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UNDER THE MICROSCOPE

The involvement of rhamnolipids in microbial cell adhesion and biofilm development – an approach for control?

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Abstract

Biofilms are omnipresent in clinical and industrial settings and most of the times cause detrimental side effects. Finding efficient strategies to control surface-growing communities of micro-organisms remains a significant challenge. Rhamnolipids are extracellular secondary metabolites with surface-active properties mainly produced by *Pseudomonas aeruginosa*. There is growing evidence for the implication of this biosurfactant in different stages of biofilm development of this bacterium. Furthermore, rhamnolipids display a significant potential as anti-adhesive and disrupting agents against established biofilms formed by several bacterial and fungal species. Their low toxicity, biodegradability, efficiency and specificity, compared to synthetic surfactants typically used in biofilm control, might compensate for the economic hurdle still linked to their superior production costs and make them promising antifouling agents.

Introduction

Our idea of bacteria's lifestyle as loner planktonic organisms has dramatically changed; we rather consider them now as highly social organisms living in communities (Shapiro 1998). Living in a sessile community (typically a biofilm) is a trait which highly increases the survival fitness of bacteria facing unpredictable fluctuating and adverse conditions in their surroundings through augmenting their rate of adaptation and defence mechanisms (Davey and O'Toole 2000).

However, from our perspective, although often beneficial, biofilms are mostly regarded as harmful as they pose serious problems in industrial or clinical environment, either acting as biofouling agents or being the cause of the most resilient chronic medical device-associated infections in hospitalized patients (Hall-Stoodley *et al.* 2004).

This has led many studies to focus on identifying potential targets to control the detrimental biofilms both in industrial and clinical settings (Davies *et al.* 1998; Donlan 2009; Rendueles and Ghigo 2012).

In spite of recognizing potential targets, no efficient antibiofilm products have found way to the market yet, and this has been largely attributed to economic issues

(Romero and Kolter 2011). It is believed that finding an agent which targets a population-level trait and hence abolishes the chance of inducing resistance against its own action is highly promising and could weigh up against the existing economic hurdles (Boyle *et al.* 2013).

Biosurfactants and their role in biofilms

Biosurfactants are amphipathic molecules produced by a variety of bacteria, fungi and yeasts. Formed of both hydrophobic and hydrophilic moieties, these molecules act as surface-active agents in the microbial world and come with structural diversity and environmental compatibility (Desai and Banat 1997). Although several important functions for biosurfactants are known, questions remain concerning their natural roles and the mechanisms behind their production (Chrzanowski *et al.* 2012).

A number of studies have addressed the role of biosurfactant production in biofilm formation by different micro-organisms (Mireles *et al.* 2001; Walencka *et al.* 2008; Rivardo *et al.* 2009; Kanmani *et al.* 2011). For example, Kuiper *et al.* (2004) have shown that production of lipopeptide biosurfactants (putisolvins) by *Pseudomonas*

putida is involved in development of its biofilm. Furthermore, putisolvins were not only able to inhibit the formation of biofilms by other *Pseudomonas* species, but also disrupt established ones. Similarly, the biosurfactant produced by *Streptococcus thermophilus* was effective in decreasing the initial adhesion of four bacterial and two yeast strains isolated from explanted voice prostheses to silicone rubber (Rodrigues *et al.* 2006a). Actually, the general potential of biosurfactants in altering cell surface properties of different micro-organisms and hence interfering with initial adhesion to solid surfaces and biofilm formation are increasingly investigated (Ron and Rosenberg 2001; Flemming and Wingender 2010; Renduelles and Ghigo 2012).

