Salicylic Acid Attenuates Biofilm Formation But Not Swarming In 
*Pseudomonas aeruginosa*

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*Pseudomonas aeruginosa*, a commonly found, nosocomial, opportunistic pathogen, is characterized by its ability to form biofilm to protect itself from various external pressures. Infection by this species is especially prominent in cystic fibrosis patients and is difficult to treat, especially with the rampant increase in antibiotic resistance. Salicylic acid has been indicated in biofilm attenuation of *P. aeruginosa* while quorum sensing plays a key role in the growth and maintenance of a biofilm. Many virulence factors in biofilm formation are also involved in motility. Here, we investigated the use of salicylic acid in biofilm and swarming motility attenuation through the quorum sensing gene, *lasR*. Overnight cultures of wildtype *P. aeruginosa* and a *lasR* deficient mutant were incubated with varying concentrations of salicylic acid. Their biofilm densities were assessed by crystal violet assay. An inversely proportional relationship between salicylic acid and biofilm density was found for both strains with a maximum inhibitory concentration of ~2 mM. Differences in biofilm density between strains were statistically significant (p<0.05) for salicylic acid concentrations above 0.25 mM, but not in the expected manner. Swarming capabilities of both strains were assessed using LB growth plates with varying concentrations of salicylic acid. Conclusive results were not obtained due to the drastic differences among replicates, but results indicate swarming motility to be independent of salicylic acid and the quorum sensing gene, *lasR*. In conclusion, salicylic acid inhibits biofilm formation in a concentration dependent manner through mechanisms other than the *lasR* quorum sensing gene.

*Pseudomonas aeruginosa* is a common gram-negative bacterium found in a variety of nosocomial infections (15). One of the key factors rendering treatment of infections difficult is the formation of biofilm. Biofilm formation in *P. aeruginosa* has been shown to protect the bacterium from antibiotics and environmental fluctuations and is characterized by an exopolysaccharide secretion (1). *P. aeruginosa* colonization in the lungs is especially problematic in cystic fibrosis (CF) patients who are prone to biofilm-type infections (15).

Both biofilm formation and production of a number of virulence factors have been shown to be controlled by intercellular communication, i.e., quorum sensing (QS) (6, 11). In *P. aeruginosa*, QS controlled genes are largely activated through binding of the LasR regulator ligand (N-(3-oxo-dodecanoyl)-homoserine lactone (3-oxo-C12-HSL)) to the LasR regulator (6). A few virulence factors produced under the control of QS are biosurfactactants; rhamnolipids and 3-(3 hydroxyalkanoyloxy) alkanoic acids which are key in swarming motility and work by decreasing the surface tension between the cell and its environment (16). Swarming motility plays an important role in the pathogenicity of *P. aeruginosa* as it enables colonization and biofilm formation on many surfaces and environments (16).

It has been observed in various studies that the plant-produced phenolic metabolite salicylic acid (SA) attenuates biofilm formation in *P. aeruginosa* (11, 15, 16). Yang *et al.* have shown that there are structural similarities between SA and the LasR regulator ligand. Thus, it is possible that the binding of SA to LasR could prevent activation of virulence factors within *P. aeruginosa*. To assess this theoretical mode of action of SA, the first part of our experiment examined biofilm formation in the presence of SA of a *lasR* mutant strain of *P. aeruginosa* PAO1 compared to the wild type PAO1 strain.

The second half of our experiment focused on the swarming capabilities of *P. aeruginosa*. The effect of SA on swarming motility of both of these strains was investigated to determine whether the presence of SA would reduce swarming in *P. aeruginosa*, and if observed, whether the reduction of swarming was mediated through SA binding to LasR.

