

Potent Synergistic Effect of Doxycycline with Fluconazole against *Candida albicans* Is Mediated by Interference with Iron Homeostasis

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Doxycycline was found to act synergistically with the antifungal fluconazole against *Candida albicans*. Combination with doxycycline converts fluconazole from fungistatic to fungicidal, prevents the onset of drug resistance, and is also effective against a clinical isolate characterized by elevated resistance to fluconazole. Investigation of the interactions between the two drugs by way of checkerboard assays indicated that doxycycline had an influence on the MIC for fluconazole, as defined by CLSI standards, only at high concentrations (200 $\mu\text{g/ml}$). However, lower concentrations were effective at eliminating residual cell growth at supra-MICs of fluconazole. Using MIC-0, defined as a drug combination resulting in optically clear wells, as an endpoint, doxycycline was found to be synergistic with fluconazole at a concentration as low as 25 $\mu\text{g/ml}$, with a fractional inhibitory concentration index of <0.5 . Doxycycline-mediated growth inhibition can be reversed by externally added iron, indicating that iron depletion may account for the synergism. Consistently, we confirmed old literature data about iron-chelating activity of doxycycline. Synergism of fluconazole with doxycycline does not appear to be mediated by calcineurin, since doxycycline further aggravates the susceptibility to fluconazole of mutants lacking the catalytic or the regulatory subunits of calcineurin. Growth in the presence of fluconazole and doxycycline is restored by an elevated dosage of *ERG11* in *Saccharomyces cerevisiae* but not in *C. albicans*, despite the full competence of the pathogen's protein to act as a suppressor in baker's yeast.

Infections by *Candida albicans* have been on the rise in the past decades, mostly due to changes in the clinical practice, such as the increased use of immunosuppressants and broad-spectrum antibacterial agents (26, 29, 47). Fungal infections represent a challenge for clinicians, because of the scarcity and sometimes the limited efficacy of antifungal drugs (1, 44). Fluconazole and other azole antifungals, targeting ergosterol biosynthesis (23), have been extensively used in the recent clinical practice thanks to their reduced toxicity and lower cost, compared to amphotericin B in its conventional and lipidic forms, respectively (44). The major disadvantage of azole antifungals, however, is their fungistatic nature, an aspect that favors the onset of drug resistance. The development of isogenic strains of *C. albicans* characterized by stepwise increased tolerance to fluconazole in patients undergoing continued treatment with this drug has been documented in several cases (34, 56, 57). For these reasons, the conversion of fluconazole from fungistatic to fungicidal via combinations with other drugs is highly desirable.

In a different perspective, tools for the detailed study of the molecular genetics of *C. albicans* have been greatly improved in recent times (6, 7, 40). Regulatable promoters are available to the research community, to induce or repress gene expression in *C. albicans*. Most of them, such as the *PCK1* and the *MAL2* promoters, allow conditional expression/repression of the gene of interest upon incubation of cells in growth media containing the repressing or the activating carbon source (3, 28). The tetracycline direct and reverse systems, which allow gene induction or repression by addition of doxycycline to the growth medium, have also been successfully used to regulate gene expression in *C. albicans* (38, 45). However, it was found that, compared to other organisms, significantly higher concentrations of doxycycline are necessary for full induction of tetracycline-inducible promoters in *C. albicans* (45).

During the course of unpublished experiments with *C. albicans*

strains carrying tetracycline-regulated promoters, we noticed that doxycycline interfered heavily with cell growth in the presence of fluconazole and decided to investigate further this unexpected interaction. We believe that researchers in the field of *C. albicans* molecular genetics may benefit from the conclusions of our studies.

MATERIALS AND METHODS

Strains and media. The strains used in this study are listed in Table 1. Most experiments were conducted with the *Candida albicans* reference strain SC5314 (18). Yeast (*Saccharomyces cerevisiae*) and *C. albicans* strains were routinely refreshed from frozen stocks at -80°C and maintained on YPD (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) plates. Experiments were carried out using minimal medium (1.7 g/liter Difco yeast nitrogen base without ammonium sulfate, 5 g/liter ammonium sulfate) supplemented with 2% glucose (synthetic defined [SD] medium), 3% glycerol, and 3% ethanol (SEG), 2% galactose (SGal), or 2% Casamino Acids (SCAA). Minimal medium was supplemented with uridine when *ura3 Δ /ura3 Δ* strains of *C. albicans* were used. RPMI 1640 medium with L-glutamine without sodium bicarbonate (Sigma) was buffered with 0.165 M morpholinepropanesulfonic acid (MOPS). Glucose at the final concentration of 2% was added only to RPMI agar medium. Fetal bovine serum (FBS) medium consisted of 10% fetal bovine serum in sterile Milli-Q water (Millipore). Media were solidified with 2% agar. Sabouraud dextrose agar medium was bought from Fluka. Minimal medium for yeast experiments was supplemented with histidine, lysine, leucine,

Received 28 October 2011 Returned for modification 3 January 2012

Accepted 27 April 2012

Published ahead of print 7 May 2012

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doi:10.1128/AAC.06017-11

TABLE 1 Fungal strains used in this study

Strain	Genotype	Comment	Source or Reference
<i>Candida albicans</i>			
SC5314	Wild type		18
CAI4	<i>ura3Δ::imm434/ura3Δ::imm434</i>		16a
AFA59b	<i>ura3Δ::imm434/ura3Δ::imm434 RPS10::pAFC89b</i>	CAI4 carrying extra copy of <i>CaERG11</i> under <i>ACT1</i> promoter	This study
AFA60a	<i>ura3Δ::imm434/ura3Δ::imm434 RPS10::Clp10</i>	CAI4 transformed with <i>URA3</i> vector	This study
AFA63a	<i>ura3Δ::imm434/ura3Δ::imm434 RPS10::pAFC92a</i>	CAI4 carrying extra copy of <i>CaERG11</i> under <i>PCK1</i> promoter	This study
AFA21	<i>ura3Δ::imm434/ura3Δ::imm434 RPS10::pPCK1-GFP</i>	CAI4 transformed with <i>URA3-PCK1</i> vector	This study
FH5		Clinical isolate, highly resistant to fluconazole	34, 57
DSY2091	<i>ura3Δ::imm434/ura3Δ::imm434 cna1Δ::hisG-URA3-hisG/cna1Δ::hisG</i>	<i>cna1Δ/cna1Δ</i> mutant	55
DSY2115	<i>ura3Δ::imm434/ura3Δ::imm434 cna1Δ::hisG/cna1Δ::hisG LEU2::CNA1::URA3</i>	<i>CNA1</i> reintegrant	55
DSY2146	<i>ura3Δ::imm434/ura3Δ::imm434 cna1Δ::hisG/cna1Δ::hisG LEU2::CNA1tr::URA3</i>	<i>CNA1tr</i> reintegrant	55
<i>Saccharomyces cerevisiae</i> BY4742	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>	Reference strain	Euroscarf

and uracil at appropriate concentrations (59). Leucine and uracil were omitted from media for the experiments shown in Fig. 5A and D, respectively. All plate tests for *C. albicans* were carried out at 37°C; plate tests for *S. cerevisiae* were carried out at 30°C. The standard concentration of fluconazole on plates was 10 µg/ml, whereas that of doxycycline was 50 µg/ml. Indications are given in the text for experiments conducted with different drug concentrations.

Fluconazole, doxycycline, tetracycline, gentamicin, neomycin, potassium cyanide, ferric chloride hexahydrate, ferric citrate, zinc sulfate, and bathophenanthroline disulfonic acid (BSP) were added, starting from concentrated solutions, to autoclave-sterilized, precooled agar media. With the exception of doxycycline, which was dissolved in 50% ethanol, all of the chemicals mentioned above were dissolved in Milli-Q water.

MIC testing in broth microdilution assays. The fluconazole MIC was determined according to the approved CLSI standard reference method for broth dilution antifungal susceptibility testing of yeasts M27-A3 (10). Inocula of *C. albicans* SC5314 and FH5 were prepared from cultures grown overnight on Sabouraud medium, adjusted to obtain final cell suspensions of 0.5×10^3 to 2.5×10^3 CFU/ml. Viable counts of the inocula were verified by plating serial dilutions on YPD plates. MIC plates were incubated at 35°C for 48 h for the reference strain SC5314 and for 24 h for strain FH5. MIC endpoints were defined as the lowest concentration of fluconazole causing a >50% decrease in optical density (prominent decrease in turbidity, or score of 2, according to CLSI guidelines) and a >90% decrease in viability compared to the drug-free control. Optical densities were recorded at 540 nm using a Molecular Devices SpectraMax Plus 384 absorbance microplate reader. Viable counts were measured by plating 100 µl of 10^{-1} to 10^{-5} serial dilutions on YPD plates. For viable count checks of optically clear wells, 100 µl of the undiluted wells' content was also plated (8, 33). Where appropriate, comparison of results obtained from plating $10^{-1}/10^{-2}$ dilutions and from 100 µl of undiluted wells' content did not evidence a significant influence of the drugs' carry-over in our experimental conditions. Experiments were repeated a minimum of three times.

