Bacteria Modify Candida albicans Hypha Formation, Microcolony Properties, and Survival within Macrophages

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ABSTRACT Phagocytic cells are crucial components of the innate immune system preventing Candida albicans mucosal infections. Streptococcus gordonii and Pseudomonas aeruginosa often colonize mucosal sites, along with C. albicans, and yet interkingdom interactions that might alter the survival and escape of fungi from macrophages are not understood. Murine macrophages were coinfected with S. gordonii or P. aeruginosa, along with C. albicans to evaluate changes in fungal survival. S. gordonii increased C. albicans survival and filamentation within macrophage phagosomes, while P. aeruginosa reduced fungal survival and filamentation. Coinfection with S. gordonii resulted in greater escape of C. albicans from macrophages and increased size of fungal microcolonies formed on macrophage monolayers, while coinfection with P. aeruginosa reduced macrophage escape and produced smaller microcolonies. Microcolonies formed in the presence of P. aeruginosa cells outside macrophages also had significantly reduced size that was not found with P. aeruginosa phenazine deletion mutants. S. gordonii cells, as well as S. gordonii heat-fixed culture supernatants, increased C. albicans microcolony biomass but also resulted in microcolony detachment. A heat-resistant, trypsin-sensitive pheromone processed by S. gordonii Eep was needed for these effects. The majority of fungal microcolonies formed on human epithelial monolayers with S. gordonii supernatants developed as large floating structures with no detectable invasion of epithelium, along with reduced gene expression of C. albicans HYR1, EAP1, and HWP2 adhesins. However, a subset of C. albicans microcolonies was smaller and had greater epithelial invasiveness compared to microcolonies grown without S. gordonii. Thus, bacteria can alter the killing and escape of C. albicans from macrophages and contribute to changes in C. albicans pathogenicity.

IMPORTANCE Candida albicans is the predominant fungus colonizing the oral cavity that can have both synergistic and antagonistic interactions with other bacteria. Interkingdom polymicrobial associations modify fungal pathogenicity and are believed to increase microbial resistance to innate immunity. However, it is not known how these interactions alter fungal survival during phagocytic killing. We demonstrated that secreted molecules of S. gordonii and P. aeruginosa alter C. albicans survival within the phagosome of macrophages and alter fungal pathogenic phenotypes, including filamentation and microcolony formation. Moreover, we provide evidence for a dual interaction between S. gordonii and C. albicans such that S. gordonii signaling peptides can promote C. albicans commensalism by decreasing microcolony attachment while increasing invasion in epithelial cells. Our results identify bacterial diffusible factors as an attractive target to modify virulence of C. albicans in polymicrobial infections.

KEYWORDS Candida albicans, Pseudomonas aeruginosa, Streptococcus gordonii, hyphal development, macrophages, microcolonies

Oral candidiasis is a superficial mucosal infection by Candida albicans that appears macroscopically as white lesions and microscopically as interconnected radiating hyphae originating from single cells termed microcolonies (1). Microcolonies are a more
virulent form of fungal growth due to their extensive hyphae that invade epithelial cells, as well as their high expression of several virulence genes, including ECE1 (encoding candidalysin, a peptide toxin critical for mucosal infection), HYR1 (encoding a hyphal wall protein that modulates phagocytic killing activity), and HWI1 (encoding a hyphal wall protein that mediates tight binding to oral epithelial cells) (2).

In the oral environment, C. albicans typically resides in complex polymicrobial communities, along with multiple bacterial and other fungal species. Interactions between C. albicans and single bacterial species may be synergistic or antagonistic and have been well described in excellent reviews (3–5). C. albicans exhibits cooperative relationships with multiple oral streptococci but has a particularly high affinity for Streptococcus gordonii (6, 7), a Gram-positive bacterium that is one of the first colonizers of the oral cavity (8). Coinfection of C. albicans and oral streptococci in a murine model of oral candidiasis increases the severity of fungal lesions (9), and C. albicans biofilms formed in vitro were synergistically increased by streptococci (7, 10). In addition to physical interactions, both species respond to signaling metabolites, including quorum-sensing molecules. For example, autoinducer 2 secreted by S. gordonii relieves farnesol-induced suppression of C. albicans hyphal formation within cospecies biofilms (7), so that C. albicans virulence and invasion into epithelial tissues is promoted. The role of S. gordonii in C. albicans hyphal morphogenesis and virulence is further supported by a transcriptional study by Dutton et al. showing that coculture of C. albicans with S. gordonii increases expression of genes required for morphogenesis (TEC1 and ALS1) and oxidative stress response (CAT1) (11).

In contrast with S. gordonii, interactions between Pseudomonas aeruginosa and C. albicans are mainly antagonistic (12, 13). P. aeruginosa is commonly isolated as part of mixed infections with C. albicans from the lungs of cystic fibrosis patients (14) and as a part of the oral microbiota (15). P. aeruginosa, a Gram-negative opportunistic rod, is becoming increasingly important in clinical settings because of its antibiotic resistance (16). Clinical isolates of P. aeruginosa are able to reduce C. albicans survival (17) and kill germinated C. albicans cells (18, 19) due to secretion of bacterial phenazines (20). Besides its candidacidal activity, P. aeruginosa influences C. albicans morphology by reducing filamentation (21), and can adhere to C. albicans hyphal cells in response to a secreted quorum-sensing molecule, 3-oxidodecanoyl-l-homoserine lactone (22). This antagonistic interaction is bilateral, since C. albicans can produce farnesol that reduces P. aeruginosa secretion of pyocyanin (23) and motility (24). Importantly, prior colonization of lungs by C. albicans increased the clearance of subsequent P. aeruginosa infection via leukocyte recruitment (25).

