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CaNAT1, a Heterologous Dominant Selectable Marker for Transformation of *Candida albicans* and Other Pathogenic *Candida* Species

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A dominant selectable marker for *Candida albicans* and other *Candida* species, which confers resistance to nourseothricin, was characterized. In a heterologous promoter system and a recyclable cassette, the marker efficiently permitted deletion and complementation of *C. albicans* genes. Neither growth nor filamentous development was affected in strains expressing this marker.

Candida albicans is the most commonly isolated invasive fungal pathogen. Molecular genetic analysis of this fungus to date is principally based on the marker *URA3*, a biosynthetic gene that complements uridine auxotrophy (5), and on a dominant selectable marker, *MPA*^{r} or *IMH3*^{r}, developed from a mutant *C. albicans* gene (2, 9).

We developed a heterologous dominant marker to investigate morphogenesis of *C. albicans*. We chose a gene conferring resistance to nourseothricin, since wild-type *C. albicans* is susceptible to moderate nourseothricin concentrations (250 to 450 μg/ml). Codon usage of the *Streptomyces noursei nat1* gene, encoding nourseothricin acetyltransferase, was adapted to that of *C. albicans* to generate *CaNAT1* (6, 8). De novo synthesis was performed by Bionexus (Oakland, Calif.). *CaNAT1* was placed under the control of the *ACT1* promoter in vector pAU34, which contains *URA3* as a selectable marker (15), generating pJK850. We transformed strain CAI4 (*ura3*/*ura3*) (5) with pJK850 to be uridine prototrophic. The resulting strains JKC435 and JKC436 were resistant to 250 μg of nourseothri-

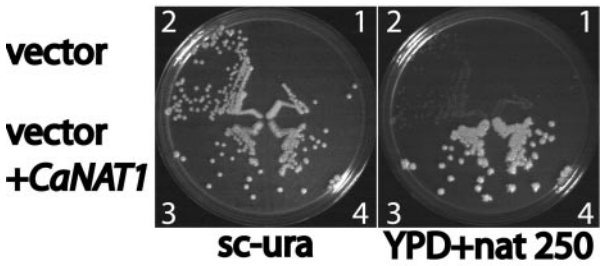

FIG. 1. *CaNAT1* confers nourseothricin resistance on *C. albicans*. The *ura3*/*ura3* strain CAI4 was transformed with the empty vector pAU34, which bears the selectable marker *URA3*, giving rise to JKC347 and JKC348, and with pAU34 containing *CaNAT1*, giving rise to JKC345 and JKC346. Transformants were streaked onto medium to select for uridine prototrophy (sc-ura). They were then replica plated onto YPD containing 250 μg of nourseothricin/ml (YPD+nat250). 1, JKC437; 2, JKC438; 3, JKC436; 4, JKC435.

