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RESEARCH PAPER



## *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.

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### KEYWORDS

ALS1; *Candida*; cross-kingdom biofilm; EFG1; oral mucosa; *Streptococcus*

## Introduction

*Candida albicans* is an important core component of the human oral mycobiota in health.<sup>1,2</sup> 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.<sup>3,4</sup> 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.<sup>5</sup> 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.<sup>5</sup>

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.<sup>6</sup>

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.<sup>5,7–10</sup> 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*,<sup>3</sup> a ubiquitous commensal of oral mucosal surfaces in healthy humans,<sup>11</sup> which lacks the ability to form biofilms on its own *in vitro* and *in vivo*.<sup>3,12,13</sup> 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.<sup>3</sup> More recently we discovered

that when these 2 commensal microorganisms grow together in mucosal biofilms, they synergize to hijack the epithelial calpain activation pathway, elicit disassembly of intercellular adherens junctions, compromise the integrity of the oral mucosal barrier and promote fungal dissemination.<sup>14</sup> It was determined that the ability to enhance *C. albicans* biofilm pathogenicity was shared among strains of the same streptococcal species.<sup>14</sup>

In this work, using established models of oral mucosal fungal-bacterial mixed biofilms, we provide novel mechanistic insights into the biofilm interactions between *C. albicans* with *S. oralis*. After interrogating the expression of the 6 master regulators of *C. albicans* biofilm growth, we identified Efg1 as a critical regulator of co-aggregation interactions with *S. oralis* that increase fungal-bacterial mucosal biofilms. We further illustrate that Als1 is a downstream effector of the Efg1 pathway promoted by *S. oralis*. This is the first report on the involvement of the Efg1 pathway in regulating cross-kingdom biofilm interactions between *C. albicans* and oral bacteria.

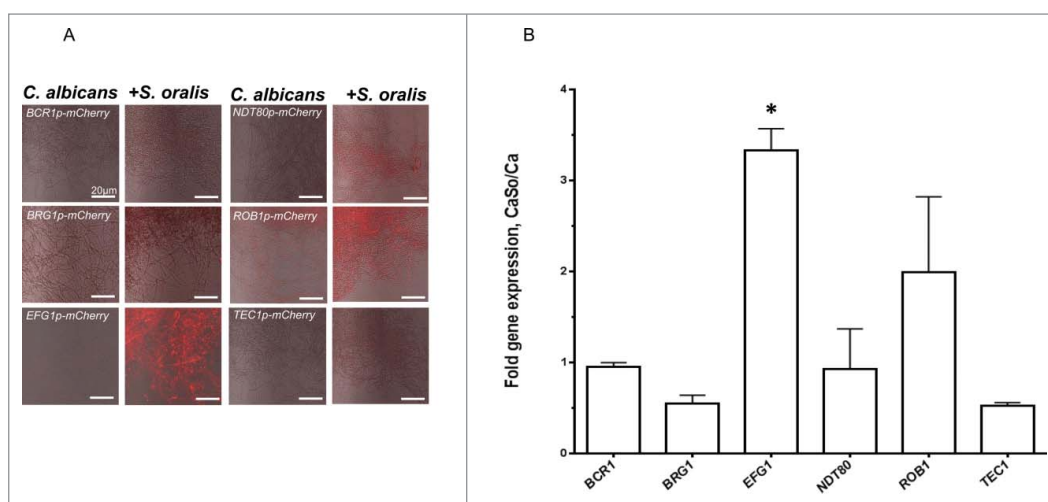
## Results

### *EFG1* transcription is activated by *S. oralis* during biofilm growth

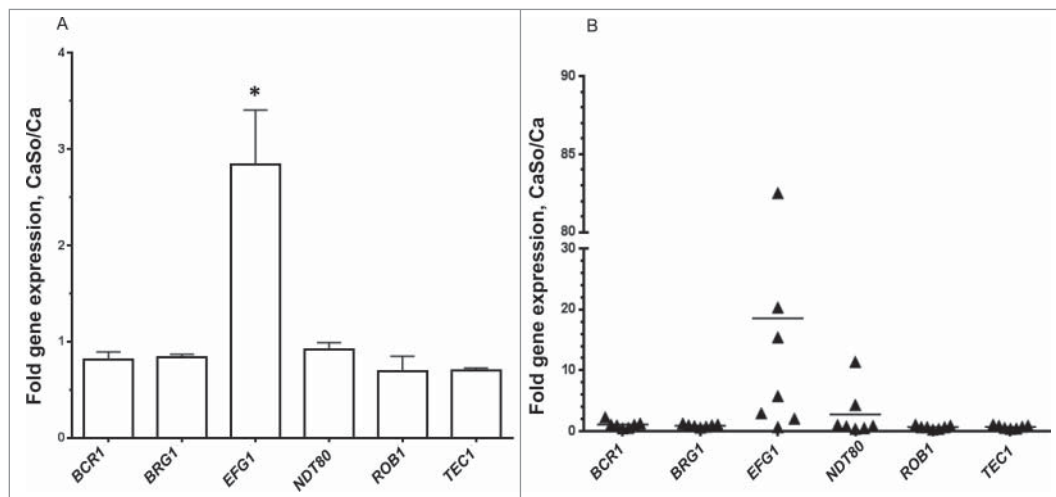
To test whether any of the *C. albicans* master transcriptional regulator(s) were activated by *S. oralis* during biofilm growth we first screened a panel of reporter strains, each driving the expression of mCherry protein under

the control of one of the 6 master transcriptional regulators.<sup>5</sup> 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 *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 *S. oralis* stimulated a significant increase in *EFG1* gene transcription in *C. albicans* in late biofilm growth stages ( $p < 0.05$ ).

