Journal of Experimental Microbiology and Immunology (JEMI) Vol. 15: 22 – 29

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

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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.**

Pseudomonas aeruginosa is a Gram-negative bacterium causing nosocomial infections in immunocompromised humans by forming a recalcitrant biofilm, providing resistance to antibiotics (14). By adhering to many different surfaces by means of virulence factors and motility, *P. aeruginosa* is able to form biofilms in the lungs, urinary tract, kidneys, and also in catheters, causing inflammation and septic shock in hospital patients (8). Biofilms are highly organized, functionally heterogeneous community structures that frequently form on biotic and abiotic surfaces to protect the bacteria from the host immune system and antibiotics via the retarding polysaccharide matrix it is embedded in (2). The sessile surface-attached community forms a structured complex after a highly regulated maturation process involving planktonic cell adhesion, microcolony and mushroom cap formation (16). These important steps in *P. aeruginosa* biofilm formation are dependent on two main bacterial appendages - the flagella and type IV pilus (2, 23) - both of which are also required for cell motility. The pathogenesis of *P. aeruginosa* and its ability to colonize environments depends on this motility; it is capable of swimming in aqueous environments (low-agar conditions), and twitching on dry environments (high-agar conditions) (24). Biofilm cap formation during maturation has been previously shown to be mediated by type IV pili binding to extracellular DNA found in the EPS (2, 16). A third form of motility affecting mushroom cap formation is swarming motility (14). Swarming is a complex motility regulated by quorum sensing that occurs on semi-solid surfaces, and requires flagella, type IV pili, and biosurfactant production (19).

To prevent infections arising from biofilm formation on implants and catheters, antibiotics are utilized at high concentrations to limited success (11). The use of an anti-biofilm therapeutic at these potential sites of bacterial colonization would be invaluable to prevent morbidity and mortality. It has been observed that the non-steroidal anti-inflammatory drug salicylic acid (SA) hinders motility and affects other virulence factors of *P. aeruginosa* (1, 5, 23) - this compound could potentially be used as a safe and effective anti-biofilm therapeutic. To elucidate the mechanism of SA attenuation of biofilm formation, we tested the effect of SA on *P. aeruginosa* motility. By

TABLE 1. Strains used, their nomenclature, and expected phenotype as provided by strain sources.

Strain	Strain number	Genotype KO	Expected Phenotype
PA14	PA14	Wild-type	Wild-type
	PA14_50290	*fliC*	Flagellin type B transposon insertion mutant
	PA14_58750	*pilB*	Type 4 fimbrial biogenesis protein PilB transposon insertion mutant
PAO1	H103	Wild-type	Wild-type
	PA1092	*fliC*	Flagellin type B transposon insertion mutant
	PA4526	*pilB*	Type 4 fimbrial biogenesis protein PilB transposon insertion mutant
	PA1430	*lasR*	Transcriptional regulator LasR transposon insertion mutant

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 swarming, 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, 100 150 mM), followed by heating to 100°C and filter sterilization using 0.2 µm pore size filters (Millipore, Cat. no. GSTF02500).

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 and inoculated with 0.1, 0.25, 0.5, 1, 25, 50, 100, or 150 mM SA in a polystyrene 96-well plate (Sarstedt, Cat. no. 82.1581.001). Growth was scored after 24 hrs incubation at 37 °C (instead of the 20 hrs time point specified), the MIC value taken as the concentration that inhibited macroscopic growth.

Biofilm assays. Biofilm formation was analyzed using an abiotic solid surface assay as described elsewhere (9). Briefly, overnight cultures in modified BM2 glucose broth (62 mM potassium phosphate buffer pH 7, 7 mM $(NH_4)_2SO_4$, 0.5% (w/v) casamino acids (Bacto Difco, Cat. no. 223030), 2 mM Mg_2SO_4, 0.4% (w/v) glucose, 10 µM $FeSO_4$) were diluted to 1.2 OD_{600} on a Beckman spectrophotometer and further diluted 1/100 in modified BM2 glucose broth mentioned above. In polystyrene microtiter plates, cultures were inoculated with 0, 5, 10, 25, 50, or 100 mM SA.

