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Effect of Farnesol on a Mouse Model of Systemic Candidiasis, Determined by Use of a *DPP3* Knockout Mutant of *Candida albicans*▽

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This work extends our previous observation that the fungus *Candida albicans* secretes micromolar levels of farnesol and that accumulation of farnesol in vitro prevents the yeast-to-mycelium conversion in a quorum-sensing manner. What does farnesol do in vivo? The purpose of this study was to determine the role of farnesol during infection with a well-established mouse model of systemic candidiasis with *C. albicans* A72 administered by tail vein injection. This question was addressed by altering both endogenous and exogenous farnesol. For endogenous farnesol, we created a knockout mutation in *DPP3*, the gene encoding a phosphatase which converts farnesyl pyrophosphate to farnesol. This mutant (KWN2) produced six times less farnesol and was ca. 4.2 times less pathogenic than its SN152 parent. The strain with *DPP3* reconstituted (KWN4) regained both its farnesol production levels and pathogenicity. These mutants (KWN1 to KWN4) retained their full dimorphic capability. With regard to exogenous farnesol, farnesol was administered either intraperitoneally (i.p.) or orally in the drinking water. Mice receiving *C. albicans* intravenously and farnesol (20 mM) orally had enhanced mortality ($P < 0.03$). Similarly, mice ($n = 40$) injected with 1.0 ml of 20 mM farnesol i.p. had enhanced mortality ($P < 0.03$), and the onset of mortality was 30 h sooner than for mice which received a control injection without farnesol. The effect of i.p. farnesol was more pronounced ($P < 0.04$) when mice were inoculated with a sublethal dose of *C. albicans*. These mice started to die 4 days earlier, and the percent survival on day 6 postinoculation (p.i.) was five times lower than for mice receiving *C. albicans* with control i.p. injections. In all experiments, mice administered farnesol alone or Tween 80 alone remained normal throughout a 14-day observation period. Finally, beginning at 12 h p.i., higher numbers of *C. albicans* cells were detected in kidneys from mice receiving i.p. farnesol than in those from mice receiving control i.p. injections. Thus, reduced endogenous farnesol decreased virulence, while providing exogenous farnesol increased virulence. Taken together, these data suggest that farnesol may play a role in disease pathogenesis, either directly or indirectly, and thus may represent a newly identified virulence factor.

Candida albicans is a dimorphic commensal fungus which is localized primarily in the gastrointestinal tract (20). It is a medically important opportunistic pathogen, particularly for immunocompromised individuals (16), and can invade a wide range of organ systems during systemic infections. For healthy, immunocompetent individuals, it is an opportunistic pathogen only. However, Calderone and Gow (6), Navarro-Garcia et al. (28), Naglik et al. (26), and Odds et al. (33) have detailed the contributions of virulence factors in candidiasis. These virulence factors could either promote *Candida* invasion or affect host defense mechanisms. It is likely that the virulence of *C. albicans* is multifactorial. Therefore, pathogenesis is the sum of the attributes of the fungus, environmental conditions, and the effectiveness of host defenses (6, 26, 28, 33). Virulence factors which have been recognized so far include the following: (i) morphogenesis—yeast-to-hypha switching is associated with pathogenesis (5), presumably because it enables tissue invasion (32) and evasion of host defense mechanisms (21); also, mutants which can be regulated in vivo in their ability to undergo the yeast-to-hypha transition were avirulent under conditions that inhibited this transition (43); (ii) phenotypic switching (47)—vaginal candidiasis is increased by high-frequency switching between white and opaque colony morphologies (48); (iii) epithelial adhesion—King et al. (17) first demonstrated that *C. albicans* cells have greater adherence than do cells of other *Candida* species, and their ability to interact with mucosal epithelia correlates with their relative virulence (6); and (iv) production of extracellular enzymes, both membrane-damaging phospholipase B (1, 22) and the secretory aspartyl proteases. Secretory aspartyl proteases are expressed during human disease and are thought to be important virulence determinants (8, 26, 39).

Recently Hornby et al. (12) identified farnesol as an extracellular quorum-sensing molecule produced by *C. albicans*, and these findings were independently confirmed by others (35, 36). Farnesol prevented mycelial development under growing conditions (12) and in a differentiation assay using three chemically distinct triggers for germ tube formation: L-proline, *N*-acetylglucosamine, or serum (12). For all in vitro assays, farnesol prevented the yeast-to-mycelium conversion, resulting in

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actively budding yeasts without influencing cellular growth rates (12). Recognizing that free farnesol is not normally detected in human plasma (41), Hornby et al. (12) proposed two competing hypotheses regarding the role of farnesol in disease pathogenesis. The first hypothesis suggested that the shift from yeasts to mycelia might be a critical step in pathogenesis and that exogenous farnesol could block this transition, thus acting as a therapeutic compound. An alternate hypothesis suggested that the farnesol excreted during infection would alter the membrane fluidity of host cells, allowing *C. albicans* to penetrate host tissues and thus indirectly acting as a virulence factor. The latter model is based on the lipophilic properties of free farnesol, which would favor membrane localization.

The idea that farnesol might promote virulence is supported by a recent study from our laboratory (27) on the pathogenicity of *C. albicans* cells pretreated with subinhibitory concentrations (0.5 to 1 μM) of fluconazole. Previous studies (14) indicated that these cells secreted 10 times more farnesol than did untreated cells. Significantly, these fluconazole-pretreated cells were also 4.2 to 8.5 times more lethal ($P < 0.001$) than untreated cells in a mouse tail vein injection model of disseminated candidiasis (27). One explanation, but certainly not the only explanation, for this enhanced pathogenicity is that it is caused in some unknown fashion by the excess farnesol excreted by the treated cells (14, 27).

The purpose of the present study was to determine whether farnesol affected the progression of disseminated candidiasis with a well-established mouse model and, if it did so, whether it would act in a therapeutic or virulence-promoting manner. We analyzed the effect of farnesol administered by different routes, both endogenous and exogenous, and concluded that farnesol excretion contributes to the virulence of *C. albicans*. This is the first time that a fungal quorum-sensing molecule has been suggested to play a role in *C. albicans* infection. It is also the first investigation of *DPP3* gene function in *C. albicans*, showing its role in farnesol production.

MATERIALS AND METHODS

***C. albicans* strain and growth conditions.** *Candida albicans* strain A72 (ATCC MYA-2430) is a well-characterized farnesol-producing and farnesol-responsive strain that has been used in previous in vitro studies (12, 13, 27, 45). This strain was originally isolated from a patient by Antonio Cassone (Rome, Italy). *C. albicans* strains 10231 and SN152 were obtained from the American Type Culture Collection (Rockville, MD) and Alexander Johnson, respectively. For challenge, *C. albicans* cells were grown for 24 to 30 h in 50 ml of mGSB medium at 30°C with aeration as previously described (12). For strains with auxotrophic requirements, mGSB was supplemented with the required amino acid at 40 μg/ml, i.e., Arg for KWN2, Arg and Leu for KWN1, Arg and His for KWN3, and Arg, Leu, and His for SN152 and KWN4. Cells were harvested by centrifugation at 4,750 × *g* for 10 min and washed three times with 50 ml of sterile, nonpyrogenic normal saline, before adjusting to the proper concentration in nonpyrogenic sterile saline (Abbott Laboratories, North Chicago) using a Petroff-Hausser counter. All strains were tested in vitro, i.e., stimulation by *N*-acetylglucosamine at 37°C (12), to be sure that their germ tube-forming ability was ≥95%. Samples were stored at 4°C overnight prior to injection. For strain A72, the 50% lethal doses (LD_{50}s) were found to be identical for cells stored overnight and cells which were used immediately. Farnesol concentrations in the culture supernatants were determined by gas chromatography/mass spectrometry as described previously (12–14).

