

Iron Salts Perturb Biofilm Formation and Disrupt Existing Biofilms of *Pseudomonas aeruginosa*

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Summary

Bacterial biofilms are thought to aid in the survivability of a variety of intractable infections in humans. Specifically, biofilm production in *Pseudomonas aeruginosa* has been shown to play a significant role in chronic infection of cystic fibrosis (CF) patients. Unfortunately, no clinically effective inhibitors of biofilm formation are available. A rapid screen of 4509 compounds for nonantibiotic biofilm inhibitors in *Pseudomonas aeruginosa* PA14 was executed in 384-well plates. Among those compounds, ferric ammonium citrate inhibited biofilm formation in a dose-dependent manner; other iron salts functioned similarly. In addition to biofilm inhibition in static culture, pre-grown biofilms could be disrupted and cleared by switching to iron-rich media in flow-chamber experiments. Furthermore, *P. aeruginosa* strains taken from the sputum of 20 CF patients showed a similar response to elevated iron levels. Previous expression-profiling analyses demonstrated that high levels of iron repress the expression of genes whose products are essential for scavenging iron and that expression of these genes is critical for virulence. Our results, combined with existing transcriptional-profiling data, now indicate that elevated iron concentrations repress the expression of certain genes essential for biofilm production in *P. aeruginosa*.

Introduction

Pseudomonas aeruginosa is a gram-negative bacterium responsible for infections that are the most frequent cause of death in cystic fibrosis (CF) patients [1]. This pathogen possesses remarkable flexibility, flourishing both independently in highly varied environments and as a parasite in other organisms. The adaptability of *P. aeruginosa* stems in part from its propensity for forming hearty, surface-associated, biocide-resistant biofilms. *P. aeruginosa* is arguably the most studied biofilm-forming bacteria, and its virulence and persistence in infections of both CF patients and other immunocompromised individuals is known to be aided by biofilm production [2]. Highlighting the general importance of biofilms in human health, recent estimates indicate that biofilms account for over 80% of microbial infections in the human body [3]. Because biofilms show such recalcitrance toward antimicrobial treatment [4–8], there is considerable interest in finding novel

methods for elimination of biofilm-producing pathogens. If compounds existed that killed biofilm bacteria efficiently or disrupted existing biofilms, then biofilm-utilizing pathogens would become much more clinically manageable either by these new biofilm-tailored antimicrobials alone or by the use of biofilm disruptors in tandem with a traditional antibiotic.

Bacterial quorum sensing is known to be intimately linked with virulence and biofilm production. Analogues of N-acyl homoserine lactones, natural quorum-sensing compounds in gram-negative bacteria such as *P. aeruginosa*, have been found to inhibit biofilm production in *P. aeruginosa* [9–11]. However, similar molecules often agonize rather than ablate quorum sensing, and the potencies and stabilities of N-acyl homoserine lactones in aqueous conditions are nonoptimal [12]. Because there are few other examples of antibiofilm compounds [13], the discovery of biofilm-specific antimicrobials to contend with pathogenic, biofilm-forming bacterial species is of particular importance. Herein, we report the discovery that iron salts are efficient inhibitors of biofilm formation in *P. aeruginosa*, disrupt pre-formed biofilms, and prevent biofilm formation in *P. aeruginosa* strains taken from the sputum of CF patients. This data serves to bolster a growing base of literature linking iron availability with virulence-factor production and biofilm formation in *P. aeruginosa*.

Results and Discussion

A microtiter plate-based assay was used to screen a collection of small molecules (see <http://scs.uiuc.edu/~phgroup/comcollections.html> for compound identities) for those that would specifically prevent or disrupt biofilm formation in *P. aeruginosa*. Known protocols for growing biofilms in 96-well plates [4, 10, 14–17] were adapted and optimized for *P. aeruginosa* PA14 in a 384-well plate format. In primary screening, *P. aeruginosa* cells were grown in 384-well plates in the presence of a functionally diverse collection of 4509 compounds. After 48 hr growth time, plates were rinsed to remove spent media, compounds, and planktonic cells, leaving only biofilm bacteria. The remaining biofilms were stained with 0.1% crystal violet stain and rinsed to remove unbound crystal violet dye. The biofilms of *P. aeruginosa* manifest themselves as rings formed at the air-liquid interface of wells in the microtiter plate. As adsorbed dye correlates directly to biofilm density, desorption of the adhered stain in each well into acidic solution allowed facile quantitation of biofilm in each well in a microplate reader at 590 nm.

