Controlling Microbial Multicellular Behaviors With Saccharide Derivatives

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Abstract

Microbial multicellular behaviors like biofilm formation and swarming motility are known to increase their tolerance against antimicrobials. From microbial standpoint, nonmicrobicidal agents that do not impede growth are tolerable and therefore, there is a lower propensity to develop resistance against such agents as compared to microbicidal ones (antibiotics). This study describes a new antibiofilm approach of using nonmicrobicidal saccharide derivatives for controlling the multicellular behaviors of gram-negative bacteria, *Pseudomonas aeruginosa* and fungus, *Candida albicans*.

*Pseudomonas aeruginosa* is known to secrete rhamnolipids, a class of biosurfactants that plays an important role in maintaining the architecture of its biofilm and promoting its swarming motility. Here we show the ability of certain synthetic nonmicrobicidal disaccharide derivatives (DSDs) to mimic the biofunctions of rhamnolipids. The *rhlA* mutant of *P. aeruginosa* is incapable of synthesizing rhamnolipids and also unable to swarm on semi-solid agar gel. When the natural ligands, rhamnolipids were externally added into the semi-solid agar gel in a concentration dependent manner, the swarming of the *rhlA* mutant reactivated at lower concentrations (10 μM) and then at relatively higher concentrations (15 μM), the swarming reactivation was reversed. When some active synthetic DSDs were tested on the *rhlA* mutant, the bacterial swarming first reactivated and then the activation reversed at higher DSD concentrations, similar to the effect of externally added rhamnolipids. Previously, a known bacterial signalling molecule has been shown to exhibit a similar concentration dependent activation and then activation reversal for light simulation by *Vibrio fischeri*. Some DSDs having disaccharide stereochemistries (cellobiose or maltose) and a bulky aliphatic tail (3, 7, 11-
trimethyl-dodecanyl) caused swarming reactivation of the *rhlA* mutant at concentrations lower than that caused by the externally added rhamnolipids.

The synthetic nonmicrobicidal DSDs were also very effective at inhibiting the adhesion of *P. aeruginosa* to polystyrene surface, and at inhibiting the bacterial biofilm formation. These DSDs were also potent dispersers of pre-formed biofilm of *P. aeruginosa*. The potent antibiofilm (inhibition and dispersion) activities were observed for those DSDs that possessed a disaccharide (cellobiose or maltose) stereochemistry and a bulky aliphatic chain such as 3, 7, 11-trimethyl-dodecanyl. These potent DSDs had half-maximal inhibitory concentrations for biofilm inhibition (IC$_{50}$) and dispersion (DC$_{50}$) comparable to those of known potent antibiofilm agents against *P. aeruginosa*. Gene-reporting assays indicate that the mechanism of action of such DSDs is not via the known *las* or *rhl* quorum sensing systems of *P. aeruginosa* but that the adhesin protein, pilin maybe a likely target of such molecules.

Biofilms formed under natural settings are usually formed by both bacteria and fungus that co-reside in the same microenvironment. Therefore, agents that can prevent mixed biofilms are desirable from a therapeutic standpoint. Despite being nonmicrobicidal to both fungal blastospores and hyphae, the synthetic DSDs were able to inhibit the biofilm formation of fungus *Candida albicans*. Microscopic evaluation showed that most DSDs did not prevent the blastospores-to-hyphae morphogenesis. The DSDs were effective at inhibiting biofilm formation of *Candida albicans* when applied within five minutes of seeding the test surface with fungal cells. Using a surface based assay it was shown that one DSD dramatically reduced the surface adhesion of *Candida albicans* hyphae. The antibiofilm activity of such DSDs against *Candida albicans* is probably due to their ability to prevent hyphae surface adhesion.
Controlling Microbial Multicellular Behaviors With Saccharide Derivatives

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Introduction

1.1 Treating microbial infections - two different approaches

The existence of strains exhibiting multidrug resistance (MDR) for many species of bacteria is a matter of grave concern and enormous magnitude in today's healthcare settings.\(^1\)\(^-\)\(^5\) The result is that, infections from many nonlife threatening species are often being cured by the most potent drugs only. To add to the woes, the pace at which newer microbicidal agents or antibiotics are being discovered and approved has been sluggish with only oxazolidinones and lipopeptides being inducted as potent new drugs in the recent years.\(^6\)\(^-\)\(^10\) Bacteria use at least three strategies to invoke MDR. These include, ability to destroy the activity of the drug compound, to undergo mutations that lead to expression of altered target proteins that do not recognize the antagonistic drug, and to deploy efflux pump mechanisms that lower cellular concentration of the drug at the active site.\(^11\)\(^-\)\(^13\) Another problem associated with bacteria is that they possess the ability to spread drug resistant genes to other species through a phenomenon known as horizontal gene transfer (HGT).\(^14\)\(^-\)\(^16\) The occurrence of HGT has ensured that over time, drug resistant genes have been disseminated from strain to strain and from species to species, resulting in strains exhibiting MDR.\(^17\)

Taking a step back and assessing the conventional approach towards treating microbial infections, brings forward the underlying common principle, "microbicidal action of drugs".\(^18\)\(^,\)\(^19\) Antibiotics target the cellular processes that are essential for bacterial survival; therefore bacteria employ the three strategies in their survival pack to overcome the effects of such microbicidal agents.\(^20\)\(^-\)\(^22\) From the human perspective, the "fightback" by microbes to our invasive
microbicidal agents has generated strains that are now sturdier and resistant to the action of most antibiotics and the occurrence of such resistant strains are on the increase due to easy transfer of genetic elements amongst strains.\textsuperscript{3,23} Therefore agents that prevent microbial infections without affecting their growth will have lower propensity to invoke drug resistance.\textsuperscript{24} To summarize, the two main problems associated with the conventional microbicidal strategies for treating bacterial infections are; (i) Antibiotics enhance the chances of inducing drug resistance in bacteria,\textsuperscript{3,25,26} (ii) Antibiotics work better when bacteria exist as unicellular organisms.\textsuperscript{27,28}

Alternatively, the newer strategies to treat bacterial infections should address the above mentioned problems associated with conventional antibacterial strategies. The issue of invoking drug resistance can be overcome or reduced with the use of nonmicrobicidal agents that do not impede bacterial cellular activities. In the present day, the notion that bacteria exist both as unicellular planktonic cells and as multicellular organisms has been widely acknowledged by the scientific community.\textsuperscript{29-31} Bacterial multicellular processes are known to be associated with most infections but are less important for bacterial survival.\textsuperscript{32} Employing strategies that look beyond bacteria as simple unicellular organisms necessitates an understanding of why and when bacteria switch between the two lifestyles.\textsuperscript{33}

1.2 Two different kinds of lifestyles that bacteria adopt: Unicellular and multicellular

Despite some earlier observations of coordinated activities, bacteria until recently were regarded as archetypal unicellular organisms.\textsuperscript{34,35} But advances in microbiology have since focused on why and when do bacteria choose a multicellular lifestyle over remaining loners.\textsuperscript{31,33} The evolutionary benefits that have been identified as being important for establishing multicellularity in bacteria include resistance from predators and better management of
To achieve these benefits, bacteria associate to form adherent clusters that give rise to various kinds of communities, both sessile and motile having different superficial morphologies.\textsuperscript{37-40} The formation of both kinds of multicellular communities is known to be initiated by different types of chemical signals that bacteria secrete, receive and interpret.\textsuperscript{41-43} The signal transduction alters bacterial gene expression and sets into motion a series of changes that eventually leads to formation of multicellular communities.\textsuperscript{44} Once multicellularity is established, the resulting communities either create sessile complex 3-dimensional architectures or motile 2-dimensional patterns that ensure division of labor and coordination of nutrients.\textsuperscript{36}

1.3 Sessile and motile multicellular behaviors

Bacteria are able to perform at least two distinct multicellular processes; (i) Formation of biofilm, a sessile lifestyle and (ii) Swarming, a motile lifestyle. While both of these processes have distinct characteristics, where one is a stationary phenomenon and the other is a motile one, there exists evidence that both these processes are intertwined and have mechanistic intermingling.\textsuperscript{38,45}

1.3.1 Formation of biofilm is a developmental process: A sessile group behavior

Biofilms are highly hydrated surface attached microbial communities having structural and metabolic division of labor and where microbes reside within a matrix of secreted extra polymeric substances (EPS).\textsuperscript{46-48} The choice of planktonic bacteria to harbor themselves within multicellular communities confers many advantages (i) Biofilm architecture and framework allows easy flow and supply of nutrients. (ii) The formation of sturdy biofilms creates a protective barrier against physical and chemical assaults. (iii) Formation of biofilms renders the
community a thousand-fold more resistant to the action of antibiotics. (iv) Different kinds of species can inhabit the same matrix as nutrient requirements for specific species are easily met by slow diffusion of small molecules across the matrix. (v) Because many species or strains reside within the same scaffold, interspecies or inter-strain transfer of genetic material can occur to spread competitive advantages against particular environmental challenges.49,50

1.3.2 Five stage differentiation of bacterial biofilms

In early reports, a biofilm was thought to be an unstructured slime of polymeric matrix which bore embedded bacteria.51 During these early years, it was not clear how such deeply embedded bacteria have access to nutrients or even oxygen. Later, with the use of confocal laser scanning microscopy (CLSM), Costerton and coworkers were the first to report that the three dimensional architecture of biofilms consists of matrix enclosed bacterial microcolonies that have voids or water-channels (Figure 1.1).47,52,53

The formation of biofilm is a developmental process involving five key stages (Figure 1.1).54 The first stage of biofilm development is the "reversible attachment" of bacteria to a surface. The planktonic bacteria at this stage are open to both mobile or sedentary lifestyle, and once environmental cues are favorable, the bacteria commit to a sessile lifestyle. Once attached onto surfaces the bacteria begin a second stage of multicellularity by secreting EPS and establishing "irreversible attachment".55,56 The composition of EPS secreted by microbes is quite diverse and varies from species to species but common elements include polysaccharides, nucleic acids, proteins and phospholipids.57,58 The matrix created by secretion of EPS helps "glue" cells together to initiate microcolony formation.52
The surface adhered bacteria then secrete chemical signals known as autoinducers into the surrounding environment to perform cell-to-cell communication, a process usually referred to as quorum sensing.\textsuperscript{59-63} Quorum sensing is carried out by bacteria to assess the cellular density of other bacteria in the environment.\textsuperscript{42,64} The assessment of cellular density is achieved by detection of secreted bacterial chemical signals in the environment.\textsuperscript{43} The increase in concentration of chemical signals in the environment is correlated with the cellular density of the bacteria. Once a threshold concentration for the chemical signals is achieved, individual bacteria transduce the signal and initiate a cascade of signaling pathways that eventually alter bacterial gene expression to initiate the third stage of biofilm formation which is the shift from planktonic lifestyle to a multicellular commune.\textsuperscript{65-68}

The sessile communities formed exhibit a fourth development stage of "maturation" where the biofilm takes complex shapes along with redistribution of bacterial cells for distinct roles and formation of water channels.\textsuperscript{69} One important change during the fourth stage is the alteration in the level of expression of many genes that are different from the genetic expression amongst planktonic bacteria.\textsuperscript{47,70}

Formation of biofilm is a lifestyle change with the underlining principle of ensuring bacterial survival and propagation.\textsuperscript{71} Therefore, like other sessile communities, biofilms too have evolved dispersive strategies to colonize new surfaces.\textsuperscript{72} This is the final stage of biofilm development that includes both passive and active dispersal strategies.\textsuperscript{73,74} In passive dispersal, chunks of the existing biofilm may erode away due to fluid shear and move to newer uncolonized surfaces. On the other hand, active dispersal, also known as seeding dispersal, of free swimming planktonic bacteria from the mature biofilms is thought to be under the control of complex cellular signaling. The mechanism by which microorganisms manipulate cellular
dispersal from biofilms is still poorly understood but the concept finds broad applications in the field of antibiofilm strategies.\textsuperscript{49}

\textbf{Figure 1.1} Different stages of biofilm development and dispersion. Insert-Criteria for developing antibiofilm strategies include interfering with one or multiple biofilm developmental stages. [Reprinted with permission from Citation:40 Bordi and de Bentzmann (2011) Hacking into bacterial biofilms: a new therapeutic challenge. Annals of intensive care, 2011, 1: 19. doi:10.1186/2110-5820-1-19; Image Credit: de Bentzmann; Copyright: © 2011 Bordi and de Bentzmann; licensee Springer. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution and reproduction in any medium, provided the original author and source are cited.

1.3.3 Numerous medical and industrial problems are associated with formation of biofilms

Until three decades ago, bacteria were primarily regarded as unicellular entities, but since then the view about bacteria has evolved to appreciate that formation of biofilms may be a preferred bacterial lifestyle. The formation of biofilms by bacteria on both biotic and abiotic
surfaces causes immense monetary losses to both medical as well as industrial setups.

*Pseudomonas aeruginosa* is the primary microbial dweller in the lungs of cystic fibrosis patients, where the bacteria's preferred mode of living is by forming biofilms. Bacteria are known to reside within oral cavities, form dental plaques and known to colonize a wide variety of medical devices by forming biofilms. Common diseases associated with formation of bacterial biofilms include burn wound infections, otitis media and bacterial endocarditis. (Table 1.1). Two important issues associated with formation of bacterial biofilms are enhanced resistance to antimicrobials and lack of strategies that can completely eradicate colonization.

**Table 1.1** Biofilm forming pathogen and the corresponding associated medical condition

<table>
<thead>
<tr>
<th>Organism</th>
<th>Biofilm-associated diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Cystic fibrosis lung infection</td>
</tr>
<tr>
<td><em>Burkholderia cepacia</em></td>
<td>Cystic fibrosis lung infection</td>
</tr>
<tr>
<td><em>Acinetobacter baumannii</em></td>
<td>Burn wound, trauma infection</td>
</tr>
<tr>
<td><em>Helicobacter pylori</em></td>
<td>Gastrointestinal infection</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Urinary, catheter infection</td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em></td>
<td>Otitis media</td>
</tr>
<tr>
<td><em>Bordetella pertussis</em></td>
<td>Respiratory infection</td>
</tr>
<tr>
<td><em>Legionella pneumophila</em></td>
<td>Legionnaires disease</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Burn wound, catheter, trauma infection</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>Sepsis, catheter infection</td>
</tr>
<tr>
<td><em>Streptococcus mutans</em></td>
<td>Dental plaques, gingivitis</td>
</tr>
<tr>
<td><em>Vancomycin-resistant enterococci (VRE)</em></td>
<td>Nosocomial infection</td>
</tr>
</tbody>
</table>

The National Institute of Health (NIH) has estimated that 65-80% percent of bacterial infections in humans are associated with microbial multicellularity. It has been reported that formation of biofilm renders the bacteria about one thousand times more resistant to the action of antibiotics. From a monetary standpoint, microbial multicellularity is causes huge losses both in medical as well as industrial set-ups. The cost of treating biofilm related infections in the United states can be upward of US $ 1 billion. Until recently, treating microbial infections
had been directed towards bacteria as an unicellular entity. But, since the appreciation of multicellularity being associated with persistent microbial infections, alternative strategies to treat such infections have been explored.

1.3.4 Common antibiofilm strategies

Since biofilm formation is a developmental process, the antibiofilm strategies developed mainly aim to arrest various stages of colonization (Figure 1.1). The antibiofilm strategies use both chemical and physical methods.

1.3.4.1 Quorum sensing inhibitors

Quorum sensing is the communication process that facilitates both inter and intra species cell-to-cell signaling that ultimately results in altered microbial gene expression leading to biofilm formation. The use of both synthetic and naturally occurring antagonists that interfere with the cell-to-cell chemical signaling in bacteria is a popular strategy to treat biofilms. These antagonists are nonmicrobicidal and hence considered to be generally safe for use as they possess lesser propensity to induce drug resistance.

1.3.4.2 Bacteriophages that degrade biofilm matrix

Bacteriophages have co-evolved within bacterial biofilms and can replicate within their host cells. Although bacteriophages are structurally larger than chemical antibiotics, they are still comparatively much smaller than bacterial cells and therefore do infect microbes within biofilms. Such bacteriophages secrete depolymerizing enzymes that reduce biofilm content by eliminating bacteria that produce the components of the biofilm matrix like EPS etc. Bacteriophages can also reside within persister cells without causing any damage to the
metabolically inactive host cells, and when such such cells became active, the bacteriophages are able to use the host genetic cycle, multiply and cause biofilm elimination.84

1.3.4.3 Surface modifications can prevent bio-fouling

The use of chemicals to create modified bio-inert surfaces that resist bio-fouling is another viable antibiofilm strategy.91-97 Such strategies are directed towards blocking of initial bacterial adhesion and hence prevent early stages of biofilm development. The surface chemical modifications make include coating with antibiotics,96 changing hydrophobicity or hydrophilicity,98,99 changing surface texture/roughness,100,101 and coating with polyethyleneglycol (PEG) derivatives.102 The Type I pili of E. Coli are important adhesin proteins that are recognize mannose moieties presented by host cells. Therefore, mannoside derivatives have been deployed as agents that interfere with the natural binding process of Type I pili to mannose epitopes present on host cells.103-106

1.3.4.4 Antibiofilm enzymes

While prevention of biofilm formation is difficult, it is still relatively easier than disrupting biofilms that have already formed, which is normally the problem on medical devices. Multiple enzymes have been used to degrade the components of the biofilm matrix, such as polysaccharide, eDNA and proteins. One such study has demonstrated the use of dispersin B to degrade the components of S. epidermidis biofilm EPS.107 One component of biofilm matrices is secreted extracellular DNA (eDNA), and hence Izano et al showed that DNAase I could be used to degrade the biofilm of S. aureus.108 Chaignon et al showed that proteases such as Proteinase K and trypsin degrade the biofilm formed by S. aureus.109
1.3.4.5 Small molecular chemical dispersers of preformed biofilms

A D-amino acid extracted from dispersing cells of *B. subtilis* was shown to degrade the existing biofilm and also prevent the formation of biofilm by *S. aureus* and *P. aeruginosa*.\(^{110}\) Another molecule isolated from *B. subtilis*, Norspermidine, was demonstrated to break down biofilm by targeting the exopolysaccharides.\(^{111}\) Nitric oxide (NO) is known to trigger biofilm dispersal in mature biofilms.\(^{112}\) Therefore, the use of low-dose of NO synergistically with a antibiotic was shown to reduce the colonization of *P. aeruginosa* in the lungs of cystic fibrosis patients.\(^{113,114}\) Apart from these agents, many of the small molecules used to prevent bacterial cell-to-cell communications (quorum sensing inhibitors) have been used effectively to disperse preformed biofilms of *P. aeruginosa*.\(^{80,115,116}\)

1.3.4.6 Physical methods for treating biofilms

Apart from using chemicals to treat biofilms, many physical and mechanical techniques are also employed to treat or prevent biofilm related infections, especially in the food industry. Ultrasonication is used to decontaminate food. Decontamination of food and food containers is often done with the use of electrolyzed water. Sometimes, prevention of adhesion or removal of adhered biofilm on surfaces is done with the use of electrical techniques.

1.3.5 Swarming on surfaces: A motile group behavior

While sessile lifestyle (biofilm formation) is critical, bacterial motility also plays an important role in microbial pathogenesis. There are different kinds of bacterial motilities that planktonic cells or a group of cells can perform. Among the various surface associated bacterial motilities, swarming is the fastest and is a coordinated multicellular phenomenon. Swarming is usually observed in both Gram-positive as well as Gram-negative bacterial strains that possess
flagella, including *Salmonella, Vibrio, Yersinia, Serratia, Proteus* and *Pseudomonas*. Apart from possession of flagella, the swarming phenomenon is also dependent on surface nutrients and surface "wettability" (surface tension). As a result of all the physico-chemical cues, the swarmer cells become hyperflagellated, elongated and undergo gene expression alteration as compared to vegetative cells. The differentiation of vegetative cells into swarmer cells suggests that swarming does not merely represent physical changes in the bacterial behavior but a more complex motile lifestyle adaption. Swarming colonies have distinct resistance to action of antibiotics.

### 1.4 Synopsis of Chapters 2 & 3

This thesis brings forward a new way to modulate the two kinds of microbial multicellular behaviors; swarming (motile) and biofilm formation (sessile). Chapter 2 describes a study in which saccharide derivatives were used for modulating the swarming motility of a Gram-negative opportunistic bacterium, *Pseudomonas aeruginosa*. Some saccharide derivatives were able to mimic the functions of a naturally secreted biosurfactant, rhamnolipids, that *Pseudomonas aeruginosa* secrete. The most potent saccharide derivatives seemed to dominate the effect that the natural biosurfactant had on swarming motility of *Pseudomonas aeruginosa*. Chapter 3 demonstrates how the nonmicrobicidal saccharide derivatives can also prevent *Pseudomonas aeruginosa* biofilm formation, adhesion and cause biofilm dispersal. The antibiofilm (biofilm inhibition and dispersion) half-maximal inhibitory concentrations (IC$_{50}$s) of the most potent saccharide derivatives were comparable to the most potent known nonmicrobicidal antibiofilm agents. Overall, Chapters 2 & 3 bring forward certain saccharide derivatives as modulators of *Pseudomonas aeruginosa* multicellular behaviors and also suggest
that the secreted biosurfactant, rhamnolipids may have more than just a physical significance in
the bacterial lifecycle.

1.5 Synopsis of Chapter 4

Prokaryotes (bacteria) are not the only organisms that exhibit biofilm formation; Biofilm
formation is a common way of colonization in eukaryotes (fungus) like Candida albicans. In
addition to the various effects of DSDs on bacterium Pseudomonas aeruginosa, Chapter 4
encompasses a study that demonstrates the versatility of saccharide derivatives to also inhibit the
biofilm formed by fungus Candida albicans. Surface-chemistry experiments show how the
antibiofilm activity of these nonmicrobicidal agents against Candida albicans is through their
ability to prevent hyphal adhesion to surfaces.
Chapter 2

Saccharide Derivatives Modulate the Swarming Motility of Pseudomonas aeruginosa

Abstract

Secretion of biosurfactant rhamnolipids by Gram-negative bacteria, Pseudomonas aeruginosa has been previously reported to be important for assisting swarming motility and for maintaining the architecture of water channels within the biofilm. In this chapter, the control of bacterial swarming by synthetic saccharide derivatives that function as rhamnolipids analogues has been reported. Few synthetic disaccharide derivatives (DSDs) were shown to be much more effective than both the naturally secreted and the externally added rhamnolipids at activating swarming of a nonswarming P. aeruginosa mutant. The study also brought forward a dose-response phenomenon where the activation of swarming in a nonswarming P. aeruginosa mutant, rhlA by some DSDs and also rhamnolipids first increases with increasing doses and after a threshold dose, the swarming activation is reversed. Previously, a bacterial cell signaling molecule, N-(3-oxohexanoyl)-3-aminodihydro-2(3H)-furanone has been shown to exhibit a similar dose-response “activity reversal” phenomenon for a different multicellular behavior, i.e light simulation. It is therefore predicted that production and secretion of rhamnolipids by P. aeruginosa probably has a greater biological significance than mere physical impact on multicellularity.
2.1 Introduction

Multicellularity offers at least three different advantages over planktonic lifestyle:\textsuperscript{38,117} (i) Increased tolerance to antagonists; (ii) Good management of limited nutrients, and (iii) Morphological differentiation helps in division of labor. The way free-living bacteria colonize a surface depends upon the immediate physio-chemical environment surrounding the bacterial population\textsuperscript{29,42,45,64,118-121} and as a consequence, multicellularity in bacteria can be manifested through either a sessile\textsuperscript{29,34,35,46-48,64,71,75,77,115} or a motile lifestyle.\textsuperscript{38,45,117,119,121-125} This chapter is dedicated to the study of how a class of small molecules modulate bacterial swarming motility.

2.1.1 Types of bacterial motility

It is important to make clear distinctions of the various types of motilities that bacteria exhibit and why amongst these motilities, swarming is unique and important from an infection standpoint.\textsuperscript{38,122} The first classification of bacterial translocation was done by J. Henrichsen, where movements by hundreds of bacterial strains were classified into five main categories: swimming, swarming, gliding, twitching and sliding (Figure 2.1).\textsuperscript{122,126-133}

While swimming motility is a flagellar dependent planktonic behavior of the bacteria in liquid medium (Figure 2.1a), swarming is a surface translocation that a group of thousands of bacteria perform in coordination (Figure 2.1b).\textsuperscript{117,122} The presence of flagella is known to be essential for the occurrence both swimming\textsuperscript{132,133} and swarming\textsuperscript{123} motilities. The direction of the rotation of the flagella decides the trajectory of the motility movement. While counterclockwise rotation ensures smooth linear trajectories with speeds of up to 40 $\mu$m/s, a clockwise rotation creates a random tumbling effect (0.1 $\mu$m/s).\textsuperscript{38,117} Swarming bacterial cells are generally multi-flagellated and longer than vegetative counterparts.\textsuperscript{38,117,122} Although swarming is the movement of choice under natural colonization on surfaces, under laboratory settings swarming is usually
observed on the semisolid gels of agar (0.5 to 1 %) that contain energy rich solid media.\textsuperscript{122} Since, within a swarmer colony, each cell can move with a speed between 2 to 10 \( \mu \text{m/s} \), swarming is considered the fastest mode of bacterial surface translocation.\textsuperscript{117}

The extension and retraction of type IV pili is known to drive the slow surface associated twitching motility exhibited by planktonic bacteria (Figure 2.1c).\textsuperscript{127} In addition to this, gliding is a surface associated motility that bacteria perform without the use of flagella or pili (Figure 2.1d).\textsuperscript{128} Beside the various models like, repulsion by macromolecule, movement of outer membrane or formation of focal adhesion complexes, no one model clearly explains the gliding movement of bacteria.\textsuperscript{117} Apart from this, the passive outward movement as a consequence of colonial growth on a surface is known as sliding motility (Figure 2.1e).\textsuperscript{129-131} The reduction of surface tension by secretion of surfactant has been observed during sliding movement.\textsuperscript{122}

Figure 2.1 Bacteria perform both unicellular and multicellular movements. (a) Swarming is multicellular movement of bacteria on a surface; (b) Individual bacteria are able to move in aqueous media by beating their flagella, known as swimming motility; (c) Twitching is a unicellular bacteria movement on a surface that is facilitated by extension of the pili; (d) Gliding

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2.1}
\caption{Bacteria perform both unicellular and multicellular movements. (a) Swarming is multicellular movement of bacteria on a surface; (b) Individual bacteria are able to move in aqueous media by beating their flagella, known as swimming motility; (c) Twitching is a unicellular bacteria movement on a surface that is facilitated by extension of the pili; (d) Gliding}
\end{figure}
movement is also a unicellular movement that proceeds through formation of focal-adhesion points; (e) Sliding is passive movement across the surface that occurs due to the growth of bacterial population. The arrows in the diagram indicates the direction of movement. [Reprinted with permission from Citation] Kearns, D. B. et al, A field guide to bacterial swarming motility. Nat. Rev. Microbiol. 2010, 8, (9), 634-644.

2.1.2. Swarming motility is a multicellular phenomenon

Both swarming and biofilm formation are bacterial multicellular behaviors that contribute to the overall survival of the microbes in the environment and therefore are essential for maintaining virulence. A switch to swarming lifestyle will generally require a coordinated merger of many chemical and physical cues. Also, a certain cell density is requisite for this multicellular behavior to be initiated. The swarming phenomenon has been observed both in gram negative as well as gram positive bacterial species. So far swarming has been observed in three bacterial families including gamma-proteobacteria, alpha-proteobacteria and firmicutes. Under laboratory settings, the water content of the agar gel is critical for observing swarming. Under laboratory settings, while some bacteria can swarm on gels composed of a wide variety of agar concentrations, usually most bacteria swarm on gels above 0.5% and below 1% agar. A concentration of below 0.5% agar is usually deemed suitable for swimming motility, while concentration above 1.5% is known to inhibit swarming.

In general the key features important for ensuring that swarming occurs include: presence of flagella, cell-to-cell communication and biosynthesis of surfactants.

A number of studies have shown that mutations that reduce flagellar biosynthesis also abolish or reduce swarming motility. Contrary to this, enhanced expression of flagella
increases swarming in certain mutant species. Therefore, it is not surprising that most swarmer cells have a peritrichous arrangement of flagella. Bacteria form a bundle with these multiple flagella and the rotation of such a bundle powers the bacteria to move on a viscous media. Some bacteria like Pseudomonas aeruginosa synthesize a lateral flagella in addition to the existing polar flagella to move on a viscous surface.

The number of cells are critical to initiate the swarming process and therefore many swarming processes are linked to the cell-to-cell communication process known as quorum sensing. The swr quorum sensing system in Serratia liquefaciens which is homologous to the lux system of Vibrio fischeri has been shown to control the swarming of Serratia liquefaciens. Autoinducers, N-acylhomoserine lactones (AHLs) are known to control the biosynthesis of a biosurfactant necessary for the swarming of a P. aeruginosa, and S. marcescens; and peptides autoinducers control surfactant synthesis in B. subtilis. One important aspect of swarming is cellular collaboration manifested through the formation of "rafts" where bacteria move side by side as groups. To the present date it is not exactly clear why the formation of rafts is necessary during swarming but it is appreciated that such a process would require communication between cells.

Secretion of surfactants by bacteria is commonly observed during the swarming process. Surfactants, being amphiphilic molecules, reduce the surface tension between the bacterial cells and the substrate, hence allowing easy translocation. Under laboratory conditions, most bacteria swarm on moist semi-solid gels containing 0.5-1.0 % agar. However, some bacteria species like Proteus, Vibrio, Rhodospirillum, and Azospirillum possess the ability to swarm effectively on solid agar gels that normally inhibit swarming, i.e >1.5 % agar. Such bacterial species are known to secrete surfactants like polysaccharides to achieve
lower surface tension and create a more conducive micro environment for swarming.\(^{134,152}\) Surfactants and polysaccharides are the major components of the slime that normally surround swarmer colonies.\(^{134}\) Production of surfactin, a surface active agent secreted by \(B.\ subtilis\) is critical for its swarming.\(^{137}\) While \(B.\ subtilis\) mutants that lacked surfactin biosynthesis lacked the ability to swarm, when externally added surfactin was introduced, the mutants were able to swarm again.\(^{137}\) \(P.\ aeruginosa\) is known to secrete a class of lipopolysaccharide based molecules, known as rhamnolipids that have been shown to be critical for its swarming process.\(^{123}\)

2.1.3 Understanding the cell-to-cell communication process

Bacteria perform cell-to-cell communication with the use of unique chemical signals known as autoinducers (AI) that they produce and secrete out into their environment.\(^{41,43,64,147,155-157}\) The accumulation of these chemical signals increases in proportion to the bacterial cell density and upon reaching a threshold concentration such signals are able to bind to the cognate receptors thereby altering population wide gene expression and inducing multicellular behaviors.\(^{29,31,43,64,118,147,155,158}\) This chemical cellular communication process also known as quorum sensing has features that are conserved amongst various species while at the same time each species has elements that are unique to its system.\(^{29,43,64,118,147,155,158-160}\)

The common elements amongst the various quorum sensing systems are;\(^{29,147}\) (i) Each system is able to synthesize a chemical signaling molecule that is secreted out and upon reaching a threshold concentration such molecules are detected and induce a response. (ii) The receptors for such signals may either exist in the cytoplasm or on cell surface membranes and signal transduction activates cooperative behaviors. (iii) Additionally, signal transduction causes autoinduction of the originally secreted chemical signal. The uniqueness of each quorum sensing
systems is manifested through the existence of signals and receptors that are structurally different for different systems but specific for each signal-receptor pair.