Mice and their inoculation with *C. albicans*. Mouse infections followed the procedures recommended by Odds et al. (34). Outbred, 4- to 6-week-old (20 to 25 g), CF-1 female mice obtained from a commercial supplier (Charles River Laboratories, Wilmington, MA) were randomly allocated to groups of five to six animals and placed in polycarbonate cages with stainless-steel wire tops, using aspen shavings as bedding material (laboratory-grade Sano-Chips; Harlan Teklad, Madison, WI), and maintained on a 12-h light/dark cycle in heated, thermostatically controlled rooms for the duration of the studies. The mice were fed a commercial rodent diet (4% Mouse/Rat Diet 7001; Harlan Teklad, Madison, WI) ad libitum. Tap water was provided in glass bottles fitted with stainless-steel nipples mounted in rubber corks. After a 7- to 10-day observation period, each group of mice was inoculated intravenously in the lateral caudal tail vein using a 27-gauge needle with 0.1 ml containing the appropriate concentration of *C. albicans* cells. The degree of clinical illness in each mouse was evaluated three times daily. In each experiment, mice that had severe clinical signs of illness were euthanized immediately by placing them in a closed chamber filled with CO_2 gas and processed for complete necropsy and collection of tissues for histopathological, microbiological, and toxicological examinations. At the conclusion of the experiment, all remaining mice were similarly euthanized for complete necropsy. The experimental protocol, housing, and care of the mice were in accordance with approved guidelines of the University of Nebraska—Lincoln Institutional Animal Care and Use Committee.

Farnesol administration. Commercial mixed isomers or *E*,*E*-farnesol (Sigma, St. Louis, MO) was diluted in 0.5% (vol/vol) of Tween 80 (Sigma) in sterile, nonpyrogenic saline. The mixed isomers were used when farnesol was administered orally. All other experiments used *E*,*E*-farnesol. The solubility of farnesol in water is only 1.2 mM (18), and therefore, Tween 80 was used to increase the concentration of farnesol which could be used. Farnesol (20 mM) was administered either orally in the drinking water (without saline) or by intraperitoneal (i.p.) injections. Mice received 1.0 ml of 20 mM farnesol (4.4 mg) by intraperitoneal (i.p.) injection. Water intake was monitored daily by the weight of the water bottles for both the control and treated mice.

DPP3 knockout. To knock out the *DPP3* gene, we adapted the strategy reported by Noble and Johnson (31) using strain SN152, which is auxotrophic for arginine, leucine, and histidine. This strategy is shown in Fig. 1. See Table 1 for the sequences of all PCR primers. The sequences of both copies of *DPP3* and their respective flanking regions are identical in the diploid *C. albicans* genome (Candida DB and CGD databases). The first step of the scheme involved amplification of the two 350-bp flanking regions of *DPP3* using a template of SC5314 genomic DNA and primers 1 and 3 or 4 and 6 (Fig. 1) in separate reactions. These primers are designated DPP1, -3, -4, and -6, respectively, in Table 1.

Plasmids pSN40 (containing *Candida maltosa LEU2*) and pSN52 (containing *Candida dubliniensis HIS1*), kindly provided by Alexander Johnson, were transformed into *Escherichia coli* cells, and following growth, the plasmids were harvested. Universal primers 2 and 5 were used to amplify the *HIS1* and *LEU2* cassettes from these plasmids (31). The 5′ tails of primers 2 and 3 and primers 4 and 5 are complementary, as is required for the fusion reaction. The upstream and downstream 350-bp sequences for *DPP3* and the respective selectable markers (*His1* or *Leu2*) were combined, and the fusion products were amplified with primers 1 and 6 (Fig. 1B).

PCR conditions to make the DNA fragments required for the *DPP3* gene disruption were as follows. In the first round of PCRs, 50-μl reaction mixtures combined 1 μl of Klen Tac LA DNA polymerase (BD Bioscience) in 1× Klen Tac buffer with 3 μl of 10 mM deoxynucleoside triphosphate, 1 μl of each primer (100 ng/μl), and 1 μl of template DNA. The template for the flanking sequences was SC5314 genomic DNA, and the templates for the *C. maltosa LEU2* and *C. dubliniensis HIS1* markers were pSN40 and pSN52, respectively. The reaction conditions were 93°C for 5 min and then 35 cycles of 93°C for 30 s, 45°C for 45 s, and 72°C for 3 min, followed by a final 72°C for 10 min. All products were gel purified on a 1% agarose gel, and the QIAquick gel extraction kit (QIAGEN) was used to obtain ultrapurified DNA fragments. The PCR conditions for the fusion reactions were identical to the above method except that the PCR elongation reaction was extended to 72°C for 4.5 min. Template DNA consisted of 1 μl of the upstream and downstream fragments and a marker DNA fragment (*C. maltosa LEU2 or C. dubliniensis HIS1*). Because of the complementary overhangs created in the initial DNA products, the fragments and markers anneal together. Primers 1 and 6 amplified the fusion reactions (Fig. 1B), whereupon the fusion PCR products were gel purified using the QIAquic gel extraction kit (QIAGEN). The *HIS1*-containing fusion product was transformed into SN152 using the lithium acetate method (Fig. 1A). Transformants were plated on selective medium (yeast nitrogen base without amino acids but with arginine and leucine) where only transformed cells will grow. KWN1 was selected.

Genomic DNA from the single-copy-deleted mutant (KWN1) was harvested and checked for the correct insertion into one copy of *DPP3* (Fig. 2, lanes B and C). After confirming the single-copy knockout, the fusion product with the *C. maltosa LEU2* marker was transformed into KWN1 to knock out the other allele of *DPP3*. Transformants were plated on selective medium (yeast nitrogen base

