S. oralis activates the Efg1 filamentation pathway in C. albicans to promote cross-kingdom interactions and mucosal biofilms

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ABSTRACT
Candida albicans and Streptococcus oralis are ubiquitous oral commensal organisms. Under host-permissive conditions these organisms can form hypervirulent mucosal biofilms. C. albicans biofilm formation is controlled by 6 master transcriptional regulators: Bcr1, Brg1, Efg1, Tec1, Ndt80, and Rob1. The objective of this work was to test whether any of these regulators play a role in cross-kingdom interactions between C. albicans and S. oralis in oral mucosal biofilms, and identify downstream target gene(s) that promote these interactions. Organotypic mucosal constructs and a mouse model of oropharyngeal infection were used to analyze mucosal biofilm growth and fungal gene expression. By screening 6 C. albicans transcription regulator reporter strains we discovered that EFG1 was strongly activated by interaction with S. oralis in late biofilm growth stages. EFG1 gene expression was increased in polymicrobial biofilms on abiotic surfaces, mucosal constructs and tongue tissues of mice infected with both organisms. EFG1 was required for robust Candida-streptococcal biofilm growth in organotypic constructs and mouse oral tissues. S. oralis stimulated C. albicans ALS1 gene expression in an EFG1-dependent manner, and Als1 was identified as a downstream effector of the Efg1 pathway which promoted C. albicans-S. oralis coaggregation interactions in mixed biofilms. We conclude that S. oralis induces an increase in EFG1 expression in C. albicans in late biofilm stages. This in turn increases expression of ALS1, which promotes coaggregation interactions and mucosal biofilm growth. Our work provides novel insights on C. albicans genes which play a role in cross-kingdom interactions with S. oralis in mucosal biofilms.

Introduction

Candida albicans is an important core component of the human oral mycobacteria in health.1,2 Under permissive host conditions this organism can form biofilms with oral streptococci on tooth or mucosal surfaces which are associated with oral diseases such as caries and oropharyngeal candidiasis.3,4 The C. albicans core biofilm transcriptional regulatory network is composed of 6 master regulators (Bcr1, Brg1, Efg1, Ndt80, Rob1 and Tec1) that control approximately 1000 downstream target genes.5 Several of these regulators have also been implicated in the control of the yeast-to-hyphal transition and robust biofilm growth almost always requires both morphotypes.5

Most oropharyngeal opportunistic infections are polymicrobial or require “cooperation” by multiple organisms, since they develop in a host habitat that harbors a large number of different bacterial and fungal species.6 Thus, although C. albicans is the most frequently isolated organism in oropharyngeal candidiasis, it is increasingly appreciated that mixed fungal-bacterial biofilms play a role in oral disease.5,7-10 Within mixed biofilm communities, fungal and bacterial cells use metabolites or cell contact-mediated signals to communicate with each other, adjust their population density, change gene expression patterns, modulate host responses and promote disease. Recent work indicated that the introduction of C. albicans to the oral cavity of mice enhances mucosal biofilm formation by S. oralis,3 a ubiquitous commensal of oral mucosal surfaces in healthy humans,11 which lacks the ability to form biofilms on its own in vitro and in vivo.3,12,13 Growth of S. oralis with C. albicans in mucosal biofilms stimulates an increase in TLR2 mucosal expression leading to TLR2-mediated proinflammatory signals and enhanced pathology.3 More recently we discovered...
that when these 2 commensal microorganisms grow together in mucosal biofilms, they synergize to highjack the epithelial calpain activation pathway, elicit disassembly of intercellular adherens junctions, compromise the integrity of the oral mucosal barrier and promote fungal dissemination. It was determined that the ability to enhance \textit{C. albicans} biofilm pathogenicity was shared among strains of the same streptococcal species. In this work, using established models of oral mucosal fungal-bacterial mixed biofilms, we provide novel mechanistic insights into the biofilm interactions between \textit{C. albicans} with \textit{S. oralis}. After interrogating the expression of the 6 master regulators of \textit{C. albicans} biofilm growth, we identified Efg1 as a critical regulator of co-aggregation interactions with \textit{S. oralis} that increase fungal-bacterial mucosal biofilms. We further illustrate that Als1 is a downstream effector of the Efg1 pathway promoted by \textit{S. oralis}. This is the first report on the involvement of the Efg1 pathway in regulating cross-kingdom biofilm interactions between \textit{C. albicans} and oral bacteria.

**Results**

**EFG1 transcription is activated by \textit{S. oralis} during biofilm growth**

To test whether any of the \textit{C. albicans} master transcriptional regulator(s) were activated by \textit{S. oralis} during biofilm growth we first screened a panel of reporter strains, each driving the expression of mCherry protein under the control of one of the 6 master transcriptional regulators. No differences in the fluorescence signals between single and mixed biofilms were observed after 4 h or 16 h (Fig. S1) of growth, although the mCherry-EFG1p reporter was fluorescing slightly higher at 4 h and 16 h, and the mCherry-NDT80p reporter was fluorescing slightly higher at 16 h in the mixed biofilms. After 36 hours of biofilm growth there was a burst of the red fluorescence signal in the mCherry-EFG1p construct growing with \textit{S. oralis}. Fluorescence of the mCherry-ROB1p strain increased somewhat, but not to levels seen with the mCherry-EFG1p construct (Fig. 1A). To confirm reporter strain activity, we performed RT-qPCR of the 6 regulator genes in biofilm cultures growing under the same conditions. As seen in Fig. 1B, these data confirmed that \textit{S. oralis} stimulated a significant increase in \textit{EFG1} gene transcription in \textit{C. albicans} in late biofilm growth stages (p<0.05).