Swarming assay. Swarming motility was evaluated on modified BM2 glucose plates (62 mM potassium phosphate buffer pH 7, 0.5% casamino acids, 2 mM $MgSO_4$, 10 µM $FeSO_4$, 0.4 % (w/v) glucose) containing 0.5% (w/v) agar (Invitrogen, Cat.#30391-023) as previously described (18). 25 or 50 mM of SA was added to plates prior to a 45 min drying period. Bacteria were inoculated as 1 µl aliquots of an overnight culture grown in BM2 glucose broth (10% (v/v) BM2, 2 mM Mg_2SO_4, 0.4% 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 swarming ($p<0.01$), 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 $\geqslant$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

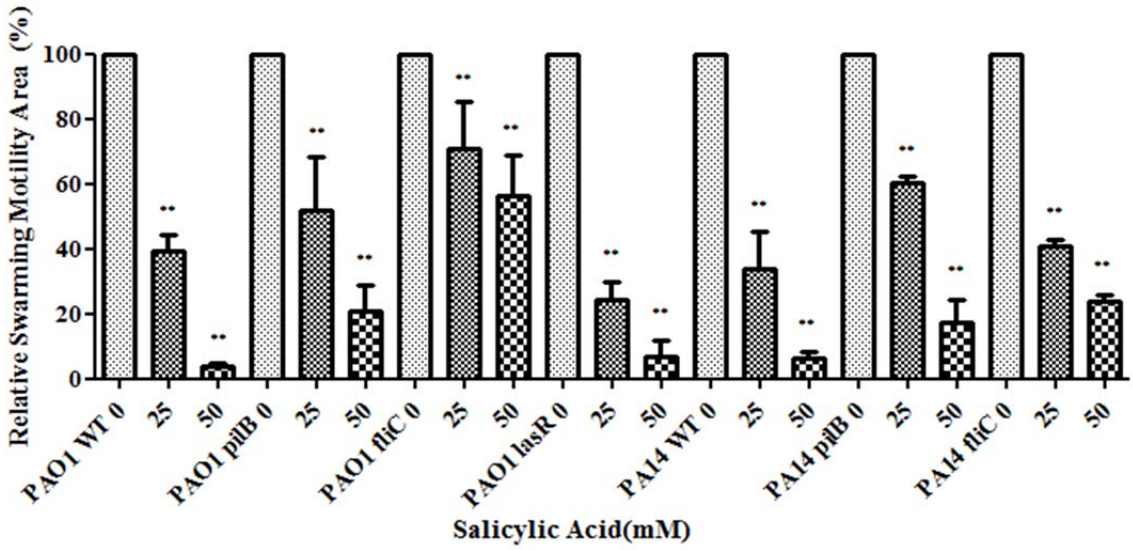

FIG. 1. Presence of biofilm formed by *P. aeruginosa* strains and mutants in the presence of SA. Biofilm formation by the indicated strains was measured after incubation in the presence of specified SA concentrations. Bars show the mean +/- SEM (n=3 independent experiments) with statistical differences (versus no SA) at P<0.01 indicated by **.

formation through a mechanism other than direct killing.

SA inhibits biofilm formation in *P. aeruginosa* in a dose-dependent manner. Increasing concentrations of SA reduced biofilm formation in all strains of *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_{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 *P. aeruginosa* strains. SA decreased the swarming ability of all *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 *P. aeruginosa*. Wild-type *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

