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Inhibition and dispersal of *Agrobacterium tumefaciens* biofilms by a small diffusible *Pseudomonas aeruginosa* exoproduct(s)

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Abstract

Environmental biofilms often contain mixed populations of different species. In these dense communities, competition between biofilm residents for limited nutrients such as iron, can be fierce, leading to the evolution of competitive factors that affect the ability of competitors to grow or form biofilms. We have discovered a compound(s) present in the conditioned culture fluids of *Pseudomonas aeruginosa* that disperses and inhibits the formation of biofilms produced by the facultative plant pathogen *Agrobacterium tumefaciens*. The inhibitory activity is strongly induced when *P. aeruginosa* is cultivated in iron-limited conditions, but it does not function through iron sequestration. In addition, the production of the inhibitory activity is not regulated by the global iron regulatory protein Fur, the iron-responsive extra-cytoplasmic function (ECF) σ factor PvdS, or three of the recognized *P. aeruginosa* quorum sensing systems. In addition, the compound(s) responsible for the inhibition and dispersal of *A. tumefaciens* biofilm formation is likely distinct from the recently identified *P. aeruginosa* dispersal factor, *cis*-2-decenoic acid (CDA), as dialysis of the culture fluids showed that the inhibitory compound was larger than CDA and culture fluids that dispersed and inhibited biofilm formation by *A. tumefaciens* had no effect on biofilm formation by *P. aeruginosa*.

Keywords

Biofilms; Inhibition; Dispersal; Iron

INTRODUCTION

There is a growing appreciation that bacteria in the environment exist primarily as multicellular communities known as biofilms. These bacterial aggregates assemble at interfaces and generally are encased in an extracellular polymeric matrix (Sutherland 2001; Whitchurch et al. 2002). In contrast to planktonically growing bacteria, the cells in a biofilm are typically more recalcitrant to antimicrobial treatment and predatory grazing, capable of maintaining extremely high local cell densities, and thus able to potentially facilitate the expression of quorum sensing controlled functions and horizontal gene transfer (Maeda et al. 2006; Horswill et al. 2007). These characteristics are of particular concern in many medically relevant contexts where biofilms often play a role in implant-associated infections, water-borne diseases, and the establishment of chronic infections (Costerton et al. 1999; Rather 2005; Lim et al. 2006). Biofilms are also problematic in a wide range of industrial and agricultural contexts where they are responsible for biofouling and spoilage (Danhorn et al. 2004; Coetser and Cloete 2005; Verran et al. 2008). Compounds that are capable of dispersing bacterial biofilms are therefore of clear practical application. One

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potential approach for the discovery and mechanistic elucidation of biofilm inhibitory compounds is to examine the competitive interactions that occur in single and multispecies biofilms. The high population density present in most biofilm communities represents a double-edged sword as bacteria in close proximity may benefit by cooperating but also compete for the limited resources available (Platt and Bever 2009; Hibbing et al. 2010). Because of this, biofilms are likely to foster intense intra- and interspecies competition among the diverse strains and species present.

Iron is a necessary nutrient for most bacteria and is one of the major limiting resources for which microorganisms compete. This element is extremely abundant but biologically unavailable in oxygen rich conditions at neutral pH, and is even more limited in the context of pathogenesis, where host organisms often sequester iron to limit the growth of pathogens (Chipperfield and Ratledge 2000; Weinberg 2009). Bacteria have evolved a range of competitive behaviors that facilitate the acquisition of iron from the environment and competing organisms. Many bacteria produce small molecules called siderophores to scavenge iron from the environment (Krewulak and Vogel 2008). In addition to their own siderophores, bacteria are often able to use those produced by other organisms as iron sources (Andrews et al. 2003; Rodionov et al. 2006). These molecules can mediate intra- and interspecies competition depending on differing iron affinities and the ability of competing organisms to use the siderophores produced by one another (Carson et al. 2000; Joshi et al. 2006; Buckling et al. 2007). In addition to the indirect competition for iron involving siderophores, *Pseudomonas aeruginosa* has been shown to kill *Staphylococcus aureus* and use the released iron (Mashburn et al. 2005). In addition to nutritional resources, bacteria also compete with one another for favorable locations in the environment. There are several facets to this form of competition, with bacteria producing adhesins to increase their chances of attachment, surface components to block invading bacteria, and secreted compounds to kill or disperse the previous biofilm inhabitants (Horie et al. 2002; Rao et al. 2006; Kolodkin-Gal et al. 2010; Martínez-Gil et al. 2010).

The opportunistic pathogen *P. aeruginosa* is a ubiquitous soil and water dwelling organism that has been intensively studied in the context of its virulence and serves as a model organism for quorum sensing and biofilm formation. *P. aeruginosa* has also been used to study multi-species competitive behaviors and is capable of producing a wide range of secreted compounds that can potentially serve to mediate competitive interactions (An et al. submitted). These compounds can function in a variety of ways to kill or inhibit the growth of competing organisms [An et al. submitted and (Schuster et al. 2003)]. At least two different compounds secreted by *P. aeruginosa* have been shown to influence biofilm formation in a competitive context. The amphipathic rhamnolipids are responsible for modulating the structure and dispersal of *P. aeruginosa* biofilm as well as for dispersing biofilms formed by *Bordetella bronchiseptica* (Boles et al. 2005; Irie et al. 2005; Glick et al. 2010). The short chain fatty acid *cis*-2-decenoic acid (CDA), also produced by *P. aeruginosa*, has been shown to disperse biofilms formed by a variety of prokaryotes and the fungus *Candida albicans* (Davies and Marques 2009).