Because *C. albicans* gene expression in biofilms may be affected by the presence of host cells,<sup>5</sup> we next tested expression of the 6 master regulators in biofilms growing on organotypic oral mucosal constructs and tongue tissues of orally infected mice. In *Candida*-streptococcal biofilms growing on the surface of organotypic constructs *EFG1* was the only gene that was significantly upregulated by *S. oralis* (Fig. 2A,  $p < 0.05$ ). Similarly, in most mice infected with both organisms, *EFG1* transcripts were higher compared with single infection, although there was variability in the magnitude of this response (Fig. 2B). Collectively these data strongly



**Figure 1.** *S. oralis* activates *C. albicans* *EFG1* gene expression in mixed biofilms. (A) *C. albicans* mCherry transcriptional regulator reporter strains were grown as biofilms on Permanox<sup>®</sup> plastic chamber slides, with or without *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 *S. oralis*, suggesting *EFG1* transcriptional activation. Bars: 20 μm. (B) *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 *C. albicans* with *S. oralis* (CaSo) over *C. albicans* (Ca) alone. Means ± SD are shown from 3 experiments. *S. oralis* stimulated a significant increase in *EFG1* gene transcripts. \* $p < 0.05$  in a comparison to other regulator genes.



**Figure 2.** *S. oralis* increases *EFG1* gene expression of *C. albicans* in organotypic tissue and mouse oral mucosal infection models. (A) Organotypic tissues were infected with *C. albicans* SC5314 with or without *S. oralis* 34, for 16h. *Candida* gene mRNA levels were analyzed by RT-qPCR. Results represent fold increase in gene expression over the single infection group. Means  $\pm$  SD are shown from twice repeated experiments, with tissue infections set up in duplicate. *EFG1* was the only gene that was significantly upregulated by *S. oralis*. \* $p < 0.05$  for a comparison to all other regulators. (B) Mice were infected with *C. albicans* or *C. albicans* plus *S. oralis* 34 for 4 d and *Candida* gene mRNA levels in tongue samples were analyzed by RT-qPCR. Results represent fold increase gene expression over the single infection group, with 6–7 mice per group. In most mice infected with both organisms, *EFG1* transcripts were higher compared with single infection, although variability in the magnitude of this response was noted.

support the hypothesis that the *Efg1* transcriptional regulator may play a role in biofilm interactions between *C. albicans* and *S. oralis*.

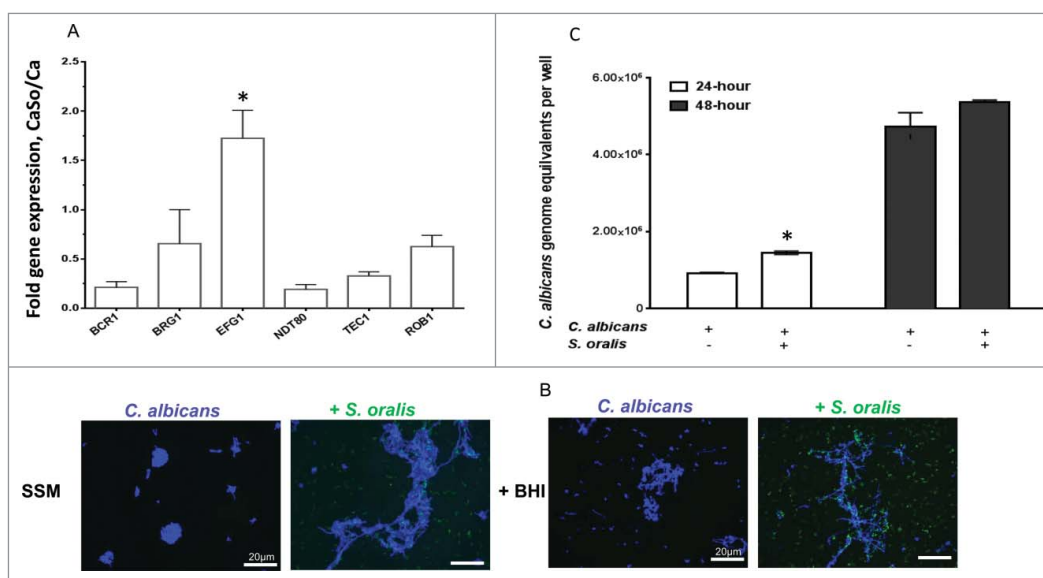
### ***S. oralis* promotes *EFG1*-dependent hyphal morphogenesis in synthetic saliva medium (SSM)**

Because *Efg1* regulates filamentation under many environmental conditions<sup>15</sup> 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,<sup>16</sup> 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.*

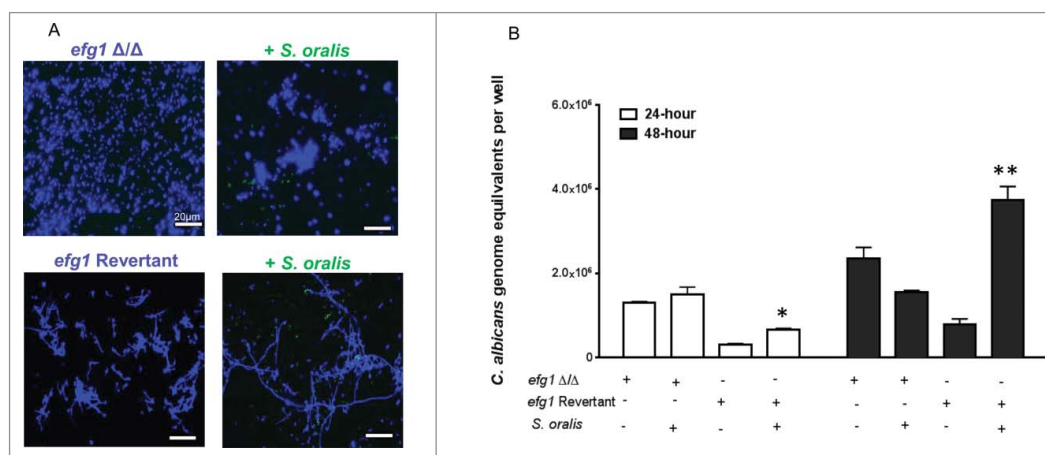
*oralis*, whereas transcription of the other regulators was either repressed or unaltered.