Each compound in the library was tested in triplicate at 50 μ M final concentrations, and compounds that exhibited at least 30% reduction in biofilm production without inhibiting bacterial growth were classified as “hits” in the primary assay. Control wells grown in the absence of compounds typically displayed <6% relative standard deviation from well to well. Six compounds (0.13% hit rate) were identified through this pri-

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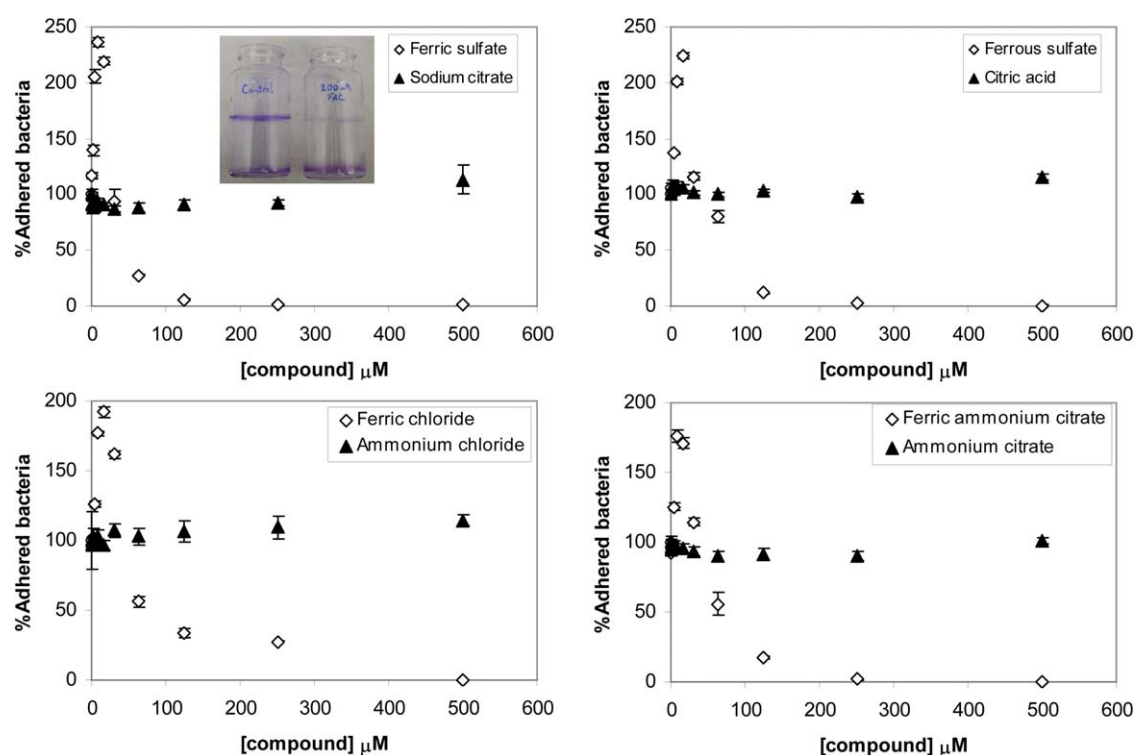


Figure 1. Dose Dependence of Iron Salts, Citrates, and Ammonium on 48 hr *P. aeruginosa* PA14 Biofilms

The amount of biofilm remaining was determined by the absorbance at 590 nm of the crystal violet dye. This dye is released into acidic solution after plates are washed to remove planktonic cells; biofilm cells remain adherent and are stained. All readings are corrected to reflect 0% and 100% controls (blank well, 0%; biofilm growth without compounds present, 100%). Error bars represent the standard deviation from the mean. Inset: static 48 hr PA14 cultures in glass at 0 and 200 μM FAC also show that biofilm fails to form at high iron.

mary screen that retarded biofilm production and gave no growth inhibition by OD_{600} . No compounds were found to enhance biofilm production. These six compounds were retested for dose dependence with the same microtiter-plate adherence assay, this time in a 96-well format. From this testing, ferric ammonium citrate (FAC) was found to provide reproducible and dose-dependent inhibition of *P. aeruginosa* biofilm formation.

We were intrigued to find, in a library containing an array of drug-like molecules, that a simple ionic compound showed moderate potency ($\text{IC}_{50} \approx 60 \mu\text{M}$) in suppressing biofilm production. Testing ammonium chloride, citric acid, ammonium citrate, and sodium citrate in dose-dependence assays showed that neither ammonium nor citrate ion were responsible for the inhibition. Conversely, when iron salts (ferric chloride, ferric sulfate, ferrous sulfate) were examined, their effects mirrored that of FAC (Figure 1). FAC was also found to exert its antibiofilm effects on glass surfaces (Figure 1, inset). Growth rates of *P. aeruginosa* PA14 were not significantly affected over the range of 0–500 μM iron (Figure 2), and control 48 hr static cultures in polystyrene tubes at low and high iron show high and low CFUs, respectively, of biofilm bacteria with plate counts (data not shown), indicating that iron does not simply interfere with CV staining of present biofilms. Based on this evidence, we determined that iron itself was the source of the inhibition of biofilm production. Con-

sidering that iron starvation (by lactoferrin chelation) had previously been found to inhibit biofilm production in *P. aeruginosa* [18], we sought to corroborate this result with additional demonstrations of excess iron inhibiting *P. aeruginosa* biofilm production.