Key components of innate immunity are phagocytic cells, including macrophages that recognize and engulf C. albicans and other microbes into phagosomes where they are killed (26–28). C. albicans, as well as bacteria, can survive phagocytic killing (29–31) by suppressing phagosomal maturation (31) or reactive oxygen species (ROS) production (32, 33). Despite the well-known relationships of C. albicans with S. gordonii and P. aeruginosa, it is unclear whether simultaneous phagocyte interactions with yeast and bacteria affects the subsequent survival of C. albicans. Phagocytosis of S. gordonii alone by activated macrophages can permit phagosomal disruption on maturation, resulting in increased bacterial survival (34), and P. aeruginosa can evade phagocytosis by modifying its motility (35, 36). It is likely that cospecies interactions within phagocytes will further modify their survival.

In this study, we investigated the effect of these two bacteria on C. albicans after phagocytosis by macrophages. We show for the first time that phagocytosis of C. albicans in the presence of selected strains of these bacteria altered the survival of C. albicans within macrophages that was associated with induction or repression of fungal hyphae. P. aeruginosa decreased fungal survival in macrophages and inhibited C. albicans filamentation and microcolony formation, while S. gordonii increased survival and C. albicans hyphal formation and their escape from macrophage. C. albicans microcolony density and biomass were also significantly altered by products of both bacteria. Phenazines produced by P. aeruginosa reduced microcolony biomass, while S.
**RESULTS**

Bacterial species modify *C. albicans* survival within murine macrophages. *S. gordonii* (strains CH1, SK12, and DL1), a Gram-positive coccus, and *P. aeruginosa* (strains PAO1, 0635, and PA14), a Gram-negative rod, were tested for their ability to modify *C. albicans* survival and escape from macrophages. Either *S. gordonii* or *P. aeruginosa* species were added with *C. albicans* so that 95 to 100% of *C. albicans* and 90 to 95% of bacteria were phagocytosed (multiplicities of infection [MOIs] of 0.1 *C. albicans*:0.1 bacteria or 0.1 *C. albicans*:1 bacteria). After 3 h of coincubation, macrophages were lysed, and internalized *C. albicans* was released and plated on antibiotic supplemented agar to remove all bacteria and obtain *C. albicans* CFU. Survival was calculated as follows: (recovered *C. albicans* CFU from macrophages/total number of phagocytosed *C. albicans* CFU) × 100. Coincubation with *S. gordonii* SK12 significantly increased *C. albicans* survival, while *P. aeruginosa* 0635 and PA14 significantly decreased *C. albicans* survival. Means ± the standard deviations (SD) of at least three independent experiments carried out in duplicate are shown. Significance was obtained using one-way ANOVA with post hoc Dunnett’s multiple-comparison test (**, *P < 0.01; ***,** P < 0.001). Labels: Ca, *C. albicans*; Sg, *S. gordonii*; Pa, *P. aeruginosa*.

gordonii culture supernatants increased biomass, but simultaneously caused loss of adhesion of fungal microcolonies to the underlying substrate and reduced epithelial invasion. Coincubation with *S. gordonii* also led to altered expression of some *C. albicans* microcolony virulence genes, suggesting that *S. gordonii* can modify microcolony virulence.
although we could not ascertain their absolute proximity. At 3 h after coinfection, neither *S. gordonii* CH1 or *S. gordonii* DL1 had significantly altered *C. albicans* survival within phagosomes compared to *C. albicans* alone (99.7%; Fig. 1, lower left panel). In contrast, coinfection with *S. gordonii* SK12 significantly increased survival (137 to 145%) of *C. albicans* after 3 h compared to the control. In contrast, cosppecies infection of *C. albicans* with *P. aeruginosa* clinical isolates (0635 or PA14) resulted in a 23% decrease in *C. albicans* survival after 3 h compared to *C. albicans* alone, which was not observed with the laboratory strain of *P. aeruginosa* (PAO1).

**S. gordonii** promoted and *P. aeruginosa* repressed *C. albicans* hyphal formation within macrophages. Since *C. albicans* is known to initiate hyphal formation in macrophages in order to escape killing (37), we next examined whether the presence of bacteria altered *C. albicans* hypha formation within the lumens of phagosomes. To compare hyphal length outside macrophages to that inside phagosomes, we used a higher MOI (0.5 *C. albicans* alone or 0.5 *C. albicans*:5 bacteria) so that some *C. albicans* (and bacteria in coinfection experiments) would not be phagocytosed, allowing us to compare germination outside macrophages with that within phagosomes. Surprisingly, the presence of *S. gordonii* DL1 (*Sg*DL1) or *Sg*SK12 both resulted in a significant (*P* < 0.01) increase in phagosomal *C. albicans* hyphal formation (89 to 86% hyphal formation with bacteria compared to 75% germination with *C. albicans* monoinfection) after 1 h (Fig. 2, left upper panel). After 2 h, there was no statistical difference between number of germinated cells between monoinfection and coinfection. However, 2 h after coinfection with *S. gordonii*, most *C. albicans* within the phagosome formed very elongated hyphae (white arrows) compared to monoinfection in which many *C. albicans* had very short hyphae (black arrows, Fig. 2, upper panel). In comparison, extracellular nonphagocytosed *C. albicans* (Fig. 2, Ca+5g panel, purple stain) formed similar long hyphae as phagosome-localized *C. albicans* in the presence of *S. gordonii*. Also, at 2 h after coinfection with *S. gordonii*, many intracellular *C. albicans* hyphae could be seen penetrating the phagosome so that the distal tips of hyphae were outside the macrophage. In monoinfections with *C. albicans* alone, most hyphae did not have sufficient length after 2 h to penetrate macrophages.

