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Tannin derived materials can block swarming motility and enhance biofilm formation in *Pseudomonas aeruginosa*

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Surface-associated swarming motility is implicated in enhanced bacterial spreading and virulence, hence it follows that anti-swarming effectors could have clinical benefits. When investigating potential applications of anti-swarming materials it is important to consider whether the lack of swarming corresponds with an enhanced sessile biofilm lifestyle and resistance to antibiotics. In this study, well-defined tannins present in multiple plant materials (tannic acid (TA) and epigallocatechin gallate (EGCG)) and undefined cranberry powder (CP) were found to block swarming motility and enhance biofilm formation and resistance to tobramycin in *Pseudomonas aeruginosa*. In contrast, gallic acid (GA) did not completely block swarming motility and did not affect biofilm formation or tobramycin resistance. These data support the theory that nutritional conditions can elicit an inverse relationship between swarming motility and biofilm formation capacities. Although anti-swarmers exhibit the potential to yield clinical benefits, it is important to be aware of possible implications regarding biofilm formation and antibiotic resistance.

**Keywords:** swarming motility; biofilm formation; *Pseudomonas aeruginosa*; tannin; cranberry

**Introduction**

With the increasing prevalence of microbial resistance to existing antibiotics, alternative strategies are needed for targeting bacterial infections of which plant products containing tannins are promising candidates (Haslam 1996; Cowan 1999; Serrano et al. 2009; de la Iglesia et al. 2010). Plant extracts can modify bacterial behavior by affecting motility, adhesion to surfaces, biofilm formation, quorum sensing and production of virulence factors such as toxins (Rasko and Sperandio 2010). A mechanistic understanding of how specific plant extracts influence multiple bacterial functions provides insights into potential clinical applications and limitations of such materials.

Bacterial motility plays a pivotal role in infection site colonization and further propagation of bacteria. The bacterium *Pseudomonas aeruginosa* undertakes three types of motility, viz. swimming, swarming and twitching. Bacteria utilize flagella to swim towards a particular surface where they can subsequently attach via bacterial adhesins such as type-IV pili, lipopolysaccharide and the flagellum itself (O’Toole and Kolter 1998; Pratt and Kolter 1998; Costerton et al. 1999; Sauer et al. 2002; Stoodley et al. 2002). Once attached, *P. aeruginosa* can spread via surface-associated swarming and twitching motilities, mediated by hyperflagellation and type-IV pili, respectively (Alm and Mattick 1995; Copeland and Weibel 2009).

Recently, the authors’ laboratory showed that derivatives of cranberry (cranberry powder (CP) and proanthocyanidin (CPAC)), pomegranate (punacalagin, PG) and green tea blocked swarming motility in *P. aeruginosa* but not the swimming or twitching motilities. Other plant-derived materials tested, including cinnamon and turmeric, did not block swarming motility (O’May and Tufenkji 2011). Swarming motility is often associated with increased virulence enabling the bacteria to rapidly colonize a new environment (Verstraeten et al. 2008; Murray et al. 2010; Tremblay and Deziel 2010). Moreover, in comparison to planktonic swimming cells, swarming bacteria are more resistant to antibiotics (Lai et al. 2009; Butler et al. 2010). It follows that anti-swarming effects of plant extracts could have clinical benefits. However, it is also important to determine whether the lack of swarming motility corresponds with the bacteria forming an enhanced biofilm.

Some bacteria are known to exhibit an inverse relationship between a migratory swarming phenotype and a sessile biofilm lifestyle (Verstraeten et al. 2008; O’Toole 2008; Shrout et al. 2011). In the case of *P. aeruginosa*, this inverse relationship has been
predominately documented using specific mutant strains that are defective in swarming motility. Examples include mutants defective in genes coding for the sensor kinase CbrA, unknown protein SadB (Catiazzo et al. 2007), inner membrane-localized diguanylate cyclase SadC (Merritt et al. 2007) and c-di-GMP phosphodiesterase BifA (Kuchma et al. 2007). Many of these genes are associated with pathways regulating the level of secondary messenger (c-di-GMP), an intracellular secondary messenger that correlates with enhanced biofilm formation and reduced swarming motility and virulence (Merritt et al. 2007; McDougald et al. 2011). In accordance with this reverse relationship, P. aeruginosa variants isolated from mature biofilms grown in a microtitre plate (Deziel et al. 2001) and in the cystic fibrosis lung (Murray et al. 2010) have demonstrated an inverse link between swarming motility and biofilm forming capacities. Moreover, P. aeruginosa isolates obtained from chronic infections in the cystic fibrosis lung, expected to be in the biofilm mode of growth (Worlitzsch et al. 2002), displayed a low capacity for swarming motility (O’May et al. 2006). Few studies have investigated this inverse relationship by the addition of nutritional factors that modify swarming motility and biofilm formation capacities (Hoffman et al. 2005; Lee et al. 2009; Babic et al. 2010; Bernier et al. 2011). The observed blocking of swarming motility by select tannin-containing materials necessitates a need to investigate how such compounds affect biofilm formation.

