Candida albicans-derived Metabolites Promote *Streptococcus mutans* Accumulation in Cross-kingdom Biofilms

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INTRODUCTION

Early childhood caries (ECC) is a highly prevalent and difficult to treat biofilm-dependent oral disease, afflicting mostly underprivileged children worldwide, resulting in annual expenditures of >$120 billion in the US alone (Dye et al. 2015; Kassebaum et al. 2015). Children affected with ECC display heavy infection with *Streptococcus mutans* (a cariogenic pathogen) accompanied by protracted feeding of dietary sugars, such as sucrose (Palmer et al. 2010), leading to rapid accumulation of virulent biofilms that cause rampant destruction of the teeth (Hajishengallis et al. 2017). These caries-causing biofilms are characterized by densely packed cell clusters (or microcolonies) that are firmly adherent and enmeshed in an extracellular matrix rich in exopolysaccharides (EPS) (Bowen & Koo 2011).

Intriguingly, *Candida albicans* (an opportunistic fungus) is frequently detected with heavy infection of *S. mutans* in plaque-biofilms from children affected by ECC. Previous studies have found that the presence of *C. albicans* enhances *S. mutans* carriage and microcolony development within biofilms, while drastically increasing the production of an EPS-rich matrix (Metwalli et al. 2013; Falsetta et al. 2014). Once together, the presence of *C. albicans* appears to activate *S. mutans* Gtfs, particularly GtfB, which is the primary exoenzyme associated with EPS production in cariogenic biofilms. However, how *C. albicans* stimulate *S. mutans* accumulation and microcolony development remain unclear. We hypothesized that chemical interactions via secreted microbial molecules from this cross-kingdom association modulate Gtf activation and *S. mutans* accumulation within biofilms. Using a novel nanoculture system and a rodent *in*

vivo biofilm model, we characterized the chemical interactors (e.g. farnesol) that enhance *S. mutans gtfB* expression and determined the role of GtfB on *S. mutans-C. albicans* plaque biofilm formation.

MATERIALS AND METHODS

Preparation of biofilm-derived conditioned medium (CM) and treatment using in vitro biofilm model. Biofilms were formed using our saliva-coated hydroxyapatite (sHA) disc model as detailed previously (Falsetta et al. 2014). The cell-free conditioned medium from single-species bacterial (**B-CM**) or fungal (**F-CM**) and bacterial-fungal (**BF-CM**) biofilms were collected at 18 h (time-point selected based on biofilm metabolic activity and microbial growth). Each sHA disc was inoculated with 2×10^6 (CFU/mL) of *S. mutans* in 2.8 mL of ultrafiltered tryptone-yeast extract broth (UFTYE) containing 1% (w/v) sucrose with or without B/F/BF-CM supplementation, and the biofilm formed at 37°C under 5% CO_2.

Quantitative chemical profiling of CM and biofilm analyses. The carbohydrate composition of CM was analyzed using high-performance anion-exchange chromatography (HPAEC), while biofilm-derived metabolites were identified and quantified through ^{1}H nuclear magnetic resonance (^{1}H-NMR). Farnesol was detected and quantified on thin-layer chromatography and high performance liquid chromatography (HPLC). The bioactivity of CM on *S. mutans*-biofilms was determined by biochemical/microbiological methods combined with RT-qPCR and high-resolution confocal microscopy (Xiao et al. 2012).

A microfluidics-based nanoculture system. To examine microcolony growth and *in situ gtfB* expression simultaneously, a P_{gtfB}::*gfp S. mutans* strain was encapsulated in polydimethylsiloxane (PDMS)-based nanoculture system (Niepa et al. 2016). Using a microfluidics fabrication method, defined cell population of *S. mutans* (~30 cells) was directly inoculated and encapsulated within a semi-permeable physical shell surrounding the bacterial culture. The nanoculture was then placed in UFTYE with or without B/F/BF-CM supplementation, and analyzed via quantitative fluorescence imaging.

In vivo rodent model. Animal experiments were performed using an established rodent bacterial-fungal biofilm model (Falsetta et al. 2014). *S. mutans* Δ*gtfB*::kan and *C. albicans* SC5314 were used to assess the role of GtfB on cross-kingdom biofilm formation on teeth.