Because \textit{C. albicans} gene expression in biofilms may be affected by the presence of host cells, we next tested expression of the 6 master regulators in biofilms growing on organotypic mucosal constructs and tongue tissues of orally infected mice. In \textit{Candida}-streptococcal biofilms growing on the surface of organotypic constructs \textit{EFG1} was the only gene that was significantly upregulated by \textit{S. oralis} (Fig. 2A, p<0.05). Similarly, in most mice infected with both organisms, \textit{EFG1} transcripts were higher compared with single infection, although there was variability in the magnitude of this response (Fig. 2B). Collectively these data strongly support the notion that \textit{S. oralis} is a key player in the enhancement of \textit{C. albicans} biofilm pathogenicity.

\begin{figure}[h]
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\caption{\textit{S. oralis} activates \textit{C. albicans} \textit{EFG1} gene expression in mixed biofilms. (A) \textit{C. albicans} mCherry transcriptional regulator reporter strains were grown as biofilms on Permanox\textsuperscript{®} plastic chamber slides, with or without \textit{S. oralis} 34 in RPMI 10%FBS, 10% BHI media for 36 hours and observed under a fluorescence microscope. A representative of 3 experiments is shown. There was a burst of the red fluorescence signal in the EFG1p-mCherry construct growing with \textit{S. oralis}, suggesting \textit{EFG1} transcriptional activation. Bars: 20 \textmu m. (B) \textit{Candida} gene mRNA levels from biofilms growing under identical conditions as in (A) were analyzed by RT-qPCR. Results represent fold increase gene expression in \textit{C. albicans} with \textit{S. oralis} (CaSo) over \textit{C. albicans} (Ca) alone. Means ± SD are shown from 3 experiments. \textit{S. oralis} stimulated a significant increase in \textit{EFG1} gene transcripts. *p<0.05 in a comparison to other regulator genes.}
\end{figure}
support the hypothesis that the Efg1 transcriptional regulator may play a role in biofilm interactions between C. albicans and S. oralis.

**S. oralis promotes EFG1-dependent hyphal morphogenesis in synthetic saliva medium (SSM)**

Because Efg1 regulates filamentation under many environmental conditions, we hypothesized that activation of EFG1 by S. oralis may promote C. albicans hyphae when growing together in the biofilm state. We first grew biofilms in nutrient-rich media, emulating the conditions that induced strong mCherry-EFG1p reporter activity. Under these conditions, C. albicans formed long hyphae and we were unable to detect differences in hyphal length in C. albicans growing with or without S. oralis microscopically (not shown), presumably because nutrient rich media alone provide ample environmental cues for robust filamentous growth. Since growth of C. albicans in SSM at 37°C occurs exclusively in the yeast form, we wondered whether S. oralis can promote filamentous growth of C. albicans in this physiologically relevant medium and whether this effect might be Efg1-mediated. Initially, we quantified the expression of each of the 6 Candida biofilm transcriptional regulators by RT-qPCR when growing together with S. oralis in SSM to confirm that EFG1 is upregulated under these conditions. As shown in Fig. 3A, under these nutrient-limiting conditions EFG1 transcripts were significantly increased (p<0.05) in the presence of S. oralis, whereas transcription of the other regulators was either repressed or unaltered.

Consistent with other reports, C. albicans remained almost exclusively in the yeast form even after 48-hours of incubation at 37°C in SSM. However, as expected, co-culture with S. oralis promoted hyphal growth and cell-cell aggregation (Fig. 3B). C. albicans growing in SSM supplemented with 10% BHI (to promote streptococcal metabolic activity) formed short hyphae which were considerably elongated in the presence of S. oralis. To provide a more quantitative measure of the positive effect of S. oralis on hyphal growth we performed qPCR of the 18 S rRNA gene and compared C. albicans biomass in biofilms with and without S. oralis under the same conditions. As anticipated, the fungal biomass, expressed as genome equivalents in each biofilm, was higher in biofilms with S. oralis although this effect was small and reached statistical significance only at the 24 h time-point (Fig. 3C).

To test whether EFG1 is required for this effect, an efg1ΔΔ strain was tested. As expected, this strain was unable to form hyphae in 10% BHI-supplemented SSM with S. oralis (Fig. 4A). In contrast, the revertant strain formed a mix of yeast and short hyphae in SSM, which were elongated when growing with S. oralis (Fig. 4A). These results were corroborated by the higher Candida biomass in biofilms with S. oralis and the revertant but not the efg1ΔΔ strains, as assessed by qPCR after 24 h-48 h of growth (Fig. 4B). Collectively, these data show...
Figure 3. *S. oralis* promotes *C. albicans* hyphae in synthetic saliva. (A) *C. albicans* SC5314 (Ca) transcriptional regulator gene mRNA levels in biofilms growing in 6-well polystyrene plates with or without *S. oralis* 34 (So) for 24h, with SSM as the sole nutrient source. RNA samples were analyzed by RT-qPCR, after 24 h of co-culture. Results represent fold increase gene expression in mixed (CaSo) over *C. albicans* alone (Ca) biofilms. Means ± SD are shown from 3 experiments. EFG1 transcripts were significantly increased in the presence of *S. oralis*, whereas transcription of other regulators was either repressed or essentially unaltered. *p*<0.05 in comparison with other regulator genes. (B) *C. albicans* SC5314 (blue) or *C. albicans* with teal protein expressing *S. oralis* 34 (green) were cultured on glass slides with SSM, supplemented with or without 10% BHI for 48 hours. *Candida* cells were stained with Calcoflour White® and cultures observed under a fluorescence microscope. Co-culture with *S. oralis* promoted hyphal growth and cell-cell aggregation under both conditions. Bars: 20 μm. (C) Fungal biomass expressed as “genome equivalents” of *C. albicans* SC5314 in biofilms with or without *S. oralis* 34. Biofilms were grown in 6-well polystyrene plates with SSM as the sole nutrient source for 24 h or 48 h. Genome equivalents were extrapolated by analyzing 18S rRNA gene copy numbers in each biofilm well with qPCR and comparing to a standard curve. Means ± SD are shown from 3 experiments. Fungal biomass was higher in biofilms with *S. oralis* consistent with hyphal growth (B), although this effect was small and reached statistical significance only at the 24 h time-point. *p*<0.05, for a comparison to *C. albicans* alone.