Checkerboard assays. The interaction of doxycycline with fluconazole was investigated by way of checkerboard tests on 96-well plates, performed according to the CLSI approved standard M27-A3 (10), with 48-h incubations for strain SC5314 and 24-h incubations for strain FH5. For SC5314, the fluconazole and doxycycline concentrations tested ranged from 0.03 to 32 µg/ml and from 3.125 to 200 µg/ml, respectively. For FH5, fluconazole concentrations ranged from 0.125 to 128 µg/ml and

doxycycline concentrations ranged from 25 to 800 µg/ml. Concentrations of doxycycline higher than 200 µg/ml were repeatedly found to cause cloudiness of the RPMI medium, altering spectrophotometric readings and the general outcome of experiments (our unpublished observations). Results obtained with such concentrations of doxycycline were therefore not considered further. Viable count determinations were performed as described above. Elimination of fluconazole tolerance, defined as incomplete growth inhibition at supra-MICs of fluconazole, by doxycycline, allowed the use of MIC-0 as an endpoint. MIC-0 was defined as the lowest drug combination resulting in optically clear wells. The fractional inhibitory concentration (FIC) index was calculated as the sum of the FICs of either drugs ($MIC_{\text{fluconazole}} + doxycycline / MIC_{\text{fluconazole}} + MIC_{\text{doxycycline}} + fluconazole} / MIC_{\text{doxycycline}}$). Drug interactions were classified as synergistic when the FIC value was ≤ 0.5 and antagonistic when the FIC value was > 4 (22, 41). FIC values that were > 0.5 but ≤ 4 were not considered informative for the establishment of interactions (22, 41). The MIC for doxycycline, which was > 200 µg/ml experimentally, was considered to be 400 µg/ml for calculation purposes. Similarly, the MIC-0 values for fluconazole and doxycycline, which were > 32 µg/ml and > 200 µg/ml, respectively, were considered to be 64 and 400 µg/ml. Experiments were repeated a minimum of three times.

MIC testing using Etest strips. Determination of fluconazole MICs using Etest strips (AB Biodisk) was carried out on RPMI-glucose plates. Overnight cultures in YPD were diluted in sterile saline to an optical density at 600 nm (OD_{600}) of 0.1 and plated using sterile swabs, following the directions from the manufacturer. Plates were incubated at 37°C for 48 h after the overlay of strips. Results of Etest measurement are normally considered in agreement with those of broth microdilution tests when discrepancies between MIC values are not higher than 2 dilutions (48).

Spot tests and resistance-to-fluconazole assays. Cultures were grown overnight in minimal glucose medium, diluted to an OD_{600} of 1, and serially diluted in presterilized, flat-bottom 96-well plates. Ten-fold (*C. albicans*) or 5-fold (*S. cerevisiae*) dilutions were spotted onto the indicated media using a spotter (Sigma). The spot test presented in Fig. 2A was performed by spotting 5 µl of each microtiter well using a multichannel pipette. Plates were incubated for the time indicated in the figure legends at 37°C (*C. albicans*) or 30°C (*S. cerevisiae*). Plates containing fluconazole and/or doxycycline were used within 24 h from preparation. All spot tests presented in this work were repeated a minimum of three times. For experiments on resistance to fluconazole, 10^4 cells of *C. albicans* SC5314

were spread on SD medium containing 128 $\mu\text{g/ml}$ fluconazole, with or without 50 $\mu\text{g/ml}$ doxycycline. Plates were incubated at 37°C for 4 days.

Determination of fungicidal activity. Determination of fungicidal activity was performed in microtiter plates using the standard CLSI M27-A3 procedure for MIC determination (10), with the exception that larger inocula of 10^4 CFU/ml were used (8, 49). Plates were incubated for either 48 or 96 h. Viable count checks were carried out by plating 100 μl of the appropriate dilutions or 100 μl of undiluted wells' content (for optically clear wells) on YPD plates. Both kinds of experiments were repeated three times.

Iron chelation assay. Iron chelation was assayed using the colorimetric SideroTec assay kit from Emergen Bio (Maynooth, Ireland), following the manufacturer's instructions. Briefly, fluconazole, tetracycline, and doxycycline at concentrations of 25, 50, 100, 200, and 400 $\mu\text{g/ml}$ were mixed with the provided reagents in flat-bottom 96-well plates and incubated at room temperature for 15 min. Plates were read at 630 nm on the above-mentioned plate reader. Decreases in absorbance are inversely proportional to iron chelation activity. Milli-Q water was substituted for the chemicals in the negative control.

Genomic screen for multicopy suppressors of susceptibility to fluconazole plus doxycycline. We transformed the *S. cerevisiae* genomic library in vector pFL44 constructed in the laboratory of F. Lacroute (60) in the yeast strain BY4742 by the lithium acetate procedure. One aliquot of each transformation mixture was plated on SD medium containing histidine, leucine, and lysine for estimation of the transformation efficiency. The remaining transformation mixture was outgrown for 4 h in liquid YPD before being spread on plates the same as those described above containing 10 $\mu\text{g/ml}$ fluconazole and 50 $\mu\text{g/ml}$ doxycycline. Truly resistant transformants were separated from false positives by further restreaking them under selective conditions and by cosegregation analysis of resistance to fluconazole plus doxycycline with the presence of a transforming plasmid. Interesting plasmids were extracted from yeast, transformed in *Escherichia coli*, and retransformed into BY4742 for confirmation of their suppressor activity. Genomic inserts of suppressor plasmids were sequenced using oligonucleotides M13F (5'-GTAAAACGACGGCCAG-3') and M13R (5'-CAGGAACAGCTATGAC-3'), identifying an ~5.4-kb fragment containing incomplete *SOD2*, *YHR007*, *ERG11*, and incomplete *STP2*. Reintroduction of pAFC88 (described further below) into BY4742 and subsequent suppression of the susceptibility to fluconazole plus doxycycline of the resulting transformants confirmed *ERG11* as the suppressor gene.

Plasmids used in this study. A HindIII-SmaI fragment, containing *ERG11* surrounded by ~730 nucleotides at its 5' end and ~670 nucleotides at its 3' end, was excised from the Lacroute library suppressor plasmid and cloned into YEplac181 digested with HindIII and SmaI (17), creating plasmid pAFC88. For overexpression studies in *C. albicans*, *CaERG11* was amplified from genomic DNA of strain SC5314 using primers CaERG11s (5'-CCCAAGCTTATGGCTATTGTTGAAACTGTCA-3') and CaERG11as (5'-GCGGCTAGCTGAATCGAAAGAAAGTTGCCG-3'). The amplified fragment was subcloned with HindIII-NheI, replacing the luciferase gene, in plasmid Cip10::ACT1p-gLUC59 (15), and in plasmid pPCK1-GFP (5), replacing the green fluorescent protein (GFP) gene. The resulting plasmids are named pAFC89b (*ACT1p-CaERG11*) and pAFC92a (*PCK1p-CaERG11*). Cloned *CaERG11* was sequenced to verify the absence of mutations.

For cross-complementation experiments, *CaERG11* was extracted with HindIII-NheI from pAFC89b, blunted with the Klenow enzyme, and subcloned into SmaI pBEVY-GL (35), creating plasmid pAFC99a.

Quantitative real-time PCR. Cells of the AFA59b, AFA60a, and AFA63a strains were grown to mid-log phase in minimal glucose medium before their RNA was extracted. For AFA63a, cells were collected and split, and each half was resuspended in either SD or SCAA medium and then outgrown for 4 h before RNA extraction. cDNA was prepared from DNase-treated RNA samples with the Promega A3500 reverse transcription kit. Quantitative PCR was performed on a StepOne Plus real-time

PCR system (Applied Biosystems) using the Kapa SYBR Fast kit (Kapa Biosystems). The fold regulation of each target gene was calculated using the threshold cycle ($\Delta\Delta C_T$) method, using expression of the *TEF1* to normalize data. The C_T value of *TEF1* was subtracted from that of *CaERG11* to calculate the ΔC_T . The ΔC_T value of AFA60a was used as a reference and was therefore subtracted from the ΔC_T value of the other samples to obtain $\Delta\Delta C_T$ values. Gene expression levels relative to the reference were expressed as $2^{-\Delta\Delta C_T}$. The primers used were *CaERG11up* (5'-TTACCTCA TTATTGGAGACGTGATG-3'), *CaERG11down* (5'-CACGTTCTCTTC TCAGTTTAATTTCTTTC-3'), *TEF1a-fw* (5'-CCACTGAAGTCAAGTC CGTTGA-3'), and *TEF1a-rv* (5'-CACCTTCAGCCAATTGTTTCGT-3').