One of the common features of the cranberry, pomegranate and green tea materials that have been shown to block swarming motility (O’May and Tufenjki 2011) is that they are all tannin derivatives. Tannins are a family of polyphenolic compounds present in a variety of plant materials where tannin composition and abundance can differ between different plants (Tarascou et al. 2010). This suggests that multiple plant materials that contain tannins may also be capable of blocking swarming motility. The cost and difficulty associated with the extraction of specific tannin derivatives and the unspecified nature of raw plant materials (eg green tea beverages) can provide challenges with research in this area. Therefore, this study investigated the effects of chemically-defined widely available tannins for their effects on both swarming motility and biofilm formation by P. aeruginosa. The compounds used were tannic acid (TA), gallic acid (GA) and epigallocatechin gallate (EGCG). Both TA and GA are found in a variety of plants including cranberry, green tea, and grape (Haslam 1996; Serrano et al. 2009). EGCG is a type of tannin compound that is abundant in green tea (Serrano et al. 2009). Undefined cranberry powder (CP) was also incorporated into experiments. Select experiments were also undertaken with cranberry proanthocyanidin (CPAC) extract, pomegranate (PG) hydrolysable tannin extract and turmeric (Turm) and cinnamon (Cinn) compounds.

Methods

Bacterial strains and media conditions

Experiments were undertaken with P. aeruginosa laboratory strain PAO1, originally isolated from a burn wound (Holloway 1955). Select experiments were performed using knockout mutants for flagella (PAO1ΔfliC), pilus (PAO1ΔpilA) or both appendages (PAO1ΔfliCΔpilA). Wild-type and mutant strains were kindly provided by M. Elimelech from Yale University, USA. Pure stock cultures were maintained at −80 °C in 30% (vol/vol) frozen glycerol solutions in 50% LB broth (tryptone 10 g l⁻¹, yeast extract 5 g l⁻¹ and NaCl 5 g l⁻¹). Frozen cultures were streaked onto LB agar (LB broth +1.5% agar [wt/vol]) and after incubation (37 °C, 24 h), a single colony was inoculated into LB broth (15 ml) to prepare bacterial inoculum for the experiments as described. All media were prepared using deionized (DI) water. Stock solutions of tannin material (described below) or sterile DI were added to the motility or biofilm media to achieve the concentration that is indicated for each experiment. Specific media are outlined in the relevant sections.

Preparation of tannin-containing materials

Tannic acid (TA), gallic acid (GA) and epigallocatechin gallate (EGCG) (Sigma-Aldrich, Canada) were solubilized to stock concentrations of 1, 5 and 2 g l⁻¹, respectively. Cranberry powder (CP; Atoka Cranberries, Canada) was kept at room temperature and stored at 4 °C protected from light, due to potential light sensitivity of tannin materials (Serrano et al. 2009). Cranberry proanthocyanidin (CPAC) was obtained from Dr Amy Howell at Rutgers University, USA. Pomegranate (PG), turmeric (Turm), and cinnamon (Cinn) compounds were obtained from Dr Navindra Seeram at the University of Rhode Island, USA. Pomegranate extract was the hydrolyzable tannin termed punicalagin. Turm was derived from the rhizome of the Curcuma longa plant. Cinn was derived from the bark of the Cinnamomum zeylanicum plant. Stock solutions of these compounds were prepared at a concentration of 100 mg ml⁻¹ in DI. Cranberry proanthocyanidin (CPAC) was obtained from Dr Amy Howell at Rutgers University, USA. Pomegranate (PG), turmeric (Turm), and cinnamon (Cinn) compounds were obtained from Dr Navindra Seeram at the University of Rhode Island, USA. Pomegranate extract was the hydrolyzable tannin termed punicalagin. Turm was derived from the rhizome of the Curcuma longa plant. Cinn was derived from the bark of the Cinnamomum zeylanicum plant. Stock solutions of these compounds were prepared at a concentration of 1.5 mg ml⁻¹ in DI. All solutions were filter sterilized using a 0.45 μm filter and stored at 4 °C protected from light, due to potential light sensitivity of tannin materials (Serrano et al. 2009). Stock solutions of tannin materials were subsequently diluted into the motility or biofilm media to yield the concentration that is indicated in each
experiment. Control media was also diluted accordingly with DI. Concentrations reported in the relevant results sections correspond to the final concentration of material within the specified medium. At the concentrations tested, the tannins did not affect the solution pH (data not shown).