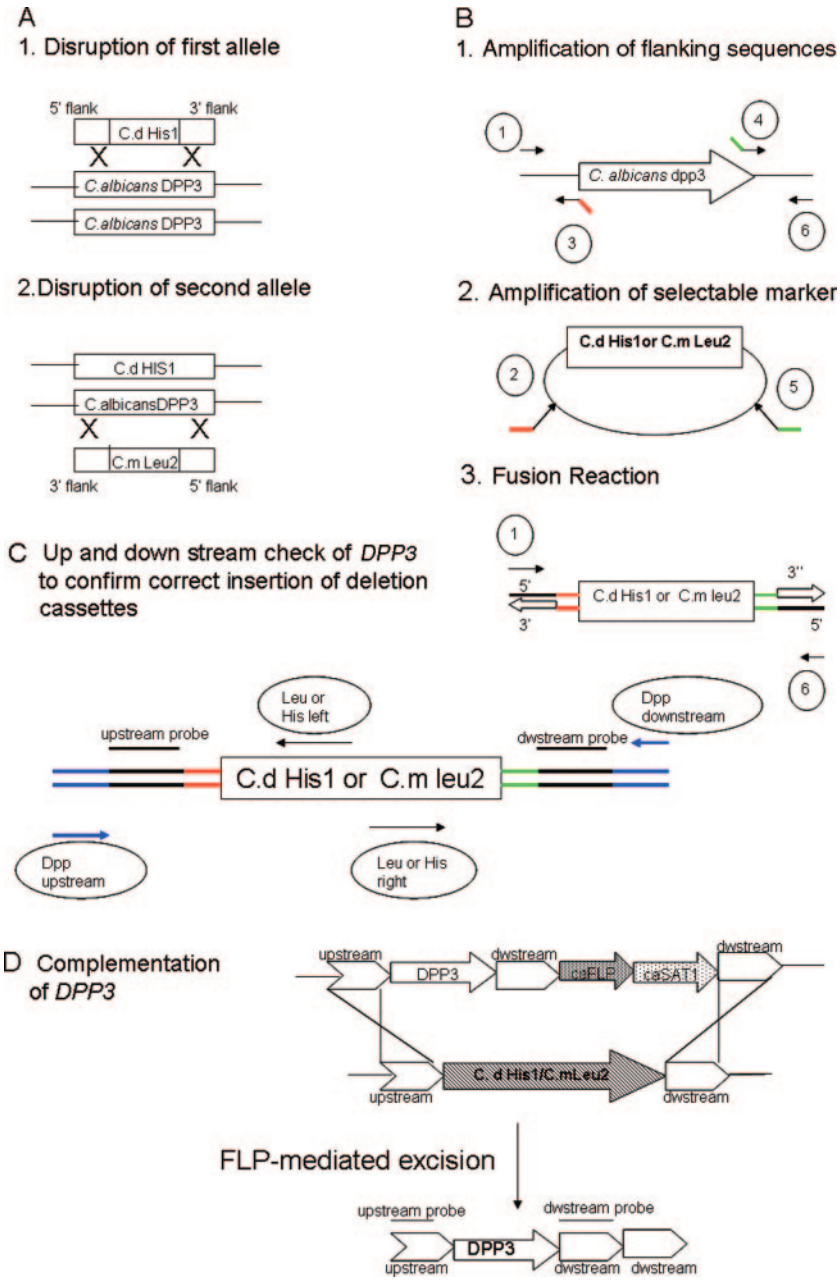

FIG. 1. Strategy used to disrupt and then reconstitute *C. albicans DPP3*. It merges the fusion PCR and heterologous marker protocols of Noble and Johnson (31) with the *SAT1* flipper protocols of Reuβ et al. (37). (A) Schematic diagram showing the method used to disrupt both alleles of *DPP3*. (B) Construction of disruption fragments for homologous recombination. Primers 1 and 3 and primers 4 and 6 were used to amplify the 350-bp flanking sequences upstream and downstream of *DPP3*, respectively. Primers 2 and 5 were used to amplify the two selectable markers. Red and green tails of primers are complementary sequences (31) needed for mutual primed synthesis in the fusion reaction. (C) 5′ and 3′ junctions of the selectable marker integration sites were confirmed using the primers mentioned. (D) Structure of the deletion cassette from pSFS2ADPP3 (top) in which the *SAT1* flipper is inserted between two downstream fragments of *DPP3*. The genomic structure of KWN2 (middle) shows that the *DPP3* allele has been replaced by one of the auxotrophic markers *His1* and *Leu*2 (cross-hatched). Complementation of the *DPP3* allele replacing the *His1* marker (after flip-mediated excision of the nourseothricin marker) was used to create KWN3 (lower). dwstream, downstream.

without amino acids but with arginine) where only transformed cells will grow. KWN2 was selected.

To confirm that the *HIS1* and *LEU2* cassettes had been inserted correctly, the following primer pairs were used to span the junction sites (Table 1 and Fig. 1C): *HIS1* Left (near primer 2) and DPP3 upstream check for the *HIS1* 5′ junction; *HIS1* Right (near primer 5) and DPP3 downstream check for the *HIS1* 3′ junction; *LEU2* Left (near primer 2) and DPP3 upstream check for the *LEU2* 5′ junction; and *LEU2* Right (near primer 5) and DPP3 downstream check for the 3′ *LEU2* junction. Confirmation of correct insertion in *DPP3* was provided by detecting PCR products of the expected size (Fig. 2). As a final check to confirm the absence of *DPP3* or a similar gene, possibly due to gene duplication in the evolutionary process, the DPP3 check left and right primers (Table 1) were used to amplify an 856-internal-base-pair region of the 921-bp sequence of *DPP3*. Genomic DNA from KWN2 was used as the test sample and genomic DNA from SN152 as the positive control. A southern hybridization confirmed the complete absence of *DPP3* (Fig. 3).

TABLE 1. Sequences of synthetic oligonucleotides used in this study

Sequence[a]	Name	Reference
GTTCCATCAAATTATTCATTC	DPP1	This study
ccgctgctaggcgcgccgtgACCAGTGTGATGGATATCTGC	Universal primer 2 (for *HIS1*, *LEU2*, and *ARG4* cassettes)	31
cacggcgcgcctagcagcggGATGAAAATGTGTAGAGTGTG	DPP3	This study
gtcagcggccgcatccctgcTCAATAGGTTACTACTTAGC	DPP4	This study
gcagggatgcggccgctgacAGCTCGGATCCACTAGTAACG	Universal primer 5 (for *HIS1*, *LEU2*, and *ARG4* cassettes)	31
AGCAGGTCAAGATGCAATG	DPP6	This study
ATTAGATACGTTGGTGGTTC	*HIS1* Left (near primer 2)	31
AACACAACTGCACAATCTGG	*HIS1* Right (near primer 5)	31
AGAATTCCCAACTTTGTCTG	*LEU2* Left (near primer 2)	31
AAACTTTGAACCCGGCTGCG	*LEU2* Right (near primer 5)	31
TGGGATAAGCCTCATACATGC	*DPP3* Upstream Check	This study
CAAAGGGAACAAGATGAAGCA	*DPP3* Downstream Check	This study
GGGGGTTTAAATCACCAACA	*DPP3* Check Left	This study
CACACATTAGTGGAAATGTC	*DPP3* Check Right	This study
GGATAAGGGCCCTCATACATGCAA	*DPP3* complement 1	This study
TCAAGCTCGAGGTAATTCAGAAAAGAA	*DPP3* complement 2	This study
CCCACCGCGGCTAGTCTTGGTCTA	*DPP3* complement 3	This study
CCATAGAGCTCATTCTTTCAAGTGGGTA	*DPP3* complement 4	This study

[a] Sequences in lower case correspond to exogenous, complementary sequences introduced for mutually primed synthesis in the second round of fusion PCR. Underlined segments introduce desired restriction sites in the process of constructing pSFS2ADPP3.

