Candidiasis represents one of the most important nosocomial infections in hospitals worldwide, affecting an increasing number of immunosuppressed as well as other at-risk patients, and *Candida albicans* remains the most common causative agent of candidiasis (1, 21). These infections are frequently associated with the formation of biofilms on the surfaces of medical devices. For example, the presence of central venous catheters is a major risk factor for candidiasis, and yeasts (mainly *C. albicans*) are the third most frequent cause of catheter-associated bloodstream infections (1, 2, 7). Because biofilms constitute a reservoir for infections and a protective environment, as cells within biofilms display increased antifungal drug resistance and protection from host immune defenses, the ability of *C. albicans* to form biofilms is considered one of its main virulence factors.

In *C. albicans*, biofilm development progresses through multiple developmental stages, with two major processes, adhesion and filamentation, playing vital roles in biofilm formation (4, 13, 31). The first stage involves attachment of *C. albicans* cells to a substrate, closely followed by cellular proliferation, hyphal development, and synthesis of exopolymERIC material, leading to the formation of an architecturally complex, three-dimensional biofilm (4, 23, 26). The confluent hyphal layers, comprising the bulk of the biofilm, form yeast cells that are continually released from the biofilms (34). This completes the biofilm developmental life cycle, as dispersed cells will eventually colonize new distal sites and the entire process can start all over again. Several molecular determinants are known to play important roles at different stages of biofilm development. Early events in biofilm formation are known to be orchestrated in part by interactions between cell wall proteins Als3 and Hwp1, which are required for complementary adhesive interactions (17, 19, 20, 36). Transcriptional factors such as Efg1p, Tec1p, and Bcr1p also play vital roles in early stages of biofilm formation. For example, Δefg1 and Δtec1 mutants are unable to undergo morphogenesis, leading to a biofilm formation defect (18, 25), while the Δbcr1 mutant is deficient in manifestations of the cell-cell adhesion important for biofilm maturation (17, 18). More recently, we showed that *C. albicans* biofilm dispersal can be controlled by manipulating levels of expression of two key morphogenetic genes, *UME6* (involved in hyphal elongation) and *PES1* (the “pescadillo” homolog involved in the reverse morphological transition, from hyphae to yeast, and lateral yeast formation) (34).

While several genes that control individual stages of biofilm formation and dispersion have been described, a common genetic determinant that might contribute throughout the biofilm life cycle has not yet been identified. Here we report on the transcriptional regulator NRG1, which influences multiple different steps during the *C. albicans* biofilm developmental cycle. This gene encodes Nrg1p, a DNA-binding protein with a zinc finger domain that functions as a negative regulator of filamentation (3, 15). Despite the functional significance of NRG1, its role during *C. albicans* biofilm growth has not yet been investigated. With the help of a genetically engineered strain of *C. albicans* in which NRG1 is under the control of a
tetracycline-regulatable promoter (30), we show that levels of expression of NRG1 exert an exquisite control over the processes of biofilm formation and dispersion in C. albicans.

**MATERIALS AND METHODS**

*C. albicans* strains and strain construction. The strains used in this study were *C. albicans* strains SC5314 (wild type), the *C. albicans* SSY50-B tetracycline-regulatable *tet-NRG1* strain, which has been previously described by our group (30), and a novel green fluorescent protein (GFP)-tagged *tet-NRG1* strain which constitutively expresses GFP under the control of the *C. albicans* ACT1 promoter, constructed as follows: a 2.4-kb fragment containing the sequence for the *C. albicans* codon-modified GFP was cut out of pMG1664 (8) (kind gift from Judith Nourse) by using EcoRI/HindIII. This fragment was then ligated into EcoRI/ HindIII-digested ClpSATSA (6) to create ClpSATSA-GFP. This plasmid was then linearized with StuI and transformed into SSY50-B (30) by using a modified electroporation transformation method (11). Digestion at the Stu site produces the RP10 homologous ends, which facilitate integration of the plasmid into the *C. albicans* genome at this locus. Nourseorthcin-resistant transformants were selected on yeast-extract-peptone-dextrose (YPD) agar plates containing 200 μg/ml of morpholinepropanesulfonic acid (MOPS) buffer, as described previously (27) and screened for GFP activity by fluorescence microscopy. To confirm the integration of the GFP construct into the *Candida* genome at the RP10 locus, DNA was extracted from transformants demonstrating GFP activity by using a commercially available kit (Masterpore; Epicentech Technologies, Madison, WI), digested with HindIII, transferred to a nylon membrane (Nytren; Schleicher & Schuell, Keene, NH), and subjected to Southern blot analysis using an established method (5) with RP10 as the probe. Stock cultures were stored in 15% glycerol at −80°C.

