RESEARCH ARTICLE

Variation in *Candida albicans EFG1* Expression Enables Host-Dependent Changes in Colonizing Fungal Populations

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ABSTRACT To understand differences in host-*Candida albicans* interactions that occur during colonization of healthy or compromised hosts, production of phenotypic variants and colonization of healthy or immunodeficient mice by *C. albicans* were studied. We showed that activity of the transcription factor Efg1p exhibited cell-to-cell variability and identified Efg1p as a major regulator of colonization. In *C. albicans* populations colonizing the murine gastrointestinal tract, average expression of *EFG1* differed depending on the immune status of the host. We propose that cellular heterogeneity in Efg1p activity allows the *C. albicans* colonizing population to differ depending on the immune status of the host, because selective pressure from a healthy host alters the composition of the population. These data are the first demonstration that differences in host immune status are associated with differences in gene expression in colonizing *C. albicans* cells. Altered gene expression in organisms colonizing immunocompromised hosts may begin the transition of *C. albicans* from a commensal to a pathogen.

IMPORTANCE In healthy people, the fungus *Candida albicans* colonizes the gastrointestinal tract and other sites without producing obvious pathology. In an immunocompromised patient, the organism can cause serious disease. The demonstration that the expression and activity of the *C. albicans* transcription factor Efg1p differs during colonization of healthy or immunocompromised mice shows that the organism adjusts its physiology when colonizing different hosts. Further, the effects of a healthy host on a heterogeneous *C. albicans* population containing cells with different levels of Efg1p activity show that selective pressure in the host can change the makeup of the population, allowing the population to respond to host immune status. The ability to sense host status may be key to the ability of *C. albicans* to colonize as a harmless commensal in some hosts but become a deadly pathogen in others.

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Candida albicans, one of the many microbes that colonize the human gastrointestinal (GI) tract, is both a commensal and an opportunistic fungal pathogen (1). *C. albicans* can become an invasive pathogen, causing life-threatening systemic candidiasis (1, 2, 3). Risk factors for development of candidemia include use of broad-spectrum antibiotics and immunosuppression or immunodeficiency (1, 4, 5).

Factors that determine whether *C. albicans* will behave as a benign commensal or an aggressive pathogen have been the focus of much study. One major determinant of *C. albicans* behavior is the status of the host immune system (3, 5, 6, 7, 8). Fungal activities, such as morphogenesis and differential gene expression, also play a role in determining the behavior of *C. albicans* in the host (9, 10, 11, 12, 13). For example, laboratory-grown hyphae (highly elongated cells that remain attached after cell division and lack constrictions at the septa) differ in their interactions with epithelial cells in comparison with laboratory-grown yeast cells, resulting in differential host responses (reviewed in reference 14). Several *C. albicans* regulatory factors that control morphogenesis in the laboratory affect GI colonization (9, 10) despite the fact that, in a previous study, fungal cells colonizing the GI tract were found to exhibit yeast cell morphology (9).

While these findings provide some insight into the interplay between host and colonizer, the fundamental question of how a change in host immune status influences colonizer behavior remains unanswered. We previously proposed that phenotypic heterogeneity in colonizing cells produces a population that can be shaped by host selective pressure, allowing the population to sense and respond to host immune status (15). To provide support for this model, we examined the role of the transcription factor Efg1p, a major regulator of *C. albicans* physiology (16, 17, 18), during colonization of the murine GI tract. Here, we show that Efg1p is an important regulator of colonization dynamics in the host and, like certain other proteins (19, 20, 21, 22, 23), exhibits cell-to-cell variation in expression and activity. We also demonstrate that the action of the host on a heterogeneous *C. albicans* population changes the composition of the population. Through this mechanism, the host is able to influence the physiology of its colonizer.