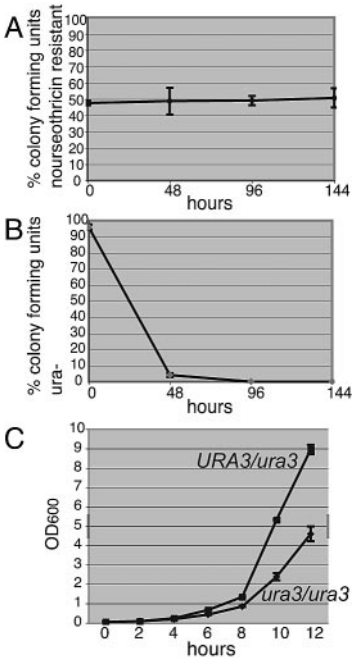

FIG. 2. Cells expressing *CaNAT1* do not exhibit a growth disadvantage. (A) Competition between wild-type SC5314 and the *DLH1*/*dlh1*::*CaNAT1* strain JKC336. Fresh cultures of the two strains were inoculated in equal parts into rich medium at a density of ca. 1 cell/ml. The numbers of total colonies and nourseothricin-resistant colonies were determined at 48 h, and the proportion of *CaNAT1*-expressing cells to wild-type cells was calculated. The cultures were diluted in fresh medium after 48 h to the starting density of ca. 1 cell/ml. This process was repeated three times. Two separate experiments were performed. Per time point, three samples were taken to determine numbers of CFU. Bars represent standard deviations. (B) Competition between strains CAF2-1 (*URA3*/*ura3*) and CAI4 (*ura3*/*ura3*). To control for the sensitivity of the competition experiment protocol, the *ura3*/*ura3* homozygote CAI4 was tested in competition with the *URA3*/*ura3* CAF2-1 heterozygote. For colony counts, cultures were plated onto YPD. They were replica plated onto medium to select for uridine prototrophy to determine the percentage of uridine-prototrophic and uridine-auxotrophic (ura$^-$) colonies. Two separate experiments were performed. Per time point, three samples were taken to determine numbers of CFU. Bars represent standard deviations. (C) Growth rates of CAF2-1 (*URA3*/*ura3*) and CAI4 (*ura3*/*ura3*). Strains were diluted from an overnight culture in 2× yeast extract–peptone–8% dextrose to an optical density at 600 nm (OD_{600}) of 0.04 in 50 ml of 2× yeast extract–peptone–8% dextrose. Growth conditions were the same as those for the competition experiments. The OD_{600} was measured every 2 h. Results of two separate experiments are shown.

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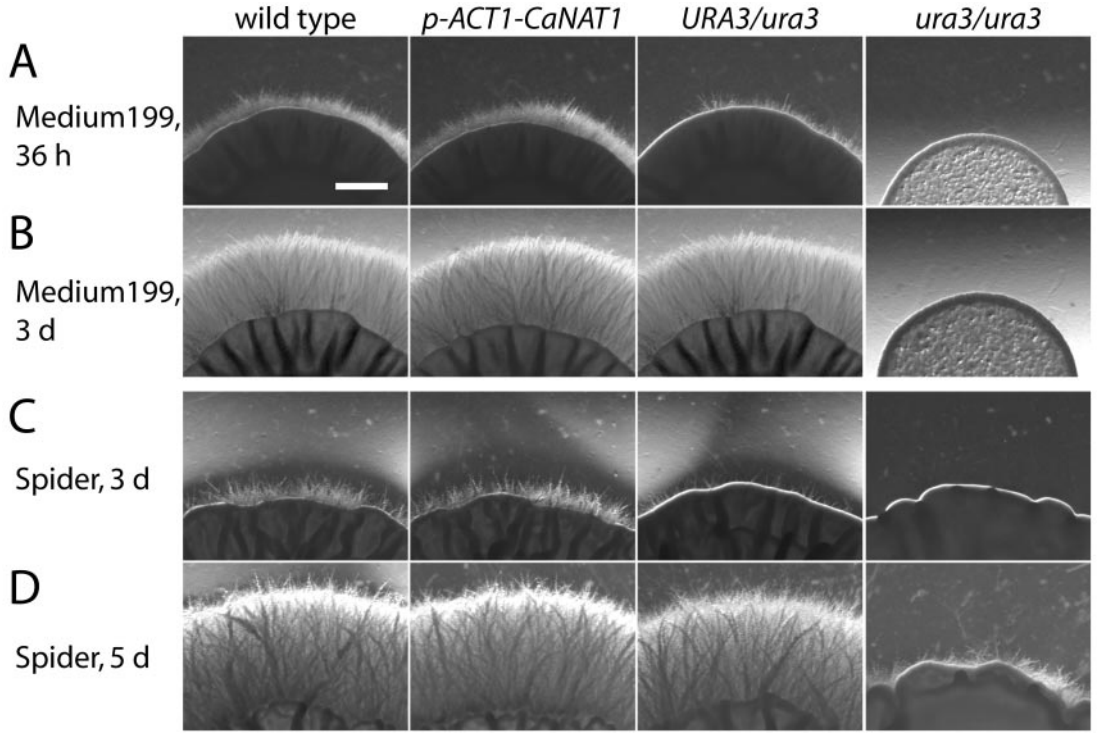

