9

BARD

FINAL REPORT

PROJECT NO. IS-3117-99

**Enhancement of Postharvest Biocontrol Activity of the
Yeast *Candida oleophila* by Overexpression of Lytic
Enzymes**

S. Droby, M. Wisniewski, M. Goldway, W. Janisiewicz, C. Wilson

2003

6586 - 0

BARD Final Scientific Report

Date of Submission of the report: 23.11.2003

BARD Project Number: IS-3117-99R

Project Title: *Enhancement of Postharvest Biocontrol Activity of the Yeast Candida oleophila by Overexpression of Lytic Enzymes*

Investigators Institutions

Principal Investigator (PI):

S. Droby

ARO, the Volcani Center

Co-Principal Investigator (Co-PI):

M. Wisniewski

AFRS, USDA-ARS, USA

Collaborating Investigators:

M. Goldway Migal Biotechnology Center, Israel

W. Janisiewicz

AFRS, USDA-ARS, USA

C. Wilson

AFRS, USDA-ARS, USA

Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.

Glucanase, Chitinase, Defensin, Adhesin, Transformation

Abbreviations commonly used in the report, in alphabetical order:

Budget: IS: \$165.000

US: \$145.000

Total: \$310.000

Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

BARD Final Scientific Report

Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted)	5	----	-----	5
Submitted, in review, in preparation	2	-----	-----	2
Invited review papers				
Book chapters				
Books				
Master theses				
Ph.D. theses				
Abstracts	2	--	--	--
Not refereed (proceedings, reports, etc.)				

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Dr. Mierav Bar-Shimon – ARO-Laboratory

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	-----	2	2	4
Longer Visits (Sabbaticals)				

Description of Cooperation:

The research conducted during this three years project was carried out in full cooperation between the two Israeli and the American laboratories. S. Droby (ARO) and M. Goldway (Migal) used to meet on a regular basis to discuss and plan for the work conducted by their teams. Numerous discussions via the phone were done to exchange protocols, explain results and plan experiments. Results were communicated to the American partners via fax and e-mail. The Israeli PI (S. Droby) met the American PI (M. Wisniewski) in August 2002 during the International Horticultural Society meeting held in Toronto, Canada and discussed with his different aspects of the research and planned for future work. Additional visit conducted on June 2003 to discuss and coordinate the final phase of the work. Meeting of the PI and the CoPI were also held during the various International meeting (e.g. American Phytopathological Society meeting, New Orleans).

BARD Final Scientific Report

Patent Summary (numbers)

	Israeli inventor (s) only	US inventor (s) only	Joint IS/US inventors	Total
Submitted	--	--	--	--
Issued (allowed)	--	--	--	---
Licensed	--	--	--	--

BARD Final Scientific Report

Table of Contents

	<u>Page</u>
Abstract	5
Achievements	6
List of Publications	9
Appendix.....	10
Unpublished Data.....	11
Reprints, manuscripts and other publications	30

BARD Final Scientific Report

Abstract

Enhancing the activity of biocontrol agents could be the most important factor in their success in controlling fruit disease and their ultimate acceptance in commercial disease management. Direct manipulation of a biocontrol agent resulting in enhancement of diseases control could be achieved by using recent advances in molecular biology techniques.

The objectives of this project were to isolate genes from yeast species that were used as postharvest biocontrol agents against postharvest diseases and to determine their role in biocontrol efficacy. The emphasis was to be placed on the yeast, *Candida oleophila*, which was jointly discovered and developed in our laboratories, and commercialized as the product, Aspire. The general plan was to develop a transformation system for *C. oleophila* and either knockout or overexpress particular genes of interest. Additionally, biochemical characterization of the lytic peptides was conducted in the wild-type and transgenic isolates. In addition to developing a better understanding of the mode of action of the yeast biocontrol agents, it was also our intent to demonstrate the feasibility of enhancing biocontrol activity via genetic enhancement of yeast with genes known to code for proteins with antimicrobial activity.

Major achievements are: 1) Characterization of extracellular lytic enzymes produced by the yeast biocontrol agent *Candida oleophila*; 2) Development of a transformation system for *Candida oleophila*; 3) Cloning and analysis of *C. oleophila* glucanase gene; 4) Overexpression of and knockout of *C. oleophila* glucanase gene and evaluating its role in the biocontrol activity of *C. oleophila*; 5) Characterization of defensin gene and its expression in the yeast *Pichia pastoris*; 6) Cloning and Analysis of Chitinase and Adhesin Genes; 7) Characterization of the rnase secreted by *C. oleophila* and its inhibitory activity against *P. digitatum*.

This project has resulted in information that enhanced our understanding of the mode of action of the yeast *C. oleophila*. This was important step towards enhancing the biocontrol activity of the yeast. Fungal cell wall enzymes produced by the yeast antagonist were characterized. Different substrates were identified to enhance there production *in vitro*. Exo- β -1,3glucanase, chitinase and protease production was stimulated by the presence of cell-wall fragments of *Penicillium digitatum* in the growing medium, in addition to glucose. A transformation system developed was used to study the role of lytic enzymes in the biocontrol activity of the yeast antagonist and was essential for genetic manipulation of *C. oleophila*. After cloning and characterization of the exo-glucanase gene from the yeast, the transformation system was efficiently used to study the role of the enzyme in the biocontrol activity by over-expressing or knocking out the activity of the enzyme. At the last phase of the research (still ongoing) the transforamtion system is being used to study the role of chitinase gene in the mode of action. Knockout and over expression experiments are underway.

BARD Final Scientific Report

Achievements:

1. Characterization of extracellular lytic enzymes produced by the yeast biocontrol agent *Candida oleophila*: The present study showed that the yeast *C. oleophila* is capable of producing and secreting various cell wall degrading enzymes including exo- β -1,3-glucanase, chitinase and protease. Exo- β -1,3-glucanase and chitinase were produced and maximized in the early stages of growth, whereas protease reached his maximum levels only after 6-8 days. Exo- β -1,3glucanase, chitinase and protease production was stimulated by the presence of cell-wall fragments of *Penicillium digitatum* in the growing medium, in addition to glucose. This study also provided evidence that *C. oleophila* is capable of secreting exo-glucanase into the wounded surface of grapefruit. The role of exo- β -1,3-glucanase (CoEXG1) in the biocontrol activity of *C. oleophila* was tested by using CoEXG1-knockouts and double-CoEXG1 over-producing transformants. In *in vitro* bioassays we found that wild-type *C. oleophila* and exo-glucanase over-expressing transformant had similar inhibitory effects on spore germination and germ-tube elongation, and both were more inhibitory to the fungus than the knockout transformant. In experiments conducted on the fruit to tests their biocontrol activity against infection of *P. digitatum*, no significant difference in inhibition were observed between transformants and untransformed *C. oleophila* cells at the high concentrations of cells used, whereas at the lower concentration of yeast cells the knockout transformants appeared to be less effective.

This work was accepted for publication in "Current Genetics" (In press, see appendix for details).

