Streptococcus gordonii Modulates *Candida albicans* Biofilm Formation through Intergeneric Communication[▽]

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The fungus *Candida albicans* colonizes human oral cavity surfaces in conjunction with a complex microflora. *C. albicans* SC5314 formed biofilms on saliva-coated surfaces that in early stages of development consisted of ~30% hyphal forms. In mixed biofilms with the oral bacterium *Streptococcus gordonii* DL1, hyphal development by *C. albicans* was enhanced so that biofilms consisted of ~60% hyphal forms. Cell-cell contact between *S. gordonii* and *C. albicans* involved *Streptococcus* cell wall-anchored proteins SspA and SspB (antigen I/II family polypeptides). Repression of *C. albicans* hyphal filament and biofilm production by the quorum-sensing molecule farnesol was relieved by *S. gordonii*. The ability of a *luxS* mutant of *S. gordonii* deficient in production of autoinducer 2 to induce *C. albicans* hyphal formation was reduced, and this mutant suppressed farnesol inhibition of hyphal formation less effectively. Coincubation of the two microbial species led to activation of *C. albicans* mitogen-activated protein kinase Cek1p, inhibition of Mkc1p activation by H₂O₂, and enhanced activation of Hog1p by farnesol, which were direct effects of streptococci on morphogenetic signaling. These results suggest that interactions between *C. albicans* and *S. gordonii* involve physical (adherence) and chemical (diffusible) signals that influence the development of biofilm communities. Thus, bacteria may play a significant role in modulating *Candida* carriage and infection processes in the oral cavity.

Human mucosal surfaces are colonized by diverse microbial communities. Current estimates suggest that over 700 different microbial species may colonize the human oral cavity (58). Synergistic, mutualistic, and antagonistic interactions occurring between microorganisms contribute to the development of polymicrobial biofilm communities (44). In particular, the ability of partner organisms to engage physically (coadherence) or metabolically is believed to be fundamental to the growth, survival, and virulence of individual species (38). *Streptococcus* bacteria are among the earliest colonizers of hard and soft oral tissues, and they express a complex repertoire of cell surface polypeptides that mediate adherence interactions (35). Some of these polypeptides, the conserved antigen I/II (AgI/II) family of cell wall-anchored proteins, which range from 826 to 1,653 amino acid residues long, are produced by most indigenous oral *Streptococcus* species (32, 55). These proteins recognize a range of host tissue proteins and cellular receptors (24, 30, 31, 36), in addition to mediating binding to specific partner microorganisms (e.g., *Actinomyces naeslundii*, *Porphyromonas gingivalis*, and *Candida albicans*) (13, 16, 28, 45).

Intergeneric microbial adherence (or coaggregation) contributes to biofilm development and results in closer proximity for cell-cell communication through the production and sensing of diffusible signaling molecules. This plays a crucial role in controlling the species composition of polymicrobial communities. In a number of *Streptococcus* species, small signaling peptides or pheromones induce development of competence

for DNA uptake (59). These peptides are autoinducing peptides and, together with bacteriocins, are able to influence biofilm development in a density-dependent and species-specific manner (46). In addition, a wide range of oral bacteria, including streptococci, carry the *luxS* gene that is involved in synthesis of autoinducer 2 (AI-2) (15). AI-2 is produced from S-adenosylmethionine in at least three enzymatic steps and is really a collective term for a group of furanones that promote cross-communication between bacteria (64). *Streptococcus gordonii*-*Porphyromonas gingivalis* (48) and *Streptococcus oralis*-*Actinomyces naeslundii* (62) dual-species biofilms are abrogated by *luxS* gene knockouts, and biofilm formation may be rescued by genetic complementation or exogenous 4,5-dihydroxy 2,3-pentanedione (DPD).

C. albicans colonizes human mucosal surfaces and is the major systemic fungal pathogen (57). Biofilm formation and virulence are both linked to the ability to transition from the yeast (blastospore) growth form to the filamentous (hyphal) growth form (23, 49). The morphological transition is affected by pH, nutrients, temperature, and a range of compounds, including small signaling molecules that either stimulate or repress hyphal formation (65, 68). Farnesol, a sesquiterpene produced by *C. albicans*, acts as a quorum-sensing molecule repressing hyphal formation at high cell density (29, 39, 60). Conversely, tyrosol, a 2-(4-hydroxyphenyl) ethanol derivative of tyrosine produced by *C. albicans*, accelerates the formation of hyphae (11), but it does not do this under noninducing conditions (e.g., high cell density). Environmental cues are sensed and coupled to transcriptional regulation (20) through mitogen-activated protein (MAP) kinase signal transduction pathways (50, 63), the adenylate cyclase (protein kinase A [PKA]) pathway (3, 37), and two-component signal transduc-

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tion (40). Morphological transition and biofilm formation are accompanied by alterations in the transcription levels of >500 genes (approximately 9% of the genome) (51, 69) and by differential expression of surface molecules, some of which are specific to the filamentous form (56). Farnesol inhibits induction of the hypha-specific genes *hwp1*, *ece1*, and *rbt1* (19).

