Bacterial GtfB Augments *Candida albicans* Accumulation in Cross-Kingdom Biofilms

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Abstract

Streptococcus mutans is a biofilm-forming oral pathogen commonly associated with dental caries. Clinical studies have shown that *S. mutans* is often detected with *Candida albicans* in early childhood caries. Although the *C. albicans* presence has been shown to enhance bacterial accumulation in biofilms, the influence of *S. mutans* on fungal biology in this mixed-species relationship remains largely uncharacterized. Therefore, we aimed to investigate how the presence of *S. mutans* influences *C. albicans* biofilm development and coexistence. Using a newly established haploid biofilm model of *C. albicans*, we found that *S. mutans* augmented haploid *C. albicans* accumulation in mixed-species biofilms. Similarly, diploid *C. albicans* also showed enhanced biofilm formation in the presence of *S. mutans*. Surprisingly, the presence of *S. mutans* restored the biofilm-forming ability of *C. albicans* *bcr1*Δ mutant and *bcr1*Δ/Δ mutant, which is known to be severely defective in biofilm formation when grown as single species. Moreover, *C. albicans* hyphal growth factor *HWPI* as well as *ALS1* and *ALS3*, which are also involved in fungal biofilm formation, were upregulated in the presence of *S. mutans*. Subsequently, we found that *S. mutans*-derived glucosyltransferase B (GtfB) itself can promote *C. albicans* biofilm development. Interestingly, GtfB was able to increase the expression of *HWPI*, *ALS1*, and *ALS3* genes in the *C. albicans* diploid wild-type SC5314 and *bcr1*Δ/Δ, leading to enhanced fungal biofilms. Hence, the present study demonstrates that a bacterial exoenzyme (GtfB) augments the *C. albicans* counterpart in mixed-species biofilms through a *BCR1*-independent mechanism. This novel finding may explain the mutualistic role of *S. mutans* and *C. albicans* in cariogenic biofilms.

Keywords: *Streptococcus mutans*, glucosyltransferase, candidiasis, glucans, dental caries, dental plaque

Introduction

Streptococcus mutans is an important cariogenic bacteria in the oral cavity (Ajdić et al. 2002; Kutsch and Young 2011). *S. mutans* colonize the tooth enamel and form pathogenic biofilms associated with dental caries, particularly in early childhood caries (ECC). Frequent consumption of dietary sugars such as sucrose is a major factor associated with ECC etiology (Hajishengallis et al. 2017). In this context, *S. mutans* becomes an important biofilm modulator capable of effectively converting dietary sucrose into acids and producing extracellular glucans using exoenzymes termed *glucosyltransferases* (Gtfs) (Bowen and Koo 2011). Specifically, insoluble glucans produced by glucosyltransferase B (GtfB) provide bacterial binding sites and form the core of the extracellular matrix of cariogenic dental plaque biofilm in vivo (Mattos-Graner et al. 2000; Bowen and Koo 2011).

However, *S. mutans* is not the sole agent associated with ECC as other organisms can contribute to its pathogenesis (Hajishengallis et al. 2017). Interestingly, *Candida albicans* is the most frequently isolated fungal organism in cariogenic plaque-biofilm associated with ECC (de Carvalho et al. 2006; Raja et al. 2010; Yang et al. 2012; Qiu et al. 2015). It has been demonstrated that *C. albicans* presence can enhance *S. mutans* growth, accumulation, and biofilm formation both in vitro and in vivo (de Carvalho et al. 2006; Pereira-Cenci et al. 2008;

Raja et al. 2010; Klinke et al. 2011; Metwalli et al. 2013; Falsetta et al. 2014). Yet, the role of *S. mutans* on *C. albicans* accumulation in this interkingdom interaction remains unclear. Biofilm formation is a major virulence attribute of *C. albicans* (Naglik et al. 2008; Dongari-Bagtzoglou et al. 2009). Expression of agglutinin-like sequences (*ALS*) and hyphal-wall protein (*HWP*) genes is important for biofilm formation (Nailis et al. 2006; Zhu and Filler 2010; Alves et al. 2014) and under the regulation of biofilm and cell-wall regulator (*bcr1*), a

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critical zinc-finger transcription factor that is essential for *C. albicans* biofilm development (Nobile et al. 2006).