***DPP3* complementation method. (i) Plasmid construction.** The pSFS2A plasmid was kindly provided by Joachim Morschhauser of the University of Wiirzburg, Germany. This plasmid has a gene deletion or complementation cassette, called the *SAT1* flipper cassette, which contains the nourseothricin marker *caSAT1* and the *C. albicans*-adapted *FLP* gene (*caFLP*), which encodes a site-specific recombinase under the control of the C. *albicans MAL2* promoter. The cassette is flanked by direct repeats of the minimal recombination target sites of the FLP recombinase. The *SAT1* flipper cassette has been designed to contain a few unique restriction sites on the left (ApaI and XhoI) and right (SacI and SacII) borders that can be used to clone target sequences for homologous recombination (37). The *DPP3* complementation cassette was constructed as follows. An ApaI-XhoI fragment of the complete *C. albicans DPP3* sequence as

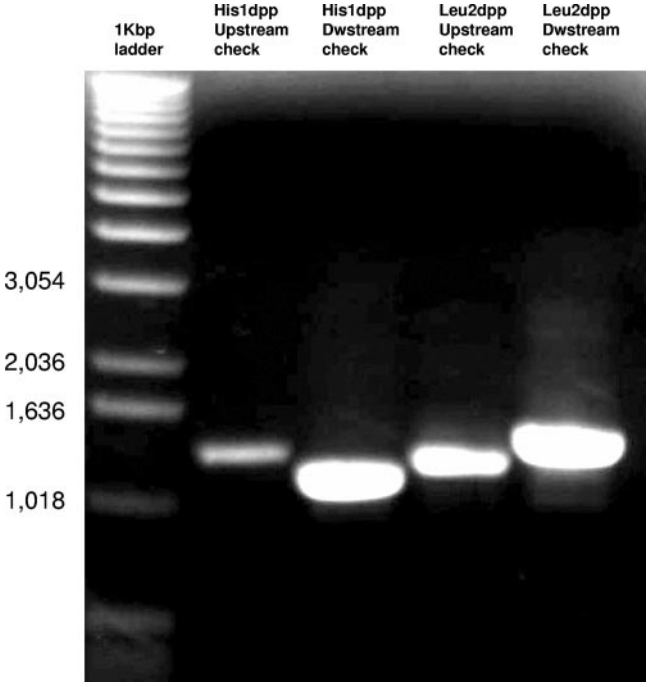

FIG. 2. Confirmation of the correct integration sites for the selectable markers used to knock out *DPP3*. His1dpp upstream check, the *HIS1* Left and *DPP3* upstream check primers were used to amplify the upstream region of the construct; His1dpp downstream check, the *HIS1* Right and *DPP3* downstream check primers were used to amplify the downstream region of the construct; Leu2dpp upstream check, the *LEU2* Left and *DPP3* upstream check primers were used to amplify the upstream region of the construct; Leu2dpp downstream check, the *LEU2* Right and *DPP3* downstream check primers were used to amplify the downstream region of the construct.

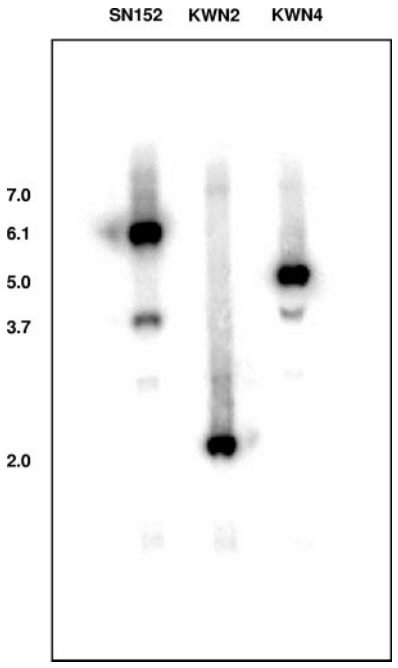

FIG. 3. Southern analysis of ClaI-digested genomic DNA of SN152, KWN2, and KWN4 with two *DPP3*-specific probes. The two probes hybridize with the bp −350 and +456 regions of the gene, which has a single restriction site for ClaI at nucleotide 514. The sizes of the hybridizing fragments (in kb) are given on the left side of the blot. Fragments corresponding to hybridization at 3.7 kb are identical in size for the parent strain (SN152) and the complemented strain (KWN4). The KWN4 fragment corresponding to the other probe, hybridizing at 5 kb, is slightly different from that of the parent strain.

well as 0.5 kb of upstream and 0.46 kb of downstream flanking sequences for the *DPP3* alleles were amplified using primers *DPP3* complement 1 and 2 (Table 1). A SacII-SacI *DPP3* downstream fragment from position +20 to +476 was amplified with primers *DPP3* complement 3 and 4 (Table 1). *DPP3* together with up- and downstream fragments was cloned on the left side of the *SAT1* flipper and the *DPP3* downstream fragment was cloned on the right side of the *SAT1* flipper (Fig. 1D) to generate pSFS2ADPP3. The *SAT1* flipper cassette and the cloned parts of the plasmids were sequenced at the University of Nebraska, Lincoln, sequencing facility to confirm accurate cloning.

(ii) *C. albicans* transformation to reconstitute *DPP3*. Transformation for complementation was done according to the method of Reuβ et al. (37). Briefly, the insert from pSFS2ADPP3 was excised as an ApaI-SacI fragment and gel purified. Approximately 1 μg (5 μl) of the linear DNA fragment was mixed with 40 μl of electrocompetent KWN2 cells and subjected to electroporation (0.2-cm cuvette; 1.8 kV). These electroporated KWN2 cells were washed with 1 ml of 1 M sorbitol, resuspended in 1 ml of yeast extract-peptone-dextrose (YPD), and incubated for 4 h at 30°C with shaking at 200 rpm. The cells were spread on YPD plates containing 200 μg/ml nourseothricin and inoculated at 30°C. Resistant colonies were picked after 1 day and reinoculated into YPD liquid medium with 200 μg/ml nourseothricin. Eight cultures from individual colonies were streaked on synthetic dextrose agar (SD)-plus-Arg, SD-plus-Arg-and-His, and SD-plus-Arg-and-Leu plates to determine which copy of *DPP3* had been reintegrated. After confirming that one of the markers introduced into SN152 had been lost, that strain was named KWN3. KWN3 was inoculated into YPD liquid medium without selective pressure to allow FLP-mediated excision of the *SAT1* flipper cassette as described by Reuβ et al. (37). Then, serially diluted KWN3 was spread on YPD plates containing 25 μg/ml nourseothricin and allowed to grow for 2 days to identify those smaller colonies that are nourseothricin sensitive due to FLP-mediated excision of *CaSAT1*. These colonies were reinoculated into yeast peptone maltose liquid medium to make sure that the *MAL2* promoter was activated for *caFLP* expression. This precaution was taken in case there were any nonexcised *CaSAT1* cassettes remaining. These cells were restreaked on YPD containing 200 μg/ml nourseothricin (where they should not grow) to make sure that FLP-mediated excision had occurred and then transformed again with the same ApaI-SacI fragment to make KWN4, with fully reconstituted *DPP3*. Nourseothricin was obtained from Werner BioAgents (Jena, Germany).

Southern hybridization. Genomic DNA from SN152, KWN2, and KWN4 was isolated for Southern hybridization. Both alleles of *DPP3* have ClaI restriction cut sites at nucleotide 514. Approximately 10 μg of DNA was digested with ClaI, separated on 1% agarose gels, and transferred to a membrane. Two gel-purified *DPP3* fragments were used as probes to confirm gene knockout and complementation. These were the upstream fragment (350 bp) generated by primers DPP1 and DPP3 and the SacII-SacI downstream fragment (456 bp) generated by primers *DPP3* complement 3 and complement 4.