FIG. 3. Expression of *CaNAT1* does not affect filamentous growth. Filamentous growth of SC5314 (*URA3*/*URA3*) (5) was compared with that of its daughter strain JKC439 (*p-ACT1 CaNAT1 URA3*/*URA3*). CAF2-1 (*URA3*/*ura3*) and its daughter strain CAI4 (*ura3*/*ura3*) (5) were used as controls for decreased filamentous growth. Fresh cultures of the strains were diluted to an OD_{600} of 0.1. Three microliters of each strain's cell suspension was spotted at equal distances from the others around the diameter of an agar plate, with six spots per plate. Spots on medium 199, pH 7, and Spider medium after various periods of incubation are shown. d, days. Bar, 1 mm.

cin/ml, as shown in Fig. 1. CAI4 was also transformed with the empty vector pAU34 to be uridine prototrophic (15). The resulting control strains JKC437 and JKC438 were sensitive to nourseothricin.

Mutant phenotype analysis requires that the marker used to generate the mutation have minimal effects on cell growth and development. To determine the effect of *CaNAT1* expression on growth, we used competition experiments (1, 7). Specifically, with the use of pJK799, *CaNAT1* was integrated at the locus of a gene, *DLH1*, whose *Saccharomyces cerevisiae* homolog is expressed only during meiosis (4). We expressed *CaNAT1* from the *Ashbya gossypii TEF1* promoter and terminator (16) in order to avoid *C. albicans* promoter and terminator sequences that might misdirect homologous recombination of constructs. The *C. albicans* SC5314 wild type and its *DLH1*/*dlh1*::*p-AgTEF1 CaNAT1 t-AgTEF1* daughter strain JKC336 were subjected to competition essentially as described in reference 7, in 2× yeast extract-peptone-dextrose (YPD) with 8% glucose to maintain the cultures in the yeast form. In control experiments, strains CAF2-1 (*URA3*/*ura3*) and CAI4 (*ura3*/*ura3*) (5) were subjected to identical competition conditions, but they were initially inoculated at a ratio of 1:20. As shown in Fig. 2, *CaNAT1* expression was phenotypically neutral with respect to growth.

In *C. albicans*, the expression level of *URA3* affects filament development and virulence (3) and is apparently influenced by position effects (2). We wished to determine the effect of *CaNAT1* expression on filamentous growth. We transformed the wild-type strain SC5314 with pJK881, in which *CaNAT1* is driven by the *ACT1* promoter (*p-ACT1*). pJK881 was derived from pJK850 by deleting a portion of the *URA3* gene so that *URA3* was no longer functional in order to avoid possible confounding effects of an extra allele of *URA3*. We compared four nourseothricin-resistant transformants with the wild-type parent. Filamentous-growth phenotypes of these five strains were indistinguishable. Figure 3 shows filamentous growth of a *p-ACT1 CaNAT1* strain and of the wild type. The *URA3*/*ura3* and *ura3*/*ura3* strains derived from SC5314 (5) served as controls for decreased filamentous growth.

In order to delete both alleles of a gene with a single selectable marker, it must be possible to eliminate the marker from the genome. Use of the FLP recombinase to recycle a *Candida* selectable marker was pioneered by Morschhäuser and colleagues (11, 13, 17). We replaced *URA3* with *CaNAT1* in the FLP cassette (11, 13). We used the *CaNAT1*-FLP cassette to delete both alleles of *CaMOS10*, a gene whose homolog is involved in yeast growth on filaments in *S. cerevisiae* (10). The final products of this procedure were strains in which both open reading frames of *CaMOS10* were replaced with the 34-bp FLP recombinase recognition target (FRT). *CaNAT1* was then used to reintegrate an intact *CaMOS10* allele by a single crossover event at the AflII site in the *CaMOS10* promoter, at which the reintegration construct was cut. Southern blots of the resulting heterozygotes and homozygotes, as well as those of reintegrants, showed genomic fragments which corresponded to the predicted *CaMOS10* alleles of each strain (Fig. 4). *CaNAT1* can thus function as the only selectable marker for gene deletion studies of *C. albicans*. A related