Strains were routinely grown on non-fragment-inducing conditions (media at 30°C) in the presence or absence of 20 μg/ml of dexamethoxycyclone (DOX). Media used for culturing both planktonic and biofilm cells were YPD (0.5% yeast extract, 1% Bacto peptone, 1% glucose), and RPMI 1640 (Sigma, St. Louis, MO) with morpholinopropane sulfonic acid (MOPS) buffer.

*C. albicans* biofilm development assays. *C. albicans* biofilms were formed in vitro under either static conditions or under conditions of flow. For static conditions, biofilms were formed in 96-well polystyrene microtiter plates as previously described by our group (22, 24). Briefly, cells were grown in YPD overnight at 37°C and resuspended in RPMI buffer with MOPS, with or without DOX (20 μg/ml) depending on the specific experimental design, at a final concentration of 10^6 cells/ml based on hemocytometer counts. An inoculum (100 μl) was added to each well of a 96-well flat-bottom plate. After 24 h of incubation at 37°C, the wells were washed with phosphate-buffered saline (PBS) three times to remove any nonadherent cells. The extent of biofilm formation was estimated by adding or omitting DOX in the growth medium (30). Cells from this strain were used to seed the wells of microtiter plates using RPMI medium and incubated at 37°C, in the presence or absence of DOX.

**Confocal scanning laser microscopy.** Biofilms grown in six-well plates for 24 h were stained with 25 μg/ml concanavalin A (ConA)-Alexa Fluor 594 conjugate (C-11253; Molecular Probes, Eugene, OR) for 1 h in the dark at 37°C. Confocal scanning laser microscopy (CSLM) was performed with a Zeiss LSM 510 upright confocal microscope using a Zeiss Achromplan 40×, 0.8-W objective. Concanavalin-A conjugate staining was observed using a HeNe1 laser with an excitation wavelength of 543 nm. For GFP visualization, an argon laser was used with 488-, 485-, and 514-nm excitation wavelengths. Images were assembled into side views using the Zeiss LSM Image Browser v4.2 software.

**RNA extraction and reverse transcription.** Total RNA was extracted from 24-h biofilms grown under static conditions in six-well microtiter plates under static conditions using the MasterPure yeast RNA purification kit (Epicentre Biotechnologies, Madison, WI). The integrity of the RNA was tested via gel electrophoresis. A total of 1 μg of RNA was treated with amplification-grade Dnase I (Invitrogen, Carlsbad, CA) and used for cDNA synthesis with the cMaster RT kit (Eppendorf AG, Hamburg, Germany) as per the manufacturer’s instructions. The following primer sets were used in conjunction with SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) and Twin.tec real-time 96-well PCR plates (Eppendorf AG, Hamburg, Germany) in an ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA): NRG1 (CAC CTC ACT TGC AAC CCC & GCC CTG CAG ATG GTC TGA) (32), ACT1 (ATG TGT AAA GCC GGT TTT GCC & CCA TAT CCT CCC AGT TGA AAA C) (33), HPWI (TCA GCC TGA CAA TCC TC & GCT GGA GTT GCC GTC TTT TC), and ALS3 (CAA CTT GGG TTA TTG AAA CAA AAA CA & AGA AAG ACA AAG CCA AGA ACA ACC T) (16). Parameters for primer design were set according to the recommendations of Applied Biosystems. Briefly, the primer sizes were between 20 and 25 bases in length, and the Tm of each primer was 58°C. The amplicons were between 90 and 110 bp in size. Each reaction mixture was set up in triplicate in a 25-μl volume with 25 ng of cDNA for 40 cycles (thermal cycling conditions were initial steps of 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min). Relative gene expression was quantified using the threshold cycle (Ct) method with the 7300 System sequence detection software with the RQ software application from Applied Biosystems (35). The target genes were normalized to the housekeeping gene ACT1. The change was calculated for each sample by using the equation 2^{-ΔΔCt}, and results from the different replicates were averaged after the 2^{-ΔΔCt} calculations.

