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Modulation of *Pseudomonas aeruginosa* surface-associated group behaviors by individual amino acids through c-di-GMP signaling

Steve P. Bernier^a, Dae-Gon Ha^a, Wajiha Khan^{a,b}, Judith H. Merritt^{a,1}, and George A. O'Toole^a

Steve P. Bernier: sbernier19@gmail.com; Dae-Gon Ha: Dae.Gon.Ha@dartmouth.edu; Wajiha Khan: wajiha_microbiologist@yahoo.com; Judith H. Merritt: judith.merritt@glycobia.com; George A. O'Toole: georgeo@dartmouth.edu

^aDepartment of Microbiology & Immunology, Dartmouth Medical School, North College St., Hanover, NH 03755, USA

^bMicrobiology Research Laboratory, Department of Microbiology, Quaid-i-Azam University, Islamabad 45320, Pakistan

Abstract

To colonize the cystic fibrosis lung, *Pseudomonas aeruginosa* establishes sessile communities referred to as biofilms. Although the signaling molecule c-di-GMP governs the transition from motile to sessile growth, the environmental signal(s) required to modulate biofilm formation remain unclear. Using relevant in vivo concentrations of the 19 amino acids previously identified in cystic fibrosis sputum, we demonstrated that arginine, ornithine, isoleucine, leucine, valine, phenylalanine and tyrosine robustly promoted biofilm formation in vitro. Among the seven biofilm-promoting amino acids, only arginine also completely repressed the ability of *P. aeruginosa* to swarm over semi-solid surfaces, suggesting that arginine may be an environmental cue favoring a sessile lifestyle. Mutating two documented diguanylate cyclases required for biofilm formation (SadC and RoeA) reduced biofilm formation and restored swarming motility on arginine-containing medium. Growth on arginine increased the intracellular levels of c-di-GMP, and this increase was dependent on the SadC and RoeA diguanylate cyclases. Strains mutated in *sadC*, *roeA* or both also showed a reduction in biofilm formation when grown with the other biofilm-promoting amino acids. Taken together, these results suggest that amino acids can modulate biofilm formation and swarming motility, at least in part, by controlling the intracellular levels of c-di-GMP.

Keywords

Amino acids; c-di-GMP; Biofilm; Swarming; Cystic fibrosis

1. Introduction

The lungs of patients with the heritable disease cystic fibrosis (CF) are an ideal niche for the development of poly-microbial infections (Harrison, 2007; LiPuma, 2010; Sibley et al., 2008, 2009). Although the susceptibility of the CF lung to chronic infections is not yet fully

understood (LiPuma, 2010), permanent colonization by microorganisms is likely due to multiple host factor deficiencies and the ability of particular microbes to grow and thrive in this environment. The nutrient-rich sputum of CF lungs was demonstrated to support high-density growth of *Pseudomonas aeruginosa*, in part through the catabolism of amino acids (Palmer et al., 2005). Furthermore, transcriptome analyses of cells grown on CF sputum revealed that expression of *P. aeruginosa* virulence-associated genes could be altered in the presence of sputum or airway liquid from CF lungs (Palmer et al., 2005; Wolfgang et al., 2004). Growth in sputum or airway liquid from the CF lung also inhibited *P. aeruginosa* motility (Palmer et al., 2005; Wolfgang et al., 2004). Together, host conditions mimicking the CF lung were shown to support growth and influence diverse *P. aeruginosa* virulence behaviors (Palmer et al., 2005; Wolfgang et al., 2004), but little is known regarding the molecular basis of this regulation.

Our previous studies have shown an inverse relationship between biofilm formation (attaching to a surface) and swarming motility (moving across a surface) (Caiazza et al., 2007; Kuchma et al., 2007; Merritt et al., 2007, 2010; O'Toole, 2008). In *P. aeruginosa*, c-di-GMP-dependent signaling is part of a regulatory network controlling surface-associated group behaviors, including swarming motility and biofilm formation (Kuchma et al., 2007; Merritt et al., 2007, 2010; O'Toole, 2008). The role for such regulation in vivo is unclear, as are the environmental cue(s) that modulate these group behaviors. Given that amino acids are abundant in the CF sputum and appear to be key for *P. aeruginosa* growth in this environment (Palmer et al., 2007), in this study we investigated whether individual amino acids would favor the establishment of *P. aeruginosa* sessile populations at the expense of swarming motility. Using physiological concentrations, we report that most amino acids promoting in vitro biofilm formation could also support swarming motility, with the exception of arginine, which boosted biofilm formation and completely repressed swarming motility. This amino acid-dependent modulation of biofilm formation and swarming motility was dependent on a specific and functional c-di-GMP signaling network. Our findings demonstrate that individual amino acids at physiological concentrations can modulate *P. aeruginosa* group behaviors.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