Figure 4. Hyphal growth stimulated by *S. oralis* is dependent on the Efg1 transcriptional regulator. (A) *C. albicans* efg1 homozygous deletion mutant (efg1Δ/Δ) and efg1 revertant were grown with or without teal protein expressing *S. oralis* 34 (green) in 10% BHI-supplemented SSM medium, on Permanox® plastic chamber slides, for 48 hours. *Candida* cells were stained with Calcoflour White® (blue) and cultures were observed under a fluorescence microscope. The revertant strain formed a mix of yeast and short hyphae in SSM, which were elongated when growing with *S. oralis*. Bars: 20 μm. (B) Fungal biomass expressed as “genome equivalents” of the efg1Δ/Δ mutant and efg1 revertant strains growing in biofilms with or without *S. oralis* 34. Biofilms were grown in 6-well polystyrene plates with SSM as the sole nutrient source for 24 h or 48 h. Genome equivalents were extrapolated by analyzing 18S rRNA gene copy numbers with qPCR in each biofilm well and comparing to a standard curve. Means ± SD are shown from 3 experiments. A higher *Candida* biomass was noted in biofilms with *S. oralis* and the efg1 revertant, but not the efg1Δ/Δ mutant, in agreement with the hyphal elongation observed microscopically in the revertant (A). *p*<0.05 and **p**<0.01, for a comparison to *C. albicans* alone.
that *S. oralis* can promote *C. albicans* hyphal growth in SSM *in vitro*, and that this effect is Efg1-dependent.

**Efg1 promotes cross-kingdom mucosal biofilms in organotypic construct and mouse models**

Given the activation of *EFG1* by *S. oralis* we next hypothesized that Efg1 plays a role in mucosal biofilms formed by the 2 organisms. Indeed, an *efg1Δ/Δ* mutant was not able to form thick biofilms with *S. oralis* on oral mucosal organotypic constructs, as neither organism exhibited robust growth in the presence of the other (Fig. 5A). In contrast, the *efg1* revertant strain promoted growth of *S. oralis* which resulted in a robust mixed mucosal biofilm (Fig. 5A). In the presence of *S. oralis* the biofilm formed by this strain was denser with longer hyphae observed extending into the submucosal compartment, compared with the single species biofilm (Fig. 5A, bottom panel, arrows). Similarly, tongue biofilms were more robust (Fig. 5B) and the oral fungal and streptococcal burdens in mice co-infected with *S. oralis* were significantly greater with the revertant strain compared with the

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**Figure 5.** (For figure legend, see page 1607.)
efg1Δ/Δ strain (Fig. 5C, p<0.05 and p<0.01, respectively). No S. oralis was retrieved from oral tissues (not shown) unless animals were also infected with C. albicans, consistent with our published work.3,14 Despite the stimulation of hyphal growth by S. oralis in SSM in vitro, S. oralis did not cause an increase in the CFU counts of the EFG1-revertant strain in vivo (Fig. 5C), consistent with our published work with the wild type strain.3 This finding may reflect the fact that CFU methods underestimate hyphal organisms.17

In both organotypic and mouse mucosae the efg1Δ/Δ mutant grew almost entirely in the yeast form regardless of the presence of S. oralis. In agreement with work published by other groups,18 the efg1Δ/Δ strain was severely deficient in colonizing the oral mucosa, as evidenced by the CFU data (Fig. 5C). Surprisingly, S. oralis promoted the mouse oral fungal burdens and tongue biofilm formation of the efg1Δ/Δ mutant (Fig. 5B,C), suggesting that additional regulators of biofilm development, are activated by S. oralis in the oral cavity of mice to compensate for the loss of Efg1. In summary, these results demonstrate that Efg1 plays a key role in promoting C. albicans-S. oralis mucosal biofilms in vitro and in vivo.

**Als1 is a downstream effector of the Efg1 pathway in dual biofilms**

We next set out to discover genes, under the transcriptional control of Efg1, that play a role in the interactions between the 2 organisms in mucosal biofilms. We first compared the expression of 3 genes with a well-established role in C. albicans-oral epithelial cell interactions (ALS1, ALS3, HWP1) between the efg1 mutant and revertant strains, in biofilms growing on mucosal constructs. Of the 3 genes tested ALS1 was the only gene with expression significantly increased by S. oralis in the revertant but not in the efg1Δ/Δ strain, suggesting that ALS1 is an Efg1-dependent gene that may play a role in cross-kingdom interactions (Fig. 6A, p<0.01). These results were further substantiated in the mouse model of oral infection where ALS1 expression was enhanced by S. oralis in the efg1 revertant but not the efg1Δ/Δ strain (Fig. 6B).

