the *CoEXG1* open reading frame. It was generated with the primers, 5'-**AGATCTGCGTGGCGGCGGC**-3' (CoPRO5) and 5'-**GGGAGATCTGCAACATTGGTGTAAG**-3' (CoPRO3). Each of the primers carried a *BglII* restriction site (shown in bold). Following *BglII* digestion, the fragment was ligated into the *BamHI* site of Yep367 (Myers *et al.*, 1986), resulting in pTA256, which carried the *CoEXG1* promoter fused to the *lacZ* reporter gene.

Construction of probes from *CoEXG1*

The *CoEXG1* internal segment was generated by PCR with the degenerate primers, 5'-CAA/TATT/CGGTTAC/TTGGGC-3' (CaEXG5-2) and 5'-CT/CTGG/AT/AG/ATGG/ATGG/ATGG/ATC-3' (CaEXG3-2), corresponding to the putative polypeptides encoded by *C. albicans XOG* nucleotides 619–636 and 1090–1109, respectively.

Synthesis of DNA fragments by PCR

PCR reactions were set up as follows: 20 pmol of each of the primers, 200 μ m of each of the dNTPs,

10 ng DNA template and 2.5 units Taq polymerase (Expand™ High Fidelity PCR System, Boehringer Mannheim) in a final volume of 100 μ l buffer. The mixture was incubated for 4 min at 94 °C, followed by 34 cycles of amplification: 2 min at 94 °C, 2 min at 47 °C for primers CaEXG5-1 - CaEXG3-1 and primers CaEXG5-2 - CaEXG3-2; 49 °C for primers CoORF5, and CoORF3 and 51 °C for the primers CoPRO5–CoPRO3, followed by 2 min at 72 °C. An additional 15 min of extension at 72 °C was performed in the final cycle. The amplified products were fractionated by agarose gel electrophoresis, purified and ligated into the pGEM-T vector (Promega).

Recombinant DNA manipulation

Transformation of *E. coli*, plasmid preparation, restriction mapping, DNA ligation, colony hybridization, Southern blotting and other DNA manipulations were executed according to standard techniques (Sambrook *et al.*, 1989). Yeast genomic DNA for Southern blots and for the library construction was prepared as described by Struhl *et al.*, (1979). DNA fragments for use as probes were labelled by the random primer procedure (Rediprime DNA Labeling System, Amersham). The nucleotide sequence was determined by the enzymatic dideoxy-chain termination method (Sanger *et al.*, 1977). Yeast transformation was performed by the lithium acetate procedure (Ito *et al.*, 1983). DNA analysis was carried out by the Genetics Computer Group package (Sequence Analysis Software Package, Genetics Computer Group, 1991, Madison, WI), version 10.

1,3- β -glucanase activity assays

4-Methylumbelliferyl- β -D-glucoside (MUG) as the substrate

Glucanase assays were initiated by adding 1 ml 0.04% MUG (Sigma) in 100 mM sodium acetate buffer, pH 5.4, to 0.2 ml growth medium, that had been separated from the cells and diluted in 0.8 ml water. The reaction was carried out at 37 °C for 30 min. Immediately following the reaction, the test tubes were placed on ice, and the liberated 4-methylumbelliferone was measured with a spectrofluorimeter (excitation at 350 nm, emission at 440 nm). One unit of activity was defined as nmol 4-methylumbelliferone released/min.

Laminarin as the substrate

Assays for the determination of glucanase activity by laminarin were carried out at 37 °C for 30 min in a reaction mixture containing laminarin, 7.8 mg/ml in 100 mM sodium acetate, pH 5.6, and stopped by heating to 100 °C for 10 min. The glucose produced was measured by the Glucose Assay Kit (Sigma), which is based on the glucose oxidase method. One unit of glucanase was defined as nmol glucose released/min.

β -galactosidase activity assay

β -galactosidase level measurements were performed according to Sambrook *et al.* (1989). Basically, transformant colonies were grown in minimal medium. After 2 days the yeast were collected by centrifugation (12 000 rpm for 5 min), washed and resuspended in 200 μ l cold breaking buffer (50 mM sodium phosphate, pH 7.4, 1 mM PMSF, 1 mM EDTA, 5% v/v glycerol). An equal volume of glass beads was added to the tube, which was vortexed for 30 s and put on ice for 30 s, alternately, eight times. The cells were collected by centrifugation (10 000 rpm for 10 min). To 40 μ l lysate, 50 μ l

reaction buffer (0.1M NaH₂PO₄, pH 7.5, 0.1 M β -mercaptoethanol, 20 mM MgCl₂, 0.2 mg/ml bovine serum albumin) and 10 μ l 50 mM orthonitrophenyl- β -galactoside were added. The mix was incubated at 37 °C until a yellow colour appeared. At this point the reaction was stopped by adding 0.9 ml 1 M Na₂CO₃. The optical density was measured at 420 nm and 550 nm, and the amount of β -galactosidase Miller units was calculated.