Consistent with other reports,<sup>16</sup> *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 *efg1Δ/Δ* 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 *efg1Δ/Δ* 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  $\pm$  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 Calcofluor White<sup>®</sup> and cultures observed under a fluorescence microscope. Co-culture with *S. oralis* promoted hyphal growth and cell-cell aggregation under both conditions. Bars: 20  $\mu$ m. (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 18 S rRNA gene copy numbers in each biofilm well with qPCR and comparing to a standard curve. Means  $\pm$  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.



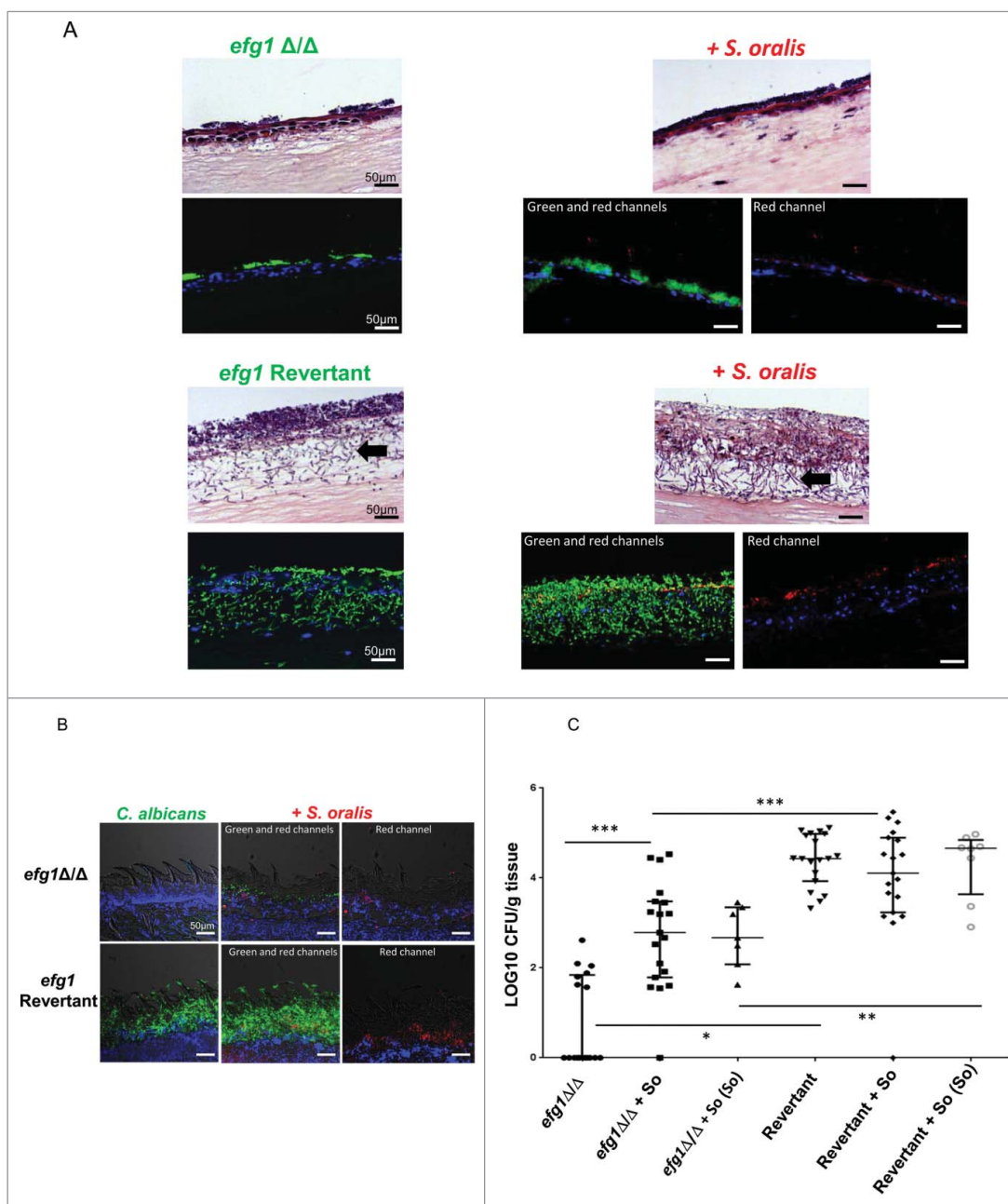
**Figure 4.** Hyphal growth stimulated by *S. oralis* is dependent on the Efg1 transcriptional regulator. (A) *C. albicans* *efg1* homozygous deletion mutant (*efg1* $\Delta/\Delta$ ) and *efg1* revertant were grown with or without teal protein expressing *S. oralis* 34 (green) in 10% BHI-supplemented SSM medium, on Permanox<sup>®</sup> plastic chamber slides, for 48 hours. *Candida* cells were stained with Calcofluor White<sup>®</sup> (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  $\mu$ m. (B) Fungal biomass expressed as “genome equivalents” of the *efg1* $\Delta/\Delta$  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 18 S rRNA gene copy numbers with qPCR in each biofilm well and comparing to a standard curve. Means  $\pm$  SD are shown from 3 experiments. A higher *Candida* biomass was noted in biofilms with *S. oralis* and the *efg1* revertant, but not the *efg1* $\Delta/\Delta$  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 *efg1* $\Delta/\Delta$  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



**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.<sup>3,14</sup> 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.<sup>3</sup> This finding may reflect the fact that CFU methods underestimate hyphal organisms.<sup>17</sup>

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,<sup>18</sup> 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**

