Development of *Streptococcus thermophilus lacZ* as a reporter gene for *Candida albicans*

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### INTRODUCTION

*Candida albicans* is a commensal yeast of warm-blooded animals and has been recognized as an opportunistic pathogen for many years. *C. albicans* can assume a variety of morphologies, including budding yeast (blastospores) as well as pseudohyphal and hyphal forms; all of these are found at sites of infection (Odds, 1988). A number of growth conditions can be utilized *in vitro* to induce the transition between morphological forms of *C. albicans*. Signals which influence the yeast to hyphal transition include temperature, pH and the nutrient composition of the growth media (Odds, 1988), and several genes with roles in the morphological transition have been identified (Liu et al., 1994; Braun & Johnson, 1997; Csank et al., 1997, 1998; Lo et al., 1997; Newport & Agabian, 1997; Alex et al., 1998; Gale et al., 1998; Timpel et al., 1998; Stoldt et al., 1997; Leberer et al., 1996, 1997; Calera et al., 2000; Brown & Gow, 1999).

Studies of transcriptional regulation in *C. albicans* have been limited by several properties of the organism; e.g. it is diploid with a little-understood sexual cycle, making conventional genetic analysis impossible (Odds, 1988). Additionally, expression of foreign genes, including reporter genes, in *C. albicans* has been complicated by the alternative codon usage of the organism. The codon CTG is read as a serine in *C. albicans* but as a leucine in other organisms (Ohama et al., 1993). *Escherichia coli lacZ*, which has been successfully adapted as a reporter gene in many organisms, contains 51 CTG codons (Blanco et al., 1992). We were particularly interested in developing a β-galactosidase as a reporter for *C. albicans* because of the extensive variety of substrates available for this enzyme. In addition, *C. albicans* is not known to have an endogenous β-galactosidase and cannot utilize lactose as a carbon source (Kwon-Chung, 1992).

Here we report the use of *lacZ* from *Streptococcus thermophilus* as a versatile reporter gene for *C. albicans*. We show that β-galactosidase activity can be readily detected by a variety of methods when *lacZ* is expressed from several *C. albicans* promoters.

### Keywords:

- β-galactosidase, yeast, fungal pathogen, gene regulation
METHODS

Strains and plasmids. pRH116 was obtained from Larry McKay (Dept of Food Science and Nutrition, University of Minnesota, St Paul) and contains Strept. thermophilus lacZ in a pBR322-based vector (Schoeder et al., 1991). The vectors utilized for LacZ expression were constructed as follows. pAU13 contains the URA3 gene from C. albicans inserted into XbaI/EcI36II-digested pBluescript KS+ (Stratagene) as an XhoI–SacI fragment from pDBV2 (Brown et al., 1996). pAU15, the MAL2 expression vector, was created by amplifying a 550 bp region upstream of the MAL2 coding sequence with the primers MAL2–5′ (GAAGGTACCATGAGGATATTTTGGCTTAGTACC) and MAL2–3′ (CTCTACCTGGATGTAGTTATTAATTTAAC) using C. albicans CAH4 genomic DNA. A 250 bp downstream region of MAL2 was amplified from C. albicans CAH4 genomic DNA using the primers UTR5′ (CCTGGGATCCTAATATACGATCAGTGCATCTAC) and MAL2–3′ (CCTGGTCTAAGAACATACGGCTTGGAGGATGTGGTT). The MAL2 upstream and downstream fragments were cloned into pAU13 to create pAU14, which is a MAL2 expression vector containing a XhoI–SalI–HindIII–Smal–BamHI polylinker following the MAL2 promoter. The HWP1 expression vector was created in a similar fashion, by amplifying an upstream region of HWP1 with the primers HWP1–5′ (GCTCTGGTACCAAAAACAGGAATTCCGGAAATC) and HWP1–3′ (CTCTTTCAAGATGGTATTTTTAAAAAGGGTTTATT). The resulting fragments were cloned into pAU13, resulting in pAU64, which contains a HWP1–EcoRI–PstI–SmaI–BamHI polylinker following the HWP1 promoter. The ACT1 expression vector was created by amplifying a 1 kb region upstream of ACT1 from C. albicans CAH4 genomic DNA with the primers ACT1–P5′ (CTCTCGGTACCAGAGCTATTAAGATCACCAGCCT) and ACT1–P3′ (CTCTTTCAAGATGGTATTTTTAAAAAGGGTTTATT). The ACT1 upstream region was amplified with the primers ACT1–U5′ (GAAGAGGATCCCTCTTAAATACGATCAGTGCATCTAC) and ACT1–U3′ (CTCTTTCAAGATGGTATTTTTAAAAAGGGTTTATT). The ACT1 downstream region was amplified with the primers ACT1–U5′ (GAAGAGGATCCCTCTTAAATACGATCAGTGCATCTAC) and ACT1–U5′ (CTCTTTCAAGATGGTATTTTTAAAAAGGGTTTATT). These fragments were cloned into pAU13 to create pAU34, which contains an XhoI–ClaI–HindIII–EcoRI–PstI–SmaI–BamHI polylinker following the ACT1 promoter region.