2. Development of a transformation system for *Candida oleophila*: At the initial phase of the project a transformation system for the yeast *Candida oleophila* was developed. The hygromycin B resistance gene, flanked by the phosphoglycerate kinase promoter and terminator of *C. tropicalis*, served as the genetic selection marker. The transformation efficacy of electroporation was almost 400 times more efficient than the lithium acetate method. While incorporation of DNA flanked by a sequence endogenous to *C. oleophila*,

BARD Final Scientific Report

transpired apparently by homologous recombination, the integration of DNA (that did not contain *C. oleophila* DNA) occurred at random.

This work was published in "Current Genetics" 40:282-287 (2001) (see appendix).

3. Cloning and analysis of *C. oleophila* glucanase gene: *CoEXG1*, 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 analyzed. Its open reading frame and putative promoter were expressed in baker's yeast, *Saccharomyces cerevisiae*. The reading frame, expressed under the inducible *GAL1* promoter, caused an increased secretion β -glucanase, and the putative promoter region activated the *LacZ* reporter gene, to which it was fused. Sequence analysis revealed that *CoEXG1* carries the signature of 5 glycohydrolases family and has putative secretion leader, as well as a high degree of identity to yeast 1,3- β - glucanases.

This work was published in "Yeast" 19:1171-1182, (2002) (see appendix).

4. Effect of under- and overexpression of *C. oleophila* glucanase gene on biocontrol activity: The relationship of β -exoglucanase in the biocontrol activity of *C. oleophila* was investigated by generating *C. oleophila CoEXG1*-knockouts and double-*CoEXG1* transformants. The knockout transformants secreted 0-0.13% of the exoglucanase activity detected in the medium of the untransformed yeast. Indicating that *CoEXG1* is the main gene responsible for production and for secreted exoglucanase. Correspondently, the double-*CoEXG1* transformants secreted approximately twice as much 1,3- β -exoglucanase as the untransformed *C. oleophila*. The biocontrol activity of the *CoEXG1*-knockout and the double-*CoEXG1* transformants against *Penicillium digitatum* did not differ from the untransformed *C. oleophila* kumquat fruit. These results implied that the 1,3- β -exoglucanase encoded by the gene *CoEXG1* is not the only gene involved in the biocontrol activity of the yeast.

This work was published in "Yeast" 20: 771-780 (2003) (see appendix).

BARD Final Scientific Report

5. Characterization of defensin gene and its expression in the yeast *Pichia pastoris*: In the present study, we describe the cloning and characterization of a defensin gene (*PpDfn1*) from a cDNA library made from winter bark tissues of peach (*Prunus persica*). A partial clone obtained from the library was extended to full length by 5' RACE. The open reading frame of 237 bp codes for a 79 amino acid peptide related to the defensin family of proteins. Sequence comparison of the encoded protein using BLAST analysis revealed significant homology to defensins from other plant species. RNA gel blot analysis indicated that the gene is seasonally-expressed in bark tissues of one-year-old shoots, and is also expressed in early fruit development. Quantitative RT-PCR and protein blot analysis largely paralleled the RNA gel blot analyses for the bark tissues. A recombinant version, rDFN1 was expressed in the yeast, *Pichia pastoris*. It was found that rDFN1 inhibited germination of the fungal pathogens *Penicillium expansum* and *Botrytis cinerea*, but not the Gram-negative bacterium *Erwinia amylovora*. The potential physiological role of PpDFN1 and its antimicrobial properties are discussed.

This work was published in "Physiologia Plantarum 119:563-572 (2003) (see appendix).

BARD Final Scientific Report

Cooperation

List of Publications:

1. Yehuda, H., Droby, S., Wisniewski, M., and Goldway, M. (2001). A transformation system for the biocontrol yeast, *Candida oleophila*, based on hygromycin B resistance. *Current Genetics* 40: 282-287.
2. Bar-Shimon, M., Yehuda, H., Cohen, L., Weiss, B., Kobeshnikov, A., Daus, A., Goldway, M., Wisniewski, M., and Droby, S. 2004. Characterization of extracellular lytic enzymes produced by the yeast biocontrol agent *Candida oleophila*. *Current Genetics* (in press).
3. Segal, E., Yehuda, H., Droby, S., Wisniewski, M. and Goldway, M. (2002). Cloning and expression of the 1,3- β -glucanase gene of a yeast biocontrol agent *Candida oleophila*. *Yeast* 19:1171-1182.
4. Wisniewski, M.E., Basset, C.L., Artlip, T.S., Webb, R.P., Janisiewicz, W., Norelli, J.L., Golway, M., and Droby, S. 2003. Characterization of defensin in bark and fruit tissues of peach and antimicrobial activity of recombinant defensin in the yeast *Pichia pastoris*. *Physiologia Plantarum* 119:563-572.
5. Yehuda, H., Droby S., Bar-Shimon, M., Wisniewski, M. and Goldway, M. 2003. The effect of under and overexpressed *CoEXG1*-encoded exoglucanase secreted by *Candida oleophila* on the biocontrol of *Penicillium digitatum*. *Yeast* 20:771-780.

BARD Final Scientific Report

Appendix

BARD Final Scientific Report

Unpublished data:

Cloning and Analysis of Chitinase and Adhesin Genes from Several Species of Yeast Used to Manage Postharvest Disease of Fruit

Introduction

Nutrient competition, parasitism, site exclusion and lytic-enzyme-action have been proposed as modes of action for yeast antagonists used as biocontrol agents (Wisniewski et al., 1991; Droby and Chalutz, 1994; Janisiewicz and Korsten, 2002). Hydrolytic enzymes such as glucanases and chitinases are a topic of interest due to their ability to degrade fungal cell walls. Adhesins, such as the *Candida albicans* ALS1-5 (agglutinin-like-sequence), are proteins demonstrated to play an essential role in the adhesion of this human pathogen to a wide variety of host surfaces, and consequently in the disease process (e.g., Hoyer et al., 1995; Hoyer et al., 1998). It is our hypothesis that adhesins may also play a role in the attachment of antagonistic yeasts to higher fungi.

To better understand the role that chitinases and adhesins may play in the mode(s) of action of biocontrol yeasts, we have undertaken a program of isolating such genes from a number of biocontrol organisms. The eventual objective of this research is to alter the expression of these genes (over-express or 'knock-out') in order to determine the effect on biocontrol activity. Presently, we describe the construction of genomic libraries of three biocontrol yeasts and the partial or full sequence information of several chitinases and adhesins from these yeasts.

Materials and Methods

Strains and Media

Yeast strains used to generate chitinase and adhesin partial clones from genomic DNA were *Candida oleophila*., *Candida saitoana*, *Pichia guilliermondii*, and *Debaryomyces hansenii*. Cultures were grown in YM liquid media at 26°C overnight with shaking.

PCR-Cloning of chitinase and adhesin gene fragments

Genomic DNA was isolated from isolates of the yeasts *C. oleophila*, *D. hansenii*, and *P. guilliermondii* using the Dr. Gentle Kit (Fisher Scientific, Pittsburgh, PA). Degenerate primers used to amplify chitinase gene fragments via PCR were as per McCreath et al. (1995), CHTdegF (5'-ATC ATG RTI TAY TGG GGI CAR AA-3'), and CHTdegR (5'-GAG CAR TAR TAR TTR TTR TAR AAY TG-3'), and spanned the catalytic domain (Figure 1). 250 ng samples of genomic DNA were subjected to 25 cycles of PCR (GeneAmp9700, PE-ABI, Norwalk, CT), with parameters of 30 secs at 95°C, 30 secs at 48°C, 1 min at 72°C, followed by a terminal cycle of 20 min at 72°C.