**Swarming assays**

Swarming assays were undertaken in Petri dishes (polystyrene, diameter of 82 mm) containing swarm agar (nutrient broth 8 g l\(^{-1}\) [Oxoid, UK] and 0.5% agar [wt/vol] (Fisher Scientific, USA)) supplemented with 5 g l\(^{-1}\) D-glucose. Swarm plates were dried for 1 h prior to inoculation as this method is reported to give the most consistent results (Tremblay and Deziel 2008). Plates were inoculated with a 5 µl aliquot of broth culture taken mid-exponential growth phase (37°C, 5 h at 200 RPM representing approximately 10\(^8\) CFU ml\(^{-1}\)). The inoculum was placed on the agar surface (center) enabling visualization of motility across the agar surface (Tremblay et al. 2007). The diameters of the swarming motility zones were measured after incubation at 37°C for 20 h.

**Biofilm assays**

Biofilm formation was predominantly examined with the standard microtitre plate model (O’Toole et al. 1999; Merritt et al. 2005; O’Toole 2011) using flat-bottom polystyrene non-tissue culture treated 96 well plates (BD Falcon, USA). To compare different short term models, select experiments were also conducted in round-bottom alternatives of the above and in both 12 × 75 mm glass and polystyrene culture tubes. A common inoculation protocol was used for all model systems. Briefly, overnight cultures (LB, 37°C, 200 RPM) were diluted 1:100 (vol/vol) into fresh LB broth (containing tannin or control solutions) yielding approximately 10\(^7\) CFU ml\(^{-1}\). The inoculum was placed on the agar surface (center) enabling visualization of motility across the agar surface (Tremblay et al. 2007). The diameters of the swarming motility zones were measured after incubation at 37°C for 20 h.

Biofilm assays were performed in LB broth cultures and set up as per the standard biofilm microplate assays. Plates were incubated at 37°C, under static or shaking (orbital shaking 44.3 RPM) conditions within the microtitre plate reader (Tecan Infinite M200 Pro, Switzerland) and OD 600 nm measurements were recorded every 20 min for 24 h. Growth was also determined under select conditions by serial dilutions (1:10) in phosphate buffered saline (PBS) and subsequent spot-inoculation (10 µl) to obtain the CFU ml\(^{-1}\).

Swarm plates (with 1.5% vol/vol agar) were supplemented with tannin-containing materials at the indicated concentrations and used to determine the effects of tannins on bacterial growth capacities under conditions relevant to the swarm assays. To do this, bacterial broth culture (identical to that used in the swarm assays) was serial-diluted (1:10, up to 10\(^{10}\)) in sterile phosphate-buffered saline (PBS), and two aliquots (10 µl) of each dilution were spot-inoculated onto duplicate plates to verify the concentrations of bacteria that were able to grow during incubation on the different plates (16 h, 37°C).

**Antimicrobial susceptibility testing**

The minimum bactericidal concentration (MBC) of tobramycin (Sigma-Aldrich, Canada) was determined in LB broth culture. Tobramycin was chosen because this antibiotic is routinely used against *P. aeruginosa* infections (Banerjee and Stableforth 2000). A stock solution of tobramycin (0.1 mg ml\(^{-1}\) DI, stored at 4°C) was diluted into solutions containing bacteria and tannins as for the biofilm assays. Aliquots (200 µl) were then transferred into triplicate wells of the standard microtitre plate (flat-bottom polystyrene non-tissue culture treated 96 well plate). Microtitre plates were incubated for 24 h under static conditions at 37°C and 10 µl aliquots from each well were spot plated onto LB agar plates to determine the MBC.

Antibiotic susceptibility was also determined on solid media using E-Test strips, plastic strips
impregnated with a concentration gradient of tobramycin (0.016–256 μg ml⁻¹) (Biome´rieux, Canada). This assay was undertaken as per the manufacturer’s guidelines except that LB agar was used to be consistent with the other assays. Briefly, cells were grown in 15 ml of LB broth for 4 h at 37°C and 200 RPM. Cells were centrifuged (7,000 RPM, 10 min 4°C) and resuspended in 0.9% saline to obtain a suspension turbidity equivalent to a MacFarland Standard of 0.5. Sterile cotton swabs were used to gently spread bacteria onto LB agar plates spiked with DI or tannin solutions. When plates were dry, E-test strips were then applied and the plates were incubated for 20 h at 37°C. The minimum inhibitory concentration (MIC) of tobramycin was read from the scale on the E-Test strip.

Statistics
Where indicated, a two-tailed Student’s t test (P < 0.05) was used to determine whether the presence of tannin-containing materials resulted in any significant differences compared to when these materials were not present.

