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Characterization of growth inhibition of oral bacteria by sophorolipid using a microplate-format assay☆

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ABSTRACT

Sophorolipid (SL) is a class of glycolipid biosurfactant produced by yeast and has potent antimicrobial activity against many microorganisms. In this paper, a microplate-based method was developed to characterize the growth inhibition by SL on five representative species of caries-causing oral bacteria. Bacterial growth on microplate in the absence and presence of varying concentrations of SL was continuously monitored by recording the absorbance at 600 nm of the cultures using a microplate reader. The results showed that SL completely inhibited the growth of the *Lactobacilli* at ≥1 mg/ml and the *Streptococci* at much lower concentrations of ≥50 μg/ml. More importantly, we further defined the mechanism of antimicrobial activity of SL by analyzing the pattern of the cell growth curves. SL at sublethal concentrations (<1 mg/ml) is bactericidal towards the *Lactobacilli*; it lengthens the apparent cell-doubling time (T_d) and decreases the final cell density (as indicated by $A_{600\ nm}$) in a concentration-dependent manner. Against the oral *Streptococci*, on the other hand, SL at sublethal concentrations (<50 μg/ml) is bacteriostatic; it delays the onset of cell growth in a concentration-dependent fashion, but once the cell growth is commenced there is no noticeable adverse effect on T_d and the final $A_{600\ nm}$. Scanning electron microscopic (SEM) study of *L. acidophilus* grown in sublethal concentration of SL reveals extensive structural damage to the cells. *S. mutans* grown in sublethal level of SL did not show morphological damage to the cells, but numerous protruding structures could be seen on the cell surface. At the respective lethal levels of SL, *L. acidophilus* cells were lysed (at 1 mg/ml SL) and the cell surface structure of *S. mutans* (at 130 μg/ml SL) was extensively deformed. In summary, this paper presents the first report on a detailed analysis of the effects of SL on *Lactobacilli* and *Streptococci* important to oral health and hygiene.

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1. Introduction

Sophorolipids (SLs) are microbial glycolipids biosynthesized by many species of yeast. The best studied yeast for the production of SL is *Starmerella bombicola* (formerly known first as *Torulopsis bombicola* and then as *Candida bombicola*). High-yield fermentative production of SL up to 400 g/l could be achieved by simultaneous addition of lipid and carbohydrate substrates to the *S. bombicola* culture (Daniel et al., 1998; Kim et al., 2009). The basic structure of SL biosynthesized by *S. bombicola* contains a sophorose molecule glycosidically bonded to a hydroxy fatty acid (Asmer et al., 1988; Nuñez et al., 2001) (Fig. 1). Depending on the fermentation conditions and feedstocks, however, the identity of the fatty acid moiety and the acetylation state of the 6′- and 6″-carbon of the sophorose group could vary (Felse et al., 2007; Ashby et al., 2008). It is apparent from the structure of SL that the molecule is amphiphilic and is likely to have an excellent surface-lowering activity, making it potentially useful as a surfactant, detergent, or emulsifier. In fact SL has been extensively researched and developed as a promising biobased substitute for the surfactants commonly used in many food- and non-food products as well as industrial processes such as environmental remediation and enhanced oil recovery (Van Bogaert et al., 2007; Shekhar et al., 2015). The commercial potential of SL is further supported by techno-economic assessment that showed viable production costs of <$1.40/lb. depending on the feedstocks (Ashby et al., 2013; Transparency Market Research, 2014).