The Strept. thermophilus lacZ gene was amplified from pRH116 using the primers LacZ5′ (GGTGTGGTTCAGTGGTTGTAAGACACTGCTAATATACGATCAGTGCATCTAC) and LacZ3′ (TCCACGAGGATCCCTCTTAAATACGATCAGTGCATCTAC) and cloned into the various expression constructs to create the following plasmids: MAL2–lacZ (pAU22), HWP1–lacZ (pAU95) and ACT1–lacZ (pAU36). Alteration of the CTG codon to TTA in lacZ was done by PCR-based mutagenesis using the primers LacZ–1631 (ATGTTGTGGTTCAGTGGTTGTAAGACACTGCTAATATACGATCAGTGCATCTAC) and LacZm5′ (GTGTGGTTCAGTGGTTGTAAGACACTGCTAATATACGATCAGTGCATCTAC) and LasZ3′. The resulting PCR fragments were mixed and served as a template for amplification with the primers LacZ–1631 and LacZm3′; this was cloned into pAU22 to give pAU160. Presence of the mutation was screened for by creation of an ApII site and confirmed by sequencing. For expression of Strept. thermophilus lacZ in Saccharomyces cerevisiae, lacZ was amplified with Pfu DNA polymerase (Stratagene) using linearized pAU22 as a template and the promoters LacZ5′ and MAL2–3′Bln (CTCTGGTACCAGGATCATGATTGTCAGTGGTTGTAAGACACTGCTAATATACGATCAGTGCATCTAC) and LacZm5′ and MAL2–3′Bln (CTCTGGTACCAGGATCATGATTGTCAGTGGTTGTAAGACACTGCTAATATACGATCAGTGCATCTAC). The resulting product was digested with XmaI and BlpI and cloned into XmaI/MluI-cut pDK20 (a gift from Doug Kellogg, Sinheimer Laboratories, Dept of Biology, University of California, Santa Cruz).

C. albicans methods. C. albicans strain CAH4 (ura3::imm434/ura3::imm434) was served as the parent strain for all manipulations (Fonzi & Irwin, 1993). Linearized DNA was transformed into CAH4 by the modified lithium acetate method (Hill et al., 1991; Gietz et al., 1995), and transformants were selected on medium lacking uridine (Guthrie & Fink, 1991). Standard recipes were utilized for all media, and carbon sources such as maltose, glucose, galactose or lactose were added at a final concentration of 2% (v/v) (Guthrie & Fink, 1991). For expression of lacZ in Sacch. cerevisiae, strain W303 (ade2-1 trp1-1 can1 102 len2-3.112 his3-11 ural3 psi+) was utilized (Guthrie & Fink, 1991). Plasmids were introduced into W303 by lithium acetate transformation (Hill et al., 1991; Gietz et al., 1995).

β-Galactosidase assays. C. albicans β-galactosidase assays were performed as described by Ausbel et al. (1992) for Sacch. cerevisiae with minor modifications. For liquid samples, 1 ml of cells was resuspended in an equal volume of Z buffer (Ausbel et al., 1992) and placed on ice. The OD600 was determined for each sample. Then 10–100 µl of cells was added to Z buffer to a final volume of 1 ml, and the cells were permeabilized with 15 µl 0.1% SDS and 30 µl chloroform. There was no appreciable change in activity when alternative methods such as toluene or glass beads were used for permeabilizing cells. Cells were equilibrated at 37°C for 5 min, then 0.2 ml ONPG (4 mg/ml) was added and the cells were mixed and incubated at 37°C. Reactions were stopped by addition of 0.5 ml 1 M Na3CO3, spun for 5 min at 10000 g and the A420 and A660 were read. Units of activity were determined by the standard equation given by Ausbel et al. (1992).