The α -proteobacterium *Agrobacterium tumefaciens* is a ubiquitous soil microorganism that is best known as the causative agent of crown-gall neoplasia on dicotyledonous plants via cross-kingdom horizontal gene transfer (Escobar and Dandekar 2003). *A. tumefaciens* can be isolated from many of the same environments from which *P. aeruginosa* can be isolated, and these bacteria have been used previously as a model for the examination of dual-species interactions in biofilms (An et al. 2006). In these assays, *P. aeruginosa* was shown to dominate the biofilms formed in both static and flowing conditions via a higher growth-rate and to further outcompete *A. tumefaciens* during stationary phase in a quorum sensing dependent fashion (An et al. 2006).

In this study, we examined the effects of compounds present in the conditioned culture fluids of *P. aeruginosa* on the biofilm formation of *A. tumefaciens*. In the work by An *et al.*, it was shown that a non-motile, aflagellate mutant of *A. tumefaciens* was able to produce slightly more adherent biomass in a continuous flow biofilm co-culture context (An *et al.* 2006). This observation led us to hypothesize that *P. aeruginosa* was producing a compound that stimulated *A. tumefaciens* to disperse from biofilms, and that the aflagellate mutant was unable to emigrate as efficiently, resulting in the increased biomass. We have found that *P. aeruginosa* produces a compound(s) that is capable of dispersing and inhibiting the formation of *A. tumefaciens* biofilms and that this activity is dramatically increased when *P. aeruginosa* is grown in iron limited conditions. We also show that the inhibition of *A. tumefaciens* biofilm formation is not due to iron sequestration, nor is it regulated by the *P. aeruginosa* quorum sensing systems or any of the other recognized mechanisms by which *P. aeruginosa* directly competes with other microbes.

MATERIAL AND METHODS

Bacterial strains, culture conditions and reagents

The strains used in this study are listed in Table 1. The *P. aeruginosa* strains were acquired from the strain collections of Matthew R. Parsek and E. Peter Greenberg. All media components and general reagents were purchased from Fisher Scientific (Pittsburgh, PA) and Sigma-Aldrich (St. Louis, MO). Both *A. tumefaciens* and *P. aeruginosa* strains were grown in *Agrobacterium tumefaciens* minimal medium with 0.5% (wt/vol) glucose and 15 mM ammonium sulfate as a nitrogen source (ATGN) (Tempe *et al.* 1977). The FeSO₄ called for in the original recipe was omitted for routine cultivation of bacteria with no effects on *A. tumefaciens* growth (Merritt *et al.* 2007). Synthetic *cis*-2-decenoic acid (CDA) was purchased from Carbosynth Limited, Berkshire, United Kingdom.

Preparation of *A. tumefaciens* and *P. aeruginosa* cell free culture fluids

Cultures of *P. aeruginosa* wild type and mutant strains and wild type *A. tumefaciens* were grown in ATGN with or without 22 μ M FeSO₄ at 28 °C with shaking to late stationary phase (for 72–96 hours). Bacterial cells were removed from cultures ranging from 5 ml to 500 ml by two centrifugations at 12,000 x g for 10 min followed by filtration through a 0.22 μ m filter (Pall Life Sciences, Port Washington, NY). Filtered culture fluid was stored at 4 °C until ready for use.

Growth and analysis of static biofilm formation assays

P. aeruginosa static culture biofilm and pellicle assays were performed as described previously (O'Toole and Kolter 1998; Friedman and Kolter 2004). Overnight cultures of *P. aeruginosa* were grown in ATGN with no added FeSO₄. These cultures were sub-cultured, to a final OD₆₀₀ of 0.05, into ATGN with or without 22 μ M FeSO₄ and with or without 50% (vol/vol) *P. aeruginosa* culture fluids resulting in four different static biofilm assay inocula. These inocula were added to 12-well polystyrene tissue culture plate wells with polyvinyl chloride (PVC) cover-slips placed upright in the wells for surface biofilm and pellicle formation. These cultures were incubated for 24–72 hours at room temperature. The surface adhered biofilm was visualized by crystal violet (CV) staining and the pellicle biofilms were photographed.

A. tumefaciens static culture microtiter plate biofilm assays were performed as previously described (Ramey *et al.* 2004). For the biofilm assays to which culture fluids were added, overnight cultures of *A. tumefaciens* were grown in ATGN without FeSO₄. Cultures were diluted to an OD₆₀₀ of 0.05 in ATGN containing 22 μ M FeSO₄ and varying concentrations of culture fluids. Concentrated ATGN was diluted with the appropriate amounts of culture

fluids and water to ensure that there was always at least a 1X concentration of nutrients in the initial biofilm inoculum. For the high iron biofilms, the final concentration of FeSO₄ was varied from 0–1100 μ M in AGTN amended with 20% (vol/vol) culture fluids, synthetic cDA preparations or unsupplemented. The inoculated cultures were incubated in PVC 96-well microtiter plates at room temperature for 48 h. The amount of planktonic growth in the wells was estimated by OD₆₀₀. The adherent biomass was stained for 10 min by the addition of 0.1% (wt/vol) CV for a final CV concentration of 0.014% (wt/vol). The stained biofilms were rinsed with distilled water and the stain was solubilized by the addition of 33% (vol/vol) acetic acid and quantified by reading the A₆₀₀. Spectrophotometric measurements of the biofilms were taken using a Bio-Tek Synergy HT microplate reader (Winooski, VT). Data are reported as the means and standard deviations of at least three technical replicates.