RESULTS

Doxycycline acts synergistically with fluconazole in a dosage-dependent manner. Growth of wild-type *Candida albicans* SC5314 cells is prevented in minimal medium containing a combination of fluconazole (10 $\mu\text{g/ml}$) and doxycycline (50 $\mu\text{g/ml}$) at concentrations at which these chemicals cause either no growth defects (doxycycline) or only have a mild one (fluconazole) when used separately (Fig. 1). Growth inhibition exerted by doxycycline in the presence of fluconazole is dosage dependent (Fig. 1A), suggesting that the action of doxycycline is specific. No growth of any kind was observed on fluconazole-doxycycline plates containing 50 $\mu\text{g/ml}$ doxycycline, even when these were incubated for up to 10 days (our unpublished observations). Tests on different media, such as RPMI-glucose, YPD, and fetal bovine serum-containing medium, produced identical results (Fig. 1B), demonstrating that growth inhibition is not dependent on the composition of the medium. Similar results were also obtained when glucose was replaced with glycerol and ethanol in the medium (Fig. 1B), indicating that growth inhibition is not dependent on the carbon source. Finally, tests in liquid media produced the same results as those on solid media (our unpublished observations).

Tetracycline acts synergistically with fluconazole with minor efficiency. Tetracyclines are antibacterial drugs that prevent the association of acylated tRNAs with the ribosome, therefore inhibiting translation (9). Since doxycycline is a derivative of tetracycline with improved pharmacokinetic properties, we decided to test whether amelioration of the antifungal properties of fluconazole was an exclusive feature of doxycycline or whether it could be extended to tetracycline.

Tetracycline proved to be also synergistic with fluconazole, albeit with reduced potency (Fig. 1C). In fact, even though an inhibitory effect of fluconazole plus tetracycline could be observed against *C. albicans*, this was less robust than that observed with doxycycline, given that a higher concentration of tetracycline was required to prevent fungal growth. Notably, the 200- $\mu\text{g/ml}$ tetracycline concentration that appeared to completely prevent growth of *C. albicans* on solid medium was considerably less efficient in liquid medium (our unpublished observations). Gentamicin and neomycin, two unrelated antibiotics that also prevent bacterial translation, showed no effect in combination with fluconazole against *C. albicans* (Fig. 1C).

Doxycycline eliminates fluconazole tolerance. Using the CLSI standard reference method (M27-A3) for broth dilution antifungal susceptibility testing of yeasts (10), the MIC for fluconazole of standard wild-type strain SC5314 was 0.5 $\mu\text{g/ml}$, whereas the MIC of doxycycline alone was >200 $\mu\text{g/ml}$. In order to verify the nature of interactions between the two drugs, fluconazole and doxycycline at concentrations ranging from 0.03 to 32 $\mu\text{g/ml}$ and from 6.25 to 200 $\mu\text{g/ml}$, respectively, were tested in all possible

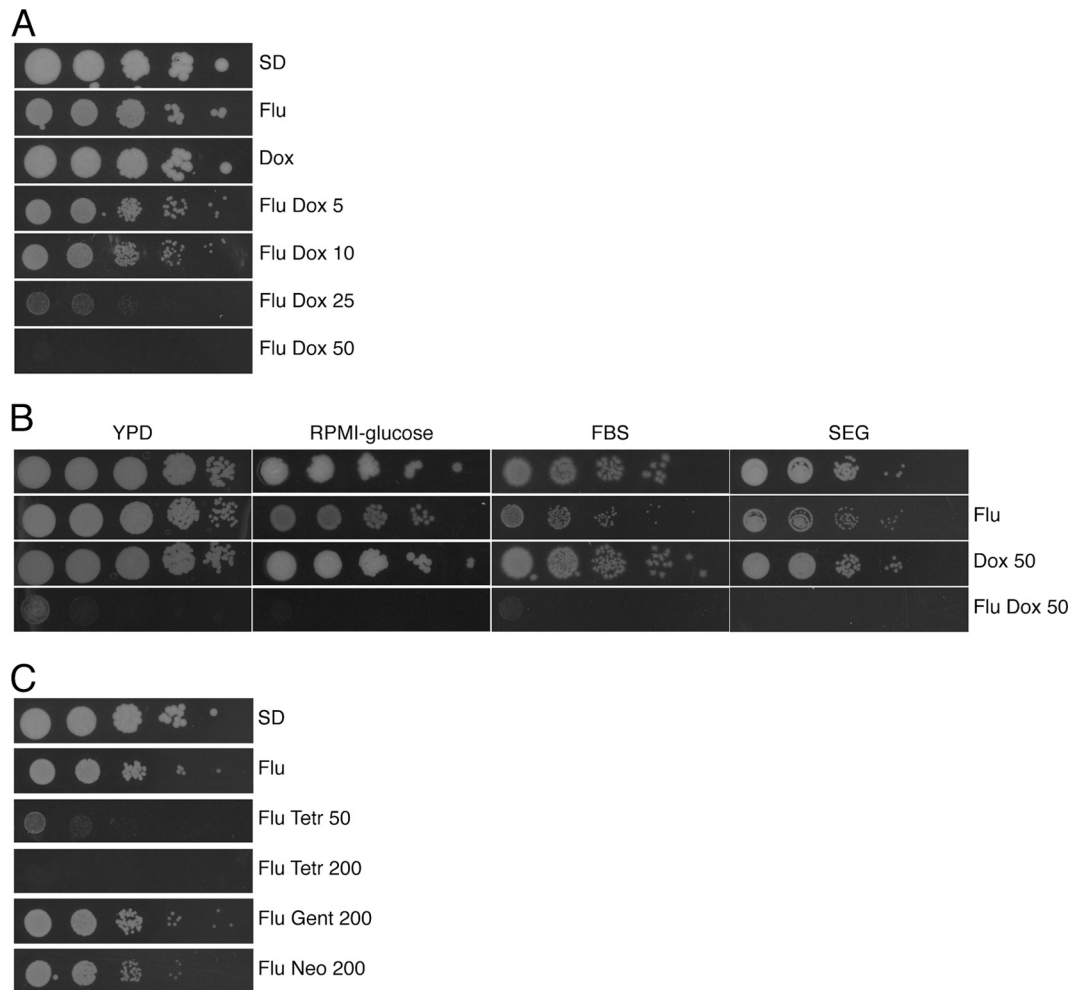


FIG 1 Doxycycline synergizes with fluconazole in a dosage-dependent and medium- and carbon source-independent manner. Cells of the *C. albicans* reference strain SC5314 were spotted onto plates containing different drug combinations. Where indicated, fluconazole (Flu) is present at 10 µg/ml, whereas the concentration of doxycycline (Dox) is expressed in µg/ml. (A) SD plates with 3 days of incubation. (B) YPD plates with 1 day of incubation, RPMI-glucose plates and FBS plates with 2 days of incubation, and SEG plates with 5 days of incubation. (C) SC5314 cells were spotted onto SD plates containing fluconazole and either tetracycline (Tetr), gentamicin, or neomycin. The concentration of the antibiotics is indicated in µg/ml. Plates were incubated for 2 days before being scanned.

concentration permutations by using checkerboard microtiter assays.

Doxycycline reduced the MIC for fluconazole 1-fold (0.25 µg/ml) when added at 200 µg/ml (not shown) and led to no detectable changes at lower concentrations (described further below). Hence, calculation of the fractional inhibitory concentration index (see Materials and Methods) produced values of >0.5 for concentrations of doxycycline that were ≤200 µg/ml, failing to indicate synergism with fluconazole (22, 41).

Growth of strain SC5314 at supra-MICs of fluconazole, a phenomenon referred to as “tolerance” (55), is commonly observed, fluconazole being only mildly fungistatic against this strain (33). These observations were confirmed in our experiments: Figure 2A shows robust recovery of cells incubated for 48 h with fluconazole alone at all concentrations tested (bottom row of cells) and decreases in viability in the presence of doxycycline. Starting from inocula of 10³ CFU/ml, viable counts indicated increases of 3 and 2 logs, respectively, for cells incubated in the absence or presence of 0.5 µg/ml fluconazole (Fig. 2B). In contrast, doxycycline fur-

ther reduced growth in the presence of fluconazole by 1 log, and resulted in optically clear wells (Fig. 2B). Addition of 25 µg/ml doxycycline stably reduced the viable counts by 1 log and was associated with a strong potentiation of the fungistatic power of fluconazole (Fig. 2B). Higher concentrations of doxycycline caused further reductions in viable counts at supra-MICs of fluconazole, to levels just above the detection limit of 10¹ CFU/ml (50 µg/ml doxycycline) (Fig. 2B) or below that (100 µg/ml doxycycline) (Fig. 2B).

