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Biofilm Formation of *Pseudomonas aeruginosa* PA14 Required *lasI* and was Stimulated by the *Pseudomonas* Quinolone Signal although Salicylic Acid Inhibition is Independent of the *pqs* Pathway

Mary Bacalso, Tony Xu, Kitty Yeung, and Denny Zheng
Department of Microbiology & Immunology, University of British Columbia

Pseudomonas aeruginosa **is a ubiquitous, opportunistic pathogen responsible for many nosocomial infections, and is often difficult to treat due to its ability to produce biofilm. Biofilms can increase resistance to antimicrobial agents by up to 1000-fold and is controlled by quorum sensing. Salicylic acid has been shown to inhibit biofilm formation in *P. aeruginosa* by acting as a competitive analogue to the LasR regulator ligand. The *las* quorum sensing system can control the activation of many different downstream systems. One such pathway *las* can control is the *pqs* pathway, where *las* can indirectly downregulate the formation of the *Pseudomonas* quinolone signal molecule (PQS), but also directly upregulate the expression of the regulator, pqsR. The PQS molecule facilitates biofilm formation and can be up-regulated through a positive feedback loop. Here, we attempted to elucidate pathways downstream of the *las* system that are affected by the inhibitory effects of salicylic acid, and whether induction of these pathways would overcome of the effect of SA. Specifically, we focused on the *pqs* system. To do this, four different strains of *P. aeruginosa* (PA14 wildtype, *lasI, pqsA,* and *pqsH* mutants) were used. The biofilm densities produced by these four strains were assessed after treating with increasing concentrations of SA and PQS combinations. Quantification of the biofilm densities was made possible with the crystal violet assay. The biofilm densities of the *lasI* mutant were reduced to approximately 10 – 20% relative to the wild type. The *pqsA* and *pqsH* mutants also had less biofilm formation compared to wildtype. An inhibitory effect of salicylic acid on biofilm formation was not observed in any strains. Addition of exogenous PQS increased biofilm density in all strains with the exception of the *lasI* mutant. In conclusion, the *pqs* pathway is not essential in *P. aeruginosa* for biofilm formation, while the *las* system is required for exogenous PQS to stimulate biofilm formation.**

Pseudomonas aeruginosa is a ubiquitous, gram-negative opportunistic pathogen. Because it thrives on most surfaces, it is often found in hospitals and medical equipment and is responsible for many nosocomial infections. Treatment of infections can often be difficult due to its ability to produce biofilm. Cells in biofilms can develop antibiotic resistance up to a thousand fold higher than planktonic cells (9). The formation of biofilm is shown to be controlled by intricate cell-to-cell communication known as quorum sensing (QS) (15).

One system in QS that has been found to control biofilm formation in *P. aeruginosa* is the *las* quorum sensing system. This system also regulates a complex hierarchy of other QS pathways that are regulators of many cellular processes (6). This regulation occurs through the binding of the activated regulator, LasR, to the promoter regions of these regulons (15). The *pqs* pathway is one such pathway that is directly regulated by the *las* system (15), and its function is to control many more downstream pathways (13). LasR upregulates the expression of PqsA/B/C/D/H and R. PqsA/B/C/D are involved in the synthesis of the precursor of PQS, which is converted by PqsH. PqsR, the regulator expressed to regulate downstream pathways, can bind to PQS or HHQ to carry out its function (15). PQS formation can also be up-regulated through a positive feedback loop as depicted in Figure 1 (15).

In a previous study, salicylic acid (SA) was shown to repress the *las* system by acting as a competitive analogue to the LasR regulator ligand (17). In another study, Da *et al.* observed lowered biofilm densities with increasing concentrations of SA until 0.25 mM, with a levelling off in decreased biofilm densities at higher SA concentrations (1).

