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A Multifunctional, Synthetic *Gaussia princeps* Luciferase Reporter for Live Imaging of *Candida albicans* Infections▽

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Real-time monitoring of the spatial and temporal progression of infection/gene expression in animals will contribute greatly to our understanding of host-pathogen interactions while reducing the number of animals required to generate statistically significant data sets. Sensitive in vivo imaging technologies can detect low levels of light emitted from luciferase reporters in vivo, but the existing reporters are not optimal for fungal infections. Therefore, our aim was to develop a novel reporter system for imaging *Candida albicans* infections that overcomes the limitations of current luciferase reporters for this major fungal pathogen. This luciferase reporter was constructed by fusing a synthetic, codon-optimized version of the *Gaussia princeps* luciferase gene to *C. albicans PGA59*, which encodes a glycosylphosphatidylinositol-linked cell wall protein. Luciferase expressed from this *PGA59-gLUC* fusion (referred to as *gLUC59*) was localized at the *C. albicans* cell surface, allowing the detection of luciferase in intact cells. The analysis of fusions to strong (*ACT1* and *EFT3*), oxidative stress-induced (*TRX1*, *TRR1*, and *IPF9996*), and morphogenesis-dependent (*HWP1*) promoters confirmed that *gLUC59* is a convenient and sensitive reporter for studies of gene regulation in yeast or hyphal cells, as well as a flexible screening tool. Moreover, the *ACT1-gLUC59* fusion represented a powerful tool for the imaging of disease progression in superficial and subcutaneous *C. albicans* infections. *gLUC59* and related cell surface-exposed luciferase reporters might find wide applications in molecular biology, cell biology, pathobiology, and high-throughput screens.

Candida albicans is responsible for a large fraction of fungal infections in humans (5) and, as such, has received considerable attention from the research community over the last two decades. *C. albicans* now represents an invaluable model for dissecting the interplay between fungal pathogens and their hosts at the molecular level (31, 32, 43, 45, 50). Studies of host-pathogen interactions have been greatly facilitated by the use of ex vivo infection models where isolated microorganisms and host cells or reconstituted tissues are brought into contact and the kinetics of pathogen and host cell responses are monitored (12, 14, 23, 36, 45). Yet, animal models remain necessary complements to ex vivo infection models, because none of these models fully reflect the development of clinical infections. Animal models allow researchers to monitor the behavior of mutant microorganisms or the expression of reporter genes in the complex environments of organs and in the presence of a fully functional or debilitated immune system (3, 20, 24).

A current limitation of animal models is the need to sacrifice animals in order to image microorganisms at the site of infection. In particular, studies aimed at evaluating whether conditions known to trigger the expression of a specific *C. albicans* gene in vitro are encountered at sites of infection have often relied on the detection of a reporter in tissue sections. Several reporter genes are available for gene expression studies of *C. albicans*, such as the *Streptococcus thermophilus* β-galactosidase *lacZ* gene (46) and the sea pansy (*Renilla reniliformis*) luciferase gene (41), but most in vivo studies have taken advantage of derivatives of the *Aequorea victoria* green fluorescent protein (GFP) gene, whose product can be detected primarily through its natural fluorescence but also through immunochemistry in tissue sections (2, 7, 16, 28). In particular, GFP fusions have been used to examine the niche-specific expression of central metabolic pathways and oxidative stress responses in *C. albicans* during disease progression (3, 10).

As the detection of GFP (and of other reporters such as β-galactosidase) is possible only in tissue sections, it is not possible to monitor in real time the spatial and temporal progression of *C. albicans* infection/gene expression in a single animal. Real-time monitoring would represent a significant advance because it would probably reveal meaningful variations in fungus/host responses that can be masked by the heterogeneous behavior of individual animals (24). Real-time monitoring might also reveal the spread of *C. albicans* to unexpected infection sites. Furthermore, real-time monitoring would reduce the number of animals required to generate

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TABLE 1. *Candida albicans* strains used in this study

Strain	Parent	Genotype	Reference
CAI4		*ura3*::λ*imm434*/*ura3*::λ*imm434*	11
SM22	CAI8	*ura3*::λ*imm434*/*ura3*::λ*imm434 ade2*::*hisG*/*ADE2-ACT1p-RLUC*	This study
BWP17		*ura3*::λ*imm434*/*ura3*::λ*imm434 arg4*::*hisG*/*arg4*::*hisG his1*::*hisG*/*his1*::*hisG*	49
CEC175	BWP17	*ura3*::λ*imm434*/*URA3 arg4*::*hisG*/*arg4*::*hisG his1*::*hisG*/*his1*::*hisG*	15
CEC161	BWP17	*ura3*::λ*imm434*/*ura3*::λ*imm434 ARG4*/*arg4*::*hisG HIS1*/*his1*::*hisG*	This study
CEC749	BWP17U	*ura3*::λ*imm434*/*URA3 arg4*::*hisG*/*arg4*::*hisG his1*::*hisG*/*his1*::*hisG PGA59*/(*PGA59-gLUC*)-*ARG4-PGA59*	This study
CEC751	BWP17U	*ura3*::λ*imm434*/*URA3 arg4*::*hisG*/*arg4*::*hisG his1*::*hisG*/*his1*::*hisG PGA59*/(*PGA59-GFP*)-*ARG4-PGA59*	This study
CA1399	CAI4	*ura3*::λ*imm434*/*ura3*::λ*imm434 RPS10*/*RPS10*::*CIp10-PGA59-gLUC*	This study
CA1398	CAI4	*ura3*::λ*imm434*/*ura3*::λ*imm434 RPS10*/*RPS10*::*CIp10-ACT1p-PGA59-gLUC*	This study
CA1435	CAI4	*ura3*::λ*imm434*/*ura3*::λ*imm434 RPS10*/*RPS10*::*CIp10-TRR1p-PGA59-gLUC*	This study
CA1400	CAI4	*ura3*::λ*imm434*/*ura3*::λ*imm434 RPS10*/*RPS10*::*CIp10-TRX1p-PGA59-gLUC*	This study
CA1434	CAI4	*ura3*::λ*imm434*/*ura3*::λ*imm434 RPS10*/*RPS10*::*CIp10-IPF9996p-PGA59-gLUC*	This study
CEC987	CEC161	*ura3*::λ*imm434*/*ura3*::λ*imm434 ARG4*/*arg4*::*hisG HIS1*/*his1*::*hisG RPS10*/*RPS10*::*CIp10-ACT1p-PGA59-gLUC*	This study
CEC971	CEC161	*ura3*::λ*imm434*/*ura3*::λ*imm434 ARG4*/*arg4*::*hisG HIS1*/*his1*::*hisG RPS10*/*RPS10*::*CIp10-HWP1p-PGA59-gLUC*	This study
CEC975	CEC161	*ura3*::λ*imm434*/*ura3*::λ*imm434 ARG4*/*arg4*::*hisG HIS1*/*his1*::*hisG RPS10*/*RPS10*::*CIp10-EFT3p-PGA59-gLUC*	This study
CEC369	CEC161	*ura3*::λ*imm434*/*ura3*::λ*imm434 ARG4*/*arg4*::*hisG HIS1*/*his1*::*hisG RPS10*/*RPS10*::*CIp10*	This study

statistically significant data sets (19). In this regard, in recent years in vivo imaging technologies have been developed that take advantage of sensitive charge-coupled device cameras to detect low levels of light emitted from luciferase reporters in vivo. Pioneering work by Contag et al. (6) demonstrated that bioluminescent *Salmonella* could be localized to specific tissues in live animals, allowing the temporal monitoring of the infection process and of the efficacy of antimicrobial treatment. This approach has now been extended to numerous pathogenic bacteria, virus, and parasites (19), and several luciferases are available for in vivo imaging, including firefly luciferase (fLUC from *Photinus pyralis*), which catalyzes light production from luciferin and ATP, and sea pansy luciferase (rLUC) and *Gaussia princeps* luciferase (gLUC), which catalyze light production from coelenterazine in an ATP-independent manner (37, 44, 47). Recently, Doyle et al. (8, 9) showed that light emitted by *C. albicans* strains expressing the firefly luciferase gene under the control of the strong *C. albicans ENO1* promoter could be detected in animals with induced vulvovaginal candidiasis that had been subjected to a vaginal lavage with a solution containing luciferin. The efficacy of an antifungal treatment could be monitored over a period of 19 days through imaging of the same group of animals. However, this in vivo reporter system did not allow detection of *C. albicans* during systemic candidiasis. As pointed out by those authors, their failure to detect light in animals despite efficient kidney colonization by luminescent *C. albicans* might have resulted from the limited permeability of hyphal cells to luciferin and the attenuation of light emitted from the kidneys by overlying tissues (8). Furthermore, the inability to detect intracellular firefly luciferase in hyphal cells represents a major limitation for further studies of *C. albicans*, since the yeast-to-hypha transition is a major virulence determinant in this species (35).