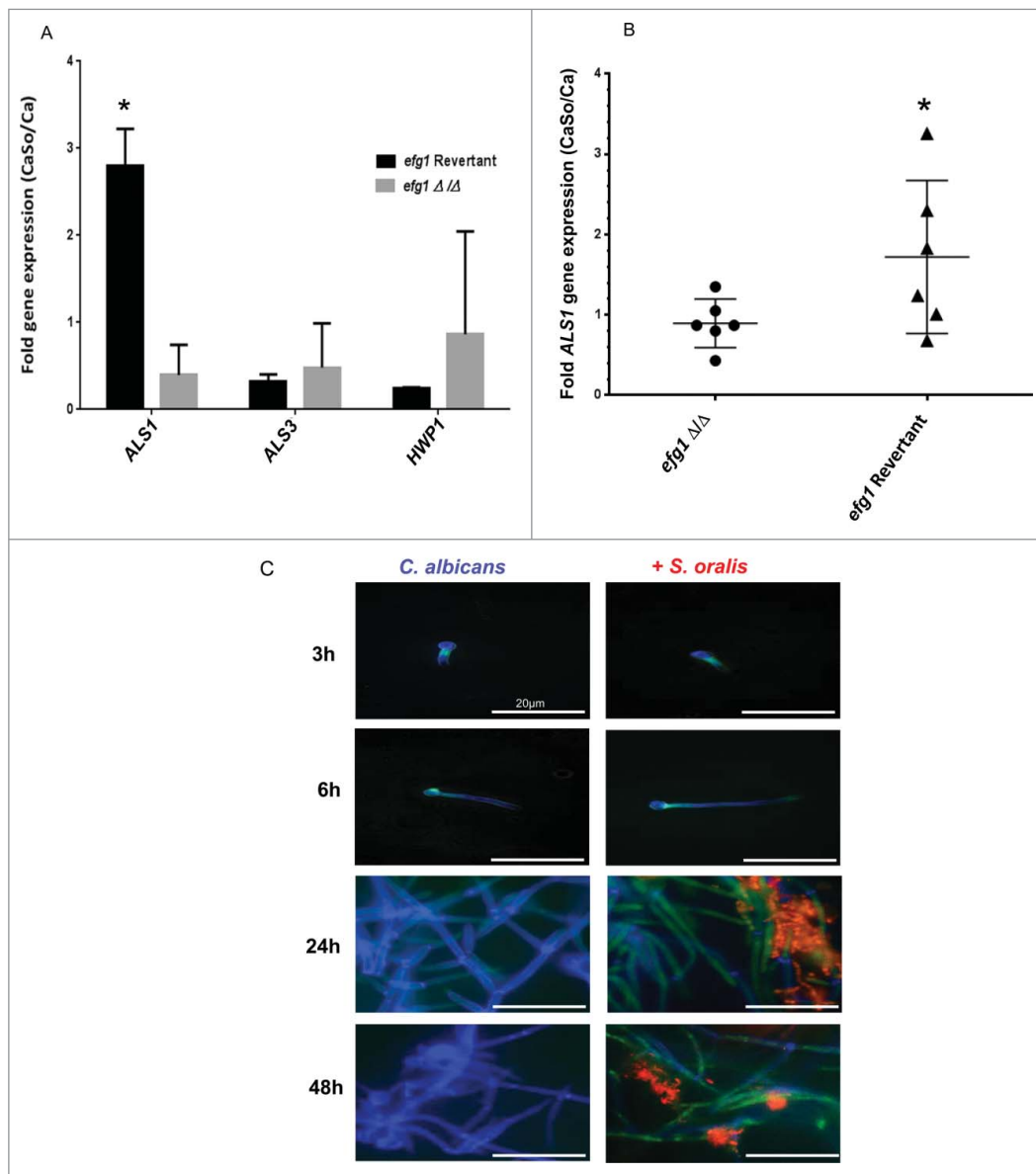
We next set out to discover genes, under the transcriptional control of Efg1, that play a role in the interactions between the 2 organisms in mucosal biofilms. We first compared the expression of 3 genes with a well-established role in *C. albicans*-oral epithelial cell interactions (*ALS1*, *ALS3*, *HWPI*) 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<sup>®</sup> 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*,<sup>19</sup> 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*

**Figure 5.** (see previous page) Efg1 promotes cross-kingdom mucosal biofilms in organotypic construct and animal models. (A) Mucosal biofilms formed by *C. albicans* (left panel) or *C. albicans* with *S. oralis* (right panel). Biofilms of the *efg1* mutant and *efg1* revertant strain were grown with or without *S. oralis* 34 on the surface of organotypic models of the oral mucosa for 16h, the time required for a well-organized biofilm to form on mucosal surfaces.<sup>7</sup> H&E staining (top panels) and fluorescence images (bottom panels) of tissue sections 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), are shown. Red and blue channel only images are used to illustrate biofilm growth by *S. oralis* in the presence of *C. albicans*. Unlike the *efg1* mutant, the *efg1* revertant promoted growth of *S. oralis* on mucosal constructs which resulted in a robust mixed mucosal biofilm. The *efg1* revertant formed longer hyphae extending into the sub-mucosal compartment, in mixed compared with single species biofilms (bottom panel, arrows). No growth of *S. oralis* was observed when inoculated alone (not shown), consistent with previous work.<sup>13,14</sup> Bar: 50  $\mu$ m. (B) Representative tongue tissue sections from mice infected with the *efg1* mutant (*efg1Δ/Δ*) and *efg1* revertant, with or without *S. oralis* 34 for 4 d. *C. albicans* (green) labeled with a FITC-conjugated anti-*Candida* antibody and *S. oralis* (red) labeled with an Alexa Fluor 568-labeled FISH probe. Mucosal cell nuclei were counterstained with the nucleic acid stain Hoechst 33258 (blue). In tissues infected with both organisms (middle and right panels), overlay of 3-color fluorescence and overlay of red and blue channels only, are used to illustrate biofilm growth of *S. oralis* with *C. albicans*. In these tissues, little biofilm growth can be seen in the *efg1* mutant with *S. oralis*. In contrast, the revertant strain formed a more robust biofilm and promoted stronger biofilm growth of *S. oralis* compared with the *efg1* mutant. No biofilm growth in mice infected with *S. oralis* alone was observed (not shown), consistent with previous work.<sup>3,14</sup> Bar: 50  $\mu$ m (C) Fungal and bacterial burdens in mice infected with the *efg1* mutant (*efg1Δ/Δ*) and *efg1* revertant (Revertant), with or without *S. oralis* 34 (So) for 4 d. Data represent log CFU of fungi, or bacteria (labeled as "So" in parentheses, on the X-axis) per gram of tongue tissue, in 3 independent mouse experiments, with 5–8 animals per group. *Candida* and streptococcal burdens in mice infected with both organisms were significantly greater with the revertant strain compared with the *efg1Δ/Δ* strain. *S. oralis* was not retrieved from oral tissues of mice infected with bacteria alone (not shown). \* $p < 0.0001$ , \*\* $p < 0.01$ , \*\*\* $p < 0.05$ .