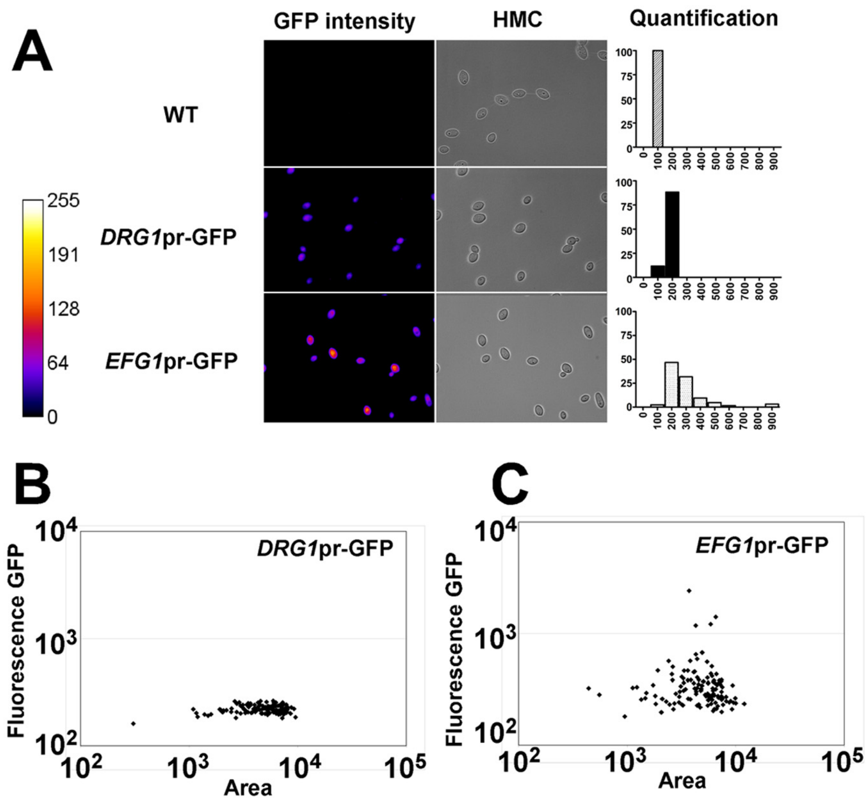

FIG 1 *EFG1* is expressed at variable levels within a population of cells. WT strains carrying *DRG1pr-GFP* or *EFG1pr-GFP* fusions were grown for 24 h at 37°C in YPD. Cells were washed, stained with Calcofluor white, and mounted as described in Materials and Methods. Fluorescence and Hoffman modulation contrast images were collected with a Nikon TE-2000 inverted U microscope. (A) Left panels, heat map of GFP fluorescence intensity. Thresholds were 200 minimum and 820 maximum, and the legend is shown at the left. Right panels, Hoffman modulation contrast (HMC). Graphs at right show the percentage of total cells (*y* axis) as a function of mean fluorescence per cell in arbitrary units (*x* axis). The experiment was repeated 3 times, and a representative experiment is shown. (B and C) Plot of mean GFP fluorescence per cell as a function of cell area for strains carrying *DRG1pr-GFP* (B) or *EFG1pr-GFP* (C).

RESULTS

Cell-to-cell variation in the levels of *EFG1* expression and activity. To test for heterogeneity in a *C. albicans* population, we analyzed the expression and activity of Efg1p. The promoter region of *EFG1* was cloned upstream of the gene encoding yeast enhanced green fluorescent protein (yEGFP) (24). As a control, the promoter of *DRG1*, a constitutive gene expressed at a level similar to that of *EFG1* (25), was similarly cloned. Expression of yEGFP was measured following growth to post-exponential phase in rich medium at 37°C. Cells carrying either fusion showed relatively low levels of fluorescence that were higher than the fluorescence of an untagged wild-type (WT) strain (Fig. 1A). Plots of fluorescence as a function of cell area showed that both strains exhibited similar ranges of cell size, and the fluorescence of cells carrying the *EFG1pr-GFP* fusion varied substantially (Fig. 1C), while cells carrying the *DRG1pr-GFP* fusion were more uniform in fluorescence (Fig. 1B). The coefficient of variance (COV) for cells expressing *DRG1pr-GFP* was 8.74%, indicating a sharp peak, while cells carrying the *EFG1pr-GFP* fusion showed a broader distribution of fluorescence (COV = 70.64%) (Fig. 1A). Therefore, cell-to-cell variation in expression from the *EFG1* promoter but not from the *DRG1* promoter was detected.

To test the hypothesis that Efg1p was differentially active in individual cells, expression of an Efg1p target gene was studied. To find target genes that were regulated by Efg1p during both laboratory growth and GI colonization, we tested candidate genes predicted by previous studies (26, 27), because some genes that are regulated by Efg1p during laboratory growth are expressed in an Efg1p-independent manner during colonization (9). We identified *FDH1* (encoding a putative formate dehydrogenase) as a gene that was negatively regulated by Efg1p in the GI tract of mice as well as during laboratory growth (see Fig. S1 in the supplemental material). To examine the activity of Efg1p as a negative regulator of *FDH1* in individual cells, the *FDH1* promoter was cloned upstream of ubiquitin-tagged yEGFP (uGFP; see Fig. S2 in the supplemental material). The majority of cells carrying the *FDH1pr-uGFP* fusion showed low fluorescence, while some of the cells showed higher fluorescence (Fig. 2A). Using ≥300 arbitrary units (AU) of fluorescence as an arbitrarily chosen threshold to define high fluorescence, 27% of the cells in a population of WT cells exhibited high fluorescence (Fig. 2A). In the absence of *EFG1*, the majority of the cells carrying *FDH1pr-uGFP* (84%) exhibited high fluorescence (Fig. 2A). Additionally, when *EFG1* was expressed from the strong promoter of the actin gene (*ACT1pr-EFG1*), expression of *FDH1pr-uGFP* was very low in most cells (Fig. 2A). Thus, Efg1p negatively regulated uGFP expression from the *FDH1* promoter, and other factor(s) contributed to the variable positive regulation of *FDH1*.

To measure *EFG1* and *FDH1* expression within the same cell, a fusion between the *EFG1* promoter and the gene encoding