Results and discussion

Production of glucanase by *C. oleophila* in culture

The ability of *C. oleophila* to secrete glucanase was determined. The secretion of the enzyme was tested by assaying the activity of glucanase in the medium at various times during growth. As evident in Figure 1, the glucanase activity identified by the ability to degrade 4-methylumbelliferyl- β -D-glucoside (MUG), continued to increase during the 54 h measured. Cell replication ceased after approximately 30 h of growth, implying that *C. oleophila* continued to secrete glucanase in

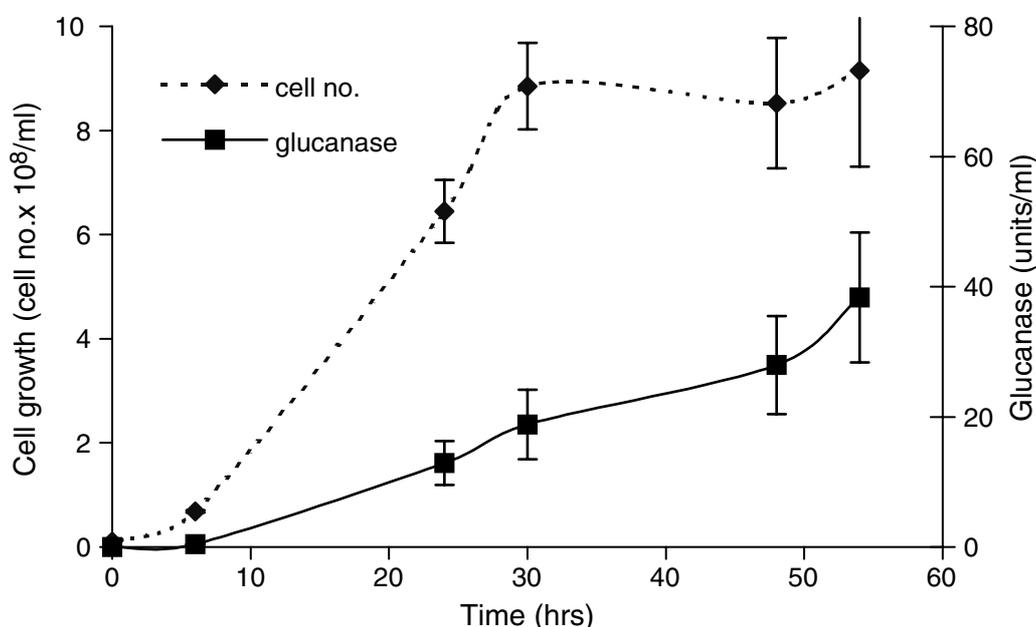


Figure 1. Activity of glucanase secreted by *C. oleophila*. *C. oleophila* was diluted to approximately 1×10^7 cells/ml from a fresh starter culture and grown at 30 °C and at 200 rpm for 54 h. A volume of 0.5 ml was sampled every 6 and 24 h of each day, in order to monitor cell concentration and glucanase activity. Each point depicts the average and standard error of four replicates

the stationary phase. Thus, as in yeasts such as *Kluyveromyces fragilis*, *Hansenula anomala* (Abdel-Al and Phaff, 1968), *Kluyveromyces lactis* (Tingle and Halvorson, 1971), *S. cerevisiae* (Farkas *et al.*, 1973; del Rey *et al.*, 1980), *Cryptococcus albidus* (Notario *et al.*, 1976a), *Pichia polymorpha* (Villa *et al.*, 1976), *Candida utilis* (Notario *et al.*, 1976b), *Kluyveromyces aestuarii* (Lachance *et al.*, 1977), *Hansenula polymorpha*, *Candida albicans* (Ram *et al.*, 1984; Molina *et al.*, 1989), *Hansenula polymorpha*, *Schwanniomyces occidentalis* and *Yarrowia lipolytica* (Esteban *et al.*, 1999 a,b); also, *C. oleophila* was discovered to secrete exoglucanase.