All bacterial strains used in this study are derived from *P. aeruginosa* PA14 genetic background. Construction of the $\Delta sadC$, $\Delta roeA$, and $\Delta sadC\Delta roeA$ mutants has been reported previously (Merritt et al., 2007, 2010). Bacterial strains were routinely grown on lysogeny broth (LB) (Bertani, 2004; Miller, 1972), which was solidified by the addition of 1.5% agar for plate medium. For biofilm and swarming assays, M63 minimal salts medium [KH_2PO_4 (22 mM), K_2HPO_4 (40 mM), $(NH_4)_2SO_4$ (15 mM)] supplemented with $MgSO_4$ (1 mM) and various carbon sources was used, as noted in the text. For all experiments performed in M63 minimal salts medium, $(NH_4)_2SO_4$ was provided as a readily available source of nitrogen. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Waltham, MA).

2.2. Biofilm formation assays

Biofilm formation assays were performed as previously described using 96-well microtiter plates (O'Toole and Kolter, 1998). Briefly, overnight (O/N) cultures grown in LB medium were diluted (1:100 v/v) into M63 medium supplemented with 1 mM $MgSO_4$ and the various carbon sources as specified in the text. Each microtiter well was inoculated with 100 μ l of freshly inoculated medium, with a minimum of three wells per condition/strain per assay, and incubated at 37 °C for 24 h. Biofilms attached to the sides of microtiter wells

were stained with crystal violet (CV) as previously described (O'Toole and Kolter, 1998). Quantification of biofilm biomass was performed by dissolution of the CV-stained biofilm with glacial acetic acid (30% v/v) and the CV solution measured at an absorbance of 550 nm. The assay was performed at least three times using a minimum of three replicates for each tested condition.

2.3. Swarming motility assays

Swarm motility plates (0.5% agar) consisted of M63 medium supplemented with 1 mM MgSO₄, 0.2% glucose plus individual amino acids, as indicated in the text. Once poured, swarm plates were allowed to dry at room temperature for 16–18 h prior to inoculation. 2.5 µl of an O/N culture grown in LB medium was spot-inoculated into the middle of the plate, allowed to dry and incubated at 37 °C for 16–18 h. The assay was performed at least three times for each condition.

2.4. Quantification of planktonic growth

O/N cultures grown in LB medium were diluted (1:100 v/v) into M63 medium supplemented with 1 mM MgSO₄ and the indicated amino acids at concentrations specified in the text. Each microtiter well was inoculated with 100 µl of freshly inoculated medium, with a minimum of three wells per growth condition, and incubated at 37 °C for 24 h – these are the same conditions used for the biofilm assays. The quantification of bacterial growth was measured at an absorbance of 600 nm.

2.5. Measurement of intracellular c-di-GMP levels

c-di-GMP levels were measured by formic acid extraction of nucleotides from planktonically grown bacteria and subsequently quantified by LC-MS, as was previously described (Merritt et al., 2010).

2.6. Statistical analysis

All statistical tests were performed using Prism 5.0 for Mac OS X (GraphPad Software, Inc.). All experiments reported here were performed at least three times.

3. Results

3.1. Amino acids modulate *P. aeruginosa* surface-associated group behaviors

Studies performed in our laboratory suggested that the use of different carbon sources in standard M63 minimal medium directly impacted the amount of biofilm formed by *P. aeruginosa* PA14 in the wells of microtiter plates (Caiazza and O'Toole, 2004). For example, we had reported previously (Caiazza and O'Toole, 2004) that providing *P. aeruginosa* PA14 with 0.4% arginine as the sole carbon source significantly enhanced biofilm formation (~3 fold; $P < 0.0001$) compared to this strain grown in 0.2% glucose or in 0.2% glucose/0.5% casamino acids (G/CAA) (Fig. 1A).

