Salicylic Acid Affects Swimming, Twitching and Swarming Motility in Pseudomonas aeruginosa, resulting in Decreased Biofilm Formation

Samuel Chow, Kevin Gu, Lucy Jiang, and Anthony Nassour
Department of Microbiology & Immunology, University of British Columbia

Pseudomonas aeruginosa is an opportunistic pathogen that causes nosocomial infections in part due to its ability to colonize many biotic and abiotic surfaces. These persistent infections are difficult to treat due to a large repertoire of virulence factors, and the ability of the organism to form biofilms. It has previously been observed that biofilm formation in several bacterial species can be attenuated by the addition of salicylic acid (SA), through an unknown mechanism. As cell motility of twitching, swimming, and swarming are proven instrumental in biofilm formation, we investigated whether or not salicylic acid affected the motility of Pseudomonas aeruginosa. Motility assays conducted on wild-type strains (PAO1, PA14), flagella (PAO1 fliC, PA14 fliC) and type IV pili (PAO1 pilB, PA14 pilB) mutants, showed that sub-inhibitory concentrations of 25 and 50 mM salicylic acid significantly decreased bacterial swarming motility. These findings correlated with significant decrease in biofilm formation when incubated with salicylic acid (SA) concentrations of 10mM and higher. Since bacterial motility necessary for proper biofilm formation was impaired in a concentration-dependent manner, these data suggest that the suppressive effect of salicylic acid on flagella-related motility led to the previously observed reductions in biofilm formation. Twitching and swimming motility showed less inhibitory results than swarming motility, due to a lack of consistent replicates across three biological repeats. Twitching motility assays showed significant decreases at 50mM SA and swimming motility assays showed significant decreases at 25 and 50 mM SA for all strains tested except for PAO1 pilB. However, further studies are needed to confirm the effect and mode of action of salicylic acid on flagella and type IV pili gene expression or functionality.
Comparing fliC mutants (flagellum knockout) and pilB mutants (type IV pilus knockout) in both PAO1 and PA14 strains to their respective wild-type strains under the presence of SA for swimming, swimming, and twitching ability, we were able to observe whether or not the loss of either of the two cellular structures affected biofilm formation.

MATERIALS AND METHODS

Strains, media, and chemicals. The bacterial strains and plasmids used in this study are as described in Table 1. _P. aeruginosa_ PAO1 and PA14 wild-type strains were obtained from the R.E.W Hancock Laboratory (Centre for Microbial Diseases and Immunity Research, UBC). The PAO1 mutants were supplied from the University of Washington _Pseudomonas_ mutant library, and PA14 mutants supplied from the Harvard University _Pseudomonas_ mutant library. All culture strains were maintained in 2% Luria Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 0.5% (w/v) NaCl) and 7% DMSO media aliquots, stored at -80°C in microfuge tubes (22). Sodium salicylate stock solution was prepared by dissolving sodium salicylate (SA)(Sigma, Cat. no. S2679-100G) in distilled water (0.1, 0.25, 0.5, 1, 25, 50, 100 μM, followed by heating to 100°C and filter sterilization using 0.2 μm pore size filters (Millipore, Cat. no. GSF02500).

Minimal inhibitory concentration (MIC) assay. MIC was assessed using the standard broth microdilution protocol as described by Weigand _et al_ with a change in incubation time from 20 hrs to 24 hrs (22). Briefly, overnight cultures were grown in Mueller Hinton broth (MHB) (Difco, Cat. no. 275710) at 37°C and diluted to yield an inoculum of approximately 1 × 10^7 CFU/ml. Cultures were further diluted 1/100 in modified BM2, 2 mM MgSO_4, 0.4% (w/v) glucose, 10 μM FeSO_4), diluted to 0.4-0.5 OD_600. Plates were incubated at 37°C for 18 hours. Photographs were taken and swarm area was assessed using ImageJ to be expressed as a percentage of inhibition.