Cloning the *CoEXG1* gene

An internal fragment of a *C. oleophila* exoglucanase gene was amplified by PCR with primers, which were constructed according to the conserved sequences in the *C. albicans* 1,3- β -glucanase gene. The fragment was determined by sequence alignment to be highly identical to other yeast exoglucanase genes. It therefore served as a probe for the Southern blot of *C. oleophila* genomic DNA. This DNA was digested with several restriction enzymes, of which digestion with *Bgl*II resulted in a single band of 5 kb. In order to clone this 5 kb fragment (which was likely to carry the entire 1,3- β -glucanase gene), a partial genomic library containing the 4–6 kb *Bgl*II fragments was constructed in the *Bam*HI site of the vector plasmid, pRS426 (Christianson *et al.*, 1992).

The partial library was transformed into *E. coli* and subjected to colony hybridization with an internal fragment of the putative glucanase gene as the probe. Two positive clones with a 5 kb insert were identified. Both clones carried an identical insert. The sequencing of 3.7 kb of the insert revealed, that a putative glucanase gene had been cloned. A full open reading frame and the putative promoter, 2.5 kb upstream of it, were identified (Figure 2). The plasmid was designated pES214 and the cloned insert *CoEXG1*.

CoEXG1 encodes for a 1,3- β -glucanase

To prove that pES214 harboured a gene that actively secreted a glucanase enzyme, this plasmid and its negative control, pRS426, were introduced into YPA84. The transformed yeast were analysed

for both the degradation of MUG and laminarin. Degradation of MUG may be interpreted as β -exoglucanase activity, and that of laminarin as exo- and/or endoglucanase activity. As shown in Table 1, the media from 18 h and 42 h cultures of YPA84 carrying pES214, degraded MUG as well as laminarin to a much higher degree than those of the negative controls.

CoEXG1 was further examined by analysing the open reading frame alone, without the promoter. Based on the sequence of the segment, the putative open reading frame was cloned under the inducible *S. cerevisiae* *GALI* promoter (designated pTA255) and introduced into a *S. cerevisiae* strain, YPA84. As can be seen in Figure 3, a significant increase in glucanase secretion was observed in the YPA84 with pTA255, following its transfer to the galactose-inducing medium. In contrast, under non-inducing conditions (in medium containing glucose), there was no increase in glucanase secretion. The negative control, YPA84 with the plasmid vector YcpIF2, secreted only minor amounts of glucanase, both in glucose and in galactose medium. Thus, it was concluded that the *CoEXG1* DNA segments encode for an exoglucanase enzyme.

The *CoEXG1* promoter is active in *S. cerevisiae*

In order to observe if the *CoEXG1* cloned segment carried an active promoter, a fragment spanning nucleotides -1700 to -1 of the sequence (Figure 2) was examined. The fragment was cloned adjacent and upstream to *lacZ* (plasmid pTA256) and the ability of the putative promoter to confer β -galactosidase production was analysed. pTA256 was examined in two *S. cerevisiae* strains, yGO535 and A303-1A. As shown in Figure 4, significant production of β -galactosidase was observed in both transformants that carried pTA256, in contrast to the negative controls, containing yEP367 (the same as pTA256, except for the addition of the *CoEXG1* promoter). This finding implies that the *C. oleophila* *CoEXG1* promoter was located within this cloned segment.

Sequence features of *CoEXG1*

The sequence of a 2080 bp fragment of *CoEXG1* is presented in Figure 2. It contains an open reading frame of 1278 bp, the putative promoter, 780 bp upstream of the 5'-end of the open reading frame

