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Antimicrobial activity and inactivation mechanism of lactonic and free acid sophorolipids against *Escherichia coli* O157:H7☆

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ABSTRACT

There has been an increasingly interest in natural antimicrobials due to concerns over long term impact of synthetic chemicals on the environment and human health. This study was conducted to investigate the antimicrobial activity and inactivation mechanism of different structural derivatives of sophorolipids (SLs) against pathogenic *Escherichia coli* O157:H7. We produced SLs from glucose and either palmitic, stearic or oleic acid via fermentation using the osmophilic yeast *Starmerella bombicola*, purified and separated the SLs into lactonic and free-acid forms and assessed their antimicrobial efficacy against 5 strains of pathogenic *Escherichia coli* O157:H7. Results showed that the antimicrobial activities of individual SLs depended on the type of SL, treatment time and concentrations, and presence of ethanol. Lactonic stearic and oleic SLs were more effective in reducing *E. coli* O157:H7 populations than the free-acid counterparts. The five strains of *E. coli* O157:H7 had different susceptibilities to SLs. Analysis using the ethidium monoazide-PCR amplification demonstrated that SLs inactivated bacteria through cell membrane damage in *E. coli* O157:H7. SLs have the potential to inactivate pathogenic *E. coli* O157:H7 in combination with low levels of ethanol. The information would be useful for food and other industries to apply natural antimicrobials to mitigate the risk of the pathogen.

1. Introduction

Outbreaks of foodborne illness have been increasing over the last few decades (CSPI, 2015). It is estimated that there are 48 million illnesses, 128,000 hospitalizations, and 3000 deaths each year due to foodborne diseases in the United States (Scallan et al., 2011). The major foodborne pathogenic bacteria involved in the foodborne outbreaks include *Escherichia coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes*. During 2003–2012, there were 390 reported *E. coli* O157:H7 outbreaks, which included 4928 illnesses, 1272 hospitalizations, and 33 deaths (Heiman et al., 2015). Current interventions to reduce pathogen contamination on food include both thermal and chemical treatments. However, consumers are becoming more aware of the health risks associated with the use of chemical preservatives in food products, and prefer natural ingredients and non-thermal processes in food production. With increased interest in the use of natural antimicrobial interventions, we studied a family of sophorolipids (SLs) (Figs. 1 and 2) for their efficacy against pathogenic *E. coli* O157:H7.

SLs are glycolipid biosurfactants, and are produced by several species of microorganisms (van Bogaert et al., 2007; Kurtzman et al., 2010). Among these species, *Starmerella bombicola* (formerly *Candida bombicola*) is the most studied SL-producing yeast, which exhibits both the highest yield and productivity (Kurtzman et al., 2010). Structurally, SLs are composed of a disaccharide sophorose which is β-glycosidically linked to a long fatty acid chain. SLs can occur in the free-acid form with a free fatty acid tail or in the lactonic form with an internal esterification between the carboxylic end of the fatty acid and the 4′ hydroxyl group of the sophorose head. In addition, minor differences in the hydroxy fatty acids occur with either 16 or 18 carbon atoms, and one or more unsaturated bonds. The structural difference in SLs may cause wide variation in their physicochemical properties (van Bogaert et al., 2011). SLs have been shown to possess various antimicrobial activities particularly against Gram-positive bacteria (Kitamoto et al., 1993, 2002; Lang et al., 1989; Ashby et al., 2011; Delbeke et al., 2016). Our earlier work (Zhang et al., 2016) demonstrated that a lactonic oleic acid SL at 1.0% reduced *E. coli* O157:H7 from an initial population of 7.1 log CFU ml^{-1} to non-detectable levels (detection limit: 1 log CFU ml^{-1}) after 2 h treatment. However, there have been limited studies dealing with direct comparison of lactone vs. free-acid forms of SLs or SLs with various types of fatty acids (length and saturation) in terms of

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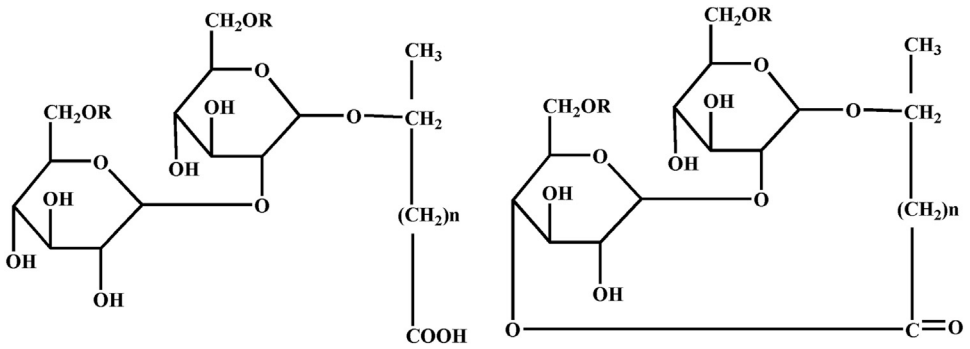

Fig. 1. Structures of free-acid (left) and lactonic (right) forms of palmitic and stearic acid sophorolipids. R = $COCH_3$. Palmitic acid sophorolipids when n = 13; stearic acid sophorolipids when n = 15.

their activities against Gram-negative food-borne pathogenic bacteria.

