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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Full Length Research Paper

Effect of co-culturing of cellulolytic fungal isolates for degradation of lignocellulosic material

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This study intended to compare the efficiency of fungal monocultures and co-cultures in the simultaneous delignification and saccharification of kitchen waste and *Eichhornia crassipes* in order to subject the hydrolysate into biofuel production. Three fungal isolates of genus *Trichoderma, Aspergillus, Pycnoporus* and an unidentified strain (F113) were grown in mono and co-cultures and the extracted enzymes were used for the degradation. Co-culture of *Trichoderma* spp with the other fungi improved its enzyme activity while the other co-cultures did not show significantly improved enzymatic degradation compared to monocultures. The highest percentage of saccharification (over total dry weight) achieved were 11.9% with kitchen waste after seven days and 9.8% with *E. crassipes* after 4 days. The drop in degradation rate normally seen after complete digestion of amorphous cellulose was not apparent probably due to the grinding of the substrates to fine particle size.

Key words: Fungal co-culture, biofuel, cellulase, kitchen waste, invasive weeds.

INTRODUCTION

Fossil fuel resources are limited and their usage leads to environmental problems. Hence, it is imperative to utilise alternative energy sources that are renewable and eco-friendly. Biofuels are promising in this regard. Second generation biofuels are produced from non-edible biomass, through degradation and fermentation.

Enzymes degrading lignocelluloses in nature include cellulases, xylanases and lignin degrading enzymes. The activity of lignin degrading enzyme is too slow for application in biofuel production (Lu et al., 2010). Thus, thermo-chemical pre-treatment is needed to overcome the recalcitrance (Margeot et al., 2009). Pre-treatment also results in degradation of hemicelluloses. Therefore, cellulases are the major enzymes involved in subsequent enzymatic hydrolysis.

Pre-treatment requires energy input and chemicals which adds up to a major component of the cost of production. The cost of enzymes also contributes significantly (Shi et al., 2009). Among microbes, some aerobic filamentous fungi are known to secrete high

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amounts of cellulases. Such strains are found in the genera *Trichoderma*, *Aspergillus*, *Pencillium* and many others (Lynd et al., 2002). The factors affecting the expression and secretion of fungal cellulases include induction, repression, metal ions, inorganic nutrients, surfactants and culture conditions (Gremel et al., 2008; Mandels and Reese, 1957; Reese and Maguire, 1969; Schmoll et al., 2005; Suto and Tomita, 2001). Cellulases belong to three classes, namely endoglucanases, exoglucanases and β-glucosidases, which act in synergy (Lynd et al., 2002). The right balance of the cellulases is crucial for optimal degradation of lignocelluloses. In *Trichoderma reesei,* large proportions of β-glucosidase remain cell-wall bound through a polysaccharide and gets released when treated with cellulase from *Aspergillus niger* (Messner et al., 1990). Endoglucanases and exoglucanases of *T. reesei* are inhibited by cellobiose whereas its β-glucosidases was inhibited by glucose (Philippidis et al., 1993). In contrast, *A. niger* secretes high amounts of β-glucosidase which is tolerant to high levels of glucose (Decker et al., 2000). For these reasons, a combination of cellulases from *T. reesei* and *A. niger* is used in the biofuel industry (Reczey et al., 1998). Co-culture of *T. reesei* and *Aspergillus* sp has been shown to result in better yield of cellulases (Ahamed and Vermette, 2008; Duff, 1985). Better utilization of substrates and formation of strong inducers for *T. reesei* cellulases by β-glucosidase of *Aspergillus* are thought to be the reasons for the increased yield (Ahamed and Vermette, 2008).

Disposal of household waste is a problem in the urban areas of Sri Lanka. Current practice is to dump the waste in the suburbs, causing unpleasant odours and health problems in those areas. Composting has been attempted in an industrial scale but has been abandoned due to operational problems (Premachandra, 2006). A possible solution to this problem could be the utilization of household waste for biofuel production. Another potential substrate is the invasive aquatic weed *Eichhornea crassipes*. Invasive weeds are probable raw materials for cellulosic biofuel production. These are non-indigenous or "non-native" plants which adversely affect the habitats and bioregions they invade economically, environmentally, and ecologically (Westbrooks, 1998). *E. crassipes is* one of a common aquatic invasive weed and another potential substrate for biofuel production. The fast growing nature of these weeds can provide raw materials in abundance for biofuel industries. Since it is less lignified, its utilisation would require low pre-treatment. If effective technologies can be developed to drive commercial products from these weeds it will be beneficial both economically and ecologically. Although a large number of microorganisms (fungi, bacteria and actinomycetes) are capable of degrading cellulose, only a few of them produce significant quantities of cell-free enzyme fractions capable of complete hydrolysis of cellulose *in vitro*. Cellulases obtained from compatible mixed cultures of fungi appear to have more enzyme activity as compared to their pure cultures and other combinations (Jayant et al., 2011).

The objective of this study was to compare the efficiency of fungal monocultures and co- cultures in the simultaneous delignification and saccharification of kitchen waste and *E. crassipes* in order to subject the hydrolysate into biofuel production.

MATERIALS AND METHODS

Isolation of cellulolytic fungi

Samples of decaying plant material, ruminant dung, decaying kitchen waste and soil were suspended in sterile normal saline, serially diluted to obtain 10^{-1}, 10^{-2} and 10^{-3} dilutions. 100 µl of each dilution was plated on potato dextrose agar (with gentamicin 50 mg/L and chloramphenicol 50 mg/L) by spread plate technique and incubated at 25°C up to a week. The fungal isolates were inoculated on Czapek dox agar without sucrose and with 1% cellulose and those showing good growth were presumed to be potential cellulose degraders and added to the culture collection. The isolates in the culture collection were screened for cellulase production by growing them without replicates as described in measurement of enzyme activities part of the work. The isolates which showed at least 0.01 FPU of cellulase activity were then tested in replicates and those with the highest enzyme activities from different genera were chosen for further study.

Measurement of enzyme activities

Production of enzymes

Fungal isolates were grown on PDA slants for 7 days. Spore suspensions were made in sterile saline, spore concentrations were adjusted to 10^{7}-10^{8}/ml and 100 µl of the suspensions were inoculated into 20 ml of a minimal medium (Mandels and Reese, 1957). Co-cultures were made by inoculating 100 µl of spore suspension from each of the relevant strain. The cultures were incubated at 28°C on a rotary shaker at 100 rpm. For initial screening, the isolates were cultured without replicates with an incubation period of 3 days. At the end of incubation, the cultures were centrifuged at 4000 g for 20 min and the supernatants were used as crude enzymes for the assays.

Total cellulase assay

Total cellulase assay was carried out using Whatmann No.1 filter paper as the substrate (Mandels et al., 1976; Ghose, 1987). Reducing sugars formed were measured by using di-nitro salicylic acid reagent (Sumner, 1921; Miller, 1959), with glucose as standard. The total cellulase activity is expressed as filter paper units/ml (FPU/ml).

Xylanase assay

Xylanase activities were measured by a method modified from Gottschalk et al. (2010) using 1% (w/v) beech wood xylan (Sigma) as the substrate. Reducing sugars formed were measured using di-nitro salicylic acid (DNS) reagent (Sumner, 1921; Miller, 1959), with xylose as standard.

β-Glucosidase assay

β-glucosidase activities were measured by using cellobiose as the substrate (Ghose, 1987; Sternberg et al., 1977). Glucose formed during the assay was measured using a commercial blood glucose meter (*One Touch Ultra 2*) based on glucose oxidase, calibrated with glucose standards in 0.05 M citrate buffer (pH = 4.8). Positive results of β-glucosidase activities were verified by high-performance liquid chromatography (HPLC) as described in enzymatic degradation of lignocellulosic materials part of this work.

Laccase assay

Laccase activities of *Pycnoporus cinnabarinus* was measured with ABTS (2,2'-azino-bis(3-ethylbenzothiazoline- 6-sulphonic acid)) as the substrate (Bourbonnais et al., 1995).

Enzymatic degradation of lignocellulosic materials

Kitchen waste and mature leaves (to represent the most recalcitrant type for degradation, as they are the most lignified) of *E. crassipes* were dried in an oven at 50°C to a constant weight. The dried material was then ground in a plant grinder and sieved through 93 µm (kitchen waste) and 50 µm (*E. crassipes*) sieve. Powdered kitchen waste (500 mg) and *E. crassipes* (200 mg) were added separately into boiling tubes, to which 6 ml of 50 mM citrate buffer (pH 4.8) was added and autoclaved at 121°C for 15 min. To each tube, 3 ml of crude enzyme from fungal monoculture or co-culture was added and incubated in a water bath at 50°C with reciprocal shaking at 100 rpm for five to seven days. Samples were withdrawn daily and total sugar concentrations were determined using DNS reagent with glucose standards (Sumner et al., 1921). The sugar components of the hydrolysate of *E. crassipes* at the end of 4th day of degradation were measured by High-performance liquid chromatography (HPLC) using agilent Hi-plex H column (p/n PL1170-6830 300x7.7 mm) at 65°C with deionised water as the mobile phase (flow rate: 0.6 ml/min, injection volume: 10 µl). Sugars were detected with an RI detector. D-cellobiose, D-glucose, D-xylose and L-arabinose (Sigma Aldrich) standards were used for calibration. High-performance liquid chromatography was not performed on kitchen waste due to the highly variable nature of its content.

Data analysis

Statistical comparisons were made by ANOVA using Minitab software (version 14). $\alpha = 0.05$ unless otherwise stated.