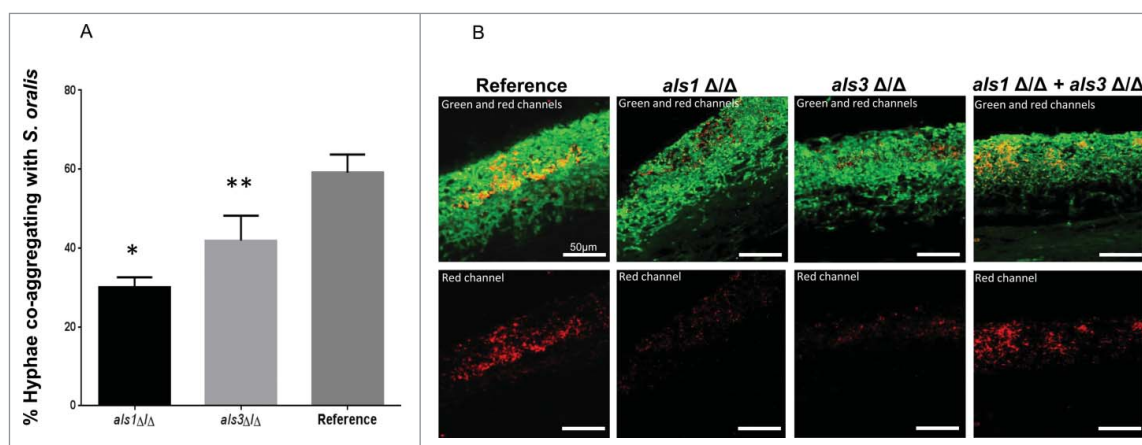


**Figure 6.** *C. albicans* ALS1 expression is increased by *S. oralis*. (A) Biofilms of the *efg1* mutant and *efg1* revertant were grown with or without *S. oralis* 34 on the surface of organotypic oral mucosal constructs for 16h. mRNA levels of 3 *Candida* genes known to play a role in the interactions with oral mucosal epithelium (*ALS1*, *ALS3* and *HWP1*) were analyzed by RT-qPCR. Results represent fold increase gene expression of each *Candida* strain with *S. oralis* over *Candida* alone. Means  $\pm$  SD are shown from technical triplicates, in 2 independent experiments. *ALS1* was the only gene with expression significantly increased by *S. oralis* in the *efg1* revertant but not in the *efg1* $\Delta/\Delta$  strain. \* $p < 0.01$  in a comparison between the *efg1* $\Delta/\Delta$  and *efg1* revertant strains. (B) *ALS1* gene mRNA expression levels in tongue tissues of infected mice analyzed by RT-qPCR. Mice were infected with the *efg1* mutant (*efg1* $\Delta/\Delta$ ) and *efg1* revertant strain with or without *S. oralis* 34 for 4 d. Results show *ALS1* gene expression levels of the mixed infection group (CaSo) relative to *Candida* (Ca) infection alone, in 6 mice per group. *ALS1* expression was enhanced by *S. oralis* in the *efg1* revertant but not the *efg1* $\Delta/\Delta$  infection group.  $p < 0.05$  for a comparison between mutant and revertant strains. (C) Als1 protein expression in single (*C. albicans*) and mixed (*C. albicans* with *S. oralis*) biofilms. *C. albicans* SC5314 was grown on Permanox<sup>®</sup> plastic chamber slides with or without *S. oralis* 34 in RPMI 10%FBS, 10% BHI media, for 3h–48 hours. Biofilms were labeled with a monoclonal antibody against Als1, followed by a secondary FITC-conjugated antibody (green). *S. oralis* (red) was labeled with an Alexa Fluor 568-labeled FISH probe and *C. albicans* (blue) was stained with Calcofluor White<sup>®</sup>. A representative of 3 independent experiments is shown. *S. oralis* increased *C. albicans* Als1 protein expression on the surface of hyphae after 24–48 h of co-culture. Bars: 50  $\mu$ m.

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.<sup>19</sup> 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





**Figure 7.** Als1 promotes co-aggregation interactions between *C. albicans* and *S. oralis*. (A) Co-aggregation assays between *S. oralis* 34 and *C. albicans* reference strain or strains lacking either *ALS1* (*als1Δ/Δ*) or *ALS3* (*als3Δ/Δ*) were performed as described in methods. Results represent percentage of hyphae co-aggregating with *S. oralis* in each microscopic field, at 40X magnification. Means  $\pm$  SD are shown from 3 independent experiments, with 8 microscopic fields analyzed per condition in each experiment. Deletion of *ALS1* or *ALS3* significantly decreased the number of hyphae co-aggregating with *S. oralis* compared with the reference strain. \* $p < 0.0001$ , \*\* $p < 0.05$  for a comparison with the reference strain. (B) Mucosal biofilms of *C. albicans* with *S. oralis* growing for 16 h on the surface of oral mucosal organotypic constructs. Tissues were inoculated with *S. oralis* 34 together with a *C. albicans* reference strain, *als1Δ/Δ* mutant, *als3Δ/Δ* mutant or a 1:1 mixture of the 2 mutants. Fluorescence images (top panel) show biofilms labeled with a FITC-conjugated anti-*Candida* antibody (green) and an Alexa Fluor 568-labeled streptococcal FISH probe for *S. oralis* (red). The red channel is individually shown in the lower panel to better visualize the bacterial biomass. Mixing the 2 mutant strains restored the *S. oralis* biofilm on mucosal surfaces, supporting a complementary functional role of Als1 and Als3 adhesins in cross-kingdom co-aggregation interactions. Bars: 50  $\mu$ m.

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 mutant 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.