**RESULTS AND DISCUSSION**

Control of *C. albicans* biofilm formation by NRG1 gene expression levels. *C. albicans* Nrg1p is a negative regulator of filamentation (3, 15). Because of the well-established link between filamentation and biofilm formation in *C. albicans*, we postulated that altering the levels of Nrg1p in the cell may affect biofilm development in *C. albicans*. For this, we utilized a genetically engineered *C. albicans* tet-NRG1 strain (SSY50-B), in which NRG1 is placed under the control of a tetracycline-regulatable promoter so that morphology can be manipulated in vitro by adding or omitting DOX in the growth medium (30). Cells from this strain were used to seed the wells of microtiter plates using RPMI medium and incubated at 37°C, in the presence or absence of DOX. *C. albicans* SC5314
biofilms were also formed in parallel and served as controls. After 24 h, the extent of biofilm formation was estimated by measuring the metabolic activity of cells within the biofilms using an XTT colorimetric assay. At least five replicate wells were prepared for biofilm formation for each condition. Results indicated that robust biofilm formation in the C. albicans tet-NRG1 strain only occurs in the presence of antibiotic, when levels of Nrg1p are low and filamentation is allowed to progress. As shown in Fig. 1, in the absence of DOX (which translates to NRG1 overexpression), the strain developed very poor biofilms, about 77% less robust than the control biofilms formed by strain SC5314 (Fig. 1A). Instead, addition of the antibiotic yielded much more robust biofilms as estimated by colorimetric readings (Fig. 1A). Although the difference between control and tet-NRG1 biofilms in the presence of DOX was significant ($P \approx 0.05$, by analysis of variance [ANOVA]), results from SEM revealed that this difference perhaps may not be sufficient for a biological difference.

As visualized by SEM, a severe defect in biofilm formation was observed for the C. albicans tet-NRG1 strain in the absence of DOX, when NRG1 was overexpressed (Fig. 1B). Only a monolayer of yeast and pseudohyphal cells was found attached to the substrate, without the presence of hyphae. These “biofilms” were very similar to those formed by the Δefg1 mutant (25). On the other hand, in the presence of the antibiotic, when NRG1 expression is low, biofilms formed by this tet-NRG1 strain appeared indistinguishable from those formed by the wild-type strain SC5314, with abundant hyphae and structural complexity.