Genomic DNA from *C. oleophila* and *P. guilliermondii* was also used as template to amplify adhesin gene fragments. Degenerate primers used to amplify these fragments were designed against the highly conserved N-terminal domain within the ALS family (Figure 1), and were designated ALS214Forward, 5'-ATG CCM TGY GTD TTY AAA TTY A-3' and ALS824 Reverse, 5'-GGA CGA TAA CCK GCA GGA ACA T-3'. 1 mg samples of genomic DNA were subjected to 25 cycles of PCR (GeneAmp9700, PE-ABI,

BARD Final Scientific Report

Norwalk, CT), with parameters of 30 secs at 95°C, 30 secs at 55°C, 1 min at 72°C, followed by a terminal cycle of 20 min at 72°C.

Products of the reactions were then cloned into pCR2.1 (TOPO-TA Cloning Kit, Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. The clones were then sequenced [BigDye Terminator Kit (PE-ABI) on an ABI310 (PE-ABI) and analyzed by BLAST (Alschul et al., 1997).

Construction and Screening of Genomic Libraries

Genomic DNA from *C. oleophila*, *P. guilliermondii*, and *C. saitoana* was isolated using the Yeast Smash and Grab DNA miniprep method (Rose et al., 1990). The genomic DNA was then partially digested using EcoR I and separated according to size over a sucrose gradient (Sambrook et al., 1989). The resulting fractions were dialyzed to remove sucrose and analyzed on a 0.6% agarose gel in 1X TAE. Fragments between 6 and 12 kb were used to construct the genomic library using the ZAP Express Predigested Vector Kit (Stratagene, LaJolla, CA). Phage were packaged using Gigapack III Gold phage extract (Stratagene). Libraries were screened for chitinase (CHT) and adhesin (ALS) genes using PCR (Amaravadi and King, 1994). Chitinase primers used to screen each genomic library were designed from consensus regions of chitinase partial sequences and the *Candida albicans* chitinases 1, 2, and 3 (i.e., proposed catalytic region). These primers were CHT101 forward, 5'-CGA TTA GCC CAG TTG ACA TTT CT-3' and CHT473rev 5'-GGG GCT GCA GAT AAG TAG TAG GT -3'. Cycling parameters were as per Amaravadi and King (1994), with an annealing temperature of 46°C. The degenerate primers used to isolate the adhesin gene fragments were also used to screen the libraries, using the parameters stated above.

The potential genomic clones were then subcloned to increase ease of sequencing. The genomic clones were subjected to PCR with nested primers, with the resulting amplicons ligated into the pCR-XL-TOPO vector via the TOPO-XL PCR Cloning Kit (Invitrogen). Sequencing was performed on the LiCor IR2 using labeled Universal M13For/M13Rev primers (LiCor, Lincoln, NE). Resulting data was then analyzed utilizing either LiCor's AlignIR software or DNastar software.

BARD Final Scientific Report

Figure 1. Schematic of chitinase and adhesin proteins from *Candida albicans*. A. Chitinase. The Ser/ Thr-rich domain is not present in C.a. CHT1. B. Adhesin. The tandem-repeat domain is variable within the family.

Results and Discussion

Partial or full-length clones of chitinase genes were successfully cloned from several yeast species. The full length chitinase clone from *C. oleophila* and the deduced amino acid sequence is illustrated in Fig. 2. The various protein domains are also highlighted. An analysis of this clone suggests that it is most like the *C. albicans* CHT1 gene, due to the lack of the extensive Ser/ Thr-rich domain found in *C.a.* CHT2 and CHT3. An overall identity of 46% at the amino acid level exists with *C.a.* CHT1, with higher homology observed in the conserved catalytic domain.

TATAGGCGCAATTGGCGCCCTCTAGATGCATGCTCGAVCVGCCGCCAGTGTGATGGATATC
TGMRGAAATCGCCCTTATAAGAAARAAGGCAATAGCCTTGGCGTTCTAAGAAAAATATGCAC
TTCTTCCCTCAGCCATAGAATACTGCACTGATTCTCTAACTTCTCAACTCGGGATTAAAGG
TAAATACATGTTTTCTCATATTACTAAAACTTTAATAACAACATGTTTGCTCACTAAAGC
AGCAAGGCGCCTAGATGTTTGTTCCTATTGGGGGCAAAACTCTGGAGGTGACCAGGGCCC
ATTAGAAATATTGTGGAAGTACTGCCGATATAATCGTTGTGTCTATTCATAAACTCGAT
L R N Y C G S T A D I I V V S F I N S I
TAGCCCAAGTTGACATTCTATAAATGGCCAAATGTAGTGGCTCATTCFAATGACGGTACCTT
S P V D I S I N G Q C S G S F N D G T L
GCATTGTCCATCTGTCCGTGAAGACATTACTTACTGTCAACAACAGGGGCAAAAAGTTCT
H C P S V G E D I T Y C Q Q Q G K K V L
TTTGAGTATCGGGGGTCAAGGTGCACAAGGATTCTCCTCAGACTCTGAAGCCACCACCTTT
L S I G G Q G A Q G F S S D S E A T T F
CGCTACCAACGTTTGGAAACAAATTTGCGGGTGTTTCTCCGACGAAAGACATTGTGACAC
A T N V W N K F A G G S S D E R P F D T
TGCTATTGTAGATGGATTGCACTTGGATTAGAAGTGGTTGGCCCAAAGGAACAGTTGC
A I V D G F D L D L E V V G P K G T V A
ATTTGGTAAGGCAATTAACTGATTGTTTGCAAAAGATACTTCTAAACCTACTACTATTATC
F G K A I K S L F A K D T S K T Y Y L S
TGCAGCCCTCAGTGTCAAGTATCCTGATGCTACTTTAAGTGATTACTTGACCCAAAGTTCC
A A P Q C Q Y P D A T L S D Y L T Q V P
AGTTGATTTACCTTTGTTCAAATCTACAACAACCTTCGTGTCAATTAGATGGTGATT
V D F T A T T F V Q F Y N N P S C A Q L G A D G D F
CAACTTTAATCTTGGTCCCAGTATGCAAGATNCTTCCCCAAATGAGAAGAACAAGAT
N F N T W S Q Y A R S S S P N E K N K I
TTTCGTTGGTTTACCGGGTAGTAGCTCTTCAAGTGGTTCTGGGTTTGTTCGCGCTCCGT
F V G L P G S S S S S G S G F V S A S V
CGTTAAGTCTCAGTGTGAAAAAATTAATGTGATCCAACTTAGGAGGTGTGTCCCTTTG
V K S Q L E K I K C D P N L G G V S L W
GGACGCTTCTCAAGTTTTCCTCTGGGTTTGCAGCAAGCATCAATCAGTCTTGAATC
D A S S S F
TATGGTTGTTAGTTCTTGTCTCTCGTTCACTCATATTCATCCGATGCTACTTCTACCTC
TGACAGTTCCACATTGGTTCCGCCCTATTATTTCAATTAGGGATCCGGTACATAGTAGC
TGCTACTGCTGATGTTCCAAATTAAGTTCACGTAATTGTTTTATCATAGATTTTGCCTCA
GGTGTCAAGCAAGCGAGGTTGCACTACTTTCCCATCTTTATCATCTATCACTAAAGTGAGG
TTCCGCGCAGCACCAAGGCTTGAATACTTTCTGCATAAGACGAAAGCCGAAAGGGCGAAT
TCCAGCACAATGGCGCGCTTAGTGTGBNCTCGAGGTGGGTACCAAGCTTGATGTATMGYT
TCGCTCTTGTCCGKCKYCGGGTCCCTCKSCTTSCYCCYCCYCCY