COEXG1 - β -1,3 glucanase *C.oleophila*

-780 TTCGATTTTTATCAAGTAGAACTCCTCTTAACTCTTCTTTTTTATCCATTCGACATAG
-720 TTTTTTTACTTGTTTTCCACGTTTAGTTTACTTTTAATCACTCGCTATCAGGTATGTAAT
-660 GAAACGGTGTCCACTGGCAAGTGGCACCACGCAGGAGAGGCTCTTGCGAAGGGAGGGCA
-600 AGAAAGGAGGGGGCTCGGGAAAATCGAAAATTACAAGCCTGCTGCCCTATGTCTTGGGGG
-540 TGATGCACGGTAGCATTAAATGCATAATCTGCAATATAATAAATACTGAATTGGATTAAT
-480 TAGAATAAGGGGTTAACTTTGGAAAAGTTATAAGAGCCCATCCAAGTCCAATCAAGCCCC
-420 AATTAATGAACTTTAGCCCTATGGTCCCTTACTGATCTTGTTTTCCAATAAACTGAAAT
-360 AACCAGCACGGAAAACCTGAAATAGCTTGAAGTGCATTTTTACTTAACTCTTCGTTCTC
-300 ACAGTATCATATACTAACAATTAAAACAAGACTACAATAAGAGAGTAAACTGAAAAATTTT
-240 TTACAAGTTTAACTACTAATTTCAAGAGAGATTCTATTTTATTTCGA^{..}CCACTATCGTAA
-180 CTATTTTAACTACCCAGTACTTGTAAACAACAGACAAAGTAGTATTGAGGTAGTAAATTG
-120 GGATAAAGTTAACTTTTTTTTTTTTATCCTAAAAAGAAGAAAAATCGAAGAAACGCGTGC
-60 ATCCAATTTTCGCATTACGCCTACTCACATAAACGTGCATAATAACTTACACATACA
1 ATGTTGCTTACATTCGCTCCTATCTTCCCTCATATCGTCTATAGTGGCTGCGCCAACG
M L L T F A P I F L L I S S I V A A P T
61 CTCCAGCTTCAAAGGAAAGTTTGGAAATGGGATTACCAAATGATAAAATCAGAGGTGTC
L Q L Q R K↑ G L E W D Y Q N D K I R G V
121 AACTTGGGTGGTGGTTTGTCCCGAACCTTATATTACACCTTCGCTCTTCTCTGTATGG
N L G G W F V L E P Y I T P S L F S V W
181 TCAAATGGTGAAGATGACCTGAACACTCCAGTAGATGAATATCACTACACTCAAAAATTG
S N G E D D L N T P V D E Y H Y T Q K L
241 GGTAAGGAAACCGCTCTCAGTCTGCTTGAAGCCCATTTGGTCCATGGTACACGGAAGCA
G K E T A L S R L E A H W S S W Y T E A
301 GACTTCGCACAAATGAAATACTTGGGAATAAACGCAGTGAGAATCCAATTGGTTACTGG
D F A Q M K Y L G I N A V R I P I G Y W
361 GCTTTCAGTTATTGGATAACGATCCATATGTGCAAGGTCAGGTAAATATTTAGATCAA
A F Q L L D N D P Y V Q G Q V K Y L D Q
421 GCTTGGAAATGGTGTAGAAACAACGGACTTTACGCTTGGGTTGACTTGACCGGTGCTCCA
A L E W C R N N G L Y A W V D L H G A P
481 GGTTCTCAAATGGGTTTGAATACTCCGTTTAAAGAGATTCTACAAGTTCAGGACGAC
G S Q N G F D N S G L R D S Y K F Q D D
541 GACGACGTTAAAGTCACTTTGGAAGTGCCTAAGACTATTGGTGCCAAATATGGTGGTCT
D D V K V T L E V L K T I G A K Y G G S
601 GACTACGAAGATGTTGTCTATTGGTATTGAATTATTAATGAACCATTTGGTCCAGTCTTA
D Y E D V V I G I E L L N E P L G P V L
661 GATATGGATGGCTTGAGACAATTCTACCAAGATGGATACTCTGAAATTAGAAACAACGAT
D M D G L R Q F Y Q D G Y S E I R N N D
721 GCGTCGAATCATACAATGCTATCATCATCCATGATGCATTCCAACAACTGACCACTAT
G V E S Y N A I I I H D A F Q Q T D H Y
781 TGGGACAATTTTATGCAAGTTTCTGGCGGATACTGGAATGTTGTTGTTGACCATCATCAC
W D N F M Q V S G G Y W N V V V D H H H

Figure 2. Nucleotide sequence and predicted amino acid sequence of the primary translation product. In the 5'-flanking region, the putative TATA element is underlined; the CAAG motif is indicated by a dashed line; potential transcription initiation sites are double-underlined (TCG/AA motif or RRYRR motif). In the coding region, the putative signal peptide is boxed and a potential processing site for a KEX2-like endoproteinase is indicated by an arrow; the signature pattern of the family 5 glycohydrolases is indicated by underlined residues