These observations indicate that tolerance to fluconazole is lost in the presence of doxycycline and that killing of *C. albicans* at supra-MICs of fluconazole occurs in the presence of >25 µg/ml doxycycline. Since in these experiments, determination of the 99.9% killing activity that classically defines the minimal fungicidal concentration (MFC) (16) was not possible, the MFC could not be used as an endpoint. Nevertheless, the ~3-log decrease in cell viability, compared to that of the drug-free control, observed at supra-MICs of fluconazole in the presence of 25 µg/ml doxycycline (Fig. 2B) met the requirements that define optically clear

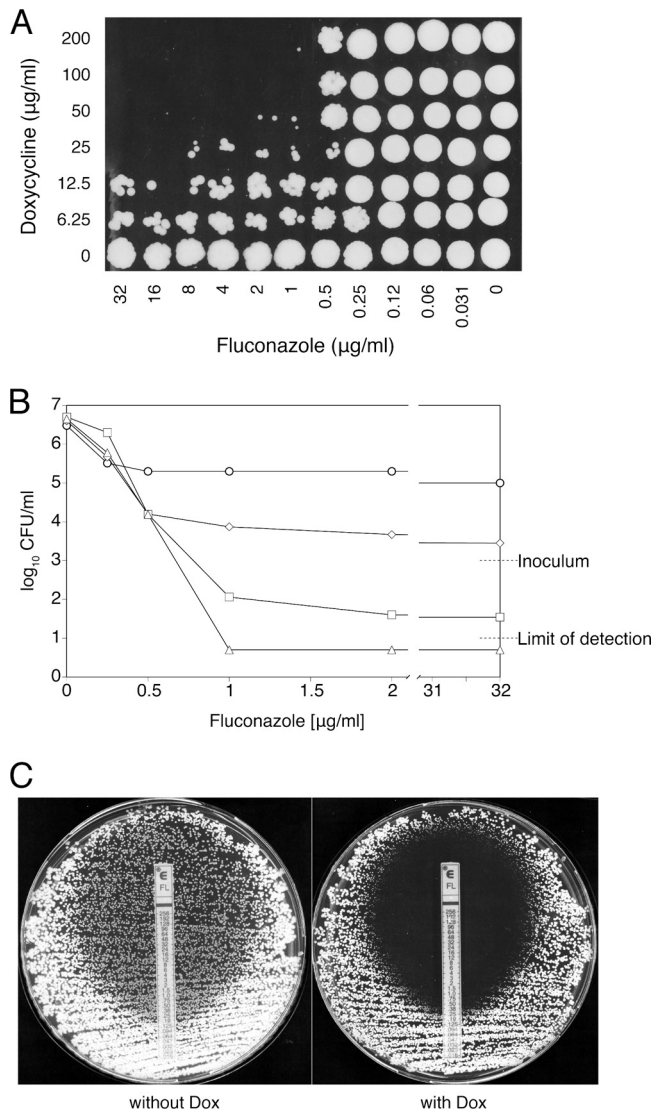


FIG 2 Doxycycline eliminates fluconazole tolerance. (A) A 5- μ l concentration of each well from one of the checkerboard assays was spotted onto a YPD plate after 48 h of incubation, to monitor the viability of drug-treated cells. The recovery plate was incubated for 2 days before being scanned. (B) Viable cell counts after 48 h of incubation in the presence of fluconazole alone or with 25, 50, or 100 μ g/ml doxycycline added (corresponding to rows 7, 4, 3, and 2 in panel A, respectively). Circles, fluconazole alone; diamonds, fluconazole plus 25 μ g/ml doxycycline; squares, fluconazole plus 50 μ g/ml doxycycline; triangles, fluconazole plus 100 μ g/ml doxycycline. Values falling below the detection limit of 10^1 CFU/ml were approximated to 0.5×10^1 CFU/ml. Mean values from three independent experiments are shown. (C) Etest measurement of the fluconazole MIC of strain SC5314 on RPMI-glucose plates. The left panel shows medium without doxycycline, and the right panel shows medium with 50 μ g/ml doxycycline.

wells according to the CLSI guidelines (10), making possible the use of MIC-0 as an endpoint. Given the MIC-0 value of fluconazole alone as >32 μ g/ml and the MIC-0 value when tested in combination with doxycycline as 2 μ g/ml, and with the MIC-0 values of doxycycline as >200 μ g/ml alone and as 25 μ g/ml in combination with fluconazole, recalculation of the FICs resulted in values of 0.031 for fluconazole and 0.063 for doxycycline. The recalculated FIC index of 0.094 indicates synergism between fluconazole and doxycycline.

Fluconazole MIC testing using Etest strips has been reported to deliver results comparable to those obtained using the broth microdilution method, within a 2-dilution margin of discrepancy (48). Consistent with the results reported above for broth microdilution MIC determinations, 50 μ g/ml doxycycline did not affect the fluconazole MIC of strain SC5314 in Etest measurements (Fig. 2C). However, the robust growth of smaller colonies within the fluconazole inhibition ellipse that is normally observed in these assays (48) was completely eliminated in the presence of the antibacterial (Fig. 2C). Clearing of the subpopulation of cells growing inside the fluconazole inhibition ellipse by doxycycline represents another piece of evidence of the interference of doxycycline with cell growth at supra-MIC fluconazole concentrations.

Taken together, results obtained by broth microdilution tests and Etests indicate that doxycycline potentiates the activity of fluconazole abrogating tolerance to the antifungal.

Doxycycline converts the action of fluconazole from fungistatic to fungicidal. Given its fungistatic nature against *C. albicans*, fluconazole is unable to eradicate fungal infections, and the presence of elevated levels of the drug tends to favor the onset of drug resistance. Experiments shown in Fig. 2 indicated that killing of *C. albicans* cells occurred upon treatment with supra-MICs of fluconazole in the presence of 50 and 100 μ g/ml doxycycline (96.5% and $>99\%$ killing, respectively, with an initial inoculum of 10^3 CFU/ml). In order to test whether killing of *C. albicans* by the fluconazole-doxycycline combination could reach the minimal fungicidal concentration (MFC) defined by 99.9% killing (16), cell survival was tested using the CLSI procedure for MIC determinations, with the modification proposed by Canton et al. (8). This consisted of using initial inocula of 10^4 CFU/ml. Sampling of 100 μ l of the well's volume under these conditions allows the detection of survival rates up to 10^1 CFU/ml, corresponding to a decrease in viability of 3 logs (99.9% killing) (49). Using this procedure, no CFU could be recovered from wells containing fluconazole at ≥ 1 μ g/ml in the presence of 100 μ g/ml doxycycline (Fig. 3A), indicating a killing activity of $>99.9\%$ of initial cells. Under similar fluconazole conditions, 50 μ g/ml doxycycline led to an average detection of 2×10^1 to 4×10^1 CFU/ml (Fig. 3A), indicating 99.6 to 99.8% killing activity. However, it has to be noted that a long incubation period (96 h) was necessary to obtain such a high percentage of killing. At standard 48-h incubations, a strong fungistatic effect was observed for 50 μ g/ml doxycycline (Fig. 3A), whereas for 100 μ g/ml doxycycline, cell viability was already below the initial inoculum (Fig. 3A). These results corroborate those presented in Fig. 2 and demonstrate that addition of doxycycline renders fluconazole fungicidal.

Doxycycline prevents the onset of resistance to fluconazole. In *Candida albicans*, resistance to fluconazole has been documented to arise via different mechanisms, including mutations in *ERG11* (encoding the drug target protein lanosterol 14α -demethylase) or increased expression of multidrug transporters and efflux pump-encoding genes such as *CDR1*, *CDR2*, and *MDR1* (reviewed in references 1, 36, and 58). In recent years, it has become clear that in patients, resistance of *C. albicans* to fluconazole develops as the result of multiple genetic alterations (30, 57).