BARD Final Scientific Report

Figure 2. Nucleotide and predicted amino acid sequences of *Candida oleophila* Cht1. The putative ATG start codon is in bold. The colored amino acid sequences reflect the scheme presented in Figure 1A (signal peptide in pink, conserved catalytic domain in yellow, and C-terminal domain in green).

A sequence comparison of the chitinase clone obtained from *C.oleophila* with reported chitinase genes from *C. albicans* is presented in Figure 3.

[illegible]

Figure 3. Alignment of the *Candida albicans* chitinases with the *Candida oleophila* chitinase. Predicted amino acid sequences, one-letter code. C.a. CHT1-3 refer to the *Candida albicans* chitinase sequences, while C.o. CHT refers to the *Candida oleophila* sequence. Color scheme as in Figure 1A (signal peptide in pink, conserved catalytic domain in yellow, Ser/ Thr-rich domain in grey, and C-terminal

BARD Final Scientific Report

domain in green). Boxed regions indicate degenerate primer sites. GenBank accession numbers: C.a. CHT1, U36490; C.a. CHT2, U15800; C.a. CHT3, U15801.v

The sequences obtained from other partial clones also revealed high homology with other chitinases and therefore most likely represent partial chitinase clones. These comparisons and a summary of subgroups within the chitinase clones obtained from our yeast species are presented in Figures 4 and Table I.

Figure 4. Alignment of partial chitinase clones from several yeast species using predicted amino acid sequences, one-letter code. Identical residues are boxed. Partial clones were generated using degenerate primers specific to the *Candida albicans* chitinase catalytic domain. C.o. is *Candida oleophila*, P.g. is *Pichia guilliermondii*, and D.h. is *Debaryomyces hansenii*.

BARD Final Scientific Report

Putative chitinase grouping	Yeast species
Group 1	<i>Candida oleophila</i> , <i>Debaryomyces hansenii</i>
Group 2	<i>Candida oleophila</i>
Group 3	<i>Pichia guilliermondii</i>
Group 4	<i>Candida oleophila</i>
Group 5	<i>Debaryomyces hansenii</i>

Table I. Summary of putative chitinase gene fragment groupings from various antagonistic yeasts. Gene fragments were from the conserved catalytic domain.

In addition to the partial and full-length chitinase genes obtained via PCR or screening of genomic libraries, partial and a near-full-length clones of and adhesion gene were obtained via PCR or screening of genomic libraries of *Candida saitoana* or *Pichia guilliermondii*. The near-full-length clone was obtained from *C. saitoana*. A comparison of a portion of the deduced amino acid sequence of this gene is presented in Figure 5. Our clone has the greatest homology with ALS1 adhesin from *C. albicans*.

Figure 5. Alignment of the *Candida albicans* adhesins with the *Candida saitoana* adhesin. Predicted amino acid sequences, one-letter code. C.a. ALS1-4 refer to the *Candida albicans* chitinase sequences, while C.s. ALS refers to the *Candida saitoana* sequence. Color scheme as in Figure 1B (signal peptide in pink, conserved N-

BARD Final Scientific Report

Terminal domain in blue). Boxed regions indicate degenerate primer sites.

GenBank accession numbers: C.a. ALS1, L25902; C.a. ALS2, AF024580 &82; C.a. ALS3, U87856; C.a. ALS4, AF024584&86.

The near-full-length clone of the adhesion gene was obtained by screening a genomic library of *C. saitoana*. The gene is quite large and the current clone is over 3.2 kb in length and is slightly truncated at the 3 prime end. We are currently performing genome walking to obtain the complete clone. The sequence and deduced amino acid of our adhesion gene is presented in Fig. 6.

```
ATGATCTTGAATCTATTGGTATTTCAATTGGATTGCAATTGTTATTGCGAAAGATAIA
M I L N L L V F S I W I A I V I A K D I
TCAGGCGTTTTCACCAGTATTAGCTCCGTTGAATGGCAACCGGGGTCTAATTATGCCTAT
S G V F T S I S S V E W Q P G S N Y A Y
GCTGCTCCGTTATTTCCAGTTGGTATATTACACTCAATTGGCAAATTGACGGTTCGGTA
A A P L F P S W Y I T L N W Q I D G S V
GCTTCACCAGGGGATACATTTTACTTGAACATGCCATGCGTGTTCAAATTCACTTCCACA
A S P G D T F Y L N M P C V F K F T S T
GTAACCACGATCGATTTAGTCGCTGGTACCACTGCCTATGCCACATGTACAATGAATGGT
V T T I D L V A G T T A Y A T C T M N G
GGAGAACTCACGGTACCTTATTCCTCGTTGAATTGTGTAGTTAATTCGGGGGTGATATCG
G E L T V P Y S S L N C V V N S G V I S
CTGGTATTTATACAGGGGATATTGTGTTCCCTGTAACCTTCAACGTTGGTGGGTCTGCAC
T G I Y T G D I V F P V T F N V G G S A
TTTCCAGTGACTTGCAATGTGCTGCTTACTTTTCTTCTGGCCCCAACACATTTAATAATG
L S S D L Q C A A Y F S S G P N T F N N
TTGTTACTTTCAACGATGGAACATAAGTATTTCCATAGTTGCTCTGGAGTTCCAATCGG
V V T F N D G T N S I S I V A L E F Q S
GATCACCCAATACCACTGAGAGAAGACCCAACCAATATGTTTACATGAATAGAGCTATT
G S P N T S G E D P T K Y V Y M N R A I
TTATCAACCAAACAGAATCAACACTTACTTATGGGTGGAACCTGTACCAATGGGATTAGT
L S T K Q N Q H L L M G G T C T N G I S
TCGGGAACCCTCGGATTTACCTTAACCTCAACAAGGACTGATCTCATGTAGTAATATCAAT
S G T L G F T L T Q Q G L I S C S N I N
GCTGATATTACCAACCAACTCAATGCTTGGTACATGCCTTTGGCTGCTTCTACTAACTTC
A D I T N Q L N A W Y M P L A A S T N F
CAATATAACCACCAGTTGTTCTACTCTGTCTTATACAATTTCAATATTCTAATATTCTGCA
Q Y T T S C S T L S Y T I S Y S N I P A
GGTTATCGGGTGTTTATGAATATGTTGATTTCTGCAACAGTGGGATCTGCCATATCGATA
G Y R V F M N M L I S A T V G S A I S I
CGTTACACCAACACTTATACTTGTGTAGGATCATCAACAGTTACCAATAATGGGTTTACG
R Y T N T Y T C V G S S T V T N N G F T
ATAAACTGGGGATCATAACCAATGGTGGGGTGAGTGGTTCTGGAAGTGCCAGTCAGGTT
I N W G S Y T N G G V S G S G S A S Q V
ATCATCACCACAAGTACATATTACAGGTTCAATTACTCAGGTTACCACATTACCATATACT
I I T T S T Y S G S I T Q V T T L P Y T
```