RESULTS AND DISCUSSION

Isolation of cellulolytic fungi

A total of 145 fungal strains were isolated from different samples. During the initial screening for cellulase activity, 35 isolates were found to have greater than 0.01 FPU/ml of activity. The isolates which showed significant cellulase activities were shown in Table 1. Most of them belong to *Trichoderma* species, while strains of *Penicillium*, *Aspergillus* and an unidentified fungal strain were also present. Fungi were tentatively identified using macroscopic and microscopic morphological characteristics. Slide culture technique was used to aid the fungal identifications.

Co-culture of fungi

Fungal isolates belonging to genera of *Trichoderma* (F1, F16,F F118), *Penicillium* (F24) and an unidentified isolate (F113) were selected for co-culturing. The selection was made to include different genera and strains with β-glucosidase activity. The isolates were divided into two groups (F1, F16 and F118) and (F24, F80, F113) and co-cultures were made in all possible combinations within each group. Cellulase and xylanase activities of the co-cultures and corresponding mono-cultures were measured (Figures 1 and 2).

Among the group containing F1, F16 and F118, the co-cultures showed lower cellulase activities compared to the corresponding monocultures. Among the group containing F24, F80 and F113, the co-culture F80 and F113 showed higher cellulase activity than F113, but the difference was statistically insignificant. The co-cultures F24/F113 and F24/F80/F113 showed significantly higher xylanase activities compared to the corresponding monocultures. Other co-cultures showed either no significant difference or reduced xylanase activity.

Degradation of kitchen waste

The isolates F24, F113 and F118 were selected for degradation of kitchen waste. The cumulative sugar contents measured at 1^{st}, 2^{nd} 3^{rd} and 7^{th} days of degradation of fresh kitchen waste was shown in Figure 3. At the end of seven days of degradation, the highest amount of sugars were released by enzymes from F24 (59.7 mg) followed by the co-culture F24/F113 (58 mg). Enzymes from the co-culture F113/F118 effected significantly higher degradation than F118 (*Trichoderma*) alone. This is probably due to the lack of secreted β-glucosidase activity by F118 being complemented by F113.

Degradation of *E. crassipes*

Enzymes from the fungal strains F24, F113, F118 and a woody mushroom from Sri Lanka (M21), identified as *Pycnoporus cinnabarinus*, were used for the degradation of *E. crassipes*. Screening revealed that some organisms were more efficient than the others. M21 was found to have a significant cellulase activity (0.21 FPU) and laccase activity (50 IU/ml). The cumulative sugar contents measured at 1^{st}, 2^{nd}, 3^{rd} and 4^{th} days of enzymatic degradation of *E. crassipes* was shown in Figure 4. The sugar contents at the 4^{th} day was shown in Figure 5 for comparison. The component sugars as

Table 1. Fungal isolates with highest cellulase activities.

Isolate no.	Total cellulase (FPU/ml)	Xylanase (IU/ml)	β-glucosidase (IU/ml)	Genus
F118	0.21	4.31	Not detected	*Trichoderma*
F80	0.16	2.03	Not detected	*Trichoderma*
F1	0.15	5.48	Not detected	*Trichoderma*
F16	0.14	5.22	0.11	*Trichoderma*
F22	0.13	5.22	0.09	*Trichoderma*
F24	0.12	4.98	Not detected	*Penicillium*
F54	0.11	1.77	Not detected	*Trichoderma*
F27	0.11	5.38	Not detected	*Trichoderma*
F98	0.10	4.79	0.05	*Trichoderma*
F10	0.09	2.14	Not detected	*Trichoderma*
F56	0.08	5.62	0.05	*Trichoderma*
F88	0.07	5.25	0.13	*Trichoderma*
F40	0.06	3.35	0.07	*Aspergillus*
F113	0.06	1.02	0.15	Unidentified

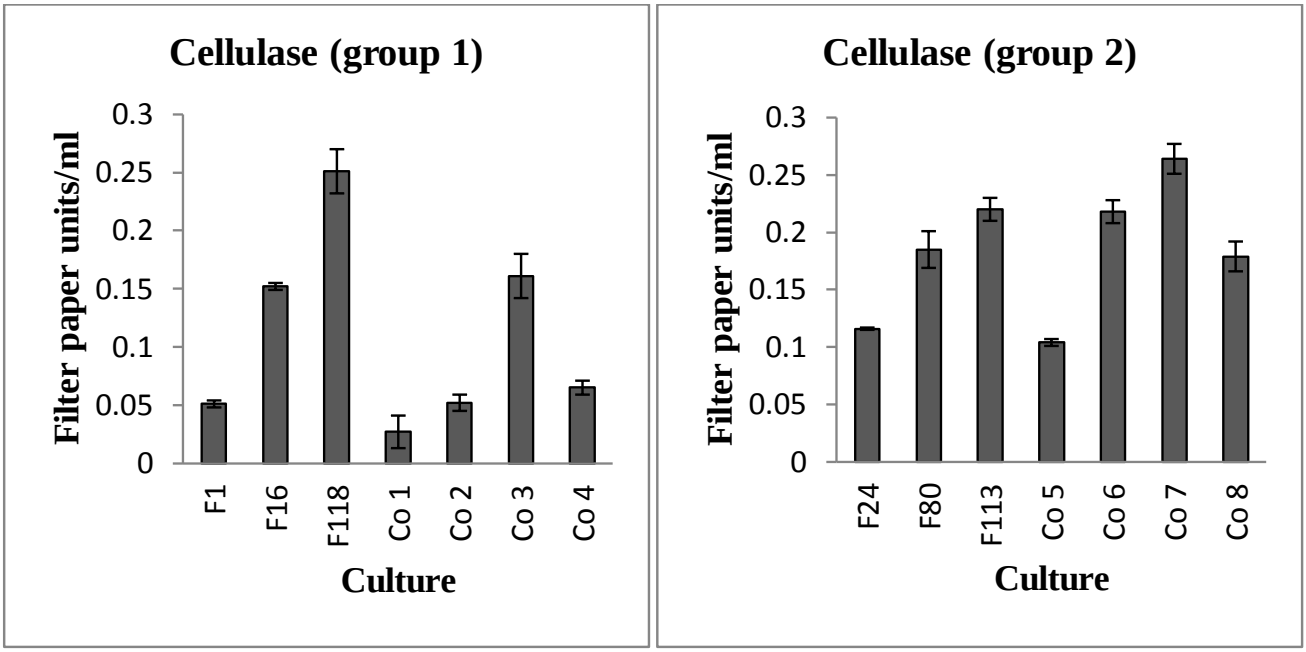

Figure 1. Cellulase activities of monocultures and co-cultures of selected fungi. Key: F1-*Trichoderma* spp., F16 - *Trichoderma* spp., F118-*Trichoderma* spp., Co1- F1 and F16, Co2-F1 and F118, Co3- F16 and F118, Co4- F1, F16 and F118, F24-*Penicillium* spp., F80-*Trichoderma* spp., F113-Unidentified, Co5- F24 and F80, Co6- F24 and F113, Co7- F80 and F113, Co8- F24, F80 and F113. Error bars indicate standard errors of the means.

measured by HPLC at the end of 4th day are shown in Figure 6. The amount of simple sugar produced and released to the medium can depend on activities of cellulase systems and physiological characteristics of particular species.

The highest quantity of total sugar content (19.6 mg) at the end of four days of degradation of *E. crassipes* was obtained with enzymes from the co-culture F113/F118. However, the mono-cultures F24, F113 and co-cultures F24/F113, F24/F118 and F24/M21 gave slightly lower quantities and the differences are not statistically significant. F118 and F113/M21 and M21 showed significantly lower degradation rate. It was noted that while the amount of xylose formed by M21 was similar to

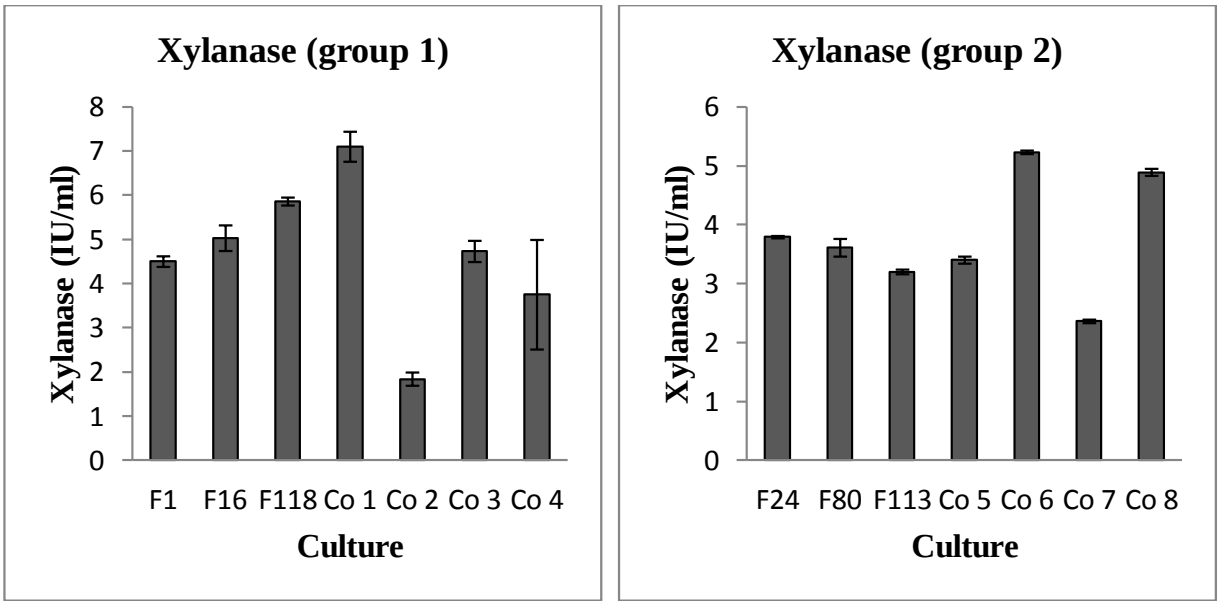

Figure 2. Xylanase activities of monocultures and co-cultures of selected fungi. Key: F1-*Trichoderma* spp., F16 - *Trichoderma* spp., F118-*Trichoderma* spp., Co1- F1 and F16, Co2-F1 and F118, Co3- F16 and F118, Co4- F1, F16 and F118, F24-*Penicillium* spp., F80-*Trichoderma* spp., F113-Unidentified, Co5- F24 and F80, Co6- F24 and F113, Co7- F80 and F113, Co8- F24, F80 and F113. Error bars indicate standard errors of the means.