The Gram-negatives bacteria use small molecular autoinducers that are either acyl homoserine lactones (AHL or AI-1) or furanosyl borate diester (AI-2)\textsuperscript{29,64,147,155,157} (Figure 2.2 (a &d)). Contrary to the small molecular autoinducer signals of the Gram-negatives, the autoinducers secreted by the gram positive bacteria are oligopeptides known as autoinducing peptides (AIP)\textsuperscript{29,64,147,155,157,161} (Figure 2.2b). The secreted AIPs are able to bind to cognate membrane bound histidine kinase receptors and the binding causes a phosphorylation cascade that ultimately results in alteration of gene expression.\textsuperscript{161-163} Certain Gram positive species like \textit{Streptomyces} employ γ–butyrolactones (Figure 2.2c) for regulation of antibiotic production and morphological differentiation.\textsuperscript{155,164}
Figure 2.2 Chemical signals or autoinducers employed by bacteria for cell-to-cell communication process. The figure shows the chemical structure of an autoinducer and the corresponding bacterial species that secretes the signal (a) Example of acyl-homoserine lactone (AHL) based autoinducers used by some Gram-negative bacteria (also known as AI-I); (b) Autoinducing peptides (AIP) deployed by some Gram-positive bacteria to achieve cellular communication; (c) The butyrolactone based autoinducer secreted by the Streptomyces species; (d) The borate diester (AI-2) type of autoinducer (AI-II) used during inter-cellular communication by some gram negative bacteria. [Reprinted with permission from Citation:155]
2.1.4 Components of a typical gram negative quorum sensing gene circuitry

All known quorum sensing systems described for gram negative bacteria are homologous to the LuxI/LuxR type quorum sensing system first described in *Vibrio fischeri* (Figure 2.3). In the LuxI/LuxR quorum sensing system the LuxI homologue is the AI synthase that couples the acyl group from specific donor proteins to the methionine on S-adenosyl methionine (SAM) and produces AHLs. The synthesized AHLs are secreted out of the cell and as the cellular population of bacteria in the milieu increases, the concentration of secreted AHLs also increases proportionally. When the external AHL concentration reaches a specific threshold, the binding of AHLs to the cognate LuxR type protein becomes effective. The LuxR-AHL binding results in the stabilization of the LuxR type protein and the ligand-receptor complex eventually activates the transcription of genes including the ones that control the production of virulence factors like biofilm formation, swarming, biosurfactant, pyocyanine, elastate B, and bioluminescence.

The quorum sensing systems in more than hundred Gram-negative bacteria species are known to be typified by the LuxI/LuxR type quorum sensing of *Vibrio fischeri*. The differences are that the AHL secreted by these systems are unique and are distinguished by different lengths of side chains (between C-4 to C-18) or different functional group (hydroxyl or carbonyl) decoration on the side chains (Figure 2.2a). Chemical diversity of the AHL chemical signals is matched by occurrence of corresponding homologous LuxR type receptor proteins. For each species the binding of the AHL signal to its
corresponding LuxR type protein is highly specific and critical for achieving intra species cell-to-cell communication. Some Gram-negative bacteria species like *P. aeruginosa* use more than one LuxI/LuxR type quorum sensing systems in tandem to carry out cell-to-cell communication.⁶⁴,¹⁵⁶,¹⁷⁴

![Diagram of quorum sensing](image)

**Figure 2.3** The components and the representation of a typical quorum sensing process performed by Gram-negative bacteria [Reprinted with permission from Citation:¹¹⁸ Bassler, B. L. *et al.*, The Languages of Bacteria. *Genes Dev.* 2001, 15, (Copyright (C) 2012 American Chemical Society (ACS). All Rights Reserved.), 1468-1480.]

2.1.5. *Swarming in P. aeruginosa*

*Pseudomonas aeruginosa* is a gram-negative bacteria and is the primary pathogen that colonizes the lungs of cystic fibrosis patients.¹⁷⁵-¹⁷⁹ *P. aeruginosa* uses many techniques for movement through either solution or on surfaces.¹²²,¹²³,¹³²,¹³⁴,¹³⁶,¹⁴⁴,¹⁸⁰ Generally, the single flagellated planktonic *Pseudomonas aeruginosa* propels through aqueous media with regular "beating" of the flagella.¹³²,¹³³,¹³⁶ Under laboratory conditions too, *P. aeruginosa* is able to
employ flagellar propulsion to swim in low-agar (<0.4 %) medium.\textsuperscript{119,123,132,133,181} Apart from using flagella, \textit{P. aeruginosa} is known to use the extension and retraction of pilus filament to move on surfaces.\textsuperscript{182} The type-IV pili in \textit{Pseudomonas aeruginosa} are also important for attachment to both epithelial cells and abiotic surfaces.\textsuperscript{183} However, the fastest mode of surface translocation that \textit{P. aeruginosa} performs is swarming.\textsuperscript{122}

The swarming colonies formed by \textit{P. aeruginosa} (PA14) are typified by having an appearance of tendrils originating from the center point of inoculation.\textsuperscript{123} (Figure 2.5g) Other strains like PAO1 swarm by forming a circular colony with not very conspicuous tendril formation.\textsuperscript{135} Like other bacteria, the important features necessary for swarming of \textit{P. aeruginosa} are presence of flagella and pili, quorum sensing dependent gene-regulation and the secretion of biosurfactants.\textsuperscript{123}

\textit{2.1.5.1 Swarming in \textit{P. aeruginosa} requires flagella}

Under the swarming mode the monotrichious \textit{Pseudomonas aeruginosa} vegetative cells usually become elongated and acquire a second polar flagella.\textsuperscript{136} Kohler and coworkers reported that a mutant strain \textit{Pseudomonas aeruginosa} PAO1, \textit{fliC} that did not synthesize any flagellum had impaired swarming.\textsuperscript{123} These investigators also discovered that another surface appendage which is responsible for twitching motility, type-IV pili was essential for swarming in strains PT623 and PT612 of \textit{P. aeruginosa}. The mutant strains of both PT623 and PT612, that is \textit{pilA} and \textit{pilR}, respectively were complete nonswarmers. Swarming was however partially restored in mutant \textit{pilR} when a plasmid encoding for expression of pili was introduced.\textsuperscript{123}
2.1.5.2 Alteration in gene expression of *P. aeruginosa* during swarming

Overhage and coworker were first to report that swarming is a bacterial lifestyle adaptation under a viscous environment. \(^{180}\) In adapting to a motile lifestyle, *P. aeruginosa* have been reported to up-regulate certain genes related to virulence and antibiotic resistance. \(^{180,184}\) On comparing swimming with swarming cells, they observed that there was a major shift in gene expression of virulence regulated genes like those encoding for Type-III secretion systems, extracellular proteases and iron transport. \(^{180,184}\) Amongst the up-regulated virulence genes, Overhage and coworkers showed that *lasB* and *pvdQ* were required for swarming motility in *P. aeruginosa*. \(^{184}\) The comparison of swimmer and swarmer cells also showed that the latter were more resistant to the action of at least three antibiotics, polymyxin B, gentamicin, and ciprofloxacin. \(^{180}\)

Deziel and coworkers compared the gene expression of nonswarming *Pseudomonas aeruginosa* PA14 cells to cells both at the center and at the tendril tips of a swarming colony. \(^{124}\) Deziel and co-workers’ screening showed the over-expression of 20 genes and down regulation of 121 genes by bacteria at tip of the tendril in comparison with bacteria in the swarm center of the swarming pattern. Using gene microarray they obtained transcriptomic profiles to show that a minority of the genes, including those for energy metabolism and transport of small molecules, were upregulated in cells found at the swarming tips. In contrast, they reported that genes responsible for virulence were repressed in cells found at the swarm edges as compared to the cells at the swarm center. This report of down regulation of virulence genes at swarming edges was distinct from the work of Overhage, where upregulation of virulence genes in cells at the swarming edge was reported in a direct comparison to cells found in broth. \(^{124,180}\) The work by Deziel and coworkers highlighted the existence of a bacterial subpopulation within a swarming
colony, a population of metabolically hyperactive swarmer cells ("hyperswarming") found at the edges and population of cells at the center of the colony that are more virulent ("hyposwarming").

Bacterial multicellularity is initiated by quorum sensing and ultimately results in altered gene expression. Since swarming motility has also been shown to exhibit altered gene expression, it is important to establish a link between quorum sensing and swarming.

2.1.5.3 The LasI-LasR and RhlI-RhlR Quorum Sensing Systems of P. aeruginosa are homologous to the LuxI/LuxR system found in Vibrio fischeri

In *P. aeruginosa*, there exist a hierarchical system of two LuxI/LuxR type quorum sensing circuits known as LasI/LasR and RhlI/RhlR, respectively that work in tandem to control group behavior of a population (Figure 2.4). The LasI/LasR system functions similar to the homologous LuxI/LuxR QS-system of *Vibrio fischeri*. The LasI is the AHL synthase that catalyzes the synthesis of autoinducer N-(3-oxododecanoyl) homoserine lactone (PAI-1). At a particular higher cellular density, the concentration of secreted PAI-1 is sufficient to exhibit effective binding to its cognate receptor protein, LasR. The LasR-PAI-1 complex initiates a signaling cascade that induces transcription of genes (like *lasB, lasA, apr*, and *toxA*) responsible for production of virulence factors. Additionally, the LasR-PAI-1 complex also auto regulates the *lasI* gene and directs it to synthesize more PAI-1, thus the name autoinducer is assigned to the N-acyl homoserine lactone (PAI-1) molecule. Additionally, the LasR-PAI-1 complex also activates the expression of *rhlI* and *rhlR* genes. The gene product of *rhlI*, RhlI catalyzes the synthesis of a second autoinducer, N-(butanoyl) homoserine lactone (PAI-2), which upon binding its cognate receptor protein, RhlR activates additional specific
target genes including \( rhlAB \) (Figure 2.4).\(^{189}\) The \( rhlAB \) operon encodes for the synthesis of rhamnolipids, a class of biosurfactants crucial for swarming motility.\(^{189}\) Therefore, the LasI-LasR/RhlI-RhlR two component quorum sensing system in \( P. aeruginosa \) regulates a cell density dependent expression of various virulence genes. The expression of such virulence genes includes secretion of toxins such as exotoxin A and exoenzyme S, proteases such as elastase, LasA protease, secretion of alginate, formation of biofilm, induction of swarming and production of surface wetting agents such as rhamnolipids.

**Figure 2.4 Biosynthesis of rhamnolipids by \( P. aeruginosa \).** The two-component LasI-LasR/RhlI-RhlR quorum sensing circuit of \( P. aeruginosa \) controls expression of various virulence genes, including biofilms formations, swarming and rhamnolipids biosynthesis.

\[ \text{Di-rhamnolipid} \]
\[ \text{Mono-rhamnolipid} \]
\[ 3-(3\text{-hydroxyalkanoyloxy})\text{alkanoic acids (HAA)} \]

\[ \text{H}O\]
\[ \text{H}O\]
\[ \text{H}O\]
\[ \text{H}O\]
\[ \text{H}O\]
\[ \text{H}O\]
\[ \text{H}O\]
\[ \text{H}O\]
\[ \text{H}O\]
\[ \text{H}O\]

\[ \text{LasR} \]
\[ \text{PAI-1} \]
\[ \text{Vfr} \]
\[ \text{lasR} \]
\[ \text{lasI} \]
\[ \text{lasB} \]
\[ \text{lasA} \]
\[ \text{toxA} \]
\[ \text{apr} \]
\[ \text{others?} \]
\[ \text{rhlR} \]
\[ \text{PAI-2} \]
\[ \text{rhlAB} \]
\[ \text{lasB} \]
\[ \text{rpoS} \]
\[ \text{others?} \]
\[ \text{RhlR} \]
\[ \text{RhlI} \]
Rhamnolipids are glycolipid biosurfactants secreted by \textit{P. aeruginosa}. These molecules play crucial role in maintaining biofilm architecture and assisting swarming.\textsuperscript{123,190,191} The PAI-2/RhlR complex activates the genes in the \textit{rhlAB} operon.\textsuperscript{174} The gene product of \textit{rhlA}, RhlA catalyses the synthesis of 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAA).\textsuperscript{174,189} RhlB is the rhamnosyltransferase protein encoded by the \textit{rhlB} gene which catalyses the reaction between dTDP-L-rhamnose and HAA to yield monorhamnolipids. A second rhamnosyltransferase RhlC encoded by the \textit{rhlC} gene adds the second dTDP-L-rhamnose moiety to mono-rhamnolipids, thereby synthesizing di-rhamnolipids (Figure 2.4).\textsuperscript{192} The composition of the biosurfactant released by \textit{P. aeruginosa} during swarming is usually a mixture of the different congeners that include di-rhamnolipids, mono-rhamnolipids and their intermediate precursor HAA.

2.1.5.4.1 Role of biosurfactant rhamnolipids in swarming

Multiple scientific reports indicate that rhamnolipids secreted by \textit{P. aeruginosa} play an important role in the two social activities displayed by bacteria, i.e swarming\textsuperscript{121,123,181} and biofilm formation.\textsuperscript{191} Scientific work elaborating the role of rhamnolipids in swarming is presented in this chapter, whilst work highlighting rhamnolipids' role in biofilm formation will be discussed in the next chapter (chapter 3, Section 3.1.3)

Kohler and coworkers used isogenic derivatives of wild type \textit{P. aeruginosa} strain PT5 in which either of the four crucial quorum sensing genes were inactivated i.e, \textit{lasI}, \textit{lasR}, \textit{rhlI}, or \textit{rhlR}.\textsuperscript{123} Their findings revealed that the mutants having inactivated \textit{lasI} and \textit{lasR} exhibited
reduced swarming after prolonged incubation on semisolid swarm gels (0.5-0.7 % agar) where as rhlI and rhlR mutants were completely unable to swarm. Kohler and coworkers findings led them to hypothesize that certain factors under the control of quorum sensing genes was crucial for swarming. Since rhamnolipids have surface activity properties that could be crucial in the surface motility, and since production of rhamnolipids is directly under the control of the rhl cell-to-cell signaling system, Kohler and coworkers further studied the surface motility of mutant rhlA. The mutant rhlA strain PT712 which is deficient in the production of rhamnolipids but can synthesize the quorum sensing signaling molecule, N-(butanoyl) homoserine lactone (PAI-2) was completely incapable of swarming on semisolid gel. Co-inoculating the rhlA mutant strain PT712 with wild type strain PT5 that can synthesize rhamnolipids, reinstated the swarming in the mutant strain. By this experiment, Kohler and coworkers identified the presence of rhamnolipids as being crucial for swarming motility in *P. aeruginosa*.

The unique feature that a population of swarming *P. aeruginosa* cells exhibit is the formation of a fractal-like pattern on the swim plate, where many tendrils formed by the bacterial population exude out from the central point of inoculation (Figure 2.5g). O’ Toole and coworkers were the first to demonstrate that the production of rhamnolipids were important for maintaining tendril formation and preventing colonization between tendrils. They observed that tendrils from two different swarming colonies avoided each other and when a nonmotile mutant of *P. aeruginosa, flgK* was placed in the course of a wild type swarm colony, the tendrils of the swarmer colony proximal to flgK mutant radiated away suggesting that both the wild type and flgK mutant secrete extracellular factors that cause repulsion between two colonies. Also, they observed that when wild type strain was inoculated onto a swarm plate containing spent supernatant prepared from a rhlA colony, the wild type strain swarmed normally, but when
grown with supernatant from a wild type colony, there was no swarming. The \textit{rhlA} mutant is unable to synthesize di-rhamnolipids, its precursor mono-rhamnolipids and HAA and is also a nonswarming strain (Figure 2.5 k). To evaluate the effect of each component on swarming, they further constructed mutants \textit{rhlB} and \textit{rhlC} lacking the rhamnosyltransferase necessary to generate mono-rhamnolipid and di-rhamnolipid, respectively. The swarming patterns of wildtype had a radial array of fine tendrils, where as both \textit{rhlB} and \textit{rhlC} swarmed with short, thick and irregular tendrils, suggesting that di-rhamnolipids were important for pattern formation. They further showed that when both wild type and \textit{flgk} mutant (nonswarming control) were co-inoculated on plates containing spent supernatant from either \textit{rhlB} or \textit{rhlC} mutant, the wild type did exhibit swarming with \textit{rhlC} supernatant but no swarming with supernatant from \textit{rhlB} mutant. With their study, O’Toole and coworkers reported that since the \textit{rhlC} mutant can produce mono-rhamnolipids and \textit{rhlB} mutant cannot, monorhamnolipids are the minimum component to cause swarming inhibition of wild type.

Deziel and coworkers made a deeper analysis of the role of each component from rhamnolipids biosynthesis (i.e HAA, mono-rhamnolipids and di-rhamnolipids) in creating swarm patterns of \textit{Pseudomonas aeruginosa}.\textsuperscript{121} Deziel \textit{et al} isolated and used pure HAA, monorhamnolipids and di-rhamnolipids in their experiments. They reported that the tendrils of a wild type \textit{Pseudomonas aeruginosa} (PA14) swarmer colony get attracted to a paper disk soaked with di-rhamnolipids and move away from a disk soaked with HAA (Figure 2.5 a & c). Further Deziel \textit{et al} exogenously added drops of either di-rhamnolipids, mono-rhamnolipids or HAA on the center of swarm plates and then inoculated these plates with either wild type strain or the \textit{rhlA} mutant. Their experiments showed that HAA inhibited the swarming of wild type and caused no restoration of swarming in the \textit{rhlA} mutant (Figure 2.5 f & j, respectively). Mono-rhamnolipids
facilitated the formation of tendrils in wild type and dispersion of bacteria away from the swarm center and no biomass accumulation at the center for the *rhlA* mutant (Figure 2.5 e & i, respectively). Contrary to HAA, di-rhamnolipids promoted formation of fine tendrils and biomass accumulation at the swarm center for both wildtype and the *rhlA* mutant (Figure 2.5 d & h, respectively). With these experiments, Deziel and coworkers concluded that, with respect to the active swarming cells, di-rhamnolipids act as attractants, HAA act as repellants and that mono-rhamnolipids are mere surface wetting agents (Figure 2.5b).

![Figure 2.5 Swarming pattern of either wild type PA14 or its rhlA mutant.](image)

*Figure 2.5 Swarming pattern of either wild type PA14 or its rhlA mutant.* Wild type PA14 inoculated on agar plates containing discs of either di-rhamnolipids, monorhamnolipids or HAA (a, b, and c, respectively). Swarm plates containing either dirhamnolipids or monorhamnolipids or HAAs or no agent inoculated with either wild type PA14 (d, e, f and g, respectively) or the rhlA mutant (h, i, j and k, respectively) [Adapted and modified with permission from Citation:121 Deziel, E., Self-produced extracellular stimuli modulate the Pseudomonas aeruginosa swarming

In order to postulate a theory for the formation of tendrils by swarming *Pseudomonas aeruginosa* cells, Deziel and coworkers further tested the rate of diffusion for di-rhamnolipids and HAA on agar plates. The results indicated that di-rhamnolipids diffuse faster and further on the agar plate as compared to HAA. Hence when swarmer cells are present at the center of an agar plate, the continuous secretion of di-rhamnolipids and HAA creates a positive concentration gradient of di-rhamnolipids/HAA. Since di-rhamnolipids diffuses faster in the agar and is an attractant for active swarming cells, there is a positive drive to move away from the slower diffusing repellant HAA. The occurrence of this positive concentration gradient creates tendrils which become more and more distinct as the cells migrate outward under continuously evolving di-rhamnolipids/HAA concentration gradient (Figure 2.6).

![Figure 2.6](image_url)

**Figure 2.6** (a) Diffusion rate for di-rhamnolipids and HAA on agar plates. (b) Positive concentration gradient of di-rhamnolipids/HAA maintains tendril pattern [Reprinted with permission from Citation:121 Deziel, E., Self-produced extracellular stimuli modulate the

2.1.6 Concentration dependent activity reversal phenomenon previously reported for bacterial cell-signaling molecule

*Photobacterium fischeri* is a marine bacteria that can emit visible light through a biochemical process known as bioluminescence. The bioluminescence in *Photobacterium fischeri* is controlled by the luciferase enzyme which is under the control of the quorum sensing system LuxI-LuxR. The LuxI-LuxR quorum sensing system is in turn activated by the cell-signaling molecules (AHLs) whose production increases in proportion to bacterial population. Upon activation, the luciferase enzyme catalyzes the oxidation of a long-chain aldehyde (RCHO) and reduced flavin mononucleotide (FMNH$_2$) resulting in the release of excess free energy in the form of blue-green light at 490nm.

Eberhard and coworkers in the classical paper isolate and characterize N-(3-oxohexanoyl)-3-aminodihydro-2(3H)-furanone (acyl homoserine lactone, AHL) as the cell-cell-signaling molecule that controls the quorum sensing of *Photobacterium fischeri*. In this work, Eberhard and coworkers demonstrated that both natural and synthetic AHLs molecules were able to simulate light production in *Photobacterium fischeri*. They also performed a dose-dependent study to understand effect of AHL concentration on light production. On doing a dose-response curve with synthetic AHLs, the bioluminescence increased over the range 0.003-3 μg/mL and then decreased at higher concentrations (Figure 2.7). The vague explanation the authors provided to describe this "activity reversal" is that the production of luciferase enzyme probably decreases as the bacteria reach the stationary phase. However, since it was first reported, the mechanism of how such an “activity reversal” operates appears to have been overlooked.
Figure 2.7 Dose-response curve for simulation of light production with increasing concentration of acyl homoserine lactone (AHL). Bioluminescence increased over the range 0.003-3 μg/mL and then decreased at higher concentrations. [Reprinted with permission from Citation:120 Eberhard, A.; Burlingame, A.; Eberhard, C.; Kenyon, G.; Nealon, K.; Oppenheimer, N., Structural identification of autoinducer of Photobacterium fischeri luciferase. Biochemistry 1981, 20, (9), 2444-2449.

2.2 Results and discussion

2.2.1 Design of synthetic saccharide derivatives library

The initial thought was to investigate if rhamnolipids exhibit a mere physical effect on swarming or whether their secretion is correlated with a more complex biological process. Rhamnolipids and its congeners are glycolipids biosurfactants and the amphiphiles have a disaccharide polar head group and greasy aliphatic tail. Due to the amphiphilic structure of rhamnolipids the initial inclination was to evaluate the effect of different types of generic surfactants (anionic, cationic, and nonionic) on bacterial swarming.194 The generic surfactants that were used included two non ionic surfactants, dodecyl-β-maltoside (DBM, 1) and
tetra(ethylene glycol) monododecyl ether (C\textsubscript{12}EG\textsubscript{4}OH), one anionic surfactant, sodium dodecyl sulfate (SDS), and one cationic surfactant, dodecyl trimethylammonium chloride (DTAC). Since microbes possess the ability to recognize a diverse range of saccharide stereochemistries,\textsuperscript{195-199} the chemical library also included amphiphiles that had a stereochemistry other than rhamnose and an aliphatic chain different from rhamnolipids (Figure 2.8). The chemical library included three disaccharide stereochemistries, cellobiose (Glc\textsubscript{β}(1→4)Glc\textsubscript{β}), maltose (Glc\textsubscript{α}(1→4)Glc\textsubscript{β}) and lactose (Gal\textsubscript{β}(1→4)Glc\textsubscript{β}), two monosaccharide stereochemistries, glucose (Glc\textsubscript{β}), and rhamnose (Rha\textsubscript{α}) and one large cyclic hepta-saccharide, β-cyclodextrin (βCD). The disaccharide derivatives (DSDs) comprising of maltose (Glc\textsubscript{α}(1→4)Glc\textsubscript{β})-based stereochemistry included dodecyl-β-maltoside (D\textsubscript{β}M), 1; saturated farnesyl-β-maltoside (SF\textsubscript{β}M), 14; and those comprised of cellobiose (Glc\textsubscript{β}(1→4)Glc\textsubscript{β})-based stereochemistry included dodecyl-β-cellobioside (D\textsubscript{β}C), 4; decyl-β-cellobioside (De\textsubscript{β}C), 2; undecyl-β-cellobioside (U\textsubscript{β}C), 3; tridecyl-β-cellobioside (T\textsubscript{β}C), 5; dodecyl-α-cellobioside (Da\textsubscript{C}), 6; undecylenyl-β-cellobioside (UD\textsubscript{β}C), 10; farnesyl-β-cellobioside (F\textsubscript{β}C), 11; saturated farnesyl-β-cellobioside (SF\textsubscript{β}C), 12; saturated farnesyl-α-cellobioside (SF\textsubscript{α}C), 13; 2-octyl-dodecyl-β-cellobioside (2-OD\textsubscript{β}C), 16; 2-octyl-dodecyl-α-cellobioside (2-OD\textsubscript{α}C), 17; and that comprised of lactose (Gal\textsubscript{β}(1→4)Glc\textsubscript{β})-based stereochemistry included dodecyl-β-lactoside (D\textsubscript{β}L), 7; saturated farnesyl-β-lactoside (SF\textsubscript{β}L), 15. The monosaccharide derivatives (MSDs) included dodecyl-β-glucoside (D\textsubscript{β}G), 8, and dodecyl-α-rhamnoside (Da\textsubscript{R}), 9. To examine the effect of a large oligo-saccharide group, dodecyl-β-cyclodextrin (D\textsubscript{β}CDS), 18, was synthesized. It is to be noted that the aliphatic derivatizations included derivatives with different length of hydrocarbons chains (2, 3, 4, 5), different degree of aliphatic unsaturation (10 and 11), and possessing or lacking methyl branches.
(1, 4, 6, 7, 12, 13, 14, 15). The methyl branched hydrocarbons were derived from hydrogenation of the farnesyl moiety to yield 3, 7, 11-trimethyl-dodecanyl group.

Figure 2.8. Structures of synthetic saccharide based derivatives that include maltose, cellobiose, lactose, glucose, rhamnose, and β-cyclodextrin stereochemistries; and hydrocarbons derived from farnesyl and “saturated” farnesyl molecules. The structures of di-rhamnolipid and mono-rhamnolipid are also shown.
2.2.1. 1 Synthesis of saccharide derivatives (SDs)

Most disaccharide and monosaccharide derivative were synthesized using the Fischer glycosylation and Zemplen deacetylation conditions mentioned in Scheme 2. 1 General synthetic route for the monosaccharide and the disaccharide derivatives involved the simultaneous peracetylation and monobromination at the anomeric position of desired sugar using a binary mixture of AcBr/AcOH, followed by Fischer glycosylation with either a synthetic or a commercially available alcohol in presence of Lewis acid catalyst. For obtaining β-anomer as the major glycosidation product, the solvent used was MeCN, where as substituting the solvent to MeNO₂ primarily gave α-anomer. The α/β anomers were purified with normal phase silica chromatography (β anomer usually eluted first). The per-acetylated saccharide-derivatives were deacetylated using Zemplen deacetylation conditions of methanolic sodium methoxide solution and neutralized further to pH ~6.5 with H⁺ ion-exchange amberlite resins.

Scheme 2.1. Synthesis of saccharide derivatives (SDs). (i) AcBr/AcOH, rt or 60 °C, ~ 1h; (ii) ROH, FeCl₃ or Hg(CN)₂, MeCN, rt, 1h; (iii) ROH, FeCl₃, MeNO₂, rt, 1h; (iv) MeONa/MeOH, 12h, H⁺ amberlite resin, Neutralize, (pH ~6.5).
2.2.1. Synthesis of oligosaccharide derivative

The synthesis of the oligosaccharide derivative involved a longer six step convergent synthesis (Scheme 2.2). The intermediates 18a and 18c were synthesized via previously reported literature procedure.\(^{201}\) Substituting one of the difluorophenoxy group on 18a with dodecylamine yielded 18b. Subsequently, the remaining difluorophenoxy group on 18b was substituted with βCD-NH\(_2\) over four days in a ternary solvent system comprising of water/DMF/acetone to yield DβCDS 18. The compound was purified by repeated precipitation from aqueous solution with acetone.

![Scheme 2.2 Synthesis of β-cyclodextrin (βCD) based saccharide derivative](image)

(i) CH3(CH2)11NH2, THF, -78\(^{\circ}\)C, ~ 12h; (ii) DMF, acetone, rt, 4days

2.2.2. All saccharide derivatives (SDs) were nontoxic to the growth of planktonic bacteria

At the onset it was necessary to establish that the observed impact of these molecules on swarming, and biofilm formation did not originate from the ability of these agents to impede bacterial growth. Also, establishing the nonmicrobicidal action was necessary to ascertain whether these SDs have the potential to change or control bacterial behavior with low or no propensity to invoke drug resistance.\(^{77}\) Therefore, the initial test after synthesis of the SDs was to
check the toxicity of these agents on the growth of planktonic bacteria in culture media. The concentration (170 μM) of SDs chosen for the toxicity studies was either relatively higher than or equivalent to the highest concentrations at which all the other bioassays (swarming in this chapter, and inhibition of bacterial adhesion and biofilm formation in chapter 3) were done. At 170 μM none of the SDs inhibited the growth of planktonic wild type *Pseudomonas aeruginosa* (PAO1) in Luria-Bertani (LB) broth (Figure 2.9).