A marker signifying biofilm production in *P. aeruginosa* is the development of resistance to killing by antibiotics [18]. Static cultures of *P. aeruginosa* were grown in 10 ml polystyrene culture tubes at varying concentrations of FAC for 48 hr before treating with 60 $\mu\text{g/ml}$ of the antibiotic tobramycin for 5 hr. This concentration of antibiotic would destroy planktonic bacteria but not those that had formed biofilms [18]. At the end of the 5 hr tobramycin treatment, the tubes were shaken and vortexed to homogenize biofilms and planktonic bacteria and then quickly diluted and plated onto fresh tryptic soy agar (TSA) plates to assess survival of the cells in each tube. Increased sensitivity to tobramycin was displayed as FAC concentration increased (Figure 3), substantiating that FAC prevented biofilm formation in a dose-dependent manner. Control experiments in the absence of tobramycin showed no inhibition of bacterial growth from 0–500 μM FAC. No synergy was detected between FAC and tobramycin; MIC tests of tobramycin on planktonic *P. aeruginosa* PA14 at 0 and 500 μM FAC result in the same value of 1.2 $\mu\text{g/ml}$.

Another common method for cultivating and examining biofilms is the use of a flow-chamber device. We

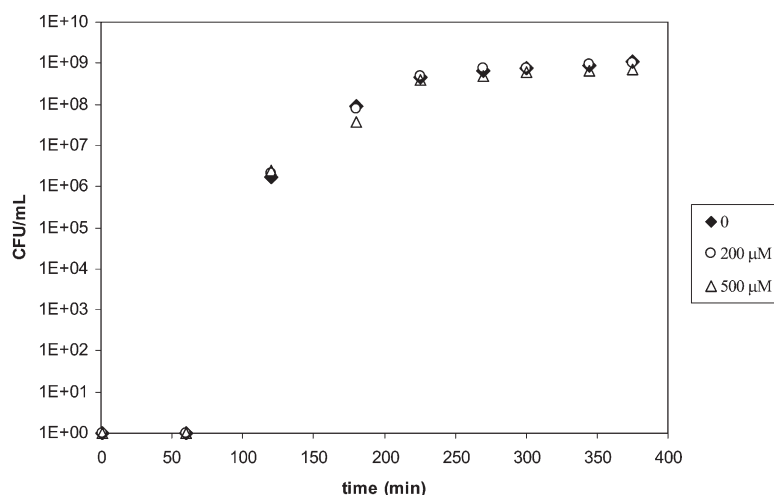


Figure 2. Growth Rate of *P. aeruginosa* PA14 in Varying Concentrations of Ferric Ammonium Citrate

A culture of PA14 at $OD_{600} = 0.5$ was diluted 1:100 into T-broth media containing varying iron. Cultures were shaken, and aliquots were taken and measured at the time points indicated. At 0 and 60 min, no significant difference from media blanks was observed. Through CFU plate counts, we determined that 3.5×10^8 CFU/ml corresponds to $OD_{600} \approx 1$.

used a commercially available flow-channel device in which biofilm grows on a microscope cover slip. Seeded into the flow chamber and allowed to grow for 5 days in 50% T-broth media under conditions of low flow (4 ml/h), *P. aeruginosa* PA14 formed robust biofilms that were easily visualized by rinsing, heat fixing, and staining with crystal violet after disassembly of the flow chamber (Figure 4A). 10-day biofilms grown in like manner were similarly robust and easily visualized by fixing and staining (Figure 4B). Conversely, attempts to cultivate biofilms in iron-rich media (50% T-broth + 200 μ M FAC) produced no biofilm growth (Figure 4C). Furthermore, biofilms that were pregrown for 5 days without iron supplementation to the media and then subjected to 200 μ M FAC in 50% T-broth for 5 additional days were completely cleared (Figure 4D). This experiment suggested that disruption of pre-existing biofilm by FAC had occurred.

P. aeruginosa PA14 was transformed with *lac*-controlled eGFP plasmid pMRP-9 in order to observe formation and subsequent disruption of biofilm in the flow

chamber in a single unequivocal experiment [18]. When grown in 50% T-broth containing 300 μ g/ml carbenicillin for plasmid maintenance and 1 mM IPTG to ensure eGFP expression, the growth and disruption of biofilm could be monitored during flow by fluorescence microscopy. Fluorescence images confirm that at 5 days (before addition of FAC), biofilm growth has proceeded readily, with confluent cell growth evident (Figure 5A). After 5 days, the chamber was fed with media containing carbenicillin, IPTG, and 200 μ M FAC. At 8 days, it was unambiguous that disruption of the biofilm had occurred (Figure 5B), and by day ten, the biofilm was completely vanquished (Figure 5C).