To provide evidence that S. oralis can increase Als1 protein expression levels we performed a time-dependent analysis of Als1 in biofilms growing on Permanox® plastic chamber slides, by indirect immunofluorescence using an anti-Als1 mAb. As seen in Fig. 6C at 24 h of biofilm growth in nutrient-rich media (RPMI, 10%FBS, 10% BHI) S. oralis increased C. albicans Als1 protein expression on the surface of hyphae, which persisted after 48 h of co-culture (Fig. 6C). In comparison, Als3 expression levels were not elevated in biofilms with S. oralis, compared with biofilms formed by C. albicans alone (Fig. S2).

Because Als1 functions as an adhesin that binds to streptococcal cell wall proteins and promotes co-aggregation interactions with C. albicans,19 we hypothesized that an als1Δ/Δ mutant would be deficient in co-aggregation interactions with S. oralis and form a mixed species biofilm with a reduced S. oralis biomass. Deletion of the ALS1 gene significantly decreased the number of hyphae co-aggregating with S. oralis compared with the reference strain, as assessed using a co-aggregation assay (Fig. 7A, p<0.0001). A significant reduction in co-aggregation interactions was also observed with an als3 Δ/Δ
mutant (p < 0.05), in line with previous observations in a related oral streptococcal species showing that both Als1 and Als3 participate in adhesion to bacterial cells and promote co-aggregation interactions. To explore the possibility that Als1 and Als3 may function as complementary co-aggregation-promoting adhesins to *S. oralis* on oral mucosal surfaces we hypothesized that whereas mutants lacking either ALS1 or ALS3 form defective...
mucosal biofilms with S. oralis, mixing the 2 mutants together would restore the mixed biofilm. As expected the biofilm mass of S. oralis growing on the surface of organotypic mucosal constructs was reduced in biofilms formed by each mutant alone, compared with biofilms formed with the reference strain. However, as predicted, a 1:1 mixture of the 2 mutants strains restored the S. oralis biofilm on mucosal surfaces (Fig. 7B), supporting a complementary function of these adhesins in cross-kingdom co-aggregation interactions on mucosal surfaces.

The ALS1 overexpressing strain in the efg1Δ/Δ background forms relatively short pseudohyphae in multiple filamentation-promoting media\textsuperscript{10} and co-culture with teal-expressing S. oralis on plastic bottom chamber slides did not affect this phenotype (Fig. 8AB). However, abundant fungal-bacterial cell co-aggregation interactions were observed both in early (Fig. 8A) and late (Fig. 8B) stages of mixed biofilm development with the ALS1 overexpressing strain, compared with the efg1Δ/Δ mutant, possibly due to high constitutive expression levels of Als1 in this strain. In co-aggregation assays in shaking flasks, flocculation was so pronounced with this strain that accurate microscopic quantitative assessments could not be made.

Because we observed more co-aggregation interactions between the ALS1 overexpressing strain and S. oralis during biofilm growth on plastic bottom chamber slides, we hypothesized that the mixed biofilm phenotype is at least partially rescued on organotypic constructs inoculated with these organisms. As seen in Fig. 8C, S. oralis promoted biofilm growth of the ALS1 overexpressing strain, however this biofilm was less robust than the biofilm formed by the reference strain, and no hyphae were observed extending into the submucosal compartment. This suggests that, in addition to ALS1, other downstream genes in the Efg1 transcriptional pathway are required to completely rescue the biofilm phenotype. Perhaps more importantly though, whereas S. oralis did not form a mucosal biofilm with the efg1Δ/Δ strain (Fig. 5A), overexpression of ALS1 in this strain background restored S. oralis biofilm to levels similar with the ones observed with the reference and efg1 revertant strains (Figs. 5A and 8C). This finding further supports the conclusion that Als1 has an important functional role in cross-Kingdom mucosal biofilm interactions between C. albicans and S. oralis.

**Discussion**

Several members of the mitis streptococcal group have been implicated in pathogenic synergy when forming polymicrobial biofilms with other bacterial or fungal organisms.\textsuperscript{3,21,22} This work builds on previously published evidence that S. oralis, a mitis group member, potentiates the virulence of C. albicans oral mucosal biofilms and that C. albicans promotes S. oralis biofilm
Because both S. oralis and C. albicans are ubiquitous colonizers of the oral cavity in healthy humans, it is important to understand how these organisms modulate the capacity of each other to form robust biofilms that enable their transition from commensals to opportunistic pathogens.

In this study we identified Efg1 and its target gene ALS1 as important regulators of cross-kingdom biofilm formation. Because both S. oralis and C. albicans are ubiquitous colonizers of the oral cavity in healthy humans, it is important to understand how these organisms modulate the capacity of each other to form robust biofilms that enable their transition from commensals to opportunistic pathogens.