Probably the best studied model to investigate the involvement of a biosurfactant in adhesion and biofilm development is production of rhamnolipids by the opportunistic pathogen *Pseudomonas aeruginosa*. Rhamnolipids, which were first discovered in 1946 by Bergström *et al.*, are surface-active glycolipids usually constituted of a dimer of 3-hydroxy fatty acids (typically from C₈ to C₁₂ length) linked *via* an O-glycosidic bond to a mono- or di-rhamnose moiety (Jarvis and Johnson 1949). Naturally produced rhamnolipids are always found as mixtures of different congeners (Déziel *et al.* 1999, 2000; Abdel-Mawgoud *et al.* 2010), and evidence shows that the individual molecules can exert different biological effects (Caiazza *et al.* 2005; Tremblay *et al.* 2007). Many functions have been attributed to these amphipathic exoproducts (see Abdel-Mawgoud *et al.* 2010 for a review); they were originally described as heat-stable haemolysins (Sierra 1960) and then intensely studied for their involvement in the assimilation of hydrocarbons (Hisatsuka *et al.* 1971) and as antimicrobials (Itoh *et al.* 1971).

In recent years, rhamnolipid production by *Ps. aeruginosa* has been increasingly shown to actually play an indispensable role in the establishment of the biofilm lifestyle, as they are involved in different stages of biofilm development, upon the earliest cell-to-surface interactions to maintenance and dispersion/disruption of the biofilm architecture (Davey *et al.* 2003). Rhamnolipids can function as virulence factor as they mediate the active dispersal of cells from biofilms, helping in the colonization of new sites and niches (Schooling *et al.* 2004). Moreover, they act as key protective agents of *Ps. aeruginosa* biofilm against phagocytes (Van Gennip *et al.* 2009). Besides, rhamnolipids display low toxicity, high biodegradability and effectiveness of their surface-active properties at wide range of temperatures, pH and salinity (Banat *et al.* 2000; Abdel-Mawgoud *et al.* 2011). The above evidence suggests that rhamnolipids might represent an attractive target to be exploited against their own producers and other biofilm-forming microbes to control the colonization of surfaces.

In this review, we discuss the role of rhamnolipids in the development of biofilms and highlight their potential not only to disperse bacteria and fungi from biofilms, but also to prevent their formation.

Rhamnolipids are actively involved in different stages of biofilm development

The first published insights about the role of rhamnolipids in the structural regulation of biofilm development showed the ambivalent influence of rhamnolipids on impediment of biofilm development of *Ps. aeruginosa* depending on the spatiotemporal action of rhamnolipids; although the exogenous addition of rhamnolipids impeded initial bacterial adhesion, it had no effect on preformed biofilm (Davey *et al.* 2003). However, endogenous production of rhamnolipids in the biofilm could interfere with the final stages of biofilm formation (Davey *et al.* 2003). Hence, it could be concluded that the stability of the biofilm structure is dependent on the production of the appropriate amount of rhamnolipids at the right moment. Indeed, rhamnolipid producers have evolved intricate regulatory mechanisms to fulfil the timely expression of genes responsible for rhamnolipid biosynthesis. In support of this, Lequette and Greenberg (2005) showed stage-specific expression of the *rhlAB* operon in *Ps. aeruginosa* biofilms. These genes encode the enzymes required for rhamnolipid biosynthesis (Ochsner *et al.* 1994a).

Moreover, regulation of rhamnolipid production in *Ps. aeruginosa* is controlled in a cell density-dependent manner through quorum sensing (Ochsner *et al.* 1994b; Ochsner and Reiser 1995) and exhibits partial cross-regulation by RpoS, which ensures precise timing of synthesis gene expression in biofilm, which is essential for the normal development of biofilm architecture (Medina *et al.* 2003).

Adherence and microcolony formation

For planktonic bacteria, initial adhesion to a surface can be regarded as the first and the most crucial step for further colonization (Palmer *et al.* 2007). Depending on the nature of the surface whether abiotic or biotic, multiple parameters, either nonspecific such as hydrophobicity, or specific such as the presence of particular molecules such as lectins, ligands or adhesins can be associated with the mechanism of adhesion (Dunne 2002). In this context, amphipathic molecules with interfacial activity such as rhamnolipids have the ability to alter cell-to-surface and cell-to-cell interactions and have the potential to diminish the ability of bacteria to adhere to the surfaces. While their overproduction inhibits biofilm development (Davey *et al.* 2003), depending on the concentration of rhamnolipids present,

the result can be different. For instance, low concentration of rhamnolipids affects the cell surface properties through increasing cell hydrophobicity by causing a release of lipopolysaccharide from the cell surface, thereby increasing the affinity for initial adherence of cells to a surface (Zhang and Miller 1994; Al-Tahhan *et al.* 2000; Raya *et al.* 2010).