Distinct clinical isolates of the organism from the sputum of 20 CF patients at a local hospital were obtained and tested for dose-dependent response to iron in biofilm production to demonstrate iron's ability to inhibit biofilm formation across multiple strains of *P. aeruginosa*. Seventeen of these strains produced significant biofilm in the microtiter plate test in 96-well plates, and dose-dependent inhibition of biofilm formation was

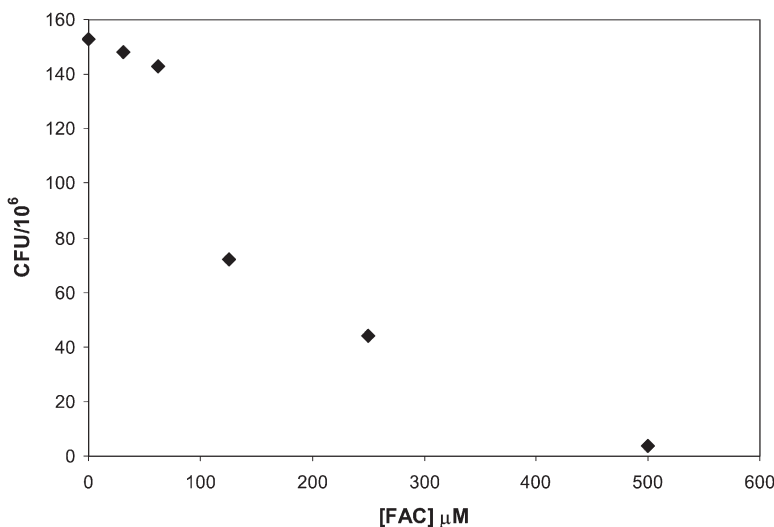


Figure 3. The Effect of Ferric Ammonium Citrate on Susceptibility of Static Cultures of *P. aeruginosa* PA14 to Tobramycin

Cells were grown for 48 hr in a range of concentrations of ferric ammonium citrate (FAC) in 10 ml polystyrene culture vials without shaking. After 48 hr, tobramycin was added to 60 μ g/ml final concentration in each vial and allowed to incubate with the cells (without shaking) for 5 hr. At the end of this incubation, the tubes were shaken and vortexed for 1 min to loosen adherent cells, and an aliquot was quickly diluted with fresh T-broth to 1:10,000–1:40,000. 10 μ l of these diluted cultures were plated onto TSA (tryptic soy agar) plates, and colonies were counted. FAC itself (0–500 μ M) showed no inhibition of bacterial growth. Controls in absence of tobramycin treatment showed $\approx 200 \times 10^6$ CFU/ml from 0–500 μ M FAC (data not shown), indicating that about 75% of the biofilm bacteria survive the tobramycin treatment.

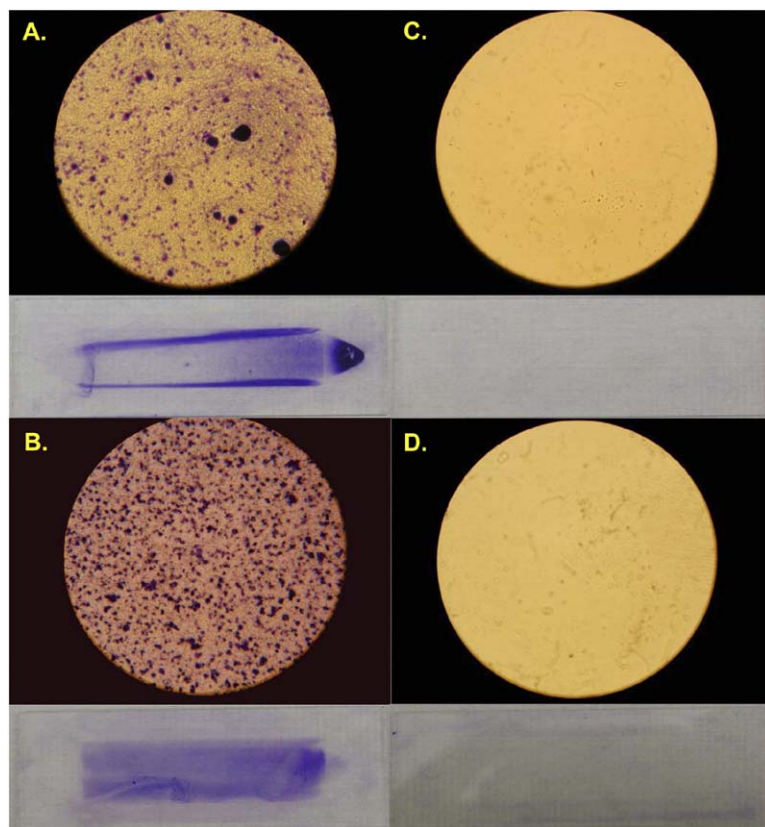


Figure 4. Macro- and Microscopic Pictures of Standard Microscope Slide Used in Flow Cell at Various Time Points

In all cases, the flow cell was disassembled after the indicated time, and the slide was stained with 0.1% crystal violet. (A) 5-day biofilm growth control. A biofilm of *P. aeruginosa* PA14 was grown in flow chamber with 50% T-broth growth media for 5 days. (B) 10-day growth control. A biofilm of *P. aeruginosa* PA14 was grown in flow chamber with 50% T-broth growth media for 10 days. (C) Ferric ammonium citrate (FAC) prevents the formation of biofilms. The flow cell was inoculated with PA14 and maintained (4 ml/hr) for 5 days in 50% T-broth + 200 μ M FAC. (D) FAC causes the disruption of existing biofilms. PA14 biofilm was grown for 5 days in 50% T-broth, and then the media feeding the cell was switched to 50% T-broth + 200 μ M FAC and grown an additional 5 days. All microscopic pictures are 400 \times magnification.

clearly evident in a vast majority of these strains (data not shown). 14 of the 17 strains (82%) displayed >40% inhibition of biofilm production by 250 μ M FAC, and 7 out of 17 (41%) revealed >80% inhibition (Figure 6). As indicated in the figure legend for Figure 6, many of these isolates were mucoid, and all isolates showed similar doubling times.