Using the same M63 medium, we observed swarming motility only when *P. aeruginosa* PA14 was grown on glucose supplemented with CAA, but not with glucose alone or when the amino acid arginine was provided as the sole carbon and energy source (Fig. 1B). Furthermore, we demonstrated that the addition of arginine to 0.2% glucose/0.1% CAA swarm agar decreased the zone of swarming motility in a dose-dependent manner (between 0 and 0.4% arginine; Fig. 1C). Together, these results indicate that biofilm formation can vary with the growth substrate provided, and furthermore, the presence of arginine both boosts biofilm formation and has a negative effect on swarming motility.

3.2. Biofilm formation by *P. aeruginosa* in response to individual amino acids

The ability of arginine to boost biofilm formation and repress swarming motility by *P. aeruginosa* PA14 prompted us to test the effects of other amino acids on these group behaviors. To place these studies within a framework relevant to this microbe, we elected to use concentrations of amino acids reported in CF sputum by Palmer and colleagues (Palmer et al., 2007). In all cases, the amino acids were tested at their measured level in CF sputum (Palmer et al., 2007) and at 2-, 4-, 8- and 16-fold above the sputum concentration as well as at 1/2 and 1/4 the measured sputum concentration. We elected to use a concentration range of amino acids around the measured average concentration based on the known heterogeneity of the CF lung likely resulting in profound nutrient gradients.

In total, 19 amino acids were evaluated for their ability to promote in vitro biofilm formation by *P. aeruginosa* PA14. The amino acids were provided as the sole carbon and energy source, and used at concentrations relevant to those found in CF sputum as outlined above. The extent of biofilm formation when *P. aeruginosa* PA14 was grown on amino acids was first compared to biofilm formation when grown on glucose (0.2%, in the absence of any added amino acids). This data set is presented in its entirety in Fig. S1.

From the data presented in Fig. S1, we identified 11 amino acids that, when provided at the maximum concentration tested here, supported biofilm formation to an extent that was equivalent to or better than that observed for growth on 0.2% glucose (Fig. S2). Next, the planktonic growth of *P. aeruginosa* PA14 on these 11 amino acids was determined. With the exception of proline, growth with the other 10 amino acids was less than that measured on glucose alone (Fig. S3). Proline promoted more planktonic growth compared to glucose. To account for these differences in growth among these 11 amino acids, the biofilm assay data were normalized to the growth of *P. aeruginosa* on the particular substrate assayed. After normalization, growth on seven amino acids including arginine, ornithine, phenylalanine, tyrosine, isoleucine, leucine, or valine as the sole carbon and energy source were shown to significantly increase the extent of biofilm formation by *P. aeruginosa* PA14 compared to growth on glucose as sole carbon source (Fig. 2). Growth on glutamate, histidine and aspartate showed a small but non-significant increase in biofilm formation, and despite the strong boost in planktonic growth, providing proline as the carbon source also resulted in a small but non-significant increase in biofilm formation. Together, these data indicated that 7/19 amino acids tested, even after accounting for differences in planktonic growth, significantly boosted biofilm formation in comparison to glucose. We focus on these seven amino acids for the remainder of this report.

Interestingly, adding glucose along with the biofilm-promoting amino acids abolished either the increase or decrease in biofilm formation observed when the amino acids were provided as the sole carbon and energy source (data not shown).

3.3. Swarming motility of *P. aeruginosa* in response to the seven biofilm-promoting amino acids

We next assessed the impact of the seven biofilm-promoting amino acids on swarming motility. For all the swarm assays reported here, glucose (0.2%) was provided in addition to the indicated amino acid, because without glucose little or no swarming was observed, likely due to the slow growth of this organism on amino acids as the sole carbon and energy source. Addition of isoleucine, leucine, valine, ornithine, phenylalanine or tyrosine to the medium supported swarming motility by *P. aeruginosa* PA14 (Fig. 3), although not to the extent observed for growth on G/CAA. When arginine was provided at 4.8 mM (Fig. 3), the lowest concentration of arginine that boosted biofilm formation (Fig. S1), *P. aeruginosa* PA14 showed no swarming motility.