![Graphs showing growth curves of PAO1 with or without agents](image)

Figure 2.9 Growth curve (24 h) of PAO1 with or without agents to confirm that the effects on swarming and biofilm formation are not due to toxic effects. Resultant concentration of each agent within wells ~ 170 μM. *Concentration of agent ~ 110 μM.*
2.2.3 The nonswarming mutant rhlA was used to screen swarming agonists and the wild type P. aeruginosa brought forward the swarming antagonists

Previously, in separate works both Deziel et al\textsuperscript{121} and O' Toole et al\textsuperscript{119} demonstrated that on supplementing the swarm agar plate (\(~0.5\%\) agar) with exogenous rhamnolipids the swarming of the inoculated \textit{rhlA} mutant was reactivated. These seminal works by others showed that either rhamnolipids were ligands for specific target proteins that control or activate swarming or that secretion of the biosurfactant rhamnolipids is simply required to reduce surface tension and enable swarming. To decipher what role rhamnolipids played in swarming (biological or physical) and to test whether the synthetic saccharide derivatives could mimic rhamnolipids, two kinds of swarming assays were constituted, swarming agonist and swarming antagonist.

A transposon mutation that impairs a gene in the rhamnolipids biosynthesis pathway of \textit{P. aeruginosa} (PAO1) generates a rhamnolipids nonproducing and therefore a nonswarming mutant strain, \textit{rhlA} (genotype, \textit{rhlA-E08::ISphoA/hah})\textsuperscript{123,202-204} Therefore, swarming agonist assays were constituted by adding various SDs into the swarm plates and then inoculating the plate with the \textit{rhlA} mutant. Agents that can mimic the functions of rhamnolipids and thereby reactivate swarming in such a nonswarming mutant would therefore qualify as swarming agonists. Contrary to this, a swarming assay with wild type PAO1 where both the naturally secreted rhamnolipids and the externally added agent are present constitutes an antagonist assay because the wild type strain can inherently synthesize rhamnolipids,\textsuperscript{123} therefore agents that have the ability to modulate swarming of such a strain will have to first compete with the natural ligand (rhamnolipids) for the putative receptor before exhibiting their effects. While inhibition of swarming by an added SD in such an antagonist swarming assay is a clear indicator of antagonistic behavior, a promotion of PAO1 swarming motility to form a bigger swarm ring is
more complex to interpret. Therefore, in the antagonist swarming assay, antagonistic behavior was primarily gauged by the ability to reduce or completely inhibit the swarming of wild type PAO1.

Firstly, the test of whether surface activity alone was critical for enabling swarming was performed by Shetye et al where, one anionic (SDS), one cationic (DTAC) and two nonionic (C<sub>12</sub>EG<sub>4</sub>OH and DβM (1)) generic surfactants were tested for their ability to reactivate swarming of the rhlA mutant, (screening for swarming agonists) (Figure 2.10a&b). The results indicated that when tested at 110 μM, three out of the four tested generic surfactants, anionic SDS, cationic DTAC and nonionic C<sub>12</sub>EG<sub>4</sub>OH were not able to reactivate the swarming of the rhlA mutant (Figure 2.10 d, e & f, respectively). However, when the disaccharide based nonionic surfactant, DβM (1) was added to the semisolid swarm gel at 110 μM, swarming of the inoculated rhlA mutant was fully restored and the swarm colony formed a pattern of radiating tendrils (Figure 2.10c).

![Images of swarm agar plates inoculated with either PAO1 (Control) or the rhlA mutant containing various generic surfactants.](image-url)
surfactants inoculated with PAO1; Swarm plate inoculated with the rhlA mutant containing; (b) No agent; (c) DβM (1); (d) DTAC; (e) SDS and (f) C12EG4OH. Concentration of the generic surfactants in the swarm plates ~ 110 μM. [Reprinted with permission from Citation:194 Shetye, G. S.; Singh, N.; Jia, C.; Nguyen, C. D. K.; Wang, G.; Luk, Y.-Y., Specific Maltose Derivatives Modulate the Swarming Motility of Nonswarming Mutant and Inhibit Bacterial Adhesion and Biofilm Formation by Pseudomonas aeruginosa. ChemBioChem 2014, 15, (10), 1514-1523.

2.2.4 Disaccharide derivatives and rhamnolipids exhibit dual functions of activating and inhibiting swarming motility of Pseudomonas aeruginosa

The antagonistic or agonistic effect of various SDs was initially screened at a constant concentration of 85 μM, except for DβC (4) and rhamnolipids, for which results from 160 μM and 30 μM are shown, respectively (Figure 2.11). The commercially available rhamnolipids used in the swarming experiments contained two congeners of rhamnolipids, dirhamnolipids (79%) and monorhamnolipid (16%) (Figure 2.8). The average molecular weight of the rhamnolipids extract calculated by using the percent composition of each cogener was 626 g/mol, and the rhamnolipids sample preparation for the swarming studies was done using this average molecular weight.

Based on the effects that the saccharide derivatives had on the swarming of both wt PAO1 and its rhlA mutant at 85μM, they can be classified into four groups (Figure 2. 11). Two SDs, DβCDS (18) and UDβC (10), that exhibited no apparent effect on the swarming motility of both the wild type PAO1 and its rhlA mutant were categorized as group I. The two SDs that were categorized as group II, DeβC (2) and FβC (11) showed weak swarming activation in rhlA mutant and slight promotion of the swarming motility in wild type PAO1. The results of one of the group II agents, DeβC (2) shown in Figure 2.11 was the effect observable at a concentration
of 160 μM and at 85 μM, \textit{De}\textsubscript{β}C (2) did not cause a noticeable effect on the swarming motility of either strains (Figure 2.12). It is noted that there was a complete abolition of bacterial growth (both PAO1 and \textit{rhlA}) on the semisolid gel (~0.5 % agar) when \textit{Fβ}C (11) was used at higher concentration of 160 μM (Figure 2.12). Contrary to the toxic effect on a swarm plate, there was no bacterial growth inhibition when planktonic PAO1 was grown in LB media containing 160 μM of \textit{Fβ}C (11) (Figure 2.9).

All of the five disaccharide derivatives (DSDs) in group III, \textit{Dβ}M (1), \textit{Uβ}C (3), \textit{Dβ}C (4), \textit{Tβ}C (5), and \textit{Da}C (6), were able cause significant tendril formation in the swarm colony of the otherwise nontendril forming wild type PAO1 strain.\textsuperscript{121,204,205} Without the presence of these five group III DSDs, the wild type PAO1(control) swarms outward with a fairly circular front, but when the DSDs were added, the swarm pattern obtained contained protrusions that radially extended outward from the central spot of bacterial inoculation. The type of tendrils formed by wild type PAO1 in presence of these five DSDs also varied slightly. Two DSDs from group III, \textit{Dβ}M (1) and \textit{Dβ}C (4) created PAO1 swarm patterns having fine straight tendrils that protruded out from the center and these tendrils itself did not branch out further. However, the three other DSDs in this group, \textit{Uβ}C (3), \textit{Tβ}C (5) and \textit{Da}C (6) caused the main radial tendrils to branch out and form a self-similar, fractal-like pattern (Figure 2.11). Among the DSDs in group III, three DSDs, \textit{Dβ}M (1), \textit{Uβ}C (3) and \textit{Dβ}C (4), were able to fully reactivate the swarming of the \textit{rhlA} mutant with a swarm area that was comparable to that of the wild type PAO1 without any agents (Control, Figure 2.11). The other two DSDs in group III, \textit{Tβ}C (5) and \textit{Da}C (6) at the tested concentration of 85 μM exhibited no apparent swarming activation of the \textit{rhlA} mutant.

The group IV consisted of six DSDs, \textit{Dβ}G (8), \textit{Da}R (9), \textit{SFβ}C (12), \textit{SFα}C (13), \textit{SFβ}M (14), and \textit{2-ODβ}C (16) that at 85 μM were able to completely inhibit the swarming motility of
wild type PAO1 (Figure 2.11). Also, the six group IV DSDs at 85 μM concentration in the swarm plate exhibited no apparent effect on the inoculated rhlA mutant (Figure 2.11).

For the swarming studies done with the rhamnolipids extract at a concentration of 10 μM there was a ~38 % increase in the swarming area of the rhlA mutant. Also, when used at 30 μM, the rhamnolipids extract caused a ~51 % decrease in swarming area for wild type PAO1 (to see the calculation of how the swarming areas and percentage increase or decrease in areas were calculated, refer to Materials and Methods section). The change in percent swarming area was obtained by comparing the swarm area in presence of the agents to that by the wild type PAO1 under the same conditions with no added agents in the swarm plate (~0.5 % agar). Apart from changing the swarm pattern qualitatively, certain DSDs also had an impact on the swarm area of either or both PAO1 and the rhlA mutant. By measuring the increase in the swarming area, the DSDs grouped as II appear to promote swarming in both PAO1 and the rhlA mutant at 85 μM. The effect of group III DSDs was more complex because at 85 μM while they inhibit the swarming of PAO1 they seem to activate the swarming of the rhlA mutant. The DSDs in group IV at 85 μM drastically reduce the swarm area of PAO1 and have no apparent effect on the swarm area of the rhlA mutant (decrease in swarm area cannot be detected because the rhlA mutant is inherently nonswarming). Three DSDs, DβL (7), SFβL (15) and 2-ODαC (17) had poor water solubilities and therefore were not tested in the swarming agonist or antagonist assays.

Two important observations from the swarming studies done with various agents were (i) Rhamnolipids inhibit swarming of PAO1 at 30 μM whereas at 10 μM they activate or promote swarming of the rhlA mutant; (ii) The DSD DβC (2) seemed to promote swarming of PAO1 and activate swarming of the rhlA mutant only at relatively higher concentration (160 μM).
Because these two agents exhibited the dual functions of either activating or inhibiting swarming at different concentrations, it was speculated that the other SDs, in general would also possess these dual-functions, and may manifest different activities at different concentrations. Therefore, a concentration dependent study with selected SDs from various groups (II, III and IV) was done next.

|            | Control | I.  | II. 
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<td><img src="image30.png" alt="Image" /></td>
<td><img src="image31.png" alt="Image" /></td>
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Figure 2.11 Four different types of swarming motility behaviors exhibited by wild type PAO1 and the \textit{rhlA} mutant on semisolid gel (~0.5 \% agar, M8 media) in presence of various DSDs (at ~85\,\mu M), except for \textsuperscript{a}De\(\beta\)C (2) (160 \,\mu M), and \textsuperscript{b}Rhamnolipids (30 \,\mu M for PAO1) and (10 \,\mu M for \textit{rhlA}). Controls are without added agents in the gel. Images were taken 24 h after inoculation with bacteria.

![Figure 2.11](image)

Figure 2.12 Semisolid gels (~0.5 \% agar) with 0 (no agent) or 85 \,\mu M concentration of De\(\beta\)C (2) or 160 \,\mu M concentration of F\(\beta\)C (11) inoculated at the center with either wt PAO1 (row 1) or \textit{rhlA} mutant (row 2). Images were taken 24 h after inoculation with bacteria.

2.2.5 \textit{Disaccharide derivatives and rhamnolipids exhibited “activity reversals” that transitioned from swarm-activating to swarm-inhibiting as the concentration was increased}

The fact that concentration of the DSD determined the overall effect on swarming was clearly observable when a switch in De\(\beta\)C (2) concentration from 85 to 160 \,\mu M increased the \textit{rhlA} swarming area by ~38\% with respect to swarming area of PAO1 without agents. The effect that different concentrations of either rhamnolipids or De\(\beta\)C (2) had on swarming of PAO1 and its \textit{rhlA} mutant alluded to two opposites bioactivities – concentration dependent activation and
inhibition of swarming motilities. Comparison of the PAO1 swarm area on a plate with no added agent (~24 cm$^2$) to a plate containing 85μM of DSD $\text{D}_\beta\text{C} (4)$ (~13 cm$^2$), showed that swarm area decreased by a value of ~46% (Figure 2.11). Therefore, intriguingly at 85μM, $\text{D}_\beta\text{C} (4)$ displayed the ability to both activate swarming in the $rhlA$ mutant and at the same time cause an overall apparent inhibition for PAO1 swarming.

It is has been reported before by Deziel and coworkers that the bacteria at the swarming tip are genetically different than bacteria in the swarm center (See section 2. 1. 5. 2). The ability of certain group III DSDs, $\text{D}_\beta\text{M} (1)$, $\text{U}_\beta\text{C} (3)$ and $\text{D}_\beta\text{C} (4)$ to activate the swarming of the $rhlA$ mutant and also induce tendril formation in PAO1 swarms suggested that newer bacterial phenotypes may be generated by different DSDs. It can be hypothesized that the tip of the tendril may be populated with swarming hyperactive and the stem of the tendril along with the center of the colony may be populated with hyposwarming phenotype, termed as "phenotypic bifurcation" here. To see if "phenotypic bifurcation" could be achieved by rhamnolipids and other DSDs and see if such an effect depends on the concentration of the agent in the plate, a dose-dependent study with rhamnolipids and selected DSDs from group II, III and IV was performed.

First, the concentration effect of the rhamnolipids extract on swarming of both PAO1 and its $rhlA$ mutant was investigated. When the concentration of rhamnolipids in the swarm plate was increased from 0 to 5 μM, the swarming of the inoculated nonswarming $rhlA$ mutant appeared to be reactivated; and the size of the PAO1 swarm ring appeared increase in area in comparison to the control (Figure 2.13, row 1). The trend in swarming reactivation for the $rhlA$ mutant by rhamnolipids extract reversed at about 10 μM, at which concentration the size of the swarming ring was maximum (~9 cm$^2$). The reversal in swarming activation beyond 10 μM ultimately
culminated with no observable swarming at 20 μM or higher by rhlA (swarm ring size was reduced to a dot). With PAO1, 10 and 15 μM of externally added rhamnolipids caused the swarming rings to appear larger than the control (with no added agents), but with 30 μM of added rhamnolipids, a significant reduction in the swarming ring was observed. Quantitatively, with 30 μM of rhamnolipids in the swarm plate, the wild type PAO1 covered an area of ~11 cm² and with no rhamnolipids the swarm area was ~24 cm². This observation indicated that, as the concentration of rhamnolipids is gradually increased, a shift from swarm-activation to swarm-inhibition occurs. The occurrence of an activity reversal with increasing ligand concentration has also been reported for another bacterial multicellular behavior, simulated light production by Photobacterium fischeri (section 2.1.6). In this classical work, Eberhard and coworkers had demonstrated that when the concentration of the AHL (ligand) is gradually increased, the production of light by Photobacterium fischeri first increases, reaches a threshold and ultimately decreases.

Similar to the effects that rhamnolipids extract had, the active DSDs (Group II, III and IV from Figure 2.11) also caused swarming promotion at lower concentration and swarming inhibition at relatively higher concentration. The dose-dependent swarming results with five DSDs using wild type PAO1 and its rhlA mutant are summarized in Figure 2.13. The images of swarming plates in Figure 2.13 containing various concentrations of DSDs clearly show the transitions from swarming activating to swarming inhibiting for both bacterial strains. For all the active DSDs, the concentrations at which the swarming transitioned from being activated or promoted to deactivated or inhibited for rhlA and PAO1, respectively were different. In general, the concentrations at which the swarming activity reversed (from activated to deactivated) for the rhlA mutant were slightly lower than the concentrations at which swarming of PAO1 were
inhibited (Table 2.1). Apart from this, the DSDs themselves could be classified into two categories; those that caused an early swarming "activity reversal" at lower concentration and those caused a late "activity reversal" at relatively higher concentrations (Figure 2.13 & Table 2.1). The low transition concentration agents included rhamnolipids, SFβM (14) and SFβC (12) that caused an activity reversal for the rhlA mutant at 10 μM, 10 μM and 8 μM; and swarming inhibition for PAO1 at 20-30 μM, 7-20 μM, 10-20 μM, respectively. The DSDs with high transition concentration, DSDs DβC (4), TβC (5) and DβG (8) exhibited the activity reversal at 45-56 μM, 40 μM and 35 μM for the rhlA mutant; and swarming inhibition for PAO1 at 20-30 μM, 20-30 μM and 35-50 μM, respectively. As visible in the swarming images with rhlA mutant (Figures 2.13), the low transition agents, SFβC (12), SFβM (14) and rhamnolipids created smaller swarming rings in comparison to the agents with high transition concentration, DβC (4), TβC (5) and DβG (8). These results imply that agents with poor swarming inhibition capabilities (for PAO1) were better swarming activators (for the rhlA mutant) than agents that had strong potencies to inhibit the swarming of PAO1. Another observation that the dose-response study brought forward was that certain DSDs beyond the transition concentration were able to induce tendril formation while others were not. Amongst the plates inoculated with the rhlA mutant, only DβC (4) induced tendril formation around ~56 μM (Figure 2.13); whereas for the plates inoculated with PAO1, tendrils formation was caused by DβC (4) at 20 μM, TβC (5) at ~30 μM, SFβM (14) at ~2 μM and SFβC (12) at ~1μM (Figure 2.13).
Figure 2.13 Images of swarming motilities of the \textit{rhlA} mutant (left) and PAO1 (right) on semisolid gel (~0.5 \% agar) containing increasing concentrations of DSDs and rhamnolipids. The concentrations are indicated above the images, and the identities of the agents are shown to the left. Red boxes highlight the swarming patterns that reveal the dominance of DSDs over rhamnolipids at comparable concentrations.

The swarming patterns of \textit{rhlA} with additional concentrations of \textit{DβC (4)}, and \textit{TβC (5)} apart from those included in Figure 2.13 are shown in Figure 2.14. Interestingly, quantitative measurement of the \textit{rhlA} mutant's swarm areas with these two DSDs, \textit{DβC (4)}, and \textit{TβC (5)}, indicated that after the first activity reversal around 50 μM, there was a second increase in swarm area at a higher concentration of 160 μM (Figure 2.14). This increase, then decrease followed by another increase in swarming area of the \textit{rhlA} mutant in response to the changing concentrations.
of the two DSDs is indicative of an oscillatory response by the bacteria. Overall the dose-response study indicated that both rhamnolipids and the active DSDs possess the unique dual abilities to both activate swarming at lower concentration, an agonistic behavior as well as inhibit swarming at relatively higher concentrations, an antagonistic behavior.

**Table 2.1.** Transition concentrations of DSDs that exhibit activity reversal from activating to inhibiting swarming motilities of the rhlA mutant and PAO1.

<table>
<thead>
<tr>
<th>Compound</th>
<th>a Transition concentration (rhlA)</th>
<th>b Transition concentration (PAO1)</th>
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<tr>
<td>Rhamnolipids</td>
<td>~10-15 μM</td>
<td>~20-30 μM</td>
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<tr>
<td>SFβM (14)</td>
<td>~10-15 μM</td>
<td>~7.5-25 μM</td>
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<tr>
<td>SFβC (12)</td>
<td>~8-12 μM</td>
<td>~10-20 μM</td>
</tr>
<tr>
<td>DβC (4)</td>
<td>c ~45-56 μM</td>
<td>~20-30 μM</td>
</tr>
<tr>
<td>TβC (5)</td>
<td>~40 μM</td>
<td>~20-30 μM</td>
</tr>
<tr>
<td>DβG (8)</td>
<td>~35-50 μM</td>
<td>~50-60 μM</td>
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<tr>
<td>SFαC (13)</td>
<td>c--</td>
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*a*From activating to inhibiting swarming motility of the *rhlA* mutant. *b*From promoting to inhibiting swarming motility of PAO1; *c*Not determined.

![Figure 2.14 Swarm agar plates with additional concentrations of DβC (4) or TβC (5) for](image-url)
their effects on the swarming of the rhlA mutant. Both the DSDs caused swarming “activity reversal.” Nonswarming mutant rhlA was inoculated at the center of the semisolid gels (~0.5 % agar). Pictures were taken 24 h after inoculation with bacteria.

2.2.6 Disaccharide derivatives, SFβC (12), SFβM (14) and DβC (4) dominated rhamnolipids at inhibiting the swarming motility of PAO1

The activation-to-inhibition “activity reversal” effect on swarming in response to concentration of both rhamnolipids and some DSDs suggests a regulatory response by PAO1 to such agents. On evaluating the potencies of the active DSDs, two separate dominating effects over rhamnolipids seem to stand out. Firstly, the swarming motility of wild type PAO1 that is facilitated by in situ secretion of natural rhamnolipids was inhibited by all the five DSDs tested, i.e SFβC (12), SFβM (14), DβC (4), TβC (5), and DβG (8). The concentrations at which each of these DSDs dominated and inhibited the in situ produced rhamnolipids were different, with SFβC (12), SFβM (14), DβC (4), being more potent than TβC (5) and DβG (8) (Figure 2.13, right column and Figure 2.15). Secondly, for PAO1, three DSDs, SFβC (12), SFβM (14) and DβC (4) exhibited stronger swarming inhibition than externally rhamnolipids at the same concentrations. The dominance of SFβC (12) over externally added rhamnolipids is evident at 20 μM, where at this concentration SFβC (12) completely reduced the swarmer colony to a dot occupying an area of 1.2 cm² which is about 5% of the 24 cm² exhibited by PAO1 without any added agents; at this concentration, the external rhamnolipids caused no apparent swarming inhibition and the swarm area was equivalent to the control (i.e. 24 cm²) (Figure 2.13). Apart from this, as tendril formation amounts to an overall reduction in swarming area, two other DSDs SFβM (14) and DβC (4) at concentrations lower than 20 μM exhibited stronger swarming inhibition of wild type PAO1 than externally added rhamnolipids. Additionally, the unique
characteristic of DSDs, SFβM (14) and DβC (4) to induce tendrils and cause phenotypic bifurcation of wild type PAO1 swarms was also not achieved by the externally added rhamnolipids at the tested concentration range (1-30 μM). A prominent activation dominance of DSD SFβM (14) over externally added rhamnolipids was also exhibited by the rhlA swarm colony. When the areas of swarm colonies at 10 μM of either SFβM (14) or external rhamnolipids were compared, the SFβM (14) caused swarming activation to generate a ring of ~38 cm² whereas only a 9 cm² ring was observed for rhamnolipids (Figure 2.13 and Figure 2.15). When viewed together, these results highlight that DSDs, SFβC (12), SFβM (14) and DβC (4) are more potent than both the in situ produced (and secreted) and the externally added rhamnolipids. The rhlA swarming results with increasing concentrations of rhamnolipids and one DSD, SFβM (14) was repeated three times to obtain an error bar for swarming area at each each concentration (see Materials and Methods, Figure S1).
Figure 2.15 Plot of the \textit{rhlA} mutant’s swarm area (after 24 h) versus increasing concentrations of rhamnolipids, D\textbeta{}C (4), T\textbeta{}C (5), SF\textbeta{}M (14), D\textbeta{}G (8) and SF\textbeta{}C (12) in the semisolid gel (~ 0.5% agar). Insert shows the curves that had low transition concentrations from swarm-activating to swarm-inhibiting. (Also see Materials and Methods, Figure S1)

2.2.7 \textit{Competition assays indicated SF\textbeta{}C (12) to be a stronger swarming inhibitor than D\textbeta{}C (4)}

While results indicate that both SF\textbeta{}C (12) and D\textbeta{}C (4) could activate and inhibit the swarming motility of \textit{P. aeruginosa}, SF\textbeta{}C (12) had a lower transition (from activating to inhibiting) concentration than D\textbeta{}C (4) (Table 2.1). From the previous dose-dependence study (Figure 2.13), it was evident that 20 μM of SF\textbeta{}C (12) did not activate the swarming motility of the \textit{rhlA} mutant. However, from these results it was difficult to speculate whether at this
concentration (20 μM), \textit{SFβC (12)} had an inhibiting effect or whether it had no effect on the swarming of the \textit{rhlA} mutant. \textit{SFβC (12)} did inhibit the swarming motility of wild type PAO1 at 20 μM and hence there was some indication that the corresponding effect on the \textit{rhlA} mutant may be primarily inhibition and not no effect. Contrary to the effect of \textit{SFβC (12)}, the swarming activation of the \textit{rhlA} mutant by 40 μM \textit{DβC (4)} was quite unambiguous. Considering the results of these DSDs, it was hypothesized that the DSDs with low transition concentrations for the “activity reversal” dominated the DSDs with high transition concentrations. In order to validate this hypothesis, a competitive swarming assay was constructed where both \textit{DβC (4)} and \textit{SFβC (12)} were introduced into the swarming gel. For this swarming assay, the bacteria of choice was the \textit{rhlA} mutant primarily because it lacks production of rhamnolipids and hence the swarming results would be unambiguous. In the competition swarming assay the concentration of \textit{DβC (4)} was kept constant at 40 μM in all the plates, but the concentration of \textit{SFβC (12)} was incrementally increased from 0.5 to 10, 20, and 40 μM (Figure 2.16) from one plate to another. The competition assay demonstrated that when 10 μM of \textit{SFβC (12)} was added into the gel containing did 40 μM of \textit{DβC (4)}, there was no inhibition of \textit{rhlA} swarming activation. But when the concentration of \textit{SFβC (12)} was increased to 20 μM, the swarming activation brought about by 40 μM of \textit{DβC (4)} was completely inhibited. Parallel to what was hypothesized, this result suggested that \textit{SFβC (12)} (having low transition concentration) is a stronger antagonist than \textit{DβC (4)} (having relatively higher transition concentration) for the putative swarming receptor. Also, the experiment confirmed that \textit{SFβC (12)} has a dominating effect on swarming at high enough concentrations for both wild type PAO1 and its \textit{rhlA} mutant.
Figure 2.16 Images of the \textit{rhlA} mutant inoculated on semisolid surface (\textendash 0.5 \% agar) containing 40 \text{$\mu$}M of DβC (4) and increasing concentrations of SFβC (12). The images were taken 24 hours after inoculation of the surface with bacteria.

\textbf{2.3 Conclusion}

A surfactant having a saccharide head and an aliphatic tail, DβM (1) was the only generic surfactant that reactivated the swarming of the \textit{rhlA} mutant. These swarming results with generic surfactants showed that while surface activity may be important for swarming, not any surfactant could instill swarming in the \textit{rhlA} mutant. Amongst the saccharide derivatives (SDs), few specific structures showed the ability to activate and inhibit the swarming motility of both \textit{P. aeruginosa} and its nonswarming mutant \textit{rhlA}. For controlling swarming motility, all the active DSDs and the rhamnolipids extract exhibited a dual function that transitioned from swarm-activating at low concentrations to swarm-inhibiting at higher concentrations. Certain DSDs, SFβC (12), SFβM (14) and DβC (4) were able to dominate the effect of both \textit{in situ} produced (and secreted) and the externally added rhamnolipids. The dominance of these DSDs over rhamnolipids is probably a result of better binding to putative swarming receptor or the ability to bind effectively to an allosteric site on the receptor. Competition swarming assay revealed that a DSD with lower transition concentration (SFβC (12)) was a better antagonist than a DSD with higher transition concentration DβC (4). Results implied that secretion of rhamnolipids is sensed...
by the bacteria and the binding of these rhamnolipids to the putative swarming receptor relays a biological response. Results also demonstrated that certain DSDs are the functional analogues of rhamnolipids and that certain DSDs overpower the effects of rhamnolipids.

2.4 Materials and methods

General experimental information for synthesis

Standard solvents and reagents were purchased from commercial sources (Sigma-Aldrich, Fisher, Acros) and used as received. Solvents were removed in vacuo using Büchi rotary evaporator below 40°C. EMD silica gel 60 F254 pre-coated plates (0.25-mm thickness) were used for TLC. Unless otherwise stated, TLC visualization was done using ceric ammonium molybdate (CAM) stain. $^1$H-NMR spectra were recorded in deuterated NMR solvents at 300 and 400 MHz ($^1$HNMR) and 75 and 100 MHz ($^{13}$CNMR) Bruker instruments respectively. $^1$H chemical shifts are reported in ppm relative to CDCl$_3$ δ 7.26, D$_2$O δ 4.80, or CD$_3$OD δ 3.31. $^{13}$C chemical shifts are reported relative to CDCl$_3$ δ 77.23 and CD$_3$OD δ 49.0. Couplings are reported in hertz (Hz). HRMS was obtained through positive ESI on Bruker 12 Tesla APEX-QE FTICR-MS with an Apollo II ion source, at Cosmic instrumentation center, Virginia. Unless otherwise stated, the % yield indicates the pure anomeric products that were successfully isolated by column chromatography resolution (the yield for mixture of anomers has not been accounted for). Anomeric configurations are assigned from the magnitude of $J_{1,2}$ (7–9 Hz for the diaxial coupling, β-configuration; 2–4 Hz indicates equatorial-axial coupling, α-configuration).$^{207}$

MALDI-TOF.$^{201}$ Matrix Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-MS). A saturated solution of 2,4,6-trihydroxyacetophenone monohydrate (matrix) (10 mg) was prepared in acetonitrile (1 mL). The saturated βCD compound solution (in water) and matrix
solution in 1:1 ratio were spotted on a MTP 384 target ground steel plate (Bruker Daltonics, Billerica, MA). Mass spectra were obtained on a Bruker AutoFlex III instrument (Bruker Daltonics, Billerica, MA). Molecular ion peak was detected in positive ion linear mode.

**Synthetic procedures and spectral data.** The synthetic procedures and spectral data for the compounds used in this work are given below.

**Synthesis of per-acetylated-bromo-sugars (Scheme 1).** The synthesis of saccharide derivatives was done by using a reported literature procedure with some modifications. Briefly, to an oven dried round bottom flask, disaccharide (maltose, lactose and rhamnose) (1.0 g, 2.8 mmol), AcBr (~3.6 mL, 44.4 mmol), and AcOH (19 mL) were added and stirred at room temperature (25 °C) for ~ 1 h. Reaction mixture was concentrated *in vacuo* at 35 °C and then co-evaporated three times with PhMe (2×10 mL, anhydrous). After removal of solvent, the flask was further heated *in vacuo* at 50 °C for 15 min to give a foamy solid, aceto-bromo sugars. The crude aceto-bromo sugars were immediately used in next step without any further purification.

The synthesis of per-acetyl-bromo cellobiose was done under more vigorous conditions. In an oven dried round bottom flask cellobiose (1.0 g, 2.8 mmol), AcBr (~7.2 mL, 88.8 mmol), and AcOH (19 mL) were added and stirred at 60 °C for ~ 1 h. The work up of aceto-bromo cellobiose (4b) involved similar steps as done for aceto-bromo lactose/rhamnose to obtain crude as foamy solid. The crude aceto-bromo sugars were immediately used in next step without any further purification.