P. aeruginosa PA14 biofilm inhibition was monitored

as a function of time at low (0 iron added) and high (250 μ M iron added) concentrations of FAC to further understand how iron might be affecting biofilm formation (Figure 7A). This assay was performed identically to the secondary screen for dose dependence except that plates were examined at varying times from 0 to 48 hr. This data shows that initial attachment is not impaired because biofilms at 0 and 250 μ M iron are virtu-

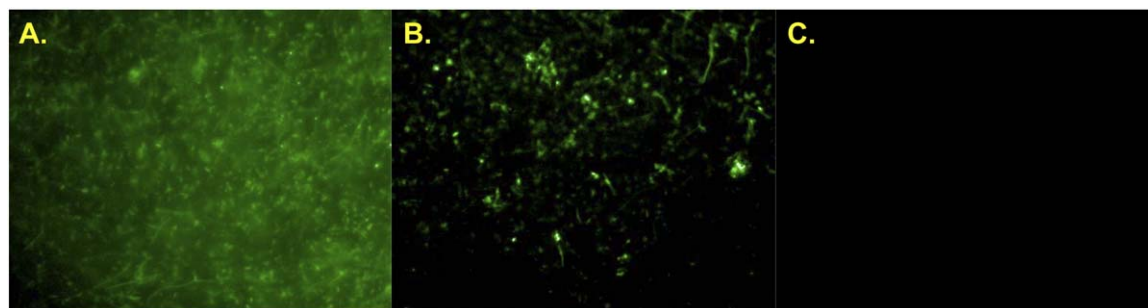


Figure 5. Pregrown Biofilms Are Disrupted by Ferric Ammonium Citrate

(A) *P. aeruginosa* PA14 harboring the plasmid pMRP-9 was grown for 5 days in the flow-cell chamber. The biofilm formed under these conditions (50% T-broth, 300 μ g/ml carbenicillin for plasmid maintenance, 1 mM IPTG for eGFP induction, flow rate = 4 ml/hr) was visualized by fluorescence microscopy.

(B) At this point, 200 μ M ferric ammonium citrate (FAC) was added to the media, and flow was continued through the chamber for 3 more days. Partial disruption of the biofilm at this day eight time point was visualized by fluorescence microscopy.

(C) Complete disruption of the PA14 biofilm was observed at day ten. Subsequent flow-cell disassembly and staining of the microscope slide (with crystal violet) after this day-ten time point confirmed the lack of biofilm (not shown). All pictures are 400 \times magnification and are representative of several locations in the center of the flow channel.

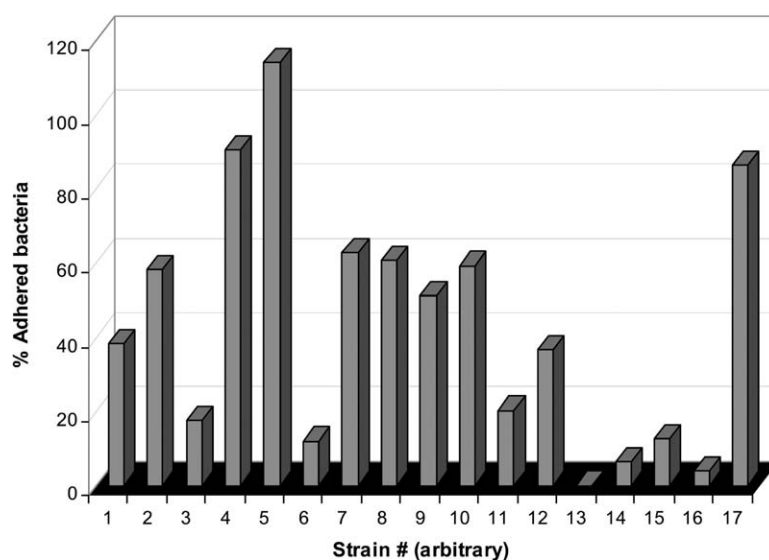


Figure 6. Effects of 250 μ M Ferric Ammonium Citrate on Clinical Isolates of *P. aeruginosa*

Ferric ammonium citrate's (FAC) effects were measured for 20 *P. aeruginosa* samples isolated from the sputum of CF patients at Carle Foundation Hospital (Urbana, IL). The bacteria were grown for 48 hr in T-broth + 250 μ M FAC. The amount of biofilm formed was graphed relative to controls where no FAC had been added. Three of the 20 isolates produced no biofilm in the assay. Among the biofilm-producing strains, isolates 1, 2, 3, 4, 5, 6, 10, 12, 15, and 16 were determined to be mucoid by visual inspection on TSA and blood agar plates. Each strain had a doubling time between 15 and 60 min, and all grew to stationary phase within 24 hr in shaken cultures at 37°C (data not shown).

ally identical at 10 hr. After initial biofilm formation, however, iron powerfully disrupts the process of biofilm formation; biofilms at 48 hr in 250 μ M iron are almost completely vanquished (as seen in earlier dose-depen-

dence experiments monitored after 48 hr biofilm growth). Also, twitching motility has been shown to be vital to the process of biofilm formation in *P. aeruginosa* [19]. Twitching motility was assessed in varying iron concentrations as previously performed [20]. Stab cultures on TSA plates with 1% agar showed impaired twitching at elevated iron concentrations at 24 hr (Figure 7B).