Physical and enzymatic treatment of the *P. aeruginosa* culture fluids

Aliquots of culture fluids were boiled or exposed to ultra violet radiation at 254 nm in a Germfree laminar airflow workstation (Ormond Beach, FL) for 60 min. Enzymatic treatment of culture fluids was performed as described previously (Berne et al. 2010). Aliquots of culture fluids were incubated with 5 μ g/ml of DNase, RNase, or Pronase beads overnight at room temperature. The RNase and DNase were inactivated by heating to 75 °C for 20 min. Pronase beads were removed by centrifugation at 18,000 x g for 1 min. The treated culture fluids were used in *A. tumefaciens* static culture biofilm assays as previously described to determine the impact of each treatment.

Dialysis of culture fluids and synthetic CDA

Aliquots of culture fluids (3 ml) and a suspension of synthetic CDA (52 mM) were placed in dialysis membrane cassettes (2000 Da and 7000 Da molecular weight cut off, MWCO; Slide-a-Lyzer, Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's instructions. The cassettes were then suspended in four liters of AT-N dialysis buffer (ATGN with the glucose omitted) overnight at room temperature with gentle agitation resulting in a 1333 fold dilution of the dialyzable molecules present in the culture fluids. The dialyzed suspensions were removed from dialysis cassettes and used in *A. tumefaciens* static culture biofilm assays to determine the remaining activity, compared to the same dilutions of undialyzed material incubated in parallel during dialysis.

Static biofilm dispersal assay

Static culture biofilms of *A. tumefaciens* were prepared as described above but incubated for only 24 h at room temperature. At 24 h post inoculation, culture fluids were added to a final concentration of 50% (vol/vol). An equal volume of ATGN was added to control wells. The OD₆₀₀ of the bacteria in the planktonic phase was measured and adherent biomass was stained by the addition of CV at 0, 5, 10, 15, and 60 min post addition. The CV was removed and the stained wells were rinsed two times with distilled water 10 min after the addition of CV. Immediately after rinsing, 33% (vol/vol) acetic acid was added and the wells were incubated for 10 min to solubilize the CV stained adherent biomass. The amount of solubilized CV was quantified by reading the A₆₀₀ as described above.

RESULTS

Pseudomonas produces a secreted inhibitor of *Agrobacterium tumefaciens* biofilm formation

A study from An *et al.* suggested that *P. aeruginosa* might produce a soluble inhibitor of *A. tumefaciens* biofilm formation (An et al. 2006). To test this, filtered cell-free culture fluids were prepared from stationary phase *P. aeruginosa* cultures (growth yields of OD₆₀₀

between 1.0 and 2.0). This culture fluid was then used as a supplement in *A. tumefaciens* biofilm formation assays. The presence of *P. aeruginosa* culture fluids resulted in a dramatic decrease in the crystal violet stained adherent biomass observed in these biofilm assays (Figure 1A). This decrease was observable with the addition of 1% (vol/vol) culture fluids and reached a maximum between 10 and 30% (vol/vol). At higher concentrations the addition of these culture fluids also inhibited the planktonic growth of *A. tumefaciens* (Figure 1B). The addition of culture fluids prepared identically from *A. tumefaciens* cultures had no effect on biofilm formation (Figure 1A and B).

The biofilm effect of the *P. aeruginosa* culture fluids was not due to nutrient depletion as the amendments were prepared in concentrated media and diluted with the addition of either culture fluids or water to provide a 1X concentration of ATGN in addition to the nutrients remaining in the *P. aeruginosa* conditioned cell-free culture fluids. In addition, the inhibition was not the result of pH changes as the non-inhibitory *A. tumefaciens* fluids and the inhibitory *P. aeruginosa* culture fluids were both pH 6.5 compared to 6.7 for un-inoculated ATGN.

Inhibitor production is regulated by iron availability, but does not act through iron sequestration

P. aeruginosa is an aggressive competitor for iron and iron levels have a significant impact on its multicellular behaviors (Banin et al. 2005; Mashburn et al. 2005). Iron levels also have a profound effect on *A. tumefaciens* biofilm formation (Hibbing and et al., manuscript in preparation). We therefore utilized a range of iron concentrations during growth of *P. aeruginosa* and tested the effect of this variable on production of the *A. tumefaciens* biofilm inhibitory activity. We observed that supplementing ATGN medium with FeSO_4 dramatically decreased production of the biofilm inhibitory activity (Figure 1A). The color of the culture fluids also changed from yellow in the unsupplemented culture to clear in the iron-replete culture, suggesting decreased production of the siderophore pyoverdine. In addition, after 72–96 h the growth yield of the *P. aeruginosa* culture with iron supplemented media increased to an OD_{600} of 2.0–5.0 (data not shown), approximately double that observed from the cultures grown in unsupplemented media.