**General synthesis of n-alkyl glycosides (cellobiosides, maltoside, rhamnoside and lactoside)**

For obtaining β-anomer as major product, the crude aceto-bromo sugars were the dissolved in MeCN (10 mL) and various alcohols (5.6 mmol, 2 equiv) were added along with two equivalents
of FeCl₃ or Hg(CN)₂. The α-anomers were obtained as major products by using MeNO₂ as solvent. The reaction mixture was stirred vigorously for about ~45-60 min at rt. After stirring at rt, aq KBr (10%, 25 mL) and then PhMe (60 mL) were added under stirring. The organic phase was washed twice with aq KBr (10%, 2×25 mL), once with aq NaHCO₃ (5%, 25 mL) and twice with H₂O (2×25 mL). The crude product was then purified by column chromatography using gradient elution (100 % hexane to 35 % ethyl acetate in hexane).

**Zemplén deacetylation.** For all acetylated sugar derivatives, the deprotection of acetyl groups were achieved by Zemplén deacetylation using MeONa/MeOH (10 mM solution) conditions followed by neutralization (pH ~6.5) over H⁺ amberlite resins. The resins were filtered off and products dried under high vacuum overnight.

**Spectral data**

![Spectral data](image)

**Acetylated decyl-β-cellobioside, ADeβC (2a)** Colorless powder, 0.302 g, 19.7 % (2-step), Rf = (0.30, 40 % EtOAc in Hexane). ¹H NMR (300 MHz, CDCl₃): δ 5.21 (m, 3H), 4.92 (q, J₁₋₂ = 8.4 Hz, 2H), 4.54-4.43 (m, 3H), 4.39 (dd, J₁₋₃ = 12.9 Hz, J₁₋₂ = 4.8 Hz, 1H), 4.13-4.02 (m, 2H), 3.87-3.74 (m, 2H), 3.69-3.56(m, 2H), 3.44 (q, J₁₋₂ = 8.4 Hz, 2H), 2.13-1.99 (s, 7 X 3H), 1.57-1.48 (m, 2H), 1.26 (br, s, 14 H), 0.88 (t, J= 6.3 Hz, 3H). ¹³C NMR (75 MHz, CDCl₃): δ 170.0, 169.9, 169.8, 169.1, 168.8, 168.6, 100.3, 100.2, 72.5, 72.2, 72.1, 71.5, 71.2, 69.8, 67.3, 61.5, 61.1, 31.4, 29.1, 29.1, 28.9, 28.8, 28.8, 25.3, 22.2, 20.4, 20.1, 20.1, 13.6. HRMS (ESI) m/z: Calcd. (C₃₆H₅₆O₁₈)Na⁺: 799.3358; Found: 799.3339, Difference: -2.4 ppm.
**Decyl-β-celllobioside, DeβC (2):** Colorless powder, 0.025 g, 87 %. \(^1\)H NMR (300 MHz, CD\(_3\)OD) \(\delta\): 4.36 (d, \(J = 7.8\) Hz, 1H), 4.28 (d, \(J = 7.8\) Hz, 1H), 3.87 (br, s, 4H), 3.69-3.47 (m, 4H), 3.40-3.31 (m 4H, overlapping with MeOH), 3.27-3.18 (m, 2H), 1.68-1.56 (m, 2H), 1.30 (br, s, 14H), 0.91 (t, \(J = 6.6\) Hz, 1H). \(^1\)C NMR (75 MHz, CD\(_3\)OD): \(\delta\) 102.8, 102.4, 78.9, 76.3, 76.0, 74.6, 73.1, 73.0, 69.5, 69.2, 60.6, 60.0, 31.3, 29.1, 29.0, 28.9, 28.8, 28.7, 25.3, 21.9, 12.7. HRMS (ESI) m/z: Calcd. (C\(_{22}\)H\(_{42}\)O\(_{11}\))Na\(^+\): 505.2619; Found: 505.2611, Difference: -1.5 ppm

**Acetylated undecyl-β-celllobioside, AUβC (3a):** Colorless powder, 0.313 g, 13.5 % (2-step), Rf = (0.52, 1:1 Hexane:EtOAc). \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\): 5.16-4.96 (m, 3H), 4.90-4.81 (m, 2H), 4.51-4.27 (m, 4H), 4.02 (t, \(J = 13.2\) Hz, 2H), 3.84-3.67 (m, 2H), 3.65-3.49 (m, 2H), 3.46-3.24 (m, 1H), 2.07-1.89 (s, 7 x 3H), 1.57-1.31 (m, 2H), 1.20 (br, s, 16H), 0.82 (br, s, 3H). \(\delta\) 170.0, 169.9, 169.8, 169.81, 168.8, 168.5, 100.4, 95.1, 72.6, 71.4, 71.2, 71.1, 70.6, 69.3, 68.2, 67.6, 67.3, 61.1, 61.0, 31.4, 29.1, 28.8, 28.8. 28.7, 25.5, 25.3, 22.2, 20.4, 20.2, 20.1, 20.0, 13.6. HRMS (ESI) m/z: Calcd. (C\(_{37}\)H\(_{58}\)O\(_{18}\))Na\(^+\): 813.3545; Found: 813.3496, Difference: -2.4 ppm
**Undecyl-β-cellobioside, UβC (3):** Colorless powder, 0.024 g, 84 %. $^1$H NMR (300 MHz, MeOD) $\delta$: 4.38 (d, $J = 7.8$, 1H), 4.25 (d, $J = 7.8$, 1H), 3.91 (br, s, 4H), 3.79-3.68 (m, 1H), 3.58-3.44 (m, 3H), 3.39-3.26 (m, 2H, overlapping with MeOD), 3.25-3.15 (m, 2H). $^{13}$C NMR (75 MHz, CD$_3$OD): $\delta$ 102.8, 102.4, 78.9, 76.3, 76.0, 74.6, 73.1, 73.0, 69.5, 69.2, 60.6, 60.0, 31.3, 29.0, 29.0, 28.8, 28.7, 25.3, 22.0, 12.7. HRMS (ESI) m/z: Calcd. (C$_{23}$H$_{44}$O$_{11}$)Na$^+$: 519.2776; Found: 519.276, Difference: -1.5 ppm

![Undecyl-β-cellobioside](image)

**Acetylated dodecyl-β-cellobioside, ADβC (4a):** Colorless powder, 0.330g, 14 % (2-step), Rf = (0.47, 42% EtOAc in Hexane). $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 5.19-5.01 (m, 3H), 4.90 (q, $J_{1-2} = 8.1$ Hz, 2H), 4.50-4.40 (m, 3H), 4.10-4.00 (m, 2H), 3.82-3.72 (m, 2H), 3.66-3.55 (m, 2H), 3.43(q, $J_{1-2} = 2.7$ Hz, 1H), 2.10-1.96 (s, 7 X 3H), 1.50 (br, s, 2H), 1.23 (br, s, 18H), 0.86 (t, $J = 6.6$ Hz, 3H). $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 170.1, 169.9, 169.8, 169.5, 169.2, 168.9, 168.7, 100.4, 100.3, 76.2, 72.5, 72.2, 72.1, 71.5, 71.2, 69.8, 67.3, 61.5, 61.1, 31.5, 29.3, 29.2, 29.2, 29.0, 29.0, 28.9, 28.8, 28.7, 25.4, 22.3, 14.1. HRMS (ESI) m/z: Calcd. (C$_{38}$H$_{60}$O$_{18}$)Na$^+$: 827.3672; Found: 827.3665, Difference: -1.0 ppm

![Acetylated dodecyl-β-cellobioside](image)

**Dodecyl-β-cellobioside, DβC (4):** Colorless powder, 0.082 g, 92.0 %. $^1$H NMR (300 MHz, MeOD) $\delta$ 4.39 (d, $J = 7.5$ Hz, 1H), 4.26 (d, $J = 8.1$ Hz, 1H), 3.85 (br, s, 4H), 3.66-3.40 (m, 4H),
3.35-3.31 (m, 2H, overlapping with MeOD), 3.29-3.16 (m, 2H), 1.65-1.54 (m, 2H), 1.27-1.22 (br, s, 18 H), 0.88 (t, J = 7.5 Hz, 3H). 13C NMR (75 MHz, CD3OD): δ 102.8, 102.4, 78.9, 76.3, 76.0, 74.6, 73.1, 73.0, 69.5, 69.2, 60.6, 60.0, 31.3, 29.0, 28.9, 28.8, 28.7, 25.3, 21.9, 12.7. HRMS (ESI) m/z: Calcd. (C24H46O11)Na+: 533.2932; Found: 533.2931, Difference: <-1.0 ppm

Acetylated tridecyl-β-cellobioside, ATβC (5a): Colorless powder, 0.345g, 14.4 % (2-step), Rf = (0.50, 1:1 Hexane: EtOAc). 1H NMR (300 MHz, CDCl3) δ 5.16-4.98 (m, 3H), 4.85 (q, J1-2 = 8.4 Hz, 2H), 4.48-4.29 (m, 4H), 4.06 - 3.97 (m, 2H), 3.82 - 3.69 (m, 2H), 3.66 - 3.50 (m, 2H), 3.44 - 3.34 (m, 1H), 2.07-1.93 (s, 7 X 3H), 1.56 -1.32 (m, 2H), 1.20 (br, s, 20 H), 0.83 (t, J = 8.7 Hz, 3 H). 13C NMR (75 MHz, CDCl3): δ 170.1, 169.9, 169.8, 169.5, 169.2, 168.9, 168.7, 100.4, 100.3, 76.2, 72.5, 72.2, 72.1, 71.5, 71.2, 69.9, 67.3, 61.5, 61.1, 31.5, 29.3, 29.2, 29.1, 28.9, 25.4, 22.3, 20.5, 20.3, 20.2, 13.7. HRMS (ESI) m/z: Calcd. (C39H62O18)Na+: 841.3828; Found: 841.3808, Difference: <-2.3 ppm

Tridecyl-β-cellobioside, TβC (5): Colorless powder, 0.052 g, 91.0 %. 1H NMR (300 MHz, MeOD) δ 4.38 (d, J = 7.8 Hz, 1), 4.24 (d, J = 7.8 Hz, 1H), 3.83 (br, s, 4H), 3.62 (dd, J1-2 = 12.0 Hz, J1-2 = 5.1 Hz, 1H), 3.56-3.44 (m, 3H), 3.37-3.27 (m, 5H, overlapping with MeOD), 3.22-3.16 (m, 2H), 1.61-1.54 (m, 2H), 1.25 (br, s, 20 H), 0.86 (t, J = 6.6 Hz, 3H). 13C NMR (75 MHz, CD3OD): δ 102.8, 102.4, 78.9, 76.3, 76.0, 74.6, 73.1, 73.0, 69.5, 69.2, 60.6, 60.0, 31.3, 29.0,
28.9, 28.8, 28.7, 25.3, 22.0, 12.7. HRMS (ESI) m/z: Calcd. (C\textsubscript{25}H\textsubscript{48}O\textsubscript{11})Na\textsuperscript{+}: 547.3089; Found: 547.3080, Difference: <-1.5 ppm

**Acetylated dodecyl-\(\alpha\)-cellobioside, AD\(\alpha\)C (6a):** Colorless powder, 0.050 g, 2.1 % (2-step)\}, Rf = (0.39, 42 % EtOAc in Hexane). \(^1\)H NMR (300 MHz, CDCl\textsubscript{3}): \(\delta\) 5.46 (t, \(J = 8.4\) Hz, 1H), 5.21 - 4.87 (m, 4H), 4.78 (dd, \(J_{1,3} = 10.8, J_{1,2} = 4.8, 1H\)), 4.56-4.34 (m, 3H), 4.16-3.87 (m, 4H), 3.73-3.63 (m, 3H), 3.44-3.35 (m, 1H), 2.13-1.99 (s, 7 x 3H), 1.65-1.56 (m 2H), 1.26 (br, s, 18 H), 0.88 (t, \(J = 7.2\) Hz, 3H). HRMS (ESI) m/z: Calcd. (C\textsubscript{38}H\textsubscript{60}O\textsubscript{18})Na\textsuperscript{+}: 827.3671; Found: 827.3663, Difference: -1.0 ppm. The spectroscopic data was consistent with a previously reported report.\(^{208}\)

**Dodecyl-\(\alpha\)-cellobioside, DaC (6):** Colorless powder, 0.010 g, 80.2 %. \(^1\)H NMR (300 MHz, MeOD) \(\delta\) 4.76 (d, \(J = 3.9, 1H\)), 4.39 (d, \(J = 7.8, 1H\)), 3.86 (t, \(J = 11.4, 2H\)), 3.79-3.76 (m, 1H), 3.71-3.63 (m, 2H), 3.60-3.43 (m, 3H), 3.40-3.31 (m, 5 H, overlapping with MeOD), 3.23 (t, \(J = 8.7, 1H\)), 1.72-1.56 (m, 2H), 1.29 (br, s, 18 H), 0.90 (t, \(J = 6.9, 3H\)). \(^{13}\)C NMR (75 MHz, CD\textsubscript{3}OD): \(\delta\) 100.4, 98.9, 72.4, 72.1, 71.3, 69.9, 69.6, 69.0, 68.0, 61.9, 61.1, 47.8, 31.5, 29.2, 29.0, 25.4, 22.3, 20.6, 20.5, 20.4, 13.7. HRMS (ESI) m/z: Calcd. (C\textsubscript{24}H\textsubscript{46}O\textsubscript{11})Na\textsuperscript{+}: 533.2932; Found: 533.2931, Difference: <-1.0 ppm. The spectroscopic data was consistent with a previously reported report.\(^{208}\)
**Acetylated dodecyl-β-lactoside, ADβL (7a):** Colorless powder, 0.282 g, 12 % (2-step), Rf = (0.40, 1:1 Hexane:EtOAc).\(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 5.35 (d, \(J = 0.6\) Hz, 1H), 5.34-5.08 (m, 2H), 4.97-4.85 (m, 2H), 4.46 (t, \(J = 17.4\) Hz, 3H), 4.14-4.04 (m, 3H), 3.62-3.48 (m, 2H), 2.17-1.97 (s, 7 X 3H), 1.61-1.47 (m, 2H), 1.22 (br, s, 18 H), 0.88 (t, \(J = 7.2\) Hz, 3H).

Spectroscopic data is consistent with reported literature.\(^{209}\) HR-MS: Calcd. (C\(_{38}\)H\(_{60}\)O\(_{18}\))Na\(^+\): 827.3671; Found: 827.3677, Difference: < 1.0 ppm.

**Dodecyl-β-lactoside, DβL (7):** Colorless powder, 0.150 g, 85 %.\(^1\)H NMR (300 MHz, CD\(_3\)OD): \(\delta\) 4.29 (t, \(J = 7.2\) Hz, 1H), 4.08 (t, \(J = 6.6\) Hz, 1H), 3.92-3.78 (m, 3H), 3.75-3.66 (m, 5H), 3.55-3.44 (m, 5H), 3.41-3.32 (m, 2H overlapping with MeOD), 3.18 (dd, \(J_{1,3} = 16.2\) Hz, \(J_{1,2} = 3\) Hz, 2H), 1.66-1.55 (m, 2H), 1.29 (br, s, 18 H), 0.90 (t, \(J = 7.2\) Hz, 3H). Spectroscopic data is consistent with reported literature.\(^{209}\) HRMS (ESI) m/z: Calcd. (C\(_{24}\)H\(_{46}\)O\(_{11}\))Na\(^+\): 533.2932; Found: 8533.2923, Difference: -1.7 ppm.

**Acetylated dodecyl-α-rhamnoside, ADαR (9a):** Colorless powder, 0.200 g, 7.3 % (2-step), Rf = (0.30, 40 %: EtOAc in Hexane).\(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 5.30 (dd, \(J = 10.1, 3.7\) Hz, 1H),
5.23 (dd, $J = 3.7$, 1.9 Hz, 1H), 5.06 (t, $J = 9.9$ Hz, 1H), 4.71 (d, $J = 1.4$ Hz, 1H), 3.9–3.85 (m, 1H), 3.68–3.64 (m, 1H), 3.43–3.40 (m, 1H), 2.15–1.99 (s, 3 X 3H), 1.63–1.56 (m, 2H), 1.33–1.30 (m, 2H), 1.28 (br s, 16H), 1.22 (d, 3H), 0.88 (t, $J = 7.1$ Hz, 3H). Spectroscopic data is consistent with reported literature.

**Dodecyl-α-rhamnoside, ADαR (9):** Colorless powder, 0.03g, 12 %. $^1$H NMR (300 MHz, CD$_3$OD): $\delta$ 4.62 (br, s, 1H), 3.76 (br, s, 1H), 3.67–3.52 (m, ~3H), 3.39–3.27 (m, overlapping with MeOD), 1.55–1.53 (m, 2H), 1.27 (br, s, 18H), 1.24 (d, $J = 8$ Hz, 3H), 0.89 (t, $J = 6$ Hz, 3H).

$^{13}$C NMR (75 MHz, MeOD): $\delta$ 102.8, 102.4, 78.9, 76.3, 76.0, 74.6, 73.1, 73.0, 69.5, 69.2, 60.6, 60.0, 31.3, 29.0, 28.9, 28.8, 28.7, 25.3, 22.0, 12.7. $^{13}$C NMR (100 MHz, CD$_3$OD): $\delta$ 104.2, 76.8, 76.5, 75.0, 74.9, 72.3, 71.8, 35.6, 33.3, 33.3, 33.2, 33.1, 33.0, 29.9, 26.3, 20.5, 17.0. HRMS (ESI): Calcd. (C$_{19}$H$_{38}$O$_5$)Na$^+$ {[M + Na]$^+$ = 369.2611; Found: 369.2610, Difference = < -1.0 ppm.

**Acetylated undecylene-β-cellobioside, UDβC (10a):** Colorless powder, 0.350g, 15.2 % (2-step), Rf = (0.53, 60% EtOAc in Hexane). $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 5.88–5.74 (m, 1H), 5.21–5.02 (m, 4H), 4.95–4.86 (m, 3H), 4.52–4.42 (m, 3H), 4.37 (dd, $J_{1,3} = 12.3$ Hz, $J_{1,2} = 4.2$ Hz, 1H), 4.12–4.02 (m, 2H), 3.86–3.55 (m, 4H), 3.44 (q, $J_{1,2} = 6.6$ Hz, 1H), 2.13–1.98 (s, 7 x 3H), 1.57–1.40 (br, s, 2H), 1.34 (br, s, 12H). $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 170.1, 169.9, 169.8,
HRMS (ESI) m/z: Calcd. (C$_{37}$H$_{56}$O$_{18}$)Na$^+$: 811.3359; Found: 811.3343, Difference: < -1.9 ppm

Undecylene-β-cellobioside, UDβC (10): Colorless powder, 0.200 g, 94.0 %. $^1$H NMR (300 MHz, CD$_3$OD) δ 5.88-5.75 (m, 1H), 5.01 (d, $J = 1.8$ Hz, 1H), 4.96 (d, $J = 1.8$, 1H, overlapped with H$_2$O), 4.42 (d, $J = 7.8$, 1H), 4.29 (d, $J = 7.8$ Hz, 1H), 3.93-3.85 (m, 4H), 3.70-3.61 (m, 1H), 3.58-3.48 (m 3H), 3.41-3.31 (m 4H, overlapping with MeOD), 3.23-3.20 (m, 2H), 2.05 (q, $J_{1-2} = 6.6$ Hz, 2H), 1.62 (q, $J_{1-3} = 13.5$ Hz, $J_{1-2} = 6.6$ Hz, 2H), 1.32 (br, s, 12H). $^1$C NMR (100 MHz, CD$_3$OD): δ 138.7, 113.3, 103.2, 102.8, 79.3, 76.7, 75.1, 75.0, 73.5, 73.4, 70.0, 69.5, 61.0, 60.5, 33.5, 29.4, 29.3, 29.2, 29.1, 28.8, 28.7, 25.7. (ESI) m/z: Calcd. (C$_{23}$H$_{42}$O$_{11}$)Na$^+$: 517.2619; Found: 517.2619, Difference: < -1.0 ppm

Acetobromo-cellobiose, AC (11a): Synthesis of acetobromo-cellobiose was done as reported earlier. Aceto-bromo cellobiose was purified using gradient elution (100 % hexane to 40 % ethyl acetate in hexane). Rf = 0.3 (40 % ethyl acetate in hexane). $^1$H NMR (300 MHz, CDCl$_3$): 6.54 (d, $J = 6.0$ Hz, 1H), 5.55 (t, $J = 9.0$Hz, 1H, 5.20-5.06 (m, 2H), 4.96 (t, $J = 6.0$ Hz, 1H), 4.78 (dd, $J_{1-3} = 9.0$ Hz, $J_{1-2} = 3.0$ Hz, 1H), 4.57-4.53 (m, 2H), 4.39 (dd, $J = 12.0$ Hz, $J = 3.0$ Hz, 1H), 4.20-4.16 (m, 2H), 4.07 (d, $J = 12.0$ Hz, 1H), 3.85 (t, $J = 9.0$ Hz, 1H), 3.71-3.67 (m, 1H), 2.15-2.00 (s, 7 X 3H).
**Acetylated farnesyl-β-cellobioside, AFβC (11b):** Glycosylation of acetobromo cellobiose with farnesol was achieved by using Hg(CN)$_2$. To a round bottom flask acetobromo-bromo cellobiose (0.5 g, 7.0 mmol) and farnesol (0.158 g, 7.0 mmol) were added and dissolved in benzene (10 mL). Hg(CN)$_2$ (0.182 g, 7.15 mmol) was then added and the mixture was allowed to stir at 50 °C for ~ 2 hours. After 2 hours H$_2$O (25 mL) was added and the organic phase was extracted with ethyl acetate (2 X 15 mL). The combined organic phase were washed with brine and dried with Na$_2$SO$_4$ (anhydrous), and concentrated in vacuo. The product was purified using a gradient solvent system (100 % hexane to 40 ethylacetate in hexane). Colorless powder, 0.143 g, 23.8 %. Rf = (0.50, 60% EtOAc: Hexane). $^1$H NMR (300 MHz, CDCl$_3$): δ 5.24 (br, s, 1H), 5.17-5.02 (m, 5H), 4.90 (q, $J_{1-2}$ = 6.9 Hz, 2H), 4.46 (br, s, 3 H), 4.35-4.25 (m, 1H), 4.16 (br, s, 2H), 4.05 (t, $J$ =14.7 Hz, 2H), 3.75(t, $J$ =10.8 Hz, 1H), 3.66-3.63 (m, 1H), 3.57-3.56 (m, 1H), 2.11-1.97 (s, 7 x 3H), 1.66-1.59 (s, 4 x 3H). $^{13}$C NMR (75 MHz, CDCl$_3$): δ 170.9, 170.7, 170.6, 170.3, 170.0, 169.7, 169.5, 142.8, 135.9, 131.1, 124.7, 124.0, 119.5, 101.2, 99.0, 73.3, 73.1, 73.0, 72.3, 72.0, 71.9, 68.1, 65.6, 62.4, 61.9, 40.1, 40.0, 27.1, 26.7, 26.1, 21.2, 21.1, 21.1, 21.0, 20.9, 18.1, 16.8, 16.4. HRMS (ESI) m/z: Calcd. (C$_{41}$H$_{60}$O$_{18}$)Na$^+$: 863.3671; Found: 863.3652, Difference: -2.3 ppm
**Farnesyl-β-cellobioside, FβC (11):** The deprotection of acetylated-farnesyl cellobioside (11b) was done using Zemplén deacetylation conditions as reported above. Colorless powder, 0.020g, 95.3 %. 1H NMR (300 MHz, CD$_3$OD): δ 5.39 (t, $J = 6.3$, 1H), 5.10 (q, $J_{1,2} = 6.9$ Hz, 2H), 4.24-4.21 (m, 4H), 3.91-3.85 (m, 3H), 3.69-3.47 (m, 3H), 3.40-3.19 (m, 7H overlapping with MeOD), 2.19-1.93 (m, 8H), 1.69 (s, 3H), 1.67 (s, 3H), 1.61 (s, 3H), 1.60 (s, 3H). 13C NMR (75 MHz, CD$_3$OD): δ 140.9, 135.3, 131.1, 124.4, 124.1, 120.9, 103.6, 101.5, 79.7, 77.1, 76.8, 75.5, 75.4, 73.9, 73.8, 70.3, 65.3, 61.4, 60.8, 39.9, 39.7, 26.8, 26.4, 24.9, 16.8, 15.5, 15.1. HRMS (ESI) m/z: Calcd. (C$_{27}$H$_{46}$O$_{11}$)Na$^+$: 569.2932; Found: 569.2926, Difference: -1.1 ppm.

**Saturated farnesol, SF (12b):** The reduction of trans-trans farnesol was done using H$_2$, Pd/C conditions using reported literature procedure (Scheme 3).$^{211}$ Briefly, a flask containing farnesol (0.887g, 3.96 mmol) and Pd/C (10% Pd) (0.1 g) in 10 mL MeOH was evacuated and filled with H$_2$ gas. The reaction mixture was allowed to stir at room temperature for ~ 24 hours, following which Pd/C was filtered off and purification was done over silica column to obtain a viscous oil as product (0.300 g, 32.9%) 1H NMR (300 MHz, CDCl$_3$): δ 3.63-3.73 (m, 2H), 1.49-1.62 (m, 4H), 1.05-1.38(m, 13H), 0.84-0.90 (m, 12H). Spectroscopic data is consistent with reported literature.$^{211}$

**Acetylated saturated farnesyl-β-cellobioside, ASFβC (12a):** Colorless powder, 0.080g, 21.0 % (2-step), Rf = (0.53, 60% EtOAc: Hexane). 1H NMR (300 MHz, CDCl$_3$): δ 5.21-5.03 (m, 3H),
4.91 (q, J$_{1-2}$ = 7.8 Hz, 2H), 4.52-4.49 (m, 2H), 4.44 (dd, J$_{1-3}$ = 8.1 Hz, J$_{1-2}$ = 1.8 Hz, 1H), 4.37 (dd, J$_{1-3}$ = 12.3 Hz, J$_{1-2}$ = 4.2 Hz, 1H), 4.06-4.02 (m, 2H), 3.92-3.83 (m, 1H), 3.77 (t, J = 9.3 Hz, 1H), 3.68-3.56 (m, 2H), 3.52-3.44 (m, 1H), 2.13-1.98 (s, 7 x 3H), 1.54-1.04 (m, 18 H), 0.88 (s, 3H), 0.86 (s, 3H), 0.85 (s, 3H), 0.83 (s, 3H). $^{13}$C NMR (75 MHz, CDCl$_3$): δ 170.7, 170.6, 170.5, 170.1, 169.8, 169.5, 169.3, 101.8, 100.8, 76.8, 73.2, 72.9, 72.8, 72.7, 72.2, 71.9, 68.8, 68.8, 62.2, 61.8, 39.6, 37.7, 37.6, 37.5, 33.0, 30.0, 29.7, 28.2, 25.0, 24.6, 23.0, 22.9, 21.1, 20.9, 20.8, 20.7, 20.0, 19.9, 19.8, 19.7, 19.5. HRMS (ESI) m/z: Calcd. (C$_{41}$H$_{66}$O$_{18}$)Na$^+$: 869.4141; Found: 869.4123, Difference: -2.1 ppm

Saturated farnesyl-β-cellobioside, SFβC (12): Colorless powder, 0.034g, 85.7 % $^1$H NMR (300 MHz, CD$_3$OD) δ 4.42 (d, J = 7.8 Hz, 1H), 4.29 (d, J = 7.8 Hz, 1H), 3.94 (br, s, 4H), 3.60-3.41 (m, 4H), 3.38-3.26 (m, 4H, overlapping with MeOD), 3.23 (t, J = 5.7 Hz, 2H), 1.75-1.13 (m, 18 H), 0.92-0.87 (s, 4 x 3H). $^{13}$C NMR (75 MHz, CD$_3$OD): δ 103.6, 103.3, 79.7, 77.1, 76.8, 75.4, 73.9, 73.8, 70.3, 68.3, 61.4, 60.8, 39.5, 37.7, 37.5, 37.4, 33.0, 29.9, 28.1, 24.9, 24.5, 22.1, 19.2, 19.1, 19.0, 18.9. HRMS (ESI) m/z: Calcd. (C$_{27}$H$_{52}$O$_{11}$)Na$^+$: 575.3401; Found: 575.3394, Difference: -1.3 ppm
Acetylated Saturated farnesyl-α-celllobioside, ASFαC (13a): Colorless powder, 0.059 g, 21.0 % (2-step), Rf = (0.48, 60% EtOAc: Hexane). $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 5.46 (t, $J = 10.2$, 1H), 5.19-5.05 (m, 2H), 4.78 (dt, $J_1 = 4.5$ Hz, $J_2 = 3.0$ Hz, 1H), 4.39 (dd, $J_{1,3} = 12.6$ Hz, $J_{1,2} = 4.8$ Hz, 1H), 4.17 (dd, $J_{1,3} = 12.3$ Hz, $J_{1,2} = 4.8$ Hz, 1H), 4.05 (dd, $J_{1,3} = 12.3$ Hz, $J_{1,2} = 2.4$ Hz, 1H), 3.96-3.89 (m, 3H), 3.42 (p, $J_{1,3} = 15.6$ Hz, $J_{1,2} = 7.8$ Hz, 1H), 2.14-1.99 (s, 7 x 3H), 1.57-1.00 (m, 18 H), 0.90-0.84 (s, 4 x 3H). $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 170.9, 170.8, 170.7, 169.7, 169.5, 101.3, 96.1, 95.9, 73.5, 72.3, 72.1, 71.5, 71.4, 70.2, 68.5, 68.2, 62.3, 62.0, 39.7, 37.8, 37.7, 36.6, 33.2, 30.1, 28.4, 25.2, 24.8, 23.1, 23.0, 21.3, 21.1, 21.0, 21.0, 19.9, 19.8, 19.7. HRMS (ESI) m/z: Calcd. (C$_{41}$H$_{66}$O$_{18}$)Na$^+$: 869.4141; Found: 869.4120, Difference: -2.4 ppm