In contrast, the presence of *P. aeruginosa* had no effect on phagosomal *C. albicans* germination at 1 h, but after 2 h *P. aeruginosa* 0635 and PA14 significantly (*P* < 0.05) reduced hyphal formation compared to monoinfection of *C. albicans* (Fig. 2, lower panel). Microscopically, coinfection with *P. aeruginosa* reduced hyphal length of *C. albicans* within the phagosome compared to extracellular *C. albicans*; and few hyphae were seen to escape macrophages at 2 h. To better quantify these observations, we next tested the ability of *C. albicans* to escape from macrophages and form microcolonies after coinfection with either *S. gordonii* or *P. aeruginosa*.

**Bacteria modify escape of *C. albicans* from macrophages and alter the size of resulting *C. albicans* microcolonies.** In order to measure relative escape ratios, macrophages were infected at an MOI of 1 macrophage: 0.1 *C. albicans*:0.1 bacteria (or 1 macrophage: 0.1 *C. albicans* for monoinfection) to ensure nearly complete phagocytosis of both yeast and bacteria, incubated together for 3 h, and then streptomycin and penicillin were added to suppress the growth of escaped or nonphagocytosed bacteria. Macrophage monolayers and fungal cells then were grown at 37°C in 5% CO2 for 17 h to permit visualization of *C. albicans* able to form microcolonies following escape from macrophages. *C. albicans* cells from monoinfections formed clearly visible microcolonies that grew on top of macrophages without damaging the majority of underlying cells (Fig. 3A), suggesting that the growth of escaped fungal cells did not result in lysis of nearby macrophages. The mean escape ratio for *C. albicans* monoinfection was 76 microcolonies per 100 added *C. albicans* (Fig. 3B). *C. albicans* and *Sg*DL1 coinfection resulted in the formation of microcolonies that were much larger and denser than for those of monoinfection alone and increased the escape ratio to 96/100. *C. albicans* and *P. aeruginosa* PA14 (*Pa*PA14) coinfection led to a much lower escape ratio of 55/100, and the microcolonies formed were much smaller and less dense. Since phenazine production by *P. aeruginosa* is known to inhibit filamentation, we examined a *P.
aeruginosa phenazine mutant (Δphz) expecting that these cells would more closely resemble monoinfection by *C. albicans*. Indeed, Δphz cells had a significantly higher escape ratio (70/100) and formed denser and larger microcolonies than did the parental *P. aeruginosa* strain, suggesting that phenazine production by *P. aeruginosa* is largely responsible for the reduction in *C. albicans* microcolony size and reduced escape from macrophages.

**Maturation of *C. albicans*-containing phagosomes and ROS production was not altered by *S. gordonii* and *P. aeruginosa***. To test whether changes in killing and escape of *C. albicans* from macrophages after coinfection with bacteria may be a result of changes in phagosomal maturation or ROS production, we measured the maturation kinetics of *C. albicans*-containing phagosomes by the loss of EEA1 (a marker of early maturation) and the acquisition of Lamp1 or lysozyme-chased dextran (markers of phagosomal maturation) (34, 38). Coinfection of macrophages with *C. albicans* and either *S. gordonii* or *P. aeruginosa* did not change the rate of phagosomal maturation, as measured by the loss of EEA1 or the increase of dextran. Acquisition of Lamp1 differed only for one strain of *S. gordonii* (SK12) (see Fig. S2), in total showing that *C. albicans* survival and escape from macrophages in the presence of bacteria was not likely to be altered by phagosomal maturation. Since production of ROS by phagosomes is an important killing mechanism (39), we also examined the total release of

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**FIG 2** *C. albicans* hypha formation within macrophages is altered in the presence of bacteria. *C. albicans* CAF2-dTomato was added in an MOI of 0.5 to RAW 264.7 macrophages in the presence of *S. gordonii* (upper panels) or *P. aeruginosa* strains (lower panels). Macrophages and *C. albicans* were incubated without bacteria as a control. Nonphagocyted *C. albicans* were stained with calciofluor white (blue), and macrophages were stained with phalloidin (green). At least 100 phagocyted *C. albicans* (red) were counted and classified morphologically as yeast or hyphae microscopically. The percentage of hyphal cells was calculated by obtaining the ratio of total *C. albicans* with hyphae/total number of *C. albicans* counted × 100. *C. albicans* long hyphae (white arrows) or short hyphae (black arrows) within macrophages are shown with or without bacteria (right panel). Experiments were carried out in duplicate, and graphs represent the means ± the SD of three independent experiments. Significance was obtained by one-way ANOVA with post ad hoc Dunnett’s multiple-comparison test (*, P < 0.05; **, P < 0.01).