Swimming assay. Swimming motility was evaluated on tryptone plates (1% (w/v) tryptone, 0.5% (w/v) NaCl) containing 0.3% (w/v) agar as previously described (22). 25 and 50 mM of SA was added to plates prior to a 30 min drying period. Bacteria were inoculated as 1 μl aliquots of an overnight culture grown in tryptone broth, diluted to 0.4-0.5 OD_600. Plates were incubated at 37°C for 24 hours, and halo diameter measurements were recorded.

Twitching assay. Twitching motility was evaluated on LB plates (LB broth + 1% (w/v) agar) as previously described (22). 25 and 50mM of SA was added to plates prior to a 1 hour drying period. Plates were inoculated by stabbing an isolated colony grown on overnight LB plates (LB broth + 2% (w/v) agar) onto the twitch plate. Plates were incubated at 37°C for 24 hours, and halo diameter measurements were recorded.

Data from both swimming and twitching assays were normalized against zero inhibition exhibited by no SA. Each 25mM and 50mM sample was then statistically analyzed against zero inhibition to determine its significance using T-test (p<0.05).

Statistical Analysis. The ANOVA test to determine differences in variance and two sample t-test (p<0.05) was used to determine any statistical significance of the biofilm assay. The t-test was used was also employed to analyze the significance of SA’s effect on swimming (p<0.01), swimming (p<0.05), and twitching motility (p<0.05). The Graphpad application was also used to analyze data collected.

RESULTS

Determination of Salicylic acid MIC for _P. aeruginosa_. To resolve whether observations in subsequent assays were due to growth inhibition or motility inhibition following SA treatment of _P. aeruginosa_, MICs and sub-inhibitory MICs were determined. MIC assay results (data not shown) indicated that SA concentrations <50 mM had no obvious effects on bacterial growth when viewed macroscopically, but concentrations ≥100 mM SA inhibited _P. aeruginosa_ growth in all wild-type lab strains and mutants, corresponding to previously reported results (1). Therefore, 25 mM and 50 mM SA were chosen as the sub-inhibitory concentrations to be used in subsequent motility studies to ensure that SA xerts its effect on _P. aeruginosa_ motility and biofilm
formation through a mechanism other than direct killing.

SA inhibits biofilm formation in \textit{P. aeruginosa} in a dose-dependent manner. Increasing concentrations of SA reduced biofilm formation in all strains of \textit{P. aeruginosa} tested after 20 hrs incubation (Fig. 1). At 0 mM SA, both wild-type strains showed greater biofilm forming capacity than their corresponding flagella and type IV pili mutants, confirming that motility is a necessary component of biofilm formation. The inhibitory effect on biofilms appeared to be dose-dependent, as there was an observed inverse relationship between decreasing biofilm formation with increasing SA concentrations; this relationship plateaus at 50 mM - 100 mM of SA where the maximum inhibitory effects were observed. PAO1 appeared to be more susceptible to the effects of SA than PA14, as the 5 mM SA treatment condition decreased biofilm formation by approximately 53% in PAO1, but only 6% in PA14. Moreover, biofilm formation in all strains reached the same levels at 100 mM SA (~0.035 A\textsubscript{595}), consistent with the MIC. From a statistical standpoint, most of the cultures exposed to SA were significantly reduced in biofilm formation with only PA14 and PA14 fliC mutant showing changes that were not statistically significant at 5 mM SA (Fig.1).