The dramatic effect of iron levels on production of the biofilm inhibitory activity from *P. aeruginosa* cultures suggested that it might be due to one or more of the siderophores produced by *P. aeruginosa*, effectively limiting iron in the *A. tumefaciens* cultures and thereby inhibiting biofilm formation (Hibbing and Fuqua, manuscript in preparation). We tested the first of these hypotheses using culture fluids prepared from cultures of *P. aeruginosa* mutants that were unable to produce pyoverdine, pyochelin (the other major siderophore produced by strain PAO1), or both of the siderophores. None of these mutants were significantly abrogated for inhibitor production (Supplemental Table 1). To test whether iron sequestration by an alternate mechanism was causing the biofilm inhibition, we added additional FeSO_4 to *A. tumefaciens* biofilm assays with or without supplemented culture fluids prepared from iron-limited wild type *P. aeruginosa* cultures. Iron concentrations from 22 μM to 220 μM FeSO_4 did not diminish the effect on the biofilm inhibitory activity of the culture fluid. Iron concentrations greater than 220 μM inhibited biofilm formation and planktonic growth regardless of the presence of the *P. aeruginosa* culture fluids (Figure 1C and D).

Mutations in the global *P. aeruginosa* iron regulators *fur* and *pvdS* do not abolish iron-responsive control of its biofilm inhibitory activity

A common global iron-responsive regulator for many bacteria including *P. aeruginosa* is Fur, the ferric uptake repressor (Vasil and Ochsner 1999). The *P. aeruginosa fur* homolog is

believed to be essential but missense mutations in this gene have been isolated that exhibit constitutive pyoverdine production and partially desensitize *P. aeruginosa* biofilm formation to iron limitation (Barton et al. 1996; Banin et al. 2005). In contrast, the deletion of the extra cytoplasmic function (ECF) σ -factor *pvdS*, involved in the response to iron limitation, resulted in the loss of pyoverdine production and formation of structurally aberrant biofilms in flow cells under iron replete conditions (Banin et al. 2005).

We hypothesized that one or both of these mutations would disrupt iron-responsive control of biofilm inhibitor production. Culture fluids were prepared from *P. aeruginosa* *furC6Tc* (a missense mutation that decreases *fur* activity) and Δ *pvdS* strains grown in minimal medium with and without 22 μ M FeSO₄. The pattern of biofilm inhibitory activity for both mutants remained iron-responsive, as culture fluids from the *furC6Tc* strain or the Δ *pvdS* mutants exhibited high inhibitory activity when prepared from iron limited-cultures and decreased inhibitory activity when prepared from iron replete cultures (Figure 2A). Planktonic growth of *A. tumefaciens* was, however, more acutely inhibited by the culture fluids from the *furC6Tc* and Δ *pvdS* mutants than by wild type *P. aeruginosa* culture fluids (Figure 2B), suggesting that these regulators potentially influence additional competitive factors.

Pre-formed *A. tumefaciens* biofilms are rapidly dispersed by *P. aeruginosa* culture fluids

The inhibition of *A. tumefaciens* biofilm formation by *P. aeruginosa* culture fluids could be due to a block in surface adhesion, the inhibition of biofilm maturation, or the inhibition could be caused by accelerated biofilm dispersal. To examine the latter possibility, we measured the effect of *P. aeruginosa* culture fluids on pre-formed *A. tumefaciens* biofilms. The addition of *P. aeruginosa* culture fluids to 24 h *A. tumefaciens* microtiter biofilm plate wells resulted in a rapid and dramatic decrease in the adherent biomass (Figure 3A). However, the addition of *P. aeruginosa* culture fluids had no effect on the planktonic culture growth of *A. tumefaciens*, identical to addition of the ATGN growth medium alone (Figure 3B). A modest level of dispersal was observed with addition of the ATGN medium alone. These experiments suggest that the *P. aeruginosa* culture fluids are capable of stimulating the dispersal of *A. tumefaciens* biofilms.

Low iron conditions increase surface adherent biofilm formation of *P. aeruginosa* while decreasing planktonic growth and pellicle formation

There are two major biofilm conformations adopted by *P. aeruginosa* in static liquid culture, solid-surface associated biofilms and air-liquid interface-associated pellicles (Friedman and Kolter 2004). In the conditions used in this study, *P. aeruginosa* grown in static cultures for 24 hours with 22 μ M FeSO₄ produced thick pellicles with limited surface-attached biofilm and robust planktonic growth. In contrast those grown in static culture with no exogenous iron demonstrated less pellicle formation and planktonic growth, while forming dense surface-associated biofilms (Figure 4). After 48 hours, pellicle formation in the 22 μ M FeSO₄ conditions had dissipated and the small amount of surface adherent biomass had further decreased. The elevated amount of surface-adhered biomass in the iron-limited conditions was still present at 72 h but the pellicle was completely absent (Figure 4).

P. aeruginosa* static biofilm formation does not respond to the culture fluids which inhibit *A. tumefaciens

In order to test the effect of the inhibitory culture fluids on *P. aeruginosa* biofilm formation, static culture coverslip biofilms were cultivated in the presence or absence of 50% (vol/vol) culture fluids with or without added 22 μ M FeSO₄. *P. aeruginosa* exhibited increased biofilm formation and decreased planktonic growth under iron limitation, but the culture fluids had no apparent impact (Figure 4). The addition of 50% (vol/vol) of these same

culture fluids completely abolishes *A. tumefaciens* biofilm formation with or without additional FeSO₄ (Figure 1A and data not shown).