To test whether the presence of doxycycline could also influence the onset of resistance to fluconazole, we plated large numbers of *C. albicans* cells on media containing a high concentration of fluconazole (128 μ g/ml), with and without doxycycline. As observed in Fig. 3B, a large number of colonies became visible on

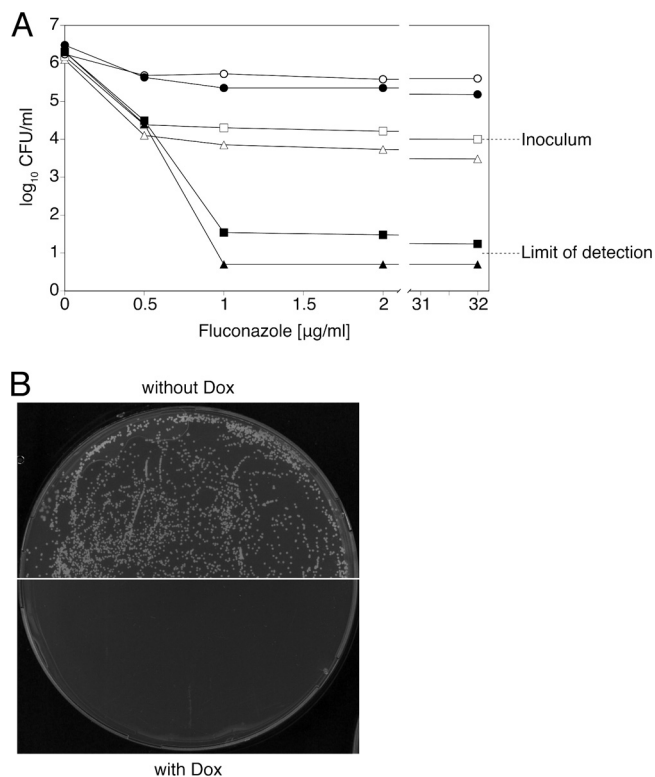


FIG 3 Doxycycline converts fluconazole from fungistatic to fungicidal and prevents the onset of drug resistance. (A) Viable cell counts after 48 h or 96 h of incubation in the presence of fluconazole alone or with 50 or 100 µg/ml doxycycline added. Open symbols, viable cell counts after 48 h of incubation; filled symbols, viable cell counts after 96 h of incubation. Circles, fluconazole alone; squares, fluconazole plus 50 µg/ml doxycycline; triangles, fluconazole plus 100 µg/ml doxycycline. Values falling below the detection limit of 10^1 CFU/ml were approximated to 0.5×10^1 CFU/ml. Mean values from three independent experiments are shown. (B) Emergence of fluconazole-resistant strains on plates containing 128 µg/ml fluconazole without (top) or with (bottom) 50 µg/ml doxycycline. Plates were incubated for 4 days before being scanned.

plates containing fluconazole only. However, no resistant colonies were present on plates that also contained doxycycline after 4 days (Fig. 3B) nor after 10 days of incubation (our unpublished observations).

Thus, the fluconazole-doxycycline combination is instrumental in the prevention of cellular resistance to fluconazole, a result that is likely to arise from the conversion of fluconazole to a fungicidal drug. In other words, cells of *C. albicans* may be killed before resistance-conferring mutations can be fixed by cell proliferation.

The fluconazole-doxycycline combination is also effective on a clinical isolate of *Candida albicans* highly resistant to fluconazole. Clinical isolate FH5, isolated from a patient who had undergone marrow transplantation (34), was described as extremely resistant to fluconazole (57). Resistance in this strain is considered to be the result of isochromosome 5L duplication and loss of heterozygosity at the *TAC1* gene locus, events that generated four copies of a nonhyperactive *ERG11* allele and four copies of the hyperactive *TAC1-7* allele (57). When FH5 was tested for growth in fluconazole-doxycycline-containing medium, it proved able to grow in the presence of the standard concentrations of 10 µg/ml fluconazole and 50 µg/ml doxycycline (Fig. 4A). However,

growth inhibition was observed when the concentration of both chemicals was raised 4-fold (Fig. 4A), although it has to be noted that, under these conditions, growth was severely retarded but not completely abolished (data not shown). Interestingly, while stepwise increases in the concentration of doxycycline alone did not affect the fitness of strain FH5, they imparted detectable growth retardation on the reference strain SC5314 (Fig. 4A) (see Discussion).

Interactions between fluconazole and doxycycline were tested by way of checkerboard tests also with strain FH5. In broth microdilution assays, 50 to 100 µg/ml doxycycline had little effect on the MIC for fluconazole of this strain, whereas a reduction from 64 to 16 µg/ml was observed in the presence of 200 µg/ml doxycycline (data not shown). Similar results were obtained by measuring the MIC for fluconazole by way of Etest strips on RPMI-glucose (Fig. 4B). The effect of doxycycline on growth at supra-MICs of fluconazole could not be established, since this phenomenon is only minimally present in FH5 (our unpublished observations).

These observations indicate that the fluconazole-doxycycline combination can also be effective against strains with increased resistance to fluconazole.

Genomewide screen for suppressors of fluconazole-doxycycline susceptibility in *Saccharomyces cerevisiae* reveals suppression by increased dosage of *ERG11*. *Saccharomyces cerevisiae* and *Candida albicans* are estimated to have diverged ~800 million years ago (21). Baker's yeast offers a number of tools for genetic studies that cannot be performed with *C. albicans*, because of the diploid nature of the latter and the absence of a known sexual cycle. For this reason, a number of studies of pathogenic fungi, including susceptibility/resistance toazole antifungals, have been conducted using *S. cerevisiae* as a tool, with the aim of translating the obtained results to pathogenic species (36, 58). In order to verify whether we could use baker's yeast for genetic studies on the susceptibility to fluconazole-doxycycline, we checked whether yeast was also susceptible to this combination. Addition of doxycycline to fluconazole plates inhibited growth of *S. cerevisiae* wild-type strain BY4742, with some differences with respect to *C. albicans*. First, a dosage of chemicals higher than the standard 10 µg/ml fluconazole and 50 µg/ml doxycycline was necessary to obtain more sensitized experimental conditions (i.e., to eliminate background growth) (Fig. 5A); second, growth in fluconazole-doxycycline was severely retarded but not completely abolished, as in the case of the pathogen (our unpublished observations). Furthermore, while we never observed the onset of stable clones of *C. albicans* resistant to fluconazole plus doxycycline, colonies of *S. cerevisiae* appeared at high frequency on plates containing this drug combination. These colonies proved to be true resistant mutants at a further analysis, since their fluconazole-doxycycline-resistant phenotype was maintained after repeated passages under nonselective conditions (absence of fluconazole plus doxycycline) (our unpublished observations).

In order to identify multicopy suppressors of fluconazole-doxycycline susceptibility, BY4742 was transformed with a yeast genomic library in a 2 µm plasmid (60), and transformations were plated on minimal medium containing fluconazole plus doxycycline. Putative suppressors were separated from false positives by restreaking them on the same medium and by allowing them to lose the transforming plasmids upon growth under nonselective conditions. Strains for which uracil prototrophy cosegregated

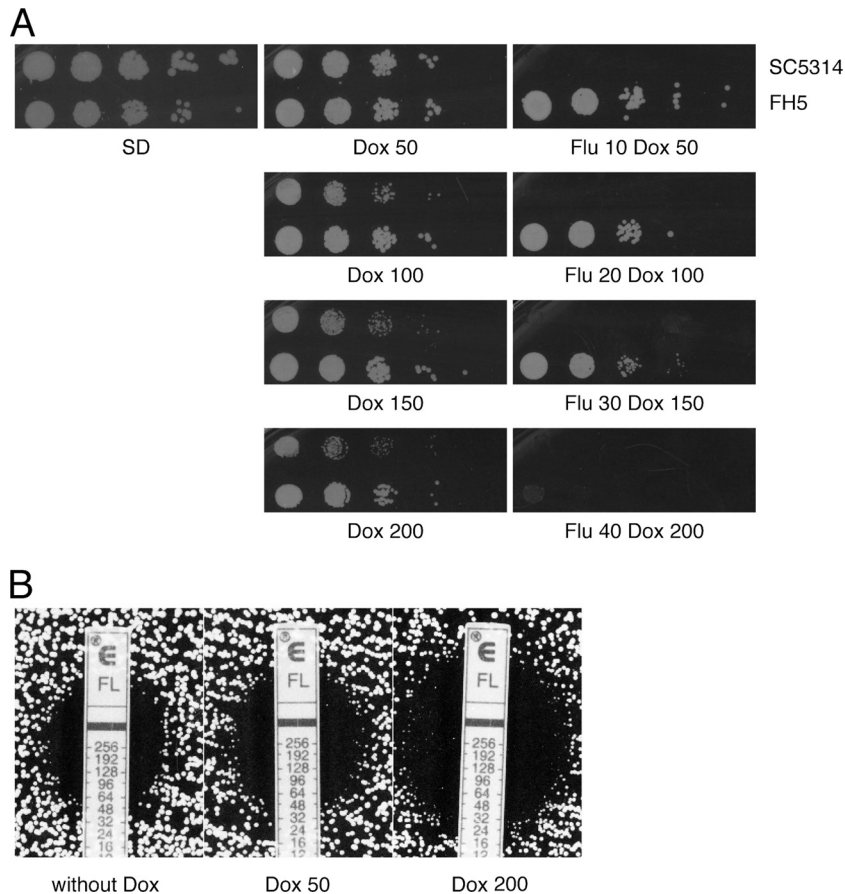


FIG 4 The fluconazole-doxycycline combination is also effective on a clinical isolate of *C. albicans* characterized by elevated resistance to fluconazole. (A) Cells of the *C. albicans* reference strain SC5314 and of the clinical isolate FH5 were spotted onto SD plates containing doxycycline, without (middle panels) or with (right panels) fluconazole. Concentrations are expressed in $\mu\text{g/ml}$. Plates were incubated for 2 days before being scanned. (B) Etest measurement of fluconazole MIC of strain FH5 on RPMI-glucose plates. The left panel shows medium without doxycycline, the middle panel shows medium with 50 $\mu\text{g/ml}$ doxycycline, and the right panel shows medium with 200 $\mu\text{g/ml}$ doxycycline.

with resistance to fluconazole plus doxycycline were analyzed further: their plasmids were extracted, amplified in *E. coli*, and retransformed into yeast to confirm plasmid-linked suppression. Two plasmids that reproducibly conferred resistance to fluconazole plus doxycycline were isolated, sequenced, and found to contain the same insert (see Materials and Methods). The insert DNA was subcloned so that the second-generation plasmid contained a yeast genomic fragment carrying a single gene. By use of this procedure, the suppressor gene was identified as *ERG11* (Fig. 5A), encoding the target of fluconazole. Overexpressed *ERG11* is already known to confer resistance to fluconazole and itraconazole in *S. cerevisiae* (24, 32), a result that was confirmed here (Fig. 5A). Hence, these results suggest that, compatibly with the synergistic hypothesis, doxycycline enhances the toxicity of fluconazole rather than adding toxicity via a different mechanism.