Filter assays were performed as described by Ausbel et al. (1992). Patches of C. albicans were replica-plated onto solid medium with an overlay of a circular Whatman filter and grown overnight. Filters were frozen in liquid nitrogen and incubated in 3 ml Z buffer with 20 µl 3% X-Gal and incubated at 37°C.

Visual screens for C. albicans were carried out by patching colonies onto X-Gal plates (Ausbel et al., 1992). The standard formulation for X-Gal plates was utilized for most applications. A slightly modified recipe proved more sensitive for detection of β-galactosidase, although strains grew more slowly on this medium (X-Gal Modified Medium, XMM). XMM contained 1.7 g Yeast Nitrogen Base (without amino acids or ammonium sulfate), 20 g glucose, 5 g ammonium sulfate and 20 g agar in 930 ml H2O. After autoclaving, 70 ml 1 M potassium phosphate pH 7.0 and 2 ml of a 20 mg ml−1 X-Gal solution were added.

RESULTS AND DISCUSSION

Assays of β-galactosidases in C. albicans

We reasoned that β-galactosidases from organisms with high percentages of adenosine/thymine genomic content might be biased against CTG codons; a search against various databases revealed two β-galactosidases that contained relatively few CTG codons. LAC4 from

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the yeast *Kluyveromyces lactis* contains only two CTG codons, while *lacZ* from the thermophilic bacterium *Streptococcus thermophilus* has only one CTG codon (Schroeder et al., 1991; Poch et al., 1992). The single leucine encoded by CTG in the *Streptococcus thermophilus lacZ* is at the extreme C-terminus of the LacZ protein in a non-conserved region of *β*-galactosidases, while one of the leucines encoded by a CTG codon in the *K. lactis* LAC4 gene is in a position conserved as a hydrophobic residue in *β*-galactosidases (Jacobson et al., 1994) (Fig. 1). Both CTG codons in *lacZ* were changed to TTA in order to specify leucines at these positions. Either wild-type LAC4 or the mutant derivatives were integrated into the *C. albicans* genome and expressed from the MAL2 (maltase) promoter. MAL2 is induced by maltose and repressed by glucose (Brown et al., 1996). No activity was detected in strains with the MAL2–LAC4 wild-type and MAL2–LAC4 mutant derivatives under inducing conditions using both liquid assays and qualitative plate assays (Fig. 1). Although LAC4 has previously been described as a possible reporter gene in *C. albicans* (Leuker et al., 1992), its usefulness in our hands appeared limited. When LAC4 was expressed from the ACT1 promoter, *β*-galactosidase activity could only be detected in a limited number of transformants; these presumably represented strains with multiple integration events (Leuker et al., 1992).

When *Streptococcus thermophilus lacZ* (unaltered in its CTG codon) or *lacZ* with the single CTG codon altered to TTA was expressed from the MAL2 promoter in *C. albicans*, *β*-galactosidase activity could be readily detected by several means (Fig. 1). Colonies of *C. albicans* expressing MAL2–lacZ turned blue on solid medium containing both maltose and the chromogenic substrate X-Gal after a few days’ growth. No such activity was detected when colonies were grown on the same medium containing glucose instead of maltose. Maltose-dependent activity was also detected by liquid assays (see below and Table 1).

### Expression of lacZ from a variety of promoters

Since *lacZ* expression could be detected from the MAL2 promoter, we examined its use for monitoring promoter activity from two other *C. albicans* genes, ACT1 and HWP1 (Fig. 2). The promoter for the ACT1 (actin) gene has previously been used for expression of foreign genes in *C. albicans* (Leuker et al., 1992). ACT1 promoter expression is constant under many conditions but has been shown to be regulated by starvation and growth phase in *C. albicans* (Delbruck & Ernst, 1993). HWP1 expression is minimal when *C. albicans* is growing in the budding yeast form and highly induced during conditions that promote filamentous growth (Staab et al., 1996). Quantitative liquid assays were performed on *C. albicans* strains expressing MAL2–lacZ, ACT1–lacZ or HWP1–lacZ from constructs that had been integrated in the genome (Table 1). Strains were grown at 30 °C in YEP-glucose or YEP-maltose (budding yeast morphology) or at 37 °C in YEP-glucose with 10% fetal calf serum (hyphal/pseudohyphal morphology).