In the present study, we have successfully circumvented most of these limitations by engineering a luciferase that becomes exposed at the cell surface and hence is readily accessible to its substrate whether *C. albicans* is in the yeast or hyphal form. This was achieved by fusing a synthetic, codon-optimized version of the gene for the naturally secreted *Gaussia princeps* luciferase (44) to the *C. albicans PGA59* gene, which encodes a glycosylphosphatidylinositol (GPI)-linked cell wall protein required for cell wall integrity (27). We confirm that the *PGA59-gLUC* gene fusion (referred to as *gLUC59*) is a convenient and powerful reporter for in vitro gene expression studies using intact yeast or hyphal *C. albicans* cells. Moreover, we show that bioluminescence imaging is a powerful tool for the detailed monitoring of the spatiotemporal behavior of cutaneous, subcutaneous, and vaginal *C. albicans* infections in live animals. The *gLUC59* reporter is also useful for the analysis of systemic infections, although the uneven distribution of the *G. princeps* luciferase substrate, coelenterazine, in live animals prevents accurate quantitative analysis of such infections.

MATERIALS AND METHODS

Strains, media, and growth conditions. All *C. albicans* strains used in this study are listed in Table 1. Strains were routinely grown at 30°C on YPD medium (1% yeast extract, 2% peptone, 2% glucose) or SD minimal medium (0.67% yeast nitrogen base without amino acids, 2% glucose, pH 5.4) supplemented with uridine (40 to 80 μg/ml), arginine (20 to 50 μg/ml), and histidine (20 μg/ml) (38). RPMI 1640 (Gibco) medium or YPD plus 10% fetal bovine serum was used to induce hyphal formation in liquid culture. RPMI 1640 medium was buffered with 50 mM HEPES, pH 7.3. To impose stress upon *C. albicans*, cells were first grown at 30°C for 48 h in SD to saturation and were then diluted to an optical density at 600 nm of 0.2 in fresh YPD and incubated at 37°C for 4 to 5 h with shaking. H_2O_2 (final concentrations of 0.1 mM to 30 mM), *S*-nitrosoglutathione (0.2 mM to 2 mM), and NaCl (0.3 M to 1 M) were added to induce oxidative, nitrosative, and osmotic stress, respectively. Heat stress was performed by transferring mid-exponential cultures from 25°C to 37°C or 42°C.

Plasmids and construction of *C. albicans* strains. All oligonucleotides used in this study are listed in Table 2. The lithium acetate procedure used to transform *C. albicans* has been described previously (48).

The BamHI-PflMI fragment of pGEM-T::(*PGA59-GFP*, *ARG4*) carrying the GFP open reading frame (27) was replaced by a BamHI-PflMI fragment carrying the *G. princeps* luciferase gene (44) that was optimized for expression in *C. albicans* using the subset of preferred codons for this organism (4, 22, 34) and synthesized by GeneArt (Regensburg, Germany), yielding plasmid pGEM-T::(*PGA59-gLUC*, *ARG4*). Strains CEC749 and CEC751 were obtained through

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′→3′)[a]
Enj_LUC_HindIII_5′	GCAGAAGCTTTATTAAAATGATGCAATTCTCATCCGCTATTATCTTATCTGC
Enj_LUC_NheI_3′	CGCCGCTAGCGCGGGATCAGGAGAAACAAAGATGTATAAGG
TTR1_Xho1_5′	ATATCTCGAGAACGGTGAGAAAGATCGTGGATTTGGTC
TTR1_HindIII_3′	ATATAAGCTTCAAAAGAAAGAAAGAAAAGAAAAAAAAGGGGC
TRX1_Xho1_5′	ATATCTCGAGATGTACTTGAGGAGTAAAGTATAGAAAAACAG
TRX1_HindIII_3′	ATATAAGCTTCTTTGAAAAAAAATATAACTATGTGTTAAGTG
IPF9996_Xho1_5′	ATATCTCGAGAACACTTGGACCTACACTCAGATCCCAAAC
IPF9996_HindIII_3′	ATATAAGCTTCCTCTAGCTTGATTAAGAACTTGAAAAATTTGG
HWP1_XhoI_5′	CGGCTCGAGAACCAAAGAGTAAAAACTTG
HWP1_Hind III_3′	CCTGAAGCTTCATATTGACGAAACTAAAAGCG
EFT3_XhoI_5′	CGGCTCGAGTTATTACTAACATATAC
EFT3_Hind III_3′	CCTGAAGCTTCATTTTCCAAAATGTTG

[a] Underlining indicates HindIII, NheI, or XhoI restriction site.

transformation of *C. albicans* strain CEC175 using SnaBI-linearized pGEM-T::(*PGA59-gLUC*, *ARG4*) and pGEM-T::(*PGA59-GFP*, *ARG4*), respectively.

CIp10::*ACT1p-yEGFP* is a derivative of the integrative CIp10 plasmid (29) that carries the yeast enhanced GFP gene (*yEGFP*) (7) downstream of a 1,024-bp fragment carrying the 5′ noncoding region of the *C. albicans ACT1* gene (2). The 1,440-bp *PGA59-gLUC* fragment was amplified from pGEM-T::(*PGA59-gLUC*, *ARG4*) using oligonucleotides Enj_LUC_HindIII_5′ and Enj_LUC_NheI_3′ (Table 2) and subcloned at the HindIII and NheI sites in CIp10::*ACT1p-yEGFP* in place of *yEGFP* to yield CIp10::*ACT1p-gLUC59* (Fig. 1). The promoter regions for *TRR1* (970 bp of the 5′ region upstream of the start codon), *TRX1* (975 bp), *IPF9996* (978 bp), *HWP1* (2,023 bp), and *EFT3* (1,556 bp) were amplified from *C. albicans* genomic DNA using oligonucleotides listed in Table 2 and subcloned at the XhoI and HindIII sites in place of the *ACT1* promoter to yield CIp10::*TRR1p-gLUC59*, CIp10::*TRX1p-gLUC59*, CIp10::*IPF9996p-gLUC59*, CIp10::*HWP1p-gLUC59*, CIp10::*TEC1p-gLUC59*, and CIp10::*EFT3p-gLUC59*, respectively. These CIp10 derivatives were linearized with StuI, integrated at the *C. albicans RPS1* locus, and proper integration was confirmed by PCR and/or Southern analysis (data not shown).

Luciferase assays. *C. albicans* cells grown in liquid culture were collected by centrifugation and washed twice in luciferase assay buffer (LA buffer) (0.5 M NaCl, 0.1 M K_2HPO_4 [pH 6.7], 1 mM EDTA, 0.6 mM NaN_3, 1 mM phenylmethylsulfonyl fluoride cocktail [Boehringer]). Coelenterazine (Molecular Probes, Eugene, OR) in LA buffer was added to a final concentration of 1.25 μM, and luciferase activity was recorded using a luminometer.

To assay *C. albicans* cells in microtiter plate format, a dilution series was prepared for each culture, and 100 μl of each dilution was deposited in black transparent-well microtiter plates and mixed with 100 μl of 2 μM coelenterazine in LA buffer. The microtiter plate was immediately placed on top of X-ray film for 2 min. Signal intensities obtained after autoradiography were quantified with AIDA 2.0 software (Raytest, Straubenhardt, Germany) to estimate the luciferase activity.

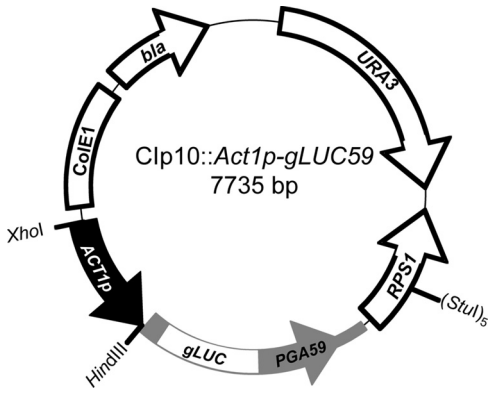

FIG. 1. Schematic representation of the Cip10::*ACT1p-gLUC59* vector. The CIp10::*ACT1p-gLUC59* vector is a derivative of the *C. albicans* CIp10 integrative vector (29), which harbors the *C. albicans URA3* transformation marker and which, upon cleavage by StuI, integrates at the *C. albicans RPS1* locus. A XhoI/HindIII fragment encompassing the *ACT1* promoter (*ACT1p*) was cloned upstream of the *gLUC59* luciferase reporter gene. *gLUC59* is a fusion between the *C. albicans PGA59* gene and the *Gaussia princeps* luciferase gene (*gLUC*). Propagation of CIp10::*ACT1p-gLUC59* is achieved in *Escherichia coli* in the presence of ampicillin (*bla*).