Overexpression of *CaERG11* is not sufficient for resistance to fluconazole and doxycycline in *C. albicans*. Isolation of *ERG11* as a multicopy suppressor in *S. cerevisiae* prompted us to test whether the corresponding gene of *C. albicans* was also capable of suppression. The *Candida* Genome database (<http://www.candidagenome.org>) indicated ORF19.922 and ORF19.8538 as the *C. albicans* *ERG11* alleles (hereafter referred to as *CaERG11*), with the corresponding predicted proteins differing at two amino

acids only. We amplified ORF19.922 and cloned it in two Clp10-derived plasmids (37), so as to obtain expression of *CaERG11* under the control of the strong constitutive *ACT1* promoter (15) or the strong, gluconeogenic carbon source-inducible *PCK1* promoter *PCK1p* (5). Both plasmids were integrated at the *RPS10* locus of the wild-type CAI4 strain of *C. albicans* and tested for suppression. The corresponding strains failed to grow in the presence of the fluconazole-doxycycline combination (Fig. 5B). Furthermore, no difference between growth of these strains and their parental ones was evidenced, even when smaller amounts of doxycycline were used (Fig. 5B) (our unpublished observations), nor was any difference in the MIC for fluconazole observed (our unpublished data). Overexpression of *ERG11* in these transformants was verified via quantitative real-time PCR and shown to be ~ 3 -fold higher in overexpression strains than that in the controls (Fig. 5C).

Finally, since overexpression of *CaERG11* failed to suppress susceptibility to the fluconazole-doxycycline combination in *C. albicans*, we decided to investigate whether this failure was to be attributed to a functional divergence of the *CaErg11* versus *Erg11* protein or to a wider difference in the physiology of *C. albicans* versus *S. cerevisiae*. For this purpose, the *CaERG11* gene was cloned in a yeast vector under the control of a galactose-inducible

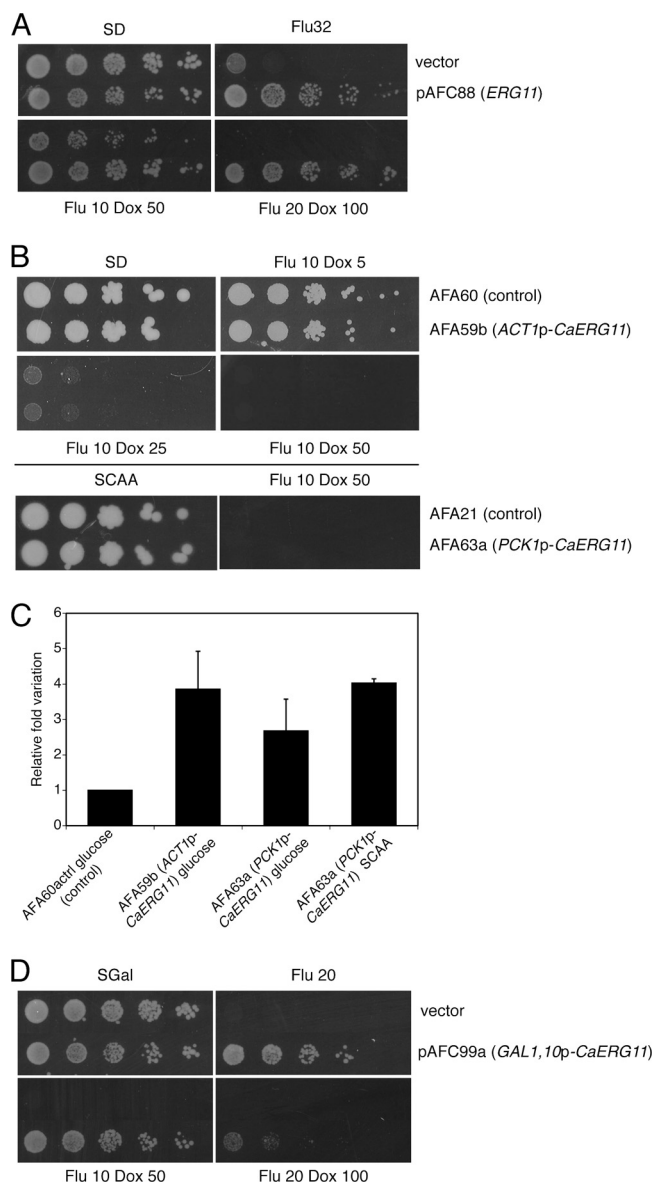


FIG 5 Overexpression of *ERG11* suppresses fluconazole–doxycycline susceptibility in *S. cerevisiae* but not in *C. albicans*. (A) Transformants of the *S. cerevisiae* reference strain BY4742 with plasmid pAFC88 (*ERG11*) or with an empty plasmid (control) were spotted onto SD plates containing combinations of fluconazole and doxycycline, at the indicated concentrations ($\mu\text{g}/\text{ml}$). Plates were incubated for 4 days before being scanned. (B, top panel) Cells of the *C. albicans* strains AFA60 (control) and AFA59b (*ACT1p-CaERG11*) were spotted onto SD plates containing the indicated concentrations of fluconazole plus doxycycline. (Bottom panel) Cells of strains AFA21 (control) and AFA63a (*PCK1p-CaERG11*) were spotted onto SCAA plates with or without fluconazole plus doxycycline. Plates were incubated for 4 days before being scanned. (C) Quantitative real-time PCR assessment of expression of *CaERG11* in strains carrying an extra copy of the gene under the control of the constitutive *ACT1* gene or of the inducible *PCK1* promoter. The graph shows mean values with standard deviations from two independent experiments. (D) Transformants of the *S. cerevisiae* reference strain BY4742 with plasmid pAFC99a (*GAL1,10p-CaERG11*) or with an empty plasmid (control) were spotted onto SGal plates containing combinations of fluconazole and doxycycline at the indicated concentrations ($\mu\text{g}/\text{ml}$). Plates were incubated for 5 days before being scanned.

promoter, and the corresponding transformants were tested for suppression of fluconazole and fluconazole–doxycycline susceptibility. As shown in Fig. 5D, yeast cells overexpressing *CaERG11* were less susceptible to fluconazole and fluconazole plus doxycycline than control cells, demonstrating that increased dosage of *CaErg11* can suppress fluconazole susceptibility in yeast but not in *C. albicans*, and, as a consequence, that the suppression activity is retained by the *CaERG11* gene. Taken together, the results on overexpression of *ERG11* in the two yeast species underline the difference existing between them in terms of their responses to the antifungal fluconazole.

Doxycycline potentiates the action of fluconazole via iron chelation. Metal chelation by tetracycline antibiotics *in vitro*, with a high affinity for Fe^{3+} ions, has been described in the past (reviewed in reference 39). Grenier and collaborators (19) proposed that the activity of tetracyclines against *Actinobacillus actinomyces-comitans* may be due not only to their antibacterial properties, but also to their iron-chelating activity. In addition, doxycycline is also known to be an inhibitor of mammalian matrix metalloproteases, an action mediated by chelation of the catalytic Zn^{2+} ions of these enzymes (20, 53).

Recovery of *Erg11*, a hemoprotein, as a multicopy suppressor of hypersusceptibility of baker's yeast to fluconazole plus doxycycline led us to hypothesize that perturbation of iron homeostasis in the presence of doxycycline may play a role in establishing the hypersusceptibility of *C. albicans* to fluconazole. To this aim, we tested whether addition of ferric iron could reverse the doxycycline effect. As can be observed in Fig. 6A and B, addition of ferric chloride or ferric citrate restored growth in the presence of fluconazole plus doxycycline, rescuing *C. albicans* as well as baker's yeast from hypersusceptibility. Importantly, growth in the presence of fluconazole plus doxycycline was not restored by the addition of zinc, nor did addition of Fe^{3+} alter growth of *C. albicans* in the presence of fluconazole alone (our unpublished observations).