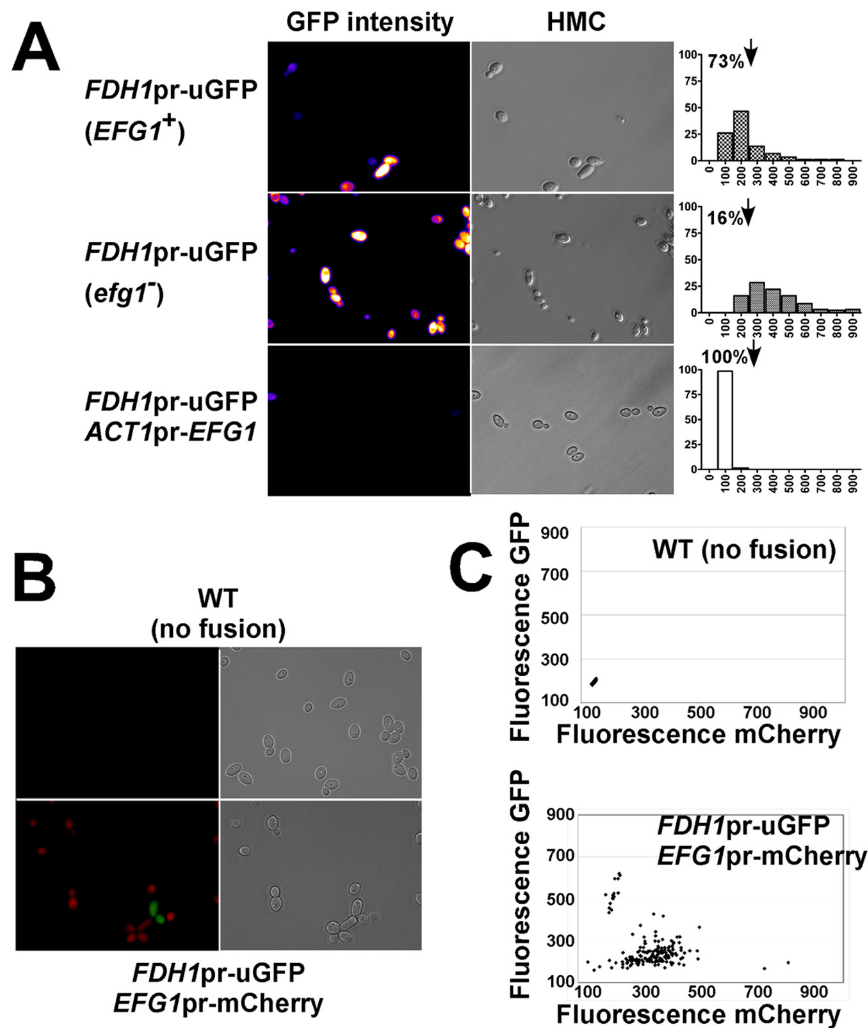

FIG 2 High expression of *FDH1* in a subpopulation of cells with low *EFG1* expression. WT, *efg1*$^-$ or *ACT1pr-EFG1* strains carrying *FDH1pr-uGFP* were grown for 24 h at 37°C in YPD. Cells were stained with Calcofluor white and visualized as described in the legend to Fig. 1. WT cells carrying either no fusion or both *FDH1pr-uGFP* and *EFG1pr-mCherry* were grown as described above and visualized without Calcofluor white staining. Fluorescence and Hoffman modulation contrast images were collected with a Nikon TE-2000 inverted U microscope. Experiments were repeated at least 2 times, and a representative experiment is shown. (A) The left panel shows a heat map of GFP fluorescence (same scale as in Fig. 1); the right panel shows an HMC image. Graphs at right show quantification of mean fluorescence per cell; the arrow demarcates 300 fluorescence units, and the number indicates the percentage of cells with fluorescence lower than 300 units. (B) The left panel shows the overlay of green and red fluorescence for the indicated strains. The right panel shows HMC image. (C) Intensity of green fluorescence (*FDH1* expression) as a function of intensity of red fluorescence (*EFG1* expression) is plotted. Each symbol indicates an individual cell. Strains are indicated above each graph.

mCherry protein (28) was transformed into a WT strain carrying the *FDH1pr-uGFP* fusion (Fig. 2B). Quantification of the fluorescence of both reporters showed that all cells with high levels of *FDH1* expression showed relatively low expression of *EFG1* (Fig. 2C); the abundance of such cells was typically 5% or less of the total population. Autofluorescence was lower than the level of fluorescence detected in the presence of these fusions (Fig. 2C). Therefore, within a population of WT cells, there is variability in Efg1p activity, and some WT cells expressed higher levels of *FDH1*, consistent with low Efg1p activity.

Cells lacking Efg1p showed increased colonization of the gastrointestinal tract and hypersusceptibility to the host response. To determine whether variable levels of Efg1p activity could affect GI colonization, a competition experiment was conducted in which mice were orally inoculated with 1:1 mixtures of a nourseothricin-resistant (nou^r) WT strain and an unmarked strain (either WT, an *efg1*$^-$ null mutant, or the complemented mutant). The WT population contains both high and low *EFG1*-expressing cells; mixing with *efg1*$^-$ null mutant cells increases the proportion of low *EFG1*-expressing cells. At various times postinoculation, fecal pellets were collected from mice, homogenized, and plated to determine the number of CFU per gram of sample (CFU/g) and the competitive index (CI; the ratio of the unmarked strain over the nou^r strain, divided by their ratio in the inoculum; see Materials and Methods). The *efg1*$^-$ null strain outcompeted WT *C. albicans* (CI > 1) at early time points (e.g., day 1; Fig. 3A; P $= 0.0001$, Mann-Whitney test). In comparison, the CI for competition between marked and unmarked WT strains was close to 1, and the CI for the complemented strain versus the marked WT strain was intermediate. At later times postinoculation, WT *C. al-*