C. albicans colonies growing on YPD plates were replicated onto 3 M filter papers. One milliliter of LA buffer containing 1.25 μM coelenterazine was then deposited on the filter paper. The filter paper was wrapped in saran film and exposed to X-ray film (Kodak) for 5 min.

Antifungal susceptibility testing. Susceptibility testing was performed by the M27-A microdilution method of the National Committee for Clinical Laboratory Standards in 0.165 M MOPS (morpholinepropanesulfonic acid)-buffered RPMI 1640 (pH 7). After 18 h of incubation of growth of a gLUC59-expressing *Candida albicans* strain (CEC987 [Table 1]) in the presence or absence of econazole (1 μg/ml) or amphotericin B (1 μg/ml), 10 μg/ml of coelenterazine substrate was added and samples were read using a luminometer (Tecan).

Antimicrobial activity of neutrophils. Heparinized venous blood from healthy donors was diluted with RPMI 1640, and white blood cells were separated by Ficoll-Hypaque density gradient centrifugation. The pellet containing neutrophils (PMNs) and erythrocytes was treated with hypotonic saline to lyse the erythrocytes. PMNs were collected by centrifugation, washed, counted, and resuspended in RPMI 1640 plus 10% fetal calf serum. Killing activity was evaluated by CFU inhibition assay and by luciferase activity of gLUC59 *Candida albicans*. A total of 10^5 PMNs were incubated in 96-well microtiter tissue culture plates with 10^4 yeast cells for 2 h. After incubation at 37°C, plates were vigorously shaken, cells were lysed by addition of 0.01% Triton X-100, 10 μg/ml of coelenterazine was added to each sample, and plates were read using a luminometer. After the reading, samples were diluted in distilled water, and cell lysates were plated for CFU on Sabouraud dextrose agar.

Animal models of infection and imaging. Seven-week-old CD1 female mice (Harlan, Milan, Italy) were housed in groups of four mice per cage. All *C. albicans* strains were grown on YPD agar and subcultured in YPD medium at room temperature for 16 h. Cells were harvested, washed twice in sterile endotoxin-free physiological saline, counted with a hemocytometer, and adjusted to the final concentration (see below) in sterile physiological saline.

The mouse model of cutaneous infection has been described previously (13). Mice were immunosuppressed with cyclophosphamide 3 days prior to infection (150 mg · kg^{-1}), the day of the infection (150 mg · kg^{-1}), and 3 days postinfection (50 mg · kg^{-1}). On the day of the infection, mice were anesthesized with 80 μl of pentobarbital (50 mg · kg^{-1}) and shaved over an area of 4 cm^2 until all fur was removed, and the skin was abraded with sandpaper until it was glistening. Twenty microliters of a 5×10^8 · ml^{-1} *C. albicans* cell suspension was deposited on the abraded area. In selected experiments, econazole or amphotericin B (Sigma) was administered intraperitoneally once daily postinfection at a dose of 10 mg · kg^{-1} of body weight. Infection was monitored at 1, 2, 3, 4, and 7 days postinfection by depositing 20 μl coelenterazine (500 μg · ml^{-1} in 1:9 methanol-phosphate-buffered saline [PBS]; Synchem OHG, Felsberg/Altemburg) on the abraded skin area and imaging the animals dorsal side up in an IVIS-200 imaging system (Xenogen Corporation, Alameda, CA) under anesthesia with 2.5% isoflurane.

For subcutaneous infection, mice were inoculated subcutaneously in the right thigh region with 100 μl of a 10^8 · ml^{-1} *C. albicans* cell suspension. After challenge and every day postinfection, mice were injected subcutaneously with

100 μl coelenterazine (500 μg · ml^{-1} in 1:9 methanol-PBS) and imaged as described above. In selected experiments, granuloma was removed histologically and then analyzed or homogenized, the fungal burden was determined in YPD agar plus chloramphenicol, and then CFU were evaluated.

The mouse model of vaginal infection has been described previously (8). Five days prior to infection a pseudo-estrus condition was induced in mice by subcutaneous injection of 0.2 mg estradiol valerate in 100 μl sesame oil (Sigma). This was repeated weekly until the completion of the study. Mice anesthetized with 2.5 to 3.0% (vol/vol) isoflurane gas were infected twice at a 24-h interval with 10 μl of a 10^9 · ml^{-1} *C. albicans* cell suspension administered from a mechanical pipette into the vaginal lumen, close to the cervix. To favor vaginal contact and adsorption of fungal cells, mice were held head down for 1 min following inoculation. Mice were then allowed to recover for 24 to 48 h, during which the *Candida* infection was established. Every day postinfection, 10 μl (1 mg · ml^{-1} in 1:4 methanol-PBS) of coelenterazine was added to the vaginal lumen. Afterwards, mice were imaged as described above. In selected experiments, mice were anesthetized with 2.5% isoflurane and then held head down, the vaginal lumen was thoroughly washed with 50 μl of saline, and the lavage fluid was monitored with the IVIS-200 system. To determine the fungal load in the vagina, the lavage solution from each mouse was serially diluted and plated on YPD agar plus chloramphenicol, and then CFU were evaluated.

The mouse model of systemic candidiasis has been previously described (33). Mice were inoculated by injection of a 200-μl yeast suspension into the lateral tail vein (final amount, 2.5×10^5 CFU per mouse) through a 27-gauge needle. At regular time intervals, mice were injected intraperitoneally with 200 μl coelenterazine (500 μg · ml^{-1} in 1:9 methanol-PBS) and imaged dorsal side up in the IVIS-200 imaging system. After the final imaging point, mice were injected with 200 μl coelenterazine (500 μg/ml in 1:9 methanol-PBS), humanely sacrificed, and autopsied in order to image organs using the IVIS-200 imaging system.

Total photon emission from selected and defined areas within the images of each mouse was quantified with the Living Image software package.

Nucleotide sequence accession number. The nucleotide sequence of the *PGA59-gLUC* gene fusion has been deposited at the NCBI under accession number FJ790493.

RESULTS

Intracellular *Renilla reniliformis* luciferase is not accessible to its substrate coelenterazine. The *rLUC* gene has been used extensively as a reporter of gene expression in *C. albicans* (1, 17, 21, 41). However, experiments using *rLUC* have been carried out using whole-cell extracts for in vitro quantification of luciferase activity. Because in vivo imaging of luciferase activity requires that the substrate must be accessible to the luciferase, we tested whether light could be detected from intact *C. albicans* cells expressing intracellular rLUC. As shown in Fig. 2A, intact *C. albicans* cells of strain SM22 expressing the *rLUC* gene under the control of the strong *C. albicans ACT1* promoter displayed a luciferase activity of 0.025 relative light units (RLU)/cell, while whole-cell extracts from the same culture displayed a 50-fold-higher luciferase activity. These data suggested that coelenterazine could not penetrate *C. albicans* cells efficiently and that luciferases that use coelenterazine as a substrate could not be exploited for in vivo imaging if localized intracellularly.

Development of a novel reporter gene that drives cell surface localization of the *Gaussia princeps* luciferase in *C. albicans*. The data presented above and those obtained by Doyle et al. (9) suggested that neither luciferin nor coelenterazine could penetrate *C. albicans* cells efficiently. Therefore, we reasoned that the successful development of a luciferase reporter suitable for in vivo imaging would depend upon the exposure of this luciferase at the *C. albicans* cell surface. For this purpose, a codon-optimized version of the *gLUC* gene was cloned in frame within the *C. albicans PGA59* gene between the codons for the second and third amino acids of the mature Pga59 protein, thereby placing the synthetic *gLUC* coding region downstream of the Pga59 signal sequence (Fig. 1). The *gLUC* luciferase gene was selected over other possible luciferase genes because it encodes a naturally secreted luciferase that is small (185 amino acids) and that does not require ATP for activity (44). During codon optimization we selected those codons that are preferentially used in *C. albicans*, and we avoided the use of CTG codons, which are translated as serine instead of leucine in *C. albicans* (4, 22, 34). *C. albicans PGA59* was selected to target gLUC to the *C. albicans* surface because it encodes a short (113-amino-acid) GPI-linked cell wall protein that has been used previously to efficiently target GFP to the *C. albicans* cell wall (27). When the resulting *PGA59-gLUC* construct was integrated at the *PGA59* locus of *C. albicans* strain BWP17U, high luciferase activities were detected from intact exponentially growing cells (3.11 RLU/cell) (Fig. 2B), and similar luciferase levels were detected from whole-cell extracts (2.60 RLU/cell) (Fig. 2B). Luciferase was also detected in the culture supernatant, although at much lower levels (data not shown). In contrast, control *C. albicans* cells containing a *PGA59-GFP* fusion integrated at the *PGA59* locus did not show any significant luciferase activity from intact (0.54×10^{-3} RLU/cell) or lysed (0.58×10^{-3} RLU/cell) cells (Fig. 2B).