3.4. A role for the SadC and RoeA diguanylate cyclases in biofilm formation and swarming repression during growth on amino acids

In *P. aeruginosa* PA14, the role of c-di-GMP signaling in inverse regulation of biofilm formation and swarming motility was demonstrated for the diguanylate cyclases (DGCs) SadC and RoeA (Merritt et al., 2007, 2010). Using the reported biofilm-deficient *sadCroeA* double mutant strain, we demonstrated a significant reduction ($P < 0.05$) in biofilm formation by *P. aeruginosa* PA14 for all seven biofilm-promoting amino acids (Fig. 4A).

Because biofilm formation by the double *sadCroeA* mutant strain was decreased in all tested conditions, we then determined the individual contribution of SadC and RoeA in this assay. Using the isogenic *roeA* and *sadC* single mutants, our genetic analysis indicated that the RoeA DGC contributes more to the biofilm formation phenotype of *P. aeruginosa* PA14 than does SadC ($P < 0.001$) when this organism is grown on arginine, leucine, phenylalanine, ornithine, tyrosine or valine as sole carbon and energy source (Fig. 4B). When grown on isoleucine, no significant difference was observed between the *sadC* and *roeA* mutants (Fig. 4B), indicating that their contribution to biofilm formation is similar in this particular growth condition. In contrast, as reported previously (Merritt et al., 2010), the *sadC* mutant showed a more robust biofilm-defective phenotype when *P. aeruginosa* PA14 was grown on G/CAA, the growth condition used to originally isolate the strain with a mutation in this gene. Thus, the DGCs encoded by the *sadC* and/or *roeA* genes appear to contribute to biofilm formation under a range of growth conditions.

As shown in Fig. 3, when arginine was provided at 4.8 mM (Fig. 3), the lowest concentration of arginine that boosted biofilm formation, *P. aeruginosa* PA14 showed no swarming motility. Similarly, when provided at the average measured concentration of 0.3 mM, arginine also repressed swarming (Fig. 4C). The *sadCroeA* double mutant, which displays a hyper-swearer phenotype when grown on G/CAA (Merritt et al., 2010) restored swarming on arginine-containing medium at both arginine concentrations tested (Fig. 4C), indicating that these DGCs participate in repression of swarming motility as well as boosting biofilm formation during growth on this amino acid.

Finally, for the other six biofilm-promoting amino acids, the double *sadCroeA* mutant also showed an increased swarming zone compared to the wild type *P. aeruginosa* PA14 (Fig. 4D), indicating that these DGC also negatively impact swarming motility on these substrates.

3.5. Biofilm-promoting amino acids modulate the level of intracellular c-di-GMP

Arginine was the only amino acid that both promoted biofilm formation and completely repressed swarming motility. The loss of swarming repression on arginine-containing medium exhibited by the *sadCroeA* mutant, combined with the observed decrease in biofilm formation for this double mutant, led us to hypothesize that arginine could modulate the level of intracellular c-di-GMP.

To test the impact of growth on arginine upon total c-di-GMP levels, we extracted this nucleotide from planktonic cells grown in the same medium used in the biofilm and swarm assays. In these experiments, cells were grown in the presence of glucose (0.2%) plus the indicated amount of added arginine. We chose to use planktonic cultures because mutations in the *sadC* and *roeA* genes negatively impact biofilm formation, and thus it is not possible to measure c-di-GMP in these mutant backgrounds using biofilm-grown cells. In addition, exponentially growing planktonic cells provided the most uniform population for analysis, in contrast to stationary phase bacteria. Further, planktonic cells are free from clumping that is common in plate-grown and stationary cells – such clumping can reduce the efficiency of

c-di-GMP extraction. However, we cannot discount that growth in a biofilm or in swarming medium might impact total and relative c-di-GMP levels compared to planktonic growth.

First, using glucose medium supplemented with a previously reported arginine concentration in CF sputum of 0.3 mM arginine (Palmer et al., 2007), we observed no significant difference in the total c-di-GMP level compared to growth on glucose alone (Fig. 5A). Note that at this concentration, arginine did not boost biofilm formation relative to growth on glucose (Fig. S1) but did repress swarming motility (Fig. 4C). However, when grown on 4.8 mM arginine, a concentration of arginine shown to both promote biofilm formation and repress swarming motility (Figs. 2 and 4), we observed a significant increase of ~1.7 fold ($P < 0.001$) in intracellular c-di-GMP (Fig. 5A).