Aside from its excellent surface-lowering ability, SL has been found to also possess other value-added properties such as antimicrobial, fibroblast skin-cell reproduction, anticancer and antiviral activities that could further add to its commercial values (Borsanyiova et al., 2016; De Rienzo et al., 2015; Dey et al., 2015; Van Bogaert et al., 2007; Kim et al., 2002; Lang et al., 1989). We recently studied the antimicrobial activity of SL against food pathogens (Zhang et al., 2016) and hides- and animal skins-degrading bacteria (Ramos et al., 2012; Solaiman et al., 2016) with a goal towards developing antimicrobial washing and

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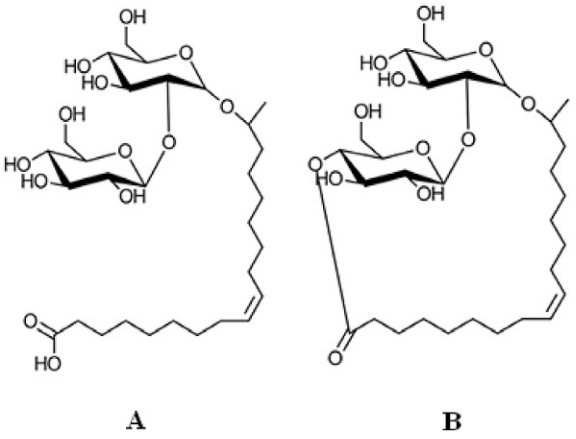

Fig. 1. Structures of SL from *S. bombicola*. The structures show SLs containing 17-hydroxy oleic acid as the lipid moiety in free-acid form (A) or lactonized to the hydroxy group at C4″ of the sophorose moiety (B). Both are shown in its un-acetylated state of the hydroxy group at the C6′ and C6″ positions.

cleaning formulae for the pertinent industries. In this paper, we report a detailed study of the antimicrobial activity of SL against tooth decay (or caries)-causing bacteria, i.e., *Lactobacillus acidophilus*, *L. fermentum*, *Streptococcus mutans*, *S. salivarius* and *S. sobrinus* that are highly relevant to the maintenance of oral care and hygiene (Loman and Ju, 2015; Prashant et al., 2007; Becker et al., 2002; Kleinberg, 2002; Tanzer et al., 2001). We also showed that the use of a microplate-based assay method (Francisco et al., 2016) is valuable in elucidating new information regarding the mode of action of the antimicrobial SL against the oral bacteria. The results not only yield information regarding the minimal inhibitory concentrations of SL against oral *Lactococci* and *Streptococci*, but more importantly showed for the first time the antimicrobial mechanisms of SL against these bacteria at sublethal concentrations.

2. Material and methods

2.1. Bacteria, growth conditions, biochemicals

All five oral bacteria studied, i.e., *Lactobacillus acidophilus* ATCC 4356, *L. fermentum* ATCC 9338, *Streptococcus mutans* ATCC 25175, *S. salivarius* ATCC 13419, and *S. sobrinus* ATCC 33478 were obtained from ATCC (Manassas, VA). *Lactobacilli* were cultured in Difco Lactobacilli MRS Broth (MRS; Becton, Dickinson and Company, Sparks, MD), and *Streptococci* were grown in Brain Heart Infusion Broth (BHI; Sigma-Aldrich, Saint Louis, MO). Cultures were grown at 37 °C with minimal rotary shaking (50–150 rpm) under atmospheric air. For agar media, 1.2% (w/v) of agar was added to the corresponding liquid broths before autoclaving.

Sophorolipid (SL) was fermentatively produced by using *Candida* (presently re-classified as *Starmerella*) *bombicola* ATCC 22214 and purified in the laboratory as described (Ashby et al., 2011). LC/MS analysis showed that 98% of the product was lactonic SL (Ashby et al., 2011). The nearly exclusive representation of the lactonic SL in the material used in this study alleviates the potential complication from the different antimicrobial potencies reported for the various forms of SL (Kitamoto et al., 2002). The purified SL was stored at ambient room temperature and atmosphere in a plastic container. All other chemicals and biochemicals were reagent-grade and were purchased from commercial sources.