A feature of *C. albicans* biofilms is that they usually consist of a mixture of morphological forms (4). On catheter disks and on plastic surfaces, *C. albicans* biofilms comprise two layers: a thin base layer of yeast cells covered by a thicker but more open hyphal layer (10). Biofilm development is inhibited by farnesol (60) but is relatively unaffected by tyrosol (1). Biofilms show reduced sensitivity to antifungal agents, but the reasons for this are not understood. Moreover, the presence of bacteria in *C. albicans* biofilms has been shown to reduce further the sensitivity of the biofilms to antifungal agents, such as fluconazole (33). *C. albicans* may be coisolated with *Staphylococcus* (67) and with *Pseudomonas aeruginosa* from other body sites (6, 47). *P. aeruginosa* appears to influence the behavior of *C. albicans* through the production of a homoserine lactone, a C_{12} compound related to farnesol, and a phenazine (22), which repress *C. albicans* filamentation and kill hyphal cells (25). *Xanthomonas campestris* and *Burkholderia cenocepacia* also produce C_{12} diffusible signaling molecules that inhibit *C. albicans* filamentation (8). This may be an aspect of control of *C. albicans* pathogenesis by bacteria at mucosal surfaces.

In the oral cavity, *C. albicans* is found in conjunction with multiple bacterial species and has been shown to adhere to or coaggregate with a range of oral *Streptococcus* species (34). Since streptococci are early colonizers of oral cavity surfaces (38), the ability of *C. albicans* to adhere to *Streptococcus* species provides an additional surface for fungal colonization. These interactions could also be important for the development of mixed-species biofilms on mucosal or prosthetic surfaces (9). *S. gordonii*, a ubiquitous human oral commensal found at multiple oral cavity sites, has multimodal interactions with *C. albicans* (28). These interactions involve AgI/II family proteins SspA and SspB, cell wall-anchored protein CshA that confers surface hydrophobicity, and streptococcal cell surface linear phosphopolysaccharides (26, 27), which are strain specific (70). It has been hypothesized that the ability of oral streptococci and *C. albicans* to interact physically and possibly chemically promotes *C. albicans* colonization in the oral cavity and the development of mixed-species communities of bacteria and fungi. In this paper, we show that *S. gordonii* enhances hyphal development and biofilm formation by *C. albicans* through intermicrobial adhesion and signaling. Understanding the processes by which microbial communities develop in the human host should help in formulating new strategies to modulate biofilm formation and control diseases associated with *C. albicans*.

MATERIALS AND METHODS

Microbial growth conditions. The strains of microorganisms used were *S. gordonii* wild-type strain DL1 (Challis), *S. gordonii* UB1360 $\Delta(sspA sspB)$ (24), *S. gordonii* UB1545 Δhsa (36), an *S. gordonii* $\Delta luxS$ mutant, a $\Delta luxS::luxS^+$ complemented strain (48), *P. aeruginosa* PAO1, and *C. albicans* SC5314. *S. gordonii* was cultivated on BHY agar (37 g/liter brain heart infusion broth, 5 g/liter yeast extract, 15 g/liter agar) anaerobically, *P. aeruginosa* was cultivated on tryptic soy agar aerobically, and *C. albicans* was cultivated on Sabouraud dextrose agar (Oxoid) aerobically; all cultures were incubated at 37°C. Suspension cultures of

S. gordonii strains with appropriate antibiotics (31) were grown in BHY medium in sealed bottles or tubes without shaking at 37°C. *C. albicans* was grown in YPD medium (5 g/liter yeast extract, 10 g/liter Bacto peptone, 20 g/liter dextrose) with shaking at 37°C. The medium that supported growth of both streptococci and *C. albicans* in biofilms contained yeast nitrogen base (Difco), 10 mM Na_2HPO_4 - NaH_2PO_4 (pH 7.0), and 0.5 g/liter Bacto tryptone (YPT medium) supplemented with 2 g/liter glucose (YPT-Glc). Pooled human saliva, collected with institutional review board approval, was mixed with dithiothreitol (2.5 mM) and centrifuged (10,000 $\times g$, 10 min) to clarify it. The supernatant was diluted 1:1 with distilled water and sterilized by filtration through a 0.22- μm -pore-size membrane. Farnesol was purchased from Sigma, and DPD was obtained from OMM Scientific Inc., Dallas, TX.