Iron-chelating activity of tetracyclines was confirmed using a colorimetric assay. We could demonstrate that both tetracycline and doxycycline chelate iron, with doxycycline showing higher affinity than tetracycline (Fig. 6C), whereas fluconazole showed no intrinsic iron-binding activity. Notably, the stronger iron-chelating activity of doxycycline correlated with its stronger synergism with fluconazole.

Potential of the action of fluconazole against *C. albicans* by iron depletion has been demonstrated by Prasad et al. (50), via the addition of the iron chelator bathophenanthroline disulfonic acid (BPS) to the growth medium. While we confirmed this observation (Fig. 6D), we also noticed that a concentration of BPS of as little as 20 $\mu\text{g}/\text{ml}$ had a negative effect on cell growth even when used alone (Fig. 6D), as also observed in *S. cerevisiae* (12).

Taken together, the observations described here indicate that iron depletion by doxycycline may underlie its synergism with fluconazole.

Synergism between fluconazole and doxycycline is not mediated by calcineurin. Despite showing no intrinsic antifungal activity of their own, the immunosuppressants cyclosporine (CsA) and FK506 were reported to be synergistic with fluconazole against *Candida albicans* (31, 33), converting fluconazole to a fungicidal drug and eliminating trailing growth (33). The synergistic action of fluconazole with CsA and FK506 is mediated by inhibi-

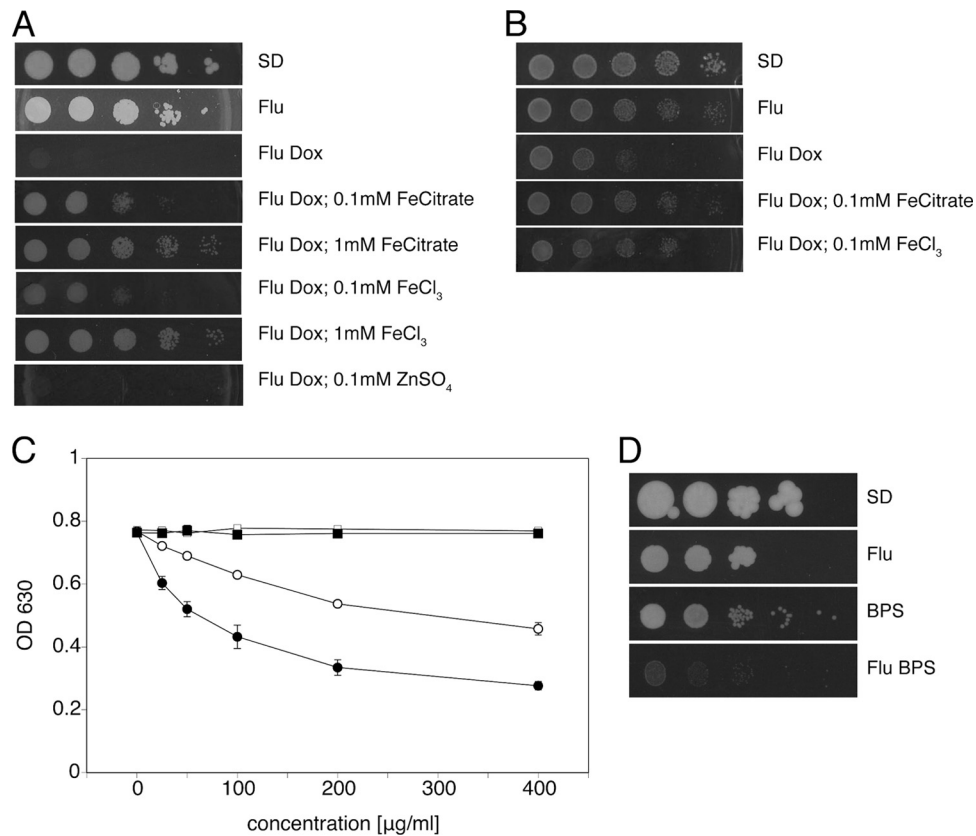


FIG 6 Doxycycline synergizes with fluconazole via iron sequestration. (A) Cells of *C. albicans* SC5314 were spotted onto SD plates containing combinations of fluconazole and doxycycline at standard concentrations, with and without ferric citrate or ferric chloride added at the indicated concentrations. Plates were incubated for 2 days before being scanned. (B) Cells of *S. cerevisiae* BY4742 were similarly spotted onto SD plates. Plate scans were taken after 2 days. (C) Colorimetric assessment of iron-chelating activity of doxycycline, tetracycline, and fluconazole. Iron chelation is expressed as a reduction of absorbance at 630 nm. Filled circles, doxycycline; open circles, tetracycline; filled squares, fluconazole; open squares, water. Mean values with standard deviations from three independent experiments are shown. (D) Cells of *C. albicans* SC5314 were spotted onto SD plates containing combinations of fluconazole (10 $\mu\text{g/ml}$) and BPS (20 $\mu\text{g/ml}$). Plates were incubated for 4 days before being scanned.

tion of the phosphatase activity of calcineurin by the immunosuppressants (13, 55).

Given the similarities between the combined action of CsA/FK506 and doxycycline with fluconazole, we decided to investigate on the possibility that a decrease in or abrogation of calcineurin activity may also result from the presence of doxycycline. For this purpose, we made use of *cna1 Δ /cna1 Δ* mutants lacking subunit A of calcineurin (55). If doxycycline exerts its action via inhibition of calcineurin, one would expect the presence of doxycycline to have no effect on the hypersusceptibility of these mutants to fluconazole. Hypersusceptibility of *cna1 Δ /cna1 Δ* mutants to fluconazole (55) was confirmed in our tests (Fig. 7). However, growth of these mutants in the presence of the antifungal was completely inhibited by concentrations of doxycycline that only mildly affected wild-type cells (Fig. 7), indicating that doxycycline further compounds the hypersusceptibility of *cna1 Δ /cna1 Δ* mutants to fluconazole. Identical results were obtained using *cnb1 Δ /cnb1 Δ* mutants (13) lacking subunit B of calcineurin (our unpublished data).

Calcineurin is inactive under normal conditions, being activated only in the presence of certain external cues (52). A strain of *C. albicans* in which calcineurin was permanently activated by removal of the self-inhibitory C-terminal domain of Cna1 was less

susceptible to fluconazole (55). While we confirmed a moderately increased tolerance to fluconazole in this strain (our unpublished observations), we also found this strain to be as susceptible to the fluconazole-doxycycline combination as an isogenic control strain carrying a reintegrated copy of wild-type *CNA1* (Fig. 7), demonstrating that constitutively active calcineurin is incapable of rescuing the cells' susceptibility to fluconazole plus doxycycline. Taken together, these observations suggest that the synergism of doxycycline with fluconazole is not mediated by inhibition of calcineurin activity.

DISCUSSION

We found that doxycycline, and to a lesser extent tetracycline, two licensed antimicrobials that prevent bacterial protein synthesis, potentiate the antifungal activity of fluconazole against *Candida albicans* in a dosage-dependent manner. Doxycycline converts the action of fluconazole from fungistatic to fungicidal and prevents the onset of drug resistance. Addition of doxycycline appears to have a major impact on prevention of fluconazole tolerance, defined as incomplete growth inhibition at supra-MICs of fluconazole. This finding is consistent with the role of doxycycline in converting fluconazole to a fungicidal drug and may also have implications in the prevention of drug resistance.

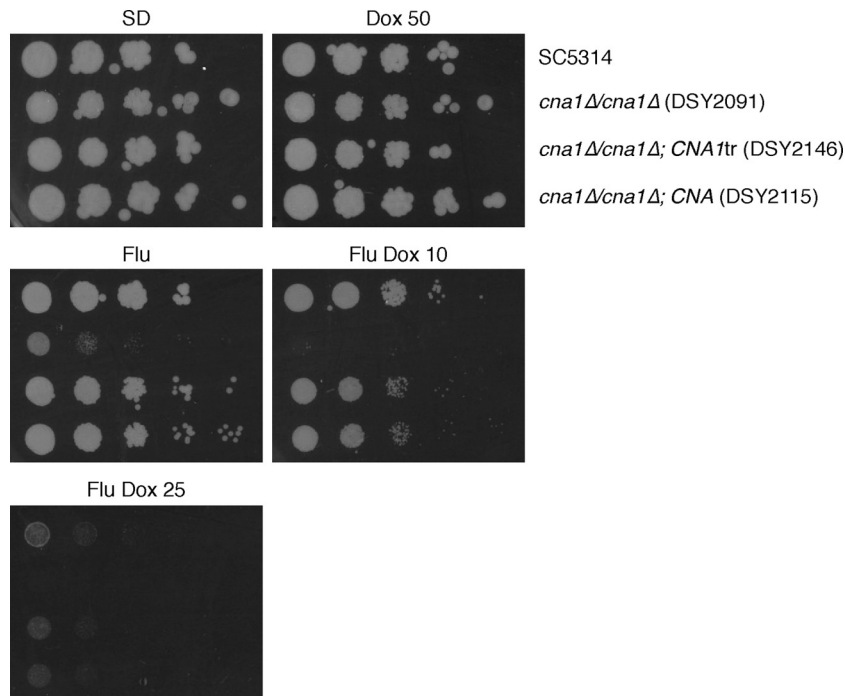


FIG 7 Synergism between doxycycline and fluconazole is not mediated by calcineurin. Cells of the indicated *C. albicans* strains were spotted onto SD plates containing combinations of fluconazole and doxycycline. Fluconazole was maintained at the standard 10- μ g/ml concentration, whereas the concentration of doxycycline was decreased as indicated (μ g/ml) to provide a sensitized background for the assay. Plates were incubated for 3 days before being scanned.