The construction reported above was obtained by fusing the *gLUC* gene at the junction between the second and third codons of the *PGA59* gene. This resulted in a fusion protein where the signal peptides of Pga59 and gLUC are in tandem upstream of the gLUC and Pga59 domains. Additional *PGA59-gLUC* gene fusions were constructed where either the gLUC signal peptide or the main Pga59 domain was omitted. The omission of the gLUC signal peptide resulted in *C. albicans* strains with no detectable luciferase activity, while omission of the main Pga59 domain resulted in *C. albicans* strains with much lower luciferase activity than with the original *PGA59-gLUC* fusion (data not shown). The expression of the codon-optimized *gLUC* gene alone, without appended *PGA59* sequences, resulted in the secretion of luciferase by the *C. albicans* cells into the culture supernatant in a manner that was dependent upon the gLUC signal peptide (data not shown). As our aim was to develop a reporter gene where the luciferase remains associated with cells and does not diffuse through the animal body following release from *C. albicans* cells, we focused our further studies on the original *PGA59-gLUC* gene fusion that included the signal peptides of both Pga59 and gLUC and that is referred below as *gLUC59* (Fig. 1).

This *gLUC59* reporter gene was subcloned into CIp10, a *C. albicans* integrative plasmid (29) downstream of the *C. albicans ACT1* promoter to generate an *ACT1p-gLUC59* gene fusion (Fig. 1). When the resulting plasmid was integrated at the *RPS1* locus in *C. albicans* strain CAI4, a strong luciferase activity (757 RLU/cell) (Fig. 2A) was detected from intact exponentially growing (data not shown) or stationary-phase cells, and high luciferase activity levels were detected from whole-cell extracts (323 RLU/cell) (Fig. 2A). The luciferase activity detected from intact cells was at least 10^4-fold higher than that detected from *C. albicans* strains expressing rLUC under the control of the *ACT1* promoter, and whole-cell extracts of the gLUC59-expressing strain had about 300 times more luciferase activity than those of the corresponding rLUC-

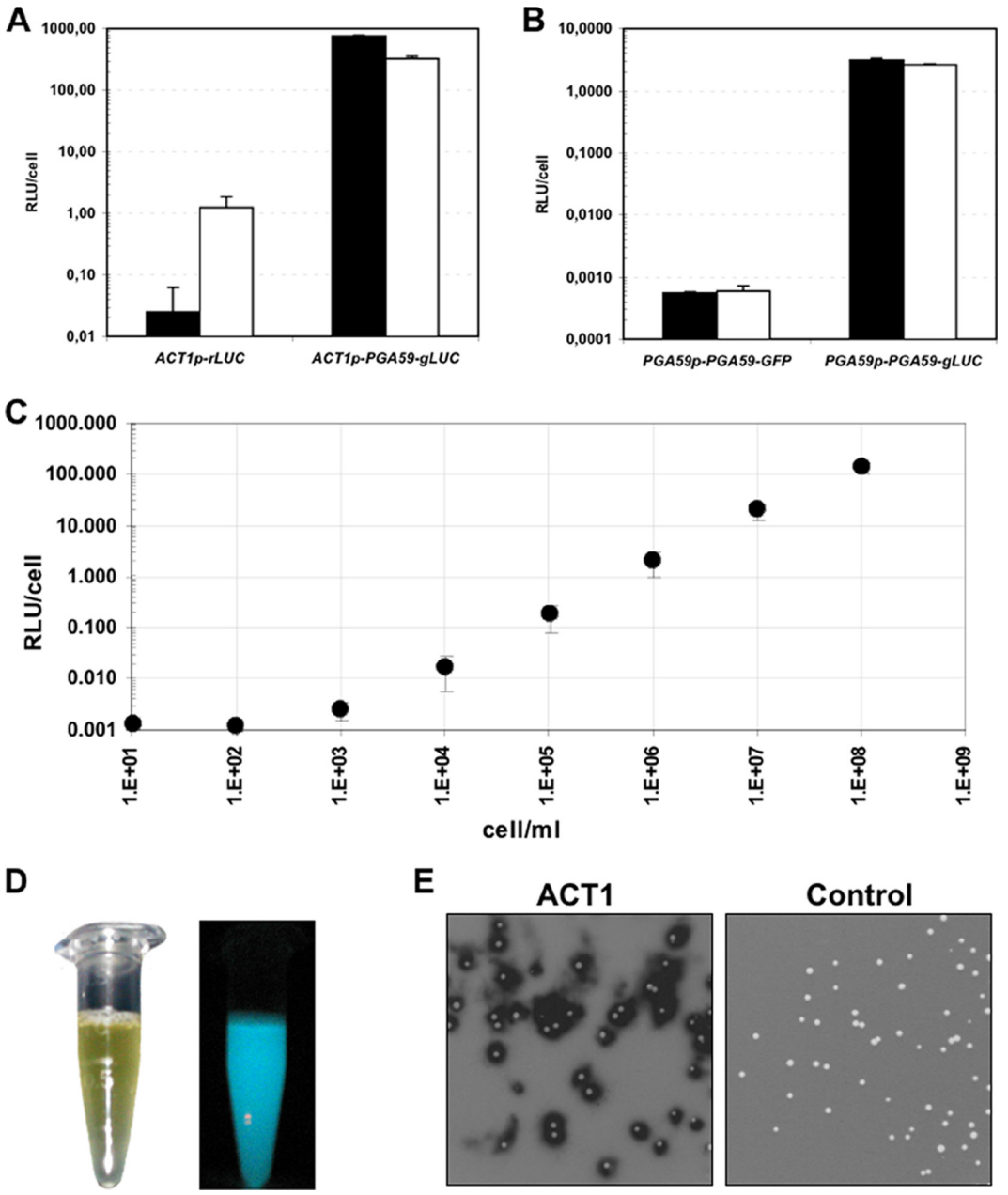

FIG. 2. Cell surface *G. princeps* luciferase (gLUC59) is a highly sensitive reporter of gene expression in *C. albicans*. (A) *C. albicans* cells expressing the *R. reniliformis* luciferase gene (rLUC) or a fusion between the *C. albicans PGA59* gene and the *G. princeps* luciferase gene (*PGA59-gLUC*) expressed under the control of the *C. albicans ACT1* promoter (*ACT1p*) were grown in YPD medium at 30°C. Luciferase activity was measured using intact cells (black bar) or whole-cell extracts (white bar) and coelenterazine as substrate. Error bars indicate standard deviations. (B) *C. albicans* cells expressing fusions between the *C. albicans PGA59* gene and the *A. victoria* GFP gene (*PGA59-GFP*) or the *G. princeps* luciferase gene (*PGA59-gLUC*) expressed under the control of the *C. albicans PGA59* promoter (*PGA59p*) were grown in YPD medium at 30°C. Luciferase activity was measured using intact cells (black bar) or whole-cell extracts (white bar) and coelenterazine as substrate. (C) Increasing numbers of *C. albicans ACT1p-gLUC59* cells grown in YPD medium at 30°C were incubated in LA buffer. Luciferase activity was measured using intact cells with coelenterazine as substrate. (D) Coelenterazine was added to a culture in YPD (at 30°C) of a *C. albicans* strain expressing the *gLUC59* reporter under the control of the *ACT1* promoter, and pictures were taken using a standard camera in the light or darkness. (E) *C. albicans ACT1p-gLUC59* (ACT1) or control cells were grown on agar plates and replicated on filter papers. The papers were soaked in a solution containing coelenterazine and autoradiographed. Images show a superimposition of these autoradiographs upon pictures of the corresponding plates.

expressing strain (Fig. 2A). Indeed, light could be detected using a luminometer from as few as a thousand gLUC59-expressing cells (Fig. 2C). Strikingly, light could even be detected by the naked eye when a culture of the gLUC59 expressing strain was incubated with coelenterazine in darkness (Fig. 2D). Moreover, colonies of the gLUC59-expressing strain could be detected by autoradiography when overlaid with coelenterazine (Fig. 2E).