Here, we examined whether the inhibitory effect of SA on biofilm formation was achieved by disrupting the production of PQS indirectly through LasR inhibition. *P. aeruginosa* wild type PA14, *lasI*, *pqsA*, and *pqsH* mutants of PA14 strain were used to test for the reproducibility of the results obtained by Da *et. al.*. *lasI* mutants have non-functional LasI proteins, thus inhibiting the regular functioning of LasR, because the

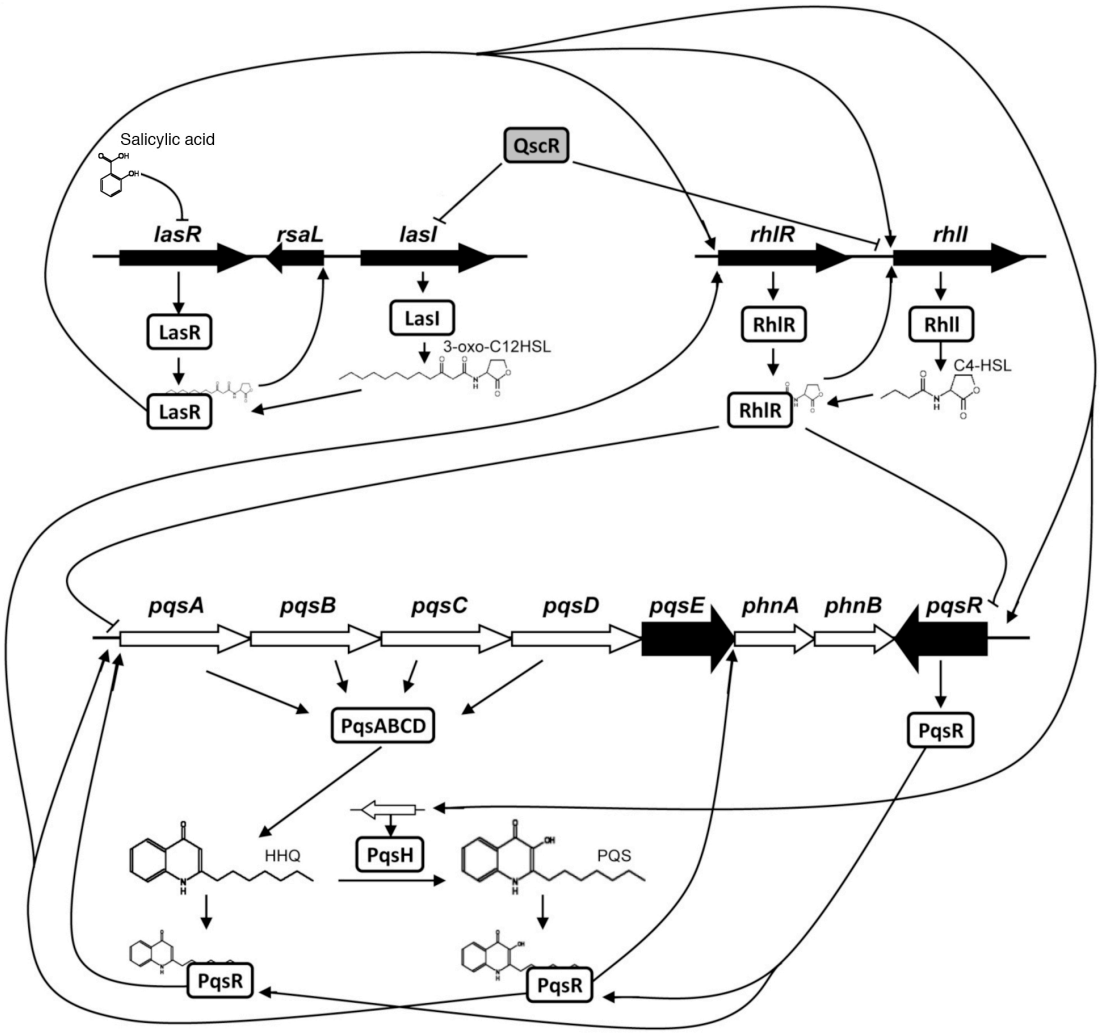

FIG 1. Diagrammatic representation of the regulation of the *pqs* pathway, the *las* system, and the *rhl* system. Arrows indicate positive regulation and bars indicate negative regulation. Adapted from Williams and Camara (15).

mutants are unable to form 3-oxo-C12-HSL, which is needed to bind LasR (2). *pqsA* mutants are, theoretically, incapable of producing HHQ, and *pqsH* mutants are unable to convert HHQ to PQS. We also investigated whether exogenous PQS could overcome the effect of SA on biofilm density with the addition of 2-heptyl-3-hydroxy-4-quinolone (PQS) in the presence of SA. This will allow us to determine if SA-dpendent reduction of biofilms can be restored by the addition of PQS. In this experiment, SA had no significant effect on biofilm formation. Furthermore, the *pqs* system was observed to have a role in biofilm formation, a function that is dependent on the *las* quorum sensing system.