Previously, we demonstrated that *S. mutans*-derived GtfB has high affinity and binding strength to the *C. albicans* cell surface (Gregoire et al. 2011; Hwang et al. 2015). The GtfB bound on the *C. albicans* surface produces glucans, which enhance the ability to harbor more viable *S. mutans* cells than single-species biofilms and accrue more biomass during mixed-species biofilm formation (Gregoire et al. 2011; Falsetta et al. 2014; Koo and Bowen 2014). The properties of *S. mutans* in mixed-species biofilms with *C. albicans* and the influences of fungal presence on bacterial biology have been explored. In contrast, understanding the role of *S. mutans* on the *C. albicans* counterpart remains incomplete. Taking this research gap into consideration, the present study aimed to characterize the effect of *S. mutans* on *C. albicans* accumulation in mixed-species biofilm. For this purpose, we took advantage of the recently developed haploid model of *C. albicans* biofilms (Seneviratne et al. 2015). The diploid genetic nature of *C. albicans* makes targeted gene deletion challenging (Noble and Johnson 2007). Discovery of the haploid nature of *C. albicans* opened a new avenue for simpler genetic manipulation where 1-step targeted gene mutations can be performed for constructing mutant libraries (Zeng et al. 2014). We screened a haploid *C. albicans* mutant library and found that *bcr1* mutant (known to defective in single-species biofilms) formed comparable biofilms to its parent strain in the presence of *S. mutans*. These findings were confirmed using diploid *C. albicans* strains (predominant form in nature), and further experiments demonstrated that GtfB from *S. mutans* plays a key role in the fungal accumulation within mixed-species biofilm. Altogether, it appears that *S. mutans*-derived GtfB augments the ability of *C. albicans* to form biofilms through a *bcr1*-independent mechanism, providing further insights into this unique and virulent bacterial-fungal association.

Materials and Methods

Microbial Strains and Culture Conditions

The haploid and diploid *C. albicans* strains used in the study are described in Appendix Table 1. *S. mutans* UA159 strain was taken from the archival collection of the oral sciences laboratory (National University of Singapore). *C. albicans* and *S. mutans* cells were grown to mid-exponential phase in ultra-filtered tryptone yeast extract medium (UFTYE; pH 5.5 and 7.0 for *C. albicans* and *S. mutans*, respectively) containing 1% (w/v) glucose and harvested by centrifugation (6,000 g, 10 min, 4°C) as described previously (Gregoire et al. 2011). The cells were then washed in phosphate-buffered saline (PBS) prior to being used for biofilm formation.

In Vitro Biofilm Development

For the *C. albicans* single-species biofilm formation, approximately 1×10^6 colony-forming units (CFU)/mL yeast cells were inoculated in UFTYE broth containing 1% (w/v) sucrose.

For mixed-species biofilm formation, approximately 1×10^6 CFU/mL of *C. albicans* yeast cells and 2×10^6 CFU/mL of *S. mutans* cells were diluted in UFTYE broth containing 1% (w/v) sucrose. The biofilms were formed in presterilized flat-bottomed 96-well microtiter plates (Greiner Bio-One) at 37°C for 24 h under aerobic conditions.

Biofilm Quantification

Three different methods were used for the biofilm quantification:

1. *XTT reduction assay*: The XTT assay was performed according to a previously optimized protocol (Seneviratne et al. 2008). The colorimetric changes of XTT were measured spectrophotometrically at 490 nm (Multiskan GO; Thermo Fisher Scientific).
2. *Crystal violet assay*: Biofilms were quantified using crystal violet (CV) assay (O'Toole 2011). Briefly, the 2% formalin fixed biofilms were stained with 1% (w/v) CV and subsequently filled with 95% ethanol. The optical density of 95% ethanol was measured at 570 nm (Multiskan GO; Thermo Fisher Scientific). XTT or CV assay provides total biomass without distinguishing *C. albicans* from *S. mutans* in mixed-species biofilms. CFU counting was performed to enumerate each species despite limitations due to the *C. albicans* yeast-hyphae transition.
3. *CFU counting method*: A dilution series of the cell suspensions from biofilms was prepared and spread plated on glucose minimal medium agar plates supplemented with suitable amino acids and 8 µg/mL gentamicin sulfate salt (Sigma-Aldrich) to prevent bacterial growth. The plates were incubated at 30°C for 48 h and the fungal colonies were counted.