Saturated farnesyl-α-celllobioside, SFαC (13a): Colorless powder, 0.020g, 95.3 % $^1$H NMR (300 MHz, CD$_3$OD): $\delta$ 4.77 (d, $J = 3.6$ Hz, 1H), 4.40 (d, $J = 8.1$ Hz, 1H), 3.92-3.88 (m, 2H), 3.81-3.63 (m 4H), 3.57-3.33 (m, 3H), 3.36-3.29 (m, 2 H, overlapping with MeOD), 3.23 (t, $J = 8.1$ Hz, 2H), 1.79-1.50 (m, 3H), 1.46-1.22 (m, 10 H), 1.21-1.03 (m, 5H), 0.92-0.86 (s, 4 x 3H). $^{13}$C NMR (75 MHz, MeOD): $\delta$ 103.9, 99.0, 98.9, 79.8, 77.1, 76.9, 73.9, 72.5, 72.3, 71.1, 70.3, 61.4, 60.8, 39.6, 37.8, 37.5, 36.7, 33.0, 30.1, 28.2, 24.9, 24.4, 22.1, 22.0, 19.2, 18.9. HRMS (ESI) m/z: Calcd. (C$_{27}$H$_{52}$O$_{11}$)Na$^+$: 575.3401; Found: 575.3395, Difference: -1.3 ppm
Acetylated saturated farnesyl-β-maltoside, ASFβM (14a): Colorless powder, 0.040g, 29.0 % (2-step), Rf = (0.41, 60% EtOAc: Hexane). $^1$H NMR (300 MHz, CDCl$_3$): δ 5.43-5.36 (m, 2H), 5.25 (t, $J = 9.3$ Hz, 1H), 5.07 (t, $J = 4.5$ Hz, 1H), 4.97-4.79 (m, 2H), 4.53-4.42 (m, 2H), 4.29-4.20 (m, 2H), 4.07-3.85 (m, 4H), 3.70-3.65 (m, 1H), 3.53-3.44 (m, 1H), 2.14-2.01 (s, 7 x 3H), 1.66-1.16 (m, 18 H), 0.89 (br, s, 12H). $^{13}$C NMR (75 MHz, CDCl$_3$): δ 169.5, 169.4, 169.2, 169.1, 169.0, 100.0, 99.8, 72.4, 72.3, 71.8, 71.6, 69.6, 68.9, 68.1, 67.6, 62.1, 61.1, 60.0, 37.1, 36.9, 32.4, 29.3, 27.5, 24.4, 23.9, 22.3, 22.2, 20.6, 20.5, 20.4, 20.3, 20.2, 19.3, 19.2, 19.1, 18.8, 13.8. HRMS (ESI) m/z: Calcd. (C$_{41}$H$_{66}$O$_{18}$)Na$^+$: 869.4141; Found: 869.4130, Difference: -1.2 ppm

Saturated farnesyl-β-maltoside, SFβM (14): Colorless powder, 0.022g, 96.0 % $^1$H NMR (300 MHz, CD$_3$OD): δ 5.17 (br, s, 1H), 4.27 (d, $J = 9$ Hz, 1H), 3.95-3.92 (m, 4H), 3.88-3.43 (m, 6H, overlap with CD$_3$OD peak), 3.27-3.20 (m, 4H), 1.79-1.13 (m, 18 H), 0.90 (s, 3H), 0.89 (s, 3H), 0.88 (s, 3H), 0.87 (s, 3H). $^{13}$C NMR (75 MHz, CD$_3$OD): δ 102.6, 101.6, 79.5, 76.1, 74.8, 73.3, 72.9, 72.5, 72.4, 69.7, 67.5, 67.4, 60.9, 60.4, 38.7, 36.9, 36.7, 36.6, 32.2, 29.1, 27.4, 24.1, 23.7, 21.3, 21.2, 18.4, 18.3, 18.2, 18.2. HRMS (ESI) m/z: Calcd. (C$_{27}$H$_{52}$O$_{11}$)Na$^+$: 575.3401; Found: 575.3399, Difference: < -1.0 ppm
Acetylated saturated farnesyl-β-lactoside, ASFβL (15a): Colorless powder, 0.059 g, 21.0 % (2-step), Rf = (0.48, 60% EtOAc: Hexane). $^1$H NMR (300 MHz, CDCl$_3$): δ 5.53-5.47 (m, 1H), 5.30-5.16 (m, 1H), 5.12-5.07 (m, 1H), 4.94-4.86 (m, 3H), 4.66-4.60 (m, 1H), 4.54-5.41 (m, 3H), 4.33-4.23 (m, 1H), 4.15-4.07 (m, 2H), 3.99-3.59 (m, 4H), 3.53-3.26 (m, 2H), 2.17-1.96 (s, 7 x 3H), 1.70-1.02 (m, 18 H), 0.88 (s, 3H), 0.85 (s, 3H), 0.84 (s, 3H), 0.82 (s, 3H). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 169.4, 169.0, 168.8, 168.5, 168.3, 168.1, 99.6, 98.3, 76.3, 76.2, 76.0, 75.7, 75.1, 72.6, 71.5, 70.8, 70.7, 70.0, 69.0, 68.4, 67.2, 67.4, 67.0, 66.2, 61.3, 61.0, 60.5, 47.1, 38.9, 36.4, 36.2, 35.3, 31.8, 28.7, 28.5, 27.0, 26.2, 23.8, 23.3, 21.7, 21.6, 20.0, 19.9, 19.9. HRMS (ESI) m/z: Calcd. (C$_{41}$H$_{66}$O$_{18}$)Na$^+$: 869.4141; Found: 869.4135, Difference: < 1.0 ppm

Saturated farnesyl-β-lactoside, SFβL (15): Colorless powder, 0.020 g, 95.3 % $^1$H NMR (300 MHz, CD$_3$OD): δ 4.41 (t, $J = 6.8$ Hz, 1H), 4.33-4.29 (m, 1H), 4.09-4.08 (m, 1H), 3.95-3.88 (m 2H), 3.83-3.76 (m, 3H), 3.76-3.66 (m, 2 H), 3.56-3.32 (m, 6H), 3.31-3.21 (m, 1H), 3.17 (d, $J = 5.2$ Hz, 1H), 1.57-1.06 (m, 18 H), 0.92 - 0.87 (s, 4 x 3H). $^{13}$C NMR (75 MHz, CD$_3$OD): δ 103.9, 103.4, 79.6, 75.4, 74.9, 73.4, 73.1, 72.0, 67.9, 66.2, 60.4, 54.4, 48.2, 48.1, 47.9, 37.1, 37.0, 36.4, 32.6, 29.5, 29.0, 27.7, 24.5, 24.0, 21.6, 18.8, 18.7. HRMS (ESI) m/z: Calcd. (C$_{27}$H$_{52}$O$_{11}$)Na$^+$: 575.3402; Found: 575.3403, Difference: < 1.0 ppm.
Acetylated-2-octyl-dodecyl-β-cellobioside, 2-AODβC (16a): Colorless powder, 0.240 g, 9.0 %
(2-step). Rf = (0.53, 60% EtOAc in Hexane). $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 5.21-5.03 (m, 3H),
4.92 (q, $J_{1,2}$ = 6.6 Hz, 2H), 4.53-4.50 (m, 2H), 4.43-4.34 (m, 2H), 4.07 (t, $J$ = 16.8, 2H), 3.81-
3.74 (m, 2H), 3.68-3.54 (m, 2H), 3.27 (t, $J$ = 6.3 Hz, 1H), 2.13-1.99 (s, 7 x 3H), 1.59-1.48 (m, 1H),
1.26 (br, s, 32H), 0.89 (t, $J$ = 6.3 Hz, 6H). HRMS (ESI) m/z: Calcd. (C$_{46}$H$_{76}$O$_{18}$)Na$^+$:
939.4923; Found: 939.4910, Difference: -1.5 ppm

2-Octyl-dodecyl-β-cellobioside, 2-ODβC (16): Colorless powder, 0.05 g, 94.0 %. $^1$H NMR
(300 MHz, CD$_3$OD): $\delta$ 4.43 (d, $J$ = 7.8 Hz, 1H), 4.26 (d, $J$ = 9.9, 1H), 3.93 (br, s, 3H), 3.84-3.78
(m, 4H), 3.70-3.49 (m, 3H), 3.42-3.31 (m, 5H, overlapping with MeOD), 3.29-3.21(m, 2H),
1.69-1.57 (m, 1H), 1.31 (br, s, 32 H), 0.92 (t, $J$ = 6.3 Hz, 6H). $^{13}$C NMR (75 MHz, CD$_3$OD): $\delta$
102.8, 79.0, 76.3, 76.0, 74.7, 74.6, 73.1, 73.0, 72.3, 69.6, 60.6, 60.2, 37.6, 31.3, 30.4, 29.4, 29.4,
29.0 (8 carbons), 28.9, 28.9, 28.7, 26.1, 22.0, 12.7. HRMS (ESI) m/z: Calcd. (C$_{32}$H$_{62}$O$_{181}$)Na$^+$:
645.4184; Found: 645.4179, Difference: < -1.0 ppm
Acetylated-2-octyl-dodecyl-α-cellobioside, 2-AODαC (17a): Colorless powder, 0.040g, 1.5% (2-step), Rf = (0.48, 60% EtOAc in Hexane). 1H NMR (300 MHz, CDCl3): δ 5.43 (t, J = 9.3 Hz, 1H), 5.11 (p, J1,3 = 18.3 Hz, J1,2 = 9.0 Hz, 2H), 4.96 - 4.90 (m, 2H), 4.77 (dd, J1,3 = 10.2 Hz, J1,2 = 3.3 Hz, 1H), 4.52 - 4.45 (m, 2H), 4.38 (dd, J1,3 = 12.6 Hz, J1,2 = 4.5 Hz, 1H), 4.15 - 4.02 (m, 2H), 3.88 - 3.73 (m, 1H), 3.69 - 3.55 (m, 4H), 3.20 (t, J = 6.3 Hz, 1H), 2.13 - 1.98 (s, 7 X 3H), 1.62 - 1.55 (m, 1H), 1.26 (br, s, 32H), 0.88 (t, J = 6.6 Hz, 3H). 13C NMR (75 MHz, CDCl3): δ 170.1, 169.9, 169.8, 169.8, 169.1, 168.8, 168.5, 100.5, 95.3, 76.8, 76.4, 72.6, 71.5, 71.3, 71.1, 70.8, 69.4, 67.6, 67.3, 61.4, 61.1, 37.3, 31.4, 30.8, 30.4, 29.6, 29.2, 29.2, 28.9, 26.3, 26.2, 22.2, 13.7. HRMS (ESI) m/z: Calcd. (C46H76O18)Na+: 939.4923; Found: 939.4910, Difference: < 1.4 ppm

2-Octyl-dodecyl-α-cellobioside, 2-AODαC (17): Colorless powder, 0.015g, 83.5% 1H NMR (300 MHz, CD3OD): δ 4.44 (t, J = 3.6 Hz, 1H), 4.27 (t, J = 3.6 Hz, 1H), 3.89 (br, s, 3H), 3.77 - 3.71 (m, 2H), 3.63 - 3.60 (m, 2H), 3.49 - 3.21 (m, 8H, overlapping with MeOD peak), 1.68 - 1.57 (m, 1H), 1.31 (br, s, 32H), 0.91 (m, 6H). 13C NMR (75 MHz, MeOD): δ 102.8, 98.4, 79.2, 76.3, 76.0, 73.0, 71.6, 71.5, 70.7, 70.3, 69.5, 60.6, 59.9, 37.5, 31.2, 30.5, 29.3, 29.3, 29.2, 28.9, 28.7, 28.6, 28.6, 28.5, 26.1, 26.0, 26.0, 21.6, 13.0. HRMS (ESI) m/z: Calcd. (C32H62O181)Na+: 645.4184; Found: 645.4178, Difference: -1.0 ppm
Difluoro phenoxy squarate, DPSq (18a) - (Scheme 2) Finely powdered 1, 2-Dihydroxycyclobutene-3,4-dione (1.10 g, 9.6 mmol), carbon tetrachloride (20 mL), and DMF (0.05 mL), were introduced into a 50 mL round-bottomed flask previously filled with argon. The mixture was frozen by immersing in dry ice-acetone and oxalyl chloride (2.10 mL, 24 mmol) was introduced at once through the condenser's outlet. Then the flask was allowed to warm to room temperature, agitation started at 300 rpm, and the flask was then lowered into an oil bath thermostatted to 50-60 °C. Regular gas evolution started immediately and was no more apparent after 50 min. After heating further for 3 h the agitation was discontinued. The solvent was removed to give 1,2-dichlorocyclobutene-3,4-dione as yellow crystal. (Yield = 1.15 g, 80 %)

(1.15 g, 7.6 mmol) of 1,2-dichlorocyclobutene-3,4-dione was dissolved in benzene (50 mL), and the solution was cooled to 0°C. 3,5-Difluorophenol (2.0 g, 15.3 mmol) was added to the above solution in one portion, followed by slow addition of excess Et$_3$N (2.090 g, 20.674 mmol), and the reaction mixture was stirred at room temperature overnight. The reaction mixture was filtered, and the residue obtained was washed with benzene to yield a white solid. The solid obtained was purified using flash silica gel column (20% EtOAc in hexanes) to give 2 (0.80 g, 27%) as a white solid. Rf = 0.59 (20% EtOAc in hexanes). Yield = {white powder, 0.282 g, 12 % (2-step)}, Rf = (0.40, 1:1 Hexane:EtOAc). 1H NMR (300 MHz, CDCl$_3$): δ 6.90-6.88 (m, 4H), 6.84-6.78 (m, 2H).

Spectroscopic data are consistent with reported values.$^{212}$

Dodecylamine-phenoxy squarate, DPSq (18b): A round bottom flask was charged with difluoro-bis phenoxy squarate (0.04 g, 1.18 mmol) and THF (5 mL, anhydrous distilled over
CaH$_2$) was added. The solution was cooled to -78 $^\circ$C in a dry-ice/acetone bath. Dodecyl amine (0.0153 g, 0.8 mmol) dissolved in THF (5 mL, anhydrous) was then added dropwise over 30 minutes. The mixture was stirred at -78 $^\circ$C for 2 hours. Column chromatographic separation using a gradient of 100 % hexane to 30 % ethyl acetate in hexane yielded product as a viscous oil, 0.025 g, 54.3 % ( Rf = 0.46 (30 % ethyl acetate in hexane). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 6.92- 6.87 (m, 2H), 6.73-6.68 (m, 1H), 3.76-3.52 (m, 2H), 1.70-1.68 (m, 2H), 1.38-1.25 (m, 18H), 0.88 t, $J$ = 6.0 Hz, 3H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 189.9, 180.2, 173.3, 171.8, 102.6, 102.5, 102.4, 102.3, 101.3, 101.0, 45.2, 31.9, 31.0, 29.6, 29.5, 29.4, 29.3, 29.1, 26.4, 26.3, 22.7, 14.1.HRMS (ESI) m/z: Calcd. (C$_{22}$H$_{29}$F$_2$NO$_3$)Na$^+$: 416.2008; Found: 416.2005, Difference: <-1.0 ppm.

**Dodecyl βCD squarate, DβCDS (18b):** Synthesis of βCD-NH$_2$ (18c) was done via a 3 step reported literature procedure.$^{212}$ The synthesized βCD-NH$_2$ (0.028 g, 0.02 mmol) was dissolved in distilled water (2 mL). To this βCD-NH$_2$ solution was added dropwise a solution of phenoxy amino dodecyl squarate (0.02 mmol ) dissolved in a binary solvent mixture of DMF (2 mL) and acetone (1 mL). The reaction mixture was allowed to stir at room temperature, monitoring with TLC to check for consumption of starting material squarate (~ 4 days) (TLC solvent system; 30 % ethyl acetate in hexane). Formation of new spot was also monitored using a polar TLC system ($i$-PrOH: EtOAc: H$_2$O: NH$_4$OH:: 5: 2: 3: 1, Rf = 0.7) followed by staining with Ceric
Ammonium Molybdate (CAM) stain. The product (DβCDS) (14) was precipitated by addition of excess of acetone (10 mL) and separated by centrifuging at 3000 rpm for 10 mins. The obtained white solid was washed several times with acetone followed by centrifugation. Colorless powder, yield 0.02 g, 56.0%). \(^1\)H NMR (300 MHz, CD\(_3\)OD): 3.95-3.64 (m, 30 H), 3.57-3.38 (m, 14H), 1.92-1.88 (m, 1H), 1.22 (br, s, 12H), 0.89 (br, s, 3H). Analyzed by MALDI and matched on [C\(_{58}\)H\(_{96}\)N\(_2\)O\(_{36}\)]Na\(^+\) at 1420.37 Expected Mass of sodium adduct: 1420.37.

**Biological assays**

**Materials and methods of biological assays.** Rhamnolipids (R95; 79:16 percent ratio of di-rhamnolipids:mono-rhamnolipids, 95 % pure, average molecular weight 626 g/mole) was obtained from Agae technologies, Corvallis, OR and was used as received. Water was purified using a Millipore Analyzer Feed System. Flat-bottomed polystyrene 96-well microtiter plates (untreated) (Costar 3370, VWR) were used to perform antibiofilm assays (biofilm inhibition, biofilm dispersion). For measuring fluorescence for adhesion and gene reporter assays, flat-bottomed 96-well microtiter plates with black walls (µClear, Greiner-One 655096, Sigma Aldrich) were used. Measurement of absorbance at 600 nm (OD\(_{600}\)) for growth assay was performed with 200 µL of culture-media in microtiter plate well on a Biotek ELx800™ absorbance microplate reader (BioTek Instruments, Inc., Winooski, VT), and the data was analyzed with Gen5™ data analysis software. Quantification of biofilm (inhibition and dispersion) was done using a crystal violet (CV) dye-based static-biofilm assay.\(^{213}\) For quantifying fluorescence for adhesion and gene reporter assays, Synergy 2 multi-mode microplate reader (BioTek Instruments, Inc., Winooski, VT) with Gen5 data analysis software
was used to detect green fluorescent protein (GFP) signal at an excitation wavelength of 500 nm and emission wavelength of 540 nm. For biofilm inhibition and dispersion, bacterial adhesion, and gene reporter assays, controls contained the same volume of deionized water without any added agents. For each biological assay, at least 3 triplicates were conducted, and data shown are the average of readings from at least 4-replicates wells. Half maximal inhibitory concentration for both biofilm inhibition and dispersion (IC$_{50}$ and DC$_{50}$) were calculated by fitting values into a logarithmic equation, $y = \text{mln}(x) + C$

**Bacterial strains and plasmids.** *Pseudomonas aeruginosa* strains PAO1 and PAO1-EGFP were obtained from Dr. Guirong Wang (Upstate Medical University). *Pseudomonas aeruginosa* transposon mutant strain PW6886 (rhlA-E08::ISphoA/hah) was obtained from two-allele library. PAO1 (plasI-LVA GFP), PAO1 (prhII-LVA GFP) strains were prepared by literature reported protocol. The plasmid plasI-LVA GFP and prhII-LVA GFP were obtained from Dr. Hiroaki Suga (The University of Tokyo) and were maintained by adding 300 μg/mL of carbenicillin in culture media. Freezer stocks of all strains were stored at -80 °C in LB media with ~20% glycerol. All strains were grown at 37 °C in a rotary-shaker (at 250 rpm) in Luria Bertaini (LB) media (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl). All biofilm inhibition, dispersion and adhesion assays were performed in LB-media and plates were incubated at 37 °C under static conditions.

**Stock solution and delivery of disaccharide derivatives (DSDs).** All but three saccharide derivatives were water-soluble. For the water soluble DSDs, no organic solvents were used to assist the mixing and delivery for all biological assays. Stock solution (~11.5 mM) of DSDs were prepared in autoclaved water and further sterilized by filtering through cellulose acetate syringe filter (0.2 μm pore size, GVS filter technology) into sterilized vials. The sterilized DSDs stock
solutions were stored at -20 °C and thawed prior to each use. Aliquots of DSD stock solutions were added to the cultures in wells. Sterile water (same volume as the DSD stock solution used) was used as positive controls (no agents) in all assays to eliminate the effect of water from the stock solution. Three agents DβL (7), SFβL (15) and 2-ODαC (17) exhibited low water solubility, and were prepared in mixed solution of sterile water (90%) and 200 proof EtOH (10%). Controls for these agents consisted of the same mixed water-ethanol solution.

**Toxicity of disaccharide derivatives against planktonic bacteria.** Overnight inoculum of PAO1 was sub-cultured to an OD$_{600}$ ~ 0.01 and the sub-culture was allowed to reach an OD$_{600}$ of ~0.1, from which 200 μL was aliquoted into the wells of a microtiter plate, followed by 3 μL of DSDs stocks (to achieve a final 160 μM). Wells without added agents were used as a positive control. The microtiter plate was shaken (250 rpm at 37 °C), and the optical density was measured after regular intervals of time over 24 h using Biotek ELx800™ absorbance microplate reader (BioTek Instruments, Inc., Winooski, VT). The OD$_{600}$ values were plotted against time to obtain a growth curve for planktonic growth with or without agents (see supporting information Figure S2).

**Swarming assays.** Semisolid gel for swarming assays were prepared using M8 medium supplemented with 0.2 % glucose, 0.5 % casamino acid, 1 mM MgSO$_4$ and 0.5 % Bacto agar.$^{204}$ For each set of swarming experiment, the plates were poured from same batch of agar and allowed to dry for 1 h before inoculation of bacteria. Bacterial culture (3 μL) (wild type PAO1 or rhlA mutant) with OD$_{600}$ between ~0.4-0.6 was inoculated on the semisolid agar gel. These “swarm plates” were incubated at 37 °C for 12 h and then incubated for additional 12 h at room temperature. After a total 24 h period, pictures of the swarming plates were taken.
Swarm area measurement. To measure swarming area, images of “swarm plates” were adjusted to keep the diameters of all the petri dishes containing the semisolid gel to a constant 4.4 inches. The adjusted images were printed and the swarming patterns were cut and weighed. With 1 cm$^2$ square of the copy paper weighing 8.0 mg, the swarming areas were calculated.

![Swarm area measurement graph](image)

**Figure 2S1.** Plot of the rhlA mutant’s swarm area (after 24 h) versus increasing concentrations of rhamnolipids and SFβM (14) in the semisolid gel (≈ 0.5% agar). The experiment was repeated on three different plates. The error bars are obtained from the mean of the three different experiments.
$^1$H and $^{13}$C NMR Spectra

$\text{DeyC (2c)}$

$^1$H NMR, 300 MHz, CDCl$_3$
\( ^1H \) NMR, 300 MHz, CD\(_3\)OD

DeβC (2)
$^{13}$C NMR, 75 MHz, CD$_3$OD
1H NMR, 300 MHz, CDCl₃
\( ^1 \text{H NMR, 300 MHz, CD}_3\text{OD} \)

\[ \text{UβC (3)} \]
$^{13}$CNMR, 75 MHz, CD$_3$OD
$^{1}$H NMR, 300 MHz, CDCl$_3$

**ADβC (4a)**
$^{13}$C NMR, 75 MHz, CDCl$_3$
$^{13}$C NMR, 75 MHz, CD$_3$OD

DβC (4)
ATβC (5a)

$^1$H NMR, 300 MHz, CDCl$_3$
$^{13}$C NMR, 75 MHz, CDCl$_3$
$^1$H NMR, 300 MHz, CD$_3$OD

TβC (5)
$^{13}\text{C NMR, 75 MHz, CD}_3\text{OD}$

$\text{TβC (5)}$
$^{1}$H NMR, 300 MHz, CD$_3$OD

DaR (9)
$^{13}$C NMR, 100 MHz, CD$_3$OD
1H NMR, 300 MHz, CDCl₃
acylated undecylene celllobioside CNMR_2

\[ ^{13}C \text{ NMR, 75 MHz, CDCl}_3 \]

AUDβC (10a)
$^1$H NMR, 300 MHz, CD$_3$OD

UDβC (10)
$^{13}$C NMR, 100 MHz, CD$_3$OD

UDβC (10)
$^{1}H$ NMR, 300 MHz, CDCl$_3$
$^{13}$C NMR, 75 MHz, CDCl$_3$

AFβC (11a)
$^1$H NMR, 300 MHz, CD$_3$OD

FβC (11)
$^{13}$C NMR, 75 MHz, CD$_3$OD
$^{1}$H NMR, 300 MHz, CDCl$_3$

SF (12b)
$^{13}$C NMR, 75 MHz, CDCl$_3$

ASF$\beta$C (12a)
$^1$H NMR, 300 MHz, CD$_3$OD

SFβC (12)
$^{13}$C NMR, 75 MHz, CD$_3$OD

SFβC (12)
$^1$H NMR, 300 MHz, CDCl$_3$

ASFαC (13a)
$^{13}$C NMR, 75 MHz, CDCl$_3$
\[^{1}H\text{NMR, 300 MHz, CD}_{3}OD\]

SFαC (13)
\[ ^{13}C \text{NMR}, 75 \text{ MHz}, \text{CD}_3\text{OD} \]
**1H NMR, 300 MHz, CDCl₃**

**ASFβM (14a)**
13C NMR, 75 MHz, CDCl3

ASFβM (14a)
$^1$H NMR, 300 MHz, CD$_3$OD

SFβM (14)
$^{13}$C NMR, 75 MHz, CD$_3$OD

SFβM (14)
$^1$H NMR, 300 MHz, CDCl$_3$

**ASFβL (15a)**
$^{13}$C NMR, 75 MHz, CDCl$_3$

**ASFβL (15a)**

---

**Current Data Parameters**
- **NAME**: 13CNMR
- **PROCED**: 1

**F2 - Acquisition Parameters**
- **Date**: 2018.12.05
- **Time**: 17:19
- **Sample**: ASFβL (15a)
- **PROBE**: 5 mm CUBIC GR
- **F1FREQ**: 75.023 MHz
- **SOLVENT**: CDCL$_3$
- **NS**: 120
- **DS**: 4
- **SNR**: 12023.651 MHz
- **FTNBLS**: 0.36673 MHz
- **AQ**: 1.3421 MHz
- **BS**: 80 MHz
- **US**: 180 MHz
- **PS**: 300.0 MHz
- **DI**: 10.0000000 MHz
- **SL**: 0.0000000 MHz
- **TOO**: 1

**--- CHANNEL F1 ---**
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- **NS**: 120
- **DI**: 10.0000000 MHz
- **PS**: 300.0000000 MHz

**--- CHANNEL F2 ---**
- **AQ**: 100.1366000 MHz
- **NS**: 120
- **DI**: 10.0000000 MHz
- **PS**: 300.0000000 MHz
$^1$H NMR, 300 MHz, CD$_3$OD

SFβL (15)
$^{13}$C NMR, 75 MHz, CD$_3$OD

SFβL (15)
Acetobromo cellobioside (4b)

$^1$H NMR, 300 MHz, CDCl$_3$
\( ^1H \text{ NMR, } 300 \text{ MHz, CDCL}_3 \)

2-AODβC (16a)

ns1 05 09 09_73_91 acetyl 2-octyl dodecyl cellulbioside
$^{13}$C NMR, 75 MHz, CDCL$_3$

2-AODβC (16a)
$^1$H NMR, 300 MHz, CD$_3$OD

2-OD$\beta$C (16)
$^{13}$C NMR, 75 MHz, CD$_3$OD

2-ODβC (16)
$^{1}H$ NMR, 300 MHz, CDCl$_3$
$^{13}$C NMR, 75 MHz, CDCl$_3$

2-AODαC (17a)
$^1$H NMR, 300 MHz, CD$_3$OD

2-ODαC (17)
$^{13}$C NMR, 75 MHz, CD$_3$OD

2-ODaC (17)
\[ 1^H \text{NMR, 400 MHz, CDCl}_3 \]

Dodecyl phenoxy sq

18b: DPSq
$^{13}$C NMR, 100 MHz, CDCl$_3$
$^1$H NMR, 300 MHz, D$_2$O

DβCDS (18)
Chapter 3

Disaccharide derivatives Inhibit and Disperse Biofilm of *P. aeruginosa*

Abstract

Bacterial sessile multicellular lifestyle leads to formation of communities known as biofilms that are highly resistant to the action of antibiotics. Agents that can prevent biofilm formation or alternatively disrupt preformed biofilms are therefore highly desired. Additionally, if such antibiofilm agents are also nonmicrobicidal against bacteria, they will be less likely to invoke resistance over time as compared to antibiotics that are microbicidal.

Here, the ability of certain disaccharide derivatives (DSDs) to inhibit *P. aeruginosa* adhesion, biofilm formation and cause dispersion of existing biofilm via a nonmicrobicidal action was evaluated. The structure activity relationship show that DSDs having disaccharide stereochemistries (cellobiose or maltose) and a bulky aliphatic tail (3, 7, 11-trimethyl-dodecanyl) were potent antibiofilm agents. The half-maximal inhibitory concentrations of few DSDs for biofilm inhibition (IC\textsubscript{50}) and dispersion (DC\textsubscript{50}) were comparable to known potent antibiofilm agents against *P. aeruginosa*. One agent, having cellobiose head and 3, 7, 11-trimethyl-dodecanyl tail (SFβC (12)) was a more potent antibiofilm agent than the natural ligands, rhamnolipids. The mechanism of action of such DSDs is not via the known las or rhl quorum sensing systems of *P. aeruginosa*. Results also indicate that the *P. aeruginosa* adhesin potein, pilin maybe a likely target for such DSD molecules.
3.1 Introduction

3.1.1 *P. aeruginosa* is a prominent pathogen associated with medical bacterial infections

The multi-drug resistance (MDR) phenomenon exhibited by a broad range of bacteria is a major health concern, and a prime bacterium in the midst of this grave problem is *P. aeruginosa*. *P. aeruginosa* is a gram negative rod shaped pathogen which is ubiquitously found in soil, water and within humans. *P. aeruginosa* is one of the most common pathogens isolated from hospital acquired infections. This bacterium is also the prime pathogen found in the lungs of patient with chronic respiratory disease, *Cystic fibrosis*. To add to the medical woes associated with *P. aeruginosa* infections, the present day strains isolated from disease organisms display resistance against most antimicrobials including, Penicillins, Ceftazidime, Carbapenems, Aminoglycosides and Ciprofloxacin.

Presently, the primary pharmaceutical approach to fight *P. aeruginosa* infections focuses on finding newer protein targets on the bacteria, against which potent antagonists can be developed and used to disrupt bacterial cellular processes and hence cause bacterial death. The major disadvantage of such a microbicidal approach is that the bacteria will over time acquire resistance against the new drug, and the futile task of finding newer targets and drugs requiring billions of dollars on research and government approvals would have to be repeated over and over again.

Contemporarily newer nonmicrobicidal approaches, that focus on the multicellular behaviors like biofilm formation and swarming (for review on bacterial biofilm formation and swarming see Chapter 1, sections 1.2 and 1.3) are being developed to treat bacterial infections while at the same time limiting the risks of inducing MDR. While no nonmicrobicidal strategy
focusing on bacterial biofilm inhibition has made it to the clinic, there is consensus that for countering MDR the future bacterial therapeutics would have to be centered around this approach. The reasons for this consensus are two; (i) Around 65-80% bacterial infections in humans are biofilm related; (ii) With the use of nonmicrobicidal agents against them, the bacteria would have a lesser or ideally no propensity to acquire resistance. With a distinct hope for a long term and probably a more permanent solution to bacterial infections, the nonmicrobicidal strategies offer some advantages over the existing microbicidal strategies. Therefore, in recent times, there has been a flurry of discoveries primarily focused on inhibiting and disrupting bacterial biofilms with agents that do not kill the microbe. A brief summary of many such nonmicrobicidal strategies has been reviewed in Chapter 1 (Section 1.3.4).