SA inhibits swarm motility in \textit{P. aeruginosa} strains. SA decreased the swarming ability of all \textit{P. aeruginosa} strains, as seen by the relative swarm motility seen in the presence of sub-inhibitory concentrations (25 and 50 mM) of SA (Fig. 2). At both sub-inhibitory concentrations, the wild-type strains and the PAO1 lasR mutant showed the least relative swarm motility area (on average about 35% and 5%) versus the motility mutants (pilB mutants on average about 50% and 20%), suggesting that the lack of motility contributes to a slightly higher resistance to SA. Also, the PAO1 fliC mutant was the least affected of all strains at 25 mM SA (~30% inhibition). At 50 mM SA, PAO1 fliC mutant was the clear exception again, as the swarming ability was the least affected at 50 mM (half the inhibition observed on average for the motility mutants). There was a greater resistance observed for PAO1 fliC mutant at 25 mM SA when compared to the PAO1 pilB mutant, as seen in the ~20% higher relative swarm area. However, both mutant strains showed much greater resistance to SA effects when compared to the PAO1 wild-type at 25 and 50 mM SA; this suggests that the flagella or type IV pilus may be major and minor targets of SA respectively in accordance with the biofilm formation results. This trend was also seen in PA14, but to a much lesser extent. All strains showed significant swarm inhibition at both sub-inhibitory concentrations of SA.

SA alters swarm motility phenotype in \textit{P. aeruginosa}. Wild-type \textit{P. aeruginosa} PA14 formed dendritic-like projections reaching the outer edges of the plate from the initial inoculation point, whereas swarm motility appeared less cohesive with shortened projections on 25 and 50 mM SA plates (Fig. 3). In the PA14 fliC mutant, there appeared to be a complete disruption of swarming motility, as inoculated aliquots

\textbf{FIG. 1. Presence of biofilm formed by \textit{P. aeruginosa} strains and mutants in the presence of SA.} Biofilm formation by the indicated strains was measured after incubation in the presence of specified SA concentrations. Bars show the mean +/- SEM (n=3 independent experiments) with statistical differences (versus no SA) at P<0.01 indicated by **.
failed to show any dendritic-like growth, resulting in small swarm areas with smooth edges. The PA14 pilB mutant swarm phenotype seemed to not be hindered by SA, as it still resembled the swarming motility of the wild-type strains. This could support the idea that flagellar locomotion is more critical to proper swarming phenotype as previously mentioned. It appeared that fliC was a poor swarmer, consistent with the observed lower biofilm formation when compared to the wild-type strain (Fig. 1), an expected finding as motility is important in biofilm formation. Similar results were observed for the pilB and fliC mutants of the PAO1 strain except that the growth was more compact and circular looking.

**SA does not inhibit swim motility to a lesser extent than swarm motility in *P. aeruginosa* strains.** When comparing the effect of SA on swarming and swimming motility, we see much greater effect of SA on swarming. However, SA did decrease the swimming ability of all *P. aeruginosa* strains tested except the PAO1 pilB mutant, as seen in the increasing percentage of inhibition seen in plates with 25 and 50 mM SA (Fig. 4). PA14 wild-type showed more inhibition than the PA14 motility mutants on average at 25 mM SA (approximately 2.5-fold higher) and 50 mM SA (approximately 2-fold higher), suggesting that the lack of motility appendage (flagella or type IV pilus) somehow induces resistance to the effects of SA. PAO1 pilB showed a consistent percent inhibition of 5% at both 25 and 50 mM SA, a 2-fold reduction in inhibition than the PAO1 wild-type. This result becomes important when comparing pilB mutants’ relative growth at 0 mM SA to the wild-type strains: pilB mutants were ~93% similar in swim halo diameter, whereas the fliC mutants were ~63% of wild-type diameters. The fact pilB mutants are less impacted by SA than wild-type strains support our swim and biofilm results. This result also indicates the importance of the flagella in swimming ability (as supported by previous studies by Skerker and Berg [19]), and may explain why the PAO1 fliC mutant exhibited the greatest percentage of inhibition at 50 mM SA (27%) versus the PAO1 WT (10%). Since the flagella apparatus is vital to swimming motility, the swimming patterns of our mutant were greatly affected regardless of SA addition. Swim halo diameters in the presence of SA were significantly inhibited at both sub-inhibitory SA concentrations for all strains tested except for PAO1 pilB.