Results
Previously, the authors showed that condensed and hydrolyzable tannin extracts derived from cranberry and pomegranate, respectively, blocked swarming motility in P. aeruginosa (O’May and Tufenkji 2011). This study expanded on those earlier findings and aimed to determine whether well-defined, commercially available tannin constituents (TA, GA and EGCG) would also block swarming motility and influence biofilm formation and antibiotic susceptibility (the structure of the compounds is shown in Figure 1). Additionally, undefined cranberry powder (CP) was used for comparison. Select experiments incorporated CPAC, PG, Turm and Cinn materials, where the former two were previously found to also block swarming motility, while the latter two exerted no effect at the concentrations tested (O’May and Tufenkji 2011).

Effects of tannin materials on swarming motility
Under control conditions (in the absence of any tannin materials), P. aeruginosa displayed proficient swarming motility and formed tendrils which migrated outwards from the point of bacterial inoculation (Figure 2), a feature characteristic of normal swarming motility in P. aeruginosa (Tremblay et al. 2007; Lindhout et al. 2009). When tannins were present in the swarm medium at different concentrations, TA, EGCG and CP blocked swarming motility (≤10% of control swarming diameter). A single large colony was observed at the point of inoculation and no tendril formation was observed (Figure 2). GA also significantly impaired swarming motility at a variety of concentrations (P < 0.05), but not as effectively as the other tannin constituents. At the highest concentration of GA tested (500 mg ml⁻¹) the swarming diameter was ~19% of the control diameter and tendril formation remained evident indicating that cells were able to swarm to an extent (Figure 2). Mid-range GA
concentrations only impaired swarming motility to \( \sim 80-40\% \) of control levels.

**Effects of tannin materials on biofilm formation**

As all of the tannin compounds tested above blocked or impaired swarming, their effects on biofilm formation were examined in the standard microtitre biofilm model. TA, ECGC and CP all significantly enhanced biofilm formation compared to biofilms seen under control conditions (\( P < 0.05 \)) (Figure 3). The compounds that enhanced biofilm formation were the ones that also effectively blocked swarming motility (Figure 2). In contrast, GA, which was the least effective at blocking swarming motility, did not significantly affect biofilm levels (Figures 2 and 3).

Staining of the attached material in the microplates revealed that the addition of TA, ECGC and CP altered the surface distribution pattern of the attached bacteria (Figure 3 insets). Under control conditions (first wells in each inset), biofilms predominated at the air-liquid interface where oxygen levels were highest, a feature characteristic of biofilm development in aerobic conditions (Yoon et al. 2002). Interestingly, in the presence of TA, ECGC and CP, biofilms also formed at the base of the wells (Figure 3 insets). These three compounds also resulted in increased intensity of the biofilm ring at the air-liquid interface (data not shown). The addition of GA did not result in any stained material at the bottom of the wells.

**Additional tannin-containing materials and their effects on biofilm formation**

Whether other tannin-containing materials exerted similar inverse effects on swarming motility and biofilm formation was also evaluated. Previously, the authors had shown that CPAC and PG tannin extracts blocked swarming motility in *P. aeruginosa*, while Cinn and Turm extracts exerted no significant effects (O’May and Tufenkji 2011). When tested for biofilm-forming effects, CPAC and PG significantly enhanced biofilm formation (\( P < 0.05 \)) and resulted in attached biofilm at the base of the well, while Turm and Cinn
did not alter the biofilm levels \( (P > 0.05) \) (Figure 4). The above data supported the theory that an inverse relationship exists between the capacity for \( P.\ aeruginosa \) to undertake swarming motility and form biofilms in the standard microtitre plate assay (Shrout et al. 2011).

**Effects of the tannin materials on growth rates**

Any effect that the tannins had on bacterial growth rates could influence findings in the swarm and biofilm formation assays. To investigate this potential interference, growth rates were determined in the presence of a concentration gradient of TA, GA, ECGC and CP. Growth was tested in the presence of TA under static conditions to parallel those of the biofilm assay \( (LB, 37^\circ C) \) by measuring turbidity changes (OD 600 nm) over time. These results suggested that addition of TA decreased bacterial growth (Figure 5A). However, under static conditions, TA enhanced biofilm development at the bottom of the well (Figure 3), which would interfere with OD measurements. Therefore, final growth yields (at 24 h) were determined by spread plating from the wells to obtain the CFU ml\(^{-1}\). The highest concentrations of any of the tannins did not significantly \( (P > 0.05) \) affect the final growth yield in comparison to the controls (Figure 5B).