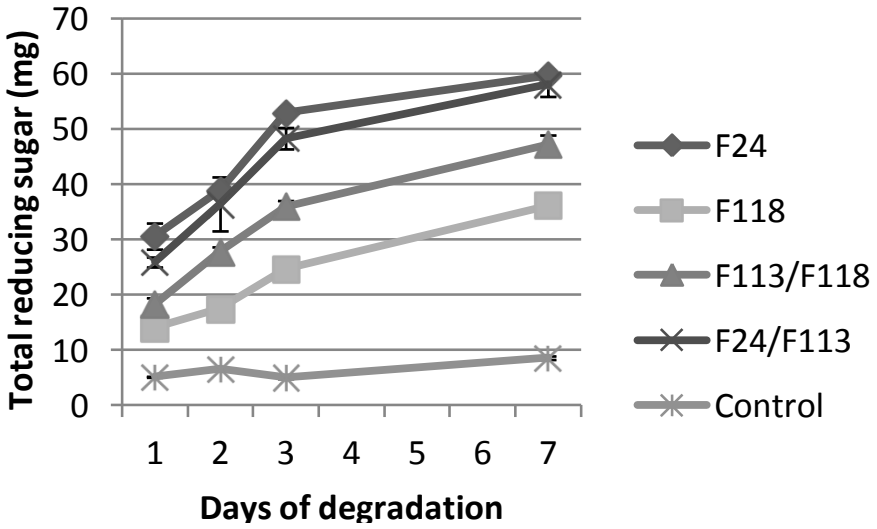

Figure 3. Total sugar content (cumulative) released from un-decomposed kitchen waste by enzymes

other isolates, the amount of arabinose formed was much lower. The percentage of maximum sugar yield over total dry weight was 11.9 and 9.8% respectively from kitchen waste and *E. crassipes*. The percentages against total polysaccharide content should be higher.

Grinding the substrate to a very fine powder (93 and 50 µm) would reduce the length of the polysaccharide chains and increase the number of free ends available for the activity of exoglucanases. It would also increase the surface area of the substrate available for enzyme activity. During the enzymatic hydrolysis of cellulose, amorphous portions are quickly degraded followed by slow degradation of crystalline regions (Mandels, 1975). Thus a change in the rate of degradation, that is, slope of the degradation curve, indicates the end of degradation of amorphous portions. This change should occur roughly

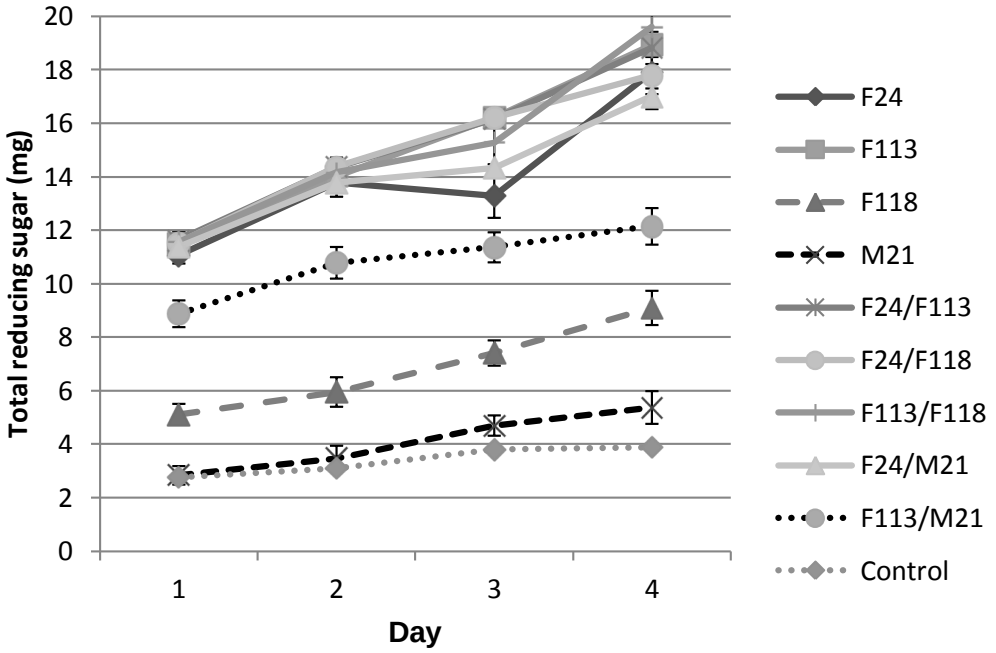

Figure 4. Total reducing sugars measured at daily intervals during enzymatic degradation of *E. crassipes*

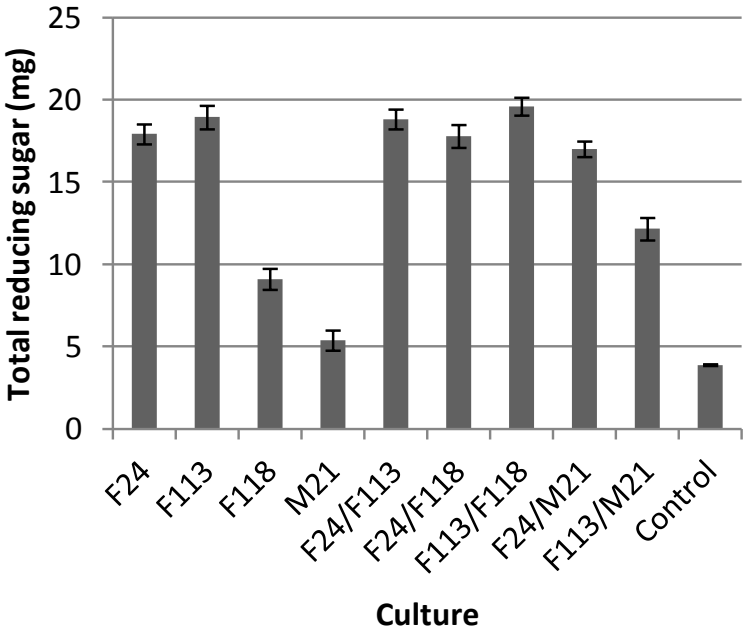

Figure 5. Total reducing sugar accumulated by degradation of *E. crassipes* by the end of 4th day of degradation

at about the same percentage of degradation for different enzymes. However, in the present study, such change of rate did not seem to occur at roughly equal percentage of degradation. This could be because at low particle sizes, the effect of enzyme loading becomes more prominent.

Conclusions

Co-culturing of *Trichoderma* with other cellulolytic fungi improved the activity of lignocellulose degrading enzymes compared to monoculture of *Trichoderma*. The co-culture

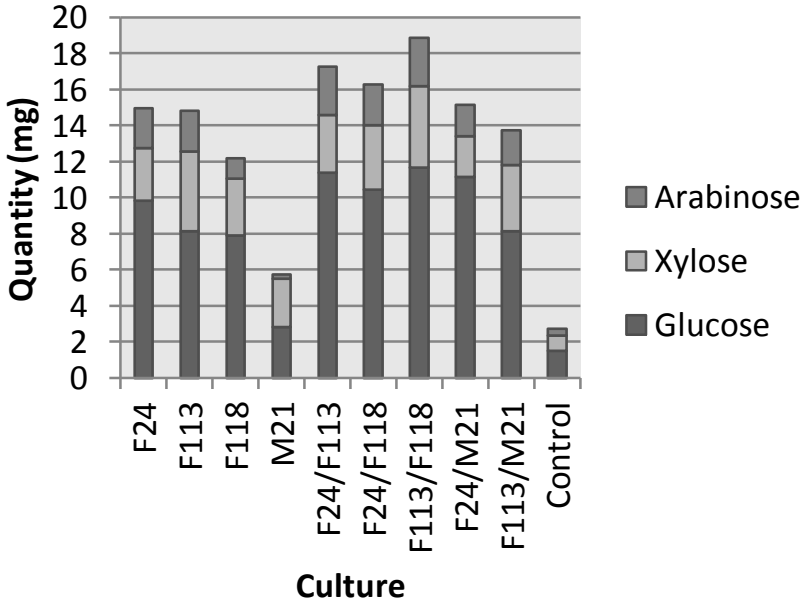

Figure 6. Quantities of glucose, xylose and arabinose of the enzyme hydrolysate of *E. crassipes* at the end of 4th day of degradation.

of other fungi did not result in significant improvement in the activity compared to corresponding monocultures. Significant percentage of degradation of kitchen waste and *E. crassipes* was achieved with the monoculture, without pre-treatment. Fine powdered material was used to eliminate the cost of pre-treatment. Enzyme from *Pycnoporus cinnabarinus* the laccase producing strain was found to be ineffective for lignocellulose degradation.