Inhibitor production is not regulated by the *P. aeruginosa* quorum sensing systems

The *P. aeruginosa* quorum sensing systems play a critical role in the regulation of its secreted virulence factors, antimicrobial agents, and anti-biofilm compounds. We therefore evaluated the possibility that the inhibitor might be regulated by quorum sensing as well. To test this hypothesis, culture fluids were prepared from five *P. aeruginosa* quorum sensing mutant strains, $\Delta lasI$, $\Delta rhII$, $\Delta lasI \Delta rhII$, $\Delta lasR \Delta rhIR$, and $pqsA::tet$. None of these mutants were found to be deficient for inhibition of *A. tumefaciens* biofilm formation or growth (Figure 5A and B).

Several recognized bioactive secreted compounds from *P. aeruginosa* are not responsible for biofilm inhibition

P. aeruginosa produces an array of secreted compounds with antimicrobial or anti-biofilm activity, including hydrogen cyanide, pyocyanin, and rhamnolipids. It seemed plausible that these compounds might be acting alone or in combination to inhibit biofilm formation by *A. tumefaciens*. To test this hypothesis, we prepared culture fluids from *P. aeruginosa* individual mutants that were unable to produce each of these compounds alone, as well as a triple mutant that could not produce any of these compounds. We found that inhibitor production was identical to wild type in all of the mutants tested (Supplemental Table 1).

The *P. aeruginosa* inhibitory activity is physically and enzymatically stable

The biofilm inhibitory activity was found to be fully resistant to boiling and exposure to UV radiation for one hour. The inhibitory activity was also completely stable over nearly a year stored at 4°C (Supplemental Table 2, data not shown). Exposure of the *P. aeruginosa* culture fluids to DNase, RNase, and Pronase enzymes for over 15 hours at room temperature did not significantly diminish the biofilm inhibitory activity, although these enzymes maintained their activity over this incubation period (Supplemental Table 2).

The inhibitory activity is predominantly due to a low molecular weight compound(s) that is distinct from CDA

To preliminarily establish a molecular size range for the inhibitory compound(s), the culture fluids were dialyzed using 2000 and 7000 Da pore size dialysis membranes. The majority of the biofilm inhibitory activity of the culture fluids was lost when dialyzed across a 7000 Da membrane, though a significant fraction of the inhibitory activity was retained when the fluids were dialyzed across a dialysis membrane with a 2000 Da cutoff (Figure 6A). In contrast, the ability of the culture fluids at high concentrations to inhibit the planktonic growth of *A. tumefaciens* was completely abrogated by dialysis across both the 7000 and the 2000 molecular size cutoff membranes (Figure 6B).

The small fatty acid *cis*-2-decenoic acid produced by *P. aeruginosa* has been previously reported to inhibit the formation and cause the dispersal of biofilms (Davies and Marques 2009). We tested the effects of CDA addition to *A. tumefaciens* biofilm assays. We found that the addition of 0.125 mM CDA was able to slightly inhibit biofilm formation and to a lesser extent planktonic growth of *A. tumefaciens* and that the addition of 0.5 mM CDA resulted in the highest degree of inhibition (Figure 7A and B). Unlike the biofilm inhibitory activity of the cell-free culture fluids, the activity of CDA was completely lost when this compound was dialyzed across a 2000 MWCO membrane (Figure 7A and B).

DISCUSSION

In this study, we provide evidence that *P. aeruginosa* produces a secreted inhibitor(s) of *A. tumefaciens* biofilm formation that functions predominantly or at least in part through stimulation of biofilm dispersal. Production of this inhibitor by *P. aeruginosa* is increased dramatically under iron-limited conditions. This increase occurs despite lower *P. aeruginosa* growth yields under iron limitation. Although it seemed reasonable that siderophores produced under iron limitation might inhibit *A. tumefaciens* biofilm formation through iron sequestration, the inhibition was not alleviated by high levels of exogenous iron. Nor was the inhibitory activity strongly regulated by the recognized *P. aeruginosa* iron-responsive regulatory systems, known quorum sensing systems, or mediated by known factors secreted by *P. aeruginosa*.

Competition for access to surfaces and for access to iron

The battery of secreted compounds produced by *P. aeruginosa* with biofilm dispersal activity suggests that, in its natural environment, *P. aeruginosa* likely competes for favorable surface environments with a diversity of other microorganisms (Irie et al. 2005; Bandara et al. 2010; Holcombe et al. 2010; Mowat et al. 2010; Pihl et al. 2010). Similarly, *P. aeruginosa* incurs the cost of producing a wide array of different siderophores to facilitate the cellular accumulation of the scarce amounts of iron to which it has access in aerobic environments. Such activity presumably provides a competitive advantage over other microorganisms in the same local environment (Poole and McKay 2003; Hibbing et al. 2010). These observations are consistent with fierce competition by *P. aeruginosa* for iron with *S. aureus*, and with *Burkholderia* spp. (Weaver and Kolter 2004; Mashburn et al. 2005).