BARD Final Scientific Report

TCGAATGGAATTGAGACGATTGTGGTCAATGTACCTACACCTACTGTACAGTTACTACC
S N G I E T I V V N V P T P T V T V T T
ACTGTTCCAACTCCATATACCACGACAGTGACTGCTACTACTGGAGGAACTGCTACTTTG
T V P T P Y T T T V T A T T G G T A T L
ATTATTGGTGTACCAACTCCACTGACGACTATTACTAGTACTTGGACTGGTTCTACTATT
I I G V P T P L T T I T S T W T G S T I
TCAACTACCACCTTTACCATTTACCCTGGTGGAATAAACTGTTGTCGTTGAAGTGCCT
S T T T L P F T T G G T K T V V V E V P
ACGCCAACAATACTGTTACTTCAACTTGGACTGGTTCTACAATTCTACAACAACATTA
T P T T T V T S T W T G S T I S T T T L
CCAGTTACTTCTGGTGGTACACAACTGTCGTGGTTGAAGTTCCAACTCCATCCACTACT
P V T S G G T Q T V V V E V P T P S T T
ATTACCAGTACTTGGGATCAAAGCACAATACTGGCACTACTACTTTACCATTTACTACTGGC
I T S T W D Q S T T G T T T L P F T T G
GGAATAAACTATTGTGGTTGAAGTTCCAACACCTTCTACTACTGTTACTAGCACATGG
G T K T I V V E V P T P S T T V T S T W
ACAGGTTTCGACGATTGAAACAATACTTTGCCGGTCACTTCTGGTGGTACCCAAACAGTT
T G S T I E T T T L P V T S G G T O T V
GTAGTCGAAGTTCCAACCCCAACCGCTACTATTACATCTACTTGGACTGGTTCCGAAGTG
V V E V P T P T A T I T S T W T G S E V
TCTACAATACTACAATACTGCAACTGAGGGTGGTACAGACATAGTGGTGGTTCGAGGTGCCT
S T T T I T A T E G G T D I V V V E V P
ACACCAATACTACTACAATACTCGACTTGGACTGGTTCCATTGTATCAACAATACTTTA
T P T T T T T S T W T G S I V S T T T L
CCATTTAGTACTGGTGTGGCATTAACTGTGGTAGTCGAAGTTCCAACCCCAACCACTACT
P F S T G V A L T V V V E V P T P T T T
ATTACATCTACTTGGACTGGTTCCGAAGTGTCTACAATACTACAATCGCTGCAACTGAAGGT
I T S T W T G S E V S T T T I A A T E G
GGTACGGATACGGTTGTTGTTGAAGTTCCAATACTACAATACTAGAGTGACATCTACTTGG
G T D T V V V E V P T P T T R V T S T W
ACTGGCTCTGAAATGTCCACTACTACCTTACCATTTAGTACTGGGGGAACATTAACCGTT
T G S E M S T T T L P F S T G G T L T V
GTTGTTGAAGTACCAACACCTACAATACTATTACGTCCACGTGGACTGGCTCCGGAGAT
V V E V P T P T T T I T S T W T G S G D
TACAACAATACTACAATCGCTGCGACAGAAGGTGGAATACTGAAACGGTGGTAGTTGAAGT
S T T T I A A T E G G T E T V V V E V
TCCAACACCTACAATACTACTGTGACTTCTACTTGGACTGGTTTGGATCAATCAATACGAC
P T P T T T V T S T W T G L D N S T T T
TATTCTTGCAAGTGAGGGTGGTACAGACGTAGTGGTGGTTGAGGTACCTACACCAACTAC
I L A S E G G T D V V V V E V P T P T T
TACTACAATACTCGACTTGGACTGGTTCAATTATATCAATACTACTTTACCATTTAGTAC
T T T S T W T G S I I S I T T L P F S T
TGGTGGGACTTTAACAGTTGTCTGTTGAAGTACCAACGCCTACCACTACTGTAACCTCAAC
G T T S T W T G S I I S I T T L P F S T
TTGGACTGGTTCCGAAGTGTCTGACAGTGACAATACTGCAACTGAAGGTGGAATACTGA
G G T L T V V V E V P T P T T T V T S

BARD Final Scientific Report

```

AACGGTGGTAGTTGAAGTTCCAACACCTACAACCTACTATAACTTCTACTTGGACTGGTTT
T W T G S E V S T V T I T A T E G G T E
GGATAATTCAACTACGACTATCATTGCAACTGAAGGTGGTACTGATACGGTGGTAGTCGA
T V V V E V P T P T T T I T S T W T G L
AGTATGCACTCCATCTACTACTATTACATCTACCTGGAGTGGTTCGGATATTTCAACGAC
D N S T T T I I A T E G G T D T V V V E
TACTATTCTAGCGAGCGAGGGTGGTACTGATACGGTGGTGGTTGAAGTGCCTACTCCAAC
V P T P S T T I T S T W S G S D I S T T
TACTACTATTACCTCAACGTGGACTGGTTCTAATATTTCAACAACCACTTTACCATTAC
T I L A S E G G T D T V V V E V P T P T
CGAGGGTGGTACTGAGACTGTCGTGGTCGAACIACCAACTCCCGTTACTACTATCACATC
T T I T S T W T G S N I S T T T L P F T
TACGTTGACTGGCTTGGAAACATCAACTTCAACATTGAATTTTACAGAAGGCGGAAGTGA
E G G T E T V V V E V P T P V T T I T S
AACTATTGAAATATTGGTTCCAACACCATTGACAACATAACATCTACTTGGACTGGCTT
T W T G L E T S T S T L N F T E G G T E
GGAAACATCAACATCAACTTTGAATTTCACTGAAGGTGGTACTCAAACAATAGAAATATT
I E I L V P T P L T T I T S T W T G L E
GGTCCCAACGGTTGTTTCTCTTAGTTCGAATCTTTTCAGAGAGTTCAAGCGATTTCGTCTTT
T S T S T L N F T E G G T Q T I E I L V
TATGTCATCCGCTGAGTCATCAACTGGTATTACCTCAGAGCTGGCCTCTTCGAGTAGTTA
P T V V S L S S I F S E S S S D S S F S
TCTAACTTCTTCATCTGAGGTTGTTAGTAGTAGTATTAGCTACTTAAGTTACACCAG
S S A E S S T G I T S E L A S S S S Y L
CTGGAAGTATTATCTTCAACCGATTTTCCTACCATATCCATGCTGCTGTCATCCTCGGGGT
T S E V V S S S S I S Y L S Y T S L E V
TTGTCTTCATCAGAATCAGAGACTTTAGACACTTTGTCTTATCTGTGACGATAAAGTAT
L S S T D F P T I S M L L S S S G L S S

GGAATTCTTTGGATCCAC
S E S E T L

```

Figure 6. Nucleotide and predicted amino acid sequences of the genomic *Candida saitoana* adhesin clone. The putative ATG start codon is in bold. The colored amino acid sequences reflect the scheme presented in Figure 1B (signal peptide in pink, conserved N-Terminal domain in blue). Blue regions indicate N-glycosylation sites, red regions indicate motifs present in the tandem repeat region, and the green areas represent the 3 prime S/T-rich region of the gene. This latter region is truncated in the present clone.