**SA inhibits twitch motility in *P. aeruginosa* strains.** SA decreased the twitching ability of all *P. aeruginosa* strains, observed in distinct decreases in twitch halo diameter in the presence of SA at 25 and 50 mM (Fig. 5). PAO1 and PA14 wild-type strains had a slighter greater percent inhibition in comparison to their respective motility mutants by ~5-10%, denoting more limited importance of motility appendages in twitch motility. Moreover, the motility mutants were more resistant to 25 mM SA than their respective wild-type strains, reinforcing the idea that the lack of motility appendages in these mutants prevents SA from exerting its full effects on motility and perhaps biofilm formation. Twitch halo diameters of all strains were significantly inhibited in the presence of SA at 50 mM SA.
DISCUSSION

As expected, the wild-type strains produced more biofilm than the motility mutants and showed greater susceptibility to SA (Fig. 1), whereas the mutant strains appeared more resistant to SA effects, as we see less dramatic reductions in biofilm formation. For example, we see a ~24% linear decrease in PA14 pilB mutant biofilm formation, versus a large 69% drop-off in PA14 as SA concentrations increase from 25 mM upwards. This supports the idea that the motility structures in the mutants are the targets of SA, as they are present in the wild-type strains but not in the knock-out mutants (12, 19). However, motility is not critical to biofilm formation, as there was still biofilm-forming capacity observed in both of the motility mutants. We observed a dose-dependent decrease in biofilm formation in the motility mutants of P. aeruginosa, suggesting that SA must have a pleiotropic effect on other factors implicated in biofilm formation, or else it would have remained consistently reduced despite increasing SA concentrations. It has been determined that SA does not affect housekeeping genes, therefore we do not need to correlate observed decreases in biofilm formation or motility inhibition to interrupting critical cellular processes for survival (4, 19). If other virulence factors were not affected, we would have seen a constant level of biofilm formation regardless of different concentrations of SA. Others have observed that several genes including rhlR and lasR, encoding for virulence factors, were down-regulated in the presence of SA (5, 19). As quorum sensing is important in biofilm formation, and rhlR and lasR are quorum sensing genes, our strains may have shown reduced biofilm-forming capacity due to the effects of SA on their quorum sensing systems. Further biofilm assays on the effects of SA on rhlR and lasR mutants still need to be done to confirm any correlation between the two variables.

In the swarm motility assay (Fig. 2), the decreased swarming area of the motility mutants lacking motility structures suggests that SA either affects some other vital processes in P. aeruginosa needed for motility, or that SA has no effect on the motility structures of P. aeruginosa. Prithiviraj et al. found P. aeruginosa secretes biosurfactants in the presence of SA to increase motility by reducing surface tension, increasing flagellar motility, improving surface colonization, and up-regulating biofilm formation (7). Therefore, it is possible that decreased biosurfactant production contributed to the overall decrease observed in swarm area by all of our strains. In terms of phenotype, both pilB mutants were still proficient in swarming motility versus their wild-type strains - this was expected, as pilB mutants have a functional flagellum necessary for swarming motility. As SA produced much less of an inhibitory effect on PAO1 fliC mutant that possesses a functional flagellum, we suspected that the flagellum could be a major target of SA. In a study on E. coli 1177, Kunin et al. (13) found that SA blocked the synthesis of flagella, suggesting that SA affects the flagella on the gene regulation level rather than inhibiting the function of the flagella. The mechanism by which SA affects P. aeruginosa flagella is unknown, but it is possible SA affects P. aeruginosa via the same mechanism as E. coli 1177. If this were true, then we can explain our observed reduction in P. aeruginosa wild-types and pilB mutants swarming ability under SA presence. However, it is well established that swarming motility also requires the type IV pilus; the lower inhibition at 50 mM SA in pilB mutants versus the wild-type strains implies that the lack of type IV pili motility appendage contributes to the higher resistance observed (1, 17). We cannot confidently conclude whether or not SA is affecting the functional type IV pili in the fliC mutants, and vice versa if SA is affecting the functional flagella in the pilB mutants, but there is support for this hypothesis when looking at our results.