MATERIALS AND METHODS

Bacterial strains, media and chemicals. The PA14 strains of *Pseudomonas aeruginosa* were derived from Dr. Robert Hancock's research laboratory in the University of British Columbia. These strains were acquired from the Harvard University PA14 mutant library developed by Liberati *et al* (12). The four *P. aeruginosa* samples tested were *lasI* (ID PA1432), *pqsA* (ID PA0996) and *pqsH* (PA2587) mutants and wild type (WT) of the PA14 strain. All cultures were prepared in Luria Bertani (LB) media composed of 1% tryptone (BD, Cat. no. 211705), 0.5% yeast extract (Difco, Cat. no. 0127-01), and 0.5% NaCl (Fisher, Cat. no. BP358-1). Sodium salicylate (Sigma, Cat. no. S2679) was used as the source of salicylic acid. 2.5 mM and 20 mM sodium salicylate stock solutions were filter sterilized using 0.22 µm sized pore filters (Millipore, Cat. no. GSTF02500). The two solutions were then pasteurized in a 75°C water bath (Fischer Scientific IsoTemp 205) for 15 minutes for further sterilization. 2-Heptyl-3-hydroxy-4(1*H*)-quinolone (Sigma-Aldrich, Cat. no. 94398) was used as the source of PQS. A 12 mM stock solution of PQS was made in 50% methanol (EMD Chemicals Inc., UN1230).

Culture conditions. Stock cultures of the strains were maintained on 1.6% LB agar (Invitrogen, Cat. no. 30341-023) at room temperature. Overnight cultures were prepared in 5 ml LB media and incubated at 37°C on a shaking platform at 150 rpm.

Biofilm production in presence of SA. Turbidity readings at 600 nm of the overnight cultures of the wild type PA14, *lasI, pqsA and pqsH* mutants were taken with the spectrophotometer (Spectronic 20) blanked using the LB media. The cultures were then diluted using the appropriate dilution to achieve a uniform starting turbidity of 0.17 OD_{600}. These were further diluted 1:400 using LB media for another overnight growth in a clear, flat-bottomed polystyrene 96-well plate (Sarstedt, 82.1581.001). The wells were filled with 80 µl of each culture in triplets and 20 µl of the appropriate diluted sodium salicylate stock to give final concentrations of 0.05 mM, 0.10 mM, 0.25 mM, 0.50 mM, 1.0 mM, 2.0 mM and 4.0 mM. Additional wells containing culture were grown without salicylate as controls. Furthermore, as a control to confirm sterility, three additional wells without culture were filled with 80 µl of LB media. Followed by thorough mixing, the plate was incubated at room temperature for 24 hours.

Quantification of biofilm with crystal violet assay. After incubation for 24 hours, the cultured cells from the microtiter plate were disposed by quickly shaking the plate over a waste tray. The plate was washed once with dH_2O and air-dried, then 125 µl of 0.1% crystal violet solution (Fisher, Cat. no. C581-25) was added to each well. The plate was stained for 10 minutes at room temperature. The crystal violet solution was removed and the plate was washed twice thoroughly with distilled water and allowed to air-dry completely. 125 µl of 95% alcohol was deposited to each well containing the solution and the plate was incubated at room temperature for 10 minutes to allow the crystal violet stain to dissolve into the alcohol. The contents of the wells were then thoroughly mixed and the absorbance at 595 nm was measured using a microplate reader (BioRad Model 3550).

Biofilm production in presence of PQS and SA. 1:400 diluted cultures were prepared as described above using overnight cultures of the PA14 wild type strains, *lasI, pqsA,* and *pqsH* mutants. 80 µL of each diluted culture was added in triplets to the wells in a clear, flat-bottomed polystyrene 96-well plate. 200 µl of distilled water was added to the outermost rows and columns of wells including any remaining wells to prevent dehydration and evaporation of the cultures. 19.5 µl of the appropriate diluted sodium salicylate stock was then added to each culture in triplets to achieve final concentrations of 0.00 mM, 0.10 mM, 0.25 mM and 1.0 mM. A final PQS concentration of 60 µM was obtained by adding 0.5 µl of PQS stock to each well. The plate was incubated at room temperature for 24 hours following thorough mixing of the solutions. Biofilm densities were then obtained using crystal violet assay as described above. Finally, absorbance was measured at 595 nm using the microplate reader.