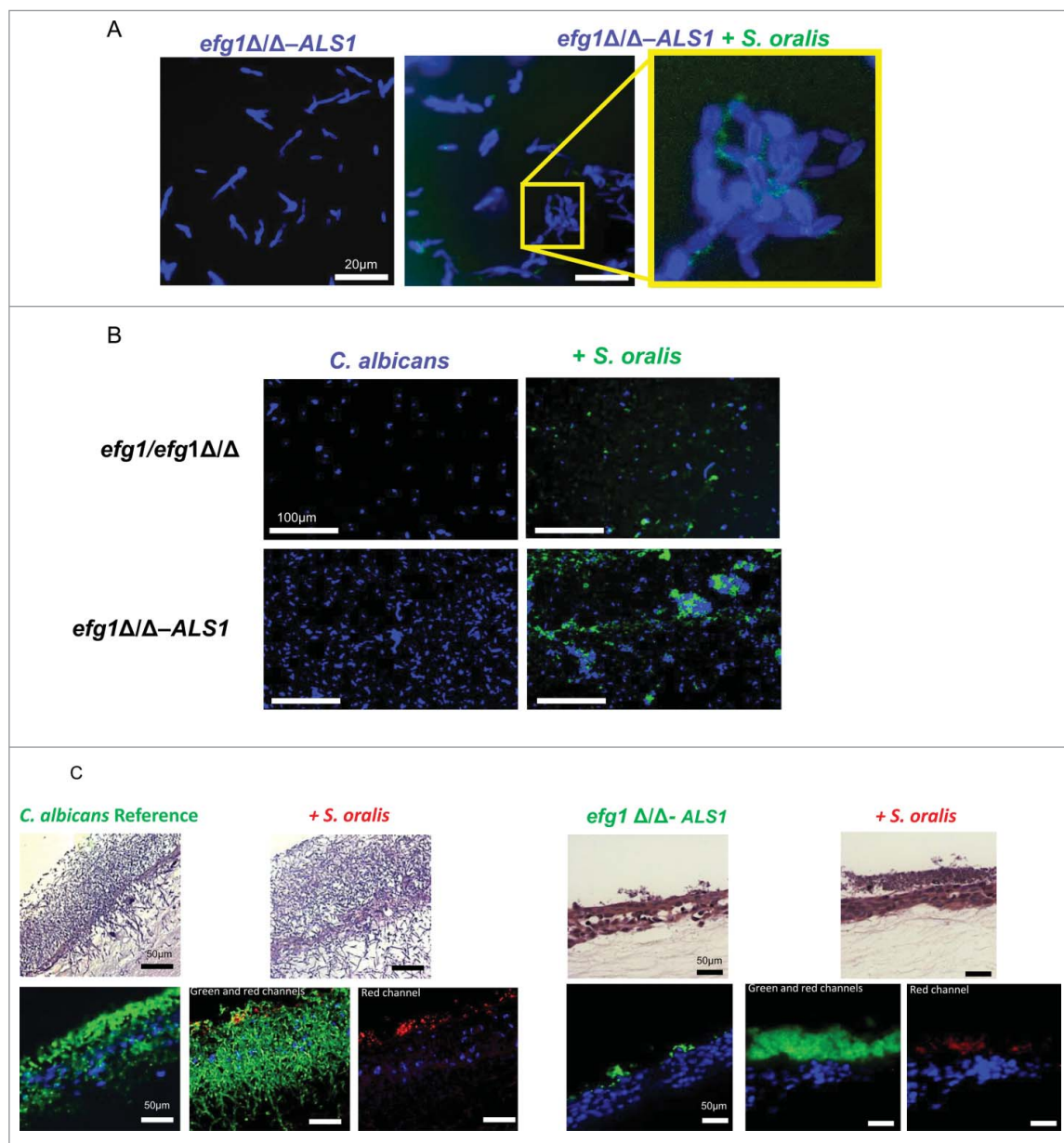
The *ALS1* overexpressing strain in the *efg1Δ/Δ* background forms relatively short pseudohyphae in multiple filamentation-promoting media<sup>20</sup> and co-culture with teal-expressing *S. oralis* on plastic bottom chamber slides did not affect this phenotype (Fig. 8AB). However, abundant fungal-bacterial 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.<sup>3,21,22</sup> 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



**Figure 8.** Overexpression of the *ALS1* gene in the *efg1*Δ/Δ background partially rescues the cross-kingdom mucosal biofilm phenotype (A-B) Co-culture of a *C. albicans* *ALS1*-overexpressing strain in the *efg1*Δ/Δ background (*efg1*Δ/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 *efg1*Δ/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 *efg1*Δ/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 *efg1*Δ/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.

formation.<sup>3,13</sup> Because both *S. oralis* and *C. albicans* are ubiquitous colonizers of the oral cavity in healthy humans,<sup>1,2,11</sup> 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

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*,<sup>23</sup> 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*.<sup>15,24-30</sup> Homozygous deletion of the *EFG1* gene in *C. albicans* strongly attenuates its response to filamentation-inducing stimuli in most liquid and solid media.<sup>30,31</sup> 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.<sup>18,27</sup> 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.<sup>19</sup> 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.<sup>3,12,13</sup>

Late biofilm stage activation of *ALS1* gene transcription in *C. albicans* biofilms has also been observed by others<sup>32</sup> 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,<sup>5</sup> 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.<sup>20,25</sup>

*ALS1* expression is completely dependent on Efg1 under many in vitro conditions<sup>20,31</sup> and Efg1-dependent *ALS1* gene expression has been shown to mediate cell-cell aggregation in *C. albicans*.<sup>33</sup> However, in biofilms growing on abiotic surfaces Als1 was found to be regulated principally by Bcr1.<sup>34</sup> 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 *efg1Δ/Δ* strain does not restore the ability to form hyphae,<sup>20</sup> 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 *rhr2Δ/Δ* mutant. However, unlike the *efg1Δ/Δ* mutant, this mutant is not defective in hyphal formation.<sup>35</sup> 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 *efg1Δ/Δ* 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,<sup>36,37</sup> hyphal adhesins such as Hwp1 and Als3 play a role in co-aggregation interactions with streptococci of the *mitis* group.<sup>38</sup> 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*.<sup>39</sup> 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.<sup>39</sup>