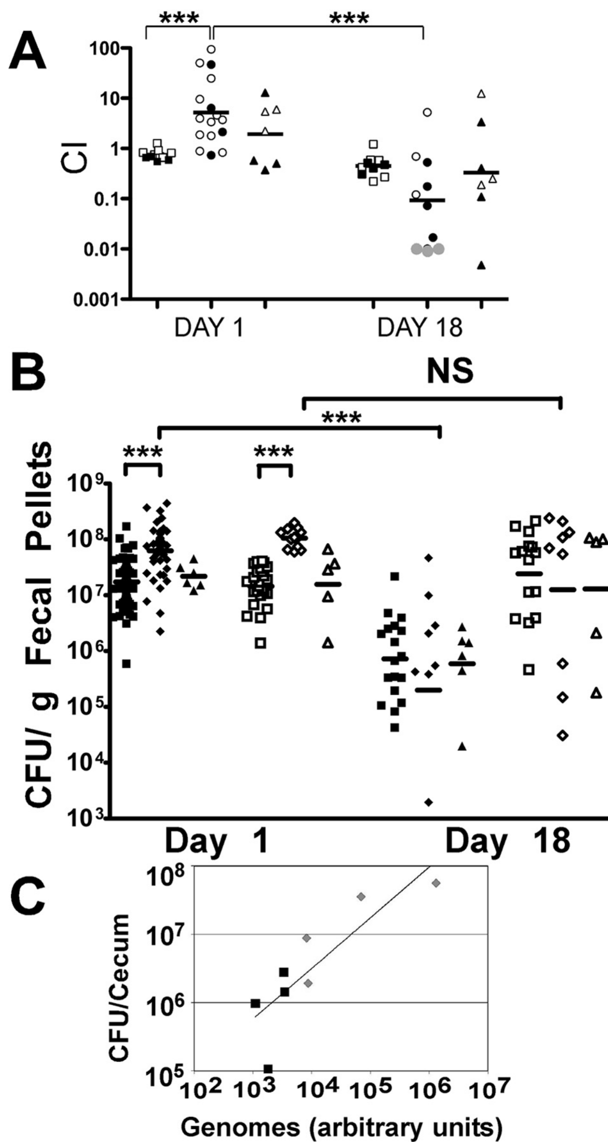

FIG 3 *efg1*⁻ cells outcompete wild-type *C. albicans* but are preferentially lost over time. (A) CI measured in fecal pellets at days 1 and 18 postinoculation. Cells of strain JPY105 (nourseothricin-resistant WT) were mixed 1:1 with either CKY101 (WT), CKY136 (*efg1*⁻ null), or HLC74 (*EFG1/efg1*⁻ complemented mutant) and inoculated by oral gavage into BALB/c (filled symbols) or Swiss Webster (open symbols) mice. Competition data are shown as the CI (the ratio of CFU for the unmarked mutant strain and the marked WT strain divided by the input ratio). Each symbol represents one mouse; bars are the geometric mean. Squares, nourseothricin^s WT/nourseothricin^r WT; circles, *efg1*⁻ strain/nourseothricin^r WT; triangles, complemented *efg1*⁻ strain/nourseothricin^r WT. Gray circles shown at a CI of 0.01 indicate samples with levels of the *efg1*⁻ strain below the limit of detection. ***, $P < 0.001$, Mann-Whitney test. (B) Colonization measured in fecal pellets at days 1 and 18 postinoculation. Single strains listed above were inoculated by oral gavage into BALB/c mice (closed symbols) or BALB/c *nu/nu* mice (open symbols). Squares, WT *C. albicans*; diamonds, *efg1*⁻ strain; triangles, complemented *efg1*⁻ strain. Each symbol represents an individual mouse, and bars are the geometric means. Samples were statistically significant by Kruskal-Wallis test ($P < 0.0001$) and Dunn's multiple comparison posttest. ***, $P < 0.001$, Mann-Whitney test for pairwise analysis (C). Colonization measured by qPCR. Cecum contents from BALB/c mice gavaged with WT or *efg1*⁻ null mutant cells were collected at day 3 postinoculation. One aliquot of cecum contents was plated and another was used for extraction of DNA. The number of CFU is plotted as a function of fungal genomes (in arbitrary units), measured using qPCR.

bicans cells began to outcompete the *efg1*⁻ mutant in fecal pellets (see Fig. S3 in the supplemental material). At day 18, the average CI for mutant/WT dropped to below 1 (Fig. 3A; P = 0.0003, Mann-Whitney test). In GI tract organ homogenates of mice sacrificed at either day 6 or day 18, the CIs were consistent with the CIs measured in fecal pellets on the same day (data not shown). Thus, at day 18, WT cells were more fit than the mutant. In summary, when both strains shared the same host, the *efg1*⁻ mutant had an initial colonization advantage over WT *C. albicans* but was preferentially eliminated at later times during colonization. We hypothesize that the *efg1*⁻ strain shows increased susceptibility to the host response that develops over time.

Results of single-strain oral gavage inoculations of BALB/c mice with WT, *efg1*⁻, or the complemented *EFG1/efg1*⁻ strains were consistent with the competition results. At early times postinoculation, the *efg1*⁻ strain exhibited increased colonization levels in fecal pellets compared to those of WT *C. albicans* (e.g., day 1; Fig. 3B; $P < 0.0001$, Mann-Whitney test). In fecal pellets collected at various times postinoculation, colonization by the *efg1*⁻ strain remained higher than WT for 6 to 9 days but then declined (data not shown). By day 18 postinoculation, the colonization of the *efg1*⁻ mutant was no longer above WT levels (Fig. 3B). Cecum homogenates from mice sacrificed on day 3 or 18 showed results that were consistent with results observed from fecal pellets (see Fig. S4 in the supplemental material). At 6 h postinoculation, WT and *efg1*⁻ null mutant cells were present at similar levels in multiple parts of the GI tract (see Fig. S5 in the supplemental material).