During these experiments we observed that the absence of DOX led to the attachment of mostly yeast cells, which seemed to indicate that the very initial adhesion phase may not be influenced by Nrg1p levels. Hence, we asked whether addition of DOX externally after initially seeding the plates would rescue the biofilm formation defect and trigger subsequent biofilm formation by these attached cells. To answer this question we seeded the wells of microtiter plates with cells of the C. albicans tet-NRG1 strain in the absence of DOX and allowed the cells to adhere for various periods of time (30 min and 1, 2, 4, and 24 h). At the specified time points, wells were washed to remove nonadherent cells and replenished with fresh medium containing the antibiotic. Then, plates were incubated for an additional 24 h and the extent of biofilm formation was estimated using the XTT colorimetric assay. We found that downregulation of levels of expression of NRG1 by the addition of DOX in the growth medium triggered biofilm formation from the yeast cells and pseudohyphae that were originally attached to the wells (Fig. 1C). Even the wells containing cells for 24 h could regain their biofilm formation ability to levels comparable to those observed for cells incubated continuously in the presence of DOX, once the antibiotic was added to the medium (Fig. 1C). These observations have important clinical ramifications, as they demonstrate that C. albicans cells may initially attach to a surface, maintain a state of “dormancy” for extended periods of time, and then proliferate and be able to form biofilms upon the receipt of the right environmental cues.

Of note, even in the absence of an extensive three-dimensional structure, attached cells already demonstrate increased levels of resistance to antifungal agents, particularly azole derivatives (9, 14), and this property may be further accentuated by the change in physiological status (nongrowing) associated with this dormant state (10, 12).

C. albicans tet-NRG1 yeast cells can get incorporated into a mixed biofilm with wild-type cells. Overexpression of Nrg1p resulted in cells that displayed filamentation defects and, as a consequence, were defective in biofilm formation. In the next series of experiments we examined whether yeast cells of the tet-NRG1 strain could incorporate into a mixed biofilm with filamentous cells of the wild-type strain. Briefly, cells of C. albicans tet-NRG1 were prepared for biofilm formation for each condition. Re-
*C. albicans* wild-type strain SC5314 and the *tet-NRG1* strain were mixed at various ratios (100:0, 75:25, 50:50, 25:75, and 0:100) and used to seed wells of microtiter plates in the absence of DOX. Plates were incubated for 24 h at 37°C to promote biofilm formation. At least three replicates were performed under each condition of biofilm development. After 24 h, the biofilms were washed, and metabolic activity of cells within the biofilms was measured in an XTT assay. As expected, the *C. albicans* tet-NRG1 strain on its own was unable to develop a robust biofilm (Fig. 2A). A modest yet statistically significant (*P* < 0.05, ANOVA) reduction was obtained in the XTT values of all mixed biofilms compared to the control biofilms formed by SC5314 cells (Fig. 2A). Also in these biofilms, increasing proportions of the *tet-NRG1* strain (from 0% to 25% to 50%) corresponded to an increase in the population of yeast cells interspersed between abundant hyphae, as observed by SEM. In fact, the biofilm containing 75% *tet-NRG1* cells was comprised of copious yeast and pseudohyphae at a proportion that appeared much higher than in the biofilm containing 100% *tet-NRG1* cells (Fig. 2B). Overall, these results suggest that cells from the *C. albicans* tet-NRG1 strain, which alone are defective in biofilm formation, can effectively be incorporated and retained as a part of a mixed biofilm along with SC5314, as hyphal elements of this wild-type strain provide for structural integrity and adhesive interactions required for biofilm development.

We note here that plating (in YPD-agar plates) of cells recovered from the biofilms formed in the wells of microtiter plates confirmed the presence of both tet-NRG1 and SC5314 strains in the mixed biofilms, since in the absence of DOX at 37°C SC5314 colonies are wrinkled whereas colonies formed by the *C. albicans* tet-NRG1 strain are smooth (results not shown).