Growth assays were also repeated under conditions of continuous shaking to further investigate the potential effects of tannins against \( P.\ aeruginosa \). Under such conditions, TA did not affect growth (Figure 5C) and subsequent staining revealed that no biofilms formed in the wells (data not shown). As with TA in the shaking conditions, GA, ECGC and CP did not impair bacterial growth yield (Figure 5D–F), although there did appear to be a slower exponential growth phase in the presence of ECGC compared to the control conditions (Figure 5E). Based on the OD measurements, GA and CP appeared to enhance the growth rates of \( P.\ aeruginosa \) (Figure 5D and F). However, in the presence of GA, \( P.\ aeruginosa \) produced a melanin pigment (data not shown) and in CP, a cranberry-bacterial aggregate was observed (data not shown) and these factors would undoubtedly influence the OD measurements. Thus, growth yields after shaking were also determined at 24 h by dilutions and spot-inoculations to obtain the CFU ml\(^{-1}\).
technique revealed that there were no measurable differences in the CFU ml\(^{-1}\) (Figure 5G). While tannins exerted subtle differences on \(P.\) \textit{aeruginosa} growth rates and yields in broth culture, it is unlikely that this accounts alone for the effects seen in the biofilm assays.

As tannins blocked or impaired swarming motility, whether tannins affected the growth capacity of \(P.\) \textit{aeruginosa} under conditions that mirrored the swarm assays was also investigated. To do this, the authors examined whether serial dilutions of bacterial broth cultures were able to grow on the swarm plates supplemented with different tannin materials (Figure 5H). In comparison to control conditions, there was no evidence of growth impairment in the presence of TA, GA, or CP as equivalent dilutions of bacteria were able to grow on tannin and control plates. Bacteria exhibited a reduced growth capacity in the presence of EGCG, but the undiluted broth culture (equivalent to that used in the swarm assay) was still able to grow. These data demonstrate that the majority of tannin-containing materials did not block swarming motility by preventing bacterial growth.

\textit{Effect of TA on biofilm formation in other models}

One of the challenges with conducting biofilm research is the variation in effects associated with different biofilm models (McBain 2009; Coenye and Nelis 2010). The effects of TA on biofilm formation in borosilicate glass tubes as opposed to polystyrene microtitre plates were also tested. In contrast to the results in the microtitre plate model, TA did not significantly alter the level of biofilm development in the glass tubes (Figure 6A). Biofilms only formed at the air-liquid interface and did not form at the base of the tubes (Figure 6A insets). Furthermore, the control levels of biofilms were higher in the glass tubes initially. Differences between the two models may reflect differences in the surface hydrophobicity, container dimensions (surface area/volume ratios) and the container geometry (flat-bottom microtitre vs round-bottom glass tube). To determine the role of these factors, biofilm formation upon exposure to TA was also tested in polystyrene culture tubes of identical dimensions to the glass tubes and round-bottom microtitre plates. TA significantly enhanced biofilm formation in the polystyrene culture tubes (Figure 6B) and the round-bottom microtitre plates (Figure 6C). Moreover, in the polystyrene models, biofilms formed at the base of the containers, unlike what was observed in the glass tubes (Figure 6 insets). This suggests that the different surface types are the factor responsible for the different effects between the models. It also demonstrates that TA did not uniformly enhance biofilm formation in all biofilm model systems and emphasizes that external factors can influence biofilm formation in different ways depending on the model.

\textit{Effects of TA on biofilm formation for flagellum and pilus mutants}

TA was used as a representative tannin to investigate the potential interactions with bacterial appendages that may account for the enhanced biofilm formation in the microplate assay. Whether mutant strains of \(P.\) \textit{aeruginosa} that did not express flagella (\(\Delta fliC\)) or pili (\(\Delta pilA\)) or both (\(\Delta fliC\Delta pilA\)) would continue to form enhanced biofilms in the presence of TA was tested. All WT and mutant strains exhibited equivalent growth rates (LB, static, 37°C) (data not shown). Mutants \(\Delta fliC\) and \(\Delta fliC\Delta pilA\) were not able to undertake swarming motility as expected. The \(\Delta pilA\) mutant was still able to undertake swarming motility comparable to the control (data not shown). The wild type and the \(\Delta pilA\) mutant (defective in type-IV pili) showed enhanced biofilm formation in the presence of TA (Figure 7) indicating that the presence of type-IV pili was not
necessary for this effect. However, ΔfliC and ΔfliCΔpilA did not form enhanced biofilms in the presence of TA implying that interactions between TA and flagella enhance biofilm formation. Such interactions may also account for the blocking of swarming motility and this will be investigated in a separate study. Interestingly, in the presence of TA, the double mutant formed significantly less biofilm compared to those seen under control conditions ($P < 0.05$).
Effects of tannins on antibiotic susceptibility

One of the undesirable consequences of bacterial biofilms is their enhanced resistance to antibiotics compared to planktonic bacteria (Costerton et al. 1999). The MBC of tobramycin (0–20 µg ml⁻¹) in the presence or absence of the maximum concentrations of the tannin derivatives used in the earlier assays was investigated. Under control conditions, no growth was evident at concentrations of tobramycin > 0.5 µg ml⁻¹. In the presence of TA and EGCG, *P. aeruginosa* was able to grow in up to 4 µg ml⁻¹ of tobramycin (Table 1). When grown in the presence of CP, bacterial growth was still evident at tobramycin concentrations up to 10 µg ml⁻¹ whereas a level of 20 µg ml⁻¹ killed all the bacteria. GA had no effect on tobramycin preventing bacterial growth (Table 1).