Scale bar, 10 μm.
ROS over 2.5 h from macrophages incubated with _C. albicans_ in combination with _S. gordonii_ or _P. aeruginosa_ strains compared to monoinfection with _C. albicans_ alone (Fig. S2). Thus, altered _C. albicans_ survival in macrophages in the presence of bacteria did not appear to be a result of altered ROS production or delayed phagosomal maturation within the initial 90 to 180 min after coinfection.

**C. albicans** microcolonies are modified by secreted molecules of _P. aeruginosa_ and _S. gordonii_. Since these data suggested a role for secreted bacterial products in altering _C. albicans_ filamentation within macrophages, we tested whether either bacteria themselves or secreted products modified microcolony formation outside macrophages. For these experiments, _C. albicans_ microcolonies were grown on solid surfaces in the presence of bacteria or bacterial supernatants to allow quantification of growth. Similar to what we observed within macrophages, _C. albicans_ microcolonies formed in the presence of three _P. aeruginosa_ strains (PAO1, Pa0635, and PA14) were smaller and had fewer hyphal projections (Fig. 4A), and addition of cell-free _P. aeruginosa_ culture supernatants (sPa) resulted in nearly 50% reduction in microcolony density for PAO1 or PA14 strains (Fig. 4B). Quorum-sensing molecules secreted by _P. aeruginosa_ play an important role during polymicrobial interactions (22, 24) and, among them, phenazines
are known to suppress *C. albicans* hyphal morphogenesis (19, 20). We therefore decided to further examine the role of phenazines by using a *P. aeruginosa* phenazine knockout (Δphz) mutant, along with synthetic the methylphenazinium analogs phenazine methosulfate (PMS) and pyocyanin (PYO) that suppress hypha morphogenesis in agar (40). The addition of Δphz cells still resulted in some reduction in *C. albicans* microcolony formation.
size, but the *P. aeruginosa* Δ*pzh* culture supernatants did not inhibit microcolony formation consistent with the loss of secreted phenazines (Fig. 4). The addition of purified phenazines PMS (5 μM) or PYO (20 μM) almost completely blocked microcolony formation (Fig. 4A), showing that phenazines suppress *C. albicans* filamentation and repress microcolony formation.

Since we observed promotion of germination by *S. gordonii* cells in macrophages, we expected a similar effect on microcolony formation. Indeed, the addition of *S. gordonii* CH1, DL1, and SK12 cells, cell supernatants, and heat-fixed (HF) supernatants all increased the size of *C. albicans* microcolonies by increasing hyphal length and number of radiating hyphae (Fig. 5A). Quantification of microcolony density showed a significant increase in density between 25 and 50% among *S. gordonii* strains and with only fresh culture supernatants compared to *C. albicans* alone (Fig. 5B, left). However, we also noticed that microcolonies formed in the presence of *S. gordonii* lost their ability to adhere to the substrate, a phenomenon we did not find with *P. aeruginosa*
experiments. Incubation with HF supernatants from each of the three *S. gordonii* strains with *C. albicans* that resulted in microcolonies that were so loosely adherent that they could not be quantitated by ImageJ. Instead, the total biomass of microcolonies was measured by crystal violet (CV) staining. The HF supernatants of all *S. gordonii* strains significantly increased *C. albicans* microcolony biomass by 3-fold compared to untreated *C. albicans* cells (Fig. 5B, right panel).

*S. gordonii* Eep contributes to *C. albicans* hypha formation and microcolony biomass. Many streptococcal signaling molecules known to affect *C. albicans* biofilm formation are small peptides and competence factors. Therefore, we first treated *S. gordonii* supernatants with trypsin (200 μg/ml) or DNase (500 μg/ml) before heat inactivation. Trypsin treatment of HF sSg CH1 significantly reduced *C. albicans* microcolony biomass, while DNase treatment had no effect. HF sSg of CH9278 missing comCDE competence regulatory system had no effect on *C. albicans* microcolony biomass. In contrast, the CH1 Δeep strain missing the zinc metalloprotease Eep significantly reduced *C. albicans* microcolony biomass, and this effect was abolished in the CH1Δeep/pSgEep1 complemented strain. *C. albicans* microcolony biomass was not altered by Todd-Hewitt (TH; 10%) medium alone. Experiments were carried out in duplicates, and the means ± the SD of three independent experiments are shown. Significance was calculated by one-way ANOVA with post hoc Dunnett’s multiple-comparison test (**, *P* < 0.01).

**FIG 6** *C. albicans* microcolony biomass is increased by a small peptide processed by *S. gordonii* Eep. *C. albicans* microcolonies were cultured using RPMI 1640 medium at 37°C in 5% CO₂, along with heat-fixed supernatants from *S. gordonii* (HF sSg) strains (10%) for 17 h, and the biomass was measured by crystal violet staining. Trypsin treatment of HF sSg CH1 significantly reduced *C. albicans* microcolony biomass, while DNase treatment had no effect. HF sSg of CH9278 missing comCDE competence regulatory system had no effect on *C. albicans* microcolony biomass. In contrast, the CH1 Δeep strain missing the zinc metalloprotease Eep significantly reduced *C. albicans* microcolony biomass, and this effect was abolished in the CH1Δeep/pSgEep1 complemented strain. *C. albicans* microcolony biomass was not altered by Todd-Hewitt (TH; 10%) medium alone. Experiments were carried out in duplicates, and the means ± the SD of three independent experiments are shown. Significance was calculated by one-way ANOVA with post hoc Dunnett’s multiple-comparison test (**, *P* < 0.01).
results show that an S. gordonii heat-resistant, trypsin-sensitive pheromone processed by Eep promotes C. albicans hyphal microcolony formation.