Once the bacteria attach to a surface, they begin clonal propagation and surface movement to form microcolonies (O'Toole and Kolter 1998). In agreement with the specific spatiotemporal role of rhamnolipids in biofilms, Pamp and Tolker-Nielsen (2007) demonstrated that rhamnolipids are necessary for the initial microcolony formation. In fact, it seems that increased hydrophobicity of cells induced by low concentration of rhamnolipids is sufficient enough to facilitate microcolony formation through enhancing aggregation of *Ps. aeruginosa* cells together (Herman *et al.* 1997; Pamp and Tolker-Nielsen 2007). While rhamnolipids seem indispensable for initial microcolony formation (Pamp and Tolker-Nielsen 2007), it is suggested that under certain conditions such as iron-limited biofilms, higher production of rhamnolipids induced by iron deficiency may contribute to increased twitching motility which, as a consequence, prevents initial microcolony formation (Patriquin *et al.* 2008). Again, it appears that the quantity of the produced rhamnolipids is a key to stability and balanced formation of microcolonies (Glick *et al.* 2010).

Proliferation and formation of the differentiated biofilm

In *Ps. aeruginosa* biofilms, at the onset of biofilm maturation, microcolonies act as platforms for formation of the stalk of the mushroom-like structures at certain foci (Klausen *et al.* 2003a,b). Then, through the emergence of a motile subpopulation, bacteria capable of migrating up the stalks form mushroom caps. For this kind of migration, bacteria require the presence of type IV pili and flagellum-mediated motility (Barken *et al.* 2008). Evidence that biosurfactant production is once again involved was presented as a *rhlA* mutant in mixed *pilA/rhlA* biofilms exhibited reduced cap formation, because of the lack of rhamnolipid production (Pamp and Tolker-Nielsen 2007). Then, once the mushroom-shaped structures are formed, another role of rhamnolipids is concerned with the maintenance of the highly hydrated structure of biofilms through prevention of colonization of the channels formed between these structures (Davey *et al.* 2003).

Detachment and dispersion of planktonic cells

Development of a biofilm typically culminates in the detachment and dispersal of cells (Kaplan 2010). This process has been mainly categorized into two different

types of events according to the nature of the cues triggering the dispersion: passive, typically shear dependent, or active, which is a dynamic and highly regulated mechanism (McDougald *et al.* 2012). Seeding dispersion is an active mechanism, where detachment of cells occurs at late stages of biofilm formation and is actively mediated by rhamnolipids (Schooling *et al.* 2004; Boles *et al.* 2005; Wang *et al.* 2013). For instance, Boles *et al.* (2005) demonstrated that rhamnolipid-mediated detachment mechanism involves the formation of cavities within the centre of biofilm structures.

All together, evidence shows that rhamnolipids play a central role in biofilm development. As these surface-active molecules are well known to be essential, along with flagella, for the type of surface group behaviour called swarming motility (Déziel *et al.* 2003; Caiazza *et al.* 2005; Tremblay *et al.* 2007), it is hypothesized that swarming occurs inside the biofilm which leads to motility-associated dispersal (Wang *et al.* 2013).

Figure 1 summarizes the various functions attributed to rhamnolipids in bacterial adhesion to surfaces and biofilm development.

From evidence to action

As biofilm development involves similar steps in most bacterial species (O'Toole *et al.* 2000) and many bacteria require the production of surface-active molecules to express swarming motility (Kearns 2010; Partridge and Harshey 2013), modulating interaction between bacteria and surfaces *via* the use of biosurfactants such as rhamnolipids is naturally considered.

