Effect of Biosurfactants on Gram-negative Bacterial Biofilm Formation
（バイオサーファクタントによるグラム陰性細菌バイオフィルム形成への効果）

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Summary

Bacterial biofilm is one mode of living that helps bacteria to survive a changing and variable environment, often under harsh conditions. When attached to submerged surfaces, bacteria cells will continue proliferating and aggregating to form biofilm structures, which they stabilize by secreting a matrix composed of extracellular polymetric substances (EPS). The EPS matrix plays multiple roles in the biological process of biofilm: it covers and helps to protect bacteria from changing environmental conditions, chemical and physical attacks. This is one reason why biofilm bacteria can be greater than 100x more resistant to antibiotics than their planktonic counterparts. On the other hand, biofilms can be disrupted with mechanical, or chemical, means. Antibiotics are frequently used in clinical settings, but besides these compounds, surface-active agents (surfactants) are also commonly used to inhibit bacteria due to their capacity to reduce the adhesion of bacterial aggregates and to dissolve the biofilm matrix. However, most common surfactants are synthesized from petrochemicals and possess varying degrees of environmental toxicity, and therefore, there is a demand for alternative products which are ecofriendly. Produced by microorganisms with natural and renewable resources, biosurfactants are considered next generation chemical surfactants for use in cosmetics, food, pharmaceuticals, and in wastewater treatment. Among some of the investigated groups of biosurfactants, sophorolipids were the first extracellular yeast glycolipids that have found practical application.

Sophorolipids (SLx) are products of oil fermentation of *Candida* species with very high yields. They are comprised of a residue of sophorose, consisting of two glucose and hydroxylated C_{16} or C_{18} fatty acid. It has been proven that SLx inhibit growth and biofilm formation of certain Gram-positive bacteria at concentration of 10 and 20 mg/mL by increasing the permeability of membrane. However, not much is known about how it affects Gram-negative bacteria.

Pseudomona aeruginosa is a Gram-negative bacterium that is an opportunistic pathogen and is often used as a model organism to study biofilm formation. It was recently highlighted by the World Health Organization as an urgent target for research and development of new antibiotics since it rapidly acquires antibiotic resistance. While the traditional methods to study antibiofilm activity, such as polystyrene-well plates, have many disadvantages because they do not mimic actual living environments of bacteria, we use microfluidics as a convenient, cost-effective platform for rapid prototyping and to allow for optical quantification.

We test the effect of sophorolipids at two points in the biofilm lifecycle on *P. aeruginosa* PAO1: 1)

at the early stage of "reversible" surface attachment; and 2) at the later stage when the biofilm has matured. We also compare the effect of application of SLx with some chemical surfactants that are often used in industrial detergents.

We find that SLx suppresses the irreversible attachment of PAO1 to clean glass surfaces in a dose dependent manner with long-lasting effects. Testing with deletion mutants for flagellar and pili, we show that SLx not only impairs the ability of pili to attach to glass but also reduces the strength of adhesins (sticky proteins). In an effort to decrease the concentration of SLx required to achieve these results, we combine SLx and fluid flow to provide shear stress to remove attached cells in microfluidic devices. The results show a synergistic combination between SLx and shear stress to wash out the attached cells more quickly and completely, than with either individually. Compared with other chemical surfactants, SLx also shows an outstanding capacity with initial attached PAO1 cells when even with a concentration of 0.01 wt%: SLx inhibits PAO1 attachment completely and the effects last ~8 h.

We demonstrate the potential of sophorolipids to disrupt mature *P. aeruginosa* biofilms in comparison to a few different chemical surfactants. Our results show that SLx can catastrophically disrupt mature biofilms at relatively lower critical micelle concentrations (CMCs) compared to other surfactants. Moreover, we demonstrate the effects of SLx on a EPS hyper-producing strain of PAO1 (Δ*wspF* mutant). We find that SLx affects the EPS structure and aggregation of PAO1 Δ*wspF*. These results show that SLx weakens the connection between EPS and the glass surface, and also the connection between molecules inside EPS. Moreover, SLx shows both a stronger effect and requires less time to become effective, which may indicate that the mechanism of its ability to washout PAO1 biofilms is different from other surfactants.

Interestingly, we find that SLx does not kill the bacteria nor does it slow their growth. Furthermore, SLx seems to inhibit pyocyanin production, which is the main virulence factor of *P. aeruginosa.* We believe that SLx represents a promising combination of anti-biofilm properties that act to suppress biofilm formation at different stages of the biofilm lifecycle without killing the bacteria. Furthermore, by using a biosurfactant, which is a more ecofriendly substance, and decreasing the amount of chemical surfactants, we can ameliorate the environmental impact of surfactants, lessen harm to aquatic life, and reduce pollution of ecosystems. We believe that combinatorial treatments could be used to clean industrial pipes and surfaces that are prone to biofilm fouling.