**Heat-fixed supernatant of S. gordonii increases the expression of C. albicans genes required for hyphal morphogenesis while decreasing the expression of adhesion genes.** Since we observed that incubation with S. gordonii HF supernatant increased C. albicans microcolony size but decreased yeast adhesion to the substrate, we next measured the expression of genes required for C. albicans filamentation (EFG1 and HGC1), virulence (ECE1), and adhesion (HYR1, EAP1, HWP1, ALS3, and HWP2) by quantitative reverse transcription-PCR (RT-qPCR). Consistent with increased filamentation and microcolony size induced by S. gordonii (Fig. 5), C. albicans expression levels of EFG1 and HGC1 genes associated with filamentation were significantly increased (2- to 7-fold) in the presence of S. gordonii HF supernatant (Fig. 7, upper panel). Intriguingly, we found that the expression of ECE1, required for virulence through candidalysin production, was significantly reduced in the presence of S. gordonii HF supernatant (Fig. 7, lower panel). Importantly, the expression of HYR1, EAP1, and HWP2 adhesins that are involved in initial attachment to surfaces was significantly decreased in the presence of all HF supernatants (Fig. 7, lower panel) and may partially account for the loss of adhesion of microcolonies to the surface that we observed. However, genes involved in some cell-cell and cell-substrate adhesion, including ALS3 and the hyphal wall protein HWP1, were upregulated, consistent with their hypha-specific expression. Together, these data confirm the increase in filamentation induced by S. gordonii, as shown by increased hypha-specific gene expression, along with altered expression levels among genes involved in adhesion.
Heat-fixed supernatants of *S. gordonii* decreased *C. albicans* microcolony adherence to oral epithelial cells while increasing microcolony size. Since adhesion and virulence genes were altered in microcolonies in the presence of *S. gordonii* supernatants, we examined whether the presence of *S. gordonii* would modify *C. albicans* microcolony adherence and invasion with oral epithelial cells. We first grew microcolonies with 1.25 to 20% *HF S. gordonii* culture supernatants (HF sSg) for 17 h and then gently washed each well to remove nonadherent cells before visualizing adherent microcolonies (Fig. 8A). Even addition of 1.25% of HF sSg resulted in some loss of adhesion compared to microcolonies grown without HF sSg; however, a subset of microcolonies had both high adhesion and invasion. Microcolonies grew on epithelial cells without *S. gordonii* were used as a control (No Sg). Scale bar, 10 μm. Experiments were carried out in duplicate and performed two times. Images represent one biological replicate.

**FIG 8** *S. gordonii* heat-fixed supernatant alters adherence of *C. albicans* microcolonies to oral epithelial cells. Oral epithelial cells were cultured in glass coverslips with DMEM/F-12 medium supplemented with 10% FBS at 37°C until reaching confluence. *C. albicans* cells and heat-fixed supernatant from *S. gordonii* DL1 (HF sSg; 1.25, 2.5, 5, 10, and 20%) were grown together at 37°C in 5% CO₂ for 17 h to form microcolonies. (A) Microcolony adhesion decreased in the presence of increasing concentrations of HF sSg. (B) Noninvasive (floating) *C. albicans* cells were stained with anti-*C. albicans* primary antibody, followed by Alexa Fluor 488 secondary antibody (shown in green). *C. albicans* hyphae invading epithelium are indicated by the unstained area (lower panel, white dotted line). The majority of microcolonies formed in the presence of HF sSg had decreased adhesion to epithelial monolayers (floating) compared to microcolonies grown without HF sSg; however, a subset of microcolonies had both high adhesion and invasion. Microcolonies grew on epithelial cells without *S. gordonii* were used as a control (No Sg). Scale bar, 10 μm. Experiments were carried out in duplicate and performed two times. Images represent one biological replicate.
DISCUSSION

*C. albicans* and *P. aeruginosa* are found together in the sputum and lungs of cystic fibrosis patients (14), while *S. gordonii* and *C. albicans* are commonly isolated together in various oral biofilms, including prosthetic surfaces in denture stomatitis (10, 43). Macrophages act as immune sentinels performing a variety of functions (44), so that macrophage depletion increased the risk for systemic candidiasis and streptococcal bacterial load, both leading to increased mortality in mice (27, 45). Furthermore, tissue-resident macrophages are important for triggering immune responses in the lung during *P. aeruginosa* pneumonia (46). Although phagocytic cells are highly effective at controlling infection, pathogenic bacteria and fungi have evolved multiple mechanisms to escape killing. *C. albicans* hypha formation within macrophages is an escape mechanism to subvert phagosomal clearance (47). Similarly, *P. aeruginosa* and certain strains of *S. gordonii* can also resist phagosomal killing in macrophages by promoting autophagy or suppressing phagosomal maturation (34, 48). Our results indicate that *C. albicans* survival in macrophages in the presence of *S. gordonii* or *P. aeruginosa* are not dependent upon changes in macrophage phagocytic uptake, phagosome maturation, or ROS production. Instead, we found that germination and hyphal production induced by coinfecting bacteria were most predictive of fungal survival within macrophages (Fig. 2), so that hyphal expansion results in phagosome membrane damage (49) and eventual escape. Thus, we found that *C. albicans* escape and subsequent microcolony formation after 17 h was proportional to the degree of hyphal promotion or suppression induced earlier (2 to 3 h) by coinfecting bacteria.