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The Lap3p aminopeptidase is not solely responsible for bleomycin resistance in *Candida albicans*

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Detoxification of the glycopeptide bleomycin is mediated by bleomycin hydrolase, a cysteine aminopeptidase identified in a variety of organisms. The opportunistic fungal pathogen *Candida albicans* is known to exhibit increased resistance to bleomycin when compared to other yeast. Presented here is the cloning of the *C. albicans* Lap3p aminopeptidase, predicted by sequence identity to be the *Candida* form of bleomycin hydrolase. *C. albicans* Lap3p is functionally capable to replace the *Saccharomyces cerevisiae* Lap3p *in vivo*. Furthermore, the *Candida* enzyme was found to function as a cysteine aminopeptidase *in vivo*. It is shown here that upon introduction into a *lap3* deletion strain of *S. cerevisiae*, the *C. albicans* Lap3p aminopeptidase does not significantly alter the response of *Saccharomyces* to bleomycin. These results suggest that *C. albicans* Lap3p does not function as the sole factor involved in bleomycin detoxification, and may require an accessory protein or co-factor in order to efficiently mediate this process in *Candida*. This study provides the first evidence of a functional description of the *C. albicans* Lap3p cysteine aminopeptidase, and provides the foundation for further mechanistic studies of the role of this protein in the cellular processes of *Candida*.

Key words: *Candida albicans*, bleomycin detoxification, Lap3p aminopeptidase, cysteine protease.

INTRODUCTION

Candida albicans is a diploid yeast that is the most common fungal pathogen of humans (Wey et al., 1988; Pfaller, 1989; Beck-Sague et al., 1993). *Candida* species typically reside as commensals in humans, and are found in over 50% of the population (Antley and Hazen, 1988). In immunocompromised patients however, mild to aggressive opportunistic infections can manifest from an imbalance of colonization. Coincident with this increase in

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Abbreviations: BCA, Bicinchoninic acid; **E-64,** *trans*-Epoxysuccinyl-L-leucyl-amido(4-guanidino)butane; **H-Arg-AMC,** H-arginine-7-amino-4-methylcoumarin·2HCl; **H-Cit-AMC,** H-citrulline-7-amino-4-methylcoumarin·2HBr; **YEP,** yeast extract/peptone; **YEPD ,** yeast extract/peptone/glucose; **YEP-Gal,** yeast extract/peptone/galactose; **Ura (-),** synthetic culture medium lacking uracil; **PMSF,** phenylmethanesulfonylfluoride; **EDTA,** disodium ethylenediaminetetraacetic acid; **DTT,** dithiothreitol; **WCE,** whole cell extract.

pathogenicity is the transition of *C. albicans* from an innocuous yeast form to a more problematic mold form (Antley and Hazen, 1988). The transition of *C. albicans* from budding yeast to mold is induced by specific environmental and host factors (Muhlschlegal et al., 1998; Calderone and Fonzi, 2001; Hube, 2004). Invasive fungal infections such as candidemia results in high health care costs, and a mortality rate that approaches 50% (Wey et al., 1988). Effective treatment options for candidiasis are hindered by the lack of knowledge regarding the mechanistic basis of pathogenicity. Therefore it is imperative to more comprehensively understand fundamental biological processes of *C. albicans*.

Proteolysis is known to play a vital role in cellular metabolic processes. The *Saccharomyces cerevisiae YCP1 (LAP3)* gene is a cysteine aminopeptidase originally isolated based on a genetic approach in which mutant cells possessing a decreased ability to hydrolyze an aminopeptidase substrate were screened (Kambouris et al., 1992; Enenkel and Wolf, 1993). Subsequent work has provided evidence that Lap3p is widely distributed throughout nature, plays a role in numerous cellular processes, and has been given numerous names based on the function for which it was identified. Enekel and Wolf (1993) showed that *LAP3* was identical to *S. cerevisiae BLH1*, a protein showing significant sequence homology to rabbit bleomycin hydrolase. The rabbit bleomycin hydrolase is responsible for inducing resistance to the anticancer glycopeptide bleomycin through a cysteine aminopeptidase activity (Sebti et al., 1987; Sebti et al., 1989; Sebti et al., 1989). Mutants in *BLH1* (orthologous to *LAP3*) demonstrate a decreased ability to detoxify bleomycin (Enenkel and Wolf, 1993). Increased bleomycin resistance is observed following introduction of the wild type *BLH1* gene in both *S. cerevisiae* (Pei et al., 1995) and mammalian cells (Wang and Ramotar, 2002). Furthermore, Xu and Johnston (1994) isolated the *GAL6* cysteine aminopeptidase (identical to *LAP3*) bound and localized to GAL4p DNA binding sites. Although *GAL6* deletion mutants are viable, Gal6p (Lap3p) is known to exert a modest negative effect on *GAL* promoter mediated transcription (Zheng et al., 1997). In humans, *BLH1* is expressed in most tissue types (Takeda et al., 1996), and functions in protection against homocysteine toxicity (Zimny et al., 2006) and progression of atopic dermatitis. A correlative association with development of astrogliosis has also been attributed to abnormalities in *BLH1* function (Montoya et al., 1998; Montoya et al., 2007). In order to provide clarity, the enzyme identified and described in the following work will be referred to as *LAP3*.

To date, the *C. albicans* Lap3p remains uncharacterized, and its function is largely inferred from sequence homology to Lap3p found in other organisms. *C. albicans LAP3* is a gene that is positively regulated by Sfu1p, a transcription factor that upregulates gene expression in iron starvation conditions (Lan et al., 2004). Based on the distribution pattern of Lap3p throughout nature, as well as the varied functions attributed to this protein, we sought to identify *LAP3* in *C. albicans*. Enzyme assays utilizing specific inhibitors demonstrated the presence of cysteine aminopeptidase activity in *C. albicans*. Using the genome sequence present in the Candida Genome Database (CGD), we have amplified the putative *C. albicans LAP3* gene and expressed it in a *S. cerevisiae* strain deleted for the *LAP3* gene. Incorporation of the *Candida LAP3* gene resulted in no remarkable growth defects in *S. cerevisiae*. Additionally, cysteine aminopeptidase activity was restored in the transformants, suggesting that the *C. albicans LAP3* is capable of functionally replacing the equivalent gene in baker's yeast. Introduction of the *C. albicans LAP3* into *S. cerevisiae* did not increase the resistance to bleomycin, suggesting that Lap3p is not the sole factor involved in bleomycin detoxification in *Candida*. This work represents the first characterization of the *C. albicans* Lap3p aminopeptidase, and provides the basis for elucidating its role in *Candida*.

METHODOLOGY

Strains and culture conditions

The *Escherichia coli* Novablue cells (EMD Millipore, Billerica, MA) were used for plasmid amplification. Bacteria were cultured in Luria-Bertani (LB) medium supplemented with the appropriate antibiotics. *S. cerevisiae* were obtained from American Type Culture Collection (ATCC, Manassas, VA) and were as follows: *Sc001* (BY4741; MATa *Δhis3 Δleu2 Δmet15 Δura3*), *Δlap3* (YNL239W; MATa *Δhis3 Δleu2 Δmet15 Δura3 Δlap3::KAN*). *Candida albicans* strains used in this study, *Ca002* (Ca1001) and *Ca004* (MYA2876). Yeast strains in either rich medium (YEP) or complete synthetic yeast culture medium (ura (-); 0.67% yeast nitrogen base without amino acids, 2 g/L yeast complete synthetic amino acid mix lacking uracil) supplemented with the appropriate carbon sources were grown at 30°C. Carbon sources glucose (D) and galactose (G) were prepared and sterilized separately from culture medium and added to a final concentration of 2% prior to use. Growth phenotypes of wild type and transformant strains were assessed by plating spot dilutions onto either rich medium or selective medium lacking uracil and supplemented with 200 µg/ml Geneticin (G418; Life Technologies, Carlsbad, CA) in order to maintain the *lap3* deletion. Plates were incubated between 1-3 days at 30°C.

Cloning of *Saccharomyces* and *Candida LAP3*

The yeast Gateway destination expression vector pYES-DEST52 (Life Technologies, Carlsbad, CA) was subjected to site directed mutagenesis using the oligonucleotide 5'CCGGTCATCATCACCATCACCATTGAGTAAAAACCCGCTGATCCTAGAGGGCC3' (Integrated DNA Technologies, Coralville, IA) in order to remove a PmeI restriction site from the 3' end of the multiple cloning site. Mutagenesis was performed using the Change-IT site directed mutagenesis kit from Affymetrix (Santa Clara, CA). Following disruption of the PmeI restriction site, a novel

PmeI site was integrated into the vector in order to create an orientation in which the inserted open reading frame would ultimately be flanked on its 3' end by a V5 epitope, followed by a protease cleavage site and finally a 6-Histidine purification tag. This was accomplished using the oligonucleotide 5'CTCGGTCTCGATTCTACGCGTGGTTTAAACACCGGTCATCAT CACCATCAC3' (Integrated DNA Technologies, Coralville, IA). The Tobacco Etch Virus (TEV) protease cleavage site was inserted into the PmeI digested pYES-DEST52 via ligation of the duplexed oligonucleotide 5'AAACCTCGAGGAAAACCTGTATTTTCAGGGCTCCTTCGGTTT 3' (Integrated DNA Technologies, Coralville, IA), and the resultant plasmid was renamed pFG91.