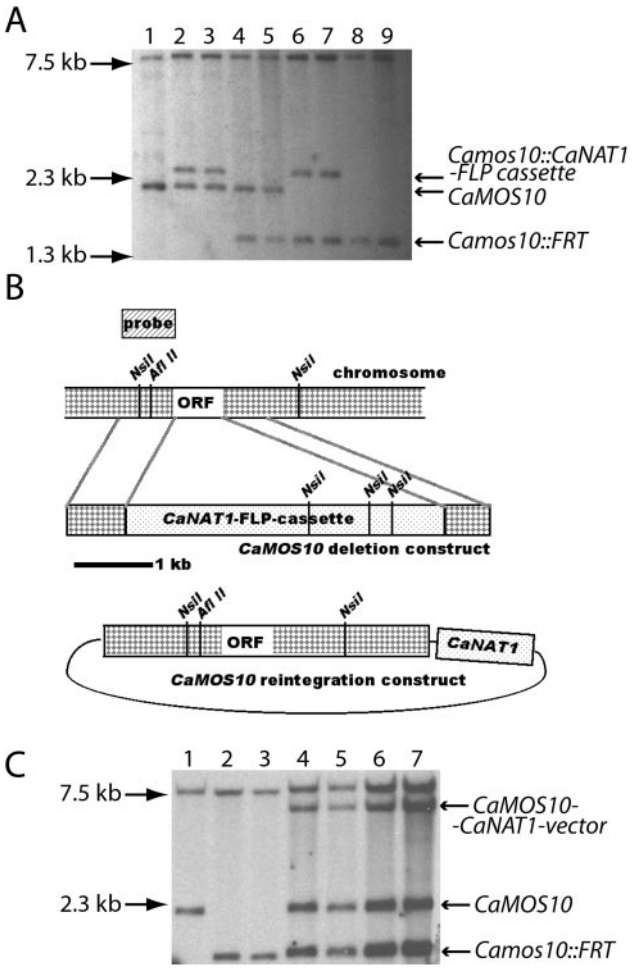

FIG. 4. *CaNAT1* can mark a recyclable cassette for deletion of both alleles of a *C. albicans* gene. (A) Genomic DNA of the following strains was digested with NsiI: lane 1, wild-type SC5314; lane 2, JKC377, and lane 3, JKC378 (*CaMOS10*/*Camos10*::*CaNAT1*-FLP cassette); lane 4, JKC380, and lane 5, JKC382 (*CaMOS10*/*Camos10*::FRT); lane 6, JKC390, and lane 7, JKC392 (*Camos10*::FRT/*Camos10*::*CaNAT1*-FLP cassette); and lane 8, JKC393, and lane 9, JKC395 (*Camos10*::FRT/*Camos10*::FRT). The *CaNAT1*-FLP cassette is FRT-*p-SAP2*-FLP-*t-CaACT1-p-AgTEF1-CaNAT1-t-AgTEF1*-FRT. Left arrows show locations of molecular weight markers. Right arrows show genomic fragments. (B) Cartoon of the *CaMOS10* locus, the deletion construct, the construct to reintegrate an intact *CaMOS10* allele, and the probe used in Southern blotting. ORF, open reading frame. (C) Genomic DNA of the following strains was digested with NsiI: lane 1, wild-type SC5314; lane 2, JKC393, and lane 3, JKC395 (*Camos10*::FRT/*Camos10*::FRT); and lanes 4 and 5, transformants of JKC393 (lane 4, JKC504, and lane 5, JKC505), and lanes 6 and 7, transformants of JKC395 (lane 6, JKC506, and lane 7, JKC507) (*Camos10*::FRT/*Camos10*::FRT/*CaMOS10 CaNAT1*).

marker, *SAT1*, was used in a large-scale gene deletion study of *C. albicans* (12). However, the investigators in that study utilized uridine and histidine auxotrophy markers in addition to the dominant marker to construct their strains. We find that recycling the *CaNAT1* marker can obviate the need for auxotrophic markers.