Colonizing WT *C. albicans* cells in the lumen of the GI tract are predominantly in the yeast form (9; data not shown). Nonetheless, to ensure that CFU measurements accurately reflected cell numbers and were not biased by different levels of filamentation in these strains, *C. albicans* genomes were measured during colonization by quantitative real-time PCR (qPCR). Cecum contents were either plated for CFU/g or used for the isolation of genomic DNA and qPCR. The number of genomes detected correlated with the CFU (Fig. 3C; $R^2 = 0.72$). WT samples occupied the lower end of both scales, while *efg1*⁻ samples occupied the higher end, confirming the conclusion of hypercolonization by the *efg1*⁻ mutant.

To test the role of the host immune response, colonization was studied in BALB/c *nu/nu* mice, which are athymic and T cell deficient. In these mice, colonization by both WT *C. albicans* and *efg1*⁻ null mutants remained high at later times postinoculation, consistent with previous results (29). At day 18 postinoculation, colonization levels in the fecal pellets (Fig. 3B; P = 0.0002, Mann-Whitney test) and cecum (see Fig. S4 in the supplemental material) of *nu/nu* mice were significantly higher than in BALB/c mice. *C. albicans* neither invaded GI tract tissue nor disseminated to deep organs in BALB/c or BALB/c *nu/nu* mice (data not shown). Therefore, the hypersusceptibility of the *efg1*⁻ strain to the immune response was not observed in this immunodeficient mouse.

To test the importance of possible differences in microbiota, in two experiments, BALB/c and BALB/c *nu/nu* mice were cohoused for 2 weeks prior to inoculation with *C. albicans*. This treatment results in sharing of the microbiota and can alter the susceptibility of a mouse to colonization by some microbes (30, 31). Cohousing did not alter the colonization dynamics of WT *C. albicans* (see Fig. S6 in the supplemental material), suggesting that differences in colonization between BALB/c and *nu/nu* mice are not dependent on historical differences in their microbiota.

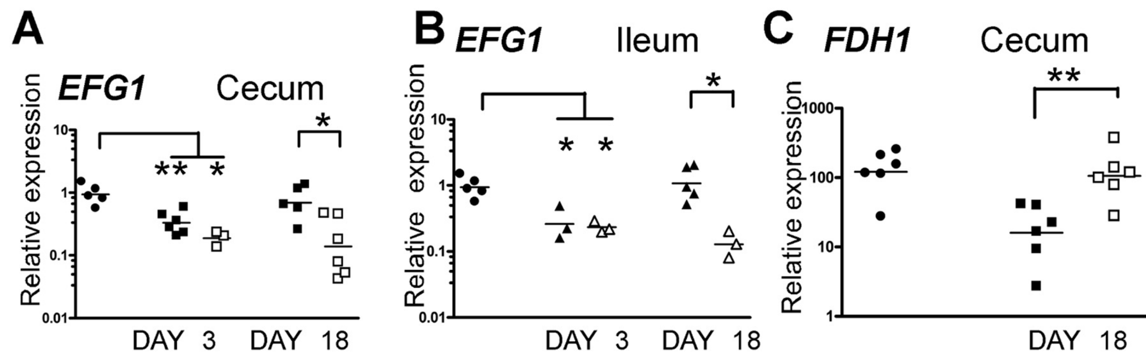

FIG 4 Transcription and activity of *EFG1* differ during colonization of healthy and immunocompromised hosts. Cells of WT strain CKY101 were recovered from organs of BALB/c (closed symbols) or BALB/c *nu/nu* mice (open symbols) on day 3 or 18 postinoculation. Expression of *EFG1* (A, B) or *FDH1* (C) was measured by qRT-PCR, normalized to *ACT1* levels, and expressed relative to expression in laboratory-grown exponential-phase cells. Each symbol represents a sample pooled from 2 (cecum contents) or 4 (ileum contents) mice. For laboratory-grown cells, each symbol represents an independently grown culture. Closed circles, laboratory-grown post-exponential-phase cells; triangles, ileum samples; squares, cecum samples. Bars represent the geometric mean; *, $P < 0.05$; **, $P < 0.01$; Mann-Whitney test.

In summary, in the absence of Efg1p, *C. albicans* hypercolonized at earlier times postinoculation. In a healthy mouse but not an immunodeficient mouse, this phenotype was unsustainable, and the mutant was less fit than WT *C. albicans* at later times postinoculation. These results suggest that, depending on the strength of the host response, selection during colonization will affect the overall Efg1p activity in the colonizing population. In this model, the differential sensitivity of individual cells in a heterogeneous population with cell-to-cell variation in Efg1p activity allows the population to respond to host status.