Hyphal and biofilm formation. *C. albicans* cells were grown in YPD medium for 16 h at 37°C with aeration. Cells were harvested by centrifugation (5,000 $\times g$, 5 min) and then suspended at an optical density at 600 nm (OD_{600}) of 0.2 (approximately 2×10^6 cells/ml) in YPT-Glc. Portions (0.5 ml) were used to inoculate wells (in a 24-well plate) containing 0.5 ml YPT-Glc and glass coverslips (diameter, 13 mm) previously coated with human salivary proteins for 1 h at 37°C. Under these conditions hyphal formation commenced after 30 min. In some experiments, *C. albicans* cells were grown in YPD medium, harvested as described above, suspended at an OD_{600} of 0.1, and incubated aerobically with shaking at 37°C in YPT-Glc to induce hyphal formation. Streptococci were grown in BHY medium for 16 h at 37°C, harvested by centrifugation (5,000 $\times g$, 10 min), and suspended at an OD_{600} of 0.2 (approximately 1×10^8 cells/ml) in YPT-Glc. Portions (0.5 ml) were used to inoculate wells containing 0.5 ml YPT-Glc and saliva-coated coverslips as described above. For dual-species biofilms, the coverslips were incubated for 1 h at 37°C with an *S. gordonii* or *C. albicans* cell suspension, and then the suspensions were aspirated. One milliliter of the appropriate medium (e.g., YPT-Glc) was added to each of the coverslips, and then the coverslips were placed into fresh wells containing 0.5 ml of the appropriate medium (e.g., YPT-Glc) together with 0.5 ml of the appropriate (*C. albicans* or *S. gordonii*) cell suspension. Biofilms that formed on saliva-coated glass coverslips were Gram stained or stained with FUN1 stain from a LIVE/DEAD yeast viability kit (Molecular Probes, Invitrogen) and visualized with a Leica DMLB or Olympus microscope. The percentages of cells forming hyphae were calculated from microscopic counts for 10 randomly selected fields of view.

Biofilms were formed on saliva-coated plastic wells by inoculating wells (in a 96-well plate) with 0.05 ml of a microbial cell suspension and 0.05 ml of YPT-Glc for single-species biofilms or with 0.05 ml of each microbial cell suspension for dual-species biofilms and incubating the preparations for 16 h at 37°C. Biofilms formed on wells were fixed with 90% methanol and stained with crystal violet, and the absorbance at 600 nm was determined as a measure of biomass (31). To visualize direct interactions with *C. albicans* cells in suspension, streptococci (OD_{600} , 1.0) were labeled with 1.5 mM fluorescein isothiocyanate in 0.05 M Na_2CO_3 containing 0.1 M NaCl (pH 7.5) for 1 h. To test the effects of *S. gordonii* spent culture medium on *C. albicans* hypha formation, supernatants from stationary-phase cultures of *S. gordonii* DL1 were diluted 1:1 with fresh YPT-Glc and sterilized by filtration (0.22- μm filter).

Phosphorylation. *S. gordonii* late-exponential-phase cells were harvested by centrifugation as described above and suspended in 0.2 volume of spent medium (5 ml, 2×10^9 cells/ml). Late-exponential-phase cells of *C. albicans* in YPD medium (5 ml, 1×10^7 cells/ml) were mixed with 5 ml *S. gordonii* cells, 5 ml spent medium alone, or 5 ml BHY medium (control). Farnesol (0.1 mM) or H_2O_2 (10 mM) was included where appropriate. In initial experiments samples were taken at 0, 10, 20, 30, and 60 min for phosphoprotein analysis (see below). To extract proteins, cells were suspended in lysis buffer (53) containing protease inhibitor cocktail (Sigma), broken with glass beads (Precellys 24 disrupter; Bertin Technologies), and centrifuged (5,000 $\times g$, 10 min), and the supernatant was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis through 10% acrylamide. Proteins were electroblotted onto a nitrocellulose membrane (Hybond) and probed with anti-phospho-p38 MAP kinase antibody (Santa Cruz Biotechnology Inc.) that reacted with *C. albicans* phosphorylated Hog1p, with anti-phospho-p44/42 MAP kinase antibody (Cell Signaling Technology) that reacted with the phosphorylated TEY signature of the Mkc1p, Cek1p and Cek2p proteins, or with anti-*Saccharomyces cerevisiae* Hog1 polyclonal antibody (Santa Cruz Biotechnology Inc.) (53). The primary antibodies bound were detected with appropriate horseradish peroxidase-conjugated secondary antibodies, and blots were developed using ECL (Amersham). Time course data showed that the levels of phosphorylation of Mkc1 and Cek1 declined after 30 min. Thus, only data for the 20-min time point are presented below.