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841 TACCAAGTTTTTCGACCAAGCAGCATTAGAGTTGCTGATTGAAGACCATATCAAGACCGCC
      Y Q V F D Q A A L E L L I E D H I K T A
900 TGTAAGTGGGGTACAACCTACAAGGATGAAGCTCATTGGAACATTGTCGGAGAATGGTCA
      C N W G T T H K D E A H W N I V G E W S
961 TCTGCCTTAACCTGATTGTGCCAAGTGGCTTAACGGGGTTGGTCATGGAGCCAGATGGTCT
      S A L T D C A K W L N G V G H G A R W S
1021 GGTAACATATGATAACTGTCCATACATTGACAGTTGTCTGTCTTACTGACTGACTGAGCGGA
      G N Y D N C P Y I D S C L S Y T D L S G
1081 TGGACTGATGAATACAAAACGAACGTTAGAAAGTATACTGAAGCTCAATTGGATGCTCGG
      W T D E Y K T N V R K Y T E A Q L D A W
1141 GAACAAGTTGGTGGTTGGTTCTTCTGGTGTGGAAGACTGAAAGTGCACCAGAATGGGAT
      E Q V G G W F F W C W K T E S A P E W D
1201 TTCCAAGCATTAAACCAATGCTGGCTTAATCCCACAACCGTTGAACGACAGACAGTATCCA
      F Q A L T N A G L I P Q P L N D R Q Y P
1261 AACCAATGTGGGTACTAAGCTCAGAACAAAAGCATAATTTCAATAAAAATAATTTATTTTC
      N Q C G Y *

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Figure 2. Continued

Table I. β -glucanase secretion by *Candida oleophila*

Time (h)	MUG (glucanase activity/l $\times 10^8$ cells)		Laminarin (glucanase activity/l $\times 10^8$ cells)	
	18	42	18	42
pES214	2.66 \pm 0.14	4.19 \pm 0.26	1.25 \pm 0.01	2.02 \pm 0.38
pRS426	0.20 \pm 0.08	0.26 \pm 0.13	0.93 \pm 0.04	0.75 \pm 0.05

Results are based on the average \pm standard error (SE) of three replicates.

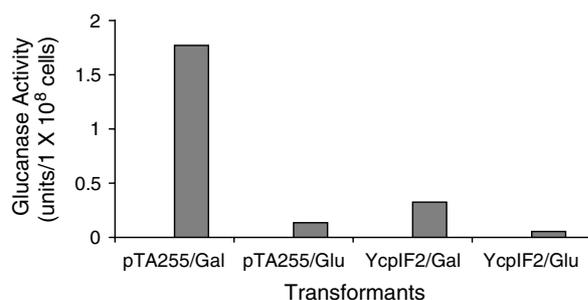


Figure 3. Glucanase expression of the *CoEXG1* open reading frame, as determined by MUG hydrolysis. The exoglucanase activity of *CoEXG1*, which was cloned under the *GAL1* promoter, was identified under both inductive and non-inductive conditions. pTA255/Gal, YPA84 with pTA255, which carries the *CoEXG1* grown in Gal medium; pTA255/Glu, YPA84 with pTA255, which carries the *CoEXG1* grown in YEPD medium; pYcplF2/Gal, YPA84 with pYcplF2, which served as a control for the induced condition; pYcplF2/Glu, YPA84 with pYcplF2, which served as a control for the non-induced condition

and a 42 bp sequence downstream of the 3'-end of the open reading frame (GenBank Accession No. AF39 3806).

The open reading frame, with a coding capacity of 425 amino acids, starts at an AUG codon, which is in a context similar to the preferred translation initiation site of eukaryotic genes, ANNATGPNNT (Kozak, 1981). At position 385–404 from this start codon is the signature pattern of the family 5 glycohydrolases, YEDVVIGIELINEPLGPVLD (Figure 5). The NEP motif in this amino acid sequence is the putative catalytic site of the protein (Chambers *et al.*, 1993).

Since the glucanase protein is secreted into the medium, it would be expected to contain a secretion signal peptide. Secretion leader sequences are hydrophobic, whereas water-soluble proteins are hydrophilic. In accordance with the hydropathy analysis of the *CoEXG1* protein (Kyte and Doolittle, 1982), it is apparent that the N-terminus is