Differential *EFG1* expression during GI colonization of different hosts. The model predicts that, during colonization of different hosts, the average expression of *EFG1* would differ because the host acts differentially on individual cells with either high or low *EFG1* expression. To test this prediction, we measured the levels of *EFG1* transcript expressed by *C. albicans* cells during GI colonization. WT *C. albicans* cells were isolated from the lumen of the cecum or ileum of WT BALB/c mice. For comparison, *C. albicans* cells were grown in laboratory medium. Quantitative real-time reverse transcription-PCR (qRT-PCR) was used to measure the level of *EFG1* transcript normalized to the transcription of the actin gene, *ACT1*. In the cecum at 3 days postinoculation, expression of *EFG1* was lower than in laboratory-grown cells (Fig. 4A; P = 0.0087, Mann-Whitney test). However, in the cecum at 18 days postinoculation, *EFG1* expression was similar to expression in laboratory-grown cells (Fig. 4A). Expression of *EFG1* in the ileum was consistent with this trend (Fig. 4B). Therefore, *EFG1* transcript levels were low during the initial adaptation of *C. albicans* to the GI tract and increased over time.

To determine whether the expression of *EFG1* was different in an immunocompromised host, *EFG1* transcript levels were measured during colonization of T-cell-deficient BALB/c *nu/nu* mice. *EFG1* transcript levels were again found to be low at 3 days postinoculation relative to levels in laboratory-grown cells (Fig. 4A and B; $P = 0.0357$ for both organs, Mann-Whitney test). However, in BALB/c *nu/nu* organs at 18 days postinoculation, *EFG1* transcript levels remained low. At this time point, *EFG1* transcript levels in fungi colonizing *nu/nu* mice were significantly lower than levels in fungi colonizing the ceca ($P = 0.0173$, Mann-Whitney test) and ileum ($P = 0.0357$, Mann-Whitney test) of BALB/c mice. Thus, colonizing populations of *C. albicans* in immunocompetent and immunocompromised hosts differed in expression of *EFG1*.

To test the hypothesis that Efg1p was differentially active in cells colonizing different hosts, transcription of *FDH1* was studied. In the cecum at day 18 postinoculation, *FDH1* expression was higher in *nu/nu* mice than in BALB/c mice (Fig. 4C; P = 0.0087, Mann-Whitney test). Thus, *EFG1* transcription and activity as a negative regulator differed between *C. albicans* cells colonizing either healthy or T-cell-deficient hosts, consistent with the proposed model.

Recapitulation of *C. albicans* colonization dynamics with a mixture of *efg1*$^-$ null cells and *EFG1*-constitutive cells. The differences in *EFG1* expression between *C. albicans* cells colonizing WT and immunodeficient mice showed that colonizing *C. albicans* populations differ under various circumstances. These differences could arise through regulated changes in *EFG1* transcription. In addition, the effects of host selective pressure exerted on colonizing cells with low Efg1p activity could provide a mechanism for producing differences in colonizing populations. To test whether the host was capable of changing the composition of a colonizing population, we created a heterogeneous population by mixing two strains that were unable to regulate their expression of *EFG1*. A strain carrying the *ACT1pr-EFG1* fusion expressed *EFG1* highly during colonization (Fig. 5A). This strain was mixed 1:1 with a drug-resistant mutant lacking *EFG1*, and the artificial heterogeneous population was orally gavaged into BALB/c mice. Initially, the *efg1*$^-$ strain outcompeted the constitutively expressing *EFG1* strain (Fig. 5B). However, over time, the CI dropped and the *ACT1pr-EFG1* strain eventually outcompeted the *efg1*$^-$ mutant (Fig. 5B), even though the *ACT1pr-EFG1*-carrying strain colonized more poorly than WT cells when inoculated singly into BALB/c mice (data not shown). At day 18 postinoculation, the *ACT1pr-EFG1* strain outcompeted the *efg1*$^-$ strain throughout the GI tract (see Fig. S7 in the supplemental material). These results show clearly that changes in the composition of the colonizing population can occur in the absence of changes in *EFG1* expression in individual cells. Thus, the effects of the host response on the makeup of the population promote the ability of a colonizing *C. albicans* population to change its pattern of gene expression in response to host immune status.