Consistent with a role for SadC/RoeA in modulating these arginine-dependent phenotypes, the double *sadCroeA* mutant strain, which results in decreased biofilm formation and increased swarming compared to the WT on arginine-containing medium (Fig. 4), showed a significant decrease ($P < 0.001$) in the level of c-di-GMP when compared to the WT when these strains were grown with 4.8 mM arginine (Fig. 5B).

In addition to arginine, several other amino acids increased biofilm formation compared to growth on glucose. Therefore, we evaluated the impact on total c-di-GMP levels when *P. aeruginosa* PA14 was grown in the presence of three of these biofilm-promoting amino acids, namely phenylalanine (at 8 mM), tyrosine (at 3.2 mM), and ornithine (at 11.2 mM). These concentrations were chosen for each amino acid based on the concentrations used in the swarming and biofilm assays above. Compared to growth on glucose, the carbon source added to all swarm assays, growth on glucose plus individual amino acids resulted in an increase in the c-di-GMP level, including ornithine (1.3-fold), phenylalanine (1.8-fold) and tyrosine (2.6-fold). Only the increase in c-di-GMP when grown on tyrosine, compared to growth on glucose, was statistically significant ($P = 0.0004$, ANOVA with Tukey's honest significant difference). These data indicate that the total amount of c-di-GMP measured under these conditions, while altered relative to growth on glucose, does not appear to directly correlate with the observed phenotypic outputs of biofilm formation and swarming.

4. Discussion

Recent studies have implicated amino acids as a key nutrient resource for *P. aeruginosa* in the context of the CF lung (Palmer et al., 2010, 2007, 2005). Thus, the goal of our study was to assess the impact of amino acids, at concentrations relevant to those measured in CF sputum, on biofilm formation and swarming motility by *P. aeruginosa* PA14. We identified seven amino acids which, even after correction for their different respective abilities to support planktonic growth, increased the extent of biofilm formed on an abiotic substratum compared to glucose (Fig. 2). Interestingly, addition of glucose to these amino acids nullified the boost in biofilm formation demonstrated for the individual amino acids, indicating potentially complex relationships among nutrient signals. Similarly, in these studies, the amino acids may provide an additional source of nitrogen in addition to the ammonia provided in the minimal medium, although it is not clear what effect, if any, the amino-acid-derived nitrogen is contributing to the biofilm and swarming phenotypes.

Arginine was the only one of the seven biofilm-promoting amino acids that also completely repressed swarming motility. This finding differs from that of Kohler et al. who also showed that leucine, tyrosine and phenylalanine did not promote swarming motility by *P. aeruginosa* PAO1 (Kohler et al., 2000). Such a discrepancy could be due to the differences in strains used, as all our studies were performed with strain *P. aeruginosa* PA14, which has been reported to have a swarm pattern distinct from the PAO1 strain (Toutain et al., 2005; Tremblay and Deziel, 2008), highlighting possible differences between these isolates.

Furthermore, the phenotypes noted may be a matter of degree, as we observed only very weak swarming motility on these same three amino acids compared to the extent of swarming motility when *P. aeruginosa* PA14 was grown on glucose supplemented with CAA (Fig. 3).

The impact of arginine on c-di-GMP levels recalls the inverse control of biofilm formation and swarming motility mediated by this nucleotide and discussed in the literature (O'Toole, 2008). That is, growth on higher concentrations of arginine (4.8 mM) stimulates biofilm formation and represses swarming motility, consistent with the similar phenotypic effects of c-di-GMP accumulation. Furthermore, we showed that the arginine-dependent biofilm and swarming phenotypes, regardless of the concentration of arginine used, required functional *sadC* and *roeA* genes, and similarly, stimulation of biofilm formation on the other six amino acids also required the DGCs SadC and/or RoeA. These data indicate that the SadC and/or RoeA DGCs likely contribute to biofilm formation and swarming motility on a variety of different growth substrates, and not just when cells are grown on G/CAA, the growth condition used to initially isolate the mutants (Merritt et al., 2007, 2010).