This study gives ample evidence supporting the importance of iron as it relates to biofilm formation in *P. aeruginosa*. Singh and coworkers showed that in the virtual absence of iron (because of chelation by lactoferrin), *Pseudomonas* cannot form biofilms [18]. Indeed, lactoferrin also inhibited biofilm formation in our static culture dose-dependence assays (data not shown), presumably by starving the bacteria of iron required for biofilm formation. Our findings have revealed that excess iron has the same biofilm-inhibiting effect, indicating that biofilm formation in *P. aeruginosa* is optimal over a limited range of iron concentrations (from \approx 1–100 μ M), above and below which the organism can exist only in a planktonic state.

The effectiveness of FAC in inhibiting biofilm growth in a majority of these clinical isolates of *P. aeruginosa* naturally arouses curiosity about a possible role for iron in the treatment of CF. In most cases, the use of iron to improve the course of bacterial infections has been discouraged because lack of iron is often a growth-limiting factor for bacterial growth in vivo [21]. Furthermore, a mutant of *P. aeruginosa* PAO1 deficient in producing the iron-chelating siderophore pyoverdine grew poorly in an immunosuppressed mouse infection model, demonstrating the importance of iron acquisition to functional pathogenesis [22]. These observations affirm the absolute requirement of iron for the growth of most bacterial species. However, a direct and provocative correlation is seen between iron deficiency in CF patients and severity of lung infection by *P. aeruginosa* [23], suggesting that a simplistic view equating iron availability with bacterial pathogenesis belies a more complex truth. In fact, in vitro growth experiments approximating conditions of the human lung showed

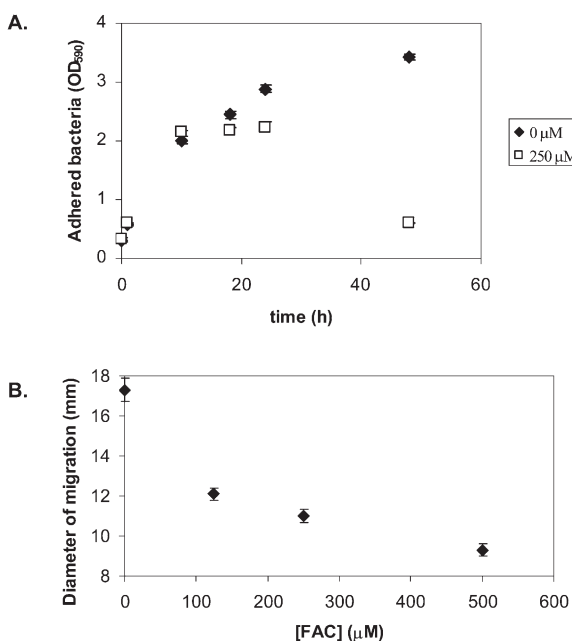


Figure 7. Mechanistic Implications of Iron Effects on Biofilm Production

(A) Time course of *P. aeruginosa* PA14 biofilm production in microtiter plates was monitored at 0 and 250 μ M iron in T-broth media. At 18 and 24 hr time points, biofilm production is stalled in 250 μ M iron compared to 0 μ M, and by 48 hr, disruption is nearly quantitative.

(B) Twitching motility of *P. aeruginosa* PA14 in varying iron concentrations. TSA plates with 1% agar were used to assess twitching motility. As the concentration of FAC increases, twitching motility decreases significantly. Error bars on both graphs represent one standard deviation from the mean.

higher *P. aeruginosa* growth under iron-limited than high-iron conditions [24]. Given that iron disrupts biofilms in vitro in a large percentage of clinical isolates of *P. aeruginosa* and that precedent exists for high concentrations of medication to be used through direct introduction to the CF lung (many CF patients inhale doses of tobramycin that would be acutely toxic if administered systemically), further investigations of the feasibility of nebulized iron treatment in the CF lung are warranted. There is some precedent for the use of metals for the treatment of specific disorders (i.e., lithium treatment for bipolar disorder), and, thus, a fair body of information about delivery strategies for metals is available [25].

Not surprisingly, two transcriptional profiling studies indicate that iron limitation induces the expression of many genes involved in iron acquisition [26, 27]: high iron concentrations (100–200 μ M) repress a number of genes involved in iron acquisition. Many of these genes are involved in the biosynthesis of two key siderophores, pyochelin and pyoverdine, that are implicated in scavenging iron, including the gene controlling pyoverdine biosynthesis, *pvdS*.