Here we test a means of biofilm attenuation through the use of salicylic acid (SA). We attempt to elucidate its mechanism of action and assess its ability to attenuate swarming in *P. aeruginosa* PAO1.
**MATERIALS AND METHODS**

Strains, media and chemicals. *P. aeruginosa* strain PAO1 was obtained from the MICB 421 culture collection (Department of Microbiology and Immunology, University of British Columbia). The PAO1 lasR mutant (strain PW3598, transposon insertion mutant) was supplied by the University of Washington *Pseudomonas* mutant library. It should be noted that UW attaches a disclaimer to all their *Pseudomonas* mutants that there is a 10% chance the mutant insertion site does not match the original assignment. All cultures and plates were prepared using Luria Bertani (LB) media (1% tryptone, 0.5% yeast extract, and 0.5% NaCl). Sodium salicylate was used as the source of salicylic acid when added to culture media (Sigma, Cat. no. S2679-100G). 10 mM sodium salicylate stock solutions were filter sterilized using 0.22 μm pore size filters (Millipore, Cat. no. GSTF02500). Filter sterilization did not appear to be a sufficient means for complete sterilization, therefore sodium salicylate solutions were subsequently pasteurized in a 75°C water bath (Fischer Scientific IsoTemp 205) for 15 minutes.

**Growth conditions.** Strain stock cultures were maintained on streaked 1.5% LB agar plates at room temperature. Overnight cultures were prepared by inoculating 5 mL volumes of LB media and incubating with aeration at 37°C on a shaking platform at 150 rpm.

**Biofilm quantification by crystal violet assay.** OD₆0₀ readings of overnight cultures of wildtype PAO1 and PAO1 lasR mutant were taken using LB media as a blank. The overnight cultures were then appropriately diluted with LB to the same starting OD₆0₀. Subsequently, these were diluted 1:400 using LB for 24 hour biofilm growth in clear, polystyrene, 96-well microtiter plates (BD Falcon, Cat. no. 351177). The outermost rows and columns of wells were each filled with 200 μl of distilled water to prevent evaporation of culture. The remaining wells were filled with 80 μl of overnight culture of either the wildtype PAO1 or the lasR mutant and 20 μl of diluted sodium salicylate stock to give final salicylic acid concentrations of 0.5 mM, 0.1 mM, 0.25 mM, 0.5 mM, 1.0 mM, and 2.0 mM. Additional wells were used to conduct a modified crystal violet assay to confirm our proposed model of regulatory control. 80 μl of overnight culture of either the wildtype PAO1 or lasR mutant was added to 20 μl of distilled water. These wells did not contain salicylic acid. Plates were incubated at room temperature for 24 hours before quantification by crystal violet staining. Following overnight incubation, planktonic cells were removed from the microtiter plates by briskly shaking the plates out over a waste tray. Wells were washed once using distilled water from a squeeze bottle. The water was then vigorously shaken out. 125 μl of 0.1% crystal violet solution was added to each well and the plates were stained at room temperature for 10 minutes. Wells set aside, as mentioned earlier for a test confirming regulatory control, were quantified in the same manner, but an additional 10 minute incubation with 1.0 mM SA was added prior to the wash and staining steps. Following staining, the crystal violet solution was dumped out and wells were washed twice with water. The plates were tapped onto paper towels and allowed to air-dry completely. 125 μl of 95% ethanol was subsequently added to each stained well and the dye was allowed to solubilize by covering the plates and incubating for 10 minutes at room temperature. The contents of the wells were mixed by pipetting up and down and the OD₆0₀ was measured using a microplate reader (BioRad Model 3550).

**Swarming assay.** LB media of 0.5% agar was prepared for use in the swarming assay. Appropriate volumes of 10 mM sodium salicylate solution were added to generate salicylic acid concentrations of 0 mM, 0.1 mM, 0.5 mM, and 1 mM. For each trial, quadruplicate plates of each salicylic acid concentration were poured for each strain and the plates were left to set for 1 hour. To generate log phase cultures for inoculation, overnight cultures were diluted 1:5 using LB and incubated at room temperature for 1.5 hours. OD₆0₀ turbidity readings were measured and necessary dilutions were performed to ensure equal inoculum concentrations for both strains. 3 μl aliquots of the log phase cultures were inoculated onto the centre of each plate that were subsequently incubated overnight at room temperature. Swarming diameters were measured 48 hours later.

**Statistical Analysis.** A two-tailed t-test was conducted assuming independent populations with different standard deviations. An α=0.05 was used as a measure of statistical significance.

**RESULTS**

Decreasing biofilm density with increasing SA.

The wild type and lasR mutant demonstrated decreasing biofilm density with increasing concentration of SA across all trials. Figure 1 shows more pronounced attenuation in biofilm density with increasing concentrations of SA over the lower ranges of SA (from 0 mM to 0.5 mM) in both strains. All trials showed a levelling off in the decrease in biofilm densities at SA concentrations above 0.5 mM.