Figure 8. Overexpression of the ALS1 gene in the efg1Δ/Δ background partially rescues the cross-kingdom mucosal biofilm phenotype (A-B) Co-culture of a C. albicans ALS1-overexpressing strain in the efg1Δ/Δ background (efg1Δ/Δ-ALS1) with a teal protein expressing S. oralis 34 strain for 45 minutes. (A) or 24 hours (B) on Permanox® plastic chamber slides, in RPMI 10%FBS, 10%BHI media. Candida cells were stained with Calcofluor White (blue) and filamentation pattern and co-aggregation interactions were observed under a fluorescence microscope. A representative of 2 experiments is shown, with conditions set up in duplicate. Fungal-bacterial cell co-aggregation interactions were observed both in early (Fig. 8A) and late (Fig. 8B) stages of mixed biofilm development with the ALS1 overexpressing strain. Bars: 20 μm (A); 100 μm (B). (C) Sixteen-hour mucosal biofilms of C. albicans (left panels) or C. albicans with S. oralis (right panels). Biofilms of the reference strain and the efg1Δ/Δ-ALS1 overexpressing strain, with or without S. oralis 34, were grown on the surface of organotypic oral mucosal surfaces. H&E staining (top panels) and fluorescence images of biofilms labeled with a FITC-conjugated anti-Candida antibody (green), an Alexa Fluor 568-labeled streptococcal FISH probe for S. oralis (red), and counterstained with the nucleic acid stain Hoechst 33258 (blue) to visualize mucosal cells, are shown. Green and red channels are individually shown in the efg1Δ/Δ-ALS1 plus S. oralis panels to better visualize the fungal and bacterial signals. A representative of 2 experiments is shown, with conditions set up in duplicate. Overexpression of ALS1 in the efg1Δ/Δ strain background restored S. oralis biofilm to levels similar to the reference and efg1 revertant strains (see Fig. 5A for comparison to the revertant strain). Bars: 50 μm.
interactions between *C. albicans* and *S. oralis*, particularly in late stages of biofilm growth. We previously reported that the master regulator Bcr1, and its downstream effector Hyr1, play a role in oral mucosal biofilms formed by *C. albicans*, however BCR1 gene expression was not activated in *Candida*-streptococcal biofilms in this work. This is showcasing the fact that fungal genes turned on during mucosal biofilm growth do not respond to a single central regulator but to different regulators, depending on the presence of other microorganisms and the stage of biofilm growth. In this work none of the 6 *C. albicans* master transcriptional regulators was strongly activated in response to early contact with bacterial cells, suggesting that they may not play a role in the initial inter-kingdom cell-cell interactions that promote mixed biofilm growth. It is possible that other, as yet unidentified *C. albicans* transcriptional regulators play a role in early mixed biofilm development steps, such as adhesion to the substratum surface. The fact that *EFG1* is strongly activated by *S. oralis* in late stages of biofilm development may suggest that a critical bacterial biomass is needed to trigger this response, perhaps through release of a quorum sensing molecule.

The Efg1 transcription regulator is one of the best-studied, multifunctional regulators in *C. albicans*.

Homozygous deletion of the *EFG1* gene in *C. albicans* strongly attenuates its response to filamentation-inducing stimuli in most liquid and solid media. The Efg1 regulator not only controls filamentous growth and biofilm formation but also the ability of *C. albicans* to adhere to the epithelial matrix protein laminin, to adhere to and invade epithelial cells, and to induce epithelial cell damage in vitro. This work highlights a novel functional role for Efg1 in promoting co-aggregation interactions between *C. albicans* and oral streptococci during late stages of mixed biofilm growth, via induction of the adhesin Als1. This was not surprising since Als1 has been shown to play a role in *C. albicans* adhesion to, and co-aggregation with *S. gordonii*, another member of the oral streptococcal mitis group. Since expression of Als1 promotes inter-kingdom co-aggregation interactions, this may be an important mechanism for *S. oralis* mucosal biofilm formation, as this bacterial species does not form single species biofilms.

Late biofilm stage activation of *ALS1* gene transcription in *C. albicans* biofilms has also been observed by others and may have a distinct functional role from early activation, in promoting cell-cell interactions within biofilms, rather than promoting adhesion to the substratum. Although recognized as an important adhesin to biofilm substrates, our finding that Als1 is upregulated in late biofilm growth stages with *S. oralis* suggests that in these biofilms the most important function of Als1 is to promote co-aggregation interactions between bacteria and hyphae. Consistent with a role in promoting co-aggregation interactions, Als1 has been shown to play a positive role in both filamentation and flocculation.  

*ALS1* expression is completely dependent on Efg1 under many in vitro conditions and Efg1-dependent *ALS1* gene expression has been shown to mediate cell-cell aggregation in *C. albicans*. However, in biofilms growing on abiotic surfaces Als1 was found to be regulated principally by Bcr1. Our finding that *ALS1* is instead regulated by Efg1 in *C. albicans*-*S. oralis* biofilms underscores the significance of environmental cues, such as the presence of bacteria in the mucosal environment, in dictating *Candida* biofilm regulatory signaling pathways.

Expression of *ALS1* from an *EFG1*-independent promoter in the *efg1ΔΔ* strain does not restore the ability to form hyphae, thus our findings that *S. oralis* only partly restored the *Candida* biofilm phenotype of this strain are not surprising. Work from Mitchell and colleagues showed that expression of *ALS1* from an *RHR2*-independent promoter fully restored biofilm formation of a biofilm-deficient *rhr2ΔΔ* mutant. However, unlike the *efg1ΔΔ* mutant, this mutant is not defective in hyphal formation. Importantly, almost complete restoration of *S. oralis* biofilm growth was noted in mixed mucosal biofilms when *ALS1* was expressed from an *EFG1*-independent promoter in the *efg1ΔΔ* strain. This further underscores the importance of Als1 in regulating co-aggregation interactions and *S. biofilm* growth, in the context of the Efg1 signaling pathway.