To test whether *CaNAT1* could be used as a selectable marker in clinical *Candida* isolates, we obtained three *C. albicans* bloodstream isolates and seven clinical isolates of other *Candida* spp. from the Boston Children's Hospital Microbiology Laboratory. None of these strains were intrinsically resistant to nourseothricin. The clinical isolates were transformed with *CaNAT1* to be nourseothricin resistant. Transformants appeared after 1 to 2 days on YPD plus 450 μg of nourseothricin/ml. At 3 days, small colonies that did not contain the *CaNAT1* sequence as detected by PCR appeared. The transformants were streaked onto YPD and replica plated onto YPD plus 450 μg of nourseothricin/ml to confirm that they

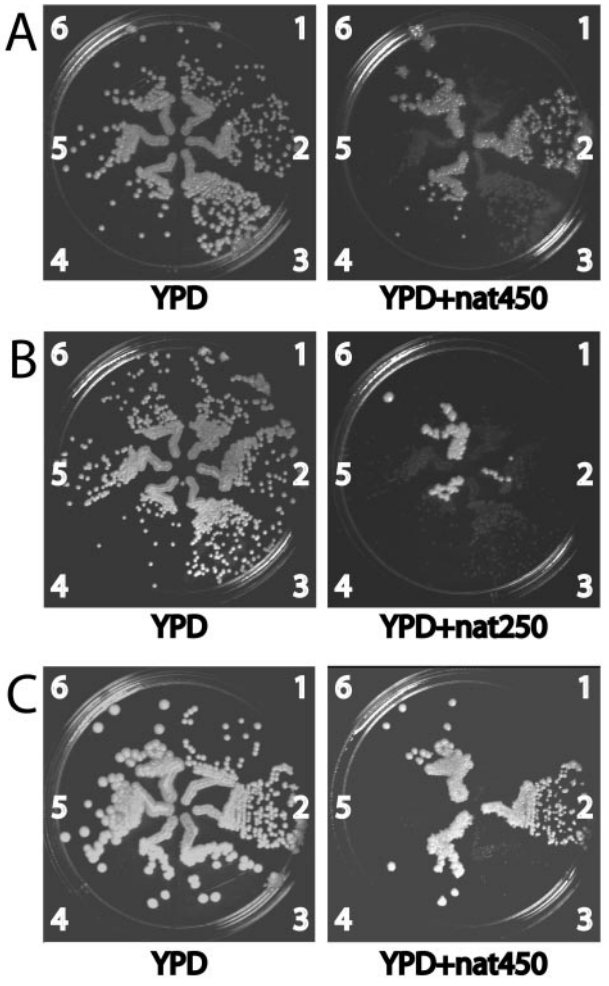

FIG. 5. Nourseothricin resistance phenotypes of clinical isolates of *C. albicans* and other *Candida* species. Clinical *Candida* isolates and their *CaNAT1*-containing transformants were streaked next to each other on YPD. They were then replica plated onto YPD with 250 (YPD+nat250) or 450 (YPD+nat450) μg of nourseothricin/ml. (A) *C. albicans* bloodstream isolates. 1, JKC8 (wild-type bloodstream isolate); 2, JKC410 (JKC8 transformed with pJK850); 3, JKC9 (wild-type bloodstream isolate); 4, JKC412 (JKC9 transformed with pJK799); 5, JKC7 (wild-type bloodstream isolate); and 6, JKC408 (JKC7 transformed with pJK850). (B) *C. parapsilosis* clinical isolates. 1, JKC355 (wild-type clinical isolate); 2, JKC414 (JKC355 transformed with pJK850); 3, JKC357 (wild-type clinical isolate); 4, JKC416 (JKC357 transformed with pJK799); 5, JKC358 (wild-type clinical isolate); and 6, JKC419 (JKC358 transformed with pJK850). (C) *Candida* species clinical isolates. 1, JKC356 (*C. lusitaniae* wild type); 2, JKC420 (JKC356 transformed with pJK799); 3, JKC361 (*C. glabrata* wild type); 4, JKC424 (JKC361 transformed with pJK850); 5, JKC359 (*C. kefyr* wild type); and 6, JKC426 (JKC359 transformed with pJK850).