Other authors have reported on alteration of *C. albicans* susceptibility to fluconazole in the presence of tetracyclines (42, 43). Odds and collaborators (42) observed a strong reduction of the MIC for fluconazole and elimination of trailing growth by doxycycline when they used diagnostic susceptibility test agar medium, but not when tissue culture-based medium was used. On the other hand, Oliver and collaborators (43) reported on a positive effect of tetracycline on a fluconazole MIC of strain SC5314 in broth microdilution tests and a negative one when MIC was measured using Etest strips. Given the similarity between the bacterial and mitochondrial ribosomes, these authors proposed that increased susceptibility to fluconazole might result from the action of tetracycline against mitochondrial translation. Consistently, they reported inhibition of respiratory growth of *C. albicans* in the presence of 200 μ g/ml tetracycline (43). We report here that the doxycycline MIC is higher than 200 μ g/ml. Furthermore, the 50- μ g/ml concentration of doxycycline used throughout this study does not affect growth of *C. albicans* on any medium tested, including medium containing ethanol and glycerol, carbon sources that can only be metabolized via mitochondrial respiration. It therefore appears unlikely that the synergistic effect of fluconazole plus doxycycline can be attributed to a perturbation of respiration by doxycycline. Furthermore, we tested whether inhibition of mitochondrial respiration could also be synergistic with fluconazole. Although a substantial growth defect could be observed in the presence of fluconazole when respiration was blocked by cyanide, this growth impairment appeared to be the sum of individual contributions of either chemical to the cells' fitness, rather than a synergistic effect, and did not resemble the strong effect of fluconazole plus doxycycline (our unpublished data).

Addition of iron to the growth medium restored growth of *C.*

albicans, as well as that of *S. cerevisiae*, in the presence of fluconazole plus doxycycline, suggesting that hypersusceptibility to the drugs in combination could be due to antibiotic-mediated titration of iron. Consistent with this hypothesis, we confirmed that doxycycline and, to a minor extent, tetracycline act as iron chelators. Interestingly, while iron was found to be a chemical suppressor of fluconazole-doxycycline hypersusceptibility in baker's yeast as well as in *C. albicans*, an increased dosage of *ERG11* rescued only the growth defect of *S. cerevisiae*. Iron depletion by doxycycline may result in lower incorporation of heme in the Erg11 protein in yeast, and an increase in the copy number of Erg11 may help the protein compete for heme. Alternatively, since iron deprivation has been demonstrated to result in downregulation of *ERG11* in *C. albicans* (25, 50), it is possible that a similar situation occurs in *S. cerevisiae* and that *ERG11* overexpression may simply restore gene expression to wild-type levels. Whatever the mechanism in baker's yeast, the situation appears to be more complex in *C. albicans*. In this organism, iron depletion as the major cause of growth inhibition has been demonstrated by reversal upon addition of ferric iron to the medium. However, the failure of an increased dosage of *CaERG11* to suppress growth inhibition in *C. albicans*, despite full competence of the corresponding protein to act as a suppressor in yeast, suggests that doxycycline-mediated iron depletion may have more severe effects in this fungus. This interpretation is also corroborated by the higher susceptibility to fluconazole plus doxycycline observed in *C. albicans* with respect to *S. cerevisiae* (see the text above and the panels with the fluconazole-doxycycline combination in Fig. 5A and B). Alternatively, lower levels of gene overexpression obtained in *C. albicans* compared to those achieved in the yeast system could provide a simpler explanation for our observations. In fact, we found that over-

expression of *CaERG11* in our *C. albicans* strains was 3-fold higher than that under normal conditions (Fig. 5C), whereas overexpression in yeast transformants carrying *ERG11* on a multicopy plasmid was ~10- to 11-fold higher than that in the untransformed control (our unpublished data). However, several observations argue against this simple threshold hypothesis for failed suppression by *CaERG11* in *C. albicans*. First, the *C. albicans* overexpressers show no signs of suppression even when mild, low-stringency conditions of fluconazole plus doxycycline are applied (Fig. 5B) (our unpublished observations). Second, if high levels of *CaERG11* were necessary and sufficient *per se* to confer a resistant phenotype, one would expect the onset of stable, spontaneous mutants of *C. albicans* resistant to fluconazole plus doxycycline at high frequency, as predicted from the frequency of mutations in single genetic loci. Mutations in *CaFKS1* conferring resistance to the antifungal caspofungin, for instance, have been reported to occur at a frequency of $<10^{-8}$ mutations per cell per generation (4). The fact that we never recovered spontaneous mutants resistant to fluconazole plus doxycycline (our unpublished observations) favors the hypothesis that multiple genetic alterations may be necessary for such resistance to occur. On the other hand, while strong suppression of susceptibility to fluconazole upon overexpression of *ERG11* in baker's yeast is reported in this article and has also been described by others (24, 32), a similar clear-cut correlation has not been established in *C. albicans*. The only published paper, to our knowledge, describing resistance to fluconazole upon overexpression of *CaERG11* in *C. albicans* reports a moderate resistance in cells with ~11-fold increased levels of *CaERG11* (14). Although certain mutations in *CaERG11* (54), as well as increased *CaERG11* mRNA levels (46, 61), have been associated with resistance to fluconazole in *C. albicans*, it has become increasingly clear in recent years that decreased susceptibility to fluconazole in this fungus derives from complex genetic alterations that comprise and are possibly not limited to genomic rearrangements and deregulated expression of several genes (11, 30, 57).

Iron depletion has been proposed to decrease ergosterol content in *C. albicans*, leading to higher fluidity in cell membranes, with consequent increased passive diffusion of fluconazole (50). Some of the results reported in this paper appear similar to those obtained using BPS as the iron chelator: addition of 200 μ M BPS lowered the MIC of *C. albicans* for fluconazole (50), and so did that for 200 μ g/ml doxycycline. Importantly, the iron chelation properties of the two chemicals at the indicated concentrations are similar (our unpublished observations). Minor differences could be due to different experimental conditions.

We report here that fluconazole and doxycycline are also effective against a clinical strain of *C. albicans* with previously acquired resistance to fluconazole, although a higher dosage of the chemicals is required in this case. While elevated dosage of doxycycline alone has a negative impact on growth of the reference strain SC5314 (without reaching the MIC), presumably by iron depletion, clinical strain FH5 is insensitive to these conditions, suggesting that the ability of this strain to thrive in a low-iron environment and resistance to fluconazole may be linked. Interestingly, transcription of the iron transporters *FRE9* and *FTR1*—the latter required for growth under low-iron conditions (51)—is upregulated in response to exposure to fluconazole (27).

Recently, a role for *C. albicans* Als3 in iron acquisition from host ferritin has been proposed (2). We therefore tested whether

als3 Δ /als3 Δ mutants were differentially susceptible to fluconazole plus doxycycline and found them to be as susceptible as their wild-type counterpart (our unpublished observations), a result that argues against a role for Als3 in the establishment of doxycycline-mediated potentiation of fluconazole activity. Similarly, our experiments argue against a role for calcineurin in this process.

From a practical point of view, since experiments with doxycycline-regulated promoters are an established reality in the field of *C. albicans* molecular genetics, we propose that the interesting results obtained using this system should be further confirmed by experiments performed using media with additional iron, in order to discriminate between phenotypes that can be attributed to altered dosage of the gene of interest and phenotypes that may have arisen from doxycycline-mediated perturbation of iron homeostasis.

ACKNOWLEDGMENTS

We thank Ilse Palmans for excellent technical assistance and Nico Vangoethem for help with the preparation of figures. We also thank Judith Berman, Joe Heitman, and Dominique Sanglard for strains.

This work was supported by SBO grant 060839 from the Flemish Institute for Science and Technology (IWT) and the research fund of the K.U. Leuven (GOA/2007/08).

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