Given the critical importance of iron to the metabolism of most organisms, it is not surprising that competitive behaviors are at least partially controlled by iron availability (Andrews et al. 2003). Indeed, while there is little work addressing the impact of iron on the regulation of antimicrobial factors, iron limitation is often used as a cue for virulence factor production, indicating that it can play a role in regulating antagonism between species (Ochsner et al. 2002). In this work, we have shown that iron limitation strongly promotes the release by *P. aeruginosa* of an inhibitor of *A. tumefaciens* biofilm formation. The addition of high concentrations of iron to *A. tumefaciens* biofilm assays in the presence of the active *P. aeruginosa* culture fluids had no effect on the inhibitory activity, suggesting that the active compound(s) does not act through iron sequestration. In addition to antagonizing the formation of *A. tumefaciens* biofilms when present throughout growth, we found that the addition of these culture fluids was able to disperse pre-formed biofilms. These data suggest that environmental iron levels play an indirect but potentially critical role in mediating the outcome of the competition between *P. aeruginosa* and *A. tumefaciens*, apparently by allowing *P. aeruginosa* to out compete *A. tumefaciens* for access to surfaces. Previous studies demonstrated that *P. aeruginosa* dominates biofilm formation in laboratory co-cultures with *A. tumefaciens* (An et al. 2006). The biofilm dispersal activity we have identified is likely to play an important part in this competitive superiority.

Iron limitation triggers a switch in the surface adherence and competitive behavior of *P. aeruginosa*

Prior studies suggest that iron plays an important role in the establishment, maturation, and dispersal of biofilms formed by *P. aeruginosa*. In flow cell biofilm conditions, the presence of iron was necessary for the formation of the large mushroom-like biofilms typically observed. Removal of iron by the addition of lactoferrin, a potent iron sequestering protein from mammals, resulted in the formation of relatively thin, flat biofilms (Singh et al. 2002; Singh 2004; Banin et al. 2005). Conversely, in static culture biofilms, the addition of

micromolar concentrations of ferric or ferrous iron salts resulted in the inhibition and dispersal of *P. aeruginosa* biofilms (Musk et al. 2005). We also observed that the addition of iron to static *P. aeruginosa* biofilm assays resulted in the formation of less dense surface adhered biofilms but an increase in the amount of pellicle formation and planktonic growth. These data suggest that in iron limited static conditions, adherence to a solid surface is a favored state for *P. aeruginosa* growth. It is of particular note that the iron limited conditions that favor *P. aeruginosa* surface adherence are also the conditions under which the production of the inhibitor of *A. tumefaciens* biofilm formation was highest. This observation suggests that *P. aeruginosa* can inhibit biofilm formation and clear existing biofilms of competing organisms to improve access to a surface under the same conditions in which adherence is a favored mode of *P. aeruginosa* growth.

Production of the biofilm inhibitory activity is not strongly regulated by iron-responsive regulators or quorum sensing

Like many species of bacteria, *P. aeruginosa* employs the Fur protein to regulate its transcriptional response to cellular iron levels (Vasil and Ochsner 1999). In *P. aeruginosa*, the *fur* gene appears to be essential; however, viable missense mutants with aberrant iron-responsive behaviors, such as PAO1 *furC6Tc*, have been isolated (Barton et al. 1996). *P. aeruginosa* also uses specific iron-responsive ECF σ -factors to control iron uptake, including *pvdS* which is responsible for controlling the production of the siderophore pyoverdine (Leoni et al. 2000). Surprisingly, deletion of *pvdS* and the *furC6Tc* missense mutation, both of which result in iron-insensitive flowcell biofilm phenotypes, had no appreciable effects on iron-stimulated production of the *A. tumefaciens* biofilm inhibitor(s). These data show that the iron-dependent regulation of inhibitor production is not controlled by the major iron-responsive regulators of *P. aeruginosa*.

P. aeruginosa uses a complex quorum sensing dependent regulatory system to control the expression of diverse biologically active compounds, and to mediate a stationary phase dominance phenotype over *A. tumefaciens* in co-culture [(An et al. 2006) and An *et al.* submitted)] These compounds can function alone or in combination to affect the physiology, behavior, and viability of competing organisms (An *et al.* submitted). We found that neither the quorum sensing-controlled effectors nor the quorum sensing systems themselves had any effect on the production of the inhibitory compound. This indicates that the previously observed dominance of *P. aeruginosa* in laboratory co-cultures with *A. tumefaciens* depends on a variety of diversely regulated *P. aeruginosa* factors including, but not exclusive to the dispersal promoting and biofilm inhibiting factor we report here.

The biofilm inhibitory factor is small, stable, and unlikely to be conferred by extracellular DNA, RNA, or proteins

To better understand the mechanism of biofilm inhibition and dispersal, and because of the potential importance and wide applicability of biofilm inhibitors, we attempted to examine the physical characteristics of the inhibitor. We showed that the inhibitory activity was stable over the course of extended storage, resistant to heat and UV-irradiation, and not degraded by nucleic acid or protein degrading enzymes suggesting that the inhibitory activity was the result of a bacterially produced small molecule. In addition, the complete loss of activity when the culture fluids were dialyzed across a 7000 MWCO membrane and partial loss of activity across a 2000 MWCO membrane suggest that the biofilm inhibitory activity may be the result of multiple small molecules, with molecular weights both greater and less than 2000 Da. However, none of the small molecules that have been shown to be produced by *P. aeruginosa* and to influence iron acquisition, biofilm formation or multi-species interactions appear to play a role in the observed inhibitory activity, with the

exception of CDA (Boles et al. 2005; Irie et al. 2005; Glick et al. 2010; Davies and Marques 2009; An et al. Submitted).