Growth Kinetics of *C. albicans* Strains

Cell concentrations of 1×10^6 CFU/mL of *C. albicans* strains were diluted in 200 µL UFTYE broth containing 1% (w/v) sucrose in 96-well microtiter plates (Greiner Bio-One). The optical density of the microbial cultures was measured at a wavelength of 600 nm (Multiskan GO; Thermo Fisher Scientific) at 30-min intervals up to 72 h at 37°C under aerobic conditions.

Confocal Laser Scanning Microscopy

Single-species and mixed-species biofilms were developed on Thermanox (Nunc) 8-well plates. Biofilms were fixed with 4% (v/v) paraformaldehyde for 24 h at 4°C and stained using propidium iodide (Invitrogen) and 0.001% (w/v) calcofluor white (Sigma-Aldrich) with modifications to a previous protocol (Cavalcanti et al. 2016). Propidium iodide (excitation/emission 535/617 nm) stained the bacteria while calcofluor white (excitation/emission 365/435 nm) stained the *C. albicans*. The biofilms were observed using an Olympus-Fluoview FV1000 TIRF confocal microscope. Z sections were collected from 4 different fields of 3 biological samples, and biofilm

biovolumes were determined using the Imaris software. Glucan formation by GtfB was visualized using Alexa Fluor 647-labeled dextran conjugate (10,000 molecular weight; absorbance/fluorescence emission maxima, 647/668 nm; Molecular Probes) as detailed previously (Klein et al. 2009).

Analysis of *C. albicans* Adhesins by Quantitative Reverse Transcription Polymerase Chain Reaction

Cells recovered from biofilms were centrifuged (10,000 *g* for 10 min) and cell pellets were used for RNA extraction using TRIzol (Thermo Fisher Scientific) reagent according to an established method (Rio et al. 2010). The integrity and purity of RNA were spectrophotometrically determined by the absorbance ratio at 260/280 nm (NanoDrop ND-1000; Thermo Fisher Scientific). Reverse transcription of RNA was performed using the M-MLV Reverse Transcriptase system (Promega). Primers used for quantitative polymerase chain reaction (qPCR) analysis are presented in Appendix Table 2. Amplification and quantification of target RNA by quantitative polymerase chain reaction were performed in MicroAmp optical 96-well plates (Applied Biosystems) using a KAPA SYBR FAST qPCR Kit (Kapa Biosystems) as described previously (Truong et al. 2016). The expression of targeted genes was normalized according to the housekeeping reference gene *PMAl* (Chau et al. 2004). Each *C. albicans* strain forming single-species biofilms was used as the reference group for gene expression analysis. Analysis of relative gene expression was achieved according to the $\Delta\Delta Ct$ method (Schmittgen and Livak 2008).

Statistical Analysis

Each error bar indicates mean $\pm$ standard deviation (SD) calculated from 3 biological replicates and 4 technical replicates. For any pairwise comparison, Student's *t* test or Mann-Whitney *U* test was performed. Kruskal-Wallis test was used for multiple comparisons. $P < 0.05$ was considered statistically significant (*).

Results

S. mutans Augments the Accumulation of Haploid and Diploid *C. albicans* in Mixed-Species Biofilms

The XTT reduction assay reading represents total biomass, including both *S. mutans* (Sm) and *C. albicans* (Ca). The XTT

Figure 1. *Streptococcus mutans* augmented the biofilm formation of haploid and diploid *Candida albicans* strains in *S. mutans*–*C. albicans* mixed-species biofilms. Biofilm formation was evaluated using (A) XTT reduction assay, (B) Crystal violet assay, and (C) colony-forming unit (CFU) counting. The presence of *S. mutans* UA159 rescued the inability of haploid *bcr1Δ* and diploid *bcr1Δ/Δ* mutants to form biofilms, showing comparable biofilm-forming ability to their respective haploid and diploid parent strains, GZY803 and SC5314. (D) Growth kinetics demonstrated that biofilm formation is not dependent on growth as *bcr1Δ* and diploid *bcr1Δ/Δ* mutants have a similar growth rate to their parent strains, GZY803 and SC5314.

assay showed an increase in the total biofilm-biomass of the mixed-species biofilm in comparison to the respective single-species biofilms of *C. albicans* (Fig. 1A; $P < 0.05$). Similar to the XTT assay, the CV assay also showed a similar pattern in the biofilm formation (Fig. 1B; $P < 0.05$). On the other hand, the haploid *bcr1Δ* and the diploid *bcr1Δ/Δ* mutants formed comparable biofilms to that of parent strains in Sm-Ca mixed-species biofilm conditions (Fig. 1A, B).