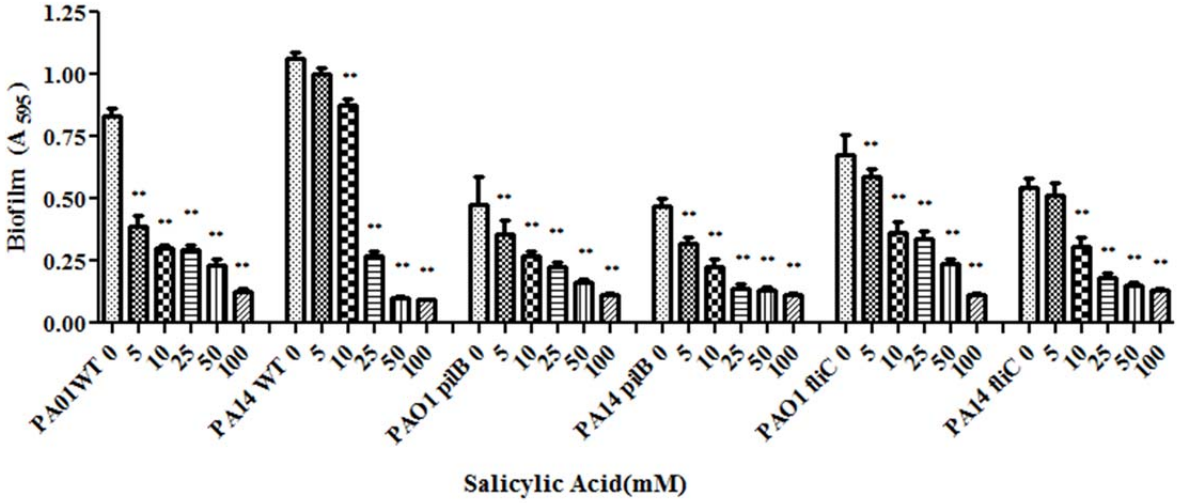

FIG. 2. Quantification of swarming motility by *P. aeruginosa* strains and mutants in the presence of SA. Relative swarming motility area of indicated strains measured after incubation in the presence of specified SA concentrations. Values are normalized to 100% motility area in the absence of SA. Bars 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 smallswarm 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 swarm 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.

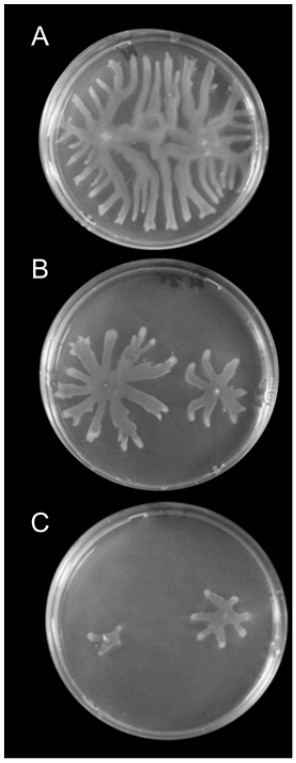

FIG. 3: Overnight swarm growths of *P. aeruginosa* in the absence/presence of SA. Swarm growth cultures grown on BM2 glucose media for 18 h in presence of SA. A) PA14 WT in the absence of SA. B) PA14 WT with 20mM SA. C) PA14 WT with 50mM SA.