To gain an even better understanding of the morphological and architectural characteristics associated with the mixed biofilms, we generated a GFP-labeled *C. albicans* tet-NRG1 strain and visualized the resulting mixed biofilms by using nondestructive CSLM. Initial experiments indicated that the integration of this reporter gene did not alter the biofilm formation defect of the tet-NRG1 strain in the absence of DOX. Single-strain and mixed biofilms (together with the wild-type SC5314 strain) were developed in six-well polystyrene plates, in RPMI
medium without DOX. After 24 h of incubation, the biofilms were stained with ConA and examined by CSLM. As expected, biofilms formed by the GFP-expressing tet-NRG1 strain were very poor, forming only a 10-µm-thick monolayer of mostly yeast cells and few pseudohyphae. A mixture of 75% SC5314 and 25% GFP-tet-NRG1 yielded a biofilm as deep (349 µm) as the one formed by the wild type only. In this mixed biofilm it was observed that GFP-tet-NRG1 cells grew mostly as yeast cells confined to the bottom layer of the biofilm, but the depth of this layer (about 20 µm) grew to twice that of the biofilms formed in the absence of strain SC5314. As the ratio of GFP-tet-NRG1 increased, so did the thickness of the bottom layer of the biofilm that it occupied. At 50% or 75% of the original proportion of the mixed-species biofilm, the green fluorescent layer measured 37 µm and 45 µm, respectively. Interestingly, a higher degree of filamentation was now observed in these basal layers, and some green fluorescent filaments were also observed extending into the middle layers of the biofilm. The overall thicknesses of the two resulting mixed biofilms (containing 50% or 75% GFP-tet-NRG1) were similar to that observed for biofilms formed by the SC5314 strain alone (Fig. 2C). Overall, it would seem that retention of yeast cells in the mixed biofilms is mostly through a mechanism of physical entrapment, where yeast cells are mostly confined to the layer closer to the substrate in which biofilms are formed, rather than through direct adhesive interactions between yeast cells of the tet-NRG1 strain and filaments of the wild-type SC5314. This picture is similar to the Δec1 mutant that stays entrapped as a basal layer in a mixed biofilm setting along with the wild-type reference strain (18).

As a validation for our experimental system to control gene expression, for these series of experiments we also wanted to make sure that the observed morphological features and extent of biofilm formation in single and mixed biofilms correlated with levels of expression for NRG1 and were indeed a consequence of our ability to manipulate levels of NRG1 gene expression in the regulatable tet-NRG1 strain. Thus, we extracted RNA from biofilms formed by the C. albicans SC5314 and tet-NRG1 strains in both the presence and absence of DOX, as well as from mixed (50/50) biofilms in the absence of DOX. As seen in Fig. 3, measured levels of gene expression for NRG1 showed excellent correlation with the presence or absence of the antibiotic in the growing medium. Moreover, as expected since expression of these adhesins is known to be dependent on Nrg1p levels (15), control of NRG1 gene expression levels also resulted in accompanying changes in levels of gene expression for HWP1 and ALS3 (Fig. 3). These observations corroborate the finding that the observed results are in direct correlation with NRG1 expression levels. Furthermore, the results indicated that Nrg1p control of biofilm formation is likely through the regulation of key target genes, including ALS3 and HWP1, encoding complementary filament-specific adhesins that play a critical role for biofilm formation in C. albicans (20). Importantly, within the biofilm context, expression of these hypha-specific adhesins is also controlled by Bcr1p and Efg1p (17, 25). Thus, similar to filamentation, biofilm formation in C. albicans is orchestrated by a complex regulatory circuitry with a seemingly high level of redundancy. From an evolutionary point of view, this high level of complexity gives further credence to the importance of the ability to form biofilms in different aspects of C. albicans biology and pathogenesis.