A novel agent capable of inhibiting and dispersing *A. tumefaciens* biofilms

A biofilm dispersing activity from *P. aeruginosa*, now identified to be CDA, was initially observed to trigger the auto-dispersal of biofilms formed by *P. aeruginosa* as well as a wide range of microorganisms including the yeast *Candida albicans* (Davies and Marques 2009). Thus, we were interested to determine if the *A. tumefaciens* biofilm-dispersing activity in iron-limited *P. aeruginosa* culture fluids would be able to disperse *P. aeruginosa* biofilms. We observed that culturing static *P. aeruginosa* biofilms in the presence or absence of 50% (vol/vol) culture fluids from iron limited cultures had no observable effect on the accumulation of surface adherent biomass. The only condition we examined that altered *P. aeruginosa* biofilm formation was the presence of added iron, which greatly reduced the accumulation of surface adherent biomass. In contrast, the biofilm formation of *A. tumefaciens* was essentially eliminated in the presence of 50% (vol/vol) culture fluids, and severely compromised at much lower amounts. A concentration of as little as 1 nM synthetic CDA was shown to be sufficient to induce the dispersal of *P. aeruginosa* biofilms and the concentration of CDA in spent medium was measured at 2.5 nM (Davies and Marques 2009). Our results, showing that over 100 μ M CDA is required for the inhibition of biofilm formation by *A. tumefaciens* and that the inhibitory activity of CDA was completely eliminated by dialysis, suggest that CDA is not the primary inhibitory compound in the cell-free culture fluids. In addition, there is no indication that CDA synthesis is stimulated under iron-limited conditions (D. Davies, personal communication). In fact, CDA is typically prepared from *P. aeruginosa* cultures grown in iron-replete media, whereas the primary activity that inhibits and disperses *A. tumefaciens* biofilms is strikingly elevated in iron-limited *P. aeruginosa* cultures (Davies and Marques 2009). Collectively, the lack of activity of the culture fluids against *P. aeruginosa* biofilms and the dialysis profile of the inhibitory activity suggest that we have identified a novel compound(s) produced by *P. aeruginosa*. The production of this compound is induced under iron limiting conditions, but the compound is distinct from siderophores, and is capable of efficiently inhibiting and disrupting *A. tumefaciens* biofilms.

Conclusions

Bacteria in the environment must compete with one another for scarce resources such as nutrients, including access to surface environments. We have found that batch cultures of *P. aeruginosa* grown in a defined medium produce a secreted factor(s) that is stimulated by iron-limitation, and inhibits *A. tumefaciens* biofilms. These same conditions also foster the formation of surface-adhered biofilms of *P. aeruginosa*. The biofilm inhibitory activity is clearly not the result of rhamnolipid production and appears to be distinct from CDA, thus likely representing a novel biofilm inhibitory activity. Future work will be directed towards identifying the chemical nature of the agent(s), its mode of action, and the regulatory basis for the iron-responsive production of the inhibitor.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The inhibitory activity of conditioned *P. aeruginosa* culture media on *A. tumefaciens* biofilm formation

Quantification of A) acetic acid solubilized CV biofilm stain, measured as A_{600} and B) the planktonic growth, measured by OD_{600} , prior to staining, from 48 h PVC 96-well microtiter plate *A. tumefaciens* C58 biofilms. Increasing concentrations of culture fluids that were prepared from *A. tumefaciens* low iron cultures (open triangle), and *P. aeruginosa* cultures from, iron replete (closed circle) and iron limited (open circle) conditions were added to the biofilm assays. Data are normalized relative to unsupplemented cultures. Quantification of C) the acetic acid solubilized CV, measured by A_{600} and D) the planktonic growth, measured by OD_{600} prior to staining, from 48 h PVC 96-well microtiter plate *A. tumefaciens* biofilms with increasing concentrations of $FeSO_4$, with (open circles) or without (closed circles) 20% (vol/vol) *P. aeruginosa* culture fluids prepared from iron-limited cultures. Data are normalized relative to the cultures with 22 μM $FeSO_4$ but with no added fluids. In all cases, values are the means and standard deviations from five wells per condition.

Figure 2. Mutants in the global iron regulators Fur and PvdS retain iron dependent inhibition of *A. tumefaciens* biofilms

Quantification of A) acetic acid solubilized CV biofilm stain, measured as A_{600} B) the planktonic growth, measured by OD_{600} prior to staining, from 48 h PVC 96-well plate *A. tumefaciens* biofilms. Increasing concentrations of culture fluids prepared from cultures of wild type *P. aeruginosa* (circles), the global iron-responsive regulatory gene missense mutant *furC6Tc* (squares) and the iron responsive ECF σ -factor mutant $\Delta pvdS$ (triangles) grown with (closed symbols) and without (open symbols) $22 \mu\text{M}$ FeSO_4 were added to the biofilm assays. Values are normalized relative to cultures with no added fluids and are the means and standard deviations from five wells per condition.

Figure 3. The addition of *P. aeruginosa* culture fluids causes the dispersal of preformed *A. tumefaciens* biofilms

Static culture 24 h biofilms of *A. tumefaciens*. At time 0, ATGN (closed circles) or *P. aeruginosa* culture fluids (open circles) were added to the cultures resulting in treatment of the preformed biofilms with 50% (vol/vol) culture fluids or ATGN. At each time point A) the wells were stained with CV, incubated 10 min, the stain was solubilized with acetic acid, and measured at A_{600} and B) the planktonic growth of the culture, OD_{600} prior to staining. Values are normalized relative to the value at time 0, are the means and standard deviations from four wells per time-point.