In addition to Als1, which is not hypha-specific, hyphal adhesins such as Hwp1 and Als3 play a role in co-aggregation interactions with streptococci of the *mitis* group. Our work with the *als1* and *als3* homozygous deletion mutants showed that both of these adhesins promote *S. oralis* mucosal biofilm growth and that their function may be complementary, if not overlapping. This finding is consistent with previous work showing that Als1 and Als3 have complementary functions in vitro and in vivo. The fact that Als1, but not Als3, was upregulated in an Efg1-dependent manner by *S. oralis* in mucosal biofilms, underlines the central role of ALS-gene regulation in identifying the manifested, as opposed to plausible function of Als adhesins in each biofilm model, an idea also supported by work from others.  

An *efg1* homozygous deletion mutant grows as short pseudohyphae with oral epithelial monolayers. Early work from our group established that hyphal growth is essential in oral epithelial adhesion, injury and induction of pro-inflammatory cytokine responses. This explains
the attenuated mucosal colonization of the efg1Δ/Δ strain in this work and the work of others. Along these lines, in the C. albicans mouse vaginitis model the efg1Δ/Δ strain grew exclusively as yeast, with mucosal damage and inflammatory responses considerably reduced, suggesting that similar to the oral mucosa, virulence in this mucosal site depends primarily on the Efg1 pathway. The fact that S. oralis promotes Efg1-mediated hyphal growth supports a possible role of this transcription pathway in the exaggerated mucosal inflammatory response and increased virulence in co-infected mice, reported previously. The role of this pathway in fungal-bacterial synergy was also demonstrated in an intra-abdominal staphylococcal-C. albicans mixed infection model where Efg1 was required for synergistic virulence.

The finding that filamentation was not promoted by S. oralis in the efg1 mutant shows that this regulatory pathway is required for hyphal growth in Candida-streptococcal biofilms. Interestingly, S. oralis was able to promote the oral colonization of the efg1Δ/Δ strain, suggesting that the requirement for Efg1 in these interactions in vivo is modified by the mucosal environment and perhaps by the presence of commensal microorganisms other than S. oralis. This is exemplified also by the fact that although the efg1Δ/Δ strain colonizes the oral mucosa of mice poorly (this work and ref. 18), it colonizes the vaginal and gastrointestinal mucosae in high numbers. Efg1 is involved in the regulation of SAP 4,5,6 genes in the GI tract, genes that are also upregulated in the Candida-streptococcal oral biofilm mouse model, further lending support to a central role of this transcription pathway in mixed infection. However, it is also important to note that, although these genes play a role in oral mucosal invasion in single-species biofilms, they were not required for the increased C. albicans tissue invasion in biofilms with S. oralis. This suggests that some genes identified as strong virulence determinants in single infection models may be dispensable in mixed biofilms of C. albicans with certain bacterial species.

We conclude that S. oralis modulates C. albicans virulence by increasing EFG1 gene expression. This, in turn increases filamentation and expression of the downstream gene ALS1, which promotes coaggregation interactions and biofilm growth. Exploring the transcriptional regulation of Candida-bacterial biofilms is novel and important, since most mucosal fungal infections occur in a polymicrobial environment. Our work provides novel insights on the regulatory control of cross-kingdom mucosal biofilms which may facilitate the transition of C. albicans and S. oralis from commensals to opportunistic pathogens.
Methods

Strains and growth conditions

*C. albicans* and *S. oralis* strains used in this study are listed in Table 1. *C. albicans* strains were routinely maintained in yeast extract peptone dextrose (YPD) agar and grown in YPD broth, aerobically, at room temperature, on a rotor shaker. *S. oralis* 34 was routinely grown from frozen stocks in brain heart infusion (BHI) medium (BD) under anaerobic, static conditions, at 37°C, 5% CO₂, one day before each experiment. A tel fluorescence protein expressing- *S. oralis* 34 strain (emitting green fluorescence at excitation wavelength 492 nm) was constructed by transforming strain 34 with the streptococcal replicative plasmid pVMTeal carrying a streptococcal codon-optimized gene for TFPI fluorescent protein. The plasmid was transformed into serum-competent strain 34 cells, plated on Todd Hewitt agar medium and excited colonies were screened for competence strain 34 cells, plated on Todd Hewitt agar at 37°C for 105 cells/mℓ. Each ml growth, 0.5 g L⁻¹ haemin, pH 7.0 supplemented with 2.5 mM DTT and type II porcine gastric mucin, 0.5 g L⁻¹. Strains and growth conditions listed in Table 1.

Biofilm growth

Growth on abiotic surfaces

To prepare the microbial inocula for biofilm growth, overnight broth cultures of each microorganism were used to inoculate new cultures in YPD (for *C. albicans*) or BHI (for *S. oralis*) broth, that were allowed to grow until late logarithmic phase. For fluorescence imaging of biofilms, microorganisms from these cultures were inoculated in 4-well Permanox® plastic chamber slides (ThermoFisher, Cat. No. 177437). Each chamber was inoculated with 10⁴ cells of *C. albicans* and 10⁵ cells of *S. oralis*. For biofilms used for DNA or RNA extraction, 10⁵ *C. albicans* and 10⁶ *S. oralis* cells were seeded in 6-well polystyrene plates. Biofilms were grown in RPMI 1640 media containing 10% FBS and 10% BHI or in SSM with or without 10% BHI. SSM consisted of 0.625 g L⁻¹ type II porcine gastric mucin, 0.5 g L⁻¹ peptone, 0.5 g L⁻¹ tryptone, 0.25 g L⁻¹ yeast extract, 0.088 g L⁻¹ NaCl, 0.05 g L⁻¹ KCl, 0.05 g L⁻¹ CaCl₂, and 0.25 mg mL⁻¹ haemin, pH 7.0 supplemented with 2.5 mM DTT and 0.5 g L⁻¹ sucrose. Biofilms were grown for 4–48 hours at 37°C, in a 5%CO₂ incubator.