Control of C. albicans biofilm dispersion by Nrg1p. We next posited that, besides its role during biofilm formation, as a
negative regulator of filamentation Nrg1p may also function during biofilm dispersion, as most cells dispersed from biofilms are in the yeast morphology (34). To test this hypothesis, we developed biofilms of strains SC5314 and tet-NRG1 on SE strips under conditions of flow using a model that, contrary to the static models, allows for the study of biofilm dispersion (33). As documented above, C. albicans strain tet-NRG1 is fully capable of developing a robust biofilm in the presence of DOX. Likely, DOX does not have any effect on biofilm formation properties of SC5314. At various time points during the course of biofilm formation (4, 8, 12, and 24 h) aliquots of flowthrough medium were collected and the number of cells released from the biofilms was enumerated. As shown in Fig. 4, the number of cells dispersed from biofilms formed by the tet-NRG1 strain in the presence of DOX was between 9,000 cells/ml (at 4 h) and 40,000 cells/ml (at 24 h). At all time points tested except for the first, the number of cells dispersed from biofilms formed by the tet-NRG1 strain was at least 1.5- to 3-fold lower than for cells released from SC5314 biofilms. After 24 h, the medium was switched to omit DOX. After just 4 h of this switch, there was a 1.5-fold increase in cell dispersion from tet-NRG1 biofilms. As time progressed, the plus-DOX-to-no-DOX switch triggered the hyphae in the biofilm to revert back to yeast cells that could easily disengage from the biofilms and disperse into the flowing medium. Dispersion in the absence of DOX was at levels that were at least 10 to 18 times higher than those observed for biofilms grown under the continuous presence of DOX. As expected, the control SC5314 biofilms showed no difference in the extent of biofilm dispersion, despite the switch to antibiotic-free medium. This adds to our previous observations on UME6 and PES1 control of biofilm dispersion (34) and also points to the complex regulatory mechanisms orchestrating this second part of the C. albicans biofilm developmental cycle.

Since mixed growth with SC5314 alleviated the biofilm-forming defects of the tet-NRG1 strain, we next questioned whether the wild-type strain’s presence in a mixed biofilm would have an impact on the extent of biofilm dispersion. For this experiment nonfluorescent SC5314 cells and GFP-labeled tet-NRG1 cells were mixed at a 50/50 ratio and allowed to adhere to SE strips. Mixed biofilms were developed under conditions of flow during 24 h, in YPD medium containing DOX. Aliquots of cells dispersed from biofilms were collected at 5, 8, and 12 h, and the proportion of fluorescent versus nonfluorescent cells in the mixture was determined. At 5 h, dispersed aliquots contained similar numbers of green fluorescent tet-NRG1 cells and nonfluorescent SC5314 cells (Table 1). As time progressed, there was a decrease in the release of tet-NRG1 cells compared to SC5314 (approximately a 30/70 ratio) (Table 1). A completely opposite result was obtained when the antibiotic was removed from the growth medium after the initial 24 h of incubation. Only 5 h after the switch to DOX-free medium, the mixed-species biofilm released a large number of fluorescent cells. By 12 h, the dispersed population contained greater than 80% green fluorescent tet-NRG1 cells and only about 20% SC5314 cells (Table 1). This indicated that overexpression of NRG1 can still lead to increased biofilm dispersion despite its presence in a mixed biofilm with the wild-type strain.

In conclusion, we found that the C. albicans transcriptional repressor Nrg1p plays an important role in biofilm formation and dispersion. This adds to its critical functions in filamentation and virulence (28–30). Together, these observations suggest that regulation of NRG1 expression could potentially lead to control of biofilm-related disseminated diseases and, additionally, point to Nrg1p as an attractive target for the development of novel antifungal agents.

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**TABLE 1. Biofilm dispersion in mixed C. albicans SC5314 and GFP-labeled tet-NRG1 biofilms**

| Time point (h) | With DOX | After switch to no DOX |
|---------------|----------|------------------------|
|               | Fluorescent (GFP-tet-NRG1) | Nonfluorescent (SC5314) | Fluorescent (GFP-tet-NRG1) | Nonfluorescent (SC5314) |
| 5             | 45.75    | 54.25                  | 70.80 | 29.20                  |
| 8             | 29.80    | 70.20                  | 77.50 | 22.50                  |
| 12            | 27       | 73                     | 80.20 | 19.80                  |

*The numbers of dispersed cells from each strain were quantified using fluorescence microscopy at different time points during formation of mixed (50:50) biofilms under conditions of flow in the presence of DOX and after a subsequent switch to medium without the antibiotic.*
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