Determination of *C. albicans* LD_{50}s. The LD_{50}s for *C. albicans* A72, SN152, KWN2, and 10231 were determined by the Kärber method (23). Strains of *C. albicans* were inoculated into mGSB medium, grown overnight at 30°C, washed three times with nonpyrogenic, sterile saline, and counted with a Petroff-Hausser counter. Five groups of five mice each were intravenously (i.v.) challenged by tail vein injection with 0.1 ml of sterile saline containing either 10^4, 10^5, 10^6, 10^7, or 10^8 yeast cells. Mortality was recorded continuously for 7 to 14 days postinoculation (p.i.), and the LD_{50} was calculated by the Kärber method (23). For *C. albicans* A72, the calculated LD_{50} was approximately 1.3×10^6 cells at 3 days p.i. This protocol was modified slightly to achieve a sublethal challenge. *C. albicans* A72 was transferred in mGSB nine times over a 9-month period. These cells still had germ tube-forming ability of ≥95%, but their LD_{50} at 3 days p.i. had increased to 1.0×10^7 cells. In this case, 1.3×10^6 cells was a sublethal injection.

For the pathogenicity experiment comparing strains SN152, KWN2, and KWN4 (50 mice total), each group of 15 mice was inoculated with 2×10^6 cells of the indicated strain. Five mice were kept as a negative control. The injection dose of 2×10^6 cells was chosen to coincide with the 3-day LD_{50} of SN152 (Table 2).

Statistical analysis. The main parameter measured was the time (h) from i.v. inoculation to death or euthanasia because of severe clinical signs. The data were plotted as Kaplan-Meier survival curves and analyzed using LIFETEST and proportional hazards regression (PHREG) procedures (42). The LIFETEST assesses model assumptions using three nonparametric tests, log-rank, Wilcoxon, and likelihood ratio tests, to determine whether two or more treatment groups are different. PHREG performs Cox regression analysis of survival data using the proportional hazards model. The resulting regression coefficients are an estimate of the exponent of the hazard ratios or relative risk.

Fluorescent farnesol in sera. In order to determine how much farnesol gets to the bloodstream and how long it remains there, we developed a high-performance liquid chromatography (HPLC) method to detect fluorescent farnesol in mouse serum. Shchepin et al. (44) designed a farnesol analog which exhibits absorption and emission near 360 and 450 nm, respectively. This molecule is biologically active in that it has 12% of the activity of farnesol in blocking the yeast-to-mycelium transition by *C. albicans* (44). However, the fluorescent farnesol was only partially soluble in 0.5% Tween 80. For this preliminary experiment, we suspended 130 mg of fluorescent farnesol in 30 ml of 0.5% Tween 80 (equivalent to 20 mM) and injected 1 ml per mouse of the solubilized portion i.p. into 27 mice. At time points of 10 min and 1, 3, 6, 12, 18, 24, 48, and 72 h p.i., three mice were euthanized and their serum (200 μl) collected. Serum of four untreated mice was collected as a negative control.

For each time point, the serum samples were pooled, extracted with ethyl acetate, and fractionated on an HPLC system (ISCO, Lincoln, NE) with a C_{18} reverse-phase column (Vydac) and a fluorescence detector (ISCO) set at an excitation of 360 nm and an emission of 450 nm. We detected twin peaks for fluorescent farnesol at ca. 13.3 and 15.3 min of retention time using acetonitrile and water for the mobile phase. The mobile-phase gradient went from 30 to 50% acetonitrile (1 to 5 min), 50% acetonitrile (5 to 15 min), 50 to 100% acetonitrile (15 to 20 min), and 100 to 30% acetonitrile (20 to 25 min). A standard curve was constructed with fluorescent farnesol added to normal mouse serum at concentrations of 2.3 to 230 μM; more than 98% was detected by HPLC.

***C. albicans* isolation and quantitative determination.** Three mice from each group were euthanized at 1, 3, 6, 12, 18, 24, or 48 h to determine the fungal burden in their kidneys. At the time of necropsy, kidneys were harvested from each mouse and placed in sterile Eppendorf tubes. The tissues were kept at 4°C until the next day, when each kidney was weighed and homogenized in 2.0 ml of nonpyrogenic sterile saline. Then, 0.1 ml of the homogenate was spread on triplicate plates of Nickerson's medium, also known as BiGGY agar, a selective and differential medium for *C. albicans* (30). After 48 h of incubation at 30°C, colony number, morphology, and color were recorded and numbers of CFU per gram of tissue was estimated. *C. albicans* appears as brown to black colonies with no pigment diffusion and no sheen (30).

TABLE 2. Altered pathogenicity by strains of *C. albicans* altered in their levels of farnesol production

Strain	Amt of farnesol produced (μg/g [dry wt])	LD_{50}[a]		
		3 days p.i.	7 days p.i.	14 days p.i.
SN152	10.9 ± 1.7	1.9×10^6	7.9×10^5	5×10^4
KWN1	1.65 ± 0.5	—	—	—
KWN2	1.79 ± 1	7.9×10^6	1.9×10^6	5×10^5
KWN3	11.5 ± 4.4	—	—	—
KWN4	21.2 ± 1.2	2×10^6	—	—
ATCC 10231	Undetected[b]	1.2×10^7	—	—

[a] LD_{50} for KWN4 was obtained from Fig. 4. All other LD_{50}s were calculated (see Materials and Methods) from experiments where mice were inoculated with five different cell densities (10^4 to 10^8 cells/ml). Note that the other two LD_{50}s obtained from Fig. 4 (2×10^6 for both SN152 after 3 days and KWN2 after 7 days) agree very well with the values from the five inoculum-level experiments. —, not done.

[b] See reference 14.

RESULTS

Reduced endogenous farnesol reduces virulence in mice. We disrupted the first *DPP3* locus with *C. dubliniensis HIS1* (Fig. 1A) and then confirmed that it had been inserted correctly by the presence of the expected upstream (Fig. 2, lane B) and downstream (Fig. 2, lane C) *HIS1*/*DPP3* junction sequences. This heterozygous mutant was named KWN1, and it was used to disrupt the other allele of *DPP3* with *C. maltosa LEU2*. This double-knockout mutant was named KWN2. We confirmed that the *LEU2* marker had been inserted correctly by the presence of the expected upstream (Fig. 2, lane D) and downstream (Fig. 2, lane E) *LEU2*/*DPP3* junction sequences. Finally, the overall success of the *DPP3* gene knockout was confirmed by its presence in SN152 and its absence in KWN2

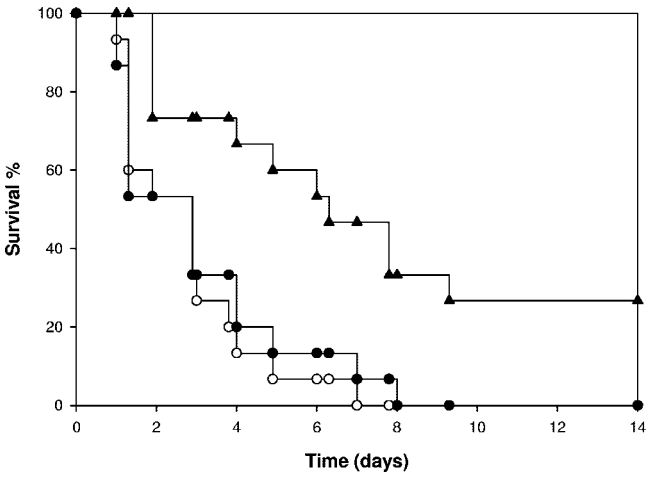

FIG. 4. Altered pathogenicity for *C. albicans* strains with *DPP3* removed and restored. Fifteen mice per group, each injected with 2×10^6 cells. ○, SN152 parent; ▲, KWN2; ●, KWN4.

as determined by both PCR (data not shown) and Southern hybridization (Fig. 3). Survival curves for the two strains were then compared with that for immunocompetent mice. The calculated LD_{50}s for strains SN152 and KWN2 are presented in Table 2. Comparison of the SN152 parent with the *DPP3* double-knockout mutant shows that the mutant produced ca. six times less farnesol and was 4, 2.4, and 10 times less pathogenic to mice at 3, 7, and 14 days, respectively (Table 2).