FIG. 1. Interactions of *C. albicans* SC5314 with *S. gordonii* DL1 in biofilms formed on saliva-coated surfaces. (A) Biomasses of 16 h-biofilms formed at 37°C in 25% human saliva (Saliva), in 25% saliva containing 0.5% glucose (Slv-Glc), in YPT medium, or in YPT medium containing 0.5% glucose (YPT-Glc) by *S. gordonii* (Sg), *C. albicans* (Ca), or both *S. gordonii* and *C. albicans* (Ca+Sg). The results are representative of three experiments, and the error bars indicate standard deviations. (B) Percentages of cells forming hyphae after 3 h of incubation at 37°C in biofilms for *C. albicans* grown in 25% saliva containing 0.5% glucose or YPT-Glc, for *C. albicans* alone (Ca), for *C. albicans* to which *S. gordonii* was added (Ca+Sg), and for *S. gordonii* to which *C. albicans* was added (Sg+Ca). The results are representative of three experiments, and the error bars indicate standard deviations (*, $P = 0.037$; **, $P = 0.012$ [statistically significant]). (C) *C. albicans* 3-h biofilm in YPT-Glc, showing mixed morphological forms, including blastospores (B), hyphae (H), pseudohyphae (P), and buds (Bu). (D) *S. gordonii* 3-h biofilm in YPT-Glc, showing chains of cocci with a relatively even surface distribution. (E) Six-hour mixed biofilm in YPT-Glc of antecedent *C. albicans* (1 h) and *S. gordonii*, with clusters of streptococci on hyphae (arrows) and around some mother cells.

RESULTS

***Streptococcus* promotes hyphal and biofilm development.** *C. albicans* and *S. gordonii* cells interact (coaggregate) through adhesin-receptor binding (28). To determine the physiological effects of this interaction on microbial community development, *C. albicans* SC5314 or *S. gordonii* DL1 single-species or mixed-species biofilms were produced in plastic wells under four different nutritional conditions. In all cases, the resulting biomass of coinoculated biofilms was greater than the sum of the biomasses of the single-species biofilms (Fig. 1A). Glucose enhanced mixed-species biofilm development in saliva or YPT medium (Fig. 1A). To investigate temporal and positional effects on biofilm formation, coverslips precoated with *S. gordonii* or *C. albicans* were incubated with the other species. Three-hour *C. albicans* monospecies biofilms in YPT-Glc showed significantly less hyphal formation (30%) than biofilms in saliva containing glucose (in which 40% of the cells produced hyphae) (Fig. 1B) ($P = 0.037$). When *C. albicans* in YPT-Glc was deposited first (Fig. 1C), subsequent addition of *S. gordonii* appeared to promote hyphal formation (Fig. 1B), and the streptococci formed clusters of chains on and around hyphal filaments and some blastospores (Fig. 1E). When *S. gordonii* was deposited first (Fig. 1D), hyphal development was enhanced (60% compared to the results when *C. albicans* was deposited first) (Fig. 1B) ($P = 0.012$), and foci of *C. albicans* communities produced extensive hyphal mats in 8-h biofilms (Fig. 1F). Somewhat similar effects were seen in saliva-glucose medium (Fig. 1B). The initial production of hyphae by deposited *C. albicans* was enhanced (Fig. 1G) compared with the production in YPT-Glc (Fig. 1C), and antecedent streptococci targeted to the fungal filaments formed dense communities (Fig. 1H). These results show that hyphal development, which is integral to biofilm formation by *C. albicans*, is promoted both by human saliva and by *S. gordonii*. Live (FUN1)-dead staining of *C. albicans*-*S. gordonii* interacting cells after 24 h revealed that they were metabolically active, and vacuoles (red) were clearly visible within the fungal cell filaments (Fig. 1I). This finding is in direct contrast to the effects of *P. aeruginosa* on *C. albicans*, where the presence of yellow or green vacuoles (Fig. 1J) suggested that the hyphal filaments died, as previously described (25).

***Streptococcus* AgI/II proteins mediate adherence to *C. albicans*.** When fluorescently labeled *S. gordonii* DL1 was incubated with *C. albicans*, heterogeneities in the binding of the bacteria at the population and cellular levels of *C. albicans* were revealed. Bacteria were found to be attached to mother

(F) Eight-hour mixed biofilm in YPT-Glc of antecedent *S. gordonii* (1 h) and *C. albicans*, showing hyphal networking on a lawn of streptococci (pink). (G) *C. albicans* 3-h biofilm in 25% saliva containing 0.5% glucose, demonstrating the increased frequency of hyphal formation compared with the biofilm shown in panel C. (H) Eight-hour mixed biofilm in 25% saliva containing 0.5% glucose of antecedent *C. albicans* (1 h) and *S. gordonii*, showing high-density hyphal formation and deposition of streptococci. (I) Live/dead staining of *C. albicans* associated with *S. gordonii* after 24 h, with metabolically active hyphae (green) containing vacuoles (red). (J) *C. albicans* and *P. aeruginosa* PAO1 after 24 h, showing metabolically inactive vesicles (yellow) and dead hyphae.