An *efg1* homozygous deletion mutant grows as short pseudohyphae with oral epithelial monolayers.<sup>18</sup> Early work from our group established that hyphal growth is essential in oral epithelial adhesion, injury and induction of pro-inflammatory cytokine responses.<sup>40</sup> This explains

**Table 1.** Organisms used in this study.

Strain	Genotype	Note
SC5314	Blood clinical isolate	51
SN425	<u>ura3Δ::λimm434::URA3-IRO1</u> <u>arg4::hisG::CdARG4</u> <u>his1::hisG</u> <u>leu2::hisG::CdHIS1</u> <u>ura3Δ::λimm434</u> <u>arg4::hisG</u> <u>his1::hisG</u> <u>leu2::hisG::CmLEU2</u>	52
<i>efg1Δ/Δ</i> / CJN2302	<u>ura3Δ::λimm434::URA3-IRO1</u> <u>arg4::hisG</u> <u>his1::hisG</u> <u>leu2::hisG::CdARG4</u> <u>efg1Δ::CmLEU2</u> <u>ura3Δ::λimm434</u> <u>arg4::hisG</u> <u>his1::hisG</u> <u>leu2::hisG</u> <u>efg1Δ::CdHIS1</u>	5
<i>efg1</i> revertant/ CJN 2318	<u>ura3Δ::λimm434::URA3-IRO1</u> <u>arg4::hisG</u> <u>his1::hisG</u> <u>leu2::hisG::EFG1-CdARG4</u> <u>efg1Δ::CmLEU2</u> <u>ura3Δ::λimm434</u> <u>arg4::hisG</u> <u>his1::hisG</u> <u>leu2::hisG</u> <u>efg1Δ::CdHIS1</u>	5
<i>EFG1p</i> -mCherry/ CJN2619	<u>ura3Δ::λimm434::URA3-IRO1</u> <u>arg4::hisG::CdARG4</u> <u>his1::hisG</u> <u>leu2::hisG::CdHIS1</u> <u>EFG1p-mCherry-FRT-FLP-SAT1-FRT</u> <u>ura3Δ::λimm434</u> <u>arg4::hisG</u> <u>his1::hisG</u> <u>leu2::hisG::CmLEU2</u> <u>EFG1</u>	5
<i>ROB1p</i> -mCherry CJN2629	<u>ura3Δ::λimm434::URA3-IRO1</u> <u>arg4::hisG::CdARG4</u> <u>his1::hisG</u> <u>leu2::hisG::CdHIS1</u> <u>ROB1p-mCherry-FRT-FLP-SAT1-FRT</u> <u>ura3Δ::λimm434</u> <u>arg4::hisG</u> <u>his1::hisG</u> <u>leu2::hisG::CmLEU2</u> <u>ROB1</u>	5
<i>BRG1p</i> -mCherry/ CJN2621	<u>ura3Δ::λimm434::URA3-IRO1</u> <u>arg4::hisG::CdARG4</u> <u>his1::hisG</u> <u>leu2::hisG::CdHIS1</u> <u>BRG1p-mCherry-FRT-FLP-SAT1-FRT</u> <u>ura3Δ::λimm434</u> <u>arg4::hisG</u> <u>his1::hisG</u> <u>leu2::hisG::CmLEU2</u> <u>BRG1</u>	5
<i>TEC1p</i> -mCherry/ CJN2616	<u>ura3Δ::λimm434::URA3-IRO1</u> <u>arg4::hisG::CdARG4</u> <u>his1::hisG</u> <u>leu2::hisG::CdHIS1</u> <u>TEC1p-mCherry-FRT-FLP-SAT1-FRT</u> <u>ura3Δ::λimm434</u> <u>arg4::hisG</u> <u>his1::hisG</u> <u>leu2::hisG::CmLEU2</u> <u>TEC1</u>	5
<i>BCR1p</i> -mCherry/ CJN2614	<u>ura3Δ::λimm434::URA3-IRO1</u> <u>arg4::hisG::CdARG4</u> <u>his1::hisG</u> <u>leu2::hisG::CdHIS1</u> <u>BCR1p-mCherry-FRT-FLP-SAT1-FRT</u> <u>ura3Δ::λimm434</u> <u>arg4::hisG</u> <u>his1::hisG</u> <u>leu2::hisG::CmLEU2</u> <u>BCR1</u>	5
<i>NDT80p</i> -mCherry/ CJN2672	<u>ura3Δ::λimm434::URA3-IRO1</u> <u>arg4::hisG::CdARG4</u> <u>his1::hisG</u> <u>leu2::hisG::CdHIS1</u> <u>NDT80p-mCherry-FRT-FLP-SAT1-FRT</u> <u>ura3Δ::λimm434</u> <u>arg4::hisG</u> <u>his1::hisG</u> <u>leu2::hisG::CmLEU2</u> <u>NDT80</u>	5
<i>efg1Δ/Δ</i> - <i>ALSI1</i> / CJN2479	<u>ura3Δ::λimm434::URA3-IRO1</u> <u>arg4::hisG</u> <u>his1::hisG</u> <u>leu2::hisG</u> <u>efg1Δ::CmLEU2</u> <u>ALSI1::AgTEF1p-NAT1-AgTEF1UTR-TDH3p-ALSI1</u> <u>ura3Δ::λimm434</u> <u>arg4::hisG</u> <u>his1::hisG</u> <u>leu2::hisG</u> <u>efg1Δ::CdHIS1</u> <u>ALSI1</u>	5
<i>als1Δ/Δ</i> / CAYC2YF1U	<u>ura3Δ::λimm434::URA3-IRO1</u> <u>als1::hisG</u> <u>ura3Δ::λimm434</u> <u>als1::hisG</u>	34
<i>als3Δ/Δ</i> / CAYF178U	<u>ura3Δ::λimm434::URA3-IRO1</u> <u>als3::ARG4</u> <u>arg4::hisG</u> <u>his1::hisG</u> <u>ura3Δ::λimm434</u> <u>als3::HIS1</u> <u>arg4::hisG</u> <u>his1::hisG</u>	34
<i>S. oralis</i> 34	Oral clinical isolate from healthy mucosa	Dr. A. Rickard
<i>S. oralis</i> 34 teal	<i>S. oralis</i> 34 carrying replicative plasmid expressing mTFP1 (fluorescent Teal Protein)	This work

the attenuated mucosal colonization of the *efg1Δ/Δ* strain in this work and the work of others.<sup>18</sup> 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.<sup>41</sup> 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.<sup>3</sup> 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.<sup>42</sup>