Statistical analysis. The quantitative data were subjected to analysis of variance (ANOVA) in the Tukey's HSD test for a multiple comparison. A pairwise comparison was conducted using student's *t*-test. Differences are considered significant with P values <0.01.

RESULTS

Our data reveal that conditioned medium from bacterial-fungal biofilm (BF-CM) significantly enhances *S. mutans* accumulation within biofilms compared to control (Fig. 1A). In contrast, no significant increase in the bacterial cell population was detected when *S. mutans* was grown in CM preparations from single-species biofilms. Using serial dilutions of BF-CM, we observed a clear trend of increasing number of viable *S. mutans*-biofilm cells (>6-fold increase at highest BF-CM content vs. no supplementation, $^{**}P<0.01$) in a dose-dependent manner (Fig. 1A), suggesting that BF-CM contains bacterial growth-inducing factors. Furthermore, confocal imaging show significant alteration in the biofilm three-dimensional (3D) architecture, resulting in enlarged bacterial cell-clusters (microcolonies; depicted in green in Fig. 1B) (~2.5-fold increase in microcolony volume vs. control, $^{**}P<0.01$) (Fig. 1B).

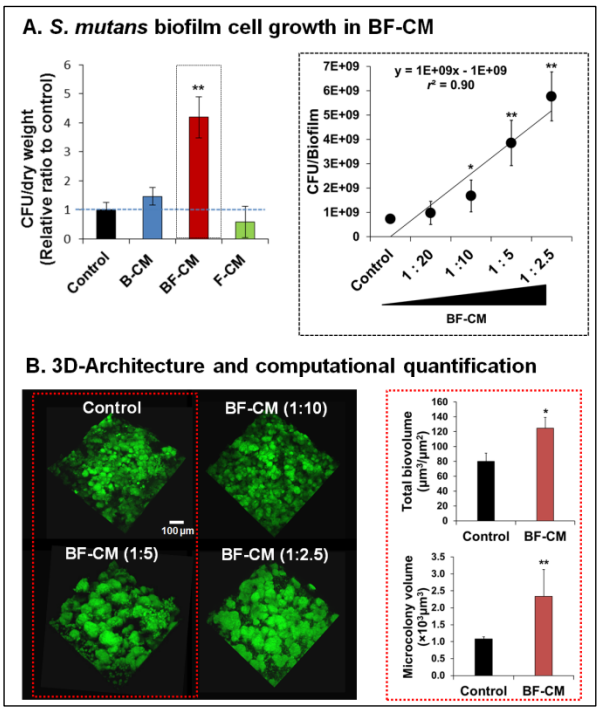

Figure 1. Influences of conditioned medium on the growth of *S. mutans* biofilm cells and microcolony development.

To further demonstrate the biological properties of BF-CM, a newly developed nanoliter-scale culturing system was used, which allows microbial growth within spatially confined yet permeable