2.2. Experimental culture set-up on microplate

Two microplate-configurations, i.e., 24- and 96-well microplates, were used as specified to monitor cell growth. Overnight cultures of test bacteria were used to inoculate the medium in wells of the microplate. For the 24-well microplate configuration, 1.9 ml of the appropriate culture medium and 0.1 ml of the overnight culture were used in each well. Regular (Falcon 35-3047 with lid, Becton, Dickenson and Co.) or side-ridged (CytoOne CC7672-7524 with lid, USA Scientific, Inc., Ocala, FL) 24-well microplates were used for comparison. When the 96-well microplate configuration was used, the experimental culture mixtures were first set up in the wells of a 24-well microplate and then 0.2 ml of the mixtures were transferred into the wells of the 96-well microplate (Corning 3995 without lid, Corning Inc., Kennebunk, ME) and sealed with AirPore Tape Sheet transparent film (QIAGEN Sciences, Valencia, CA) for subsequent cell growth monitoring. Cell growth was monitored by measuring the absorbance at 600 nm ($A_{600\ nm}$) of the test culture in the wells for 2 days at 37 °C in a TECAN Infinity M200 Pro microplate reader (Morrisville, NC) controlled by a TECAN i-Control computer program. Absorbance was recorded every 1–1.5 h (as specified in the results) either without or with a 5-min orbital shaking (3 mm radius) prior to each absorbance reading. The correlation of $A_{600\ nm}$ to the number of viable cell counts were established separately using *L. acidophilus* and *S. mutans* as representative strains for the *Lactobacilli* and the *Streptococci* tested in this study (Fig. S2).

A 45% (w/v) SL stock solution (prepared in 70% v/v ethanol or EtOH) was prepared and stored at 4 °C in a refrigerator. We had previously established that SL used in this study could be solubilized in 70% v/v EtOH at up to a concentration of 45% (w/v) SL at room temperature (unpublished data). We further found that the 45% (w/v) SL in 70% v/v EtOH remained in solution on long-term storage at room temperature or 4 °C. All SL working solutions were prepared from the 45% (w/v) SL stock solution by appropriately diluting it in 70% *v*/v EtOH. SL working solutions were then added at a volume of 5–50 μl into the experimental cultures to achieve the desired final test concentrations. It is noted that at these volumes of addition, the final concentrations of EtOH never exceeded 1.75% v/v in the cultures; Supplemental Fig. S1 verifies that the growth inhibitory effects of 1.75% v/v EtOH alone towards all of the test bacteria was not significant in comparison to the effects of SL. It is further noted that SL at the test concentrations used in this study did not result in any observable precipitation in the cultures.

The log-phase of the growth curves was used to determine the apparent cell-doubling times (T_d; h) of the cultures. Linear regression was used to construct a straight line through the data points in the log-phase of the growth curves, and the inverse of the slope of the linear equation is then defined as the apparent cell-doubling time (T_d).

All data points represent the average of determinations from two separate biological replicates except otherwise stated. Average and standard deviation (S.D.) were calculated using Microsoft Excel software.

2.3. Scanning electron microscopy (SEM)

In one SEM experiment (Figs. 6 and 7), twenty (20) μl of bacterial cultures at the end of the 2-day growth studies were withdrawn and placed on round micro-cover glass slides (d = 12 mm; Thermo Scientific, Portsmouth, NH) previously cleaned in acetone. In another SEM experiment (Fig. 8), cells of stationary-phase cultures were harvested and resuspended in equal volume of fresh medium; they were then incubated with SL at the indicated concentrations for 1–2 days. Twenty (20) μl of the treated cells were spotted on glass slides as mentioned. The slides were incubated at room temperature (R.T.) for 30 min to allow the bacterial cells to adhere to the glass surface. A volume (50 μl–1 ml) of 2.5% glutaraldehyde (Electron Microscopy Sciences or EM Sciences, Hatfield, PA) was added to completely cover the previously spotted bacterial culture, and the cells were fixed for 30 min at R.T. The samples were then rinsed twice for 30 min each with 2–3 ml of a 0.1 M imidazole solution (EM Sciences), followed by 30 min washes each of 2–3 ml 50, 80 and 90% EtOH (The Warner-Graham Company, Cockeysville, MD). Final washes of 3 × 2 ml of 100% EtOH were performed before the samples were subject to critical point drying (CPD). CPD was performed by stacking the samples separated by cloth, in a