RESULTS

Biofilm density independent of SA concentration. To attempt to reproduce the effect of SA on biofilm formation, the four strains were incubated with increasing concentrations of SA. In general, the three mutants displayed lower biofilm densities for all the concentrations of SA compared to that of the PA14 wild type strains. This can be observed in figure 2. The *pqsH* and *lasI* mutants both displayed lower densities than the wild type; however, the densities of the *lasI* mutant were consistently much lower than those of the wild type, at roughly 10-20% of its densities. SA in biofilm attenuation was not evident in any of the strains. No significant increase or decrease in densities is present for the *lasI* and *pqsH* mutants, while the wild type and the *pqsA* mutant each showed an increase in densities at 0.5 mM and 1.0 mM SA, respectively.

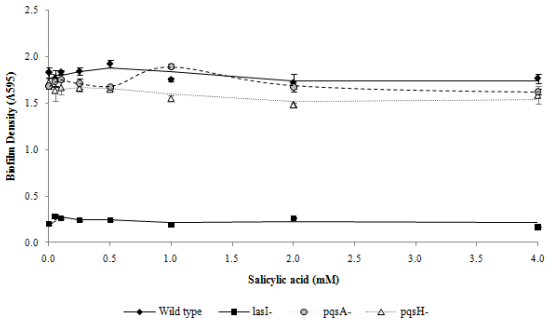

FIG. 2. Effects of varying concentration of salicylic acid during growth on biofilm density of *Pseudomonas aeruginosa* mutants. Average of three replicates is shown. Error bars represent standard error.

Increased biofilm density in the presence of PQS. To look at whether the inhibitory effect of SA is a result of an inhibition of the *pqs* system, exogenous PQS was added to each strain in the presence or absence of SA. Figure 3 shows that biofilm density is significantly increased for the *pqsA* and *pqsH* mutants, when compared to the biofilm density in only SA treated conditions (figure 2), in the presence of 60 µM of exogenous HHQ at all SA concentrations. The densities of the two mutants increased by roughly 30% for all SA concentrations when in the presence of PQS. The PA14 wild type strains also displayed significant increases in the biofilm densities. However, the increase was not as high as the two *pqs* mutants, increasing by only roughly 10% for SA concentrations of 0 mM, 0.10 mM, and 0.25 mM. An increase was also observed at 1.0 mM SA but the increase was insignificant. On the contrary, the densities of the *lasI* mutant decreased by around 40% for all SA concentrations when in the presence of PQS.

DISCUSSION

Our results indicate that mutation in the *lasI* gene severely impaired *P. aeruginosa* PA14's ability to produce biofilm (Fig. 2). The biofilm density of the *lasI* mutant is only about 10 to 20% compared to that of the wild type strain. This finding was expected, as previous studies suggested that biofilm formation in *P. aeruginosa* is regulated by QS mechanisms (14, 15), and it is known that many QS-controlled genes are largely activated by the *las* system (8). LasR, the target for SA competitive inhibition, is still present in the *lasI* mutant; however this mutant is missing LasI, the protein that produces 3-oxo-C12-HSL, the regulator ligand for LasR. Therefore, LasR is inactivated regardless of whether SA is present (Fig. 1).