To understand how *S. gordonii* and *P. aeruginosa* alter *C. albicans* filamentation, we examined bacterial effects on microcolony formation outside macrophages and found that the addition of heat-fixed supernatants of *S. gordonii* significantly altered fungal biomass. Although previous work found that *S. gordonii* cells promote fungal hyphal morphogenesis via direct contact (50), we found that *S. gordonii* supernatant alone was effective in promoting microcolony formation. Thus, while physical interaction between fungal and bacterial cells may provide synergy, *S. gordonii* binding to *C. albicans* is not required for this effect on filamentation. We also found that *S. gordonii* competence factors are not involved. Instead, a heat-stable, trypsin-sensitive small peptide processed by the Eep metalloprotease appears to be crucial for hyphal elongation in *C. albicans* microcolony formation. Although the *S. gordonii* pheromone autoinducer 2 has been suggested as one diffusible molecule affecting filamentation of *C. albicans* (7), to our knowledge this is the first instance of an Eep processed pheromone signal peptide affecting fungal growth. In addition to potential lipoprotein signal sequences known to be cleaved by Eep, any protein signal peptide encoded in the *S. gordonii* genome could potentially be cleaved by Eep, including a large number of unannotated small hydrophobic peptides. Thus, the range of possible targets of Eep processing and the numbers of released peptides are numerous. Eep has similarities to proteases processing certain pheromone precursors in *Enterococcus faecalis* (42, 51); thus, it is likely that Eap may generate related small peptides from other bacteria that may similarly influence *C. albicans* filamentation. Further studies are needed to determine with more detail the range of bacteria able to influence microcolony formation.

The Dongari-Bagtzoglou group previously showed that the presence of oral streptococci increases the ability of *C. albicans* to invade organotypic models of oral mucosa (9) and that *Streptococcus oralis* can activate *C. albicans EFG1* gene expression (a key inducer of hyphae and microcolonies) that increases polymicrobial biofilm (52). In addition, coinfections of *S. oralis* and *C. albicans* increase mucosal fungal invasion (53), and coculture of *C. albicans* with *S. gordonii* induces the expression of fungal filamentation genes and hyphal adhesins, including Als1, Hyr1, and Eap1 (11), some of which we showed are important for microcolony adhesion (54). However, we found that *S. gordonii* supernatants induced a dual phenotype, with the majority of *C. albicans* microcolonies becoming enlarged and detached from an underlying epithelium, while some microcolonies (30%) had less biomass but were highly invasive. This subpopu-
lation of invasive cells may be a result of a microenvironment in which fungal cells have low exposure to secreted bacterial products that cause detachment or, alternatively, some fungal cells might become hyperinvasive in response to *S. gordonii* when in close contact with epithelium. Further work is needed to understand this phenotype. We found that the floating dense microcolonies had increased in the expression of ALS3 and HWP1, hypha-specific cell adhesion proteins. It is possible that in the context of these *S. gordonii* modified microcolonies, Als3 and Hwp1 function in cell-to-cell adhesion rather than cell-to-substrate adhesion, resulting in the phenotype of a denser and larger mass of hyphae that are not attached to the underlying surface, potentially increasing dissemination of *C. albicans* to distant sites. However, *S. gordonii* might also promote *C. albicans* commensalism by repressing expression of virulence genes involved in epithelial invasion (*ECE1*) and decreasing expression of genes required for adhesion (*HYR1*, *EAP1*, and *HWP2*). What differences exist in gene expression between these two microcolony phenotypes induced by *S. gordonii* remains an important question.

In contrast, *P. aeruginosa* induced a striking reduction of *C. albicans* microcolony size that was replicated when purified phenazines were added, suggesting that these secreted compounds are responsible for the repression of *C. albicans* hypha formation and possibly reduced survival within macrophages. Nonlethal concentration of phenazines secreted by *P. aeruginosa* are known to suppress *C. albicans* respiration and to acidify the extracellular pH, resulting in suppression of filamentation (40). Important differences have been found among *P. aeruginosa* clinical isolates in their ability to secrete phenazines and their quorum-sensing inducers (24, 55). The production of phenazines is promoted in environments of low oxygen and nutrient deprivation (56) and by the presence of *C. albicans* (57), suggesting that these molecules may be more functional in confined spaces, such as the lumens of macrophages while phagocytosed together with *C. albicans*, compared to the more open environment of respiratory mucosa where phenazines are easily inactivated (58).

Interkingdom signaling is a major contributor in development of the microbiome. The identification of *C. albicans* genes modified by *S. gordonii* diffusible factors represents an attractive target to modify virulence of polymicrobial biofilms. Also, potential identification of new fungal pathways affected by *S. gordonii* or *P. aeruginosa* may suggest a broad basis for the regulation of fungal biofilms by cocolonizing bacteria.