3.1.2 Motile and sessile multicellular behaviors in P. aeruginosa are inversely regulated

When bacteria land on a surface, they make a calculated choice between forming a sessile community or moving further ahead on the surface in search of more conducive microenvironments for colony formation (Figure 3.1). It is therefore conceived that both these multicellular processes, sessile (biofilm formation) and motile (swarming) are controlled by a common set of genes, whose expression inversely regulates the two bioprocesses. Many such genes and gene products have been identified in P. aeruginosa whose role is to make that calculated choice between motile and sessile life styles. In P. aeruginosa, the presence of surface attachment defective (SadB) protein is critical for both biofilm formation and swarming motility. While SadB protein was shown to be critical for progression from reversible to irreversible attachment during biofilm formation, this cytoplasmic protein was also shown to be important for sensing rhamnolipids, which are crucial virulence factors necessary during swarming. O’Toole et al created a mutant (WT/pSadB) strain of type P. aeruginosa by inserting a plasmid
(pSadB$^+$) that caused overexpression of SadB protein. When the biofilm formation of such a mutant strain was quantified and compared to the biofilm content of wild type, there was a 3 fold increase in biofilm formation. Contrary to the enhanced biofilm formation, the swarming motility of such a SadB overexpressing mutant was reduced by 2.5 fold. When the gene encoding for SadB was deleted, the resulting mutant strain was hyperswarming. These results by O'Toole et al alluded to the notion that the sadB gene inversely regulates biofilm formation and swarming in P. aeruginosa. Further studies by O'Toole et al showed that another gene known as sadC, which expresses the membrane-localized diguanylate cyclase protein inversely regulated biofilm formation and swarming motility in P. aeruginosa. Later studies by O'Toole et al identified another gene bifA that can also inversely regulate swarming and biofilm formation, and they tentatively assigned the gene product of this gene, BifA as functioning upstream to the previously identified SadB protein.

![Figure 3.1 Inverse regulation of swarming and biofilm formation.](image)

[Modified and Reprinted with permission from Citation: Caiazza et al, Inverse regulation of biofilm formation and swarming motility by Pseudomonas aeruginosa PA14, J. Bacteriol. 2007, 189, 3603-3612]
3.1.3 Apart from being critical during swarming, rhamnolipids are important biomolecules secreted by P. aeruginosa during biofilm formation

The biosynthetic pathway for the secretion of rhamnolipids by P. aeruginosa has been discussed in Chapter 2, section 2.1.5.4. Also, the biological importance of rhamnolipids secretion by P. aeruginosa with respect to swarming motility has been discussed in chapter 2, section 2.1.5.4.1. Secretion of rhamnolipids is also associated with the sessile mode of living, i.e biofilm. The biofilm architecture is quite complex and the component of its matrix varies from species to species. However, it is well documented that most biofilm architectures are "mushroom" shaped that contain voids and channels. The voids and channel are critical elements of biofilm architecture and it is thought that such interconnected channels are important for supplying nutrients, oxygen and water throughout the community. Davey et al showed using fluorescence microscopy that while both the wild type and the rhlA mutant strain of P. aeruginosa were able to form a biofilm architecture containing macrocolonies separated by voids, only the wild type strain was able to maintain the voids over six days, where as the rhlA mutant could not. In comparison to wild type, the critical element that the rhlA mutant cannot secrete are rhamnolipids, hence they concluded that secretion of rhamnolipids during biofilm formation of P. aeruginosa is necessary for ensuring formation of sturdy biofilm. Apart from the role in maintaining voids in the biofilm, Gilbert et al showed that over production of rhamnolipids by P. aeruginosa could lead to dispersal of bacteria from the biofilm.
3.1.4 Bacteria employ surface proteins known as adhesins to recognize and bind carbohydrate epitopes found on host cells

The initiation of many infections begin with adherence of bacteria to a host surface with the use of certain surface-exposed bacterial proteins known as adhesins. These bacterial adhesin proteins initiate adherence by recognizing many different kinds of carbohydrate epitopes present on host surfaces. The adhesin proteins used by bacteria may be present either on the cell surface (lectins)\(^1\) or on specialized appendages (pili and fimbriae). Four different kinds of fimbriae present on *E.Coli* (P, type 1, S, and F1C) are known to exhibit affinity for galabiose (Galα-(1-4)-Gal), mannose, sialic acid ligands, and GalNAcβ(1-4)Gal, respectively.\(^2\) Galabiose epitope is also known to be recognized by *Streptococcus suis* (S. suis), a gram positive bacteria that causes meningitis in human.\(^1\) It has also been reported that *H. pylori*, a common stomach bacterium recognizes a prominent carbohydrate epitope (2’3-sialyllactose, Neu5Acα(2-3)Galβ(1-4)Glc) found on human cells.\(^1\)

*Pseudomonas aeruginosa* use surface proteins as well as adhesins to interact with carbohydrates moieties on host surfaces. Proteins used by *Pseudomonas aeruginosa* for surface adhesion include flagellin (on flagella), pilin (on pili) and lectins (LecA and LecB).\(^2\) While the flagella proteins on *P. aeruginosa* can bind to carbohydrate epitopes found on mucin protein and to glycolipids (asialo-GM1) on mammalian cells, pilin protein has a specificity for D-GalNac-β-(1→4)D-Gal-β disaccharide moiety found on glycosphingolipids.\(^2\) The *P. aeruginosa* lectins, LecA and LecB, on the other hand are known to be specific for binding to galactose and fucose moieties, respectively. Molecular intervention of bacterial adhesion process via the use of synthetic carbohydrate ligands also known as the "antiadhesive strategy" is therefore viable therapeutic approach for preventing infection. Reymond and co-workers have demonstrated that...
multivalent fucosyl-peptide dendrimers targeting LecB could be used as molecular intervention to inhibit and disperse *P. aeruginosa* biofilm on steel coupons.228

In *P. aeruginosa*, pilin protein is known to mediate up to ~90% of the adhesion events. The pili assisted epithelial cell binding domain is located between residues 128-144 on the C-terminal region of PilA, the pili structural protein.229 Irwin and co-workers have demonstrated that the presence of Type IV pili is critical for adherence of *P. aeruginosa* to steel coupons. Also, synthetic pili peptide sequence (residues 128-144) can also bind to polyvinyl chloride (PVC) and polystyrene (PS) surfaces.230 The binding specificity of D-GalNac-β-(1→4)-β-D-Gal disaccharide ligand with minimum binding amino acid sequence of *P. aeruginosa* pili (peptide sequence PAK(128-144)) was also studied and it was determined that increasing the hydrophobicity of disaccharide side chain increased the binding to the PAK(128-144) pili peptide.231

Here, the three key points to summarize are; (i) Swarming and biofilm formation in *P. aeruginosa* are inversely regulated; (ii) Rhamnolipids secretion by *P. aeruginosa* is important for building sturdy biofilms; (iii) *P. aeruginosa* is able to recognize and hence bind with ligands that offer various saccharide stereochemistries. In Chapter 2, the use of certain saccharide derivatives (SDs) to modulate *P. aeruginosa* swarming was shown. Also, the results show that the modulation of swarming by such SDs was probably a result of them mimicking the functions of rhamnolipids. Since, SDs mimicked rhamnolipids during swarming of *P. aeruginosa*, and since swarming and biofilm formation in *P. aeruginosa* are inversely regulated, the potencies of such nonmicrobicidal agents (i.e SDs) to inhibit *P. aeruginosa* surface adhesion and biofilm formation and cause dispersal of preformed biofilm was evaluated in this chapter. As *P. aeruginosa* has many surface proteins that can recognize different saccharide epitopes, a preliminary study to identify the putative target protein of such SDs was also carried out.
3.2 Results and Discussion

The rationale of synthesizing the chemical structures in Figure 3.2 is the same as mentioned in Chapter 2 (Section 2.2.1). In summary, saccharide derivatives (SDs) comprising of different saccharide stereochemistries and various aliphatic structures were included in the study. The structure-activity relationship between structural details and corresponding effects on biofilm inhibition and bacterial adhesion was investigated.

The antibiofilm activities (biofilm inhibition and dispersion) of the SDs and rhamnolipids were screened by using the crystal violet (CV) based static biofilm assay. In this assay, wild type *Pseudomonas aeruginosa* (PAO1) was grown in LB-media with or without 160 μM SDs or rhamnolipids at 37 ºC in the wells of microtiter plates for 24 hours. After the initial incubation period of 24 hours, the biofilm formed on the bottom of the wells was stained with crystal-violet dye and quantified by measuring the absorbance at 600 nm (OD$_{600}$) (for procedure see Materials and Methods). Comparing the content of biofilm in control wells (no added SD) to that of wells containing various SDs, gave the percent inhibition. The initial analysis of the SDs’ antibiofilm (both inhibition and dispersion) activities were assessed at a concentration of ~ 160 μM. In the dispersion assay, the SDs or rhamnolipids were added after the initial incubation period of 24 hours and then the plates were incubated for additional 24 hours before quantification with CV-dye. Those agents that exhibited >50% antibiofilm activities were further analyzed in a concentration dependent manner to obtain half-maximal inhibitory concentrations for biofilm inhibition (IC$_{50}$) and dispersion (DC$_{50}$). Additionally, the antibiofilm activities of a few SDs were also examined via fluorescence based biofilm assay (for procedure see Materials and Methods).
Further, the ability of SDs to prevent initial bacterial adhesion to polystyrene surface was analyzed using a fluorescence based adhesion assay. Here a strain of *P. aeruginosa* that constitutively expresses green fluorescent protein (PAO1-EGFP) was inoculated in the wells of a microtiter plate for 2 hours with LB media as the nutrition source along with (160 μM) or without various SDs or rhamnolipids. After the initial adhesion period, the amount of fluorescent bacteria (PAO1-EGFP) on the surface of the microtiter plate was quantified by measuring the fluorescence at 510 nm. The ability of pili peptide (minimum binding segment of pilin protein) to reduce the potency of a SD was also analyzed.

The quantitative values of all bioassays (biofilm inhibition & dispersion, IC$_{50}$, DC$_{50}$ and bacterial adhesion inhibition) are the average obtained from four identical wells. Each experiment was repeated at least 3 times, each time giving similar results (repeat results are not shown).
Figure 3.2. Structures of synthetic saccharide based derivatives that include maltose, cellobiose, lactose, glucose, rhamnose, and β-cyclodextrin stereochemistries; and hydrocarbons derived from farnesyl and “saturated” farnesyl molecules. The structures of di-rhamnolipid and mono-rhamnolipid are also shown.
3.2.1 Effect of generic surfactants on biofilm formation

Swarming motility had exhibited no correlation with the surface activity of the molecule (see Chapter 2, Section 2.2.3). The preliminary screening of four generic surfactants (dodecyl-β-maltoside, DβM (1); tetra-(ethylene glycol) monododecyl ether, C12EG4OH; sodium dodecyl sulfate, SDS; and dodecyl trimethyl ammonium chloride, DTAC) for the ability to reactivate the swarming of the rhlA mutant (see Chapter 2, Section 2.2.3), showed that only DβM (1) alone was able to exhibit reactivation, similar to the natural ligand, rhamnolipids while other surfactants could not. Further, to assess whether surfactant action had an effect on biofilm formation, the four generic surfactants were also tested for the ability to inhibit P. aeruginosa biofilm. The biofilm inhibition assay in presence of 110 µM of various generic surfactants showed that only one molecule, DβM (1) exhibited significant reduction in biofilm content (Figure 3.3).

![Figure 3.3 Inhibition of P. aeruginosa (PAO1) biofilm by 110 µM of generic surfactants; Dodecyl-β-maltoside (DβM, 1, -ionic surfactant); tetra (ethylene glycol) monododecyl ether](image)

Figure 3.3 Inhibition of P. aeruginosa (PAO1) biofilm by 110 µM of generic surfactants; Dodecyl-β-maltoside (DβM, 1, ionic surfactant); tetra (ethylene glycol) monododecyl ether
(C\textsubscript{12}EG\textsubscript{4}OH, nonionic surfactant); sodium dodecyl sulphate (SDS, anionic surfactant); dodecyl trimethyl ammonium chloride, (DTAC, cationic surfactant). Only DβM exhibited antibiofilm activity at tested concentration. Percent PAO1 biofilm remaining after 24 hours of inoculation with (110 μM) or without various generic surfactant. The percentages were obtained by comparing biofilm content of control (no agent) to biofilm content in presence of an agent. Bacteria were cultured in LB-media. Error bar is standard error of the mean from 4 replicates.

3.2.2 Structure-activity relationship (SAR) for biofilm inhibition

(a) Structure and size of sugar head group is critical for biological activity

Initial screening of saccharide derivatives (SDs) at a concentration of 160 μM brought forward following SARs. Saccharide derivatives with a monosaccharide moiety and having a aliphatic chain of twelve carbons (glucose; DβG (8) and mono-rhamnoside; DaR (9)) had poor antibiofilm activities (Figure 3.4). In contrast to the poor potencies of monosaccharide derivatives (MSDs), a SD having a disaccharide stereochemistry (maltose) and a twelve carbon aliphatic chain DβM (1) reduced the biofilm content by ~50% (Figure 3.4). Saccharide derivative with a large cycloheptasaccharide moiety (β-cyclodextrin) and a twelve carbon aliphatic chain (DβCDS (18) was incapable of exhibiting any biofilm inhibition, in fact this agent seemed to increase the biofilm content slightly (Figure 3.4). Therefore, amongst the various tested SDs, the one with a disaccharide stereochemistry seemed to work best at inhibiting the biofilm of P. aeruginosa at a concentration of 160 μM.
(b) Inhibition of Pseudomonas aeruginosa (PA) biofilm by disaccharide derivatives (DSDs) is sensitive to stereochemistry of the sugar head group

Antibiofilm potencies of three different disaccharide derivatives (DSDs) each bearing a twelve carbon aliphatic chain with either lactose (Galβ(1→4)Glc, \textbf{DβL (7)}; poor solubility in water), cellobiose (Glcβ(1→4)Glc, \textbf{DβC (4)}) or maltose (Glcα(1→4)Glc, \textbf{DβM (1)}) head group revealed that cellobiose stereochemistry, Glcβ(1→4)Glc outperformed other stereochemistries (Figure 3.4). While lactose stereochemistry severely compromised the antibiofilm activity, cellobiose stereochemistry exhibited ~ 62% biofilm inhibition. In general, cellobiose derived DSD compared better than those of maltose derived DSD having similar aliphatic chains (~46% biofilm inhibition activity).

(c) Chain length of twelve carbons is optimum for observing maximum biofilm inhibition

In order to evaluate the importance of aliphatic chain length, the aglycone moiety of SDs with cellobiose stereochemistry was systematically altered from 10 to 13 carbons. On comparing the effect of carbon chain length on biofilm inhibition activity, DSD with an aliphatic chain length of ten carbons, \textbf{DβC (2)} was inactive, where as those with eleven \textbf{UβC (3)}, and twelve \textbf{TβC (5)} carbons had moderate activity (29 % and 40 %, respectively), while DSD with twelve carbons \textbf{DβC (4)} exhibited best activity (62 %) (Figure 3.4). This reliance of activity on aliphatic chain length of the chemical signal is also observed during quorum sensing, when each species is able to produce, secrete and recognize its own chemical signal based on the length of the aliphatic chain.\(^{167,232}\) In Gram-Negative bacteria, different species are able to distinguish their own chemical signal from that of others by recognizing the difference in the length of acyl side chain of acyl-homoserine lactone (AHL) auto inducers (chemical signals).\(^{233-236}\) Not surprisingly,
the AHL cognate receptor (LuxR type protein) is homologous amongst different species.\textsuperscript{167,233} The optimization of DSD antibiofilm activity with aliphatic chain length of twelve carbons is therefore an indication of a ligand-receptor type of phenomenon.

(d) Introducing short methyl branches off the main aliphatic chain enhanced the antibiofilm activity of DSDs greatly with low IC\textsubscript{50} values (as low as \textasciitilde 9.9 \textmu M)

The effect of aglycone part of the DSD was further evaluated by introducing short methyl branches coming off the main aliphatic chain. This aliphatic chain was obtained by hydrogenating farnseol molecule (3,7,11-trimethylundodeca-2,6,10-trien-1-ol) to obtain saturated farnesol (3, 7, 11-trimethyl-dodecanyl) group. At 160 \textmu M, DSDs with saturated farnesyl aliphatic chain and cellobiose head group, SF\textbeta C (12) and SF\textalpha C (13) were strong inhibitors (up to \textasciitilde 80\%) of \textit{P. aeruginosa} biofilm (Figure 3.4). In an attempt to further optimize antibiofilm activity, DSDs bearing saturated farnesyl aliphatic chain and either maltose or lactose groups were synthesized (SF\textbeta M (14) and SF\textbeta L (15), respectively). Synthetic lactose analog exhibited poor solubility in water and hence was not tested for antibiofilm activity. But SF\textbeta M (14) at a concentration of 160 \textmu M exhibited antibiofilm activity of around 60\% (Figure 3.4). Contrary to the enhanced antibiofilm activities of DSDs with saturated farnesyl chain, the antibiofilm activity of cellobiose based DSD with unsaturated farnesyl aliphatic chain F\textbeta C (11) was a meager \textasciitilde 12\% (Figure 3.4). This decrease in antibiofilm activity when unsaturation is introduced into the aliphatic chain (leading to less bulky chain structure) was also consistent for another set of DSDs; i.e D\textbeta C (4) (62\% inhibition) and UD\textbeta C (10) (13\% inhibition) (Figure 3.4). Also, the effect of anomeric position of the aliphatic chain (from $\beta$ to $\alpha$) on biofilm activities was difficult to predict; for example at 160 \textmu M, D\textbeta C (4) gave 62\% inhibition where as to DaC (6) gave only
28% inhibition, but on comparing another set of anomers, i.e SFβC (12) and SFαC (13), SFβC (12) gave 76 % inhibition where as SFαC (13) gave 82 % inhibition (Figure 3.4).

(e) Polyol-derivatized hydrocarbons with the alkyl chain having lipid-like structure are not potent antibiofilm agents

Aliphatic chain with methyl branches seemed to enhance bioactivity, therefore the bulkiness of the aliphatic chain was further increased by attaching a 2-octyldodecyl chain, forming two lipid-like DSDs (2-ODβC (16) and 2-ODαC (17)). However, when tested at a concentration of 160 μM, this modifications lead to structures with poor antibiofilm activities (33% and 15%, respectively) (Figure 3.4).

(f) At a concentration of 160 μM, rhamnolipids seemed to enhance biofilm production

Since, SDs were able to mimic the swarming biofunction of rhamnolipids, and since rhamnolipids also help maintain biofilm architecture, a comparitive study between antibiofilm activities of SDs and rhamnolipids was also performed. At 160 μM of rhamnolipids the biofilm content seemed to increase (Figure 3.4).
**Figure 3.4** Biofilm inhibition potencies of DSDs and rhamnolipids quantified by CV-dye based assay. Percent PAO1 biofilm remaining after 24 hours of inoculation with bacterial culture supplemented with (160 μM) or without various DSDs or with rhamnolipids. The percentages were obtained by comparing biofilm content of control (no agent) to biofilm content in presence of an agent. Bacteria were cultured in LB-media. Error bar is standard error of the mean from 4 replicates.

3.2.3 Antibiofilm ability of certain DSDs were qualitatively assessed by performing a confocal microscopy assay

The antibiofilm activities of five DSDs, **SFβC** (12), **SFαC** (13), **DβC** (4), **DeβC** (2) and **DβL** (7) were also qualitatively validated by using confocal laser scanning microscopy (CLSM) to measure the amount of fluorescent bacteria, PAO1-EGFP adhered on stainless steel coupons grown in LB-media for 24-h supplemented with either of the five DSDs.237 The CLSM micrographs (Figure 3.5) showed that 160 μM of **SFβC** (12), **SFαC** (13) and **DβC** (4) decreased the fluorescence signal significantly as compared to the steel coupon placed in just LB-media. This decrease in fluorescence signal by three DSDs indicated a lesser bacterial cell density on the surface of the steel coupons as compared to control steel coupon. The mean thickness of biofilm obtained from Z-stacked images showed a much thinner biofilm coverage on steel coupons placed in media containing 160 μM of **SFβC** (12), **SFαC** (13) and **DβC** (4). In contrast, with 160 μM of **DeβC** (2) or **DβL** (7), biomass surface coverage had similar thickness as that of control without the agents. These fluorescence results were consistent with the results of CV-dye based assays for biofilm inhibition.
Figure 3.5 Biofilm inhibition activities of DSDs was also validated qualitatively by a
**confocal fluorescence based biofilm assay.** Steel coupons inoculated with PAO1/EGFP for 24
h with (~160 μM) or without various disaccharides derivatives (DSDs). After 24-h, the steel
coupons with biofilms were viewed under confocal laser scanning microscope (CLSM). Scale
bar ~ 76 μM. Reduced fluorescence due to the presence of DSDs (in comparison to the Control)
indicates lesser amount of biofilm on the steel coupons.

3.2.4 *Rhamnolipids antibiofilm activity increases and then decreases with increasing doses
exhibiting an “activity reversal”*

The antibiofilm activities of those DSDs that exhibited >50% inhibition at 160 μM along
with rhamnolipids extract were further probed in a concentration dependent manner to obtain the
half-maximal inhibitory concentrations (IC$_{50}$) of these agents (Figure 3.6). As concentration of
rhamnolipids were increased, the antibiofilm activity initially increased and then declined sharply tracing a bell shaped or an “activity reversal” profile. The antibiofilm activity of rhamnolipids increased gradually to a maximum value of ~65% at a concentration of 42 μM followed by decrease to 46% at 85 μM and then to -14% at 160 μM with respect to biofilm formed in control. The unusual “activity reversal” profile of rhamnolipids observed during antibiofilm assays was also observed during a dose dependent study with many SDs and rhamnolipids itself during swarming assays. The unusual dose dependent bell shaped graph of rhamnolipids prevented calculation of its IC$_{50}$. Rhamnolipids is a glycolipids and another lipid-like molecule included in this study was 2-ODβC. Consistently, the antibiofilm activities of both these lipid-like structures at relatively higher concentration (160 μM) is rather low. However, unlike the bell-shaped profile of swarming dose-dependence, none of the four DSDs, SFβC (12), SFαC (13), SFβM (14) and DβC (4) exhibited any "activity reversal". Instead, the biofilm inhibition of these four DSDs increased over a concentration range and then leveled off at a particular maximum value. The calculated half maximum inhibitory concentrations (IC$_{50}$s) of the four DSDs were; ~9.9 μM for SFβC (12); ~ 32 μM for SFαC (13); ~72 μM for DβC (4); and ~102 μM for SFβM (14). The dose-dependence study demonstrates SFβC (12) is more potent antibiofilm agent than rhamnolipids at all the tested concentrations. This antibiofilm dominance over rhamnolipids is also shown by the other three DSDs, SFαC (13), SFβM (14) and DβC (4) but only at concentrations higher than 42 μM. The low IC$_{50}$ values of the four DSDs are comparable to some of the most powerful synthetic agents that have been reported to nonmicrobicidally inhibit $P$. aeruginosa biofilm.$^{115,213}$
Figure 3.6 Disaccharide derivatives' (DSDs) and rhamnolipids' dose-response curves for *P. aeruginosa* (PA01) biofilm inhibition. The biofilm was allowed to form with increasing concentrations or without agents for 24-h old biofilm and then quantified by staining with crystal violet (CV) dye. The images on the right show the CV-dye stained biofilms formed on the wells of the microtiter plate for each agent and each concentration tested. Error bar is standard error of the mean from 4 replicates.

3.2.5 Strong biofilm inhibitors were also good biofilm dispersers

Both in industrial and medical settings one commonly encounters biofilms that have already formed and dispersing these preformed biofilms is usually a daunting task. Therefore, while discovering agents that prevent onset of biofilm formation is important, identifying agents that are able to disperse preformed biofilms is more critical from application standpoint. With this objective in mind, the ability of DSDs to disperse 24-h old PA01 biofilm was evaluated. In general (with a few exceptions), the dispersing activities of the DSDs displayed an overall similar trend as the SAR for biofilm inhibition (Figure 3.7). When tested at a concentration of 160 μM against 24-h old biofilms, the agents with good antibiofilm activities, *SFβC* (12), *SFαC* (13), *SFβM* (14) and *DβC* (4) also displayed good biofilm dispersing abilities...
(>60% dispersion as compared to control). At 160 μM, the rest of the DSDs barring DβCDS (18) had biofilm dispersion activities ranging between 20-60%. The saccharide derivative, DβCDS (18) was unique as it was the only tested agent that seemed to increase biofilm content when it was added to a 24-h old biofilm.

**Figure 3.7 Biofilm dispersion potencies of DSDs and rhamnolipids quantified by CV-dye based assay.** PAO1 biofilms were grown in microtiter plates without agents for 24-h in LB-media and then fresh LB-media supplemented with various DSDs or rhamnolipids (160 μM) was added. The percent PAO1 biofilm remaining after inoculating for another 24 hours with (160 μM) or without various DSDs or rhamnolipids were calculated by comparing biofilm content of control (no agent) to biofilm content in presence of an agent. Error bar is standard error of the mean from 4 replicates.

3.2.6 *The potent DSDs strongly dispersed the preformed biofilm of P. aeruginosa while rhamnolipids could not*

The moderately active antibiofilm rhamnolipids were not able to display much dispersing ability against 24-h old biofilms. The highest biofilm dispersion activity of 34% displayed by
rhamnolipids was at a concentration of 42 μM beyond which the activity continuously dropped (only 17% dispersion at 85 μM) (Figure 3.8). Contrasting the poor dispersive bioactivity of rhamnolipids, the dispersion dose-response curve of three active DSDs, SFβC (12), SFαC (13) and SFβM (14) brought forward structures that exhibited half-maximal dispersion concentration (DC₅₀) values that compare favorably to known dispersive agents of *Pseudomonas aeruginosa* biofilm.⁸⁰,¹¹⁵ The DC₅₀ values calculated for SFβC (12) was ~44 μM, for SFαC (13) was ~89 μM and for SFβM (14) was ~126 μM.

![Figure 3.8 Disaccharide derivatives' (DSDs) and rhamnolipids' dose-response curves for dispersion of 24-h old *P. aeruginosa* (PAO1) biofilms.](image)

Figure 3.8 Disaccharide derivatives' (DSDs) and rhamnolipids' dose-response curves for dispersion of 24-h old *P. aeruginosa* (PAO1) biofilms. PAO1 biofilms were grown in microtiter plates without agents for 24-h in LB-media and then fresh LB-media supplemented with various DSDs or rhamnolipids was added at different concentrations. The percent PAO1 biofilm remaining after inoculating for additional 24 hours with or without various DSDs or rhamnolipids was calculated by comparing biofilm content of control (no agent) to biofilm content in presence of an agent. Error bar is standard error of the mean from 4 replicates.
3.2.7 Some DSDs and even rhamnolipids reduce the initial adhesion of bacteria to a polystyrene surface

Adhesion of bacteria to biotic surfaces is usually the first step of infection; the first step of biofouling on abiotic surfaces is also the initial attachment of bacteria. Therefore, agents that prevent this initial attachment of bacteria to surfaces offer potential therapeutic and industrial benefits. To evaluate the DSDs' bacterial adhesion inhibition potencies, a mutant strain of *P. aeruginosa* that constitutively expresses green fluorescent protein (PAO1-EGFP) was inoculated for 2 hours in LB media with (160 μM) or without various SDs or rhamnolipids. After the initial adhesion period, the amount of fluorescent bacteria (PAO1-EGFP) adhered on the surface was quantified by measuring the fluorescence at 510 nm. With the exception of two DSDs, DaR (9) and DßCDS, the overall SAR trend of bacterial adhesion inhibition was consistent with the trend obtained from biofilm inhibition (Figure 3.9). Another disparity between biofilm inhibition and bacterial adhesion inhibition was the activity of rhamnolipids, while at 160 μM exhibited negative biofilm inhibition, it showed 39% inhibition of bacterial adhesion. The DSDs that had good biofilm inhibition and dispersion activities, also displayed good bacterial adhesion inhibition potencies including 77% for SFαC (13), 66% for SFβC (12), and 70% for SFβM (14).
Figure 3.9 Percent bacterial adhesion inhibition potencies of the various DSDs and rhamnolipids. Percent PAO1-EGFP remaining adhered on the wells of polystyrene microtiter plate after 2 hours from inoculation with bacterial culture supplemented with (160 μM) or without various DSDs or with rhamnolipids. Fluorescent signal emitted by the bacteria (PAO1-EGFP) at 510 nm was quantified to measure the amount of bacteria on the surface. The percentages were obtained by comparing PAO1-EGFP content of control (no agent) to PAO1-EGFP content in presence of an agent. Error bar is standard error of the mean from 4 replicates.

3.2.8 Bioactivities of DSDs depend on structural details

Important results from activation of swarming (Chapter 2), inhibition (IC$_{50}$) and dispersion (DC$_{50}$) of biofilm (Chapter 3) are summarized in Table 3.1. Analyzing this data reveals that potent antibiofilm DSDs (SFβC (12) & SFβM (14) having low IC$_{50}$ and DC$_{50}$) generally activated the swarming of the rhlA mutant at a relatively lower concentration than DSD that had poor antibiofilm activities (TβC (5) and DβG (8)). Also, the potent antibiofilm DSDs were the only agents that exhibited dominance over rhamnolipids during swarming motilities. While others have shown that under natural settings, rhamnolipids help maintain biofilm architecture of
the results in this study showed that adding low concentration of rhamnolipids externally inhibits biofilm formation. The addition of rhamnolipids externally into the growing media could add to the in situ secreted rhamnolipids that the bacteria secretes, hence the local concentration of this biosurfactant may increase dramatically, leading to a possible washing effect on the biofilm. The dose-response curves of rhamnolipids depicts "activity reversal" for both swarming activation and biofilm inhibition. This a first documentation for the effect of different concentrations of rhamnolipids on biofilm formation and swarming. The "activity reversal" phenomenon has been noticed earlier by others while studying concentration effects of bacteriologic signalling molecules on light simulation (another bacterial multicellular behavior). The reversal in antibiofilm activity at higher doses of rhamnolipids underscores the chances of this being a physical phenomenon because the surface activity should only increase with higher doses.