Indeed, Irie *et al.* (2005) demonstrated the ability of rhamnolipids to disperse preformed biofilms of *Bordetella bronchiseptica*. Moreover, the potent antibiofilm activity of rhamnolipids was evaluated against several microbial species associated with biofilm formation on voice prostheses and silicone rubber in the presence and absence of adsorbed rhamnolipids (Rodrigues *et al.* 2006b). The anti-adhesive activity of rhamnolipids at different concentrations was significant against all the strains and depended on the micro-organism tested, with a maximal initial reduction of adhesion rate (66%) reported for strains of *Streptococcus salivarius* and *Candida tropicalis* (Rodrigues *et al.* 2006b). Furthermore, rhamnolipid conditioning of the silicon rubber caused a reduction of 48% in cell adherence of tested *Staphylococcus epidermidis*, *Strep. salivarius*, *Staphylococcus aureus* and *C. tropicalis* strains.

Further evidence on efficiency of rhamnolipids to inhibit initial adhesion of bacteria was recently reported by Sodagari *et al.* (2013). In this study, three Gram-negative species *Ps. aeruginosa*, *Ps. putida* and *Escherichia coli* and

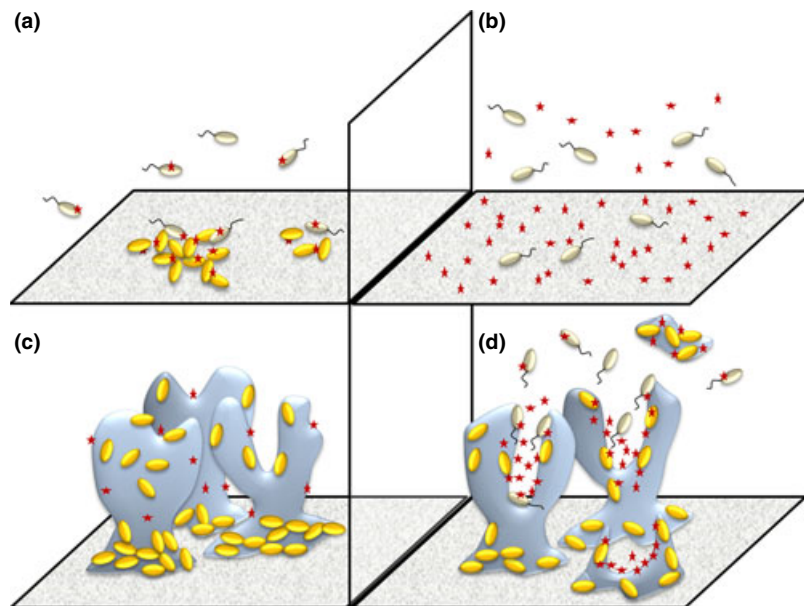


Figure 1 Representation of rhamnolipid implication in different stages of *Pseudomonas aeruginosa* biofilm development. (a) Low concentration of rhamnolipids increase affinity of cells for initial adherence to surfaces through increasing cell's surface hydrophobicity; (b) Presence of high concentrations of rhamnolipids in surrounding medium prevents attachment of cells and further microcolony formation; (c) At proliferation stage, rhamnolipids are actively involved in the maintenance of the complex-differentiated architecture of the biofilm; (d) At late stages of biofilm development, rhamnolipids promote seeding dispersal of motile cells. The red stars represent rhamnolipids.