CFU counting method demonstrated that GZY803 and SC5314 had significantly higher *C. albicans* cells compared to the *bcr1Δ* and *bcr1Δ/Δ* mutants in single-species biofilms (Fig. 1C). There was no significant difference in the growth kinetics of the *C. albicans* *bcr1Δ* and *bcr1Δ/Δ* mutant strains with their respective parent strains (Fig. 1D). Therefore, the difference in biofilm formation between *C. albicans* parent and *bcr1* mutants was not due to the growth kinetics. Rather, the presence of *S. mutans* significantly augmented the accumulation of the *C. albicans* counterpart in Sm-Ca mixed-species biofilm (Fig. 1C). Surprisingly, *C. albicans* *bcr1Δ* and *bcr1Δ/Δ* mutants, which were defective in single-species biofilm formation, showed an increase in biomass (Fig. 1A, B) and viable cell numbers in Sm-Ca mixed-species biofilms (Fig. 1C; $P < 0.05$). CFU counting showed that *S. mutans* cells in the Sm-Ca mixed-species biofilm were significantly higher compared to the *S. mutans* single-species biofilm (Appendix Fig. 3; $P < 0.05$). There was no statistical significance between the number of *S. mutans* cells in the Sm-*bcr1Δ/Δ* and Sm-SC5314 mixed-species biofilms.

Figure 2. Microscopic comparison of haploid and diploid *Candida albicans* biofilms in the presence and absence of *Streptococcus mutans*. *C. albicans* in the single- and mixed-species biofilms were fixed and stained with calcofluor white (in blue), and the *S. mutans* in mixed-species biofilms were stained with propidium iodide (in red) for confocal laser scanning microscopy (CLSM) visualization (images I–IV and IX–XII). Images V–VIII and XIII–XVI represent only the *C. albicans* cells in the single- or mixed-species biofilms. **(A)** The presence of *S. mutans* notably enhanced the biofilm formation of *bcr1Δ* (IV and VIII) and *bcr1Δ/Δ* (XII and XVI) mutants in comparison to the absence of *S. mutans* (II, VI and X, XIV). Haploid and diploid parent strains, GZY803 (III and VII) and SC5314 (XI and XV), also showed a slight increase in biofilm formation in the presence of *S. mutans*. **(B)** The biovolumes of *C. albicans* single- and mixed-species biofilms were compared using the Imaris software.

Microscopic Comparison of Haploid and Diploid *C. albicans* Biofilms in the Presence of *S. mutans*

Confocal laser scanning microscopy (CLSM) imaging corroborated the foregoing findings, providing evidence on the increased biofilm formation of *C. albicans* in the presence of *S. mutans* (Fig. 2A). *C. albicans* *bcr1Δ* (Fig. 2A.II, A.VI) and *bcr1Δ/Δ* (Fig. 2A.X, A.XIV) formed sparse biofilms compared to the haploid-parent GZY803 (Fig. 2A.I, A.V) and diploid-parent SC5314 (Fig. 2A.IX, A.XIII), respectively. Consistent with colorimetric and microbiological data, the presence of *S. mutans* enhanced the biofilm formation of *C. albicans* haploid-parent (Fig. 2A.III, A.VII) and haploid-*bcr1Δ* (Fig. 2A.IV, A.VIII) as well as diploid-parent (Fig. 2A.XI, A.XV) and diploid-*bcr1Δ/Δ* (Fig. 2A.XII, A.XVI). It was noteworthy that there was a drastic increase in the biofilm formation of *bcr1Δ* (Fig. 2A.IV, A.VIII) and *bcr1Δ/Δ* (Fig. 2A.XII, A.XVI) in the presence of *S. mutans* compared to single-species biofilms of *bcr1Δ* (Fig. 2A.II, A.VI) and *bcr1Δ/Δ* (Fig. 2A.X, A.XIV). The visual observations were supported by the quantitative analysis of biovolumes of *C. albicans* cells using the Imaris software in both single- and Sm-Ca mixed-species biofilms (Fig. 2B; $P < 0.05$). Taken together, *S. mutans* appears to augment the biofilm formation of haploid and diploid *C. albicans* in Sm-Ca mixed-species biofilms, including *bcr1Δ* and *bcr1Δ/Δ* mutants, suggesting that the enhancement of the *C. albicans* counterpart is independent of *BCR1*.