We conclude that *C. albicans* Pga59 can be used to efficiently target gLUC to the *C. albicans* cell surface and that the Pga59-gLUC protein is accessible to its substrate.

gLUC59 is a convenient reporter for gene expression studies in vitro. The use of firefly luciferase as a reporter for in vivo imaging is in part limited by the poor diffusion of luciferin through the cell wall and membrane of *C. albicans* hyphae (8, 9). Therefore, we tested whether gLUC59 activity could be detected on intact *C. albicans* hyphae as well as yeast cells. *C. albicans* strain CA1398 expressing *gLUC59* under the control of the *ACT1* promoter was grown for 3 hours in YPD at 30°C (yeast phase) and in YPD at 37°C in the absence or presence of 10% fetal bovine serum (hyphal phase), and gLUC59 activity was measured. As shown in Fig. 3A, gLUC59 activity was detected on intact yeast and hyphal cells. Therefore, *gLUC59* can serve as a reporter for gene expression irrespective of the morphological state of *C. albicans*.

In order to test whether *gLUC59* could be used as a reporter of gene expression under a variety of conditions, a set of *gLUC59* promoter fusions were constructed and integrated at the *C. albicans RPS1* locus. These fusions carried the promoters from the *TRX1*, *TRR1*, *IPF9996*, *HWP1*, and *EFT3* genes upstream of *gLUC59* (Table 1). *TRX1*, *TRR1*, and *IPF9996* encode a thioredoxin, a thioredoxin reductase, and a protein of unknown function, respectively, and their expression has been shown to be upregulated in response to oxidative stress (10). *HWP1* encodes a GPI-linked cell wall protein that is specifically expressed in hyphal cells (42). *EFT3* encodes translation elongation factor 3 and is upregulated in some infection models (23, 30, 50).

As shown in Fig. 3A, the expression of the *TRX1p-gLUC59* fusion increased as the concentration of hydrogen peroxide was raised to 5 mM. Above this concentration, gLUC59 levels decreased. In contrast, gLUC59 levels remained unchanged when the cells expressing this *TRX1p-gLUC59* fusion were exposed to nitrosative or heat stress or induced to undergo the yeast-to-hypha transition (Fig. 3A). Similar data were obtained using a *C. albicans* strain expressing the *TRR1p-gLUC59* fusion (data not shown). Moreover, no significant increase in cell surface luciferase activity was observed when a *C. albicans* strain carrying the *ACT1p-gLUC59* fusion was exposed to oxidative stress. Therefore, the luciferase levels detected in intact *C. albicans* cells expressing *TRX1p-gLUC59* or *TRR1p-gLUC59* fusions accurately reflected our published observation that the *C. albicans TRX1* and *TRR1* genes are induced specifically in response to oxidative stress (10). A similar regulatory profile was observed for the *IPF9996p-gLUC59* fusion, although this gene was expressed at lower levels than the *TRX1* fusion (Fig. 3A). Again this was consistent with our previous observation that *IPF9996* is expressed at relatively low levels. Indeed, in our hands the *IPF9996* promoter was not sufficiently active to drive GFP expression at detectable levels (B. Enjalbert and A. J. P. Brown, unpublished data). However, in this study *IPF9996p-gLUC59* expression levels were orders of magnitude above the limit of detection.

Morphogenesis was induced in cells carrying the *ACT1p-gLUC59*, *EFT3p-gLUC59*, and *HWP1p-gLUC59* fusions in RPMI medium at 37°C, and luciferase activities were recorded at different time intervals (Fig. 3B). While *EFT3p-gLUC59* expression levels remained relatively constant over the course of the experiment, the *HWP1p-gLUC59* fusion was strongly upregulated in response to hyphal induction, as expected (42). These data indicated that, unlike the *fLUC* reporter (8), *gLUC59* can be used to monitor the expression of hypha-induced genes under hypha-inducing conditions.

Taken together, these data showed that *gLUC59* is an exceptionally sensitive reporter that can be used to monitor the expression of diverse *C. albicans* promoters under a variety of experimental conditions without the need to prepare *C. albicans* cell lysates.

gLUC59 is a convenient reporter of cell viability. Prior to conducting animal experiments using a *C. albicans gLUC59*-expressing strain, we tested whether killing or growth inhibition of such cells by antifungals or PMNs resulted in changes in luciferase activity. Growth of *C. albicans* strain CA1398 carrying the *ACT1p-gLUC59* fusion in the presence of econazole (1 μg/ml) or amphotericin B (1 μg/ml) reduced luciferase activity by 82% or 99%, respectively (data not shown). Moreover, killing of *C. albicans* CA1398 cells by PMNs was associated with a decrease in luciferase activity that correlated strongly with cell counts (Fig. 4). Therefore, gLUC59 provided a reporter of viable *C. albicans* cells.

gLUC59 can be used to monitor the progression of superficial *C. albicans* infections in live animals. We then tested whether gLUC59 could be used to monitor the course of different types of *C. albicans* infection. Mice were infected with *C. albicans* strain CA1398 carrying the *ACT1p-gLUC59* fusion (gLUC59 strain below) or the control strain CA1399, which did not express *gLUC59.* Preliminary studies showed that these gLUC59 and control strains were equally pathogenic in the mouse model of systemic infection (data not shown).

Different experimental infections were used, such as subcutaneous, cutaneous, vaginal, and systemic infections. Subcutaneous inoculation was performed in the right thigh region through injection of 10^7 gLUC59 or control cells, and these infections were imaged at various time points over a 1-week period (see Materials and Methods). Figure 5A and B show representative examples of images and luminescence data obtained for an uninfected mouse, a mouse infected with gLUC59, and a mouse infected with the control strain. For the gLUC59 strain a progressive increase in luminescence was observed from day 1 to 5 postinfection, and this observation was reproducible ($n = 5$). This signal was higher than those obtained with mice infected with the control strain or with uninfected mice. The luminescence signals observed for mice infected with the gLUC59 strain decreased at days 6 and 7 postinfection but remained higher than those observed for the control mice. A typical granuloma started to develop at the site of inoculation at 1 day postinfection. Histological analyses revealed the presence of a cellular infiltrate composed predominantly of neutrophils, macrophages, some lymphocytes, and yeast and hyphal cells (data not shown). *C. albicans* cells were quantified in a CFU assay, showing that the fungal burdens increased between the first and fourth days of infection and subsequently decreased (Fig. 5C), consistent with the luciferase detection in live animals.

Cutaneous infections (13) were examined in immunosuppressed mice using 10^7 gLUC59 or control cells. After 1 day of infection, selected groups of mice were treated intraperitoneally with econazole or amphotericin B (once daily for 4 days),