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,

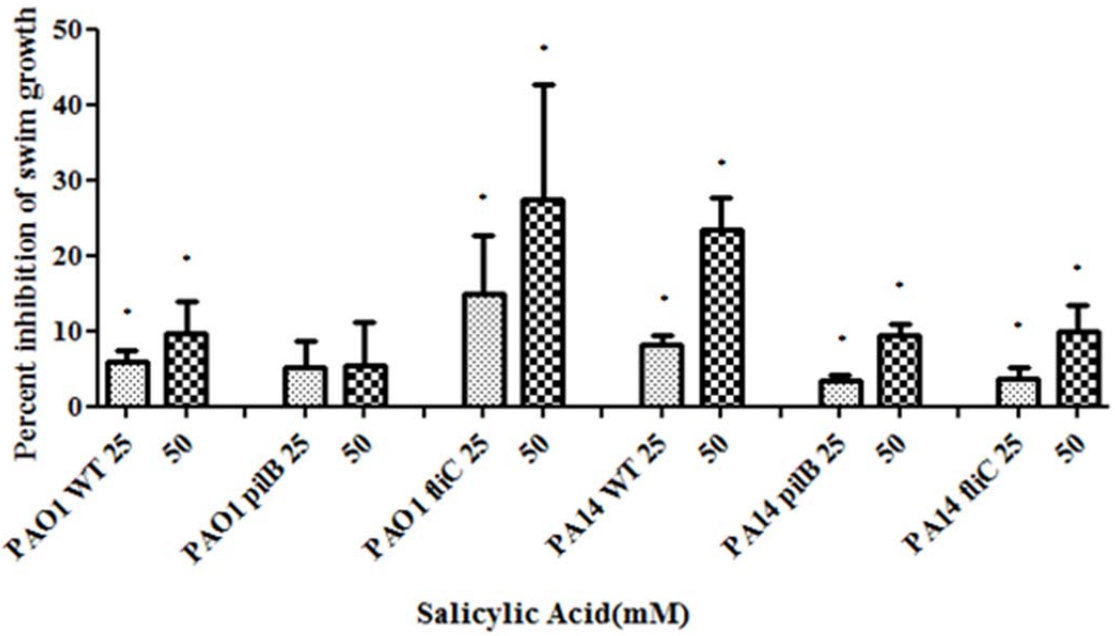

FIG. 4. Quantification of the inhibition of the formation of swimming halo by *P. aeruginosa* strains and mutants in the presence of SA. Percent inhibition of swim motility growth of indicated strains measured after incubation in the presence of specified SA concentrations. Values are normalized to no inhibition exhibited by the no SA controls in each strain. Bars show the mean +/- SEM (n=3 independent experiments) with statistical differences (versus no SA) at $P<0.05$ indicated by *.

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 pilus 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,

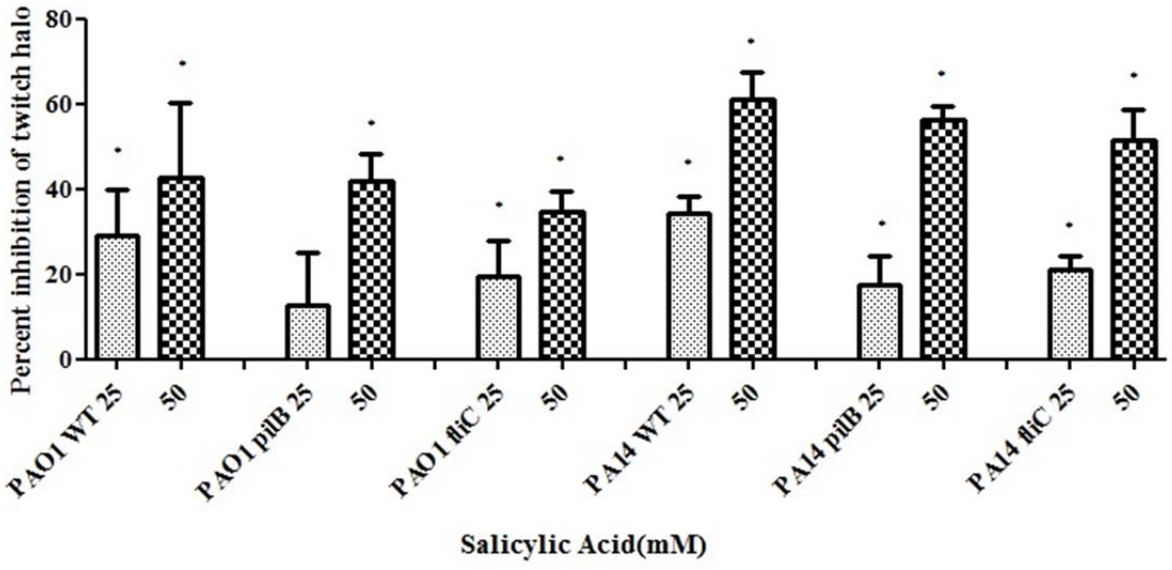

FIG 5. Quantification of the inhibition of the formation of twitching halo by *P. aeruginosa* strains and mutants in the presence of SA. Percent inhibition of twitch halo diameter of indicated strains measured after incubation in the presence of specified SA concentrations. Values are normalized to no inhibition exhibited by the no SA controls in each strain. Bars show the mean +/- SEM (n=3 independent experiments) with statistical differences (versus no SA) at P<0.05 indicated by *.

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 swarming, 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 Gellatly 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.

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