S. mutans Enhances the Expression of *C. albicans* *HWP1*, *ALS1*, and *ALS3* in Mixed-Species Biofilms

qPCR analysis showed that the presence of *S. mutans* enhanced the expression of *C. albicans* *HWP1* (Fig. 3A), *ALS1* (Fig. 3B), and *ALS3* (Fig. 3C) genes in Sm-Ca mixed-species biofilms compared to the single-species biofilms. In Sm-Ca mixed-species biofilms, *HWP1* expression was significantly increased in both haploid *bcr1Δ* and diploid *bcr1Δ/Δ* mutants as well as in their respective parent strains, GZY803 and SC5314 ($P < 0.05$). *ALS1* and *ALS3* expressions were significantly increased in both haploid-parent strain GZY803 and diploid-parent strain SC5314 and in the *bcr1Δ/Δ* mutant ($P < 0.05$). However, there was no significant increase in *ALS1* and *ALS3* expression in the *bcr1Δ* mutant in the presence of *S. mutans*.

GtfB Enhances *C. albicans* Biofilm Formation

From the above observations, *S. mutans* appeared to produce an “effector molecule” that promoted *C. albicans* accumulation in mixed-species biofilms. Previous studies have demonstrated that *S. mutans*-derived GtfB is a key mediator associated with *C. albicans* and development of mixed-species biofilm (Gregoire et al. 2011; Falsetta et al. 2014). Gtfs can use sucrose to synthesize glucans, which are the main components of extracellular matrix in cariogenic biofilms (Kuramitsu 1993). Therefore, we examined the formation of glucans in biofilms using an established fluorescence-labeling method detailed previously (Xiao et al. 2012) (Appendix Fig. 1). As expected, Sm-Ca biofilms showed the presence of abundant glucans (Appendix Fig. 1A.I–A.IV), whereas the polysaccharides were absent in all *C. albicans* single-species biofilms (Appendix Fig. 1B.I–B.IV). Hence, we postulated that GtfB produced by *S. mutans* can influence the ability of *C. albicans* to form biofilms. We used purified, cell-free GtfB enzyme preparation in sterile buffer as described previously (Koo et al. 2002). *C. albicans* single-species biofilms were formed in the presence of 5 μg/mL of enzymatically active GtfB. This GtfB amount was selected following a dose-dependent study (Appendix Fig. 2). Interestingly, GtfB enhanced the biofilm formation by *C. albicans* parental strain as well as *bcr1Δ/Δ* mutant (Fig. 4A–C; $P < 0.05$) and their haploid counterparts (Appendix Fig. 5). Notably, extracellular glucans (in red) were detected in *C. albicans* biofilms formed in the presence of GtfB, as shown by confocal imaging (Fig. 4D). In contrast, *S. mutans* strain defective in *gtfB* expression is incapable of

enhancing *C. albicans* biofilm formation (Appendix Fig. 6). Previous studies have shown that GtfB can bind to both yeast and hyphal cells of *C. albicans* (Gregoire et al. 2011; Hwang et al. 2015). Therefore, it is possible that GtfB bound to *C. albicans* cells may modulate the enhanced fungal biofilm formation when grown together with *S. mutans*. To further confirm this modulating effect of GtfB on *C. albicans* biofilm formation, an *ira2Δ/Δ* mutant previously shown to be defective in biofilm formation (Seneviratne et al. 2015) was used for similar experiments (Appendix Fig. 4). GtfB produced by *S. mutans* was able to stimulate the biofilm formation of the *ira2Δ/Δ* mutant, confirming our observation on *bcr1Δ/Δ*.