In general, the mutant strain was found to have higher levels of biofilm formation across the different concentrations of SA. Biofilm densities were found to be most similar between strains in the control group (0 mM SA) as compared to the other SA concentrations tested.

Figure 1 shows that the amount of biofilm measured varied with the concentration of SA with an approximately inversely proportional relationship for both the wild type and the lasR mutant, and with a maximum inhibitory concentration of ~2 mM SA (no further decrease in biofilm density was observed at concentrations of SA > 2 mM). The measured absorbances from SA concentrations of 0 mM to 0.50 mM are significantly different from each other, with p<0.05 for both strains, although the difference becomes insignificant (p=0.05) when comparing the 0.5 mM level treatment biofilms.

Figure 2 shows percent decrease in biofilm in the wildtype and the lasR mutant. The difference in biofilm measured between strains was significantly different (p<0.05) for SA concentrations of 0.25 mM and upward.
for all trials, but for lesser concentrations this difference was not significant (p>0.05).

Swarming motility independent of SA concentration. The lasR mutant did not present a lower degree of swarming motility as compared to the wild type strain as would be expected by disruption of the lasR gene (Figure 3). While a concentration dependent trend was not observed in the swarming diameters of the wild type strain, Figure 3 shows the average swarming diameters for the lasR mutant did appear to decrease with increasing concentration of salicylic acid exposure. However, as a result of the high variability between the diameters of the different replicates, dependence of swarming diameter on the concentration of salicylic acid for both the wild type and lasR mutant cannot be considered significant due to a high degree of overlap between 95% confidence intervals.

DISCUSSION

Biofilm formation and swarming motility have been shown to be closely connected and regulated by a large set of overlapping genes (7). The exact mechanisms are unclear and previous literature is contradictory in regards to the relationship between these two phenomenon and the factors that affect them (10). One popular hypothesis is that QS is a major regulator of both biofilm formation and swarming motility (14). It is suggested that critical concentrations of particular signaling molecules serve as a substitute for diffusibility and cell density (15). Through sensing of these critical concentrations, QS systems can trigger a shift in behavior at the community level (14). Previous studies have also indicated that SA disrupts all three P. aeruginosa QS systems (las, rhl, and pqs) (15). In addition, it has been observed that SA alters the structure and composition of a PA lasR mutant (disrupted by a Tn insertion at lasR) (15). In our study, while both strains are highly sensitive to even small concentrations of SA, the inhibitory effect is greatly diminished at increasing concentrations (Figure 2). This observation lends support to the hypothesis that SA inhibits biofilm formation at the level of regulation, which is sensitive to small changes in inhibitory molecules. If the effect were a chemical or physical one (i.e. SA disrupting the extracellular matrix directly) we would expect to see a continuing downward trend in biofilm inhibition, as opposed to the observed plateau, since SA has been shown to neither be bacteriostatic nor bacteriocidal. To confirm this, a modified CV assay was conducted which revealed no measurable decrease in the absorbance between the treated and untreated wells, supporting our hypothesis that the effects of SA are due to altered regulation.

It was also observed that there was a significant difference in biofilm density in all SA concentrations greater than 0.25 mM. If SA-dependent biofilm inhibition were solely due to disruption of QS, we would expect the curve for the lasR mutant in Figure 1 to be flat, and for the wild type curve to begin at a level above the mutant, and to descend and converge with the mutant curve. However, because the QS mutant also exhibited a SA-dependent decrease in measured biofilm, this suggests the inhibitory effect of SA depends on more than just QS disruption, which appears to play only a relatively small role in the cellular response to SA. Despite this, because the lasR mutant displayed a significantly different response to the SA treatment than seen in the wild type, the results support that QS disruption plays at least an indirect role in the effect of SA on biofilm formation. Furthermore, it is interesting to note that SA inhibited wild type...
biofilm formation more so than in the QS mutant. This suggests that some aspect of a functional QS system may in fact itself be inhibiting biofilm formation in the wild type strain. This is supported by Davey et al., who found that rhl mutants produce significantly more biofilm than wild type strains grown on media containing glucose. Our results are consistent with these observations as the rhl QS system is under hierarchical control by the las QS system (7).