Figure 4. *P. aeruginosa* biofilm formation is not influenced by its own culture fluids but is impacted by iron levels

P. aeruginosa

static culture biofilms were grown in 12-well microtiter dishes with PCV coverslips sitting vertically in the wells. After incubating for 16 h, 40 h, and 64 h the coverslips were removed and the adherent biomass was stained with CV, and the OD₆₀₀ of the remaining planktonic culture was measured. The bacteria at the air-liquid interface were disrupted and photographed. The border between the pellicle suspended at the air-liquid interface (the lighter colored regions) and the planktonic bacteria (the darker colored regions) are marked with a white arrow. The images shown are representative of three coverslips. The OD₆₀₀ values presented are the mean and (standard deviation) from three wells.

Figure 5. Quorum sensing deficient mutants direct wild type levels of biofilm inhibition

A) acetic acid solubilized CV biofilm stain, measured as A_{600} and B) the planktonic growth, measured by OD_{600} prior to staining, from 48 h PVC 96-well plate *A. tumefaciens* biofilms. Increasing concentrations of culture fluids prepared from wild type (closed circles) and the five quorum sensing deficient mutants used in this study, $\Delta lasI$ (closed squares), $\Delta rhII$ (closed triangles), $pqsA::tet$ (open circles), $\Delta lasI \Delta rhII$ (open squares), and $\Delta lasR \Delta rhIR$ (open triangles), prepared from iron limited cultures were added to the biofilm assays. Values are normalized relative to cultures with no added fluids and are the means and standard deviations of five wells per condition.

Figure 6. Dialysis of *P. aeruginosa* culture fluids reveals a low molecular size for the active compound(s)

A) acetic acid solubilized CV biofilm stain, measured as A_{600} and B) the planktonic growth, measured by OD_{600} prior to staining, from 48 h PVC 96-well plate *A. tumefaciens* biofilms. Increasing concentrations of culture fluid that was either untreated (closed circles) or dialyzed across 2000 Da (open squares) or 7000 Da (closed squares) dialysis membranes into 4 liters of AT-N buffer were added to the biofilm assays. Values are normalized relative to cultures with no added fluids and are the means and standard deviations from five wells per condition.

Figure 7. CDA has a dialyzable biofilm inhibitory activity

A) acetic acid solubilized CV biofilm stain, measured as A_{600} and B) the planktonic growth, measured by OD_{600} prior to staining, from 48 h PVC 96-well plate *A. tumefaciens* biofilms. Increasing concentrations of CDA that was either untreated (closed circles) or dialyzed across 2000 Da (open squares) dialysis membrane into 4 liters of AT-N buffer were added to the biofilm assays. Values are normalized relative to cultures with no added CDA and are the means and standard deviations from three wells per condition.

Table 1**Bacterial strains**

Strain Name	Genotype	Reference
<i>A. tumefaciens</i> C58	Wild type	(Watson et al. 1975)
<i>P. aeruginosa</i> PAO1	Wild type	(Pesci et al. 1997)
<i>P. aeruginosa</i> PTL5032 + pMRP9- 1	<i>pchA</i> - + <i>gfp</i> (Carb)	U. of Washington (Jacobs et al. 2003) and (Kaneko et al. 2007)
<i>P. aeruginosa</i> $\Delta pvdA$ + pMRP9-1	$\Delta pvdA$ + <i>gfp</i> (Carb)	(Banin et al. 2005; Kaneko et al. 2007)
<i>P. aeruginosa</i> $\Delta pchEF \Delta pvdD$ + pMRO9-1	$\Delta pchEF \Delta pvdD$ + <i>gfp</i> (Carb)	Urs Ochsner, Replidyne Inc., Louisville, CO and (Kaneko et al. 2007)
<i>P. aeruginosa</i> <i>furC6Tc</i>	<i>furC6Tc</i>	(Barton et al. 1996; Ochsner et al. 1999)
<i>P. aeruginosa</i> $\Delta pvdS$	$\Delta pvdS$	(Ochsner and Vasil 1996)
<i>P. aeruginosa</i> $\Delta hcnB$	<i>hcnB::ISlacZ/hah Tet</i>	Dingding An, Unpublished
<i>P. aeruginosa</i> PTL47305	<i>phzA1::ISphoA/hah Tet</i>	U. of Washington (Jacobs et al. 2003)
<i>P. aeruginosa</i> $\Delta rhlAB$	$\Delta rhlAB$ <i>Gm</i>	(Shrout et al. 2006)
<i>P. aeruginosa</i> AHP4C-GFP	<i>hcnC::ISlacZ/hah phzA1::ISphoA/hah rhIB::hah miniTn7-gfp2- ωGm</i>	Colin Manoil (Jacobs et al. 2003)
<i>P. aeruginosa</i> PAO-JP1	$\Delta lasI$	(Pesci et al. 1997)
<i>P. aeruginosa</i> PDO100	$\Delta rhII$	(Pesci et al. 1997)
<i>P. aeruginosa</i> PAO-JP2	$\Delta lasI \Delta rhII$	(Pesci et al. 1997)
<i>P. aeruginosa</i> $\Delta lasRA \Delta rhIR$ -GFP	$\Delta lasRA \Delta rhIR$ mini Tn7-gfp3-Sm Ω Km	Michael Givskov
<i>P. aeruginosa</i> PTL17628	<i>pqsA::Tet</i>	U. of Washington (Jacobs et al. 2003)