### MATERIALS AND METHODS

**C. albicans and bacterial cultures.** *C. albicans* and bacteria strains are listed in Table 1. *C. albicans* CAI-4 or SC5314 wild-type (WT) were cultured overnight in yeast extract-peptone-dextrose (YPD; BD Difco) broth supplemented with 50 μg/ml of uridine (Sigma-Aldrich) at 30°C in an orbital shaker at 220 rpm. Cultures were diluted to an optical density at 600 nm (OD₆₀₀) of 0.3 to 0.4 in fresh YPD medium and allowed to reach an OD₆₀₀ of 0.6 to 0.7. The cells were then washed twice by centrifugation at 2,800 × *g* for 5 min, with phosphate-buffered saline (PBS; pH 7.4; Corning), counted in a hemocytometer,
and suspended in the required medium prior to experiments. Heat-killed yeast cells were prepared by incubation at 65°C for 30 min. Strains of *S. gordonii* were routinely maintained in Todd-Hewitt (TH) agar (BD Biosciences) at 37°C in a humidified incubator with 5% CO₂ for experiments. *S. gordonii* CH9278 and CH1Δep strains were maintained in TH agar supplemented with 250 μg/ml spectinomycin (Sigma-Aldrich), and *S. gordonii* complement CH1Δep/pSgEepI was kept in TH agar supplemented with 250 μg/ml spectinomycin and 5 μg/ml erythromycin (Sigma-Aldrich). All three mutant strains were grown overnight in TH broth without antibiotics prior to use. *P. aeruginosa* strains were kept in Luria-Bertani (LB) agar (BD Biosciences) and cultured in broth in an orbital shaker at 220 rpm at 37°C. To evaluate the role of phenazines, *P. aeruginosa* qphA strains, characterized by the deletion of the two redundant 7-gene operons phzA1-phzG1 (phz1) and phzA2-phzG2 (phz2) encoding the biosynthetic enzymes responsible for phenazine production, were compared to the PA14 parental strain. Each bacterial culture was grown for 10 h, diluted in fresh medium, and allowed to reach mid-log phase (OD₆₀₀ ~ 1) prior to use. The cultures were then spun down at 2,500 × g for 3 min and washed two times in 1× PBS and counted using a hemocytometer. *S. gordonii* cells were briefly sonicated for 30 s on ice to break bacterial chains into individual bacteria prior to counting. Fresh-filtered *S. gordonii* (SSg) or *P. aeruginosa* (SP) culture supernatants were collected by centrifugation after log-phase growth and filtered using a 0.20-μm syringe filter (Corning, Inc.). Heat-fixed *S. gordonii* culture supernatants (HF SSg) were obtained by boiling fresh-filtered supernatant of *S. gordonii* strains for 15 min and then stored at 4°C until use. When indicated, fresh-filtered *S. gordonii* supernatant was treated with DNase (500 μg/ml; Sigma-Aldrich) or trypsin (200 μg/ml; Sigma-Aldrich) for 10 min at 37°C with gentle shaking before heat treatment.

**Phagosomal survival and escape of *C. albicans* during coinfection with bacteria.** Survival of *C. albicans* in macrophage phagosomes was performed as described previously (59) with modifications. Murine RAW 264.7 macrophages from the American Type Culture Collection (ATCC TIB-71) were seeded (5 × 10⁵ cells/ml) in 24-well plates (Corning, Inc.) with RPMI 1640 supplemented with L-glutamine (Corning) and 10% fetal bovine serum (PBS; Seradigm). The cells were activated using 10 ng/ml gamma interferon (BioLegend) for 12 h prior to experiments. *C. albicans* and bacterial cultures were each grown to mid-log phase as described above, added simultaneously at ratios of 10 macrophages:1 *C. albicans*:1 bacteria (MOI of 0.1:0.1) or 10 macrophages:1 *C. albicans*:10 bacteria (MOI of 0.1:1), and then incubated for 3 h at 37°C and 5% CO₂. For survival assays, 0.25% SDS (Thermo Fisher Scientific) and sterile water were added to lyse macrophages and release phagocytosed *C. albicans* and bacteria. Lysates were serially diluted and cultured for 24 h at 30°C on yeast-dextrose-peptone agar supplemented with 50 μg/ml streptomycin and 50 U/ml penicillin (Sigma-Aldrich) to remove bacteria and obtain *C. albicans* CFU. The percentage of survival was determined as follows: (recovered *C. albicans* CFU after phagocytic cell lysis/total number of phagocytosed *C. albicans*) × 100. For escape assays, 10 macrophages:1 *C. albicans*:1 bacteria (MOI of 0.1:0.1) were incubated together for 3 h at 37°C and 5% CO₂ to allow for phagocytosis of yeast and bacterial cells, and then 50 μg/ml streptomycin and 50 U/ml penicillin (Sigma-Aldrich) were added to each well to suppress the growth of nonphagocytosed bacteria or bacteria released by the lysis of macrophages. Macrophage monolayers were incubated at 37°C in 5% CO₂ for 17 h to permit visualization of *C. albicans* able to form microcolonies. Microcolonies on the surface of macrophage monolayers were imaged using a Zeiss Axio microscope, and the total number of microcolonies per well was counted. Assays were performed in duplicates, and results are representative of at least three independent experiments.