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.<sup>41,43</sup> Efg1 is involved in the regulation of *SAP* 4,5,6 genes in the GI tract,<sup>43</sup> genes that are also upregulated in the *Candida*-streptococcal oral biofilm mouse model,<sup>14</sup> 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<sup>44</sup> they were not required for the increased *C. albicans* tissue invasion in biofilms with *S. oralis*.<sup>14</sup> 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 *ALSI1*, 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 Cat. No. 211059) under aerobic, static conditions, at 37°C, 5% CO<sub>2</sub>, one day before each experiment. A teal 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 TFP1 fluorescent protein.<sup>45</sup> The plasmid was transformed into serum-competent strain 34 cells, plated on Todd Hewitt agar medium and excited colonies were screened for fluorescence intensity on a ChemiDoc MP Imaging System (Bio-Rad). After 2 successive passages in Todd Hewitt broth and selection, plasmid DNA was recovered from one of the brightest colonies and retransformed into parental *S. oralis* 34 cells to create strain *S. oralis* 34 teal.

### 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<sup>®</sup> plastic chamber slides (ThermoFisher, Cat. No. 177437). Each chamber was inoculated with 10<sup>4</sup> cells of *C. albicans* and 10<sup>5</sup> cells of *S. oralis*. For biofilms used for DNA or RNA extraction, 10<sup>5</sup> *C. albicans* and 10<sup>6</sup> *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<sup>-1</sup> type II porcine gastric mucin, 0.5 g L<sup>-1</sup> peptone, 0.5 g L<sup>-1</sup> tryptone, 0.25 g L<sup>-1</sup> yeast extract, 0.088 g L<sup>-1</sup> NaCl, 0.05 g L<sup>-1</sup> KCl, 0.05 g L<sup>-1</sup> CaCl<sub>2</sub>, and 0.25 mg mL<sup>-1</sup> haemin, pH 7.0 supplemented with 2.5 mM DTT and 0.5 g L<sup>-1</sup> sucrose.<sup>15</sup> Biofilms were grown for 4–48 hours at 37°C, in a 5%CO<sub>2</sub> 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.<sup>46</sup> 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 10<sup>6</sup> cells of *C. albicans* or 10<sup>7</sup> 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 × 10<sup>5</sup> 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% CO<sub>2</sub> incubator for 16h. These conditions have been optimized to result in well-organized, mature mucosal biofilms.<sup>7</sup>

### 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.<sup>3,14</sup> 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 × 10<sup>8</sup>/mL] and/or bacteria [2.5 × 10<sup>9</sup>/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<sup>®</sup> 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.<sup>3,14</sup>

### RNA extraction and RT-qPCR

Fungal RNA from abiotic biofilms, mucosal constructs or mouse tongue tissues was extracted as described previously, with minor modifications.<sup>14,47</sup> 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 beat 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 RNeasy 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 CFX96 cyclor 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 AGAACTGATTTGCAGT-GATGG, Als1-R TGAGGATTCATTGCTATCTGG; Hwp1-F TGGTCCAGGTGCTTCTTCTT, Hwp1-R GGTTGCATGAGTGGAAGTGA; Als3-F CCACTTCA-CAATCCCAT, Als3-R CAGCAGTAGTAGTAACAG-TAGTAGTTTCAT; Ndt80-R GGTTGTCTTGCTGGTT GAGGC, Ndt80-F ACACCTCAGCAGCCACATTTG; Efg1-F TATCACGTGAATATTTCCAGGG, Efg1-R CTGACTGTTTCGTTTGATTTGG; Tec1-F GCTCAG-TAGCTTCACAAGTGC, Tec1-R AGTAGGTGGAA-CAAAAGTGCC, Brg1-F GGGTTATTCCACGCTAAA TTG, Brg1-R TATTCTTCGACCGTTCTCCC, Rob1-F AGCCAAAACATGAATACCACG, Rob1-R TTCTTGT GGTGTTGGTTTCGTC Bcr1-F CCCCCAGTATCAAG-CATAACAG, Bcr1-R ATCGTGAAGTTCGATACTTT GG. The *EFB1* translation elongation factor gene was used as an internal control and data were normalized and calculated by  $\Delta\Delta C_q$  method.

#### DNA extraction and *Candida* quantification by qPCR

For quantitative assessment of *C. albicans* hyphae in biofilms growing on abiotic surfaces, qPCR was performed. Fungal DNA was extracted using the Fungi DNA Isolation Kit® (NORGEN Cat. No. E5038), according to the manual. Primers specific for the 18 S rRNA gene of *C. albicans* were used (18S-F GGATT-TACTGAAGACTAACTACTG, 18S-R GAACAA-CAACCGATCCCTAGT), with an annealing

temperature of 59°C. The amplification protocol and genome equivalent calculations were described in detail elsewhere.<sup>48</sup> 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,<sup>49</sup> 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](http://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<sup>23</sup>) / (15 × 10<sup>6</sup> \* 1 × 10<sup>9</sup> \* 650). PCR was performed with a BIO-RAD CFX96 cyclor 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.<sup>3,7,14</sup> To visualize epithelial cell nuclei and *C. albicans* simultaneously, Hoechst 33258 and a FITC-conjugated anti-*Candida* antibody (Meridian Life Science Cat. No. B65411F) were used, respectively. In mucosal biofilms *S. oralis* 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.<sup>19,36,37</sup> 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')<sub>2</sub> 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,<sup>50</sup> and later modified by Hoyer and colleagues,<sup>19</sup> 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 MgCl<sub>2</sub>, and 0.1 mM CaCl<sub>2</sub>. 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<sup>®</sup> plastic chamber slide, hyphae were stained with Calcofluor White<sup>®</sup> 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<sup>®</sup> 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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