Interestingly, in *P. aeruginosa*, there is a direct correlation between the expression of iron acquisition genes and virulence-factor production and pathogenesis. For example, in addition to its role in scavenging iron, secreted pyoverdine signals for the production of virulence factors that are critical to the ability of this pathogen to cause disease [28]. Transcription of *pvdS* is directly regulated by iron levels; thus, the PvdS protein is only synthesized (and, hence, pyoverdine is only produced) under conditions of iron starvation [26, 28]. Although not without controversy, evidence indicates that low levels of free iron in the lung are important for *P. aeruginosa* virulence in CF [23, 24, 29–31].

Among the most important virulence factors in *P. aeruginosa*, and the one actively investigated in this manuscript, is the formation of biofilms [32]. Biofilm formation is a complex process of differentiation in which many changes in gene expression occur [5, 6]. Many of these changes have been shown to be mediated through quorum sensing, a positive-feedback process in which secreted signaling molecules called AHLs (n-Acyl Homoserine Lactones) are monitored by the bacteria as a sensitive measure of their localized population density [33–35]. Specifically, *P. aeruginosa* uses 3-oxo-dodecanoyl homoserine lactone and butyl homoserine lactone as its primary and secondary quorum-sensing signals. At sufficient population density (and corresponding AHL concentrations), the quorum sensing system directs *P. aeruginosa* to form biofilms.

Intriguingly, the quorum sensing system itself is subject to some degree of regulation by iron concentration. Gene microarray studies have shown that the mRNA of *MvfR*, a protein required for synthesis of adequate levels of 3-oxo-dodecanoyl homoserine lactone (the primary AHL signal in *P. aeruginosa*) is repressed considerably in iron-replete compared to iron-limited growth conditions [26]. Thus, *mvrR* can function as an iron-sensitive trigger to modulate quorum sensing in *P. aeruginosa* and, by so doing, can inhibit production of bacterial biofilms. Of course, genetic control of biofilm formation, virulence, and iron acquisition are com-

plex, interlinked, and poorly characterized. Other iron-regulated mechanisms [36, 37] could well be important to biofilm formation in *P. aeruginosa*.

Significance

Biofilms play an important role in most bacterial infections of the human body. Particularly, *Pseudomonas aeruginosa* uses biofilms to evade immune defenses and exert its pathogenesis in cystic fibrosis (CF) patients. Precious few compounds have been developed that target biofilm bacteria, and these compounds have potency and stability problems that will likely preclude any eventual clinical application. A facile screen to test putative inhibitors of biofilm formation in *P. aeruginosa* PA14 has been developed and used to screen a collection of 4509 diverse compounds in search of potent modulators of biofilm formation. It was determined that ferric ammonium citrate (FAC) inhibited biofilm formation in a dose-dependent fashion and disrupted previously formed biofilms in flow-channel experiments. Through additional testing on strains of *Pseudomonas aeruginosa* from CF patients, iron's effects were found to be general: 14 of 17 strains that formed significant biofilm also displayed a dose-dependent decrease of biofilm production in response to FAC. These results add to the mounting evidence indicating that iron levels dictate virulence of *P. aeruginosa*: extreme iron starvation (through total chelation of iron) inhibits biofilm production, moderate iron concentrations allow for biofilm production and are critical for virulence, and now we show that high concentrations of iron powerfully inhibit biofilm production, likely through the repression of various genes involved in iron scavenging and quorum sensing.

Experimental Procedures

Bacterial Strains and Plasmids

P. aeruginosa PA14 was a kind gift of Professor Roberto Kolter, Harvard Medical School. Plasmid pMRP-9, which carries carbenicillin resistance and *lac*-inducible eGFP expression, was a kind gift of Professor E.P. Greenberg, Department of Microbiology at the University of Iowa. The 20 *P. aeruginosa* clinical strains were collected from the sputum of 20 CF patients at Carle Foundation Hospital in Urbana, Illinois. Each strain was subcultured twice on tryptic soy agar (TSA) plates and stored as a glycerol stock. The strains were given the arbitrary designations C11–C120.

384-Well Plate Screen for Biofilm Inhibition

The 4509 compounds screened were collected from a variety of sources within the Department of Chemistry at the University of Illinois; the full list of compounds with structures can be viewed at <http://scs.uiuc.edu/~phgroup/comcollections.html>. All compounds were arrayed in 384-well plates as 10 mM stock solutions in DMSO. *P. aeruginosa* PA14 was grown in T-broth (10 g tryptone, 5 g NaCl per liter) to a OD_{600} of 0.8 and diluted by a factor of 40 with fresh T-broth media. 100 μ l of this diluted bacterial suspension was placed in each well of the first 23 columns of a 384-well plate, leaving the final column for control wells. Half of the control wells were filled with the bacterial suspension as positive controls with no compound treatment, and the other eight were left empty as a negative control for the staining procedure. Using a 384-well pin transfer device (V & P Scientific, San Diego CA), we transferred ~ 0.25 μ l of the compounds from the compound plate into the wells of dilute bacterial suspension (the 24th column in the compound

plates was left without compounds to correspond to the experimental control wells). Compounds were pin transferred into each plate two times to reach 50 μ M concentration. The library was screened in triplicate by this procedure.