The first question we attempted to answer through this study is whether the inhibitory effect of SA on

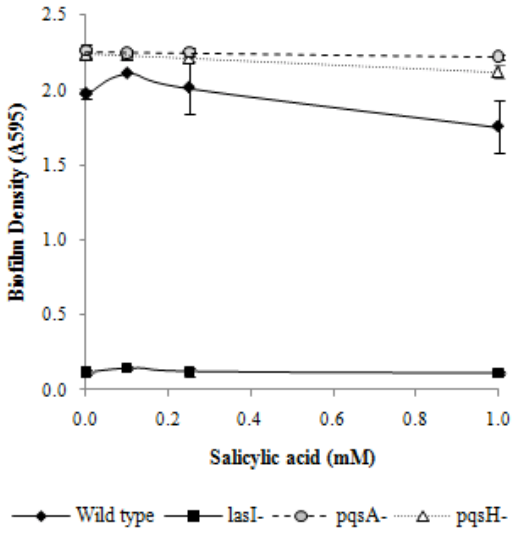

FIG. 3. Effects of salicylic acid and PQS during growth on biofilm density of *Pseudomonas aeruginosa* mutants at varying concentrations of salicylic acid. Concentration of PQS is constant at 60μM. Average of three replicates is shown. Error bars represent standard error.

biofilm formation observed in past studies (1, 13) is achieved by disrupting the production of PQS indirectly through the *las* system. Here, it was seen that the *pqsA* and *pqsH* mutants produced biofilms of significantly lower densities as compared to the wild type PA14 strains (Fig. 2), but the biofilm densities of both *pqs* mutants were still significantly higher than that of the *lasI* mutant. This suggests that the *pqs* system is not essential for *P. aeruginosa* biofilm formation. which is a reasonable outcome as LasR has been shown to affect 331 genes downstream besides being able to up-regulate the production of PQS, and apparently the essential genes for biofilm production are further downstream (13). Some of these regulated pathway component can be found in in Figure 1. Nevertheless, as depicted in figure 3, presence of PQS still shows significant increase in biofilm formation in *pqsA* and *pqsH* mutants.

However, we did not see a significant reduction in biofilm density for any of the 4 strains when they were subjected to increasing concentrations of SA (Fig. 2). This observation was problematic, as a similar past study by Da *et al.* showed that even 0.25 mM of SA caused a significant decrease in biofilm density in wild type PAO1 *P. aeruginosa* in comparison with control cultures after 24 hours of incubation (1). A potential explanation is that PA14 strains are less responsive to SA inhibition than PAO1 strains and needs to be incubated for a longer period of time before the difference in biofilm density becomes significant. This reasoning is supported by the result of Prithiviraj *et al.*'s study done using PA14 wild type cells, in which the difference between the biofilm density of the cultures grown under different concentrations of SA did not become significantly different until after 40 hours (13).

It was also observed that the biofilm density of the wild type and the *pqsA* mutant increased significantly in response to 0.5 mM and 1.0 mM of SA, respectively (Fig. 2). This anomaly may be due to a separate regulator, QscR which is known to bind weakly to 3-oxo-C12-HSL (11). When QscR is bound to 3-oxo-C12-HSL, it down-regulates the expression of LasI, thus completing a negative-feedback loop (Fig. 1). Since 3-oxo-C12-HSL binds to both LasR and QscR, and SA fits its binding site on LasR, it is possible that SA is also able to bind QscR and competitively inhibit it (10, 11). In that case SA should remove QscR inhibition and increase the expression of *lasI*, leading to the synthesis of more 3-oxo-C12-HSL, which has been shown to increase biofilm formation when added exogenously (2). However, even if this assumption is true, because only two data points showed an increase in biofilm density, it could also be assumed that QscR does not have an uniform effect in response to SA, but no further inference can be made as much of the details about QscR regulation is still uncertain and the effects of different concentrations of SA on its function is unknown. Furthermore, QscR is able to repress the expression of RhlI (12). The *rhl* system is known to negatively regulate the production of PQS, which stimulates biofilm production (3, 15). The interplay between QscR, the *las* and *rhl* systems could also contribute to the observed fluctuation.

Given the results to answer the first experimental question, it was not necessary to look at the whether exogenous PQS would reverse the inhibitory effect of SA. However, this was still carried out, and from the results, it was still possible to see a connection between *pqs* system and biofilm formation. From figure 3, we can see that PQS addition resulted in greater biofilm formation in the *pqsA* and *pqsH* mutants than wild type *P. aerguinosa*. Interestingly, when comparing figure 2 and figure 3, it is evident that the addition of 60 uM of exogenous PQS increased the biofilm density significantly for *pqsA* and *pqsH* mutants under all concentrations of SA used, but not for the *lasI* mutant. These results allow us to conclude that PQS up-regulation of biofilm formation is dependent on the *lasI* gene as its absence prevented any increase in biofilm formation from PQS addition. For the wild type PA14 strains, PQS increased biofilm density significantly at SA concentrations of 0, 0.1 mM, and 0.25 mM. The difference was insignificant at 1.0 mM of SA, but an increase was still observed. The large standard deviation at that data point was likely due to the fluctuation of the crystal violet assay, as crystal violet is known to stain negatively charged cellular components