Figure 7 represents an alignment comparison of the partial clones we obtained from three of the yeast species by PCR-cloning. In subsequent work we will screen the genomic library of *Pichia guilliermondii* in order to obtain additional adhesin clones.

BARD Final Scientific Report

1	M	P	C	V	F	K	F	T	S	T	T	I	N	L	V	A	G	T	S	Y	A	T	C	T	M	N	S	G	E	L	T	V	P	Y	S	S	L	N	C	V	V	N	S	G	V	I	S	C.s-a			
1	M	P	C	V	F	K	F	T	S	T	T	E	V	E	L	K	V	D	S	T	A	Y	A	S	C	S	F	I	A	G	E	L	L	D	Y	S	E	L	K	C	I	V	L	P	T	I	K	K	C.s-b		
1	M	P	C	V	F	K	F	T	S	T	T	E	V	E	L	K	V	D	S	T	A	Y	A	S	C	S	F	I	A	G	E	L	L	D	Y	S	E	L	K	C	I	V	L	P	T	I	K	K	C.s-c		
1	M	P	C	V	F	K	F	T	S	T	T	E	V	E	L	K	V	D	S	T	A	Y	A	S	C	S	F	I	A	G	E	L	L	D	Y	S	E	L	K	C	I	V	L	P	T	I	K	K	C.s-d		
1	M	P	C	V	F	K	F	T	A	N	T	P	T	V	Q	L	K	V	N	N	D	V	Y	A	T	C	N	F	Y	S	G	E	I	I	T	T	Y	S	E	L	Q	C	T	M	S	D	L	V	N	D	P.g-a
1	M	P	C	V	F	K	F	T	A	N	T	P	T	V	Q	L	K	V	N	N	D	V	Y	A	T	C	N	F	Y	S	G	E	I	I	T	T	Y	S	E	L	Q	C	T	M	S	D	L	V	N	D	P.g-b
1	M	P	C	V	F	K	F	T	A	N	T	P	T	V	Q	L	K	V	N	N	D	V	Y	A	T	C	N	F	Y	S	G	E	I	I	T	T	Y	S	E	L	Q	C	T	M	S	D	L	V	N	D	P.g-c
1	M	P	C	V	F	K	F	T	A	N	T	P	T	V	Q	L	K	V	N	N	D	V	Y	A	T	C	N	F	Y	S	G	E	I	I	T	T	Y	S	E	L	Q	C	T	M	S	D	L	V	N	D	P.g-d
51	T	G	I	Y	T	G	D	I	V	F	R	V	T	F	N	V	G	G	S	A	L	A	N	D	L	Q	C	D	A	Y	E	S	S	G	P	N	T	F	N	N	V	L	T	F	T	D	G	T	N	S	C.s-a
51	S	V	T	Y	S	G	K	I	S	F	P	I	T	F	N	S	G	G	S	N	N	P	V	D	L	K	C	A	K	A	Q	S	S	G	L	D	D	V	K	Q	Q	V	T	W	S	D	G	S	K	K	C.s-b
51	S	V	T	Y	S	G	K	I	S	F	P	I	T	F	N	S	G	G	S	N	N	P	V	D	L	K	C	A	K	A	Q	S	S	G	L	D	D	V	K	Q	Q	V	T	W	S	D	G	S	K	K	C.s-c
51	S	V	T	Y	S	G	K	I	S	F	P	I	T	F	N	S	G	G	S	N	N	P	V	D	L	K	C	A	K	A	Q	S	S	G	L	D	D	V	K	Q	Q	V	T	W	S	D	G	S	K	K	C.s-d
51	Q	T	D	V	S	G	T	L	T	V	E	M	F	F	N	T	G	L	T	A	N	A	V	D	L	T	N	A	K	C	F	K	A	G	-	-	-	D	N	T	V	T	F	T	D	G	N	N	E	P.g-a	
51	Q	T	D	V	S	G	T	L	T	V	E	M	F	F	N	T	G	L	T	A	N	A	V	D	L	T	N	A	K	C	F	K	A	G	-	-	-	D	N	T	V	T	F	T	D	G	N	N	E	P.g-b	
51	Q	T	D	V	S	G	T	L	T	V	E	M	F	F	N	T	G	L	T	A	N	A	V	D	L	T	N	A	K	C	F	K	A	G	-	-	-	D	N	T	V	T	F	T	D	G	N	N	E	P.g-c	
51	Q	T	D	V	S	G	T	L	T	V	E	L	A	F	N	V	G	G	S	A	N	D	V	D	I	E	G	A	N	C	Y	T	A	G	-	-	-	Q	N	T	V	T	F	T	D	G	N	N	Q	P.g-d	
101	I	S	T	V	A	A	A	N	F	N	R	E	H	P	I	P	G	G	E	D	P	N	N	Y	V	Y	M	N	R	A	V	L	A	T	R	Q	N	Q	H	L	T	M	S	G	T	C	T	N	G	I	C.s-a
101	L	S	V	-	-	N	A	D	F	L	G	G	Q	-	L	Y	S	-	P	D	F	S	I	I	A	Y	R	N	R	N	I	P	S	L	D	R	Q	Q	H	F	M	F	G	G	D	C	P	N	G	Y	C.s-b
101	L	S	V	-	-	N	A	D	F	L	G	G	Q	-	L	Y	S	-	P	D	F	S	I	I	A	Y	R	N	R	N	I	P	S	L	D	R	Q	Q	H	F	M	I	G	G	D	C	P	N	G	Y	C.s-c
101	L	S	V	-	-	N	A	D	F	L	G	G	Q	-	X	X	X	-	V	D	F	S	I	I	A	Y	R	N	R	N	I	P	S	L	D	R	Q	Q	H	F	M	F	G	G	D	C	P	N	G	Y	C.s-d
97	I	S	T	-	-	N	V	N	F	N	G	G	Q	-	M	L	G	P	L	D	P	N	N	I	I	V	G	S	R	F	V	P	S	L	N	T	L	Q	H	F	L	A	A	G	K	C	D	N	G	Y	P.g-a
97	I	S	T	-	-	N	V	N	F	N	G	G	Q	-	M	L	G	P	L	D	P	N	N	I	I	V	G	S	R	F	V	P	S	L	N	T	L	Q	H	F	L	A	A	G	K	C	D	N	G	Y	P.g-b
97	I	S	T	-	-	N	V	N	F	N	G	G	Q	-	M	L	G	P	L	D	P	N	N	I	I	V	G	S	R	F	V	P	S	L	N	T	L	Q	H	F	L	A	A	G	K	C	D	N	G	Y	P.g-c
97	I	S	R	-	-	N	V	N	F	N	S	G	A	-	T	A	G	N	I	D	T	D	Q	I	I	Y	S	S	R	V	I	E	T	L	N	K	L	Q	H	Y	T	V	A	G	S	C	D	N	G	Y	P.g-d
151	S	S	G	T	L	G	F	T	L	T	Q	Q	G	T	I	S	C	S	N	I	N	A	D	I	T	N	Q	L	N	A	W	Y	M	P	L	A	A	S	T	N	F	Q	Y	T	T	S	C	S	T	L	C.s-a
147	K	S	G	S	L	G	F	T	I	N	-	T	G	V	-	D	C	S	T	A	G	K	G	I	T	D	A	L	N	A	W	Y	L	P	M	S	-	S	S	D	A	T	F	T	T	S	C	E	G	D	C.s-b
147	E	S	G	S	L	G	F	T	I	N	-	T	G	V	-	D	C	A	T	A	G	K	G	I	T	D	A	L	N	A	W	Y	L	P	I	S	-	S	S	D	A	T	F	T	T	C	E	G	N	C.s-c	
147	K	S	G	S	L	G	F	T	I	N	-	T	G	V	-	D	C	S	T	A	G	K	G	I	T	D	A	L	N	A	W	Y	L	P	M	S	-	S	S	D	A	T	F	T	T	S	C	E	G	D	C.s-d
144	T	S	G	T	L	G	I	T	V	D	N	T	N	L	-	D	C	S	T	L	Q	A	Q	I	T	L	Q	L	N	D	W	F	F	P	E	S	-	S	Q	D	F	N	F	Q	T	T	C	T	G	N	P.g-a
144	T	S	G	T	L	G	I	T	V	D	N	T	N	L	-	D	C	S	T	L	Q	A	Q	I	T	L	Q	L	N	D	W	F	F	P	E	S	-	S	Q	N	F	N	F	Q	T	T	C	T	G	N	P.g-b
144	T	S	G	T	L	G	I	T	V	D	N	T	N	L	-	D	C	S	T	L	Q	A	Q	I	T	L	Q	L	N	D	W	F	F	P	E	S	-	S	Q	N	F	N	F	Q	T	T	C	T	G	N	P.g-c
144	S	S	G	T	L	A	F	T	T	S	N	T	R	V	-	D	C	S	T	Y	H	A	O	I	T	L	O	L	N	S	W	S	F	F	E	N	-	T	O	D	F	N	F	Q	T	S	C	S	G	N	P.g-d
201	S	Y	T	I	S	Y	S	N	V	P	A	G	Y	R																																		C.s-a			
194	E	Y	R	V	D	F	E	N	V	P	A	G	Y	R																																		C.s-b			
194	E	Y	R	V	D	F	E	N	V	P	A	G	Y	R																																		C.s-c			
194	E	Y	R	V	D	F	E	N	V	P	A	G	Y	R																																		C.s-d			
192	T	F	T	A	T	Y	N	N	V	P	A	G	Y	R																																		P.g-a			
192	T	F	T	A	T	Y	N	N	V	P	A	G	Y	R																																		P.g-b			
192	T	F	T	A	T	Y	N	N	V	P	A	G	Y	R																																		P.g-c			
192	T	F	S	V	T	Y	N	N	V	P	A	G	Y	R																																		P.g-d			