C. albicans LAP3 was amplified by PCR from *Ca002* genomic DNA, using the oligonucleotide pair 5'CACCATGGGTTCCAACAC3'/5'AGCTAAAGCACCCATAGG3' (Integrated DNA Technologies, Coralville, IA). The *LAP3* fragment was then ligated into the Gateway entry vector pENTR-D-TOPO (Life Technologies, Carlsbad, CA). The resultant plasmid was renamed pENTR-Ca LAP3. This plasmid possessed the *C. albicans LAP3* gene flanked by the *attL* recombination sequences to facilitate its transfer into the pFG91 destination vector (which contains the corresponding *attR* acceptor sites).

pFG91 was then mixed with pENTR-Ca LAP3 and a recombination event was carried out between the 2 plasmids using the LR clonase recombination enzyme (Life Technologies, Carlsbad, CA). Following recom-bination the generated plasmid contained the *C. albicans LAP3* gene immediately upstream and in frame with a V5 epitope, TEV cleavage site, and 6-Histidine tag. This plasmid was confirmed by sequencing (Genewiz, South Plainfield, NJ) and renamed pFG99.

Yeast transformations

The *S. cerevisiae* strain *Δlap3* (ATCC #YNL239W), which is deleted for *LAP3*, was transformed with pFG99 in order to address the possibility of functional replacement of the *S. cerevisiae LAP3* gene with the orthologous gene from *C. albicans*. Transformations were carried out by a standard protocol that utilized lithium acetate and polyethylene glycol (Ito, et al., 1983). Transformed cells were plated on selective media and allowed to grow at 25°C for four to eight days.

Whole cell extracts and enzyme assays

Fungal whole cell extracts from *Ca002*, *Sc001*, and *ScΔlap3,* along with strains transformed with an empty vector or pFG99 were generated as described previously (Xu and Johnston, 1994) and quantitated by absorbance at 562 nm using the BCA Protein Assay kit (Pierce, Rockford, IL). Measurement of cysteine aminopeptidase activity in whole cell extracts was performed by modification of the protocol described in Xu and Johnston (1994). Briefly, 50 µg WCE was mixed with 25 µl 4X assay buffer (200 mM KH_2PO_4, 200 mM $NaHPO_4$, pH 7.5, 8 mM ethylenediaminetetraacetic acid (EDTA), 40 mM DTT), and pre-warmed to 30°C. Sterile water was used to bring the final reaction volume to 100 µl. The reaction was initiated upon the addition of 5 µl of 0.5 mM H-Arg-7-amino-4-methylcoumarin hydrochloride (H-Arg-AMC; Bachem, Torrence, CA) or H-Citrulline-7-amino-4-methylcoumarin hydrobromic acid (H-Cit-AMC; Bachem, Torrence, CA). Reactions were performed in triplicate and allowed to progress at 30°C for 2 h.

Fluorescence of liberated AMC corresponded to aminopeptidase activity and was measured in a BioTek Synergy2 microplate reader, using excitation wavelength of 340 nm and emission wavelength of 465 nm. Specific protease inhibitors (Enzo Biosciences, Farmingdale, NY) were incubated with WCE for 15 min prior to the start of the reactions.

Bleomycin detoxification

To assess detoxification of bleomycin by different strains, cells were grown in the appropriate medium to mid logarithmic phase, pelleted and washed in cold sterile water. Ten-fold dilutions of each strain were spotted on culture plates containing 1, 2.5 or 5 µg/ml bleomycin (Bleocin; EMD Millipore, Billerica, MA). Plates lacking bleomycin were used as positive controls for growth. Plates were incubated at 30°C for 24-72 h (depending on colony density) and bleomycin detoxification was scored by visual inspection of the colony survival following incubation.

Additionally, sensitivity to bleomycin was measured in liquid cultures. Cells in early logarithmic phase were washed in cold water and resuspended in an equal volume of medium supplemented with 1, 2.5, or 5 µg/ml bleomycin (Bleocin; EMD Millipore, Billerica, MA). Cells were induced with 2% galactose containing medium for 2 h, and treated with the indicated concentrations of bleomycin for 2 h. Cells were plated on selective medium, ura (-), as previously described, with the exception that glucose was the sole carbon source. Plates were scored for survivors after 2 days of growth at 30°C.

Western blot analysis

A *S. cerevisiae Δlap3* strain harboring the *C. albicans LAP3* overexpression plasmid pFG99 was used to visualize the expression pattern of Lap3p. Cells were grown to early log phase and either induced in ura (-) selective medium supplemented with 2% galactose or left uninduced in ura (-) medium with 2% glucose. Alternatively, YEP supplemented with either 2% glucose (uninduced) or 2% galactose (induced) was also used to address Lap3p protein expression.

Ten µg whole cell extract taken from each growth condition was run on 10% Tricine SDS-polyacrylamide gels and transferred to nitrocellulose (BioRad, Hercules, CA) using submerged blotting apparatus (Idea Scientific). *C. albicans* Lap3 was visualized by a standard immunoblot method using a mouse antibody generated against the V5 epitope (Sigma-Aldrich, St. Louis, MO).

RESULTS

C. albicans LAP3 functionally replaces *LAP3* in *S. cerevisiae*

Sequence analysis of the *C. albicans* genome predicted an open reading frame encoding a putative amino-peptidase possessing significant sequence homology to the *S. cerevisiae LAP3* gene (Candida Genome Database). The corresponding gene product, however has remained uncharacterized.

Using a SIM (Swiss Institute of Bioinformatics) amino acid sequence alignment, it was discovered that there is a 41% sequence identity (over 87% of the protein) with the *S. cerevisiae* Lap3p aminopeptidase (Figure 1). Of note is that essential active site amino acids found in *S. cerevisiae*, in particular the active site triad of Cysteine 73, Histidine 369 and Asparagine 392 (Joshua-Tor et al., 1995) are highly conserved in the *Candida* protein.

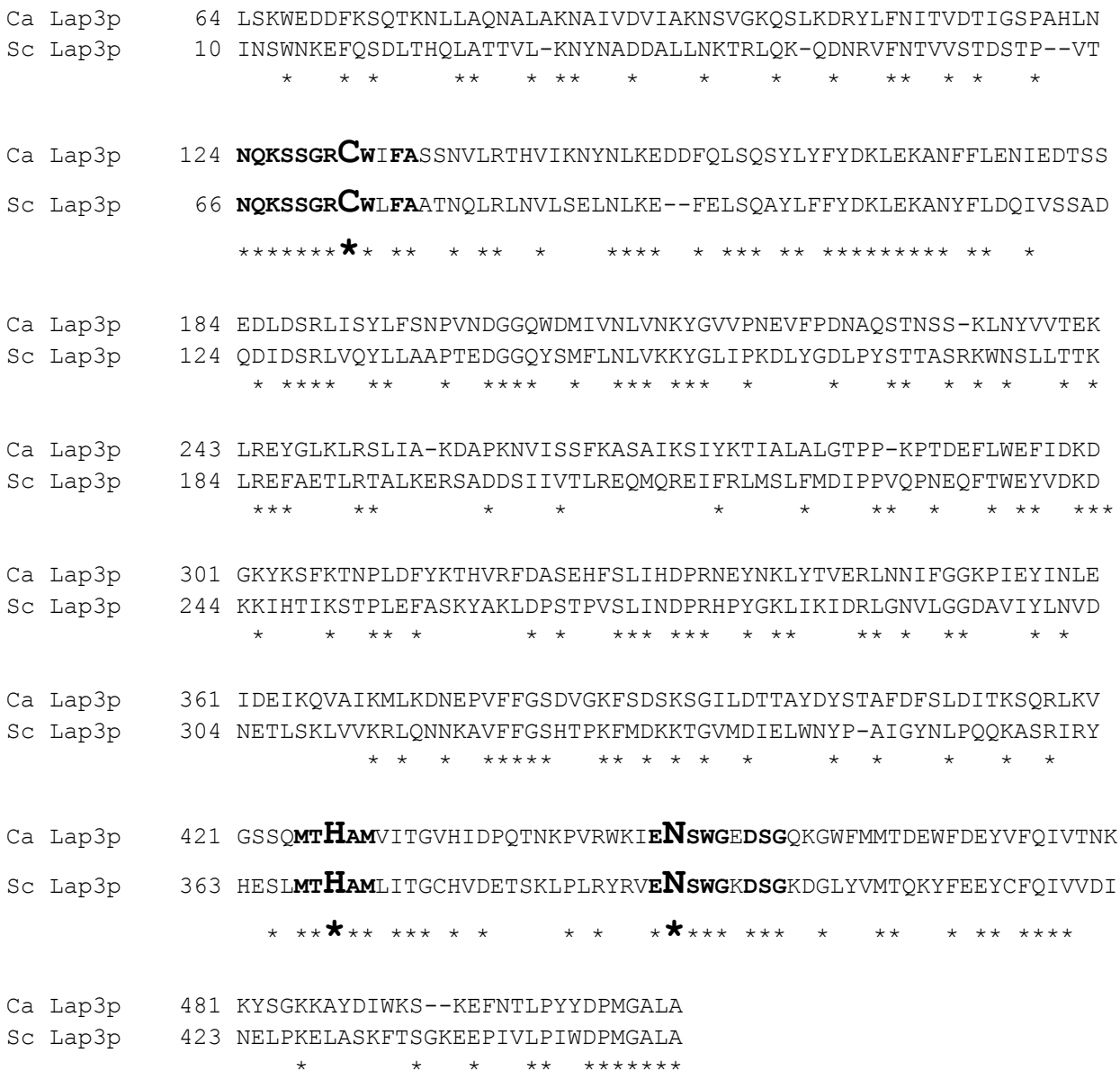

Figure 1. Amino acid sequence analysis of *Candida albicans* Lap3p. Comparison of the *S. cerevisiae* and *C. albicans* Lap3p amino acid sequences was carried out using the SIM amino acid sequence alignment program (Swiss Institute of Bioinformatics). Data shown represents 87% of the *C. albicans* Lap3p amino acid sequence in which there is 41% identity to the well-characterized *S. cerevisiae* Lap3p sequence. Amino acids denoted with asterisks (*) indicate highly conserved amino acids between the 2 proteins. Enlarged amino acids are those found in the active site triad of the *S. cerevisiae* protein, and represent those amino acids necessary for peptidase activity. Note the highly conserved nature of the *Candida* amino acid sequence at these positions, as well as in the regions surrounding the active site triad (in bold).