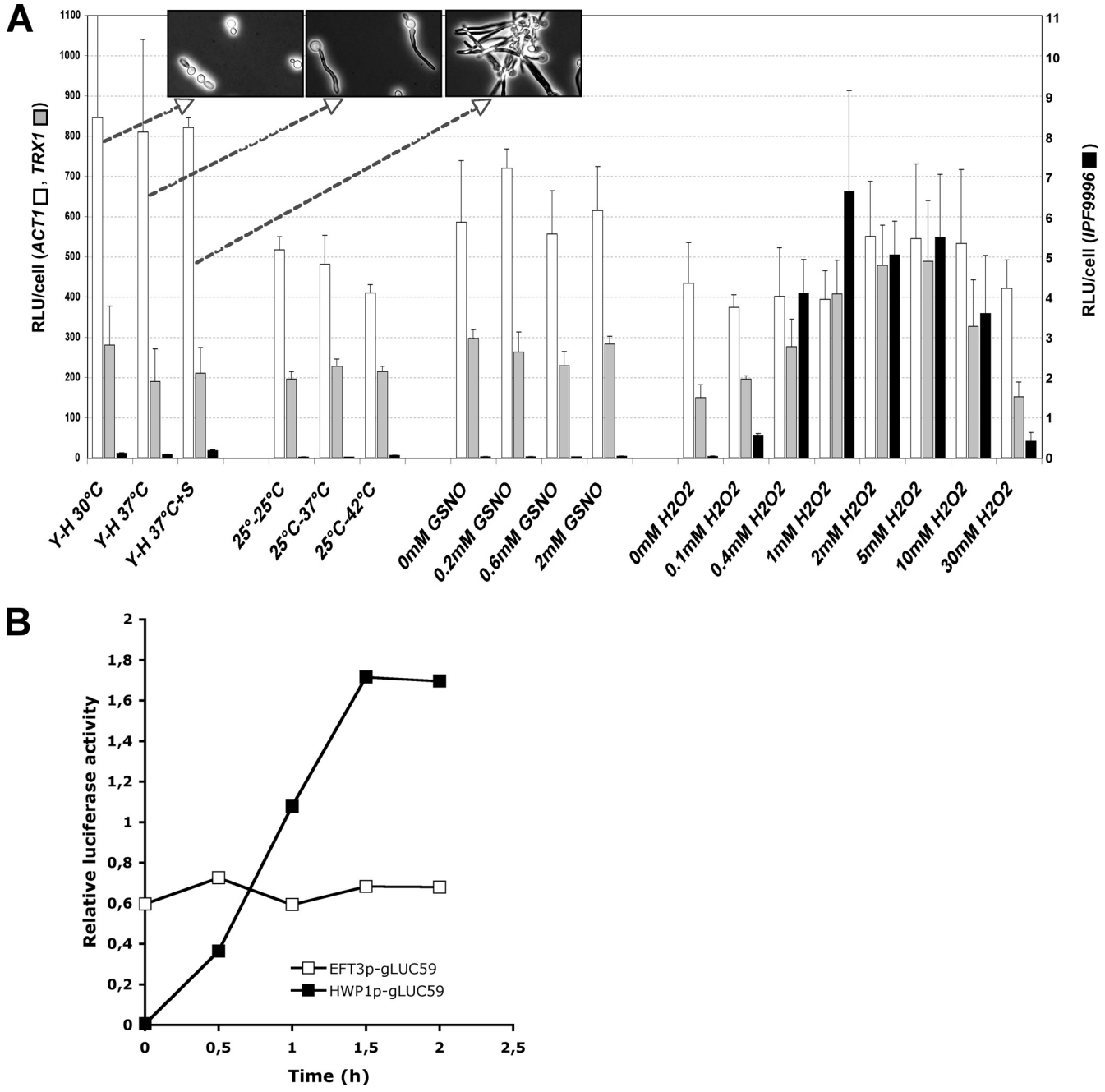

FIG. 3. *gLUC59* is a convenient reporter for gene expression studies in vitro. (A) *C. albicans* strains expressing *ACT1p-gLUC59*, *TRX1p-gLUC59*, or *IPF9996p-gLUC59* fusions were grown under a range of conditions and luciferase activities recorded using intact cells: yeast cells in YPD at 30°C (Y-H 30°C), hyphal cells in YPD at 37°C (Y-H 37°C), hyphal cells in YPD containing 10% serum at 37°C (Y-H 37°C+S), control and heat-shocked yeast cells in YPD (25°C-25°C, 25°C-37°C, 25°C-42°C), yeast cells subjected to nitrosative stress (*S*-nitrosoglutathione [GSNO], 0 to 2 mM), and yeast cells subjected to oxidative stress (H_2O_2, 0 to 30 mM). Insets show the morphology of *C. albicans* cells expressing the *ACT1p-gLUC59* reporter when grown under yeast- or hypha-inducing conditions. Note that different scales are used for the *IPF9996p-gLUC59* strain compared with the other strains. Error bars indicate standard deviations. (B) *C. albicans* strains expressing *ACT1p-gLUC59*, *EFT3p-gLUC59*, and *HWP1p-gLUC59* fusions were grown in RPMI medium, and luciferase activities were recorded using intact cells at different time points. Luciferase activities for cells expressing the *EFT3p-gLUC59* and *HWP1p-gLUC59* fusions were normalized to those for *ACT1p-gLUC59* cells under equivalent conditions to correct for time-dependent increases in culture density.

and the infections were imaged at various time points. Figure 6 shows a representative example of the images obtained for four mice infected with control or gLUC59 cells and treated with econazole or amphotericin B. Strong gLUC59 signals were maintained for over 1 week in the untreated mice infected with the gLUC59 strain. In contrast, these signals disappeared rapidly in mice treated with econazole or amphotericin B (Fig. 6). As expected, no significant signals were

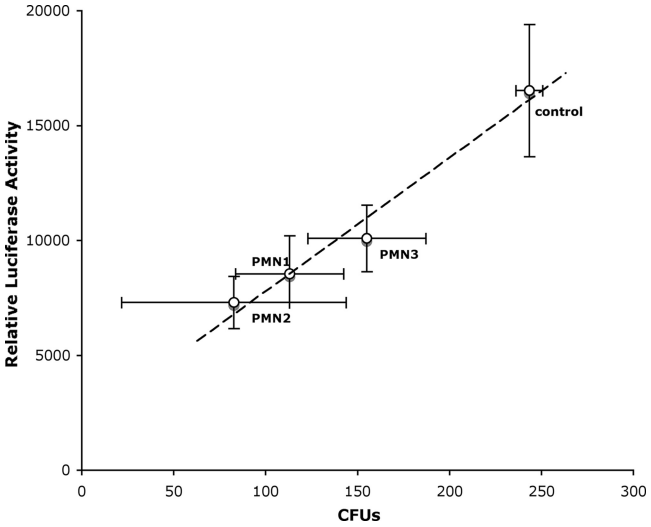

FIG. 4. gLUC59 is a convenient reporter of cell viability. *C. albicans ACT1p-gLUC59* (CA1398) cells were incubated in the presence of three independent preparations of PMNs for 2 h at 37°C. Luciferase activity and CFU were subsequently recorded and were highly correlated ($r = 0.975$). Error bars indicate standard deviations.

observed in mice infected with the control strain. These observations confirm that the gLUC59 reporter can be used to examine the impact of antifungal drugs upon these infections.

Next we examined the utility of the gLUC59 reporter for vaginal infections (8). Significant luminescence signals were evident at 4 to 6 days postinfection for mice infected with the gLUC59 strain, and these signals declined by day 8 postinfection (Fig. 7A). In contrast, only weak luminescence was observed for mice infected by the control strain (Fig. 7A). In selected experiments a vaginal wash was performed on the infected mice, and luminescence in the recovered fluid was assayed. Strong luminescence was observed in vaginal fluids obtained from gLUC59-infected mice, whereas no significant signal was seen in mice infected with the control strain (Fig. 7B and C). Fungal burdens in these vaginal fluids were also determined and confirmed that the luminescence signal correlated strongly with the presence of *C. albicans* cells (Fig. 7C).

Taken together, these data showed that the gLUC59 reporter can be used to image cutaneous, subcutaneous, and vaginal *C. albicans* infections and the impact of antifungal treatments.

Applicability of gLUC59 for monitoring *C. albicans* systemic infections. To test whether gLUC59 could be used to monitor the spread of *C. albicans* during the progression of systemic infections (33), mice were injected via the tail vein with *C.*