This hypothesis is made problematic, however, by the observation that the 0 mM SA treatment resulted in very similar absorbencies for both strains (Figure 1). This contradicts both Davey et al. and other previous studies, which have, conversely, shown that QS mutants exhibit a significantly impaired ability to form biofilms (4, 15). One explanation, offered by Shroot et al., suggests that the QS-dependency of biofilm formation is in turn dependent on nutritional requirements. These authors found that QS mutant biofilms grown on media that contain glucose as a sole carbon source are nearly identical to wild type biofilms, which could explain our results at 0 mM SA. The other SA treatments, however, cannot be explained by this phenomenon alone.

Since the inhibitory action of SA on biofilm formation is not directly dependent on QS disruption as suggested by our results, there must be some other mechanism present. For example, there is evidence that suggests biofilm formation is heavily dependent on the second messenger c-di-GMP, which is responsible for regulating flagellar assembly and secretion of extracellular polysaccharide (7). If SA interacted with some component of the c-di-GMP pathway, this could explain the differential response of the QS mutant and the wild type to SA, since the control of QS and c-di-GMP regulons have a certain degree of overlap (7).

Another possibility is that the observed biofilm inhibition is due to iron starvation, since SA has moderate iron chelating properties (5). Studies using lactoferrin as a chelating agent have been shown to drastically reduce biofilm formation (13). However, lactoferrin has very high affinity for iron and is a large globular protein, whereas SA is a small soluble molecule and not nearly as efficient in sequestering iron away from PA’s own iron chelators, which themselves have a very high affinity for iron (8).

Swarming motility has been shown to be important for the early stages of biofilm formation (12). Operating under our initial hypothesis that QS disruption is the predominant mode of action of SA, we expected to see a SA-dependent reduction in the swarming diameter of P. aeruginosa colonies, with complete abolishment of swarming in the QS mutant, since previous studies have shown gene products of the QS regulons to be necessary for swarming motility (2). However, our data in Figure 3 shows that both the wild type and the QS were capable of swarming under the given experimental conditions. Colony morphology was compared to previous literature (7) and displayed hallmark signs of swarming motility, such as a terracing effect in which multiple concentric rings of growth are visible (data not shown). Despite exhibiting clear swarming behavior, it was not possible to draw any comparison between the two strains, or even between different treatment levels of SA, as the swarming diameters were too small and exhibited too much variability to be considered significant (p>0.1). Our swarming result in the QS mutant does contradict previous literature (2). However, as discussed above, many behaviors of P. aeruginosa are highly sensitive to growth conditions, and swarming motility is independent of QS when grown on glucose (12), resulting in tiny swarming colonies similar to ones observed in this experiment. It is possible that the SA treatment affected some other sensing mechanism of P. aeruginosa, but based on our results, we are unable to draw that conclusion with certainty.

The field of biofilm formation and swarming motility are still in their infancy, and while there are many theories regarding their underlying mechanisms, they are often contentious and matched by contradictory evidence. Our study found that SA inhibits biofilm formation in a concentration dependent manner, and that QS disruption is not the predominant mechanism, as previously thought. Furthermore, despite our swarming assay yielding statistically insignificant results in terms of SA-dependent inhibition, it still provided additional evidence that QS is not crucial for swarming motility. It cannot be stressed enough, however, that our results are only valid under the specific growth conditions used in this experiment, as the growing body of evidence in the biofilm/swarming field is making it increasingly clear that these behaviors can manifest in strikingly different ways when subjected to different growth conditions.

**FUTURE DIRECTIONS**

To further explore the mechanism of SA action, it would be interesting to investigate the effect of SA on other pathways that are known to regulate biofilm formation and swarming motility, such as the one that synthesizes the second messenger c-di-GMP. Iron starvation and the chelating effects of SA could also explain some of the observed effects of SA on biofilms, and it would be interesting to quantify how important this factor is. Finally, any future experiment conducted on this topic would need to carefully consider the carbon source employed, as this seems to have a drastic effect on the induction of biofilm/swarming regulatory genes. Ideally this would include investigating behavior...
on multiple types of media drawing on different carbon sources, such as glutamate, succinate, citrate, and glucose, all of which seem to generate distinctly different responses in the context of biofilm formation and swarming motility.

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REFERENCES