GtfB Increases the Expression of HWPI, ALSI, and ALS3 in *C. albicans* Biofilms

We postulated that GtfB is responsible for the increased expression of *HWPI*, *ALSI*, and *ALS3* in *C. albicans* biofilms when grown as mixed-species biofilms. Parent *C. albicans* strain SC5314 and the *bcr1Δ/Δ* biofilms in the presence of GtfB exhibited a significant increase in the expressions of the *HWPI* (Fig. 5A), *ALSI* (Fig. 5B), and *ALS3* (Fig. 5C) genes in the presence of GtfB ($P < 0.05$); similar gene expression enhancement was observed using the equivalent haploid *C. albicans* strains (Appendix Fig. 7). The ability of GtfB to enhance the expression of the aforementioned genes in biofilms of *C. albicans* SC5314 parent isolate as well as *bcr1Δ/Δ* mutant shows that the expression happens through a *BCR1*-independent mechanism.

Discussion

Previously, we and others demonstrated that the presence of *C. albicans* promotes *S. mutans* cells accretion and GtfB production in mixed-species biofilm grown in sucrose (Pereira-Cenci et al. 2008; Falsetta et al. 2014). Here, we found that *S. mutans* also augments *C. albicans* accumulation in mixed-species biofilms, providing

Figure 3. *Streptococcus mutans* increase relative gene expression of *Candida albicans* hyphal wall protein (*HWPI*) and adhesins (*ALSI*, *ALS3*). Quantitative reverse transcription polymerase chain reaction analysis was performed for *C. albicans* genes (A) *HWPI*, (B) *ALSI*, and (C) *ALS3*. Target genes were normalized with the *C. albicans* housekeeping gene *PMA1* using the $\Delta\Delta Ct$ method. Single-species biofilms of each *C. albicans* strain were used as reference biofilm to compare the relative expression of above genes in the respective *C. albicans* strain in the *C. albicans*–*S. mutans* mixed-species biofilm. The fold change of the gene of interest for each strain forming mixed-species biofilms of the same strain. Bars show the average and the standard deviation (SD) within each group. The presence of *S. mutans* UA159 helped haploid *bcr1Δ* and diploid *bcr1Δ/Δ* mutants and their respective parent strain GZY803 and diploid SC5314 to increase the expression of (A) *HWPI*, (B) *ALSI*, and (C) *ALS3* genes, which are under the direct regulation of *bcr1* transcription factor.

Figure 4. Glucosyltransferase B (GtfB) increases biofilm formation in *Candida albicans*. The biofilm formation in the presence of GtfB was evaluated using (A) visual observation, (B) XTT reduction assay, (C) colony-forming unit (CFU) counting, and (D) confocal microscopy. Confocal laser scanning microscopy (CLSM) images show *C. albicans* cells stained with Calcofluor white (in blue) and GtfB-derived glucans labeled with Alexa Fluor 647-labeled dextran conjugate (in red). Adding cell-free, purified GtfB significantly increased biofilm formation in *C. albicans*. This figure is available in color online.

Figure 5. Glucosyltransferase B (GtfB) increases relative gene expression of *Candida albicans* hyphal wall protein (*HWP1*) and adhesins (*ALS1*, *ALS3*). Quantitative reverse transcription polymerase chain reaction analysis was performed to evaluate the relative expression of *C. albicans* (A) *HWP1*, (B) *ALS1*, and (C) *ALS3* genes using the $\Delta\Delta C_t$ method. Target genes were normalized using the *C. albicans* housekeeping gene (*PMA1*). Single-species biofilms of each *C. albicans* strain developed without the supplementation of GtfB were used as the reference biofilm to compare the expression of the above genes in *C. albicans* strains forming biofilms in the presence of GtfB. Bars show the average and the standard deviation (SD) within each group. Cell-free, purified GtfB increased the expression of (A) *HWP1*, (B) *ALS1*, and (C) *ALS3* genes in haploid *bcr1Δ/Δ* and diploid *bcr1Δ/Δ* mutants and their respective parent strains GZY803 and diploid SC5314.

further evidence of a synergistic interaction between the 2 organisms. Strikingly, we also observed that *C. albicans* strains lacking the *bcr1* transcription factor were capable of forming enhanced biofilm in the presence of *S. mutans* or by GtfB itself. This observation suggests that GtfB secreted by *S. mutans* is able to restore the biofilm-defective phenotype of *C. albicans*. These findings support the clinical and in vivo observations that *C. albicans* and *S. mutans* develop a symbiotic relationship allowing coexistence and mixed-biofilm formation on tooth surfaces, an atypical habitat for *C. albicans* (de Carvalho et al. 2006; Raja et al. 2010; Yang et al. 2012; Falsetta et al. 2014).