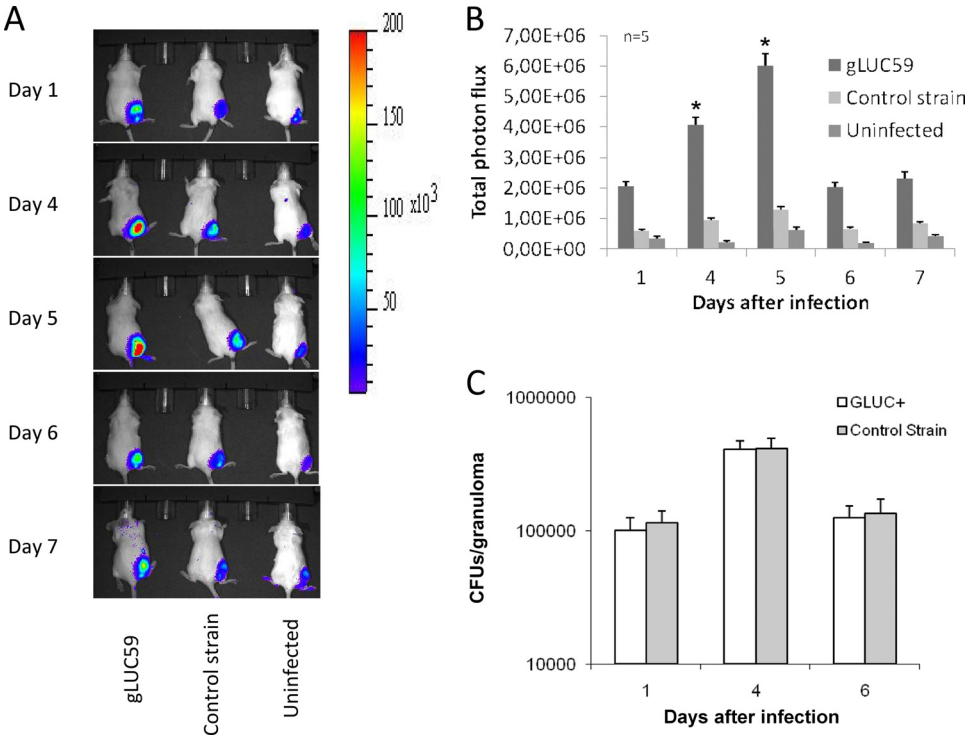

FIG. 5. In vivo imaging of mice injected subcutaneously with *Candida albicans ACT1p-gLUC59*. Mice were inoculated subcutaneously in the right thigh region with 10^7 *ACT1p-gLUC59* (CA1398) or control (CA1399) cells. On days 1, 4, 5, 6, and 7 postinfection, mice were injected at the same site with 50 μg of coelenterazine and imaged in the IVIS-200 imaging system under anesthesia with 2.5% isoflurane. (A) Representative images of untreated mice and mice infected with *ACT1p-gLUC59* or control strains. (B) Histogram of the means and standard deviations of total photon emissions from the infected regions obtained for five mice. Data that differ significantly from those obtained at day 1 are shown (Student *t* test, $P < 0.05$). (C) Mean (+ standard deviation) CFU recovered from granulomas at 1, 4, and 6 days postinfection. Data are expressed as CFU/granuloma. Data at day 4 differ significantly from those obtained at day 1 (Student *t* test, $P < 0.05$).

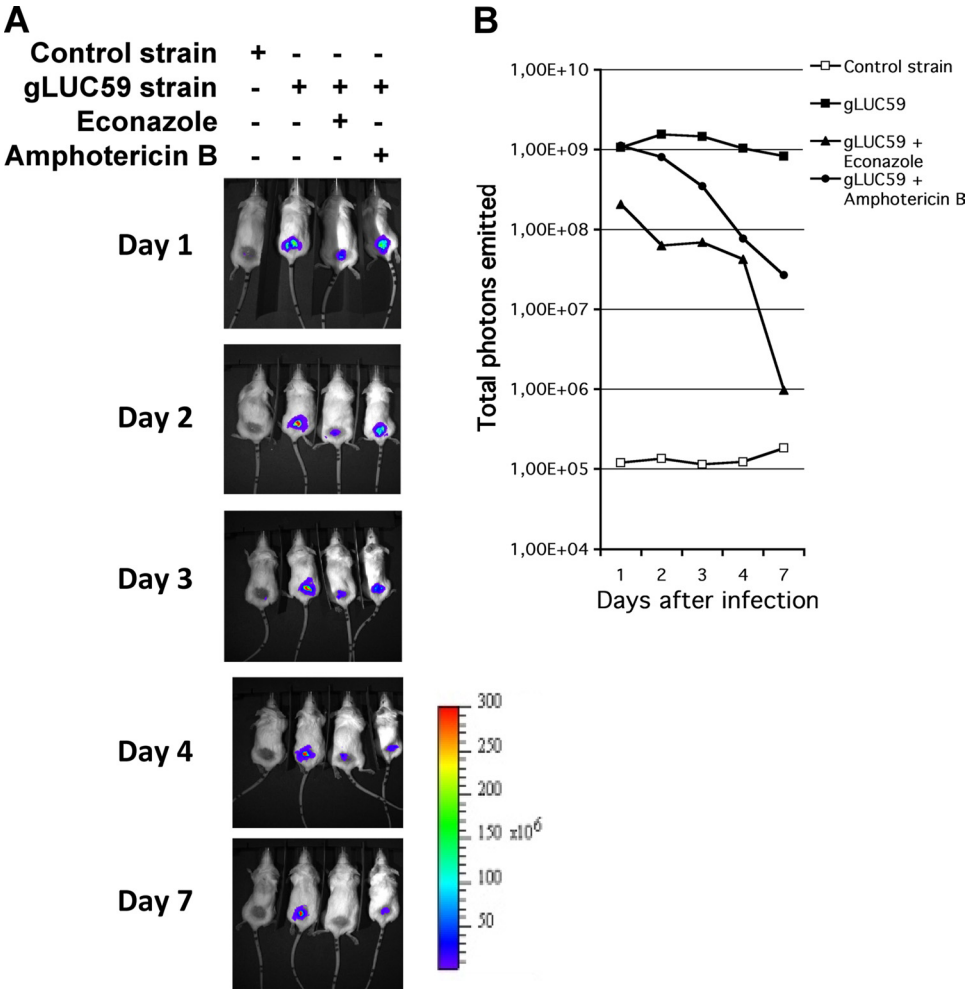

FIG. 6. In vivo imaging of mice infected cutaneously with *Candida albicans ACT1p-gLUC59*. Immunosuppressed mice were anesthesized and shaved over an area of 4 cm^2 until all fur was removed, and the skin was abraded with sandpaper until it glistened. A total of 10^7 *ACT1p-gLUC59* (CA1398) or control (CA1399) cells in 20 μl were deposited on the abraded area. Econazole and amphotericin B were administered intraperitoneally at a dose of 10 mg/kg of body weight once daily postinfection. Infection was monitored on days 1, 2, 3, 4, and 7 postinfection by depositing 10 μg coelenterazine on the abraded skin area and imaging the animals dorsal side up in the IVIS-200 imaging system under anesthesia with 2.5% isoflurane. (A) Representative images of mice infected with *ACT1p-gLUC59* or control strains and treated with econazole or amphotericin B. (B) Histogram of total photon emission from the infected regions observed in panel A.

albicans cells carrying the *ACT1p-gLUC59* or control fusion and imaged every day. As expected, luminescence was detected in the kidney area for mice infected with the gLUC59-expressing strain. However, similar levels of luminescence were also seen for the control strain and for mice that had not been infected by *C. albicans* (data not shown). This suggested that coelenterazine was unstable under these conditions, allowing emission of light independently of any gLUC59 luciferase activity. Moreover, when mice were injected with coelenterazine and subsequently autopsied, light was not detected from the kidney. Luminescence was detected only when the kidneys of mice infected with the *ACT1p-gLUC59* strain were homogenized and incubated with coelenterazine (data not shown). As expected, no significant luminescence was observed from the homogenates from control kidneys. Therefore gLUC59 did not appear to be a convenient reporter for imaging systemic *C. albicans* infection.

DISCUSSION

In this study, we have developed a novel reporter gene for *C. albicans*, i.e., *PGA59-gLUC* (also referred to as *gLUC59*). This reporter takes advantage of the *C. albicans PGA59* gene, which encodes a GPI-linked cell wall protein (27), and the naturally secreted *G. princeps* luciferase (44). This *gLUC59* construction was designed to localize the Pga59-gLUC protein to the *C. albicans* cell surface. Characterization of *C. albicans* strains expressing *ACT1p-gLUC59*, *TRR1p-gLUC59*, *TRX1p-gLUC59*, *IPF9996p-gLUC59*, *HWP1p-gLUC59*, and *EFT3p-gLUC59* fusions under a variety of experimental conditions, including morphogenetic and stress conditions, demonstrated the reliability, versatility, and sensitivity of this novel reporter.

gLUC59 has significant advantages over the other luciferase reporters that have been developed for gene expression studies of *C. albicans*. gLUC59 is 300 times more sensitive than the