other than biofilm (5), such as exopolysaccharide present on the surface of sessile bacterial in the culture. The increase in biofilm density in the wild type PA14 strains, *pqsA* and *pqsH* mutants brought by the addition of PQS is consistent with the finding of Diggle *et al.*, who showed that 60 uM of exogenous PQS caused a hundred-fold increase in the surface coverage of *P. aeruginosa* (3). However, because SA did not significantly reduce the biofilm density of any strains, we are unable to draw a valid answer for the experimental question based on our results.

Knowing that PQS does indeed play a role in biofilm formation, we then moved on to look at whether HHQ also has an effect on biofilm density. HHQ is the precursor of PQS and is known to be able to activate PqsR, by binding to it as PQS does (16). PqsA is one of the enzymes essential for the synthesis of HHQ, while PqsH catalyzes the conversion of HHQ to PQS. Our results show that the presence of HHQ does not affect biofilm density, as no significant differences between the biofilm densities of the *pqsA* and *pqsH* mutants at all concentrations of SA were observed (Fig. 2). Instead, the role of PQS for activing PqsR seems to be the more important role in initiating biofilm formation, as both *pqsH* and *pqsA* mutants formed levels of biofilm with lower density than wild type. A number of recent studies have examined the role of HHQ and generated inconsistent findings, with some stating that PQS-dependent signaling can only be activated in the presence of HHQ, while other studies deny such dependency (4, 15, 16).

The fact that exogenous PQS had no effect on the *lasI* mutant presented an interesting finding. It was known that PQS could up-regulate the production of *P. aeruginosa* virulence determinants elastase, lectin A and pyocyanin, and *rhl*-dependent phenotypes are abolished in the absence of PQS signaling (4, 15). However, no previous studies have demonstrated that exogenous PQS would have no effect when the strain tested is lacking a functional *las* system. Our study suggests that the *las* system is essential in order for PQS to stimulate biofilm production. The nature of the dependency of PQS on the *las* system is difficult to infer, as the effects of PQS are an area of intensive research, and still relatively little is known in this field.

To conclude, our study showed that the *las* system is essential for proper biofilm formation. This is inconsistent with Da *et al.*'s finding but it is coherent with their hypothesis (1). We showed that the *pqs* system was not essential for biofilm formation, a finding previously shown by Xiao et al. (16). The main problem of our study is that SA did not have a significant inhibitory effect on biofilm formation in any of the 4 strains we tested. Nonetheless, the first experimental question can still be addressed. It is certain that any decrease in biofilm density caused by LasR inactivation cannot be attributed to the consequent down-regulation of PQS production alone, even though PQS does play a part in biofilm formation. We were unable to assess the second experimental question because no SA inhibition was observed and we therefore could not consider the second half of our study as a "rescue" experiment. However, we succeeded in showing that exogenous PQS caused generally significant increase of biofilm density in *pqsA* and *pqsH* mutants and the PA14 wild-type *P. aeruginosa*, which is consistent with Diggle *et al.*'s observation. Interestingly, out study also showed that a functional las system is needed in order for exogenous PQS to stimulate biofilm formation.

FUTURE DIRECTIONS

To continue to investigate whether exogenous PQS could overcome the inhibitory effect of SA on biofilm formation, it may be beneficial to use a more sensitive assay to quantify biofilm density, such as ruthenium red, since it appeared that the fluctuation in our data could be greater than the variance we are measuring, which largely obscures the trend we are seeking in the results (7). Incubating the cultures for a longer period of time, for 40 hours for example, might allow the trend of SA reduction of biofilm density to become significant for *P. aeruginosa* PA14, as suggested by the data from Prithiviraj *et al.* (13). The role of QscR in regulating biofilm density in response to increasing concentrations of SA is also worthy of further investigation using a *qscR* mutant strain.

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