Therefore, sequence analysis strongly suggests that the *Candida* Lap3p is orthologous to the *Saccharomyces* Lap3 protein.

To address its cellular functions in fungi, the *C. albicans LAP3* gene was cloned into a Gateway Destination (Life Technologies, Carlsbad, CA) yeast expression vector. The resultant inducible expression plasmid (pFG99) was introduced into a *Saccharomyces* strain deleted for *LAP3* (*Δlap3*), and verified for protein production by western blot analysis (Figure 2C). Transformation of pFG99 (*CaLAP3*) into *Δlap3* demonstrated no measurable effects on yeast growth on a rich culture medium (Figure 2A). Similar results were obtained when transformants were selected on a uracil deficient culture medium, which was used to ensure expression of the *C. albicans LAP3* gene present on

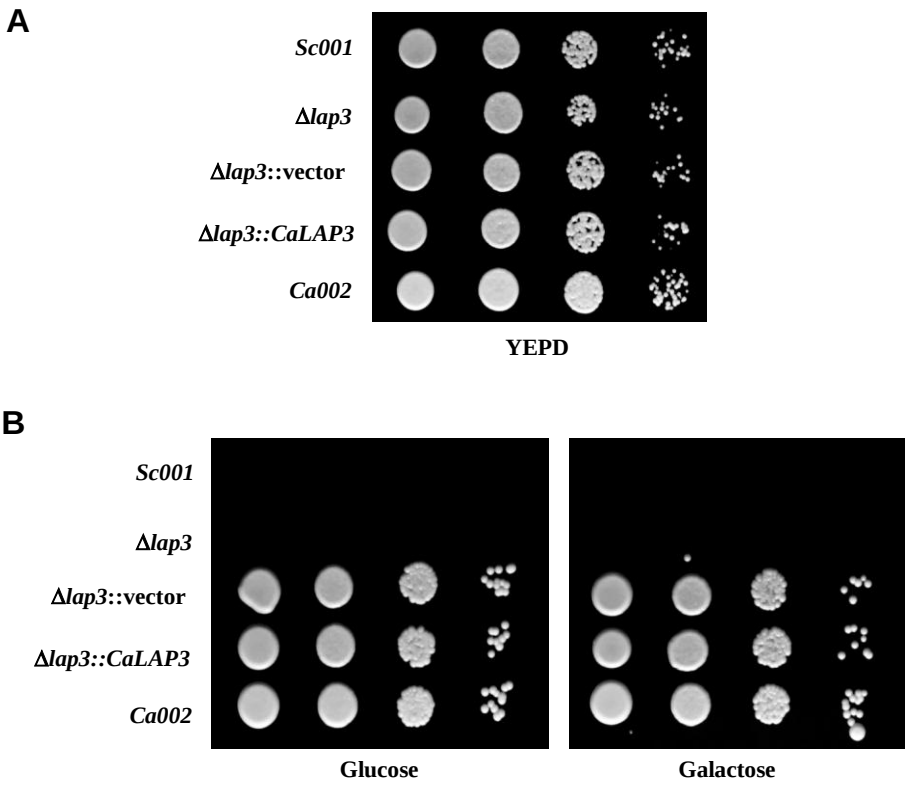

Figure 2. *Candida albicans LAP3* serves as a functional equivalent of the *Saccharomyces cerevisiae LAP3* gene. *S. cerevisiae* deleted for the *LAP3* (*Δlap3*) gene were transformed with either empty vector or an expression vector containing *C. albicans LAP3*. Following selection of transformants, cells were spotted in 10-fold serial dilutions on rich medium (A), or synthetic medium lacking uracil (B) supplemented with 200 µg/ml Geneticin. Untransformed *Δlap3* cells, along with wild type *S. cerevisiae* (*Sc001*) and *C. albicans* (*Ca002*) were included on each plate. Panel B demonstrates representative data from spot dilutions on medium supplemented with either glucose or galactose. Plates were maintained at 30°C for two days prior to assessing growth patterns. (C) Western blot analysis was carried out in order to visualize the expression of *C. albicans* Lap3p in *S. cerevisiae* wild type and mutant strains. *Δlap3::pFG99* cells were either grown for 2 h in uninducing conditions (2% glucose) or induced by washing the cell pellet and resuspending the cells in culture medium supplemented with 2% galactose. WCE generated from each cell pellet were used as the input in SDS polyacrylamide gel electrophoresis. Following transfer to nitrocellulose, the expression of *C. albicans* Lap3p in *S. cerevisiae* was observed using an anti-V5 epitope antibody (Sigma-Aldrich, St. Louis, MO).

pFG99 (Figure 2B). Additionally, upon overexpression of the *CaLAP3* (Figure 2B, galactose), growth patterns of the transformed strains appeared to be unaffected.

Lap3p orthologs are found in a number of eukaryotes (Sebti et al., 1987; Ferrando et al., 1996; Takeda et al., 1996; Schwartz et al., 1999) and are known to exhibit

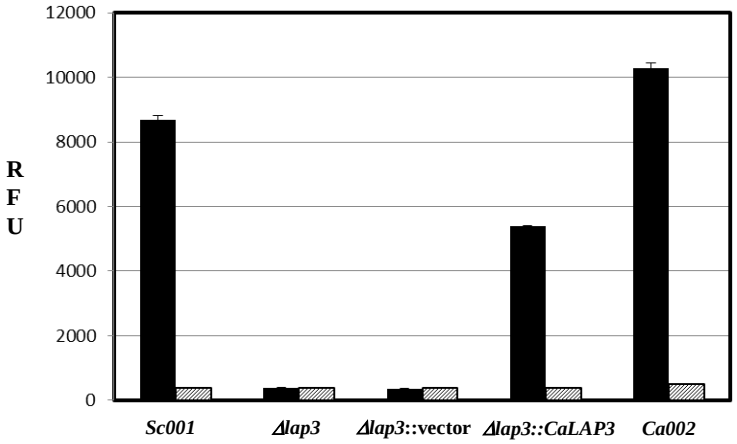

Figure 3. Enzyme activity of *C. albicans* Lap3p in *S. cerevisiae*. Cleavage of a fluorogenic cysteine aminopeptidase substrate was measured by incubating H-Arginine-AMC with 50 µg whole cell extracts from *Sc001*, *Ca002*, *Δlap3* and *Δlap3::CaLAP3* strains. The yeast strain *Δlap3::vector* was used as a control against transformation induced activation of peptidase activity in the *lap3* deletion strain. Cross-hatched bars represent WCE that were treated with the cysteine protease specific inhibitor E-64 for 5 min prior to initiation of the reaction. Reactions were conducted at 30°C for 2 h. Experiments were repeated 3 times, and results reflect the specific hydrolysis of the fluorogenic substrate in relative fluorescence units (RFU).

aminopeptidase activity against the fluorogenic substrate H-Arg-AMC, and to a lesser extent H-Citrulline-AMC. As demonstrated in Figure 3, a whole cell extract from a *S. cerevisiae* strain containing the *Candida LAP3* gene as the only source of the Lap3p aminopeptidase exhibited activity against H-Arg-AMC comparable to the activity of wild type *Saccharomyces* and *Candida* strains. Importantly, transformation of the *Δlap3* strain with the empty vector did not significantly affect aminopeptidase activity against H-Arg-AMC. Incubation of the yeast whole cell extracts with the cysteine protease specific inhibitor E-64 resulted in significant decrease of Lap3p activity in whole cell extracts. Similar results were obtained using the H-Cit-AMC substrate (data not shown), suggesting that the *Candida* Lap3p enzyme possesses general aminopeptidase properties similar to other characterized Lap3p orthologs.

Candida Lap3p functions as a member of the cysteine aminopeptidase family

In order to further characterize the *Candida* Lap3p aminopeptidase, a series of experiments were conducted in which inhibitors to different proteases were incubated with the *Candida LAP3*-expressing *S. cerevisiae* strain. As evidenced in Table 1, use of cysteine protease inhibitors either eliminated (E-64) or greatly reduced (leupeptin) detectable Lap3p activity. Interestingly, inhibitors directed against other types of proteases demonstrated little to no effect against *C. albicans* Lap3p. More specifically, incubation of whole cell extracts with bestatin (inhibitor of metalloproteases and leucine aminopeptidases), pepstatin (inhibitor of aspartyl proteases) and PMSF (inhibitor of serine proteases) had little effect on *C. albicans* Lap3p activity. Additionally, extracts incubated with EDTA or $MgCl_2$ (factors involved in metalloprotease activity) are not affected in their Lap3p activity. Taken together, the results in Table 1 further support the hypothesis that Lap3p functions as a cysteine aminopeptidase.