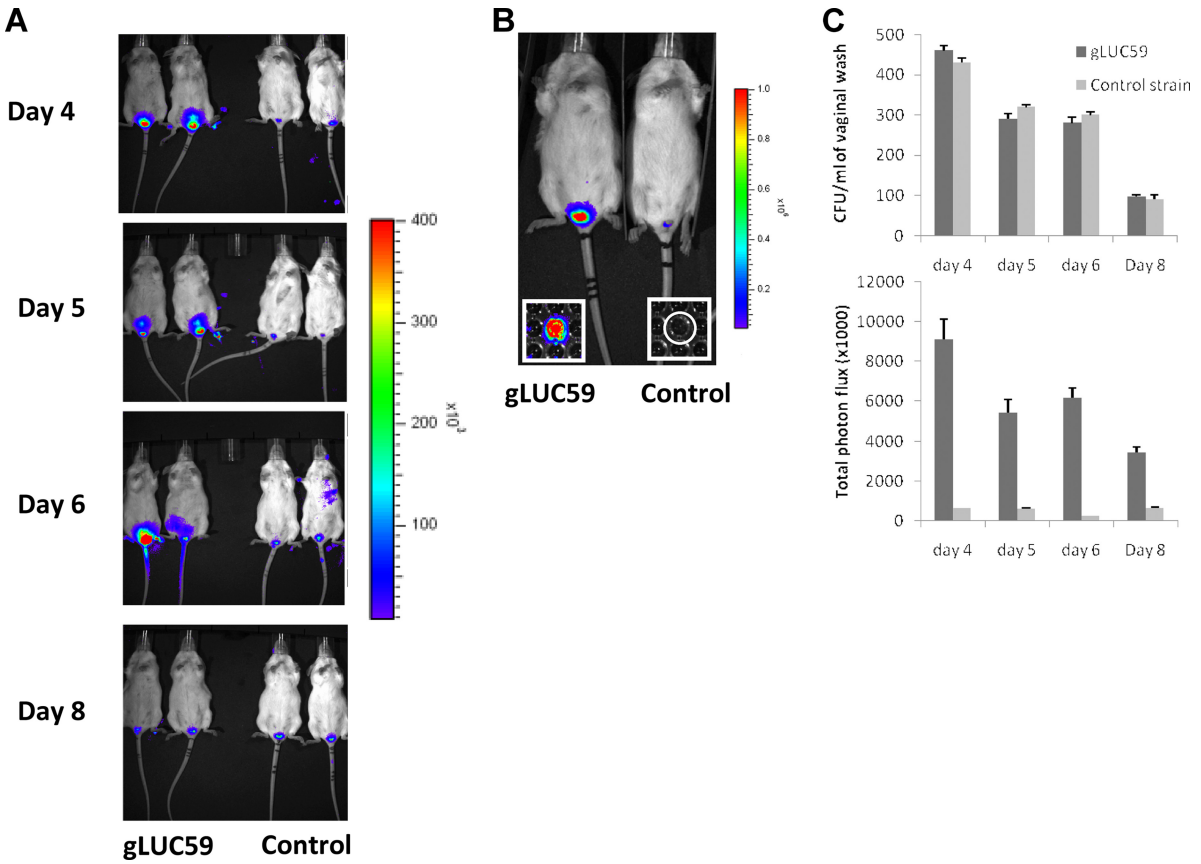

FIG. 7. In vivo imaging of mice vaginally infected with *Candida albicans ACT1p-gLUC59*. (A) Mice under pseudo-estrus conditions were infected for two consecutive days with 10 μl of a 10^9-cell/ml suspension of *ACT1p-gLUC59* (CA1398) or control (CA1399) cells into the vaginal lumen. Starting on the first day postinfection and every day thereafter, mice were treated intravaginally with 10 μg of coelenterazine and imaged in the IVIS-200 imaging system under anesthesia with 2.5% isoflurane. (B) Representative example of the luciferase activity associated with the vaginal lavage of mice infected for 5 days and whose vaginal lumens were thoroughly washed with 50 μl of PBS using a mechanical pipette. The lavage fluid was monitored with the IVIS-200 imaging system (insets). (C) Quantification of CFU (upper panel, mean + standard deviation) and luciferase activity (lower panel, mean + standard deviation) in vaginal lavage fluid of two mice infected for 4, 5, 6, and 8 days.

Renilla reniliformis luciferase (41) (Fig. 2). Cell lysates must be prepared to assay expression of the *Renilla reniliformis* luciferase. In contrast, the cell surface exposure of gLUC59, and hence its accessibility to its substrate coelenterazine, permits direct measurement of expression levels through luciferase assays on intact *C. albicans* cells (Fig. 2). Furthermore, gLUC59 expression could be detected under all conditions tested, including hyphal development (Fig. 3), a condition where the firefly luciferase did not prove to be a convenient reporter (8). In principle, given the sensitivity and ease of use of gLUC59, this reporter could be exploited in a wide variety of future applications. For example, gLUC59 could be used for screening *C. albicans* mutants with altered gene expression or for the detailed dissection of *C. albicans* promoter regions. In this regard, we have shown that gLUC59 luciferase activity can be effectively monitored on agar plates and in microtiter plate assays (Fig. 2), thereby facilitating the implementation of high-throughput screens based on differential gLUC59 expression levels in *C. albicans*.

The primary aim of this study was to develop a reporter gene for the imaging of *C. albicans* infections in vivo. We envisaged that this reporter could be used to examine the spread of infections, the efficacy of antifungal treatments, or the expression of infection-associated genes during disease progression. Our results showed that a *C. albicans* strain expressing *gLUC59* under the control of the constitutive *ACT1* promoter could be used to image subcutaneous and cutaneous infections (Fig. 5 and 6). This represents the first experimental examination of such infections using imaging approaches. Cutaneous and subcutaneous *C. albicans* infections are common dermatological conditions that are exacerbated by several factors, including infancy, pregnancy, old age, disorders of immunity, immunosuppressive therapy, antibiotics, endocrine diseases, and carcinoma (18). In certain subpopulations the prevalence of cutaneous candidiasis has increased in recent years because the number of immunocompromised patients has increased (26). Our gLUC59 models of cutaneous and subcutaneous *Candida* infection allow real-time monitoring of yeast colonization. Importantly, the bioluminescence measurements correlated with yeast counts, suggesting that the gLUC59 system provides an excellent tool for monitoring the course of such infections as well as the efficacy of vaccine antigens or pharmacological treatments. This is particularly relevant given that local infections can be difficult to treat and may persist for months, despite treatment.

We also showed that the *C. albicans ACT1p-gLUC59* strain is an effective tool for the imaging of vaginal infections (Fig. 6). Vulvovaginal candidiasis affects 75% of women at least once in their reproductive years and is equally common in immunocompetent and immunocompromised women (39, 40). It can present as a chronic infection or have regular relapses. Recurrent or chronic vaginal candidiasis is a distressing problem for women and an expensive issue for public health. The management of these patients with recurrent vaginitis is a major challenge, and several studies are directed toward the development of vaccination strategies and new antifungal drugs (25). The ability to monitor yeast colonization in real time with a sensitive reporter will significantly enhance the repertoire of tools available for the analysis of vulvovaginal infections. This infection model has been studied using a luciferase-based imaging system (8). However, that approach had limitations because it was not able to reveal hyphal cells, thus underestimating the extent of infection (8). In contrast, using gLUC59 we were able to monitor both morphological forms of *C. albicans* in vulvovaginal infections, thereby providing a more accurate model of mucosal infection.

Despite repeated attempts, we were unable to image systemic infections using *C. albicans ACT1p-gLUC59* strains in live animals. We obtained promising images of luminescence emission from the kidney regions of live mice systemically infected with these strains. However, similar images were obtained for control mice infected with *C. albicans* strains lacking *gLUC59*, probably through the autoluminescence of coelenterazine. In contrast, when autopsied animals were imaged, only those infected with *C. albicans ACT1p-gLUC59* strains showed a significant luminescent signal in the kidney, the main target organ of *C. albicans* upon systemic infection. Therefore, current approaches for the live imaging of systemic *C. albicans* infections with gLUC59 appear to be limited by the autoluminescence of coelenterazine as well as by the uneven distribution of substrate throughout the mouse and the deep-seated location of the kidneys, which precludes efficient visualization of photons from this organ through the mouse body. The use of stable coelenterazine derivatives such as ViviRen did not overcome the problem of coelenterazine autoluminescence (not shown), possibly because these derivatives require processing by intracellular esterases in order to be used by luciferases. In this regard, firefly luciferase uses luciferin, which is intrinsically more stable than coelenterazine. Therefore, the targeting of firefly luciferase to the *C. albicans* cell surface using Pga59 signals might represent a suitable alternative to gLUC59. Doyle et al. (8) have shown that intracellular firefly luciferase in *C. albicans* does not permit in vivo imaging of systemic *C. albicans* infections, possibly because of the limited diffusion of luciferin across the cell walls/membranes of infecting hyphae. Thus, the targeting of firefly luciferase to the *C. albicans* cell surface might also provide a means of circumventing this limitation. However, firefly luciferase uses ATP for the conversion of luciferin into light (37), and therefore the efficient monitoring of firefly luciferase activity in vivo might be limited by the availability of ATP in extracellular fluids. Thus, future experiments should be aimed at improving the mode of administration of coelenterazine and its stability in vivo in order to take advantage of the exciting properties of gLUC59 for the real-time monitoring of *C. albicans* infections in live animals. Meanwhile gLUC59 does provide a sensitive tool for the analysis of systemic infections in autopsied animals.

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