Figure 7. Alignment of partial adhesin clones from *C. saitoana* and *P. guilliermondii*. Predicted amino acid sequences, one-letter code. Identical residues are boxed. Partial clones were generated using degenerate primers specific to the *Candida albicans* adhesin conserved N-terminal domain. *C.a.* is *Candida saitoana* and *P. g.* is *Pichia guilliermondii*.

BARD Final Scientific Report

The Role of ribonucleases produced by *Candida oleophila* in the mode of action

Introduction

There are a number of studies that imply that ribonucleases could potentially inhibit the growth of molds. For example, Galiana et al. (1997) reported that immediately after inoculation with the pathogenic fungi, extracellular RNase NE was expressed in the tomato leaf, suggesting that this enzyme be involved in the defense mechanism of the plant. Hugot et al. (2002) demonstrated that RNase NE inhibited hyphal elongation of zoospores of *Phytophthora parasitica* and conidia of *Fusarium oxysporum*. In this study we discovered that *C. oleophila* secretes a ribonuclease, and therefore hypothesized that a ribonuclease may be involved in the biocontrol activity against molds.

Purified T2-ribonuclease of *Aspergillus oryzae* inhibited spore germination of *Penicillium digitatum*. In order to learn if ribonucleases have an effect on spore germination, purified T2-ribonuclease of *Aspergillus oryzae* and ribonuclease A of bovine pancreas (as a control, shown to inhibit fungus development [Galiana et al., 1997]) were added to spores of *P. digitatum*. Both ribonucleases inhibited spore germination when in PDB, but not in YPD (Fig. 2 and Table 1). However, RNase A was more effective than T2-RNase of *A. oryzae*.

BARD Final Scientific Report

Figure 1. Inhibition of spore germination by ribonucleases. Purified T2-ribonuclease of *Aspergillus oryzae* (Sigma), 300 units/ml or ribonuclease A from bovine pancreas (Sigma), 0.8 units/ml were added to PDB and to YPD, and double dilutions executed in the respective media. To 90 μ l of diluted medium 10 μ l of *P. digitatum* spores (5×10^5 /ml of 50% PDB) were added. These mixtures were incubated at 18 °C overnight. At least 100 spores per sample were observed microscopically and counted to determine the percent of spore germination in the tested medium relative to that in the corresponding dilution of the reference medium (medium that had not been exposed to yeast cells). The percent of inhibition was calculated as follows: $100 \times (A - B) / A$, where A is the percent of germinated spores in a specified dilution of medium and B is the percent of germinated spores in the tested medium of the same dilution. Each value is the average of two experiments.

BARD Final Scientific Report

Table 1. Spore germination inhibition by ribonuclease in PDB and YPD.

	Medium	Spore Germination Inhibition (%)
T2 RNase	PDB	71 ± 4
	YPD	4 ± 4
RNase A	PDB	86 ± 1
	YPD	2 ± 2

The experiment was carried out as described in Fig. 1. The results are from three experiments and are expressed as the mean $\pm$ standard error.

Candida oleophila secretes ribonuclease. Cultures of *C. oleophila* have ribonuclease activity, when grown in PDB. Media of *C. oleophila* cultures, grown for 1, 2 and 6 days in YPD and in PDB at 30 °C with 200 rpm were examined for their RNase activity. As evident in figure 2, ribonuclease activity was found in each of the PDB-*C. oleophila* cultures of 1,2 and 6 days, whereas very little of this activity was observed in the YPD-*C. oleophila* cultures of the same ages.

BARD Final Scientific Report

Fig. 2 Ribonuclease activity in *C. oleophila* cultures. A volume of each culture (without cells) was mixed with an equal volume of RNA (extracted from the fungus-*Phanerochaete chrysosporium*), incubated together at 37 °C for 15 minutes and run on a TAE-ethidium bromide gel.