Interestingly, while 4.8 mM arginine increased biofilm formation, c-di-GMP levels, and repressed swarming motility, the average measured CF sputum concentration of arginine, 0.3 mM, only had a significant effect on repressing swarming motility. These findings are in agreement with our previous data indicating the lack of a linear correspondence between cellular c-di-GMP levels and phenotypic output (Merritt et al., 2010). That is, while both 0.3 mM and 4.8 mM arginine repress swarming motility and this repression requires the documented DGCs SadC and RoeA, only in the higher concentration of arginine could we detect a difference in c-di-GMP levels (Fig. 5). While additional studies would be required to establish arginine as a direct environmental signal that impacts c-di-GMP production, this study highlights the importance of available nutrients in regulating bacterial behaviors.

The concentration range of arginine used in these studies is based on several factors, including published studies indicating serum arginine levels in the micromolar range (Grasemann et al., 2008), the production of low millimolar arginine by immune cells during infection (Talaue et al., 2006), the measured K_m of human arginase at low millimolar concentration (Alarcon et al., 2006) and the known heterogeneity of the CF lung. Therefore, we believe that the selected broad range concentration of arginine is physiological relevant for *P. aeruginosa* in a context of CF lung infections.

In addition to its unique ability to both stimulate biofilm formation and repress swarming, arginine is one of only two known substrates, along with pyruvate (Eschbach et al., 2004; Schreiber et al., 2006), to be fermented by *P. aeruginosa* (Eschbach et al., 2004; Vander Wauven et al., 1984). Thus, growth in the presence of arginine provides a means for this microbe to generate ATP via substrate level phosphorylation in the absence of oxygen. Given the growing appreciation that the CF lung might represent an anoxic or microaerophilic environment (Alvarez-Ortega and Harwood, 2007; Worlitzsch et al., 2002), it is tempting to speculate that arginine might provide a resource for this microbe to survive in the context of the CF lung. It is important to note that our findings do suggest that other amino acids, such as phenylalanine, ornithine and tyrosine, which do not support anaerobic growth, also boost biofilm formation and c-di-GMP levels at the expense of swarming motility.

The rise in antibiotic-resistant bacterial infections has recently led researchers to focus on unconventional anti-infective targets, such as virulence determinants (Cegelski et al., 2008; Rasko and Sperandio, 2010) and carbon metabolic pathways (Brown et al., 2008). Our study

indicates that interfering with the availability and/or metabolism of key host-derived nutrients may have a profound impact on a variety of bacterial phenotypes related to disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix. Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.resmic.2011.04.014.

Fig. 1.

Amino acids impact surface-associated group behaviors of *P. aeruginosa*. (A) 24-h biofilms of *P. aeruginosa* PA14 grown in M63 minimal medium supplemented with 0.2% glucose (Glc), 0.2% glucose/0.5% casamino acids (Glc/CAA) or 0.4% arginine (Arg). Arginine was used at 0.4% in this experiment based on our previous study (Caiazza and O'Toole, 2004). The bar graph represents the quantification of biofilms formed in the wells of a microtiter plate stained with crystal violet (CV), the CV solubilized in glacial acetic acid (30% v/v) and the CV solution measured at 550 nm. Shown are CV-stained wells (top) and quantification of biofilms (bottom). Data represent the mean $\pm$ standard error of the mean (SEM) of four replicates normalized to 0.2% glucose values. The dotted line represents the mean value of biofilms formed by *P. aeruginosa* PA14 when grown on glucose (0.2%) shown for comparison. The asterisks indicate values significantly different from 0.2% glucose-grown biofilms by one-tailed unpaired *t* test: **P* = 0.001 and ***P* = 0.0001. (B) Ability of *P. aeruginosa* PA14 to swarm over semi-solid surfaces (0.5% agar) containing 0.2% glucose, G/CAA, or 0.4% arginine. (C) Repression of *P. aeruginosa* PA14 swarming motility by arginine in a dose-dependent manner. Swarm motility plates contained 0.2% glucose/0.1% CAA with various concentrations of arginine (from 0 to 0.4%). In this experiment, the concentration of CAA was reduced from 0.5% to 0.1% to better discern the repressive effects of arginine. Shown are pictures of swarm plates incubated for 16–18 h at 37 °C.

Fig. 2.