Pathogenicity was restored when *DPP3* was reconstituted (Fig. 4). The first reconstituted strain (KWN3) regained a single copy of *DPP3* while losing the *His* marker. The *HIS* marker was the first to be integrated in place of one of the *DPP3* alleles (creating KWN1) and the first to be replaced (creating KWN3). Next, KWN3 was transformed, regaining a second copy of *DPP3* while losing the *Leu* marker. KWN4, like SN152, is auxotrophic for Arg, His, and Leu. The accuracy of these genetic manipulations was confirmed by two methods: PCR amplification of the His or Leu junctions and Southern hybridization. Southern hybridization (Fig. 3) confirmed the absence of *DPP3* in KWN2 and the reintegration of *DPP3* in KWN4. For SN152, probes for the up- and downstream regions of *DPP3* (Fig. 1D) hybridized in the southern blot at approximately 6.0 kb and 3.7 kb. The same probes for KWN2 hybridized to fragments at approximately 2 kb and 7 kb, respectively (Fig. 3). SN152 and the complemented strain (KWN4) had one hybridization band of the same size at 3.7 kb (Fig. 3), but the probe corresponding to the 6-kb fragment in SN152 hybridized with a fragment of approximately 5.0 kb in the reconstituted strain KWN4. One reason that the hybridization bands are of different sizes for SN152 and KWN4 (Fig. 3) is that during complementation the *SAT1* flipping technique (37) causes the 460-bp downstream fragment to be duplicated. However, any doubt regarding the correct integration of *DPP3* is removed by the disappearance of the auxotrophic markers which disrupted the gene originally (Fig. 1).

For the reconstituted strains, the farnesol production level was restored to parental levels in KWN3 and doubled in KWN4 (Table 2). With regard to mouse pathogenicity (Fig. 4), the SN152 and KWN4 survival curves were not different from each other ($P < 0.96$), but they were both significantly different ($P < 0.0014$ or 0.0017, respectively) from the KWN2 group. The PHREG hazard ratio estimates indicated that SN152 and KWN4 cells had 4.1 and 4.2 times higher lethality than KWN2 cells, respectively.

Mutants altered in farnesol production have unaltered cell and colony morphology. The four strains altered in their farnesol production levels, KWN1 to -4, appeared normal in their dimorphic abilities. That is, at 30°C they grew as yeasts in both YPD and mGSB media (12) and at 37°C they formed ≥95% germ tubes in 2.5 mM *N*-acetylglucosamine buffer (12) and filamentous colonies on spider medium (24). No further tests on cell morphology were conducted.

Additionally, *C. albicans* ATCC 10231, a natural strain which produces farnesoic acid (35) but not farnesol (14), also exhibited normal cell and colony morphology. However, it did exhibit reduced pathogenicity. The LD_{50} dose for strain 10231 after 3 days was 1.2×10^7 cells (Table 2). The reduced virulence of 10231 compared to those of other wild-type strains was also observed by Kretschmar et al. (19).

Nontoxicity of exogenous farnesol. The safety of farnesol administered orally in the drinking water (20 mM without saline) and intraperitoneally (1 ml of 20 mM) was evaluated. Because farnesol was diluted in Tween 80, the effect of i.p. administration of Tween 80 alone was also evaluated. Farnesol and Tween 80 had a negligible toxic effect on the mice. In each experiment, the weight of individual mice and the consumption of water in control and treated groups were recorded daily for 14 days. Irrespective of the route of administration and the concentration of farnesol and Tween 80, there was no diarrhea or mortality. Furthermore, there were no significant differences in either weight gain or water consumption between the control and treated groups throughout the 14-day observation period. Additionally, significant gross changes were not seen in control and treated mice examined at necropsy on day 14 p.i. These results are consistent with those of a previous study (2), which found that the LD_{50} of i.p. farnesol for mice was 2.95 g/kg of body weight, which corresponds to 75 mg for a 25-g mouse. For comparison, 1 ml of 20 mM farnesol contains only 4.4 mg of farnesol.

Exogenous oral farnesol increases mouse mortality. The effect of farnesol by oral delivery was investigated (Fig. 5). Seven mice per group were challenged with an LD_{50} dose of *C. albicans.* The first group received *C. albicans* and regular drinking water, while the second group received farnesol orally (20 mM farnesol–Tween 80 in the drinking water). Figure 5 demonstrates enhanced mortality ($P = 0.036$) for the group receiving oral farnesol. Thus, exogenous farnesol acts to enhance the virulence of *C. albicans*.

Exogenous i.p. farnesol increases mouse mortality. Farnesol given i.p. affected both the onset of mouse mortality and the percent survival (Fig. 6). Forty mice per group were inoculated i.v. with 1.3×10^6 *C. albicans* cells, with a 1.0-ml i.p. injection of either 20 mM farnesol or 0.5% Tween 80–saline (vol/vol). Farnesol enhanced the lethality of the systemic *Candida* infection; the onset of mortality was 30 h sooner for mice injected with farnesol (Fig. 6). In all cases the kidneys showed the same histopathology regardless of the route (oral versus i.p.) of farnesol delivery. The data were analyzed according to the log-rank, Wilcoxon, and likelihood

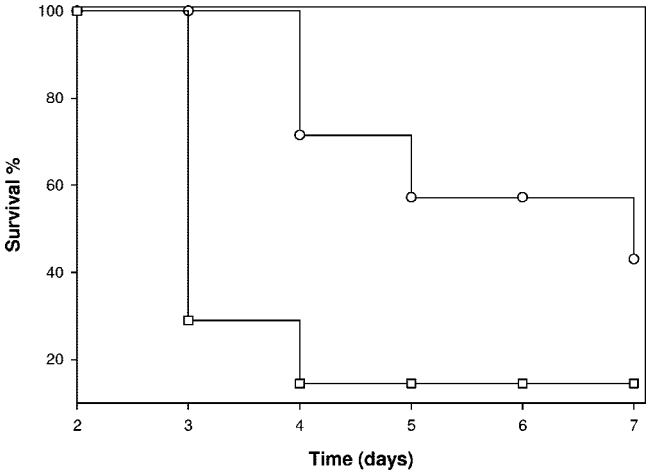

FIG. 5. Effect of orally administered mixed isomers of farnesol on mouse mortality caused by intravenous administration of wild-type *C. albicans* A72. ○, LD_{50} (1.3×10^6) of *C. albicans*-only control; □, LD_{50} of *C. albicans* with farnesol in drinking water; each group contained seven replicates. Farnesol oral and i.v. control groups showed 100% survival.