FIG. 2. Physical interactions of *S. gordonii* with *C. albicans*. (A) Light microscopy (upper panel) and corresponding fluorescence (lower panel) images of fluorescein isothiocyanate-labeled *S. gordonii* attached to hyphal filaments (H) and mother cells (MC) but not to some blastospores (B) (arrows). (B) Biomasses for 16-h biofilms (formed at 37°C in 25% saliva containing 0.5% glucose [Slv-Glc] or YPT-Glc) of *S. gordonii* DL1 (Sg), *S. gordonii* UB1360 $\Delta(sspA sspB)$ (Sg sspAB), or antecedent *S. gordonii* and *C. albicans* (Sg+Ca). The results are representative of three experiments, and the error bars indicate standard deviations (*, $P = 0.0015$ [statistically significant]). (C to F) Light microscopy (left panels) and corresponding fluorescence (right panels) images of *C. albicans* hypha-forming cells mixed with fluorescein isothiocyanate-labeled *S. gordonii* DL1 (C), *S. gordonii* UB1360 (D), *S. gordonii* UB1360/pUB1000sspB+ (E), and *S. gordonii* UB1545 Δhsa (F). (G and H) Three-hour biofilms of antecedent *C. albicans* with *S. gordonii* DL1 (G) or UB1360 (H). The arrow in panel G indicates a close association of *S. gordonii* DL1 cells with hyphae, which is less apparent in panel H, which is a lower-magnification image showing streptococcal cells distributed across the field.

cells (blastospores) producing hyphae and to hyphal filaments (Fig. 2A). However, some individual blastospores did not have attached bacteria, and regions on the fungal cell surface, especially stretches along the hyphae, appeared not to support *Streptococcus* attachment (Fig. 2A). Previously, adherence of *C. albicans* ATCC 10261 cells to *S. gordonii* DL1 has been thought to involve recognition by *C. albicans* of the streptococcal cell wall-anchored AgI/II family proteins SspA and SspB (26, 28). Extending this observation, fluorescently labeled cells of *S. gordonii* UB1360 $\Delta(sspA sspB)$, which is isogenic with wild-type DL1 but deficient for production of SspA and SspB, interacted only weakly with *C. albicans* SC5314 (Fig. 2D) compared with wild-type DL1 cells (Fig. 2C). Complementation of strain UB1360 with plasmid pUB1000-sspB+ (24) expressing SspB restored *C. albicans* binding (Fig. 2E), while *C. albicans* recognition by strain UB1545 Δhsa , which is deficient in pro-

duction of an irrelevant cell wall-anchored adhesin (30), was unaffected (Fig. 2F). The biofilms formed by the AgI/II mutant strain UB1360 had slightly greater biomasses than those formed by wild-type DL1 (Fig. 2B), as reported elsewhere (71), but the data were not statistically significant. However, mixed-species biofilms of coinoculated *S. gordonii* UB1360 and *C. albicans* in YPT-Glc had reduced biomasses compared with *S. gordonii* DL1-*C. albicans* biofilms (Fig. 2B) ($P = 0.0015$). Microscopically, strain UB1360 streptococcal cells were found to be, in general, less closely associated with *C. albicans* filaments (Fig. 2H) than wild-type DL1 cells, which formed societies around some blastospores and along hyphal filaments (Fig. 2G). Thus, mixed-species biofilm formation appears to be promoted by cell-cell contact mediated by *S. gordonii* SspA and SspB, but it does not depend entirely on expression of these streptococcal proteins.

***C. albicans* responds to *Streptococcus* signaling molecules.** We next investigated if an *S. gordonii* $\Delta luxS$ mutant, in which production of AI-2 was ablated (48), promoted *C. albicans* biofilm formation. This *luxS* mutant is partially defective in biofilm formation (48), and the mutation is corrected by transcomplementation with *luxS*+. Biofilms of the $\Delta luxS$ strain did not have biomasses that were significantly different (Fig. 3A) from the biomasses of strain DL1 biofilms, but mixed biofilms containing the $\Delta luxS$ mutant and *C. albicans* had significantly (~35%) reduced biomasses (Fig. 3A) ($P = 0.013$). The numbers of *C. albicans* cells producing hyphae were also approximately 30% lower in the presence of the *S. gordonii* $\Delta luxS$ strain than in the presence of the wild type (Fig. 3B), and the hyphae were generally shorter (Fig. 3D) than the corresponding wild-type hyphae (Fig. 3C). Complementation of the $\Delta luxS$ mutant restored the ability of *S. gordonii* to promote *C. albicans* hyphal formation (Fig. 3E). When suspended in stationary-phase cell-free culture supernatant from *S. gordonii* DL1, some *C. albicans* cells were induced to form hyphae (Fig. 3F). However, *C. albicans* did not form hyphal filaments when it was suspended in the $\Delta luxS$ mutant culture fluid (Fig. 3G).

To determine if this stimulatory effect might be due to AI-2 directly, we tested the effects of adding exogenous chemically synthesized DPD (a precursor of AI-2) at a range of concentrations (0.4 nM to 80 μ M) on *C. albicans* hyphal and biofilm development. Exogenous DPD had no significant effect on biofilm biomass or the number of cells producing hyphae (data not shown), although hyphal extension in 2-h biofilms was marginally increased (data not shown). Exogenous DPD (concentration range, 8 to 800 nM) did not rescue the $\Delta luxS$ mutant deficiency (Fig. 3B) for forming mixed biofilms (results not shown). Thus, DPD does not appear to effectively substitute functionally for *S. gordonii* AI-2. There may be other molecules which are secreted by wild-type *S. gordonii* and not by the $\Delta luxS$ mutant that are effective enhancers of hyphal formation.