Increased bleomycin sensitivity in *Candida* is not solely attributed to Lap3p function

In studying the effects of DNA repair components in *C. albicans*, Garcia-Prieto, et al. (2010) demonstrated that *Candida* is more sensitive than *Saccharomyces* to the anticancer glycopeptide bleomycin. In that study it was hypothesized that there may be a previously uncharacterized bleomycin hydrolase activity present in *C. albicans*. The Lap3p aminopeptidase is thought to be solely responsible for bleomycin detoxification in *S. cerevisiae* (Pei*, et al.*, 1995) and mammalian cells (Wang and Ramotar, 2002), which prompted a test of whether the *C. albicans* Lap3p was functionally distinct in such a way that it would impart increased bleomycin resistance in a *Δlap3 S. cerevisiae* strain. To address this possibility,

Table 1. Effects of protease inhibitors on *Candida albicans* Lap3p activity.

Inhibitor	Final Concentration	Activity (%)	
		H-Arg-AMC	H-Cit-AMC
None	-	100	100
E-64	10 µM	0	0
Leupeptin	10 µM	5	9
Bestatin	10 µM	81	87
Pepstatin	1 µM	73	72
PMSF	1 mM	78	72
EDTA	2 mM	103	120
DTT	2 mM	109	108
$MgCl_2$	5 mM	101	107

Effects of protease inhibitors on *Candida albicans* Lap3p activity. Characterization of the *C. albicans* Lap3p was performed by mixing 50 µg WCE per reaction with 4X assay buffer (200 mM KH_2PO_4, 200 mM $NaHPO_4$, pH 7.5, 8 mM EDTA, 40 mM DTT), and pre-warmed to 30°C. Sterile water was used to bring the final reaction volume to 100 µl. Specific protease inhibitors were incubated with WCE for 15 min prior to the start of the reactions and the reactions were initiated by addition of H-Arg-7-amino-4-methylcoumarin hydrochloride (H-Arg-AMC) or H-citrulline-7-amino-4-methylcoumarin hydrobromic acid (H-Cit-AMC). Reactions progressed at 30°C for 2 h. Fluorescence of liberated AMC corresponded to aminopeptidase activity and was measured by excitation at 340 nm and emission at 465 nm. 100% activity corresponds to cleavage of 1 nM substrate/min.

the yeast strains described above were used in spot dilution test on rich medium (YEPD) plates containing 1, 2.5 or 5 µg/ml bleomycin. As shown in Figure 4A (top left box), YEPD plates lacking bleomycin provided evidence that the strains do not exhibit noticeably different growth rates in this type of assay. Culture plates containing either 2.5 or 5 µg/ml bleomycin (Figure 4A, bottom panels) showed the varied response to bleomycin by *S. cerevisiae* and *C. albicans*. In these conditions, *C. albicans* retained robust growth characteristics, but the growth of *S. cerevisiae* was inhibited by the presence of bleomycin to the extent that no detectable colonies were present. Each of the *S. cerevisiae* strains, whether *LAP3* was present or not, demonstrated the ability to grow in plates containing 1 µg/ml bleomycin (Figure 4A, top right panel).

Interestingly, introduction of the *C. albicans LAP3* into the Δ*lap3* strain did not impart a significant difference in resistance to bleomycin by *S. cerevisiae*. Expression of *C. albicans* Lap3p derived from the episomal expression is “leaky” on a glucose containing culture medium, as evidenced by western blot analysis (Figure 2C), but may not have reached a critical amount to exert any effect on bleomycin detoxification.

To address the possibility that there is simply not a sufficient quantity of recombinant *C. albicans* Lap3p produced in *S. cerevisiae* to adequately detoxify bleomycin, culture plates lacking uracil (supplemented with galactose as the sole carbon source) as the sole carbon source were used in a spot dilution assay. Under these conditions, overexpression of the *C. albicans* Lap3p is observed (Figure 2C).

In Figure 4B (top left panel), no apparent difference in growth pattern was obvious when comparing the *S. cerevisiae* and *C. albicans* strains. It should be noted that the lack of growth in the *Sc001* and Δ*lap3* lanes was expected due to the lack of a *URA3* producing plasmid in each of those uracil deficient strains.

As in Figure 4A, *S. cerevisiae* strains were incapable of growth in 2.5 or 5 µg/ml bleomycin, regardless of *C. albicans LAP3* presence. Additionally, overexpression of recombinant *C. albicans LAP3* did not increase resistance to 1 µg/ml bleomycin in *S. cerevisiae*, suggesting that *C. albicans* Lap3p is not the sole factor involved in bleomycin detoxification in *Candida*.

Based on the collection of evidence suggesting that bleomycin resistance *in vivo* is significantly increased following introduction of recombinant Lap3p (Pei et al., 1995; Wang and Ramotar, 2002), a liquid culture bleomycin resistance assay was employed.

Following exposure to 1, 2.5, or 5 µg/ml bleomycin in liquid culture, yeast cells were plated on bleomycin-free agar plates. In this experimental design, a measure of yeast colony forming units following bleomycin exposure was determined.

As shown in Figure 5, wild type *C. albicans* exhibited ability to grow in up to 5 µg/ml bleomycin. In stark contrast, growth of each of the *S. cerevisiae* strains was severely impaired. Surprisingly, presence of either *S. cerevisiae* Lap3p (*Sc001*) or recombinant *C. albicans* Lap3p (*Δlap3::CaLAP3*) resulted in no detectable increase in bleomycin resistance when compared to strains deleted for the *LAP3* open reading frame. Even at the lower bleomycin concentrations (1 µg/ml), no signi-

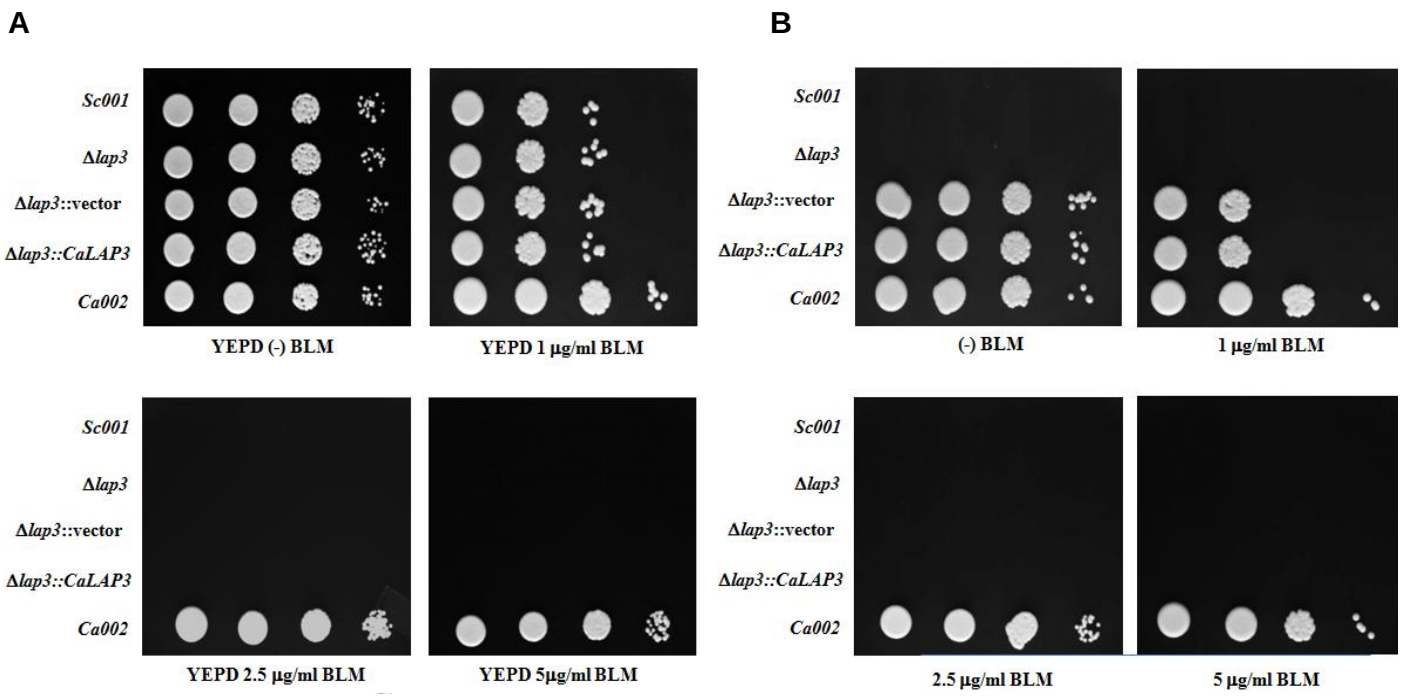

Figure 4. Sensitivity of *C. albicans* and mutant *S. cerevisiae* to bleomycin. Serial dilution assays were used to assess the relative sensitivity to bleomycin in *S. cerevisiae cells* expressing the *C. albicans LAP3* gene. Cells were spotted in 10-fold serial dilutions on rich medium (YEPD-Panel A) or synthetic medium lacking uracil (Panel B) supplemented with 2% galactose, 200 µg/ml Geneticin and either 1, 2.5 or 5 µg/ml of bleomycin in the molten agar. Untransformed *Δlap3* cells, along with wild type *S. cerevisiae* (*Sc001*) and *C. albicans* (*Ca002*) were included on each plate. Plates were incubated at 30°C for 72 h. The data is representative of three independent assays.

ficant difference in growth patterns of the *S. cerevisiae* strains was observed. Taken together with the results shown in Figure 4, we conclude that the *C. albicans* Lap3p is not solely responsible for imparting bleomycin resistance in all fungal systems.