The motility mutants (Δ*fliC*, Δ*pilA* and Δ*fliC Δ*pilA*) were also assessed for antibiotic susceptibility in LB broth ± TA. In the absence of TA, all mutants exhibited the same susceptibility as the WT strain. As for the WT strain, TA enhanced the MBC for Δ*fliC* and Δ*pilA* mutants, but not for the Δ*fliC Δ*pilA* double mutant (Table 1).

Antibiotic susceptibility was also assessed on solid agar medium using E-Test strips. In this model, GA, TA, and EGCG did not significantly alter the MIC of tobramycin required to impair bacterial growth. However, CP significantly enhanced the MIC of tobramycin (P < 0.05) (Figure 8).

Figure 6. Effect of TA on biofilm formation in (A) glass and (B) polystyrene culture tubes and (C) microtitre plates with round bottom wells. Values shown represent the mean ± SD of three experiments with triplicate tubes or wells per experiment. *denotes significantly larger biofilms compared to when no TA is present (P < 0.05). Figure insets show representative images of the stained biofilms.

Figure 7. Effects of TA concentration gradient on *P. aeruginosa* biofilm formation using wild-type strain (WT) and motility mutants. Values shown represent the mean ± SD of three experiments, with triplicate wells per experiment. * denotes significantly larger or lower biofilms compared to control conditions (P < 0.05).
Previously, it was reported that cranberry, pomegranate and green tea derivatives can block the swarming motility of *P. aeruginosa* (O’May and Tufenkji 2011). Herein, this finding has been expanded to demonstrate that defined tannin constituents present in multiple plant materials (such as TA and EGCG) can also block swarming motility (Figure 2), emphasizing that a variety of tannin-containing materials are likely to mediate anti-swarming effects. To gain insights into applications of anti-swarming effectors, it is crucial to unravel whether the blocking of surface-associated motility corresponds with an enhanced sessile biofilm lifestyle. This study used the standard microtitre plate biofilm assay to show that tannins can exert inverse effects between swarming and biofilm formation capacities. Firstly, (1) TA, ECGC, CP, CPAC and PG blocked swarming motility and enhanced biofilm formation; (2) GA, turmeric and cinnamon did not completely block swarming motility and did not enhance biofilm formation (Figures 2 and 3). The ability of the anti-swarming compounds to enhance biofilm formation may be concerning as the biofilm mode of growth is associated with enhanced resistance to antibiotics and the host immune response (Costerton et al. 1999). Indeed, key tannins that blocked swarming motility enhanced bacterial resistance to tobramycin (Table 1).

As emphasized in this study, a variety of tannin-rich plant extracts would be expected to stop the swarming motility of *P. aeruginosa*. Indeed, *P. aeruginosa* swarming is impaired by ginseng (Wu et al. 2011) and other plant extracts including cranberry, pomegranate, green-tea, blueberry, strawberry, oregano and basil (Vattem et al. 2007; Zahin et al. 2010; O’May and Tufenkji 2011). Plants have also been reported to impair swarming motility in other actively swarming bacteria such as in *Escherichia coli* with cranberry derivatives (Hidalgo et al. 2011a) and pomegranate materials (Asadishad et al. 2012), *Proteus mirabilis* with TA (Smith 1975), *Lithrea molleoides* plant extract (Carpinella et al. 2011) and cranberry derivatives (the authors’ laboratory, unpublished data). The anti-swarming properties of plant materials could have beneficial effects under some circumstances as swarming permits rapid propagation across a surface (Tremblay and Deziel 2010) and has been associated with enhanced virulence (Merritt et al. 2007; McDougald et al. 2011). However, the lack of