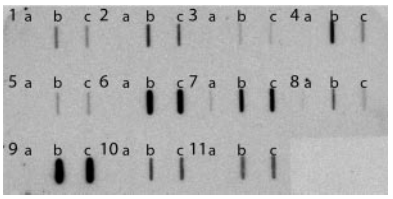

FIG. 6. Nourseothricin-resistant strains transformed with *CaNAT1* contain the *CaNAT1* sequence. Genomic DNA from untransformed parent strains (a) and from two each of their nourseothricin-resistant transformants (b and c) was slot blotted and probed with a portion of the *CaNAT1* open reading frame. The strains were as follows: 1a, SC5314; 1b, JKC406; 1c, JKC336; 2, 3, and 4, *C. albicans* bloodstream isolates (2a, JKC7; 2b, JKC406; 2c, JKC375; 3a, JKC8; 3b, JKC410; 3c, JKC411; 4a, JKC9; 4b, JKC412; and 4c, JKC413); 5, 6, and 7, *C. parapsilosis* clinical isolates (5a, JKC355; 5b, JKC414; 5c, JKC415; 6a, JKC357; 6b, JKC416; 6c, JKC417; 7a, JKC358; 7b, JKC418; and 7c, JKC419); 8 and 9, *C. lusitaniae* clinical isolates (8a, JKC356; 8b, JKC420; 8c, JKC421; 9a, JKC360; 9b, JKC422; and 9c, JKC423); 10, *C. glabrata* clinical isolate (10a, JKC361; 10b, JKC424; and 10c, JKC425); and 11, *C. kefyr* clinical isolate (11a, JKC359; 11b, JKC426; and 11c, JKC407).

were nourseothricin resistant. Their growth phenotypes are shown in Fig. 5. In *C. parapsilosis* transformants, nourseothricin resistance was not clonally stable, as shown in Fig. 5B. We did not examine whether the *CaNAT1*-carrying plasmid was maintained episomally in this species, with loss of the plasmid under nonselective conditions.

PCR was used to confirm that the nourseothricin-resistant strains which grew after transformation with *CaNAT1* were actually transformed with *CaNAT1* and were not spontaneous nourseothricin-resistant mutants. The PCRs for all nourseothricin-resistant transformants yielded a product of the expected size, while none of the reactions for the untransformed parents yielded this product (data not shown). In addition, we subjected 22 nourseothricin-resistant *Candida* transformants and their 11 untransformed parent strains to slot blotting to detect the *CaNAT1* gene. As shown in Fig. 6, all tested transformants were found to contain a sequence that hybridized to a portion of the *CaNAT1* open reading frame. DNA of a *C. parapsilosis* clinical isolate appeared to hybridize with the *CaNAT1* probe in the slot blot. Southern blotting of this strain and its *CaNAT1*-transformed daughters showed no *CaNAT1*-sized band in the clinical isolate and showed the expected band in the transformants; in addition, a faint cross-hybridizing band of higher molecular weight than the *CaNAT1*-sized band was seen in these strains and in the positive and negative controls (data not shown). Our results demonstrated that transformation with *CaNAT1* conferred nourseothricin resistance on clinical isolates of several *Candida* species, as well as on *S. cerevisiae* (data not shown). *C. lusitaniae* and *S. cerevisiae* are the most distantly related hemiascomycetes that we transformed with *CaNAT1* to be nourseothricin resistant (14). *CaNAT1* is expected to function in other ascomycetous species within this span of evolutionary distance.

We have shown that *CaNAT1* can function as a selectable marker in *C. albicans* and in several other *Candida* species. It is phenotypically neutral with respect to growth and filament development under our experimental conditions. We anticipate that the use of *CaNAT1* will expand the scope of molecular genetic analysis to clinical isolates of many pathogenic *Candida* species.

Nucleotide sequence accession number. The nucleotide sequence of *CaNAT1* has been deposited in GenBank under accession number AY854370.

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