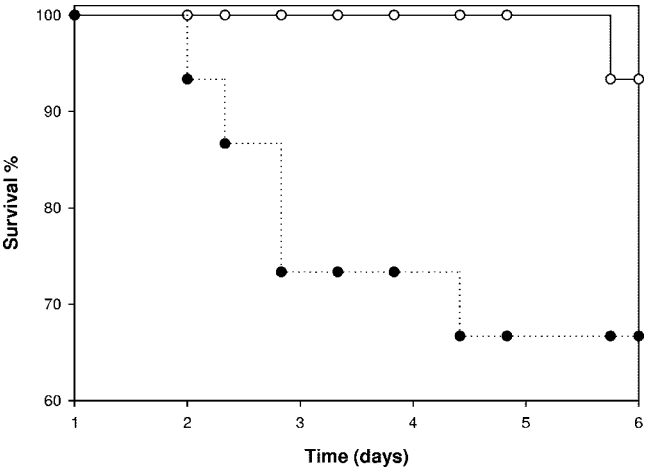

FIG. 7. Effect of i.p. administered *E,E*-farnesol on mouse mortality caused by sublethal levels of wild-type *C. albicans* A72. Each group contained 15 replicates. ○, sublethal *C. albicans* given i.v. with a single i.p. injection (1 ml) of 0.5% Tween 80 in sterile saline; ●, sublethal *C. albicans* with a single i.p. injection (1 ml) of 20 mM farnesol. The farnesol i.p. and 0.5% Tween 80 i.p. controls (with no *C. albicans*) showed no mortality.

ratio tests. All three tests revealed that the survival curves with and without i.p. farnesol differed from each other ($P = 0.031$). In all mouse experiments (Fig. 4 to 7), the farnesol-only, Tween 80-only, and no-treatment control groups (five mice per group) showed no mortality and had similar patterns of weight gain.

A second experiment (Fig. 7) used 15 mice per group with a sublethal *C. albicans* challenge. Both groups were challenged with *C. albicans* and a 1-ml i.p. injection of either farnesol or 0.5% Tween 80–saline (vol/vol). Mice administered farnesol i.p. started to die 4 days earlier, and by day 6 p.i. the percent survival was five times lower than that for the no-farnesol control mice (Fig. 7). The effect of i.p. farnesol administration on percent survival was significant ($P = 0.048$). From this experiment we conclude that farnesol alone is responsible for the enhanced *C. albicans* pathogenesis.

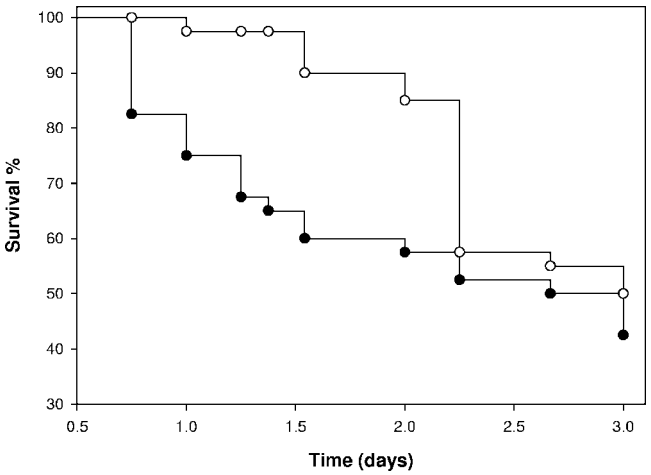

FIG. 6. Effect of i.p. administered *E,E*-farnesol on mouse mortality caused by intravenous administration of wild-type *C. albicans* A72. Each group of treatments had 40 replicates. All control groups contained five replicates. ○, *C. albicans* LD_{50} (1.3×10^6) dose, i.v. only; ●, *C. albicans* LD_{50} and 1 ml of i.p. 20 mM farnesol. Farnesol i.p. control and the 0.5% Tween 80 i.p. control never showed mortality.

Time course of fluorescent farnesol in serum. A fluorescent analog of farnesol with five conjugated double bonds (44) was used to determine how much i.p. farnesol gets into the bloodstream and how long it remains there. Serum samples were analyzed by HPLC. The fluorescent farnesol was detected 10 min following i.p. injection. The levels reached a maximum (ca. 1 μM) after 1 h, were barely detectable at 6 h, and had completely disappeared by 12 h. This rapid appearance and disappearance of the fluorescent farnesol is likely relevant for disease progression, particularly in the early stages following i.p. injection of farnesol (Fig. 6). Robert et al. (38), using very similar experimental conditions, found that 98% of the injected *C. albicans* cells had been cleared from the bloodstream within 3 min and they were almost all gone within 15 min. For Fig. 6 and 7, the farnesol i.p. injections occurred ca. 5 min after the tail vein injections of *C. albicans*.

Exogenous i.p. farnesol speeds up kidney colonization. We examined kidney colonization over time for 48 h p.i. (Fig. 8). Two groups of 24 mice were administered *C. albicans* i.v. accompanied by 1.0 ml of either 0.5% (vol/vol) Tween 80–saline or 20 mM farnesol i.p. Kidneys from mice with i.p. farnesol had $>2 \times 10^4$ CFU/g by 12 h, whereas those from mice without farnesol required 48 h to reach this level (Fig. 8). By this time the entire kidney was fully colonized by *C. albicans*, both grossly and by histopathology. Farnesol i.p. did not alter *C. albicans* morphology in kidneys. Extensive filamentation was observed for all organs examined, i.e., kidney, liver, spleen, and brain. Finally, we note the congruence in timing for the disappearance of fluorescent farnesol from the serum by 12 h, coinciding with the more-rapid kidney colonization (Fig. 8), and more rapid onset of lethality (Fig. 5).

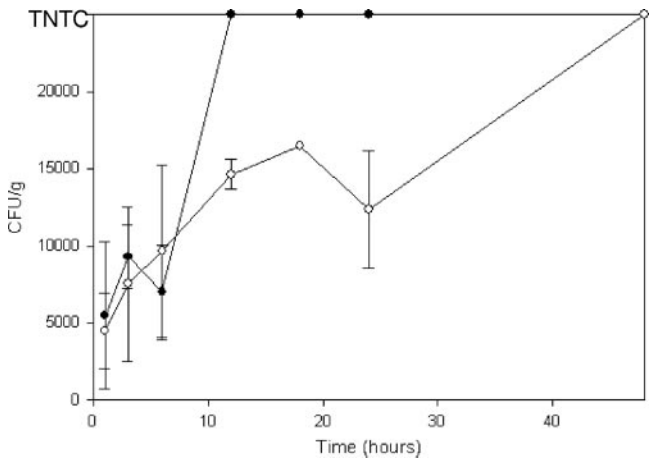

FIG. 8. Time course of kidney infection by *C. albicans* A72 with or without i.p. farnesol. ○, *C. albicans*, 1.3×10^6 cells, i.v. only; ●, *C. albicans*, i.v., with 1 ml of 20 mM farnesol i.p. Each value is the average for three kidneys from three mice. Undiluted kidney homogenate (100 μl) was plated onto BiGGY agar plates (30). Values of 20,000 CFU/g of kidney correspond to actual plate counts of ≥800 CFU, deemed too numerous to count.