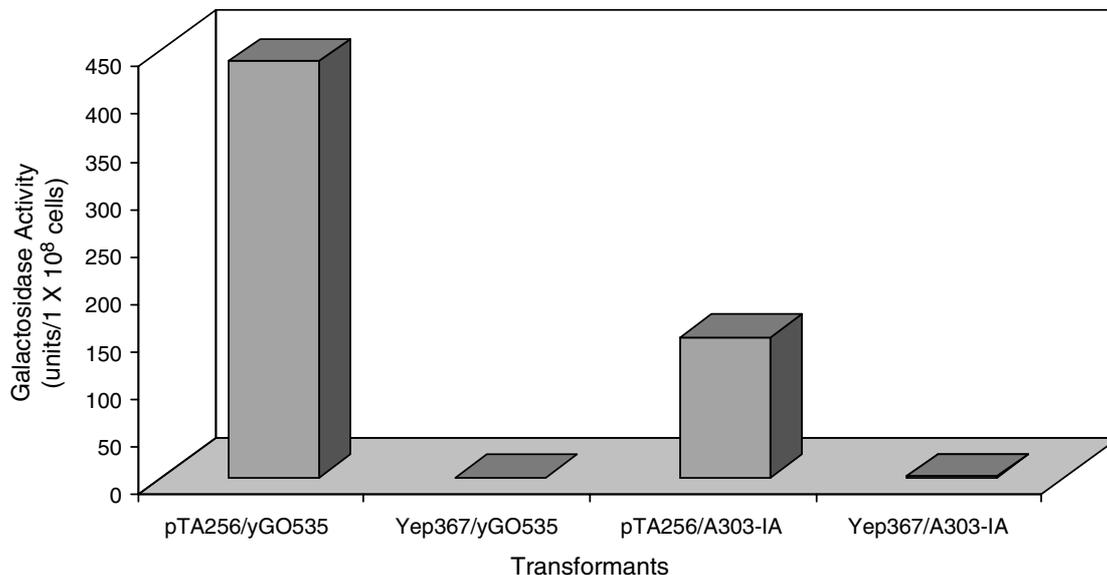


Figure 4. β -galactosidase induction by the *CoEXGI* promoter in *S. cerevisiae*. pTA256 carrying the *CoEXGI* putative promoter region fused to *lacZ* was analysed in two *S. cerevisiae* strains, yGO535 and A303-IA. Yep367 (pTA256 without the *CoEXGI* promoter) served as the negative control. Activity was determined by measuring β -galactosidase activity, with ONPG as the substrate (Sambrook et al., 1989)

hydrophobic, whereas the rest of the protein is hydrophilic (Figure 6). The first 26 amino acids constitute the putative leader signal, typical of secreted proteins (von Heijne, 1984). Its most likely cleavage site is located after residues RK, 26 amino acids from the N-terminus. This site may be digested by the endoprotease Kex2 (Bussey, 1988). The putative cleaved product is of 400 amino acids, with a predicted molecular weight of 48 774 Da and a calculated pI of 4.32.

On the route to secretion, proteins may be glycosylated. Yeast exoglucanases were found to have several glycoforms due to differential glycosylation of the primary translation product (Larriba et al., 1995). However, potential N- or O-glycosylation sites (Hubbard and Ivatt, 1981) are not present in the *CoEXGI* protein.

In the promoter region, instead of the putative TATA box with the canonical sequence TATAAA (Ponticelli and Struhl, 1990), a missing form, at position -290 , was identified. A pyrimidine-rich block, common to yeast promoters, was detected between nucleotides -301 and -322 . The motifs TCG/AA and RRYRR (a pyrimidine flanked by two purines), both of which are considered potential transcription sites (Hahn et al., 1985), were discovered at positions -195 and -218 , and -76 ,

-119 , -128 , -143 , -155 , -196 and -258 , respectively (Figure 2).

Downstream of the open reading frame and 28 nucleotides from the stop codon, a sequence beginning with TAAATAAT was sited, which contains part of the AATAAA element, usually encountered 10–30 nucleotides upstream of the polyadenylation site (Proudfoot and Brownlee, 1976) (Figure 2).

Comparison of *CoEXGI* to other yeast exoglucanase genes

When comparing the entire open reading frame of *CoEXGI* to that of glucanases of other yeasts, it was found that the highest identity was to the exoglucanase encoding gene, *SoEXGI*, from *S. occidentalis* and the lowest to the exoglucanase encoding gene, *ScEXG2*, from *S. cerevisiae*, 64.2% and 41.9%, respectively (Figure 7).