3.2.9 A more stringent structural requirement on DSD is needed for controlling biofilm formation than for controlling swarming motility

Some structures that activated rhlA swarming motility including DeβC (2), UβC (3), DβG (8) and TβC (5) exhibited poor antibiofilm activities. On the contrary some structures including 2-ODβC (16) and DaR (9) had poor antibiofilm capabilities but seemed to effectively inhibit the swarming process of wild type PAO1. However, the DSDs that had good antibiofilm activities like SFβC (12), SFαC (13) and SFβM (14), were generally strong inhibitors of PAO1 swarming. Thus, the structure-activity relationships (SAR) indicate that poor antibiofilm agents do not have a predictive effect on swarming, whereas effect of good antibiofilm agents on swarming was generally predictive. The SAR also revealed that combination of certain glycone and aglycone elements generally generated DSDs with poor capabilities to modulate both swarming and
biofilm formation. These elements include short aliphatic chain, DeβC (2), aliphatic chains with unsaturation, UDβC (10) and FβC (11), sugar head groups that are either small monosaccharides, DβG (8) and DaR (9) or large cyclic oligosaccharide, DβCDS (18). Together, these results suggest that the swarming motility, bacterial adhesion and biofilm formation are intertwined together, and designs of disaccharide derivatives can control selectively some or all of these three biological activities.

Table 3.1. Transition concentrations of DSDs that exhibit activity reversal from activating to inhibiting swarming motilities of the rhlA mutant and PAO1, and IC50 and DC50 of DSDs for PAO1 biofilm inhibition and dispersion. Antibiofilm activities (inhibition & dispersion) at 160 µM of various agents.

<table>
<thead>
<tr>
<th>Compound</th>
<th>a Transition concentration (rhlA)</th>
<th>b Transition concentration (PAO1)</th>
<th>IC50 (PAO1)</th>
<th>DC50 (PAO1)</th>
<th>Antibiofilm activity 160 µM (PAO1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhamnolipids</td>
<td>~10 µM</td>
<td>~20-30 µM</td>
<td>c--</td>
<td>d--</td>
<td>-14%</td>
</tr>
<tr>
<td>SFβM (14)</td>
<td>~10 µM</td>
<td>~7.5 µM</td>
<td>~102 µM</td>
<td>~126 µM</td>
<td>60%</td>
</tr>
<tr>
<td>SFβC (12)</td>
<td>~8 µM</td>
<td>~10-20 µM</td>
<td>~9.9 µM</td>
<td>~44 µM</td>
<td>76%</td>
</tr>
<tr>
<td>DβC (4)</td>
<td>~45-56 µM</td>
<td>~20-30 µM</td>
<td>~72 µM</td>
<td>d--</td>
<td>62%</td>
</tr>
<tr>
<td>TβC (5)</td>
<td>~40 µM</td>
<td>~20-30 µM</td>
<td>d--</td>
<td>d--</td>
<td>40%</td>
</tr>
<tr>
<td>DβG (8)</td>
<td>~35-50 µM</td>
<td>~50-60 µM</td>
<td>d--</td>
<td>d--</td>
<td>46%</td>
</tr>
<tr>
<td>SFαC (13)</td>
<td>d--</td>
<td>d--</td>
<td>~32 µM</td>
<td>~89 µM</td>
<td>82%</td>
</tr>
</tbody>
</table>
From activating to inhibiting swarming motility of the rhlA mutant. From promoting to inhibiting swarming motility of PAO1; rhamnolipids exhibits “activity reversal,” IC<sub>50</sub> was not calculated; not determined, weak antibiofilm activities.

3.2.10 DSDs do not interfere with the known las and rhl quorum sensing circuits of P. aeruginosa

The onset of multicellularity in bacteria is controlled by quorum sensing. For this quorum sensing process, bacteria use small molecule chemical signals to communicate with one another. Therefore, one effective antibiofilm strategy is to make use of chemical mimics of quorum sensing molecules that interfere with cellular communication and hence disrupt biofilm formation. To identify antagonists for the two quorum sensing circuits of P. aeruginosa, las and rhl, gene reporter assays were constructed for reporting activation or suppression of such systems. In this assay, the plasmids, plast-LVAgfp and prhl-LVAgfp were transformed into the wild type strain PAO1 to report activity against the las and rhl systems, respectively. Natural autoinducers N-3-(oxododecanoyl)-L-homoserine lactone (PAI-1) and N-(butyryl)-L-homoserine lactone (PAI-2) bind the las and rhl gene product, respectively, resulting in expression of green fluorescent protein (GFP) encoded by the plasmids. Agents that antagonise the las or rhl system, will have to compete with the naturally secreted autoinducers and as such cause a decrease in the expression of GFP. When two potent antibiofilm agents, SFβC (12) and SFαC (13) were tested for antagonistic activity against the two reporter strains, PAO1/plast-LVAgfp and PAO1/prhl-LVAgfp, both agents did not seem to change the expression of GFP (Figure 3.10). Hence, these agents do not interfere with either the las or the rhl quorum sensing circuit of P. aeruginosa.
Figure 3.10 Gene-reporter assay with two reporter strains of Pseudomonas aeruginosa, PAO1/plasI-LVAgfp and PAO1/prhlI-LVAgfp, showed that DSDs did not interfere with either the las or the rhl quorum sensing systems. Fluorescence (GFP) signal by PAO1 having plasmid; (A) plasI-LVAgfp and (B) prhlI-LVAgfp in the absence and in the presence of SFαC (13) and SFβC (12). Fluorescence signals were corrected for cell density by dividing with OD$_{600}$ of bacterial cell culture. Errors bar is a standard error of the mean of the readings from 4 replicate wells.

3.2.11 Competitive biofilm inhibition assay with one DSD and synthetic pili peptide reduced antibiofilm potency of the DSDs

The involvement of pili in promoting the swarming motility of *P. aeruginosa* has been documented by Kohler and coworkers.\textsuperscript{123} Randall and coworkers have shown that the receptor domain on pilin protein that recognizes carbohydrate moieties on tissues cells is a 17-amino acid residue on the C-terminal region (residues 128-144). This 17-amino acid residue has been shown to be semi-conserved amongst various strains of *P. aeruginosa* with two specific conserved cysteines (at position 129 and 142) in all strains being oxidized to form a di-sulfide loop.\textsuperscript{229} Randall and workers have also shown that both the pilin receptor domain and it's synthetic
peptide is also critical for enabling bacteria to adhere onto abiotic surface.\textsuperscript{183,225,243} To date the minimum binding carbohydrate epitope that binds both the pilin receptor domain and it's synthetic peptide has been shown to be D-GalNac(1→4)-β-D-Gal moiety.\textsuperscript{199,244} The pili assisted epithelial cell binding domain is located between residues 128-144 on the C-terminal region of PilA, the pili structural protein.\textsuperscript{229} To explore the identity of the putative receptor for such DSDs and rhamnolipids, the minimum seventeen amino acids residues receptor domain (N'-Ac-AKSTQDMFTPKGCDN-OH-C\textsuperscript{3})\textsuperscript{245,246} of \textit{P. aeruginosa} strain PAO was synthesized via a solid phase strategy. In order to replicate the disulfide loop formed by natural receptor domain, the synthesized pili peptide was subjected to oxidizing conditions to oxidize the cysteines at positions 129 and 142 to form a intra-molecular disulfide loop (called as pili peptide henceforth). To serve as a control a scrambled (N'-Ac-AKSTQDMFTPKGADN-OH-C\textsuperscript{3}) version of the pili peptide was also synthesized where in the cysteines at positions 129 and 142 position were exchanged to alanines (here in referred to as scrambled pili peptide). Both the synthetic peptides were tested along with a potent antibiofilm DSD, SF\textit{aC} (13) at concentration of 85 μM in a competitive antibiofilm assay against \textit{wt P. aeruginosa} (PAO1) (see Figure 3.11). The DSD, SF\textit{aC} (13) by itself exhibited ~70% biofilm inhibition, but when 85 μM of pili peptide was added along with 85 μM of SF\textit{aC} (13), the antibiofilm activity of the DSD dropped to only ~30% inhibition. Contrary to the effect of pili peptide, when 85 μM of both SF\textit{aC} (13) and the scrambled pili were added to the culture, DSD still seemed to inhibit close to 65% of the biofilm. These results suggest that the synthetic pili peptide likely interferes with the binding between the the DSD SF\textit{aC} (13) and the natural pili receptor domain on the bacteria, whereas the scrambled version of the synthetic pili peptide had little interference.
Figure 3.11 Crystal violet (CV) stained PAO1 biofilm within the wells of the microtiter plate after biofilm was allowed to develop for 24 hours in LB-media without any agents and in LB-media that is supplemented with SFαC (13) ~85 μM; SFαC (13) ~85 μM + Pili peptide ~ 85 μM; SFαC (13) ~85 μM + Scrambled pili peptide ~ 85 μM; Pili peptide ~ 85 μM; Scrambled pili peptide ~ 85 μM.

3.2.12 Mechanistic understanding

Biofilm inhibition and swarming results brought forward some key mechanistic points. Rhamnolipids are amphiphilic molecules and their role in biofilm formation and swarming has always been debated to either be biological or physical. Similarly, the effects on biofilm formation and swarming by various DSDs, which are amphiphilic in structure could have been hypothesized to either mere washing effect due to surfactant properties (physical) or biological in which a receptor on bacterial surface may bind to these molecules leading to either an antagonistic or an agonistic signaling event. However, few results in this study clearly suggested
that the effects by both rhamnolipids and DSDs on the various bioactivities of \textit{P. aeruginosa} are probably of biological nature. Firstly, generic surfactants that lacked amphiphilic structure with sugar head group (C$_{12}$EG$_5$OH, DTAC and SDS) exhibited no effects on biofilm formation and swarming, but DβM (1) possessing a maltose head group activated swarming as well as inhibited biofilm formation.\textsuperscript{247} Secondly, bioactivities of each DSD were sensitive to its structural details. Thirdly, swarming activation reversal caused by increasing concentrations of both rhamnolipids and the active DSDs indicated a trait of a cell-signaling molecule. A similar concentration dependent activity reversal has been reported before for N-(3-oxohexanoyl)-3-aminodihydro-2(3H)-furanone molecule, which was eventually identified as a bacterial signaling molecule.\textsuperscript{120}

The gene reporter assays showed that neither of the two quorum sensing circuits of \textit{P. aeruginosa} were perturbed by the two most active DSDs, SFβC (12) and SFαC (13). Preliminary studies indicate that the receptor of such DSDs and also rhamnolipids maybe the cell surface adhesins, pilin protein.

\textbf{3.3 Conclusion}

The SAR brought forward specific structural elements that were important for imparting potent bioactivities to disaccharide derivatives (DSDs). The DSDs with either cellobiose or maltose stereochemistries having a bulky aliphatic tail, 3, 7, 11-trimethyl-dodecanyl were both good inhibitors of \textit{P. aeruginosa} adhesion and biofilm formation and also potent dispersers of pre-formed (1-day old) biofilm. Making structural changes on DSDs revealed that alteration in the disaccharide stereochemistry produced lesser variation in bioactivity than changes made to the aglycone part of the molecule. From previous chapter (Chapter 2), it was evident that the most active DSDs and also rhamnolipids caused a swarming activation at lower concentrations followed by a reversal in swarming activation at higher concentrations for the \textit{rhlA} mutant.
Contrary to the effect on swarming, the concentration studies with most active DSDs for biofilm inhibition and dispersion showed that the bioactivities plateaued off at higher concentrations with no indication of reversal. However, in a dose-dependent study with the naturally secreted molecule rhamnolipids being added externally for dispersion assay showed an activity reversal phenomenon. Surfactant action associated with amphiphilic structures like DSDs and rhamnolipids was shown to be less likely to cause such biological events. These DSDs did not perturb the known las and rhl quorum sensing circuits of *P. aeruginosa*. Therefore, the mode of action of such amphiphilic molecules is through a yet to be identified pathway. Preliminary studies showed that these molecules could be recognizing the adhesin protein pilin present on *P. aeruginosa* pili appendage. The search for putative receptor/s for such DSDs molecules and also rhamnolipids is ongoing. Importantly, the ability of such DSDs molecules to control *P. aeruginosa* motile lifestyle, i.e adhesion, biofilm formation and dispersion and also motile behavior, i.e swarming, while at the same time being nonmicrobicidal to the growth of the bacteria offers a viable alternative strategy to fight bacteria related problems with lower propensity to cause multi-drug resistance (MDR).

3.4 Materials and methods

**Bacterial strains and plasmids.** *Pseudomonas aeruginosa* strains PAO1 and PAO1-EGFP were obtained from Dr. Guirong Wang (Upstate Medical University). *Pseudomonas aeruginosa* transposon mutant strain PW6886 (rhlA-E08::ISphoA/hah) was obtained from two-allele library.202 PAO1 (*plasI*-LVAgfp), PAO1 (*prhlI*-LVAgfp) strains were prepared by literature reported protocol.214 The plasmid *plasI*-LVAgfp and *prhlI*-LVAgfp were obtained from Dr. Hiroaki Suga (The University of Tokyo) and were maintained by adding 300 μg/mL of carbenicillin in culture media. Freezer stocks of all strains were stored at -80 °C in LB media.
with ~20% glycerol. All strains were grown at 37 °C in a rotary-shaker (at 250 rpm) in Luria Bertini (LB) media (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl). All biofilm inhibition, dispersion and adhesion assays were performed in LB-media and plates were incubated at 37 °C under static conditions.

**Stock solution and delivery of disaccharide derivatives (DSDs).** All but three saccharide derivatives were water-soluble. For the water soluble DSDs, no organic solvents were used to assist the mixing and delivery for all biological assays. Stock solution (~11.5 mM) of DSDs were prepared in autoclaved water and further sterilized by filtering through cellulose acetate syringe filter (0.2 µm pore size, GVS filter technology) into sterilized vials. The sterilized DSDs stock solutions were stored at -20 °C and thawed prior to each use. Aliquots of DSD stock solutions were added to the cultures in wells. Sterile water (same volume as the DSD stock solution used) was used as positive controls (no agents) in all assays to eliminate the effect of water from the stock solution. Three agents DβL (7), SFβL (15) and 2-ODαC (17) exhibited low water solubility, and were prepared in mixed solution of sterile water (90%) and 200 proof EtOH (10 %). Controls for these agents consisted of the same mixed water-ethanol solution.

**Crystal violet (CV) dye based-biofilm inhibition assay.**

Overnight culture of PAO1 were sub-cultured in LB media to obtain an OD$_{600}$ of 0.01, and further cultured to reach an OD$_{600}$ of ~ 0.1. The bacterial culture (200 µL) was added into the wells of a microtiter plate, followed by predetermined volumes from DSDs stocks. The microtiter plate was then wrapped in GLAD Press n’ Seal® (Saran wrap) and further incubated under stationary conditions for 24 h at 37 °C. After incubation, the culture media was discarded by careful pipetting, and the wells were washed once with 200 µL sterile water and the plate was dried at 37 °C in the incubator for 30 mins. Surface attached biofilms in the wells were then stained with 200 µL of 0.1% aqueous
solution of crystal violet (CV) for 30 mins at ambient temperature. The CV stain was then removed by pipetting, and each well was washed with 200 μL sterile water. The resulting surface attached CV stain in the wells was then solubilized by adding 100 μL of 30 % AcOH solution (in water) and mixing gently by pipetting. Adding 100 μL of 30% AcOH solution only solubilizes the biofilm attached to the bottom of the well surface (termed as “surface attached biofilm”) and not the “liquid-air interface” biofilm that is formed at the interface of culture media (200 μL) and air. The amount of biofilms were inferred and quantified by measuring absorption at 600 nm (OD$_{600}$) of the AcOH solution. A negative control that contained only the media without bacterial inoculum and without agents was also stained. The OD$_{600}$ values of the negative control were subtracted from the OD$_{600}$ values of the wells containing bacteria and treated with DSDs. The difference in OD$_{600}$ values of wells with DSDs and wells with no agent (positive controls) was divided by the OD$_{600}$ value of control to obtain percent biofilm inhibition.

**Antiadhesion assay.** Overnight culture of PAO1-GFP (supplemented with 300 μg/mL of carbenicillin) in LB medium was sub-cultured (250 rpm and 37 °C) to an OD$_{600}$ of 0.01. When the OD$_{600}$ of the sub-culture reached ~ 0.1, 200 μL aliquot of the sub-culture was transferred to the wells of 96-well black microtiter plate, followed by designated volumes of DSD stock solutions to achieve desired concentrations of DSD. Positive controls used sterile water in place of DSD stock solution. The black microtiter plate was wrapped by Saran wrap (GLAD Press n’ Seal®) and incubated at 37 °C for 2 h. The bacterial culture was then discarded by pipetting, and each well was washed once with saline water (0.85 w/v% aqueous NaCl). Aliquot of fresh LB medium (200 μL) was then added into the wells and the fluorescence was measured at an excitation wavelength of 500 nm and an emission wavelength of 540 nm by using Synergy 2
multi-mode microplate reader with Gen5 data analysis software. The absorption from wells containing just LB medium was subtracted from wells treated with DSDs.

**Confocal laser scanning microscopy (CLSM) based biofilm inhibition assay.** Steel coupons (316L, thickness 0.012", 1 in. × 1 in.) were washed with EtOH, dried under a stream of nitrogen and then sterilized in an autoclave. Overnight culture of PAO1/EGFP (OD$_{600}$ ~ 1.0) grown in LB media (supplemented with 300 μg/mL of carbenicillin), was sub-cultured to OD$_{600}$ ~ 0.01, and further cultured to an OD$_{600}$ of ~ 0.1. Aliquots of 600 μL PAO1/EGFP subculture was added into the wells of a 24-well microtiter plate, followed by 9 μL of stock solutions of DSDs to obtain the concentration of 160 μM. Bacterial sub-culture with no agent served as positive controls. Sterile steel coupon were then placed into the wells and the plates were wrapped with Saran wrap and incubated for 24 h at 37 °C under static conditions. The steel coupons were then removed and washed by dipping in PBS buffer twice and then gently dabbed on a bleached paper (KIM wipes) to remove the excess solution. The dried steel coupons were viewed under a confocal laser scanning microscope (CLSM). Images were taken at 4 random locations on each steel coupon. The Z-stacked images were also taken to determine the relative thickness of the biofilm.

**Biofilm dispersion assays.** Crystal violet dye-based assay for quantifying dispersion of 1-day old PAO1 biofilms were similar to the biofilm inhibition assay. Wells of the microtiter plate were inoculated with 200 μL PAO1 subculture (OD$_{600}$ ~ 0.1). The plates were incubated at 37 °C for 24 hours to allow biofilm formation. After 24 hours, bacterial culture was gently pipetted out and predetermined volumes of DSDs were added followed by addition of 200 μL of fresh LB medium. The microtiter plate was then incubated for an additional 24 hours at 37 °C. The amount
of the biofilm in the wells was measured using the same procedures for biofilm inhibition assays with the same control solutions.

**Dose dependence assays for biofilm inhibition and dispersion.** Disaccharide derivatives (DSDs) that were most potent at 160 μM (exhibiting > 60% biofilm inhibition and dispersion) were selected for determining the dose dependence. For both biofilm inhibition and dispersion assays, predetermined volumes of DSD stock solutions were added to the assigned wells to achieve a concentration between 0.5 to 170 μM. Percent biofilm inhibition of dispersion was calculated by dividing the difference in biofilm content (absorbance at 600 nm, OD<sub>600</sub>) between wells with DSDs and control wells (no agents) by biofilm content of controls. The percent biofilm inhibition or dispersion was plotted versus the concentrations of DSDs. The graph was curve fitted using equation \( y = m \ln (x) + C \), where \( y \) is % of inhibition (or % dispersion) and \( x \) is the concentration. The half maximal inhibitory concentration for both biofilm inhibition and dispersion (IC<sub>50</sub> and DC<sub>50</sub>) were obtained by solving for \( y = 50 \).

**Synthesis of oxidized pili peptide PAO<sub>(128-144)ox</sub> and scrambled pili peptide**

**PAO<sub>(C129A/C142A)Scrambled</sub>.** Synthesis of the peptides were done according to previously reported procedure. Fmoc-based solid phase peptide synthesis was used and the purification was done by reversed-phase high performance liquid chromatography (HPLC). Air oxidation was employed to oxidize the two cysteines residues in PAO<sub>(128-144)</sub> sequence to generate a di-sulfide linkage. For this a dilute solution (6mg/ 4mL) of unoxidized pili peptide was made PBS buffer (pH ~7.8) containing 10 % DMSO, and solution was stirred in air and monitored by reversed phase HPLC and MALDI-TOF. Following the oxidation of pili peptide, purification was done over reverse phase HPLC. PAO<sub>(128-144)ox</sub> sequence is N’Ac-A-C-K-S-T-Q-D-P-M-F-T-P-K-G-C-D-N-OH-C’. While for generating scrambled pili peptide, cysteines at positions 129 and 142
were replaced with alanines to generate the following sequence; PAO\textsubscript{(C129A/C142)Scrambled}; N’Ac-A-A-K-S-T-Q-D-P-M-F-T-P-K-G-A-D-N-OH-C.
Chapter 4

Synthetic Disaccharide Derivatives Inhibit the Biofilm Formation of *Candida albicans* by Preventing Hyphal Adhesion

Abstract

Disaccharide derivatives (DSDs) with either cellobiose or maltose head groups and having either n-alkyl, farnesyl, hydrogenated farnesyl or 2-octyl dodecyl aliphatic chains were used to nonmicrobicidally inhibit biofilm formation of *Candida albicans*. The tested DSDs did not inhibit the growth of either fungal blastospores or hyphae. Microscopic evaluation showed that none of the tested DSDs (except FβC (1) prevented hyphal germination. The DSDs were only effective at inhibiting the biofilm of *Candida albicans* when applied within five minutes of seeding the test surface with fungal cells. The half-maximal inhibitory concentrations (IC₅₀s) of 14.7 and 21.5 μM for some DSDs like saturated farnesyl-β-maltoside (SFβM, 6) and 2-octyl dodecyl-β-cellobioside (2-ODβC, 7), respectively are comparable to IC₅₀s of known nonmicrobicidal agents that inhibit *Candida albicans*’ biofilm. Tri(ethylene glycol) terminated self-assembled monolayers (SAMs) on gold surface are able to resist *Candida albicans* hyphal adhesion where as pentadecanethiol SAMs allow hyphal adhesion. Using a modified gold surface that contained patterns of pentadecanethiol SAMs surrounded by regions of tri(ethylene glycol) terminated SAMs it was shown that in a media that supports hyphae formation (RPMI-1640), addition of one DSD, saturated farnesyl-α-cellobioside (SFαC, 5) dramatically reduced the adhesion of *Candida albicans* hyphae onto the pentadecanethiol SAMs. It is therefore predicted that, the DSDs’ ability to prevent initial adhesion of *Candida albicans* hyphae translates into their ability to inhibit biofilm formation.
4.1 Introduction

Both bacteria and fungi can reside harmoniously in the same environment including on mucosal surfaces like skin, the oral cavity, and the gastrointestinal tract.\textsuperscript{248} The co-colonization of bacteria and fungi is particularly common in the respiratory tracts of chronic lung-diseased patients. A number of pathogenic fungi like, \textit{Candida},\textsuperscript{249} \textit{Aspergillus},\textsuperscript{250} \textit{Cryptococcus},\textsuperscript{251} \textit{Trichosporon},\textsuperscript{252} \textit{Coccidioides},\textsuperscript{253} and \textit{Pneumocystis}\textsuperscript{254} have been identified as biofilm dwellers. Importantly, the biofilms of these pathogenic fungi have been reported to display severe to mild resistance against the common antifungals like, fluconazole, amphotericin B, nystatin, and voriconazole. In the United States alone, an estimated \$2.6 billion dollars is spent annually to treat fungal related hospital nosocomial infections.\textsuperscript{255} Unfortunately, the increase in the occurrence of fungal biofilm within medical settings parallels the rapid increase in the use of more number of medical devices and implants. The most common fungal commensal known to infect medical equipments is \textit{Candida albicans}.\textsuperscript{256,257} The opportunistic pathogen \textit{Candida albicans} is the most common fungi associated with hospital acquired infections, particularly on medical devices, implants, tissue surfaces, oral and vaginal mucosal epithelial cells.\textsuperscript{258,259} Presently, the approximate mortality rate for device related \textit{Candida albicans} infections in the United States is about \%30.

4.1.1 Biofilm development in \textit{Candida albicans} involves morphogenesis

\textit{Candida albicans} is a dimorphic fungi that exhibits at least two kinds of morphological cells, budding blastospores and filamentateous hyphae. The onset of fungal biofilm progresses through various stages, similar to the step-by-step biofilm formation process exhibited by bacteria. One important difference between bacteria and fungus, is that upon surface attachment fungal cells known as blastospores form filamentous structures known as hyphae that invade into the surface,
where as biofilm dwelling bacterial cells do not show any such morphogenesis. The next step in biofilm progression is secretion of extracellular matrix which coincides with acquisition of drug resistance. Under normal biofilm progression, small oval shaped yeast cells, blastospores first form germ tubes that give rise to pseudohyphae and ultimately tubular hyphae.260 The existence of dimorphic cells (blastospores and hyphae) within fungal biofilms was confirmed via the genetic studies done by Finkel et al.255 Douglas et al, showed that dimorphic morphogenesis was true under both natural and laboratory settings (Figure 4.1).261 The final step in Candida albicans’ biofilm development is the release of yeast cells to initiate colonization of newer surfaces. It has been reported that the biofilm development in Candida albicans is under genetic control and many such genes may display opposite regulation depending upon which step of biofilm is involved i.e. formation or dispersion.255

![Figure 4.1 Dimorphic nature of Candida albicans where both blastospores and hyphae co-exist is evident in both (a) in vitro and (b) in vivo biofilm. The scanning electron microscopic (SEM) micrographs of in vitro biofilm (a) shows lower region comprising of blastospores, middle region containing mainly hyphae and an upper region consisting of both hyphae and blastospores. The in vivo biofilm sample obtained from a rat catheter model (b) shows a mixture of blastospores and hyphae. [Reprinted with permission from Citation:8 Finkel, J. S.; Mitchell, A.](#)

4.1.2 Farnesol is the key quorum sensing molecule

The occurrence of cell-to-cell communication via secretion of various chemical signals during biofilm formation by bacteria has been well documented. Similar cellular communication process through the use of chemical signals has also been reported in many fungal species. The first autoregulatory molecule that was identified to play a crucial role in *Candida albicans* signaling process was 3,7,11-trimethyl-2,6,10-dodecatrienoate (farnesoic acid). By adding the farnesoic acid into the growth media, Oh and coworkers showed that 3,7,11-trimethyl-2,6,10-dodecatrienoate was able to prevent the morphological differentiation from yeast to hyphae. After the discovery of the farnesyl based autoregulatory signal, Ramage and coworkers employed trans-trans farnesol molecule for effective inhibition of *Candida albicans* biofilm. The biofilm inhibition ability of farnesol was mediated through its ability to prevent yeast to hyphae morphogenesis. Later, Alem and coworkers discovered a second QS molecule secreted by *Candida albicans*. This, second QS chemical signal was an alcohol derived from tyrosine. Apart from these two chemical signals, many small molecules like, phenylethyl alcohol, dodecanol and nerodiol have been identified as autoregulators secreted by *Candida albicans* during QS and biofilm formation. The later three molecules are also known inhibitors of hyphal formation therefore, these agents have good biofilm dispersion properties. Similar to genetic regulation of virulence genes in bacteria, the secretion of many extracellular factors responsible for both intercellular and cell-to-substrate adhesion by *Candida albicans* are controlled by a set of genes. Two such *Candida albicans* genes regulate the expression of hyphal wall proteins (Hwp1) and adhesin Eap1. While the expression of *hwp1* is
critical for formation of hyphal wall protein, adhesin Eap1 mediates binding of fungal cells to other cells and to surfaces. Both, hyphal wall proteins (Hwp1) and adhesin Eap1 have become targets for effective therapeutics against *Candida albicans*.

**4.1.3 Interspecies interactions between fungus and bacteria can be cooperative as well as uncooperative**

From clinical perspective, the mixed biofilm formed by bacteria and fungi probably has more adverse effects than biofilm formed by a single species alone. One work consistent with the medical perspective about mixed biofilms was done by Azoulay, E. *et al* in which they showed that the risk of contracting ventilator associated pneumonia (VAP) caused by *Pseudomonas aeruginosa* was increased when the patients respiratory tract was colonized with *Candida spp*. Therefore, exploring ways to treat mixed biofilms is important primarily because dual infection by bacteria and fungus are known to lead to increased mortality rates. Mechanistically, co-existence of bacteria and fungi can exhibit many diverse interactions, that affect either microbial physiology or growth or both, and of these interactions some are symbiotic but most are inhibitory. The autoregulatory signal farnesol secreted by *Candida albicans* has been reported to inhibit the formation of biofilm by *Staphylococcus aereus*. Likewise, the autoinducers secreted by *Pseudomonas aeruginosa* have been shown to prevent *Candida albicans* hyphal formation and hence inhibit biofilm formation.

**4.1.4 Three broad classes of fungicidal agents against Candida albicans**

Overall antifungal agents can be classified into three broad classes; echinocandins, polyenes and azoles. The mode of action for all the three types of antifungal agents is via blocking some fungal metabolic activity or by targeting an important structural element on yeast cells. Apart from theses traditional antifungal agents, Cheng *et al* isolated and characterized
Flocculosin (octadecyl cellobiosides) which is a class of cellobiose-based lipids from a fungus, *Pseudozyma flocculosa* and reported that such agents possessed antifungal activities. Mimee et al used flocculosin isolated from *Pseudozyma flocculosa* for effective antibacterial activity. Cellobiose based molecule isolated from *Trichosporon porosum* by Nifantiev and co-worker was shown to have antifungal activity too.