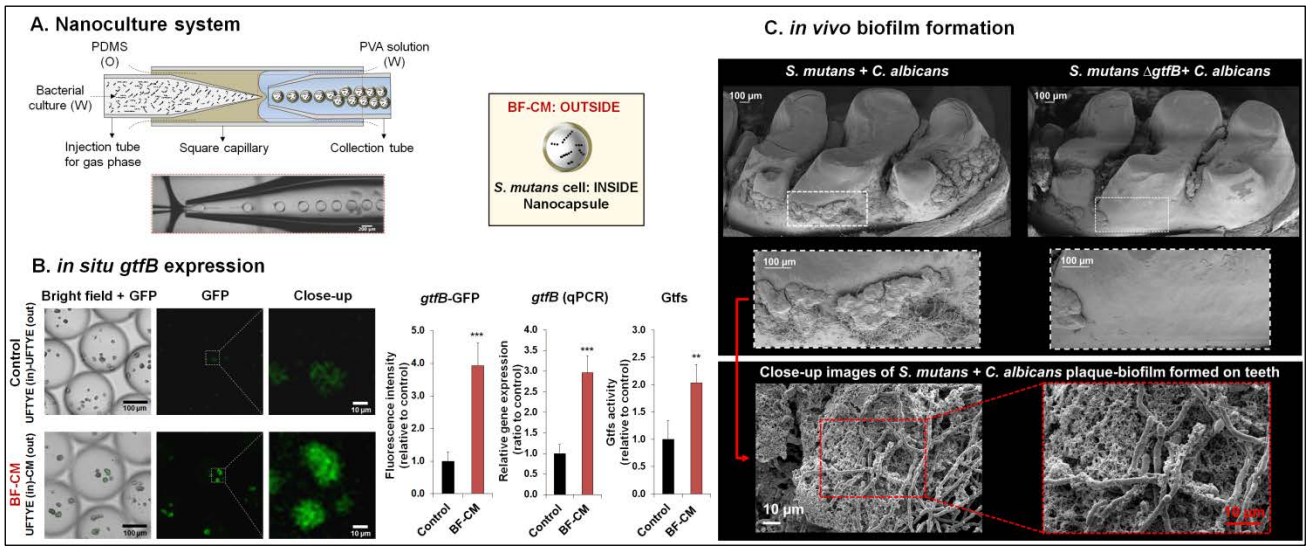

Figure 2. Microcolony assembly and *gtfB* expression *in situ* using a microfluidics-generated nanoculture system and *in vivo S. mutans-C. albcans* plaque biofilm formation using *gtfB* defective *S. mutans.*

microcapsules, mimicking biofilm microenvironment (Niepa et al. 2016). Since this analytical platform allows chemical fluxes between internal and external environments, it was determined whether *S. mutans* cells grown within the nanoculture respond to bioactive molecules in the BF-CM placed outside of the microcapsules (Fig. 2A). Using a *S. mutans* P_{gtfB}::*gfp* strain, we demonstrate that BF-CM stimulates bacterial cells to develop into microcolonies while inducing *gtfB* expression *in situ* (Fig. 2B). The observed effect of BF-CM on *gtfB* expression was also confirmed via qPCR (~3-fold increase vs. control, Fig. 2B), which also resulted in increased Gtf activity (~2-fold increase vs. control, Fig. 2B). Thus, we have further investigated the importance of GtfB in fungal-bacterial biofilm accumulation on teeth using a rodent model. We made a striking observation that GtfB is essential for the plaque-biofilm formation *in vivo* (Fig. 2C).

Having shown enhancement of *gtfB* expression *in vitro* by BF-CM and the key role of *S. mutans* GtfB for bacterial-fungal biofilm formation *in vivo*, we have pursued the identity of the biomolecules that influence bacterial accumulation and Gtf activity. Metabolite profiling and chromatographic analyses of BF-CM reveal the presence of farnesol (~25 µM, Fig. 3A1), a quorum-sensing molecule from *C. albicans* that is commonly understood to exhibit antibacterial activity (Fig. 3A). Surprisingly, farnesol levels (25 µM) detected in BF-CM enhance *S. mutans* cell growth, microcolony development, and Gtfs activity (Figs. 3B&C) in a manner similar to that observed with BF-CM. However, higher concentrations (>100 µM) of farnesol inhibited *S. mutans* growth (Fig. 3B). Thus, we propose that farnesol is a potential key modulator in this cross-kingdom interaction, and that *S. mutans* growth responds non-monotonically to farnesol concentration (Fig. 3B). Since the amount detected in BF-CM (~25 µM) promotes both *S. mutans* growth and Gtfs activity, further experiments are conducted to determine whether farnesol can stimulate microcolony