BCR1 is a key regulator for *C. albicans* biofilm development, and *bcr1Δ/Δ* mutant is biofilm defective (Nobile and Mitchell 2005; Nobile et al. 2006). Major functional downstream targets of *bcr1* include *HWP1*, *ALS1*, and *ALS3* genes that encode cell surface proteins. Overexpression of these genes in *C. albicans* *bcr1Δ/Δ* mutant restored the biofilm formation (Nobile et al. 2006; Nobile et al. 2012). Interestingly, *C. albicans* *bcr1Δ* and *bcr1Δ/Δ* mutants were able to form comparable biofilms to their respective parent strains GZY803 and SC5314 in the presence of *S. mutans*. Hence, it appears that the presence of *S. mutans* enables *C. albicans* to bypass the *BCR1*-dependent biofilm defect during the formation of Sm-Ca mixed-species biofilms. Moreover, *S. mutans* also increased the *HWP1*, *ALS1*, and *ALS3* expressions of *C. albicans*. These findings suggest that *S. mutans* increases the expression of the

above genes in a *BCR1*-independent mechanism, which may partly explain the comparable biofilm formation of *BCR1* mutants in Sm-Ca mixed-species biofilms. A recent study is in agreement with our findings where *S. mutans* and *Streptococcus sanguinis* together were able to increase the *ALS3* and *HWP1* gene expressions in *C. albicans* during mixed-species biofilm formation (Cavalcanti et al. 2016).

Notably, GtfB by itself increased *C. albicans* biofilm formation. Introducing GtfB allowed *C. albicans* diploid *bcr1Δ/Δ* mutant to form comparable biofilms to wild-type SC5314. *IRA2* is another hyphal-independent, biofilm-specific regulator upstream to *BCR1* in *C. albicans* (Seneviratne et al. 2015). Interestingly, observations on *ira2Δ/Δ* mutant in the present study further confirmed the augmented effect of GtfB on *C. albicans* biofilm formation. Moreover, addition of GtfB increased the expression of *HWP1*, *ALS1*, and *ALS3* in *C. albicans* SC5314 as well as *bcr1Δ/Δ* mutant. These findings provide supportive evidence that *S. mutans*-derived GtfB may be the reason for enhanced *C. albicans* accumulation in Sm-Ca

mixed-species biofilms. Previous studies have demonstrated that GtfB can bind to both *S. mutans* and *C. albicans* (Vacca-Smith and Bowen 1998; Hwang et al. 2015) but with different avidity. Intriguingly, the binding strength and binding stability of GtfB to the *C. albicans* surface were several folds higher compared with the enzyme adhesion to *S. mutans* (Hwang et al. 2015). Hence, there may be a highly specific interaction between *S. mutans* GtfB and the cell-wall component in *C. albicans*, as suggested previously (Gregoire et al. 2011; Hwang et al. 2015), that may also induce the expression of *HWP1*, *ALS1*, and *ALS3* genes. However, future studies are required to identify the binding sites of GtfB and the downstream regulation of genes activated upon binding. We are generating targeted gene mutations using the genetic flexibility of the haploid system, which will be examined in the haploid *C. albicans*-*S. mutans* mixed-species biofilm model established here. Inclusion of mutants affecting the *bcr1* pathway and GtfB-defective *S. mutans* strains as well as nonmutant streptococci may help to further elucidate the mechanisms of this bacterial-fungal interaction.

These findings shed new light on the previously observed *C. albicans* and *S. mutans* synergism in biofilms associated with early childhood caries. Furthermore, the presence of *C. albicans* on the tooth surface may become a fungal reservoir for mucosal fungal infections and systemic mycoses in compromised host populations. Hence, targeting GtfB may be a promising strategy to counter the formation of pathogenic bacterial-fungal biofilms.

Author Contributions

K. Ellepola, contributed to conception, design, data acquisition, analysis, and interpretation, drafted the manuscript; Y. Liu, contributed to data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; T. Cao, contributed to data interpretation, drafted the manuscript; H. Koo, C.J. Seneviratne, contributed to conception, design, data analysis, and interpretation, drafted the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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