***S. gordonii* modulates *C. albicans* signaling pathways.** Hyphal formation by *C. albicans* at high cell density is suppressed by farnesol, which accumulates in the medium during growth (19). We found that exogenously added 30 μ M farnesol was sufficient to inhibit *C. albicans* hyphal formation in suspension (Fig. 4A) by >90%. However, in the presence of *S. gordonii* DL1 the inhibitory effect of 30 μ M farnesol on hyphal formation was effectively suppressed (Fig. 4B). We believe that this effect was not due to adsorption or inactivation of the farnesol

FIG. 3. Effect of *luxS* on mixed-species biofilm formation. (A) Bio-masses for 16-h monospecies biofilms (37°C in YPT-Glc) of *S. gordonii* DL1 (*Sg* wt), the Δ *luxS* mutant (*luxS*), or the Δ *luxS*(*luxS*⁺) complemented strain (*luxS*⁺) or for *S. gordonii* DL1 coinoculated with *C. albicans* (*Sg*+*Ca*). The results are representative of three experiments, and the error bars indicate standard deviations (*, $P = 0.031$ [statistically significant]). (B) Percentages of *C. albicans* cells forming hyphae (3-h biofilm) alone (*Ca*), with antecedent *C. albicans* and *S. gordonii* (*Ca*+*Sg*), or with antecedent *S. gordonii* and *C. albicans* (*Sg*+*Ca*). The results are representative of three experiments, and the error bars indicate standard deviations (**, $P = 0.0008$ [statistically significant]). (C to E) Biofilms (3 h) of antecedent *C. albicans* and *S. gordonii* DL1 (C), the Δ *luxS* mutant (D), or the Δ *luxS*(*luxS*⁺) mutant (E). (F and G) Suspensions (3 h at 37°C) of *C. albicans* SC5314 with cell-free culture supernatant from *S. gordonii* DL1 (F) or the Δ *luxS* strain (G).

by *S. gordonii*. When 1×10^6 *C. albicans* cells/ml in a dialysis sac (1 ml) was incubated in YPT-Glc (200 ml) containing 1×10^8 *S. gordonii* cells/ml and 30 μ M farnesol, hyphal formation by *C. albicans* was still inhibited (data not shown). Concentrations of farnesol greater than 100 μ M were deleterious to streptococcal growth. Exogenous DPD did not relieve the farnesol suppression of hyphal formation (not shown).

A number of signal transduction pathways in *C. albicans* are associated with regulation of hyphal morphogenesis and biofilm formation through environmental sensing (7). Accordingly, we investigated the effects of *S. gordonii* DL1 on activation of MAP kinases Mkc1, Cek1 and Cek2, which impact morphogenesis (12, 53), and Hog1, which is activated in response to osmotic, heavy metal ion, and oxidative stresses (18) and influences cell wall biogenesis (2). Under the experimental conditions used, it was confirmed that Mkc1, Hog1, and Cek2 were phosphorylated in response to oxidative stress (10 mM

FIG. 4. Effects of *S. gordonii* on *C. albicans* signaling pathways. (A and B) *C. albicans* suspensions in YPT-Glc incubated for 4 h at 37°C, showing inhibition of hyphal formation by 30 μ M farnesol (A) and suppression of 30 μ M farnesol inhibition by *S. gordonii* DL1 (B). (C) Western immunoblot analysis of effects of 10 mM H₂O₂ or 0.1 mM farnesol (FnI) on phosphorylation of *C. albicans* MAP kinases after 20 min in the absence (left three lanes) or presence (right three lanes) of *S. gordonii* DL1. MAP kinases were detected with anti-phospho-p44/42 MAP kinase antibody (Mkc1p, Cek1p, Cek2p), anti-phospho-p38 MAP kinase antibody (Hog1p), and an anti-SchHog1 polyclonal antibody (Hog1) control. Experiments were repeated three times, and representative data from one experiment are shown.

H₂O₂) (Fig. 4C), while Mkc1 was activated weakly in response to farnesol (100 μ M). Coincubation of *C. albicans* with *S. gordonii* DL1 cells led to activation of Cek1 (Fig. 4C). The presence of *S. gordonii* cells also suppressed the H₂O₂-induced phosphorylation of Mkc1, but not the H₂O₂-induced phosphorylation of Hog1. However, Hog1 was activated in response to farnesol in the presence of *S. gordonii* (Fig. 4C). Spent culture supernatant from *S. gordonii* did not have these effects (data not shown). Thus, the activities of three MAP kinases (Mkc1, Cek1, and Hog1) in *C. albicans* differentially respond to the presence of, or contact with, *Streptococcus* bacteria in the environment.