**Evaluation of phagosome maturation in macrophages.** Macrophages were seeded in 12-well culture plates with cover glass (Azer Scientific) and activated as described above. Phagosomal maturation was measured (34) with some modifications. *C. albicans* and bacterial cells were counted, and added to macrophages at an MOIs of 1 and 10, respectively. Nonphagocytosed *C. albicans* cells were stained with calcifluor white (CW; Sigma-Aldrich) for 2 min on ice to differentiate them from phagocytosed cells. Cover glasses were washed, returned to fresh RPMI 1640 medium, and maintained at 37°C. Cells were sampled at indicated time points and immunostained for lysosomal associated membrane protein 1 (LAMP1) (clone 1D4B; Developmental Studies Hybridoma Bank, University of Iowa) or early endosomal antigen 1 (EEA1; Cell Signaling Technology) and Alexa 594-conjugated donkey anti-rat or anti-rabbit secondary antibody (Jackson ImmunoResearch). Alexa Fluor 488-dextran (Invitrogen) was added with a chase of 2 h to ensure all dextran was in lysosomes prior to performing phagocytosis experiments as described above. In all cases, macrophages were costained with Alexa Fluor 488-conjugated phalloidin (Invitrogen). At least 50 phagosomes were observed per time point using a Zeiss Axio Observer Z1 inverted fluorescence microscope (Carl Zeiss, Germany) and ZEN 2011 (blue edition) software. Phagosomes of internalized *C. albicans* (no CW stain) with total surrounding staining were counted as positive, and phagosomes with incomplete or nonexistent staining were counted as negative. The percentage of label-positive phagosomes was calculated as the number of positive phagosomes/total number of phagosomes counted × 100.

**Evaluation of hypha formation within macrophages.** Fluorescent *C. albicans* CAF2-dTomato and unlabeled bacterial strains were added to macrophages at MOIs of 0.5 and 5, respectively, spun to allow contact, and incubated at 37°C and 5% CO₂ for 2 h. These MOIs were used so that some yeast and bacterial cells would not be phagocytosed and the formation of hyphae extracellularly could be compared to that of phagocytosed yeast. The phagocytic index for *C. albicans* cells was evaluated as previously described (59) with or without added bacteria to ensure that bacteria did not alter the phagocytic indices of yeast cells. Extracellular *C. albicans* was stained with CW, cells were fixed with 4% paraformaldehyde, and then macrophages were permeabilized and stained with phalloidin. A minimum of 100 phagocytosed *Candida* cells was counted using a Zeiss Axio microscope and classified as yeast or...
hyphae. The percentage of hyphal cells was calculated by obtaining the ratio of total hyphal form C. albicans/total number of Candida counted × 100.

**C. albicans microcolony formation.** C. albicans microcolonies were formed by seeding 100 cells in 12-well plates using RPMI 1640. Whole S. gordonii or P. aeruginosa bacterial strains (1,000 cells per well), Sg or Sp (10% final volume), or heat-fixed (HF) Sg (1.25 to 20% final volume) were added to wells, followed by incubation at 37°C and 5% CO₂ for 17 h to form microcolonies. For experiments with phenazines, purified pyocyanin (30 mM) (PYO; Cayman Chemical) and phenazine methosulfate (25 mM; PMS; Acros Organic) were diluted in H₂O to reach a final concentration after addition to wells (PYO [20 μM] and PMS [5 μM]). Microcolonies were imaged, and density was measured with ImageJ using inverse gray values/μm² (2). The biomass of microcolonies grown with HF Sg was determined by crystal violet (CV) staining as described previously (60). The total absorbance (A₄₀₅) was obtained by subtracting negative controls (no cells) from experimental samples of destained solutions using a FlexStation 3 multimode microplate reader (Molecular Devices).

**Microcolony invasion on oral epithelial cells.** TR146 epithelial cells, a buccal epithelial squamous carcinoma cell line from the European Collection of Authenticated Cell Cultures (ECACC), were grown on glass coverslips to confluence in 1:1 Dulbecco modified Eagle medium (DMEM)/F-12 medium supplemented with 10% FBS. Invasion of epithelial cells by C. albicans microcolonies was performed as described previously (2) with or without 10% HF Sg. After 17 h of incubation, microcolonies were photographed with white light using an InGenius imaging system (Syngene). The medium covering the cells was then aspirated, and monolayers were washed and stained as described previously (2).

**Microcolony RNA isolation and qRT-PCR.** C. albicans microcolonies formed in the presence of HF Sg were collected and pelleted by centrifugation at 10,000 × g for 5 min to isolate RNA as previously described (2). Total RNA was further purified by using an RNeasy minikit (Qiagen, Hilden, Germany) and quantified with a NanoDrop One (Thermo Scientific). Total RNA was used to quantitate HWP1, HWP2, ECE1, HYR1, EAP1, ALS3, EFG1, and HGC1 gene expression using primers listed in Supplemental Table S1. Total cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad). All samples were prepared with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and cycled using a CFX Connect Real Time system (Bio-Rad). Data were analyzed with CFX Maestro software (Bio-Rad). The relative fold changes in gene expression were calculated using both C. albicans actin and GAPDH (gyceraldehyde-3-phosphate dehydrogenase) as controls.

**Statistical analysis.** Data were analyzed by a Student t test or one-way analysis of variance (ANOVA) with a post ad hoc Dunnett’s multiple-comparison test using Prism 7 (GraphPad Software, La Jolla, CA) at a significance level of P < 0.05 for all experiments.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**FIG S1**, EPS file, 2.7 MB.

**FIG S2**, EPS file, 2.8 MB.

**TABLE S1**, DOCX file, 0.02 MB.

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