In the MAL2–lacZ strains, *β*-galactosidase activity was significantly induced (> 20-fold) by growth in maltose compared to growth in glucose; activity was detected at only background levels in glucose (Table 1), where background levels are defined as the very low levels of *β*-galactosidase activity observed in the absence of the *Streptococcus thermophilus lacZ* reporter gene. Expression of

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**Fig. 1.** *β*-Galactosidase genes tested for activity in *C. albicans*. *K. lactis LAC4* (Poch et al., 1992) and *Streptococcus thermophilus lacZ* (Schroeder et al., 1991) were placed under control of the maltose-inducible *C. albicans* MAL2 promoter (Brown et al., 1996) and integrated into the *C. albicans* genome. *β*-Galactosidase was scored by visual assays on medium containing X-Gal and maltose and by liquid assays on cells grown in medium with maltose (Ausubel et al., 1992). For the LAC4 and *lacZ* genes, mutant derivatives were also constructed in which the CTG codons were changed to TTA. CTG is decoded as serine in *C. albicans* and as leucine in other organisms (Ohama et al., 1993). The approximate locations of the CTG codons in LAC4 and lacZ are shown.
MAL2–lacZ was not induced significantly under conditions that promoted filamentous growth (Table 1). We noticed that approximately 10% of strains transformed with our lacZ constructs expressed significantly higher levels of β-galactosidase than did the majority; these strains were assumed to represent multiple integrants and were not studied further. Examples of this are shown in Fig. 2. Additionally, approximately 5% of strains did not express detectable levels of β-galactosidase; these presumably resulted from integration into the genome at sites other than the intended locus or from rearrangements that occurred subsequent to transformation.

Expression of the ACT1–lacZ construct was constant during all growth conditions, varying less than 1.5-fold during all growth conditions (Table 1). The ACT1 gene is known to be downregulated by starvation and during growth-phase changes; these conditions were not tested for the ACT1–lacZ fusion (Delbruck & Ernst, 1993; Swoboda et al., 1994). In contrast, the HWP1–lacZ construct was strongly induced by growth under filamentous conditions, including YEP-glucose + 10% fetal calf serum at 37 °C. Compared to growth in YEP-glucose, the HWP1–lacZ construct was induced approxi-
mately 250-fold by growth in YEP-glucose + 10% fetal calf serum. This is consistent with measurements of RNA levels of HWP1 in cells grown in YEP-glucose or YEP-glucose + 10% fetal calf serum at 37°C (data not shown). HWP1-lacZ was also induced to a lesser degree by substitution of maltose for glucose in the growth medium (see Table 1 and Fig. 2), and utilization of maltose as a carbon source has been reported to be an inducer of filamentous growth in C. albicans (Odds, 1988).

In order to optimize enzymic assays for the Strep. thermophilus β-galactosidase, we performed assays at various temperatures and pH values. The temperature optimum of Strep. thermophilus LacZ was found to lie between 45 and 50°C; enzymic activity fell sharply at temperatures higher than 50°C. Activity of the enzyme was 2-2-fold higher at 45°C than at 30°C. The pH optimum of the enzyme was between 6.8 and 7.2.

Expression of lacZ does not allow growth on lactose as a sole carbon source

Since expression of ACT1-lacZ was independent of carbon source, this allowed a test of whether C. albicans expressing β-galactosidase could utilize lactose as a sole carbon source. We grew C. albicans SC5314 (wild-type) and C. albicans CA361 (ACT1::lacZ) on minimal medium with glucose (SG), minimal medium with lactose (SL) and minimal medium without any carbon source (S). No growth of SC5314 or CA361 was observed on SL or S medium, while both strains grew on SG medium (data not shown). A similar phenotype has been noticed for Sacch. cerevisiae strains expressing β-galactosidase, and expression of a lactose permease was necessary before lactose could be utilized as a carbon source (Sreekrishna & Dickson, 1985).