The most important criterion for distinguishing between viable and dead or irreversibly damaged bacterial cells is membrane integrity (Nocker et al., 2006). It is believed that bio-surfactants such as SLs target the cell membranes of bacteria (Dengle-Pulate et al., 2014; Zhang et al., 2016). Ethidium monoazide (EMA) is a DNA-intercalating dye with the azide group allowing covalent binding of the chemical to DNA upon exposure to strong visible light (Rudi et al., 2005; Wang and Mustapha, 2010; Elizaquível et al., 2014). Cells with a compromised membrane, when exposed to EMA, allow the dye to penetrate the membrane, and bind to their DNA (Nocker et al., 2006). Photo-induced cross-linking inhibits PCR amplification of DNA from dead or membrane-compromised cells. Live cells with intact membranes have the ability to exclude DNA-binding dyes. Therefore, DNA from intact live cells, protected from reactive EMA before light exposure, is not affected by the EMA pretreatment. As a result, PCR amplification of DNA purified from the intact live cells is not inhibited. The EMA coupled with PCR amplification has been used to study cell membrane permeability (Elizaquível et al., 2014; Xie et al., 2011).

This study was conducted to evaluate antimicrobial activities of various structural derivatives of SLs against *E. coli* O157:H7 and their mode of action in altering membrane permeability using the EMA-PCR method.

2. Materials and methods

2.1. Source of chemicals

Glucose, oleic acid, stearic acid and palmitic acid were from Sigma-Aldrich (St. Louis, MO, USA). Absolute ethanol was from Warner Graham Company (Cockeysville, MD, USA). Ethidium monoazide was from ThermoFisher Scientific (Waltham, MA, USA).

2.2. Bacterial strains

Five outbreak-associated *E. coli* O157:H7 strains (RM6535, RM7386, RM1484, 06F00475, and Sakai) were used in the study. *E. coli* O157:H7 RM6535 (2006 Iceberg lettuce outbreak), RM7386 (Romaine lettuce outbreak), RM1484 (apple juice outbreak), *E. coli* O157:H7 Sakai (sprout outbreak) and 06F00475 (spinach outbreak) were provided by Dr. Robert E. Mandrell (USDA, ARS, WRRC).

Cultures of each individual strain were propagated on tryptic soy agar (TSA: Difco Laboratories, Detroit, Mich., USA) at 37 °C and maintained at 4 °C until use. Prior to the inoculum preparation, individual strains were grown in 10 ml tryptic soy broth (TSB: Difco Laboratories) for 18–20 h at 37 °C with agitation (100 rpm) using a shaking incubator (Incu-Shaker Mini, Benchmark Scientific, South Plainfield, NJ, USA). After centrifugation at 4000 rpm for 10 min in a Sorvall Lynx 4000 refrigerated centrifuge (Kendro Laboratory Products, Newtown, CT, USA), the cultures were suspended in phosphate buffered saline (pH 7.2), and combined to form a cocktail of *E. coli* O157:H7 unless used for the study of individual strains (see below). Cell populations of the *E. coli* cocktails were ~9.2 log CFU ml^{-1}.

2.3. Synthesis of SLs

SLs were synthesized from *S. bombicola* ATCC 22214 using palmitic, stearic and oleic acids as co-feedstocks, followed by extraction and purification (Ashby et al., 2011; Solaiman et al., 2015). Briefly, 10 L of a *Candida* growth media (CGM; 10% w/v glucose, 1% w/v yeast extract and 0.1% w/v urea) was prepared in a 12-L capacity vessel of a benchtop bioreactor (Bioflo 3000 Batch/Continuous Bioreactor, New Brunswick, NJ, USA). Following sterilization by autoclaving and then cooling to 26 °C, palmitic, stearic and oleic acids (200 ml, technical grade, Sigma-Aldrich, St. Louis, MO, USA) were added to the medium to a final concentration of 2% (v/v). A stock inoculum culture (previously prepared and stored in −80 °C freezer) was thawed and added to the 10-L CGM (containing 2% palmitic, stearic or oleic acids) medium to initiate the fermentation. The bioreactor was set at the following parameters: 26 °C, an impeller speed of 700 rpm, 2 L/min aeration, and no pH control. On day 2 of the fermentation, 7.5% (w/v) of granular glucose and 2% (v/v) of palmitic, stearic or oleic acids were added to the

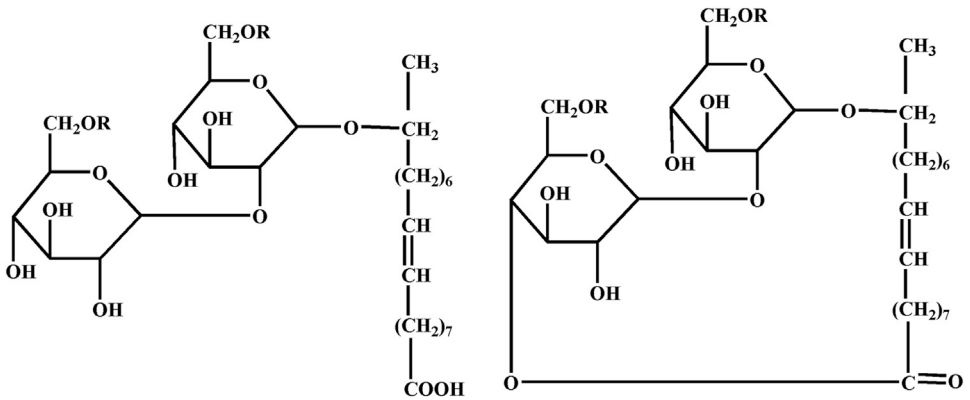

Fig. 2. Structures of free-acid (left) and lactonic (right) forms of oleic acid sophorolipids. R = $COCH_3$.

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