### Table 1. Growth of *P. aeruginosa* in tannin compounds and a concentration gradient of tobramycin.

<table>
<thead>
<tr>
<th>Tannin</th>
<th>Concentration</th>
<th>Tobramycin concentration (µg ml⁻¹)</th>
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<tbody>
<tr>
<td>WT + Control</td>
<td></td>
<td>+ + + + + + - - - -</td>
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<tr>
<td>WT + TA</td>
<td>100 mg l⁻¹</td>
<td>+ + + + + + - - - -</td>
</tr>
<tr>
<td>WT + GA</td>
<td>500 mg l⁻¹</td>
<td>+ + + + + + - - - -</td>
</tr>
<tr>
<td>WT + EGCG</td>
<td>200 mg l⁻¹</td>
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<tr>
<td>WT + CP</td>
<td>10 mg ml⁻¹</td>
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<tr>
<td>ΔflIC</td>
<td></td>
<td>+ + + + + + - - - -</td>
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<tr>
<td>ΔflIC + TA</td>
<td>100 mg l⁻¹</td>
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<tr>
<td>ΔpilA</td>
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<tr>
<td>ΔpilA + TA</td>
<td>100 mg l⁻¹</td>
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<tr>
<td>ΔflICΔpilA</td>
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<tr>
<td>ΔflICΔpilA + TA</td>
<td>100 mg l⁻¹</td>
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Note: + corresponds with the ability of *P. aeruginosa* to grow; − corresponds with no bacterial growth. Values shown are representative values of three experiments where duplicate wells were tested per experiment.

Figure 8. Minimum inhibitory concentration (MIC) of tobramycin against *P. aeruginosa* was determined using the E-Test method on LB agar. Values shown represent the mean ± SD of three experiments with duplicate plates per experiment. * denotes significantly higher MIC compared to that seen under control conditions (P < 0.05). Concentrations of compounds correspond as follows: TA (100 Mg l⁻¹), GA (500 mg l⁻¹), EGCG (200 mg l⁻¹), CP (10 mg ml⁻¹).
swarming can correspond with enhanced biofilm formation (Figures 3 and 4).

The inverse relationship between swarming and biofilm formation capacities has been well documented using mutants deficient in swarming motility capacities (Caiazzia et al. 2007; Kuchma et al. 2007; Merritt et al. 2007; Verstraeten et al. 2008; Shrout et al. 2011). This study supports this inverse relationship, showing that the conditions that blocked swarming also enhanced biofilm formation. Other examples of externally added materials exerting these effects include indole (Lee et al. 2009), arginine (Bernier et al. 2011), tobramycin (Hoffman et al. 2005; Babic et al. 2010), and iron (Deziel et al. 2003; Banin et al. 2005; Berlotti et al. 2005). This inverse relationship theory is also supported by Landry et al. (2006) who reported that surfaces which promoted biofilm development also reduced surface-associated motility. For example, glass surfaces pre-conditioned with mucin coatings resulted in greater biofilm development by *P. aeruginosa* and reduced swarming motility in comparison to that seen with other coatings. The inverse relationship between motile and sessile behaviors also occurs in other organisms including *E. coli* (Garrett et al. 1999; Ulett et al. 2006; Pesavento et al. 2008), *P. mirabilis* (Jones et al. 2005) and *Salmonella enterica* serovar Typhimurium (Mireles et al. 2001). In the authors’ laboratory, CPAC also reduced swarming motility in *E. coli* (Hidalgo et al. 2011a) and caused a down-regulation of the genes *ymgC* and *ymgA* (Hidalgo et al. 2011b) that normally function in biofilm repression (Lee et al. 2007a).

Although there is evidence that effectors exert an inverse relationship between swarming and biofilm formation capacities, not all studies are in agreement. For example, both swarming motility and biofilm formation were found to be impaired by the fatty acid anteiso-c15:0 (Inoue et al. 2008) and ginseng (Wu et al. 2007a). Tannin-rich plant extracts of *Terminalia catappa* (microtitre plate, LB) (Taganna et al. 2006; La et al. 2010), *E. coli* (microtitre plate, LB) (Pinzon-Arango et al. 2011), *S. mutans* (saliva coated hydroxyapatite disks, tryptone-yeast broth) (Koo et al. 2010) and *S. epidermidis* (contact lenses, TSB) (Leshem et al. 2011). The majority of these studies have not used *P. aeruginosa* and this may partially explain the differences in the results. Indeed, Habash et al (1999) found that urine from individuals consuming cranberry juice reduced adhesion of *E. coli*, but not *P. aeruginosa* to silicone rubber. Along similar lines, EGCG (50–100 µg ml⁻¹) impaired the growth of Gram-positive *Staphylococcus* isolates but concentrations of more than 800 µg ml⁻¹ were required to impair growth of *P. aeruginosa* (Yoda et al. 2004).