Growth on oral mucosal constructs

The development of 3-dimensional oral mucosal constructs which faithfully mimic non-keratinized human oral mucosa have been described in detail elsewhere. Briefly the organotypic constructs consist of a TERT-2-immortalized human oral keratinocyte cell line (OKF6) seeded on collagen type I-embedded fibroblasts (3T3 fibroblasts, ATCC). Tissues are airlifted to ensure epithelial differentiation and stratification. Microbial inocula were prepared as done for abiotic surface biofilms. Each tissue was inoculated with 10⁵ cells of *C. albicans* or 10⁷ cells of streptococci or a combination, in 25 μL of infection medium. When 2 strains of *C. albicans* were used to simultaneously inoculate a tissue surface, 5 × 10⁵ cells of each strain were used/tissue. Infection media consist of DMEM, supplemented with L-glutamine, hydrocortisone, ITES, O-phosphorylethanolamine, adenine and triiodothyronine. Mucosal biofilms grew at the air-liquid interface at 37°C in a 5% CO₂ incubator for 16h. These conditions have been optimized to result in well-organized, mature mucosal biofilms.

Mouse model of co-infection

Six- to 12-week-old female C57BL/6 mice were purchased from the Jackson Laboratory and used in these experiments (Animal protocol #100358–0215). The *C. albicans*-*S. oralis* 34 mouse oral co-infection model has been described in detail elsewhere. Briefly, mice were immunosuppressed with cortisone acetate (225 mg/kg, subcutaneously, Sigma Cat. No. C3130–5G), on the first and third day of the infection period. Microbial inocula were prepared as described above. A cotton pellet, saturated with 100 μL microbial suspension of each organism or their combination (yeast cells [6 × 10⁹/mL] and/ or bacteria [2.5 × 10⁹/mL]), was placed sublingually, under general anesthesia for 2 h. Fresh suspensions of microorganisms were added daily in the drinking water. Mice were killed on day 4 post-inoculation and tissues were harvested for mRNA, CFU or histological analysis. For CFU determinations tongues were excised, weighed and homogenized. Undiluted and diluted homogenates were plated on Sabouraud dextrose agar (Becton-Dickinson, Cat. No. 211584) supplemented with chloramphenicol (Sigma, Cat. No. C0378), or Mitis-Salivarius® agar supplemented with 1% Tellurite (Difco Cat. No. 229810), for *C. albicans* and *S. oralis* CFU counts, respectively. Tongue homogenates plated from uninfected animals show no fungal or bacterial colony growth on these solid media.

RNA extraction and RT-qPCR

Fungal RNA from abiotic biofilms, mucosal constructs or mouse tongue tissues was extracted as described previously, with minor modifications. Mouse tongues were dissected sagittally along the mid line and one half
was used to extract fungal RNA. Tissues were homogenized using a POLYTRON® homogenizer and the supernatants were beaten by using 0.5 mm zirconium beads (BeadBug® prefilled tubes, Sigma-Aldrich, Cat. No. Z763772), mixed with 4°C UltraPure Phenol:Chloroform:isoamyl Alcohol (Invitrogen, Cat. No. 15593–031) in 1:1 vol:vol ratio. RNA was purified using the RNaseasy Mini Kit® which includes a DNase treatment step (Qiagen Cat. No. 74104). A second DNase treatment was performed by using the TURBO DNA-free® Kit (Thermo Fisher Scientific Cat. No. AM1907). RNA concentrations and quality were determined using a NanoDrop®. cDNA was synthesized with SuperScript III CellDirect cDNA Synthesis kits® (Invitrogen Cat. No. 18080–051).