DISCUSSION

We have described the first experimental evidence regarding the cloning and functional analysis of the *Candida albicans* Lap3p aminopeptidase. The gene that encodes this protein (*LAP3*) is orthologous to the *S. cerevisiae LAP3* and mammalian *BLH1* genes, which encode a cysteine aminopeptidase that functions in detoxification of bleomycin *in vivo*. Utilizing a *S. cerevisiae lap3* deletion strain, we have provided evidence that *Candida* Lap3p aminopeptidase is capable of functionally replacing the proteolytic activity of the *Saccharomyces* Lap3p.

Enzyme inhibitor profile studies verified that *C. albicans* Lap3p does indeed function as a cysteine aminopeptidase. Introduction of the *Candida* Lap3p demonstrated no significant negative effects on growth of the organism. We have used *in vivo* cell survival assays to demonstrate the increased bleomycin resistance properties of *C. albicans* when compared to *S. cerevisiae*. Furthermore, introduction of the *Candida* Lap3p did not significantly influence bleomycin resistance in *S. cerevisiae*.

The *Streptomyces vertillicus*-derived glycopeptide bleomycin has long been used as an anticancer chemotherapeutic (Lazo, 1999). Shortly after the discovery of bleomycin-resistant cell lines (Lazo et al., 1989) the rabbit bleomycin hydrolase (*BLH1*) protein was identified as the responsible protein in this response (Sebti and Lazo, 1987). Identification of an orthologous protein in *S. cerevisiae* (*YCP1/BLH1/GAL6/LAP3*) prompted further genetic studies, but has yielded reports that differ in describing the response of *lap3*-deletion strains when compared to wild type strains. Upon deletion of the *BLH1* (Enenkel and Wolf, 1993) or *GAL6* (Xu and Johnston, 1994) gene, cells exhibited hypersensitivity to bleomycin.

It has also been reported that strains deleted for *yBLH1* exhibited an equivalent level of bleomycin resistance as did wild type strains (Kambouris et al., 1992; Wang and Ramotar, 2002). Deletion of *LAP3* in *Saccharomyces* does not significantly influence the response of those cells to bleomycin. Our findings were consistent in both culture plate-based tests of resistance as well as in liquid culture, and suggest that a second protein (aside from Lap3p) may exist that functions in bleomycin

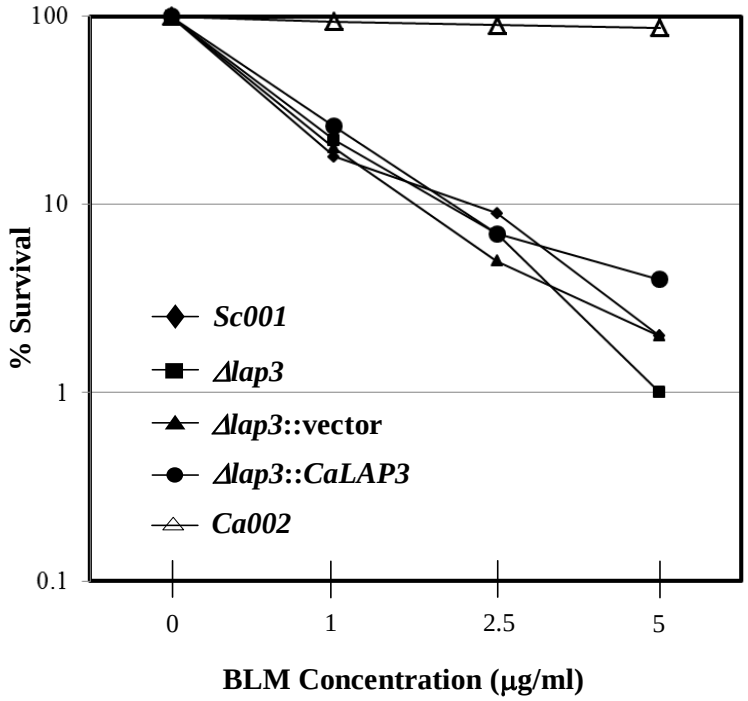

Figure 5. Cell survival of wild type and mutant yeast strains following exposure to bleomycin. Cells from the *Sc001*, *Ca002*, *Δlap3, Δlap3::vector* and *Δlap3::CaLAP3* strains were grown to mid log phase in synthetic medium lacking uracil and supplemented with 2% glucose. Cell pellets were washed in cold water and induced using 2% galactose for 2 h, and resuspended in with 1, 2.5 or 5 µg/ml of bleomycin for 2 h. Two hundred µl from each condition were plated on culture medium lacking uracil and supplemented with 2% glucose. Plates were scored for survivors after 2 days of growth at 30°C.

detoxification in baker's yeast. Although a second bleomycin hydrolase has not been of identified in yeast, the Lap aminopeptidases represent a family of 4 aminopeptidases that were isolated from the same mutant screen (Trumbly and Bradley, 1983). Analysis of the Lap aminopeptidase family revealed that activity of Lap1p, 2p and 4p greatly decreases upon introduction of EDTA to the assay system. Reactivation aminopeptidase activity was observed following addition of various cations, suggesting that these 3 Lap enzymes function as metallopeptidases. Interestingly, Lap3p is not classified as a metallopeptidase and possesses very little amino acid similarity to the 3 other members of the Lap family, suggesting that it is unlikely that members of the Lap family other than Lap3p function in detoxification of bleomycin. Through the use of active site Gal6p mutants, Zheng and Johnston (2008) demonstrated that hydrolysis of bleomycin was linked to the cysteine aminopeptidase activity of the protein both *in vivo* and *in vitro*. Evidence has been presented that describes changes in the Gal6p/Lap3p substrate specificity based on the carboxy-terminus of the enzyme (Zheng et al., 1998). It is possible that functional redundancy with regard to bleomycin resistance can exist with another cysteine aminopeptidase.

Conflicting reports also exist regarding the effect of Lap3p expression on bleomycin detoxification. Cells expressing high levels of Ycp1p demonstrate increased resistance to bleomycin than do those expressing normal levels of the protein (Kambouris et al., 1992). Additionally, introduction of yeast Ycp1p resulted in an increase in bleomycin resistance of mammalian cells (Pei et al., 1995).

Wang and Ramotar (2002), however presented data to show that addition of Blh1p imparts no additional resistance to bleomycin *in vivo*. This outcome was obtained when bleomycin hydrolase was either expressed at normal levels or over-expressed. Data shown in Figures 4 and 5 demonstrate that over-expression of *C. albicans* Lap3p in a *S. cerevisiae lap3* deletion strain does not significantly increase the bleomycin resistance properties of *Saccharomyces.* The *C. albicans* protein may require a secondary factor not present in *Saccharomyces* in order to exert its bleomycin

detoxification effects. Alternatively, a possibility may arise in which *C. albicans* cells exhibit resistance to killing by bleomycin by physically excluding the drug from cells.

Jayaguru and Raghunathan (2007) used bleomycin-treated cells to investigate the possibility of targeting the splicing machinery of *C. albicans* as a method by which to design new antifungals. Additionally, in studies of *Candida* DNA repair processes; it was found that bleomycin was capable of killing *C. albicans* cells at concentrations greater than 5 µg/ml (Garcia-Prieto et al., 2010). We have also found that concentrations at or over 10 µg/ml bleomycin serve to induce cell death of *C. albicans* (data not shown). Taken together, it would seem unlikely that *C. albicans* exhibits greater resistance to bleomycin simply by excluding it from the cell.

Finally, the ability of the *C. albicans* Lap3p to bind DNA may be a key determinant in its activity against bleomycin. The Gal6p was originally identified bound to the *GAL1/10* promoter region (Xu and Johnston, 1994), and Zheng and Johnston (1998) demonstrated that *S. cerevisiae* strains housing a DNA binding mutant Gal6p were decreased in their capacity to neutralize bleomycin. The DNA binding properties of the *C. albicans* protein have yet to be determined.

Although the *Candida* Lap3p is predicted to have an overall more negative charge than the *Saccharomyces* enzyme (CGD), this does not appear to be the sole determinant to nucleic acid binding by Lap3p-orthologous proteins. As an example, although the rat bleomycin hydrolase enzyme is able to bind single stranded Gal4 binding site (Takeda et al., 1996), this enzyme does not associate with DNA cellulose.

In this study it was proposed by the authors that there may be an accessory factor required for localization onto DNA. In *Escherichia coli*, the LexA protein is also known to be a DNA binding protease, but its association with DNA demonstrates an absolute requirement for the co-factor RecA (Kim and Little, 1993). In either case, we believe it is imperative to fully characterize the nucleic acid binding properties of *Candida* Lap3p to further understand the implications of this activity in fungal cellular processes.

Numerous cellular functions other than bleomycin detoxification have been described for yeast Lap3p and its orthologs (Koldamova et al., 1998; Montoya et al., 1998; Zimny et al., 2006; Montoya et al., 2007; Kamata et al., 2011) Furthermore, cysteine proteases throughout nature have demonstrated a role in a large variety of processes, ranging from signaling pathways in many organisms to programmed cell death (Degterev et al., 2003). Our results presented here raise further questions regarding the nature of the function of the *Candida* protein. Our evidence presented here provides the first evidence of cloning and functional characterization of the *C. albicans* Lap3p, and opens the door for dissecting its function(s) in *Candida* physiology.

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