In addition to variations in bacteria and strains between studies, one of the challenges with undertaking biofilm research is that different biofilm models and medium conditions can elicit different results (McBain 2009; Coenye and Nelis 2010). Indeed, in this study, it was found that TA-biofilm effects differed in short-term models incorporating polystyrene or glass surfaces (Figures 3 and 6). For the purpose of this study, the authors focused on the polystyrene microtitre plate model and LB medium as these are commonly used in biofilm research. In addition, this study supports this inverse relationship, showing that TA-biofilm effects differed in short-term models incorporating polystyrene or glass surfaces (Figures 3 and 6). For the purpose of this study, the authors focused on the polystyrene microtitre plate model and LB medium as these are commonly used in biofilm research on *P. aeruginosa* (O’Toole et al. 1999). However, variations would be expected when using different biofilm models and medium conditions. This also emphasizes that when examining the ability of a compound to impair biofilm formation, it ideally should be evaluated using multiple model systems. It would be interesting to further explore tannin effects on biofilm formation using additional surfaces such as polyvinylchloride and polypropylene. Moreover, conducting experiments under continuous-culture conditions would provide key additional insights.

The capacity of tannins to bind proteins is likely to influence the results in a number of microbiological assays (Hagerman and Butler 1981; Haslam 1996; Serrano et al. 2009). In the present study it was found that mutants lacking flagella did not form enhanced biofilms in the presence of TA (Figure 7), suggesting that TA-flagellum interactions contributed to the enhanced biofilm development. This is not surprising given that TA itself is a component of a flagellin stain (Leifson 1951). Tannin-flagellum interactions could also be a key mechanism by which tannin constituents block swarming motility. Tannins could be cross-linking the flagella thereby preventing swimming or binding to flagella causing significant drag forces. Of the tannins tested, it appears that the tannins with the greatest degree of polymerization and higher molecular weight (Figure 1) were the ones that effectively blocked swarming motility (Figures 1 and 2). Additional research with complemented mutants and other
experiments are needed to further elucidate the mechanisms by which tannins modify the behavior of *P. aeruginosa*. Although GA did not block swarming motility, it did significantly impair it, suggesting that GA, or compounds with similar properties, could have applicative benefits. Unlike the other compounds affecting swarming motility, GA did not enhance biofilm formation or antibiotic resistance, suggesting that further research could unravel application of GA-incorporated materials.

The possibility of enhanced biofilm formation could be problematic in a clinical setting due to a potential increased resistance to antimicrobial compounds. Indeed, in this study, it was found that TA, EGCG and CP enhanced the MBC of tobramycin (Table 1) in broth culture. Additionally, when CP was incorporated into agar, it enhanced the MIC of tobramycin when using E-Test strips on solid surfaces but the effects were not as strong as those seen in bacterial broth culture, nor did this occur with TA or EGCG (Figure 8). Differences in the results between the broth/MBC or agar/E-Test methods could be attributable to the changes the tannins exerted on the biofilm phenotype in broth culture (enhanced biofilm formation) compared to bacteria growing on an agar surface. Furthermore, if the tannins were reducing the bioactivity of tobramycin by binding to the antibiotic, then this tannin-antibiotic interaction would be more evident in broth/MBC assay as opposed to the agar/E-Test assay. The effects seen with mutants lacking flagella, pili or both provide support for both of the above phenomena (Table 1). For example, TA also enhanced the MBC for mutants lacking either flagella or pili. As discussed above, the pili mutant continued to form enhanced biofilms in the presence of TA, thus enhanced MBC could correspond with the biofilm phenotype. In contrast, the flagella mutant did not form enhanced biofilms in the presence of TA. This suggests that the presence of TA alone could reduce tobramycin bioactivity, but TA did not affect the MBC of the mutant lacking both flagella and pili. Further analyses are needed to unravel the mechanisms by which tannins influence antibiotic susceptibility.

The anti-swarming properties of tannins may exert clinical benefits under some situations but this study emphasizes that caution needs to be applied with regards to the possible effects of enhanced biofilm formation and antibiotic resistance. Tannin extracts may serve greater benefits in situations where bacterial motility is important for colonization and infection. Examples include the urinary tract where motility up the tract is an important factor contributing to pathogenesis (Siitonen and Nurminen 1992; Walters et al. 2012). However, tannins may be less effective in other situations such as the cystic fibrosis lung, where bacteria become trapped within the thick mucus and form a biofilm (Worlitzsch et al. 2002). As these studies were conducted in vitro it would be necessary to confirm whether similar effects occur in vivo. Biofilm enhancing effects seen in vitro may not always result in negative in vivo outcomes. Lee et al. (2007b, 2009) found that indole and its derivative 7-hydroxyindole decreased swarming in *P. aeruginosa* and also concurrently enhanced biofilm formation and antibiotic resistance, similar to that reported for the tannins in this manuscript. Importantly, this compound resulted in reduced virulence using an in vivo animal model of pulmonary colonization emphasizing that the anti-swarming and virulence effects can yield beneficial effects in vivo despite the possibilities of enhanced biofilm development (Lee et al. 2007b, 2009). Further analyses will underpin the mechanisms by which tannins modify bacterial behavior assisting in the guidance of various plant extracts for their use in clinical applications.

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