Qualitative assays of β-galactosidase activity

Growth of cells on medium containing X-Gal provides a useful method of screening large numbers of cells for the expression of β-galactosidase activity under particular sets of conditions. When plated on the appropriate medium containing X-Gal, activity was easily detectable from C. albicans strains expressing MAL2-lacZ (CAU221, HWP1-lacZ (CAU951) and ACT1-lacZ (CAU361) (Fig. 2). After 2 d, colonies expressing the HWP1-lacZ fusions turned blue on X-Gal+10% fetal calf serum, but remained white on X-Gal+glucose or X-Gal+maltose (not shown). However, after 5 d, strains with the HWP1-lacZ fusion began to turn blue on all media (Fig. 2). This result was expected since HWP1 expression is highly induced under a variety of conditions that promote filamentous growth, including starvation, a condition that arises in colonies upon prolonged growth (see below). Colonies of the ACT1-lacZ fusion strain turned visibly blue on all media as soon as 2 d, and developed further after 5 d (Fig. 3). Colonies of the MAL2-lacZ fusion strain remained white on X-Gal+glucose medium (Fig. 2). The longer developing time of the MAL2-lacZ fusion strains compared with the other strains is consistent with the lower level of expression as detected by the liquid assays (Table 1).

In addition to its usefulness in distinguishing expressing from non-expressing colonies, growth on X-Gal medium can also be used to discern changes in gene expression that occur within a colony. Fig. 3 depicts a streak of a C. albicans strain containing the HWP1-lacZ fusion that had been grown on X-Gal+maltose for 3 d. Most of the streak is white, consistent with the lack of expression of HWP1 by budding cells. However, in the centre of the streak, particularly in areas of wrinkled appearance, groups of cells have begun to express lacZ. Microscopic analysis revealed that many of the cells taken from the centre of the streak were filamentous while almost all those taken from around the edge were in the budding form. It seems likely that conditions in the centre of the streak, perhaps depletion of nutrients, had induced filamentous growth, which is easily monitored by expression of the HWP1-lacZ fusion. No such patterns of expression were seen in colonies of C. albicans expressing the ACT1-lacZ fusion.

Although growth on X-Gal medium is a useful monitor of lacZ expression, it can require several days for the colour to develop. For more rapid detection of β-galactosidase activity, colonies grown on solid medium can be transferred to paper filters, permeabilized by freezing in liquid nitrogen and incubated with X-Gal (Ausubel et al., 1992). Results with filter assays matched results of the liquid β-galactosidase assays and plate assays (not shown). Positive reactions were obtained after only a few hours’ incubation at 37°C for C.
β-exhibit regulated pansy luciferase (Srikantha et al., 1996), that contain the GAL1 promoter and integrated in the genome readily exhibited regulated β-galactosidase activity as determined by filter assays (Guthrie & Fink, 1991). When grown on YEP-galactose, 88% of the transformants produced β-galactosidase activity within 1 h, while no activity was observed when transformants were grown on YEP-glucose. No activity was detected from Sacch. cerevisiae stains containing the GAL1.10 vector alone. Thus, Strept. thermophilus lacZ can be shuttled between C. albicans and Sacch. cerevisiae and used as a reporter gene in both yeasts.

The results presented in this paper show that lacZ from Strept. thermophilus is a useful reporter gene for the human pathogen C. albicans. Other reporter genes have been developed for C. albicans, including the sea pansy luciferase (Srikantha et al., 1996), the K. lactis β-galactosidase LAC4 (Leuker et al., 1992), the URA3 gene from C. albicans (Myers et al., 1995) and the yeast-enhanced green fluorescent protein (yEGFP; Cormack et al., 1997). These reporter genes have been used effectively for studies of transcriptional regulation in C. albicans (Wirsching et al., 2000; Srikantha et al., 1996, 1997; Stoldt et al., 1997; Leuker et al., 1997). Strept. thermophilus LacZ combines the advantages of sensitivity, simplicity of qualitative assays, and ease of colorimetric visual screens in growing colonies of C. albicans.

E. coli lacZ has proved to be a highly versatile reporter gene in Sacch. cerevisiae and has been used to study many aspects of signal-transduction pathways, gene regulation and other cellular processes (Guarente & Ptashne, 1981; Rose et al., 1987; Guarente, 1983; Burns et al., 1994). We believe that the Strept. thermophilus lacZ reporter gene can be used for many of these same purposes in C. albicans.

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