There was a similarly high degree of inhibition observed in the wild-type and lasR strains in comparison to the motility mutants. Theoretically,
knocking out quorum sensing would cause swarm motility to decrease as cell-density dependent processes would be inhibited. As this is not the case, we assumed that the lasR mutant showed wild-type swarming because the other quorum sensing system, the rhl system, compensated for the lacking lasR system (3). Also, there was a lack of congruency between degree of inhibition in the PA14 and PAO1 fliC mutants since the swarming motility of the PA14 mutant was affected to a greater extent. This may have been because both the PA14 and PAO1 fliC mutants are similar in their inability to swarm, and calculating the area of motility was difficult since the calculated inhibition was sensitive to small decreases in swarm area. With this point in mind, we can explain why the data went against our expectations that the fliC mutants would be less resistant to SA effects (since they are lacking a functional flagellum).

Similarly, all PAO1 and PA14 strains in the swim and twitch motility assays were susceptible to increasing concentrations of SA. This suggested that either SA had pleiotropic effects on the motility of *P. aeruginosa*, or that SA affected another mechanism required for swimming/twitching (similar to the swarm explanation proposed above). In the swim assay, both the PA14 fliC and pilB mutants were more resistant to SA effects than the wild-type at all SA concentrations. This went against our hypothesis for the pilB mutants, as swimming is independent of type IV pili (21); therefore, the pilB mutants were expected to exhibit the same behavior as its respective wild-type, especially since the pilB mutants showed high similarity of swimming ability when compared to the wild-type when SA was not present (data not shown). The mechanism by which this result arose is not clear, but it is known that the ability of PA14 to attach to abiotic surfaces via its type IV pili is inhibited by SA, which may have affected its ability to swim (20). However, swim halo diameters of all strains except for PAO1 pilB were significantly inhibited at both concentrations of 25 and 50 mM SA (p<0.05), supporting our hypothesis that motility inhibition affects biofilm formation. Likewise, twitching motility is flagella-independent, so we expect the fliC mutants to exhibit the same behaviour as the wild-type strains (21). However, both wild-type strains exhibited slightly greater percentage inhibition in twitching ability at 50 mM SA. Therefore,
we would hypothesize there is a mechanism by which there is greater resistance to SA in fliC mutants, but determining this fell outside the scope of our experiment. All twitch halo diameters in the presence of SA were significantly reduced at 50 mM SA (p<0.05). Both motility mutants exhibited less susceptibility to SA at 25 mM versus the wild-type, suggesting that the lack of flagella and type IV pili mediates the resistance of P. aeruginosa to SA. As a note, it has been shown that another compound, rosmarinic acid, has an anti-swimming effect on P. aeruginosa, but not directly on biofilm formation (6). Similar motility and biofilm assays with rosmarinic acid could prove to have a more specific role on the motility of P. aeruginosa rather than the pleiotropic effects of SA.

Overall, we found that there is a direct inhibitory effect of SA on swimming, swimming, and twitching motility in both PAO1 and PA14 lab strains, but we cannot conclude whether or not SA preferentially targets one mechanism or motility system in P. aeruginosa. The motility and biofilm assays in this present study illustrated the pleiotropic effect SA had on biofilm formation, as sub-inhibitory concentrations of SA had significant effects in decreasing swarm, swim and twitch motility for all strains tested - in comparison to the wild-type, the motility mutants showed significantly greater resistance to SA for all types of motility dependent on flagella and type IV pili. Henceforth, we believe that motility is impaired by SA explain observed differences in swarming motility, as gene differences could lead to differences in their structural and functional characteristics.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. William Ramey and Matt Mayer for their assistance during the experiment, and the critical reading of the manuscript. The authors would like to thank Shaan Gelliaty and the R.E.W Hancock Lab for donating the strains required for the experiment. This project was financially supported by the Department of Microbiology and Immunology, University of British Columbia.

REFERENCES

vitro and on the foreign-body response in vivo. Biomater. 27:5018.