The most extensive research on genes encoding exo-1,3- β -glucanase has been in *S. cerevisiae*, in which three exo-1,3- β glucanase encoding genes have been identified. Two genes, *ScEXGI/BGLI* and *ScEXG2*, are expressed during mitotic growth and the third, *SSGI*, is expressed in meiotic cells (San Segundo et al., 1993). *ScEXGI* is secreted,

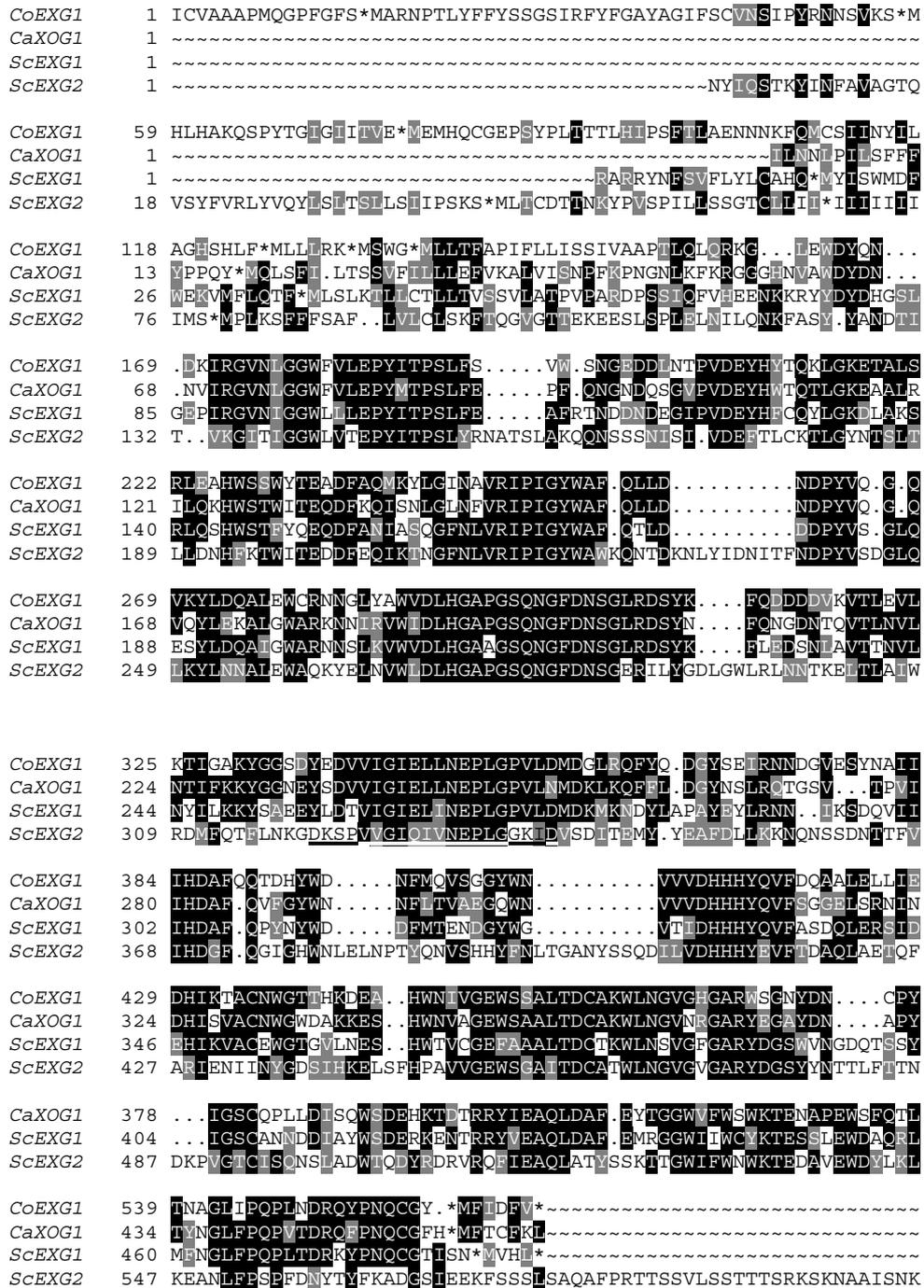


Figure 5. Multiple sequence alignment. The putative protein sequence of *CoEXG1*, *C. oleophila EXG1* gene; *CaXOG*, *C. albicans XOG* gene; *ScEXG1*, *S. cerevisiae EXG1* gene; *ScEXG2*, *S. cerevisiae EXG2* gene, were aligned using the CLUSTAL program (Higgins and Sharp, 1988). Black boxes indicate identical residues in at least three sequences. Half-toned boxes indicate conservative substitutions. The glycosyl hydrolases family 5 signature is underlined. Numbers refer to the adjacent amino acid residue in each line