P. aeruginosa biofilm formation in response to individual amino acids. Shown is the quantification of 24 h biofilms formed in the wells of a microtiter plate stained with CV, the CV solubilized in glacial acetic acid (30% v/v) and the CV solution measured at 550 nm. The biofilm data shown here are normalized to their respective planktonic growth (OD₆₀₀) on each amino acid used as sole carbon and energy source (see Supplemental Fig. S3), and represent the mean $\pm$ SEM of at least five replicates. The dotted line represents the mean value of biofilms formed by *P. aeruginosa* PA14 when grown on glucose (0.2%) shown for comparison. Amino acids that significantly boosted biofilm formation as sole carbon and energy source when compared to growth on 0.2% glucose are shown in white bars. Also shown are growth-normalized biofilm formation data for the amino acids that supported biofilm formation to an extent not significantly different from glucose (black bars). Amino acids were used at the following concentrations (mM): proline (27.2), glutamate (24), histidine (8), aspartate (12.8), isoleucine (17.6), arginine (4.8), leucine (25.6), phenylalanine (8), ornithine (11.2), tyrosine (3.2) and valine (17.6). These amino acid concentrations represent 16-fold the value previously quantified in CF sputum (Palmer et al., 2007), except for tyrosine that is used at 4-fold over the average measured sputum concentration. These concentrations were selected for the seven biofilm-promoting amino acids because they supported robust biofilm formation when they were provided as sole carbon and energy source (Fig. S1). Asterisk indicates values significantly different from 0.2% glucose by one-way analysis of variance (ANOVA) corrected with the Dunnett's multiple comparison Post test ($P < 0.05$).

Fig. 3.

The impact of biofilm-promoting amino acids on swarming motility. Swarming motility of *P. aeruginosa* PA14 in response to the seven biofilm-promoting amino acids. Shown are pictures of swarm plates after 16–18 h of incubation at 37 °C. Glc/CAA was used as a positive control for swarming motility. In these studies, the medium contained 0.2% glucose supplemented with individual amino acids at the following concentrations (mM): isoleucine (17.6), leucine (25.6), valine (17.6), arginine (4.8), ornithine (11.2), phenylalanine (8), and tyrosine (3.2). These concentrations are the same as those evaluated for the biofilm formation assay represented in Fig. 2.

Fig. 4.

The role of c-di-GMP on biofilm formation and swarming repression. (A) 24 h biofilms of *P. aeruginosa* PA14 (WT) and the *sadCroeA* double mutant on the indicated growth substrate. The biofilm data shown are normalized to their respective final planktonic growth (OD₆₀₀) and represent the mean $\pm$ SEM of at least three replicates. (B) 24 h biofilms of single *sadC* or *roeA* mutants on the indicated growth substrate and the values normalized relative to the WT strain. Data represent the mean $\pm$ SEM of at least three replicates. The asterisks indicate values significantly different from the WT (panel A) or *sadC* mutant (panel B) using two-way ANOVA corrected with the Bonferroni Post test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (C) Shown are images of swarm plates for *P. aeruginosa* PA14 (WT) and the *sadCroeA* double mutant taken after 16–18 h of incubation at 37 °C. Swarming assays performed on medium supplemented with either glucose/CAA (G/CAA), as previously reported (Merritt et al., 2010) or glucose 0.2% supplemented with arginine (G/Arg, 0.3 or 4.8 mM Arg, as indicated). (D) Swarming assays performed on medium containing 0.2% glucose supplemented with either isoleucine, leucine, phenylalanine, ornithine, tyrosine or valine using the same concentrations described in Fig. 3.

Fig. 5.

Quantification of the intracellular level of c-di-GMP. (A) Shown is the relative concentration of the total extracted c-di-GMP level for the WT strain of *P. aeruginosa* PA14 grown on glucose (0.2%) plus the indicated concentration of arginine. The dotted line represents the mean value of total c-di-GMP extracted from *P. aeruginosa* PA14 when grown on glucose (0.2%) shown for comparison. The asterisk indicates a value significantly different from the glucose-only condition by the two-tailed unpaired *t* test (* $P < 0.001$). (B) Shown is the relative concentration of the total extracted c-di-GMP level for *P. aeruginosa* PA14 (WT) and the *sadCroeA* double mutant grown on glucose (0.2%) plus 4.8 mM arginine. The asterisk indicates a value significantly different from WT *P. aeruginosa* PA14 by two-tailed unpaired *t* test (* $P < 0.001$). Data represent the mean $\pm$ SEM of three replicates.