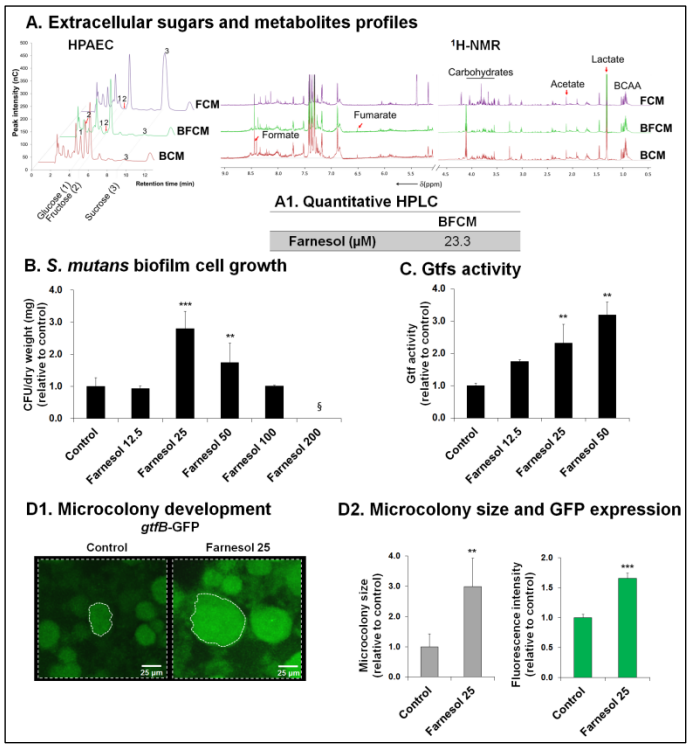

Figure 3. Chemical composition of BF-CM and influences of farnesol at levels found in BF-CM on Gtfs activity and *S. mutans* biofilm formation.

development and *gtfB* expression (via P_{gtfB}::*gfp*). Intriguingly, farnesol induces the formation of enlarged microcolonies (~3-fold increase vs. control, $^{**}P<0.01$) with elevated expression of *gtfB* ($^{***}P<0.001$) (Figs. 3D1&2) at similar levels to those observed with BF-CM. Together, our data suggest that farnesol may be an important molecule mediating this cross-kingdom interaction, with a potential role in regulating *S. mutans* growth and microcolony assembly within cariogenic biofilms.

DISCUSSION

Our findings reveal an intriguing cross-kingdom interaction whereby a fungal quorum sensing (QS) molecule (farnesol) stimulates bacterial accumulation and activates its gene expression within biofilms. Farnesol triggers up-regulation of *gtfB* and its Gtf activity, while enhancing microcolony formation by *S. mutans*. The glucans produced by GtfB can provide bacterial binding sites for *S. mutans* accumulation, and also serve as a structural scaffold that is essential for microcolony development (Xiao et al. 2012). The microcolonies are critical for biofilm virulence and caries pathogenesis because they promote localized acidic microenvironments that cause acid-dissolution of the adjacent tooth enamel (Koo et al. 2013). Elevated GtfB levels in human saliva and increased amounts of insoluble glucans in plaque-biofilms have been associated with caries activity in ECC-affected children (Vacca-Smith et al. 2007; Parisotto et al. 2015). Excitingly, we found that GtfB is indeed critical for the bacterial-fungal plaque-biofilm formation *in vivo*. Thus, an intriguing concept may arise where an extracellular QS molecule from a fungus may trigger the virulence of a bacterial pathogen by enhancing the expression of an exoenzyme (GtfB) associated with dental caries (Koo et al. 2013).

Farnesol is an important chemical mediator regulating *C. albicans* yeast-to-hyphae transition while exhibiting antibacterial activity (Hornby et al. 2001; Koo et al. 2003). However, we demonstrated that *S. mutans* growth responds non-monotonically to farnesol concentration. At low farnesol levels (~25 μM) found in the conditioned medium from co-cultured biofilms, it enhances bacterial cell growth and triggers *gtfB* expression/activity within biofilms, which correlated with microcolony development by *S. mutans*. In contrast, higher concentrations (>100 μM) of farnesol inhibited *S. mutans* growth, consistent with previous observations of its antibacterial effects (Koo et al. 2003). These observations suggest a well-controlled mechanism to maintain farnesol at levels that promote a symbiotic fungal-bacterial relationship, and prevent *C. albicans* from killing *S. mutans*. In parallel, our findings also suggest new strategies for

manipulating this pathogenic bacterial-fungal interaction. For example, high concentration of farnesol can be used to inhibit both *S. mutans* growth and *C. albicans* transition to hyphae, which could dampen the virulence of this polymicrobial biofilm associated with a costly and difficult to treat childhood disease.

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Author's contribution

D.K. and H.K. developed the concept and designed experiments; D.K. carried out experiments with conditioned medium via biochemical/microbiological analyses combined with RT-qPCR and confocal microscopy. D.K., T.H.R.N. conducted generation of nanoculture and confocal imaging. B.-H.L., A.W., and R.M.M performed the metabolomics experiments; D.K., H.K. performed *in vivo* animal experiment. A.W., R.M.M. and H.K contributed reagents/materials/analysis tools; D.K. and H.K. co-wrote the abstract. All authors discussed the results and revised abstract.