DISCUSSION

This work demonstrates that farnesol, both endogenous and exogenous, accelerates the mortality of candidemia. Past studies have shown that *C. albicans* produces farnesol in vitro (12) and that increased production of farnesol in vivo is accompanied by increased virulence of *C. albicans* (27). In this regard, we previously showed that *C. albicans* cells pretreated with 0.5 to 1.0 μM fluconazole produced 6 to 12 times more farnesol (14) and were 4.2 to 8.5 times more lethal (27) than untreated cells. To substantiate this observation on the importance of farnesol, we sought an alternate method for studying the pathogenicity of *C. albicans* cells differing in their farnesol excretion levels. This method employed a knockout mutant defective in the phosphatase which converts farnesyl diphosphate to farnesol (11, 49, 50). Two such genes have been identified for *Saccharomyces cerevisiae*, *DPP1* (50) and *LPP1* (49). Their homologs in *C. albicans* are *DPP3* and *DPP2*, respectively (Candida DB database). We chose to knock out *DPP3*, because in *S. cerevisiae* (11) the rates for dephosphorylation of farnesyl diphosphate catalyzed by particulate fractions from the *lpp1*Δ, *dpp1*Δ, and double-knockout *lpp1*Δ *dpp1*Δ strains were 85, 25, and 8%, respectively (11).

We used the gene deletion system developed by Noble and Johnson (31). This system has the essential feature that the multiply auxotrophic parent (SN152) is still pathogenic for mice (31), and its LD_{50} is close to that of strain A72 (27). We have now shown the following: (i) a double-knockout strain with a mutated *DPP3* gene, which encodes a farnesyl-PP phosphatase (11), produced 6 times less farnesol and was ca. 4.2 times less pathogenic than its SN152 parent; (ii) when *DPP3* was reconstituted, both farnesol production and pathogenicity were regained; (iii) farnesol is harmless by itself; (iv) mice challenged with *C. albicans* in the presence of i.p. farnesol died earlier and had a lower percent survival ($P < 0.03$) than did control mice receiving *C. albicans* with an i.p. injection of Tween 80; and (v) the more-rapid onset of mortality among mice administered farnesol concurrently with *C. albicans* correlated with a more-rapid kidney colonization as measured by higher numbers of CFU of *C. albicans* on kidneys. For these reasons we suggest that farnesol fits the definition of a virulence factor (7) and that it be added to the list of virulence factors for *C. albicans* (9, 28, 33). Although we prefer the idea that *DPP3* exerts its effect on pathogenicity through a change in farnesol levels, we cannot rule out other cell activities that may require the Dpp3 isoprenoid phosphate phosphatase.

C. albicans is a diploid organism. In the progression from SN152 to KWN1 and KWN2, we knocked out one copy of *DPP3* and then the other, while for KWN3 and KWN4, we restored one copy and then another. For *C. albicans* SC5314, the two copies of the *DPP3* structural gene have identical sequences. Yet the farnesol production levels (Table 2) for these mutants suggest that the gene removed in making KWN1 was active whereas that removed in making KWN2 was not and, by extension, KWN3 and -4 were both reconstituted with active genes. The likelihood of this scenario is supported by the high frequency of haploinsufficiency mutants found by Uhl et al. (51) in a transposon mutagenesis study. For these mutants, knocking out a single copy of the gene is sufficient to generate the observed phenotype (51). Our farnesol production data (Table 2) suggest that *DPP3* fits this category.

Our evidence suggests that farnesol, as produced by A72 and most other strains of *C. albicans* (14), contributes to fungal pathogenicity. In contrast, farnesoic acid, as produced by strain 10231 (35), does not. In our parallel tests, the LD_{50} dose for A72 (27) was ca. 10-fold lower than that for 10231 (Table 2). Perhaps some of the *C. albicans* strains found by others to be naturally attenuated in mouse virulence (33) are also deficient in farnesol production. This difference in effectiveness between farnesol and farnesoic acid may be because farnesol is lipophilic and able to cross membranes, whereas farnesoic acid is ionic. Indeed, animals such as rats and mice remove farnesol by converting it to farnesoic acid and a highly soluble farnesol-derived dicarboxylic acid prior to excretion in the urine (3).

It has been established that dimorphism is strongly associated with fungal invasiveness and dissemination for both animal models (25, 43) and, via histopathology, human patients (6). With regard to farnesol's mode of action as a virulence factor, no firm conclusions can be made yet. However, five ideas seem pertinent. First, farnesol is an autoregulator of *Candida* dimorphism in vitro (12). There is, of course, no assurance that the in vitro mode of action preventing germ tube formation and the in vivo mode of action are related. Indeed, the predominant cell types in infected kidneys are filamentous, i.e., pseudohyphae and hyphae. Second, farnesol could promote invasiveness by altering the membrane fluidity of host cells. The lipophilic properties of free farnesol would favor its membrane localization. For instance, Cole et al. (10) describe the likely intercellular transmigration (termed persorption) whereby yeast cells of *C. albicans* cross the mucosal barrier into the bloodstream. As another case in point, Saidi et al. (40) found that gingival fibroblasts lost viability when treated with >10 μM farnesol. Third, if farnesol contributes to the lysis of even a few red blood cells, the iron made available could enhance pathogenicity. Animals often sequester iron in an attempt to starve iron-requiring pathogens (52), and thus,

red blood cell lysis would make critical iron supplies available to those pathogens. *C. albicans* does not produce an iron-binding siderophore (15), but it can use hemin as an iron source (15). The bacterium *Staphylococcus aureus* uses hemolysin to punch holes in host red blood cells and then acquires iron from the hemoglobin released (46). Perhaps *C. albicans* uses farnesol to punch holes in host red blood cells for similar reasons. Fourth, farnesol could protect *C. albicans* cells from destruction following ingestion by professional phagocytes by countering their oxidative burst. Westwater et al. (53) showed that in vitro farnesol protected yeast cells from oxidative stress from both hydrogen peroxide and superoxide anion-generating agents. Fifth, farnesol could interfere with aspects of host defense necessary for the animals to survive pathogenesis. It is known that *C. albicans* infections delay induction of proinflammatory cytokines, thus giving *C. albicans* an advantage during invasion compared with other fungi. For instance, the survival of *Candida glabrata*-infected mice was associated with rapid induction of three proinflammatory cytokines, tumor necrosis factor alpha, interleukin-12, and interferon gamma (4), and of equal importance, the lack of induction for the anti-inflammatory cytokine interleukin-10 (4). Thus, farnesol could interfere with the induction of appropriate cytokines by the host. Finally, these possible mechanisms are not mutually exclusive; farnesol could have multiple modes of action during pathogenesis which are operative at different times.

In summary, we have identified the first gene, *DPP3*, having a role in farnesol production by *C. albicans*, made a knockout mutant of that gene, and shown that the gene product farnesol acts as a virulence factor as well as a quorum-sensing molecule (29). Thus, if there is any antifungal therapeutic potential for farnesol (35) or its analogs (45), they would likely be restricted to topical applications.

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