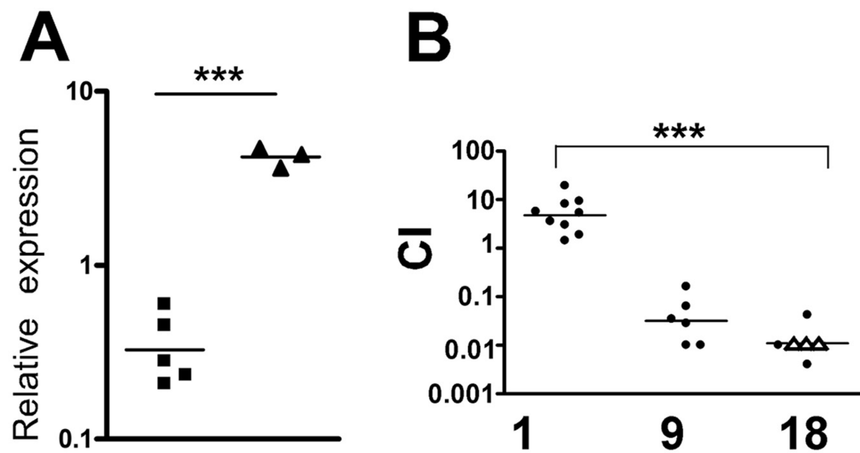

FIG 5 Cells with forced high expression of *EFG1* outcompete *efg1*$^{-}$ cells over time. (A) BALB/c mice were inoculated by oral gavage with strains CKY101 (WT) or JPY104 (*ACT1pr-EFG1*). Expression of *EFG1* in cells recovered from the cecum at day 3 postinoculation measured by qRT-PCR, normalized to *ACT1* expression, and shown relative to expression in laboratory-grown exponential-phase cells. Each symbol represents a sample pooled from 2 mice. Squares, WT *C. albicans*; triangles, *ACT1pr-EFG1* strain (B). CI determined in the fecal pellets at days 1, 9, and 18 postinoculation. JPY106 (*efg1*$^{-}$ nou^{r}) and JPY104 (*ACT1pr-EFG1* cells) were coinoculated into BALB/c mice at a 1:1 ratio by oral gavage. Competitive index (CI; ratio of the *efg1*$^{-}$ strain/*ACT1pr-EFG1* strain divided by the input ratio) is shown. Each symbol represents one mouse; bars are the geometric mean. Open triangles shown at CI of 0.01 indicate samples with levels of the *efg1*$^{-}$ strain below the limit of detection.

DISCUSSION

The results presented in this communication demonstrate that over time, *C. albicans* populations colonizing healthy or compromised hosts became different from each other in terms of gene expression. The differences in colonizing cells occurred despite the fact that tissue invasion and fungal dissemination were not observed in T-cell-deficient mice under the conditions of these experiments. Alterations in the expression of *EFG1* and the increased colonization levels seen in T-cell-deficient mice may represent a first step toward the initiation of disease, which could occur if the host were to become further compromised. Further study of the progression from colonization to disease will require use of immunodeficient mice that are susceptible to invasive or mucosal disease.

Results also showed that Efg1p is an important regulator of *C. albicans* colonization. Since Efg1p is a transcriptional regulator of a large number of genes (26, 27), many differences in gene expression are expected in response to changes in *EFG1* expression. Previous observations showed that fungal cells colonizing the GI tract as commensals exhibited yeast cell morphology and expressed adhesins and other virulence factors (9, 10). Expression of many of these factors was Efg1p independent in the GI tract (9), in contrast to their Efg1p-dependent expression under laboratory conditions (26, 27). Therefore, in the GI tract, WT cells of both morphologies and cells with low Efg1p activity are expected to express factors that allow them to interact with host epithelial cells.

We previously showed that the *EFG1* homolog *EFH1* is also an important regulator of colonization (9). Unlike *EFG1*, expression of *EFH1* is upregulated during growth in the GI tract relative to laboratory growth. In the absence of Efh1p, cells show enhanced persistence during colonization, in contrast to the hypersusceptibility of the *efg1*$^{-}$ null mutant. Thus, the two transcription factors have different effects on colonization.

We propose that WT cells with low Efg1p activity, arising due to natural variation in Efg1p activity from cell to cell, provide a mechanism that allows the colonizing population to respond to host immune status. Upon entry into a naive or compromised host, low-Efg1p-activity cells promote growth to higher levels. Over time in a healthy host, selective pressure leads to preferential loss of low-Efg1p-activity cells and an increased proportion of cells with higher Efg1p activity. We propose that under all conditions, the colonizing population is heterogeneous. Thus, if a change in host status occurs, diminishing the selective pressure on the population, a higher representation of low-Efg1p-activity cells would result, poising the population to initiate pathogenic interactions. We use the term "immunosensing" to describe the ability of colonizing *C. albicans* populations to change in response to host status (15).

The production of heterogenous phenotypic variants within a population has been described in other systems, including bistability in phenotypic state in *Bacillus subtilis* and the generation of persister cells in bacteria and *C. albicans* (32, 33, 34, 35, 36, 37). In the opportunistic fungal pathogen *Candida glabrata*, individual cells exhibited different levels of Epa1p, an important adhesin, and the different levels were maintained for several cell cycles (23). Switching has also been observed between two states of *C. albicans*, termed white and opaque, leading to differential mating capabilities (38, 39, 40, 41, 42). The ability to generate phenotypic variants increases diversity within a population and gives a subset of cells a survival advantage under stressful conditions, ensuring the survival of the population.

Efg1p negatively regulates its own transcription through a histone deacetylase-dependent mechanism (43). Transcriptional repression due to high levels of Efg1p would eventually cause a reduction in Efg1p levels, followed by relief of repression. Through this mechanism, subpopulations of cells with high and low levels of Efg1p could be generated. Simulations and modeling show that negative autoregulatory loops can promote oscillations in protein abundance (44, 45). For example, mammalian Hes1 protein, a transcription factor that binds to its own promoter and negatively regulates its own expression, oscillates in abundance with a 2-h

periodicity (46). In a more complex system involving at least two proteins, the human tumor suppressor p53 and its negative regulator Mdm2 both show oscillatory behavior following activation of p53 in response to DNA damage (47). Thus, oscillatory expression of *EFG1* in nonsynchronized cells could generate cell-to-cell variation in Efg1p activity, contributing to the responsiveness of *C. albicans* populations to host immune status.

MATERIALS AND METHODS

Strains. *C. albicans* strains used in this study are listed in Table S1 in the supplemental material. Details of strain construction, plasmid construction, and strain growth are provided in Materials and Methods in the supplemental material. The promoter regions of the *EFG1* and *DRG1* genes were amplified and cloned upstream of the genes encoding either yEGFP (24) or mCherry (28), while the *FDH1* promoter was cloned upstream of a ubiquitin-green fluorescent protein gene fusion. For expression of *EFG1* from the *ACT1* promoter, the *EFG1* open reading frame (ORF) was cloned onto plasmid Clp10 ACT1p-gLUC59 (48, 49).