DISCUSSION

Biofilm formation is central to colonization of oral cavity surfaces by *C. albicans*. In vitro, biofilm formation occurs through initial adherence of *C. albicans* cells to a surface, firmer anchorage, formation of hyphae (or pseudohyphae), and proliferation of cells (10, 49). The molecular basis for the initial surface adherence is not entirely understood. For the saliva-coated surfaces utilized in this biofilm study, there are indications that *C. albicans* protein adhesins recognize salivary

components (33). However, it is unclear how initial surface contact leads to firmer anchorage and to sending the signals for undergoing morphogenetic development. It seems that the cell wall may serve as the initial contact point, with tension generated on the fungal cell membrane resulting in opening of mechanosensitive ion channels and activating G-protein-coupled receptors (42). An early response to surface contact in *C. albicans* is activation (phosphorylation) of MAP kinase Mkc1 (43). This is required for normal biofilm development and also for invasive hyphal growth (41). Hyphal formation appears to be critical in the development of *C. albicans* biofilms, and mutations in transcription factor genes (e.g., *EFG1*, *CPH1*, *TEC1*, and *BCR1*) or hyphal cell surface protein genes (e.g., *ALS3* and *HWPI*) result in severe defects in biofilm formation (56).

Previous work has shown that a number of strains of oral streptococci are able to specifically coaggregate with *C. albicans* (34). The results described here extend these observations to show that *S. gordonii* is able to adhere to hyphae and mother cells of *C. albicans* that are forming an early biofilm. Interestingly, the deposition of *S. gordonii* cells on hyphae and mother cells was not uniform. Streptococci did not adhere to some hyphae, and attachment to the hyphal filaments was often localized. These observations suggest that there is heterogeneity of receptor expression within the *C. albicans* cell population and spatially on the hyphal filaments. Likewise, *C. albicans* cells are able to deposit on an *S. gordonii* early biofilm. Such depositions seem to accelerate the frequency and extent of hyphal formation compared with those for *C. albicans* cells attached to a surface coated only with saliva. Associated with these interactions was the formation of a mixed-species biofilm with a biomass at least twofold greater than that of a *C. albicans* single-species biofilm. These results might be more revealing if we were able to develop an accurate method for determining the biomass contributed by each component. The ability of these two organisms to show enhanced growth when they are present together has potential clinical implications for the formation of biofilms on mucosal or hard surfaces, such as dental prostheses (61), and for influencing longer-term carriage of *C. albicans*.

A major physical interaction between *S. gordonii* and *C. albicans* SC5314 depended upon the streptococcal AgI/II polypeptides SspA and SspB. The adherence of *S. gordonii* *sspA sspB* mutants to *C. albicans* was decreased (28), and mixed-species biofilms had reduced biomasses compared with the biomasses of wild-type biofilms. The phosphopolysaccharide produced on the cell surfaces of some oral streptococci (70) acts as an additional receptor for *C. albicans* (27). However, *S. gordonii* DL1 does not produce significant amounts of surface phosphorylated polysaccharide. These results suggest that mixed-species biofilm formation is enhanced by, but not dependent upon, expression of the SspA and SspB polypeptides.

The presence of *S. gordonii* cells appeared to induce earlier and more extensive hyphal formation by *C. albicans*. This was mediated in part by soluble factors produced by *S. gordonii* and present in the spent culture medium of the wild type but not in the spent culture medium of a *luxS* mutant. The biofilm formation by the mutant was not significantly affected itself, but mixed biofilms with *C. albicans* had significantly reduced bio-

masses. These observations imply that AI-2 has a role in *Candida-Streptococcus* interactions. It has been shown that during the first 6 h of biofilm formation by *C. albicans*, 41 open reading frames were expressed more highly than they were in planktonic cultures (51). Nine of these open reading frames were involved in sulfur metabolism (*MET3* was upregulated 23-fold), which in turn is linked with oxidative metabolism. The methionine and cysteine biosynthetic genes were some of the most prominent genes that were upregulated. It is interesting that production of AI-2 occurs in the methionine salvage pathway in *S. gordonii*. A potential link between these observations is provided by the observation that AI-2 can trigger an oxidative stress response in *Mycobacterium avium* and lead to increased biofilm formation (21).

Other possible diffusible signals for differentiation of *C. albicans* in response to streptococci are metabolic end products. The possibilities that lactic acid, the major fermentation end product for *S. gordonii* growing on glucose, and an acidic pH influenced hyphal development were considered. However, we tested the effects of acidic pHs (down to pH 5) and of up to 50 mM lactate on hyphal formation and biofilm development, but both low pH and lactate were found to be inhibitory to hyphal formation (data not shown). Recently, it has been observed that hyphal formation by *C. albicans* in YPD medium is induced by 0.5 to 10 mM H₂O₂ (52). *S. gordonii* spent culture medium contains up to 0.15 mM H₂O₂ (5), a concentration that is below the levels shown to be effective in inducing hyphal formation. This concentration of H₂O₂ might be insufficient to induce the core stress response in *C. albicans* (17, 66). However, where streptococci are in close contact with or attached to *C. albicans* cells, it is possible that the localized concentrations of H₂O₂ might be above the threshold. The same might be true for effects mediated by AI-2. Taken together, therefore, these observations suggest that hyphal formation might be enhanced in the presence of streptococci, at least in part, by the concerted actions of AI-2 and H₂O₂ on the *C. albicans* oxidative stress response.