BARD Final Scientific Report

Saccharomyces cerevisiae T2-RNase knockout – a model for assaying the role of T2-RNase in the biocontrol of molds.

RNase activity. Three isogenic strains, which are intended as controls, Y03254 (lys2), Y07202 (trp), and, Y07186 (thr4), were grown in YPD and PDB at 30 °C and 37 °C for three days. The media from these cultures were tested for RNase activity. As revealed in figure 3, Y07186 and Y07202 cultures had more RNase activity when grown at 37 °C than at 30 °C. Y03254 cultures also showed this same phenomenon (data not shown). These results coincide with results from MacIntosh et al. (2001). On the other hand, cultures grown in PDB secreted a material that caused the RNA to remain in the wells, especially in cultures grown at 37 °C . Presently, the T2 knockout is being tested.

Figure 3. A volume of each culture was mixed with an equal volume of RNA (extracted from the fungus- *Phanerochaete chrysosporium*), incubated together at 37°C for 15 minutes and run on a TAE-ethidium bromide gel.

BARD Final Scientific Report

Biocontrol activity. The spore germination inhibition ability against *P. digitatum*, *Penicillium expansum* and *P. italicum* spores, was examined in Y07186 and Y03254, two of the three *S. cerevisiae* strains. The assay was executed in the same manner as in the biocontrol assay mentioned above. Y07186 and Y03254 cultures grown in PDB inhibited spore germination of the three molds, in contrast to cultures grown in YPD, which had no effect on the spore germination (Table 2). These results correspond with those of the purified T2-RNase and RNase A, which also prevented spore germination only in PDB and not in YPD. Apparently, RNase is less active in YPD. Cultures grown at 30 °C tended to have more biocontrol activity than the 37 °C cultures, although the opposite seemed to be true in the case of the *P. italicum* spores. The T2-RNase knockout, Y02129 will shortly be assayed.

Table 2. Spore germination inhibition by *S. cerevisiae* cultures

Strain	Temp. (°C)	Medium	Inhibition of spore germination (%)		
			<i>P. digitatum</i>	<i>P. expansum</i>	<i>P. italicum</i>
3254	30	PDB	34 ± 11	55 ± 19	49 ± 15
	37		27 ± 7	34 ± 1	80 ± 14
	30	YPD	0	2 ± 0	0
	37		0	5 ± 0	3 ± 2
7186	30	PDB	57 ± 4	78 ± 8	93 ± 3
	37		30 ± 5	48 ± 9	95 ± 5
	30	YPD	0	1 ± 1	0
	37		3 ± 2	3 ± 0	4 ± 3

The values are the results of two experiments and are expressed as the mean ± standard error.

BARD Final Scientific Report

T2-RNase gene in *C. oleophila* genome. By means of PCR with degenerative primers, that were derived from relatively homologous sequences in T2 RNase of *C. albicans* (encoded by *RBT-7*) (Braun et al., 2000) and *S. cerevisiae* (Rny1) (MacIntosh et al. 2001), part of the gene for a T2 RNase *C. oleophila* was amplified, cloned and sequenced. The partial sequence including one of the active sites, CAS 1, of this gene is shown in figure 4. Since T2-RNase is secreted into the medium by *S. cerevisiae*, it is likely that the ribonuclease secreted by *C. oleophila* is a T2-RNase. This stands to be proven by a *C. oleophila*-T2-RNase knockout.

```

P G G I M L Q T Q F W D Y Y P P
TGCCTGGGGGTATAATGCTACAGACGCAGTTCTGGGACTATTACCCCCC

                                CAS1
                                ▼
I G P N D T F T L H G L W P D N
GATTGGACCCAATGACACGTTACCTTGCATGGGTTGTGGCCCGACAAC

C D G T Y E Q F C D D S L N V A
TGTGATGGAACGTACGAGCAATTCTGTGACGATAGCTTGAACGTTGCC

N V T D I I V N E F K D P E L Y
AATGTTACGGACATCATTGTCAATGAGTTTAAGGACCCAGAACTCTAC

Q T M T S I W K N F N G N D

CAGACGATGACTTCGATCTGGAAGAACTTCAACGGGAACGACGA
```

Figure 4. T2 RNase - partial nucleotide sequence and deduced peptide. The sequence was retrieved with the degenerative primers, YPL1degA- AAC TCK TGT TGT TTT GAA and YPL1degB- RCA MGT RCC ATG TTT RTT. The conserved active site, CAS 1, is underlined.

BARD Final Scientific Report

The fact that RNase activity is found in *C. oleophila* - PDB cultures and not in *C. oleophila* - YPD cultures made it possible to perform an experiment that would indicate if this RNase is also associated with the hyphae development of *P. digitatum*, and therefore also with the biocontrol of *P. digitatum* by *C. oleophila*. The development of *P. digitatum* spores was assayed in the two media – 1) a three-day YPD-*C. oleophila* culture and 2) a three-day PDB-culture in which the yeast cells had been eliminated by centrifugation. The development of the spores in these cultures was compared to the development of the spores in the uncultured medium, YPD and PDB, respectively. YPD-*C. oleophila* cultures inhibited the development of the spores, more than PDB-*C. oleophila* cultures, implying that the RNase secreted by *C. oleophila* is not the major factor in the biocontrol of this mold (Table 3). This same test was carried out on medium from one- and two-day cultures of *C. oleophila* YPD and PDB, in which little, if any, inhibition of the spore development was evident (data not shown). Since this RNase is also secreted into the medium within 24 hours, the lack of spore inhibition in medium from cultures of 24-48 hours, also indicates that RNase is not connected with the biocontrol of *P. digitatum* by *C. oleophila*. Still it is possible that other substances in the two media mask the true results.

Table 3. Spore germination inhibition by *C. oleophila*

Medium	Spore germination inhibition (%)
PDB	27 ± 17
YPD	82 ± 16

These experiments were carried out as were the other above spore germination inhibition experiments. The values are the results of at least three experiments and are expressed as the mean ± standard error.

BARD Final Scientific Report

References

- Braun, BR, WS Head, MX Wang and AD Johnson. Identification and characterization of TUP1-regulated genes in *Candida albicans*. *Genetics* 156:31-44, 2000.
- Galiana, Eric, Philippe Bonnet, Sandrine Conrod, Harald Keller, Franck Panabieres, Michel Ponchet, Alain Poupet and Pierre Ricci. RNase activity prevents the growth of a fungal pathogen in tobacco leaves and increases upon induction of systemic acquired resistance with elicitor. *Plant Physiol.* 115: 1557-1567, 1997.
- Hugot, Karine, Michel Ponchet, Antoine Marais, Pierre Ricci and Eric Galiana. Tobacco S-like RNase inhibits hyphal elongation of plant pathogens. *Molecular Plant-Microbe Interactions* 15: 243-250, 2002.
- MacIntosh, Gustavo C., Pauline A. Bariola, Ed Newbigin and Pamela J. Green. Characterization of Rny1, the *Saccharomyces cerevisiae* member of the T₂ RNase family of RNases: Unexpected functions for ancient enzymes? *PNAS* 98: 1018-1023, 2001.