4.1.5 Antibiofilm strategies developed so far

Farnesol is the cell-signalling molecule of *Candida albicans* and its secretion controls induction of hyphal phase. Ramage *et al* demonstrated that biofilm formation could be prevented when farnesol was applied in the early stages of biofilm formation. As farnesol prevented biofilm formation via blocking germ tube formation, Nickerson *et al* performed a comprehensive structure activity relationship (SAR) study with farnesol analogues to understand how structural changes affected germ tube formation in *Candida albicans*. In that study, a chemical library of forty molecules that differed from farnesol either in head group, or aliphatic chain structure, or in chain unsaturation, or in occurrence of hetero atoms in the backbone were studied for their effect on germ tube formation. Of the forty molecules, only twenty two molecules inhibited the germ tube formation by >50%, and the rest did not even come close to the activity of farnesol. Amongst the better twenty two molecules, the activity of the most potent agent was only ~7% of the activity exhibited by farnesol. The extracellular factors of both bacteria and fungi can affect the physiology and morphology of each other and vice-versa. The prime example of this inhibitory relationship between bacteria and fungus was reported by Hogan *et al* when they showed that two auto inducers secreted by *Pseudomonas aeruginosa*, 3-oxo-C12 homoserine lactone (HSL) and C12-acyl homoserine lactone (AHL) can inhibit hyphal germination in *Candida albicans*. Inhibitory relationship was further elucidated by Holcombe *et al* who
showed that supernatants taken from *Pseudomonas aeruginosa* cultures could decrease the ability of *Candida albicans* to form biofilms on silicone.\textsuperscript{282} Dispersion of *Candida albicans* biofilms by an amphiphilic molecule, dodecyl glucoside was reported by Dusane *et al.* Apart from the antibiofilm agents mentioned here, many other small molecules have also been applied to prevent biofilm formation and disperse existing biofilms of *Candida albicans*.\textsuperscript{283-285} Amongst these, the antibiofilm activities of a class of small molecules isolated from natural sources by Cichewicz *et al.* against *Candida albicans* are the the most potent reported to date.\textsuperscript{286,287}

In the previous chapters (2&3), the effective use of disaccharide derivatives (DSDs) for modulating multiple multicellular behaviors of bacteria *Pseudomonas aeruginosa* was reported. Here, these synthetic DSDs were functional mimics of a class of amphiphilic molecules, rhamnolipids secreted by *Pseudomonas aeruginosa*. Since external factors secreted by both bacteria and fungus have been shown to impact the morphology and physiology of one another, it was but logical to test the effect of DSDs on *Candida albicans*. Since certain DSDs were potent inhibitors of *Pseudomonas aeruginosa* biofilm, their corresponding effect on *Candida albicans* biofilms formation was evaluated. Second compelling reason to test DSDs on *Candida albicans* stemmed from their structural details. The most potent DSDs against *Pseudomonas aeruginosa* biofilm were composed of cellobiose head group and a farnesyl aliphatic tail. The effect of both farnesyl based molecules and cellobiose based molecules on *Candida albicans* has been document earlier. Primarily, many fungicidal agents have been shown to posses cellobiose moieties while farnesol is a known cell-signalling molecule secreted by *Candida albicans*. But a study to understand the effect of structures possessing both the farnesyl and cellobiose moieties on *Candida albicans* morphology and physiology had never been done before.
4.2 Results and Discussions

4.2.1 Structural design of disaccharide derivatives (DSDs) selected for biofilm inhibition of *Candida albicans*

**Figure 4.2 Synthetic disaccharide derivatives (DSD) used in this study.** Inset: Structure of *Candida albicans* quorum sensing molecule (QSM), farnesol.

The structural design of the agents incorporated disaccharide head group (either cellobiose or maltose) and an aliphatic tail (either n-alkyl, or farnesyl, or saturated farnesyl, or lipid). The DSDs that had cellobiose stereochemistry include, farnesyl-β-cellobioside, FβC (1); dodecyl-β-cellobioside, DβC (3); saturated farnesyl-β-cellobioside, SFβC (4); saturated farnesyl-α-cellobioside, SFαC (5) and 2-octyl-dodecyl-β-cellobioside, 2-ODβC (7); and those with maltose stereochemistry included, dodecyl-β-cellobioside, DβC (3); saturated farnesyl-β-maltoside, SFβM (6) (Figure 4.2). The DSDs could also be classified via their aliphatic tail; having a farnesyl tail; FβC (1, cellobiose based); or those having a saturated farnesyl tail; SFβC
(4, cellobiose based); SFαC (5, cellobiose based) and SFβM (6, maltose based); or those having n-alkyl tail; DβM (2, maltose based); and DβC (3, cellobiose based); or the one having a branched lipid tail; 2-ODβC (7, cellobiose based); Since farnesol is a known QSM secreted by \textit{Candida albicans} that has been shown to inhibit biofilm formation, it was also included in the chemical library as a control. Synthesis of all DSDs molecules was done according to previously reported route and the synthetic procedures and characterizations are mentioned in Chapter 2 (Materials & Methods).

4.2.2 \textit{None of the DSDs were toxic to the growth of Candida albicans blastospores}

At the onset it was necessary to establish whether the effect on \textit{Candida albicans} biofilm formation by these DSDs did not originate from their ability to impede growth. For this, the toxic effect of DSDs was evaluated on the growth of \textit{Candida albicans} blastospores grown in yeast-peptone-dextrose (YPD) media. The concentration of DSDs selected 340 μM, was higher or equal to the highest concentration at which all the other bioassays were done (fungal susceptibility assay, biofilm inhibition assay, adhesion assay etc). The growth of blastospores with or without 340 μM of various DSDs in YPD media was monitored by observing absorbance at 600 nm (OD$_{600}$). The growth curves (Figure 4.3) so generated over 24 hours revealed none of the tested DSDs impeded the growth of \textit{Candida albicans} blastospores at 340 μM.
**Figure 4.3 Candida albicans growth curves in YPD medium.** Growth was monitored in the presence (340 μM) or in absence of various DSDs by measuring the optical density of the culture at 600 nm (OD600) over 24 hours.

4.2.3 *The disaccharide derivatives (DSDs) were not fungicidal*

The fungicidal activity of the DSDs on *Candida albicans* was done according to the standard protocol outlined in the NCCLS 2002 CLSI M27-A2 method (micro-broth dilution assay).\(^{290}\) The fungal susceptibility assay was done in RPMI-1640 media and the highest concentration of the DSDs (340 μM) were serially diluted to obtain a minimum inhibitory concentration (MIC\(_{90}\)). Here again, like the growth studies with blastospores, the highest concentration tested was either higher than or equal to the concentrations at which biofilm inhibition studies were done. Images of the micro-broth dilution assay results after 24 hours (Figure 4.4) shows that all the DSDs did not inhibit the growth of *Candida albicans* in the tested concentration range. With no effect on growth of blastospores and no fungicidal activity, the
subsequent effect of these DSDs on the biofilm formation of *Candida albicans* cannot be attributed to the inhibitory effect on growth.

4.2.4 Most DSDs did not inhibit *Candida albicans* hyphae formation

Although, the DSDs were not fungicidal nor had any inhibitory effect on the growth of blastospores, it was also necessary to establish whether such agents affected the germination of hyphae. Aliquots from the microbroth dilution susceptibility assays after 48 hours of inoculation (Section 4.2.3) with few of the DSDs were viewed under a microscope to physically characterize the fungal morphology. Two concentrations of DSDs selected for being viewed under the microscope were 340 and 40 μM and the fungal morphology within cultures containing these concentrations were compared to that of control (observed with 0 μM concentration of the agent). The content of the cultures with the mentioned concentrations was aspirated and then a drop of the culture was gently dispensed atop a microscope slide and viewed under the microscope. The optical micrograph images of some of these DSDs obtained at the two
concentrations (340 and 40 μM) and at 0 μM (control) in RPMI-1640 media are shown in Figure 4.5. Amongst the four tested DSDs, FβC (1), DβC (2), SβC (4) and SαC (5) none (except one, FβC (1)) seemed to inhibit the hyphal formation of *Candida albicans* at both the selected concentrations. However, FβC (1) did seem to prevent hyphae formation at 340 μM. Therefore, overall, it could be concluded that all DSDs were nonfungicidal at tested concentration range and one DSD prevented hyphal formation at 340 μM.

![Figure 4.5 Optical micrograph images of Candida albicans grown in RPMI-1640 media with (at 340 or 40 μM) or without (0 μM; control) DSDs.](image)

The aliquots were obtained from microbroth dilution antifungal susceptibility assays after 48 hours of inoculation. Scale bar ~76 μm
4.2.5 DSDs were screened for biofilm inhibition potencies at 170 μM using a semi-quantitative XTT-dye based assay \(^{291,292}\)

The quantification of *Candida albican* biofilm was done by using a XTT-based semi-quantitative analysis. Since, *Candida albican* under goes yeast to hyphae morphogenesis over time, therefore, it was important to establish what stage of biofilm development did the DSDs inhibit. Therefore, *Candida albican* cells were allowed to adhere for two different time-frame (seeding of cells), 5 minutes and 4-hours (see Materials and Methods for detailed procedure). After the seeding time, wells were washed with PBS buffer and fresh media containing 170 μM of various DSDs was introduced in experimental wells where as no agent was added to control wells. The biofilm was allowed to form for 24 hours, following which quantification was done by adding XTT dye, then storing the microtiter plates in the dark for 3 hours followed by measuring the absorbance of the culture at 490nm.\(^{267,286,292}\) The results of the XTT-dye based quantification are shown in Figure 4.6. When the initial seeding time of 4 hours is allowed, none of the DSDs were able to inhibit biofilm formation of *Candida albicans*. However, when the *Candida albicans* cells were seeded for only 5 minutes followed by addition of fresh media and 170 μM of various DSDs, the percent biofilm inhibited by all agents was > 60 %.

The biofilm inhibition potency of two DSDs, **SFβC (4)** and **SFβM (6)** at 170 μM was particularly high, >85 %.
Figure 4.6 *Candida albicans* percent biofilm inhibition caused by various DSDs with two different seeding times (5 minutes & 4-hours). The biofilm was allowed to form for 24 hours in RPMI-1640 media with or without the agent and then the quantification was done using XTT-dye assay. Error bars are the mean standard deviation of biofilm inhibition from 4-replicate wells.

4.2.6 Dose response assays brought forward potent DSDs

After getting preliminary evidence that with initial seeding time of five minutes, 170 μM of the most DSDs could effectively inhibit *Candida albicans* biofilm, a dose-response curve for all agents was plotted to obtain the half-maximal inhibitory concentrations (IC$_{50}$) for inhibition of *Candida albicans* biofilm. The IC$_{50}$ values of DSDs for inhibiting *Candida albicans* biofilm are shown in Table 4.1. The corresponding dose-response curves have also been plotted (see Materials and Methods). The dose-response study brings forward highly potent antibiofilm agents with SFβM (6) having IC$_{50}$ ~ 14.7 μM and 2-ODβC (7) having IC$_{50}$ ~ 21.5 μM (Table
4.1). We note that structure activity study reveals that DSDs with bulky aliphatic and branched aliphatic chains were generally more potent than DSDs with n-alkyl or unsaturated chains. The activity of $F\beta C$ (1) with $IC_{50} \sim 129 \mu M$ was lesser than all of the DSDs having hydrogenated farnesol aliphatic chain $SF\beta C$ (4), $SF\alpha C$ (5) and $SF\beta M$ (6). Therefore, reducing the number of double bonds in the aliphatic chain from three to zero increased the potency and lowered the $IC_{50}$ for $SF\beta C$ (4) to $\sim 14.7 \mu M$ (31% reduction in $IC_{50}$ value as compared to $F\beta C$ (1)). Apart from inhibition, none of the DSDs exhibited any ability to disperse the pre-formed (24-hours old) biofilm of *Candida albicans*.

<table>
<thead>
<tr>
<th>Agents</th>
<th>$IC_{50}$ (μM)</th>
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<tbody>
<tr>
<td>$SF\beta M$ (6)</td>
<td>14.7</td>
</tr>
<tr>
<td>$2-$OD$\beta C$ (7)</td>
<td>21.5</td>
</tr>
<tr>
<td>$SF\beta C$ (4)</td>
<td>71.5</td>
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<td>$D\beta C$ (3)</td>
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</tr>
<tr>
<td>$SF\alpha C$ (5)</td>
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</tr>
<tr>
<td>$D\beta M$ (2)</td>
<td>122.0</td>
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<tr>
<td>$F\beta C$ (1)</td>
<td>129.0</td>
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</table>

**Table 4.1.** Half-maximal inhibitory concentrations ($IC_{50}$) for inhibition of *Candida albicans* biofilm by various DSDs. DSDs were immediately added after seeding the microtiter plate wells with *Candida albicans* cells ($1 \times 10^5$ cell/mL) for 5 minutes.

4.2.7 *DSD prevent surface adhesion of Candida albicans hyphae*

The next step was to understand the mechanism of action of such DSDs on *Candida albicans* biofilm. Results had indicated that the DSDs did not inhibit growth of *Candida albicans* blastospores or hyphae but yet they were effective at inhibiting its biofilm when applied within five minutes of seeding the wells with cells. As, DSDs had exhibited good antiadhesion property against bacteria *P. aeruginosa* (Chapter 3), the antiadhesive ability against fungus *Candida*
albicans was therefore evaluated. For this, a surface-study was employed where firstly, a modified gold surface was created by micro-contact printing the patterns of alkane terminated self-assembled monolayers (SAMs) surrounded by regions of tri-(ethylene glycol) terminated SAMs on gold. Secondly, the adherence of Candida albicans blastospores and hyphae on such a modified gold surface in presence and in absence of a DSD was studied. In a study performed by Luk and workers previously, it was shown that SAMs with tri(ethylene glycol) termination resist attachment of C. albicans hyphae but not blastospores, where as SAMS terminating with alkyl groups allow adherence of both blastospores and hyphae. Using the adhesion assay, the effect of a DSD, SFαC (5) on adhesion of C. albicans blastospores and hyphae to modified gold surface was evaluated. For observing the effect of SFαC (5) on attachment of blastospores, the C. albicans cells were grown in yeast-peptone-dextrose (YPD) growth media whereas for analyzing the effect on hyphal attachment, the cells were grown in a media that supports hyphae i.e RPMI-1640 media. The control contained no agent where as the experimental well contained 170 μM of DSD, SFαC (5). The adhesion of C. albicans blastospores and hyphae to microcontact printed gold surface in presence and in absence of 170 μM SFαC (5) was observed at two different time points 3 and 6 hours (Figure 4.7). C. albicans grown on gold slides under these conditions was viewed under phase contrast microscope. The phase contrast micrographs are reported in Figure 4.7. From the micrographs, it is evident that at 6 hours, both in presence and in absence of 170 μM of SFαC (5) blastospores appear to adhere everywhere on the microcontact printed gold surface. At 6 hours, under a condition that supports hyphae, dense square patterns were formed on alkane terminated SAMs for control experiment. Whereas, addition of 170 μM of SFαC (5) caused significant reduction in hyphal adhesion onto alkane terminated SAM surface as compared to control. The reduction in adherence of hyphae to
modified gold surface in presence of SFαC (5) could be due to either microbicidal action of such an agent on C. albicans cells or prevention of blastospores to hyphae morphogenesis or such an agent preventing hyphae adhesion. From growth studies in presence of 340 µM of SFαC (5), it is evident that the DSD neither inhibits the growth of blastospores (Figure 4.3) nor the growth of hyphae (Figure 4.4 & 4.5). Therefore, SFαC (5) inhibits surface adhesion of hyphae which possibly leads to inhibition of surface attached biofilm.

**Figure 4.7. Analysis of antiadhesive property of DSD, SFαC (5) against Candida albicans blastospores and hyphae.** Phase contrast optical micrographs of Candida albicans grown in medium supporting either blastospores (left box) or hyphae (right box) growth in presence (170 µM) or in absence of DSD, SFαC (5). Candida albicans were grown on gold slides having squares patterns of pentadecanethiol SAMs surrounded by regions of tri-(ethylene glycol) terminated SAMs. Scale bar ~ 76 µm. Candida albicans sells were inoculated for mentioned time-frame and then viewed under phase contrast microscope.
4.3 Conclusion

This study brought forward a new way to inhibit biofilm formation of fungus *Candida albicans* by making use of DSDs. The growth assays indicated that such DSDs do not inhibit the growth of both blastospores and hyphal cells. Adhesion assay using modified gold surface showed that one DSD, SFαC (5) prevented surface adherence of hyphae. The antibiofilm activity of DSDs against *Candida albicans* was only apparent when these agents were applied within 5 minutes of seeding the surface with fungus cells. Results suggest that the DSDs are not effective antibiofilm agent when applied after 4-hours because longer seeding times would allows hyphae to form and adhere to the surface. Therefore, the antibiofilm activity of such DSDs is probably due to their ability to prevent initial adhesion of hyphae to the surface. Some agents like, SFβM (6) and 2-ODβC (7) exhibited potent antibiofilm activities with IC₅₀s of 14.7 and 21.5 μM, respectively. The “antihyphal adhesion” property of such DSDs could be used as an approach to design new therapeutics that prevent *C. albicans* colonization.

4.4 Materials and Methods

**General experimental information for synthesis.** Synthesis of a DSDs used here are reported in Chapter 2 under Materials and Methods. Fluconazole and trans-trans-farnesol were bought from Sigma Aldrich.

**General information for biological assays**

**Chemicals.** Phosphate buffer saline (PBS), 1-Pentadecanethiol, sulfuric acid (95-98%), (3-N-morpholino) propane sulfonic acid (MOPS) were purchased from Sigma-Aldrich. The piranha cleaning reagents, hydrogen peroxide (30%) and methanol were purchased from Fisher chemical (Fair Lawn, NJ). 200-proof ethanol used for washing and cleaning glass-slides were purchased
Gold deposition was done on piranha cleaned Fisher’s finest premium microscope slides (Pittsburgh, PA). For preparing yeast peptone dextrose (YPD) media to support growth of blastospores, yeast extract was purchased from Fulka (St. Louis, MO), bacto peptone was purchased from BD and Co (Sparks, MD) and dextrose was purchased from Sigma (St. Louis, MO). Media supporting hyphae was prepared from commercially available RPMI-1640 media containing glutamine and NaHCO₃ by buffering with 0.165 M MOPS to a pH (~6.9-7.1). Flat bottom 96-well microtiter plates (untreated) (Costar 3370) and U-bottom 96-well microtiter plates (nontissue culture, BD-falcon) were used for biofilm inhibition and fungicidal assays respectively. For assays involving patterned gold slides 24-well plate were purchased from Nunc (Thermofisher Scientific, Pittsburgh, PA). Synthesis of tri(ethylene glycol)-terminated undecanethiol was done by previous group member.

**Fungal strains and growth media condition.** Clinical isolate of *C. albicans* SC5314 were obtained from Dr. Dacheng Ren (Syracuse University). To culture blastospores, a loopful of *C. albicans* SC5314 glycerol stock maintained at -80°C was cultured in 5mL yeast peptone dextrose (YPD) media (1%, w/v yeast extract; 2%, w/v of peptone, 2%, w/v of dextrose in autoclaved water) overnight in orbital shaker (250 rpm) overnight at 35 °C to produce phase of budding blastospores. For experiments involving surface adhesion, working stocks of blastospores were prepared by subculturing overnight culture to an optical density OD₆₀₀ ~ 0.05 as measured by Biotek ELx800 absorbance plate reader which uses Gen 5TM data analysis software. Hyphal induction was achieved by harvesting cells from 5 mL of overnight YPD culture by centrifugation at 3000g for 5min at room temperature, followed by discarding supernatant, resuspending yeast cells in 20 mL of sterile PBS buffer (pH ~7.4) twice followed by addition of prewarmed (37°C) RPMI 1640 media (20 mL) buffered to pH 7.04 with 0.165M MOPS. From
this suspension, a working cell suspension containing $1 \times 10^5$ cell mL$^{-1}$ of *C. albicans* was obtained by doing a 1:100-fold dilution with buffered RPMI 1640 media followed by counting cells with a hematocytometer.

**Effect of disaccharide derivatives on growth of *Candida albicans*:** Yeast cells were grown overnight in yeast-dextrose peptone (YPD) media at 35 °C with constant shaking (250 rpm). The overnight culture was subcultured to an initial optical density at 600 nm (OD$_{600}$) of ~0.05 measured by Biotek ELx800 absorbance plate reader which uses Gen 5TM data analysis software. The sub culture was then grown in presence (340 μM) or in absence of various DSDs to a final OD$_{600}$ of ~1.0 under constant rotatory conditions (250 rpm) at 35 °C. The OD$_{600}$ at various time intervals were plotted to obtain a growth curve (Figure 4.3).

**Micro-broth based anti fungal susceptibility assay**

*Prepapring the microtiter plates and delivering agents.* The DSD agents were prepared as 11.5 mM stock solution in sterile water. To the wells of 96-wells microtiter plate was added 100 μL of RPMI-1640 media (buffered to pH ~7.00 by adding MOPS buffer). To the wells in column 11 was added additional 100 μL of RPMI-1640 media. Aliquots of various DSD stock solutions were added to wells in column number 11 at double the required concentration. The media in column 11 wells was aspirated ~ six times with the use of a pipette set at withdrawing 100 μL of solution. After mixing the DSD stock solution six times with the RPMI-1640 media, 100 μL of the mixture was withdrawn an added into the adjoining wells on the left (i.e column 10). The contents were aspirated and again 100 μL was removed and added to wells of column nine and so on. Following this serial dilution of DSD, the extra 100 μL of solution remaining after last the dilution was discarded away. Appropriate amount of water was added into wells serving as
positive controls. The micro-titer plate containing RPMI-1640 media with serially diluted DSD agent was now ready to receive 100 μL of *Candida albicans* working culture stock.

*Culturing C. albicans for fungicidal studies.* Yeast cells were grown in YPD media (5mL) at 35 °C with constant shaking (250 rpm) for ~16 hours. After 16 hours the yeast cells were harvested by centrifuging at 3000g for 5 mins at room temperature. The harvested cells were washed twice (20 mL) with PBS buffer solution (pH ~ 7.4, 10 mM). After washing, the yeast cells were suspended in 20 mL of RPMI-1640 media to obtain the culture stock. The stock culture was diluted 100 times and cells were counted using a hematocytometer to a final cell count of 2 X 10⁵ cells/mL to obtain a working stock of culture. Aliquots (100 μL) of the working culture stock were then added into each well of the microtiter plate having serial dilution of the DSD agent. The resultant culture in each well was mixed gently by swirling the solution with clean pipette tips. The plates were then covered with lids and placed at 35 °C for 48 hours. After 48 hours, the amount of cellular growth in each well was visually inspected and compared to the wells having no agent (positive control). Images of the wells after 48 hours of growth were taken with a camera. Micro-titer plate containing serially diluted flucanazole was also optimized to serve as a control.

*Inspecting for hyphal growth.* For observing the growth inhibition of hyphae in presence of various DSDs, 3 μL of culture from the wells (340, 60 and 0 μM) of the micro-broth dilution experiments were spotted on a microscope slide and viewed under a microscope (Olympus BX51 microscope) and images were taken under 10X zoom using a mounted Olympus wide zoom camera.
**XTT-based biofilm inhibition assays.**

*Preparation of the reagents for the assay.* A semi-quantitative XTT [2, 3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide]-reduction assay was used to quantify biofilm formed by *C. albicans* SC5314. For this, XTT was prepared as 0.5 mg/mL solution in 10 mM sterile PBS buffer (pH ~7.4) and filtered through 0.22 μm filter, aliquoted (5 mL) into vials wrapped with aluminum foil and stored at -80 °C in the dark. A solution of menadione (5 mM) in acetone was also prepared, aliquoted as 50 μL working stock into eppendorf tubes and stored at -80 °C. During the day of quantitative measurement for biofilm, a freezer aliquot of both XTT and menadione was removed, thawed to room temperature prior to use. Leftover solution was never reused for a subsequent assay. Stock solutions of all antibiofilm agents were prepared in sterile water (11.5 mM), filtered through a 0.22 μm filter and stored at -20 °C. A stock solution of trans-trans-farnesol was also prepared (11 mM) in MeOH to serve as a control for biofilm assay.

*Serial dilution of DSDs for dose-response assay.* Aliquots (100 μL) of *C. albicans* (1 x 10⁵ cell mL⁻¹) from a working culture stock prepared in RPMI-1640 media were seeded into the wells of a 24-well microtiter plate. Two different seeding times, 5 mins and 4 hours were chosen. After the seeding time, the culture was removed and the wells were washed and aspirated with 200 μL of PBS buffer (10 mM, pH~7.4) to remove nonadherent cells. Following this, fresh media (RPMI-1640) (200 μL) containing the highest concentration of agent (340 μM) was added to wells in column number 11. Fresh media (100 μL) was also added to the remaining wells (column 2-10). Serial dilution of the agent was performed by mixing with pipette the content of column 11 gently, then removing 100 μL of culture media and adding to wells in column 10. Again the contents of column 10 were mixed gently and 100 μL was removed and added to the
next column (9) and so on. After serial dilution of column 3, the extra 100 μL was discarded. Column 2 served as positive control. Column 12 content only RPMI-1640 media to serve as a negative control. Following the serial dilution of agent, the microtiter plate was wrapped in GLAD press (saran wrap) and incubated at 37 °C under static conditions for 24 hours.

**XTT-reduction assay.** Prior to each assay, 1 μL of thawed menadione was added to the thawed XTT solution (5 mL). After 24 hours incubation, the culture was removed and the wells of the microtiter plates were gently washed and aspirated twice with 100 μL of PBS buffer solution to remove nonadherent and planktonic cells. Following this 100 μL of XTT/menadione solution was added into the wells and the plates were incubated in the dark for 3 hour at 37 °C. The surface attached metabolically active cells can reduce XTT into a water soluble orange dye, formazan. A semi-quantitative measurement of surface attached biofilm was therefore made by measuring the absorbance from different wells at 490 nm and comparing the $A_{490}$ to the readings obtained from the wells without any agent.

![Figure S1. Schematic depicting XTT-dye based biofilm assay for *Candida albicans* with two different seeding times (5 minutes and 4 hours).](image-url)
Figure S2. Dose-response curves for inhibition of *Candida albicans* biofilms by various disaccharide derivatives (DSDs).

Adhesion studies with a modified gold surface

*Piranha Cleaning of Glass Substrates.* Piranha solution (7 parts of concentrated sulfuric acid and 3 parts of hydrogen peroxide (30 % in water)) was used to clean glass substrates prior to gold deposition. For this, the glass substrates were first washed with 200-proof ethanol and dried under a stream of nitrogen, followed by dipping the slides for 45 min in a piranha solution maintained at 70 °C. Following this, the piranha solution was allowed to cool to room temperature and slowly poured off. Multiple washes (~20 times) of the slides was done with deionized water, followed by 10 washes with ethanol and 10 washes with methanol. The slides
were dried off by placing under a stream of nitrogen gas and then kept in the oven overnight at 100 ºC. The dried slides were subsequently used for gold deposition.

**Gold deposition on gold slides.** An electron beam evaporator system (Thermionics, Port Townsend, WA) was used to deposit a semi-transparent film of gold on the piranha cleaned glass slides. Deposition of titanium followed by gold was done by placing the glass slides in a holder such that the deposition angle was 45° to the glass surface. The first coating applied on glass to enhance adhesion of gold was that of titanium (~70 Å). Following deposition by titanium, gold was deposited on the surface at a rate of 0.1 Å/s to achieve a final gold thickness of ~280 Å.

**Micropatterning of pentadecanethiol SAMs surrounded by tri(ethylene glycol) terminated alkanethiols.** Previous protocol reported by Luk et al. was used to create micropatterned regions of pentadecanethiol SAMs surrounded by tri(ethylene glycol) terminated alkanethiols. Briefly, the gold deposited glass slides were cut into 1 cm x 1 cm squares and washed with 200 proof ethanol followed by drying under a stream of nitrogen gas. A polydimethylsiloxane (PDMS) stamp having size (380 X 380 μm²) washed with 200 proof of ethanol and dried under nitrogen gas. The clean PDMS stamp was dabbed with a thin film of 2 mM ethanolic solution of pentadecanethiol and later stamped for 20 seconds on the gold slides. Following the stamping the glass slide was rinsed with ethanol, dried under nitrogen and then immersed in 2mM ethanolic solution of tri(ethylene glycol) terminated undecanethiol for 18h. After 18 h, the micropatterned gold slides (called gold slides henceforth) were removed from the solution, rinsed with ethanol and dried under a stream of nitrogen gas.
Adhesion of blastospores and hyphae on micropatterned gold slides.

Three separate 24-well microtiter plates were prepared such that each plate contained four micropatterned gold slides in the inner four wells. Two wells in each plate was inoculated with YPD culture containing blastospores (OD$_{600}$ ~ 0.05; 600 μL), while in the remaining two wells were inoculated with $C.\, albicans$ (1 x $10^5$ cell mL$^{-1}$) in RPMI-1640 media (600 μL). In each plate, one well with YPD yeast inoculum was supplemented with $SFαC$ (5; 170 μM) and one well with RPMI inoculum was also supplemented with $SFαC$ (5; 170 μM). The remaining wells containing no agents served as controls for YPD and RPMI conditions, respectively. In the remaining outer wells, sterile water (600 μL) was added to limit evaporation and drying from the inner wells. The microtiter plates containing the gold slides and culture media were then wrapped in GLAD Press n’ Seal® (saran wrap) and further incubated under stationary conditions at 37 °C. The time of incubation was noted and for observation. The gold slides were removed from the media after three, six and twenty four hours and washed gently by slowly dipping in
sterile water once followed by dabbing the edge of the gold slide on a kim wipe to remove excess water. The gold slides were viewed under phase contrast microscope (Motic, AE31, equipped with Infinity 2 Camera) under 4X magnification.
Chapter 5

Future directions

One future direction of the *Pseudomonas aeruginosa* project (chapters 2 & 3) would be to create activity based probes to identify the target protein. Results in this thesis have indicated that the three bioactivities, i.e. biofilm inhibition, adhesion inhibition and swarming modulation associated with the saccharide derivatives are less sensitive to the sugar stereochemistry in comparison to the aliphatic structure. Hence, making chemical modifications like epoxides on the sugar head groups are less likely to reduce the molecule's activity when compared to the same change done to the aliphatic chain. In future, sugar derivatives having epoxide modification could serve as good activity based probes for making covalent linkage with the active site.

Once the receptor protein has been identified, the next goal would be target validation, identification of the active site and ultimately performing binding studies. Structure of active site and identification of key interactions will be important for creating more optimized saccharide structures.

Rhamnolipids and its aliphatic chain variants are produced by a few bacteria including *Pseudomonas aeruginosa, Burkholderia glumae* and *Burkholderia pseudomallei*, and such secreted rhamnolipids have also been used to inhibit the biofilm formation for a wide range of other bacterial species, including *Rhodococcus erythropolis, L. monocytogenes, Staphylococcus aureus, Bacillus pumilus*. These findings call for a deeper understanding of how rhamnolipids function with respect to their specific biological activities that other generic surfactants and biosurfactants cannot perform. Because of the versatile bioactivities, this class of secreted
molecules has the potential to be developed as nonmicrobicidal therapeutic agents that can control bacterial behaviors without invoking drug resistance.
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