Morphogenesis in *C. albicans* is positively regulated by the cyclic AMP-dependent pathway (Fig. 5A) and PKA activity, with phosphorylation of the Efg1p transcriptional regulator. A parallel route is a conserved MAPK pathway involving Cek1p (Fig. 5A). Phosphorylation of Cek1p was observed to be an early response of *C. albicans* to the presence of *S. gordonii*. Conversely, two major proteins phosphorylated in response to H₂O₂ stress, Mkc1p (54) and Hog1p (Fig. 5A), were not activated, and addition of *S. gordonii* cells actually reduced activation of Mkc1p in response to H₂O₂. These observations are in keeping with stimulation of filamentation by streptococci and argue against the hypothesis that H₂O₂-induced oxidative stress is the main cause of increased hyphal and biofilm development in the presence of streptococci. There was also evidence for abrogation of farnesol-induced phosphorylation of Mkc1p by *S. gordonii*. This is consistent with relief of quorum-sensing-mediated repression of hyphal formation by streptococci. The possibilities are that *S. gordonii* blocks or inactivates farnesol receptors and/or induces intracellular signaling in *C. albicans* that overrides the farnesol signal. Farnesol is proposed to affect the activity of the Ras1-Cdc35-PKA-Efg1-dependent signaling pathway (14) (Fig. 5A). It is possible, therefore, that *S. gordonii* impacts the PKA pathway to repress

FIG. 5. Summary of environmentally responsive signaling pathways and *Streptococcus* interactive mechanisms in *C. albicans*. (A) Pathways associated with hyphal development or biofilm formation, based on information from references 7, 14, 39, 43, and 63. Various environmental cues (top) interact with cell surface receptors to activate intracellular signaling pathways, leading to transcription factor modulation. cAMP, cyclic AMP; MAPKKK, MAP kinase kinase kinase; MAPKK, MAP kinase kinase; MAPK, MAP kinase; AC, adenylate cyclase; PM, plasma membrane. (B) Proposed interactions of *S. gordonii* with *C. albicans* resulting in stimulation of hyphal development and biofilm formation. *Streptococcus* cells produce diffusible signals (DS) mediated in part through activity of *luxS*, which might include metabolic by-products, such as H_2O_2 . The effects of these diffusible signals may be intensified by contact signals (CS) generated through attachment of *S. gordonii* to *C. albicans* receptors expressed on mother cells or hyphae. Streptococci also appear to suppress the inhibitory effects of farnesol (Fnl) on hyphal formation, which does not involve farnesol inactivation. This may suggest that an alternative signal response generated through recognition of *S. gordonii* overcomes the repression of morphogenesis as a result of the farnesol-responsive signaling pathway.

farnesol inhibition. The use of *C. albicans* mutants (e.g., *ras1/ras1* and *ssk1/ssk1* mutants) in future studies should help to clarify this, together with the use of transcriptional microarrays for both species of microorganisms.

In summary, adherence of *S. gordonii* DL1 to *C. albicans* SC5314 appears to primarily involve the *Streptococcus* AgI/II polypeptides SspA and SspB recognizing *C. albicans* cell wall receptors, the nature of which is currently under investigation. Within a biofilm, close cell-to-cell contact provides a shorter range over which signals may be transmitted and received. Response circuits triggered by potential threshold concentrations of signaling molecules would therefore be more readily activated (Fig. 5B). Thus, the potential for AI-2 or small molecules, such as H_2O_2 , to act as signaling mechanisms between

Streptococcus and *C. albicans* could be enhanced by close juxtaposition of the different species within the biofilm. The consequences of the interactions between *S. gordonii* and *C. albicans* are very different from consequences of the interactions between *P. aeruginosa* and *C. albicans* (25). *P. aeruginosa* secretes homoserine lactones, which behave like farnesol analogs and inhibit hyphal formation, effectively killing *C. albicans* hyphae. Conversely, complex physical and chemical interactions between *S. gordonii* and *C. albicans* lead to a synergy in biofilm formation, which is manifested by enhanced hyphal production and increased biomass. In the oral cavity these interactions could be significant for promoting more rapid biofilm formation on denture surfaces or increased hyphal penetration of mucosal surfaces cocolonized by *Candida* and *Streptococcus*. Further investigation of the mechanisms of communication between these species may impact strategies to control *C. albicans* colonization where mixed-species biofilms may form more rapidly, may be harder to disrupt, and may have altered susceptibilities to antimicrobial drugs.

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