All RT-qPCR was performed with a BIO-RAD CFL96 cycler and the IQTM SYBR® Green Supermix kit (BIO-RAD Cat. No. 1708880). Mastermix, cDNA and primers (0.5 μM) were mixed in a total volume of 20 μL per reaction. The amplification program included an initial denaturation step at 95°C for 5 min, followed by 40 cycles of 95°C for 45 s and 58°C for 30 s. A minus reverse transcriptase control was used in all PCR reactions to confirm that amplification of any residual genomic DNA is negligible. The primers used to amplify Candida genes were, Als1-F AGAAGTATTTGCGATGATGAG, Als1-R TGAGATCTTGGTATCTTG; Hwp1-F TGGTCCAGGTTCTTGTTT, Hwp1-R GGTTGAGTGGGAACTGA; Als3-F CCACCTCAACATCCCATCAT; Als3-R CAGCGTAGTAGACAGTAGTAGTTTTCT; Ndt80-R GGTTGTCCTTGCATTGTTGAGG, Ndt80-F ACACCTCAGGAGCCACATTTG; Efg1-F TATCAGTGAATTTCCCAGG, Efg1-R CTGACTGTTCGTTGATTGG; Tec1-F GCTCACAGTTTCTCCAACACTGC, Tec1-R AGTGTAGGGACAATAGGC, Brg1-F GGGTATTTACCGCTAAAATTG, Brg1-R TATTTCTACCGCTTCCCTGGC, Rob1-F AGCCCAACAATGATACCGCC, Rob1-R TATTTGTTGCTCGTGTCGCCC, Bcr1-F CCCCGATCGATCATAGACACAC, Bcr1-R ATCGTGAAGTTCGATACTTG, with an annealing temperature of 59°C. The amplification protocol and genome equivalent calculations were described in detail elsewhere.48 Briefly, the qPCR was performed using the thermocycling instructions recommended for the SYBR Green PCR Master Mix (95°C for 30 s and 40 cycles of 30 s at 95°C and 30 s at 59°C). Since the organism has variable numbers of 18S rRNA gene copies per genome,19 genome equivalents (or fungal biomass) were calculated based on standard curves obtained after amplifying 10-fold serial dilutions of fungal DNA isolated from overnight 37°C YPD broth cultures (strain SC5314). To convert nanograms of DNA in overnight cultures to genome equivalents, we took into account that the C. albicans SC5314 haploid genome size is ~15 Mbp (www.candidagenome.org). Assuming the average base pair weight in fungal cells to be 650 Daltons, cell numbers were calculated according to the formula: cell number = (ng of DNA * 6.022 × 10^23) / (15 × 10^6 * 1 × 10^9 * 650). PCR was performed with a BIO-RAD CFL96 cycler and the IQTM SYBR® Green Supermix kit (BIO-RAD Cat. No. 1708880).

**Fluorescence imaging**

Protocols for immunofluorescence labeling of C. albicans and S. oralis biofilms were described in detail previously.3,7,14 To visualize epithelial cell nuclei and C. albicans hyphae in single-species and mixed abiotic biofilms with established specificity was visualized by FISH with the Streptococcus-specific oligonucleotide probe STR405, conjugated to Alexa 546. In abiotic surface biofilms Candida cells were stained with Calcofluor White® (Sigma Cat. No. 18909–100ML-F) for 5 minutes. Als1 and Als3 protein expression in single-species and mixed abiotic surface biofilms were tested by indirect immunofluorescence labeling using monoclonal anti-Als1 and anti-Als3 antibodies with established specificities and protocols developed by the Hoyer laboratory.19,36,37 Briefly, biofilms were fixed in 4% paraformaldehyde, followed by washing and a blocking step with 15 μl/ml normal goat serum for 15 min. Immuno-labeling was performed with 18 μg/ml of purified anti-Als antibody, followed by a FITC-conjugated goat anti-mouse IgG F(ab')2 fragment-specific antibody (Jackson ImmunoResearch Cat. No. 115–096–006). Als protein immuno-labeling was followed by FISH with the Streptococcus-specific oligonucleotide probe STR405, conjugated to Alexa 546 to visualize S. oralis, and Calcofluor White® to visualize C. albicans hyphae. Fluorescence images were captured using a Zeiss Axio Imager M1 microscope, using a 63x oil immersion objective.
Co-aggregation assays

Experiments to quantitatively assess co-aggregation between C. albicans strains and streptococci were performed as originally described by the Jenkinson laboratory, and later modified by Hoyer and colleagues, with the exception that instead of FITC-labeled bacteria we used a teal-expressing S. oralis strain. Briefly, C. albicans yeast cells from an overnight YPD broth culture were inoculated into RPMI1640–10% FBS medium for 3 h to form hyphae. S. oralis late-log phase inocula were prepared as described in biofilm growth assays. Harvested bacterial and fungal cells (10:1 ratio) were suspended in co-aggregation buffer (pH 8.0) which consisted of the following: 1 mM Tris HCl, 0.15 M NaCl, 0.1 mM MgCl2, and 0.1 mM CaCl2. Co-aggregation interactions were allowed to take place for 1 h, at 37°C, and 0.1 mM CaCl2. Co-aggregation interactions were expressed as percentages of the total number of hyphae counted, as determined as originally described by the Jenkinson laboratory,50 and later modified by Hoyer and colleagues,19 with the exception that instead of FITC-labeled bacteria we used a teal-expressing S. oralis strain. Briefly, C. albicans yeast cells from an overnight YPD broth culture were inoculated into RPMI1640–10% FBS medium for 3 h to form hyphae. S. oralis late-log phase inocula were prepared as described in biofilm growth assays. Harvested bacterial and fungal cells (10:1 ratio) were suspended in co-aggregation buffer (pH 8.0) which consisted of the following: 1 mM Tris HCl, 0.15 M NaCl, 0.1 mM MgCl2, and 0.1 mM CaCl2. Co-aggregation interactions were allowed to take place for 1 h, at 37°C, in a shaking flask. 150 μL of cell suspension from each flask was transferred into a well of an 8-well Permanox® plastic chamber slide, hyphae were stained with Calcofluor White® and observed under a fluorescence microscope. The numbers of hyphae with bacterial binding were expressed as percentages of the total number of hyphae counted, as determined in 8–10 microscopic fields (40X)/per condition, in 3 independent experiments.

Statistical analyses

Animal experiments used groups of 5–8 mice and were independently repeated 3 times, unless noted otherwise. Pair-wise comparison of gene expression data was performed using the non-parametric Mann-Whitney test, or Student’s t-test when data points were normally distributed. Fungal and bacterial burdens with different combinations of strains were analyzed using ANOVA or the Kruskal-Wallis test, when data did not pass the normality test. Analyses were performed using the Graph-Pad Prism® software. Statistical significance for all tests was set at P < 0.05.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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