Animal models. All experiments were done in compliance with regulatory guidelines defined by the Tufts University IACUC committee. Female 18- to 20-g Swiss Webster mice (Charles Rivers Laboratories) or 5- to 7-week-old BALB/c and BALB/c *nu/nu* mice (NCI) were treated with antibiotics (tetracycline, 1 mg/milliliter; streptomycin, 2 mg/milliliter; gentamicin, 0.1 mg/milliliter) as described (9). Mice were tested for fungal contamination prior to each experiment. Mice were inoculated with 0.1 ml *C. albicans* cells at 5×10^8 cells/milliliter by oral gavage. Colonization was tested over time by collecting fresh fecal pellets and plating homogenates on YPD agar plus streptomycin (100 μg/milliliter) and ampicillin (50 μg/milliliter) (YPD-SA). Mice were sacrificed at various time points postinoculation to determine levels of colonization in cecum contents and cecum wall and ileum homogenates. Combined results from at least 2 experiments are shown for all data. Homogenates of kidney, liver, and tongue were plated on YPD-SA. No colonization of these organs was detected in any experiment. Mann-Whitney, Kruskal-Wallis, and Student's *t* tests were done using GraphPad Prism software with *P* values of <0.05 as the cutoff for significance.

For competition experiments, colonies on YPD-SA plates were replica plated onto YP-sucrose-nourseothricin, and the ratio of drug-sensitive to drug-resistant cells was measured. The competitive index equals CFU *efg1*$^-$/CFU WT or *ACT1pr-EFG1* at time *x* divided by CFU *efg1*$^-$/CFU WT or *ACT1pr-EFG1* in the input. For *efg1*$^-$/WT competitions, the WT strain was nou^r. For *efg1*$^-$/ *ACT1pr-EFG1* competitions, the *efg1*$^-$ strain was nou^r.

Microscopy. To determine fluorescence, strains were grown to post-exponential phase at 37°C in YPD for 24 h. Cells were resuspended in a 1-mg/milliliter solution of Calcofluor white for 5 min and then washed twice in phosphate-buffered saline (PBS). To disrupt clumps, cells were diluted and shaken for 5 min at maximum speed using a Tissuelyser II (Qiagen). Cells were visualized using a Nikon Eclipse TE2000-U inverted microscope and a Roper Scientific charge-coupled-device (CCD) black-and-white camera using NIS-elements-BR software. GFP images were taken with a 460- to 490-nm filter, and Calcofluor white staining was detected with a UE-2E/C filter. mCherry was visualized with a G-2E/C filter. Photographs were taken at 90× magnification. Image analysis was performed using the ImageJ program (NIH) to quantify GFP fluorescence per cell. Eighty to 200 cells were imaged per strain, and experiments were repeated in triplicate. COV were used to measure the broadness of the distribution and were based on the image quantification (COV = average fluorescence/standard deviation × 100).

RNA and cDNA preparation. RNA was extracted from laboratory-grown samples using mechanical disruption and an RNeasy minikit (Qiagen) with on-column DNase I digestion. Samples of mouse GI tract contents were filtered through 250-μm polypropylene mesh (Small Parts, Inc.) and then pelleted and extracted using the Purelink kit Trizol extraction procedure (Invitrogen) with DNase I digestion. cDNA was generated with Superscript II or III reverse transcriptase (Invitrogen) and an oligo(dT) primer by following the manufacturer's procedures. Further details are provided in Materials and Methods in the supplemental material.

qRT-PCR. Primers used to detect expression of genes are listed in Table S2 in the supplemental material. qRT-PCR reactions were performed with SYBR green reaction mix (Qiagen) with reference dye ROX using an MX3000p or MX4000 instrument (Stratagene). Each sample was tested in triplicate, and melting curve analysis and agarose gel electrophoresis were used to confirm primer specificity. Samples tested with no cDNA or cDNA from an *efg1*$^-$ strain using *EFG1* primers showed no detectable signal. Standard curves were generated, and all results were normalized to the level of actin expression in each respective sample. All samples are presented normalized to the level of expression for each respective gene in wild-type cells grown in YPD at 30°C to an optical density of 1.

qPCR. Cecum contents, including fungal cells, from BALB/c mice collected at day 3 postinoculation were diluted with PBS. One aliquot was plated on YPD-SA as described above, and another was pelleted and weighed. DNA was extracted from 70 mg (wet weight) of pelleted cecum contents by bead beating in phenol-chloroform, followed by chloroform extraction and ethanol precipitation (9). The DNA was further purified using a Qiagen DNeasy kit and RNase digestion. The amount of *C. albicans* DNA present in each sample was determined using qPCR as described (9).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00117-12/-/DCSupplemental.

Text S1, DOC file, 0.1 MB.
Figure S1, TIF file, 0.9 MB.
Figure S2, TIF file, 0.8 MB.
Figure S3, TIF file, 0.1 MB.
Figure S4, TIF file, 1.1 MB.
Figure S5, TIF file, 0.1 MB.
Figure S6, TIF file, 1 MB.
Figure S7, TIF file, 0.9 MB.
Table S1, DOC file, 0.1 MB.
Table S2, DOC file, 0 MB.

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