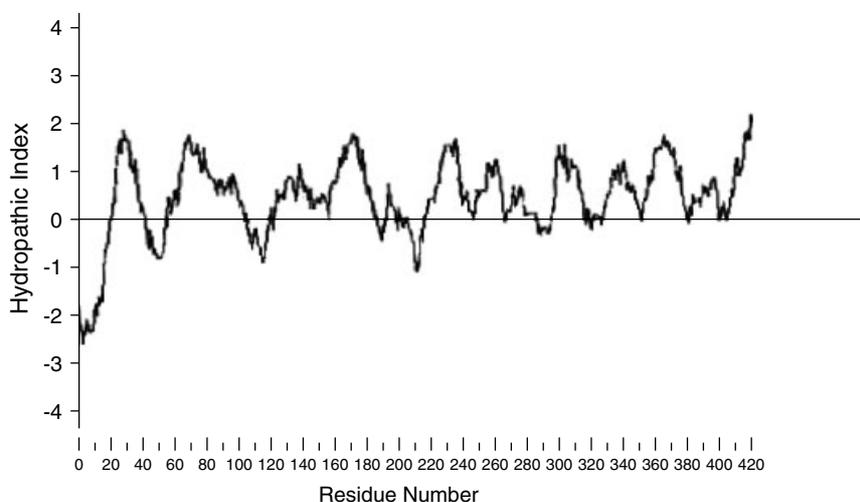


Figure 6. Hydropathy profile of the putative *CoEXG1* protein. The primary translated product of 425 amino acids is presented. Peaks above the midpoint line are hydrophilic regions and those below are hydrophobic. Calculated according to Kyte and Doolittle (1982)

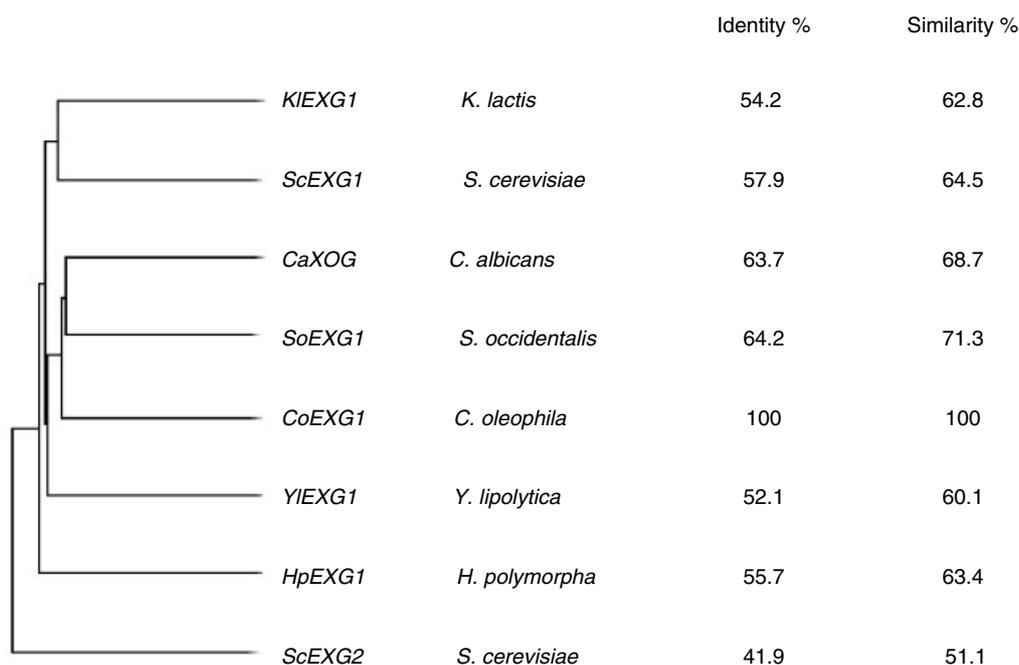


Figure 7. Dendrogram analysis of *C. oleophila* *CoEXG1* and related exo-1,3-glucanases from other yeasts. The degree of relatedness between *CoEXG1* and each homologue protein is shown as the percentage of identity or similarity for the whole length of the sequence, and has been calculated from pairwise sequence alignments, with gaps to maximize homology. The degree of similarity was calculated by taking into account conservative substitution

whereas *ScEXG2* is not (Nebreda *et al.*, 1986, 1987). Among the *S. cerevisiae* exoglucanases, *CoEXG1* has a higher degree of identity to *ScEXG1*, the secreted exoglucanase.

The cloning of *CoEXG1*, which is the first gene to be cloned from *C. oleophila*, adds a new gene to the exoglucanase yeast family, which in further investigations may help shed light on

the role of the secreted exo-1,3- β glucanase in biological control.

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