Plates were incubated in a humidified chamber at 37°C for 48 hr and then examined via a multiwell plate reader at OD₆₀₀. Wells that showed no turbidity were classified as antibiotics and dismissed from further analysis as biofilm disruptors. After noting all antibiotics, the plates were gently rinsed under lukewarm tap water to remove planktonic and loosely adherent organisms. The plates were then shaken dry and each well of each plate stained with 110 μ l of a 0.1% crystal violet solution in water. After allowing the stain to adhere to the biofilms for 5 min, each plate was again rinsed gently under lukewarm water until no more stain could be rinsed from the plate. Each plate was again shaken dry, inverted, and allowed to dry thoroughly for 15 min. Finally, 120 μ l of a 30% acetic acid solution was pipetted into each well to desorb the adhered stain back into solution. After allowing 30 min for the adhered stain to dissolve into the destaining solution, the biofilm in each well was quantified by reading the absorbance of the plates at 590 nm. A similar procedure, scaled up, was used to test the glass vials as substrates for biofilm inhibition, with the same dilute bacterial suspension being utilized.

Dose-Dependent Effect of Iron Salts on *P. aeruginosa*

A similar method was used to test dose dependence of iron salts and controls in 96-well plates. Bacteria were grown and diluted as above, and 150 μ l dilute bacterial suspension was placed in each well of a 96-well plate (except the final column, again reserved for positive and negative controls). Compounds were added to the wells via multichannel pipette as serial dilutions. The bacteria were grown for 48 hr and analyzed similarly, with 160 μ l CV stain per well and 180 μ l 30% acetic acid per well for the destaining/quantitation step. Strains of *P. aeruginosa* isolated from the sputum of CF patients were analyzed in an identical fashion.

Susceptibility of *P. aeruginosa* to Tobramycin in Varying [FAC]

P. aeruginosa PA14 were grown to OD₆₀₀ = 0.8 and diluted 1:40 into 10 ml plastic tissue culture vials, 2 ml per vial. FAC was added to each dilute culture to the desired concentration. The cultures were grown for 48 hr at 37°C without shaking. After 48 hr, tobramycin was added to 60 μ g/ml total concentration in each vial, and each was left undisturbed for 5 hr. After 5 hr, vials were vortexed and shaken to homogenize cultures and aliquots quickly diluted and plated onto TSB agar plates to count remaining viable bacterial colony forming units (CFU).

Flow-Cell Experiments

P. aeruginosa PA14 was grown overnight to stationary phase (OD₆₀₀ = 1–3) in T-broth media to visualize the effects of FAC on flow-cell biofilms. About 5 ml of this culture was used to fill the flow cell (BioSurface Technologies Corporation, FC81 flow cell with Fisherbrand variable-flow peristaltic pump) via sterile syringe, and the culture was left in the cell without flow for 90 min. At this time, 50% T-broth media was flowed through the chamber at 4 ml/hr. After 5 days (Figure 4A) or 10 days (Figure 4B), the flow cell was disassembled and the standard microscope slide that forms one of the walls of the flow chamber was rinsed gently but thoroughly under lukewarm tap water, heat fixed, and stained for 1 min with 0.1% crystal violet stain. Excess stain was rinsed away, and the slide was shaken dry before light microscopy and photographs were recorded. The same protocol was employed for experiments utilizing 50% T-broth, 200 μ M FAC (Figure 4C). For the biofilm disruption experiment (Figure 4D), the media was changed after 5 days to 50% T-broth, 200 μ M FAC. After 5 more days, the flow cell was disassembled, and the slide was stained as above.

P. aeruginosa PA14 was transformed with pMRP-9 to directly visualize the formation and disruption of biofilms within the flow-cell chamber. PA14-pMRP-9 were grown overnight to stationary phase (OD₆₀₀ = 1–3) in T-broth media supplemented with 300 μ g/ml carbenicillin. Approximately 5 ml of this culture was used to fill the flow cell via sterile syringe, and the culture was left in the cell without flow for 1.5 hr. At this time, media flow (50% T-broth, 300 μ g/

ml carbenicillin, 1 mM IPTG) was begun through the chamber at a rate of 4 ml/hr. As described in the text and legend for Figure 5, after 5 days, the chamber was examined microscopically with an Axiovert model fluorescence microscope. At this point, the media was changed to 50% T-broth, 300 μ g/ml carbenicillin, 1 mM IPTG, and 200 μ M FAC, the flow was continued, and the cell was monitored by fluorescence microscopy after 3 and 5 more days.

Acknowledgments

We wish to thank Professor Roberto Kolter (Harvard Medical School) for supplying the *P. aeruginosa* PA14 strain, Carle Foundation Hospital (Urbana, IL) for the gift of the clinical isolates of *P. aeruginosa*, and Professor E.P. Greenberg (University of Iowa) for the gift of the pMRP-9 plasmid DNA. This work is supported by the Science and Technology Center Program of the National Science Foundation under Agreement Number CTS-0120978.

Received: February 19, 2005

Revised: April 29, 2005

Accepted: May 6, 2005

Published: July 22, 2005

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