two Gram-positive species *Staph. epidermidis* and *Bacillus subtilis* were assessed for their ability to establish biofilm on hydrophilic glass and hydrophobic octadecyltrichlorosilicone (OTS)-modified glass in the presence of two different concentrations of rhamnolipids. Rhamnolipids significantly reduced the attachment of all but *Staph. epidermidis* on both glass and OTS-modified glass. For *Staph. epidermidis*, rhamnolipids reduced the attachment on OTS-modified glass but not on glass. Several mechanisms might occur, and the authors investigated potential ones. For instance, rhamnolipids were found ineffective in modifying substratum surface properties and in facilitating the detachment of already attached cells, whereas rhamnolipids could inhibit the growth of *B. subtilis*, *Staph. epidermidis* and *Ps. aeruginosa* PAO1 but not the growth of *E. coli*, *Ps. putida* and *Ps. aeruginosa* E0340. Also, rhamnolipids were found effective in changing the cell surface hydrophobicity of the tested strains, although no clear effect was observed on *B. subtilis*. Despite the observed trends on cell detachment, the authors did not find any correlation with the potential mechanisms by which rhamnolipids influence cell detachment, and hence, the responsible mechanism(s) remained to be elucidated.

With regard to the biofouling problems, such as reduced heat transfer across heat exchanger surfaces, caused by bacterial biofilms in industrial settings exposed to the marine environment, Dusane *et al.* (2010) investigated the ability of rhamnolipids to inhibit adhesion and disrupt preformed *Bacillus pumilus* biofilms. The effectiveness of rhamnolipids to impair adhesion of *B. pumilus* cells to microtitre plates varied from 46 to 99% depend-

ing on the concentrations of the rhamnolipids investigated ($0.05\text{--}100\text{ mmol}^{-1}$). The minimal inhibitory concentration (MIC) of rhamnolipids against planktonically growing *B. pumilus* was 1.6 mmol^{-1} , while a disruption of up to 93% of preformed *B. pumilus* biofilms was achieved at 100 mmol^{-1} , showing the effectiveness of rhamnolipids as promising compounds for inhibition/disruption of marine biofilms.

Apart from the efficacy of rhamnolipids against bacterial biofilms, a number of recent studies have also demonstrated the activity of rhamnolipids against fungal biofilms. Compared to conventional synthetic surfactants being used in medical settings such as cetyl trimethyl ammonium bromide (CTAB) and sodium dodecyl sulfate (SDS), Dusane *et al.* (2012) tested the potential of rhamnolipids to prevent biofilm formation and disrupt pre-established biofilms of the yeast *Yarrowia lipolytica*. In their study, precoating of microtitre plate wells with rhamnolipid effectively reduced *Y. lipolytica* biofilm formation by 50% as compared to CTAB, which inhibited by 29%, and SDS which decreased biofilms by <10% at their respective MIC values. Moreover, rhamnolipid displayed 55% dispersion of *Y. lipolytica* biofilms (formed for 3 days in microtitre plate wells and treated for 1 h), while 35% disruption and 40% disruption were observed with CTAB and SDS, respectively, at their respective MIC values.

Further evidence on effectiveness of rhamnolipids to disrupt fungal biofilms was recently shown by evaluating the potential of rhamnolipids to disrupt fungal biofilms of *Candida albicans* formed on polystyrene surfaces (Singh *et al.* 2013). Anti-adhesive activity of rhamnolipids

on *Candida* cell adhesion was showed to be concentration dependent. About 50% of the cells remained adhered to 96-well plate after 2 h of treatment with 0.16 mg ml⁻¹ of rhamnolipid; while up to 90% reduction in pre-established *C. albicans* biofilm on polystyrene surface was observed with rhamnolipid treatment at concentration of 5.0 mg ml⁻¹ (Singh *et al.* 2013).

Conclusion

Taken together, the available literature supports the potential of rhamnolipids produced by *Ps. aeruginosa* as anti-adhesive and dispersing agents effective against established bacterial and fungal biofilms. Besides direct use in solution to disrupt already established biofilms in clinical and industrial settings, embedding of materials with rhamnolipids or surface coating might represent a promising approach to prevent the initial adhesion of bacteria and fungi. The environmental friendliness and specificity of biosurfactants might compensate for the economic hurdles still linked to their superior production costs as compared to the costs of synthetic surfactants.

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Conflict of interest

No conflict of interest declared.

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