Uracil influences quorum sensing and biofilm formation in *Pseudomonas aeruginosa* and fluorouracil is an antagonist

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Summary

*Pseudomonas aeruginosa* is an ubiquitous, opportunistic pathogen whose biofilms are notoriously difficult to control. Here we discover uracil influences all three known quorum-sensing (QS) pathways of *P. aeruginosa*. By screening 5850 transposon mutants for altered biofilm formation, we identified seven uracil-related mutations that abolished biofilm formation. Whole-transcriptome studies showed the uracil mutations (e.g. *pyrF* that catalyses the last step in uridine monophosphate synthesis) alter the regulation of all three QS pathways [LasR-, RhlR- and 2-heptyl-3-hydroxy-4-quinolone (PQS)-related regulons]; addition of extracellular uracil restored global wild-type regulation. Phenotypic studies confirmed uracil influences the LasR (elastase), RhlR (pyocyanin, rhamnolipids), PQS and swarming regulons. Our results also demonstrate uracil influences virulence (the *pyrF* mutant was less virulent to barley). Additionally, we found an anticancer uracil analogue, 5-fluorouracil, that repressed biofilm formation, abolished QS phenotypes and reduced virulence. Hence, we have identified a central regulator of an important pathogen and a potential novel class of efficacious drugs for controlling cellular behaviour (e.g. biofilm formation and virulence).

Introduction

The diverse small signal molecules used for quorum sensing (QS) (Camilli and Bassler, 2006) transform independent cells into specialized cell communities (Battin et al., 2007). These small molecules are used to regulate biofilm formation (Davies et al., 1998; González-Barrios et al., 2006). The ubiquitous pathogen *Pseudomonas aeruginosa*, which is one of the major causes of chronic lung infections of cystic fibrosis patients (Stover et al., 2000) and a major cause of hospital-acquired infections, thrives in many environments due to its exquisite gene regulation which consists of myriad two-component systems (Stover et al., 2000). In this organism, expression of many genes is regulated via three distinct QS systems.

The Las QS system is activated by *N*-(3-oxododecanoyl)-L-homoserine lactone (3OC12-HSL), and the LasR transcription factor controls expression of 3OC12-HSL synthesis (*lasI*) and virulence factors (e.g. *lasAB*, *toxA* and *apr*) (Wagner et al., 2004). The Rhl system is activated by *N*-butyryl-HSL (C4-HSL), and the RhlR transcription factor controls expression of C4-HSL synthesis (*rhlI*, *rhlAB*, *lasB*, and pyocyanin production (Wagner et al., 2004). The Las and Rhl QS systems regulate more than 300 genes (Schuster et al., 2003). The *Pseudomonas* quinolone signal (PQS, 2-heptyl-3-hydroxy-4-quinolone) also controls the production of pyocyanin, rhamnolipids and elastase (Diggle et al., 2006). The hierarchy of QS regulation is that the LasRI controls RhlRI regulation, and the PQS system is related to both the LasRI and RhlRI systems (Diggle et al., 2006). Hence, these QS systems regulate virulence factors such as extracellular enzymes (LasA protease, LasB elastase and alkaline proteases), metabolites (pyocyanin and hydrogen cyanide), and biofilm formation that cause persistent infections by *P. aeruginosa*.

Ribonucleic acid secondary messengers, including cyclic adenosine monophosphate (cAMP) (Baker and Kelly, 2004), cyclic guanosine monophosphate (cGMP) (Baker and Kelly, 2004) and cyclic-diguanosine monophosphate (c-diGMP) (Cotter and Stibitz, 2007), serve as signals for diverse biological functions. c-diGMP regulates virulence factors and biofilm formation in *P. aeruginosa* (Cotter and Stibitz, 2007), and cAMP regulates various *in vivo* bacterial functions by binding to intracellular receptors and protein kinases (Baker and Kelly, 2004). In humans, uridine-5'-triphosphate (UTP) is an extracellular...
signal that regulates a broad spectrum of cell functions via the P2Y2 receptor, a G protein-coupled membrane receptor that regulates phospholipases and mitogen-activated protein kinases (Lazarowski and Boucher, 2001). Hence, ribonucleic acid signals are important biological regulators.

By screening thousands of transposon mutants to discern genes related to biofilm formation, we discovered that uracil influences virulence, biofilm formation, and other QS-controlled phenotypes that are regulated by well-studied QS pathways (e.g. LasR, RhlR and PQS). Whole-transcriptome analysis indicated that strains with an altered uridine monophosphate (UMP) synthesis pathway had hundreds of QS genes repressed, and transcription of these genes was restored by exogenous uracil. We then hypothesized that uracil analogues may inhibit biofilm formation which led to the discovery that the well-studied 5-fluorouracil (5-FU) is a potent inhibitor of P. aeruginosa biofilm formation; hence, 5-FU shows promise for preventing biofilm formation.

Results

Biofilm screening identifies UMP-related genes

To understand how P. aeruginosa regulates biofilm formation, we screened 5850 mutants that cover 4596 of the 5962 predicted PA14 genes (Liberati et al., 2006) to discern which genes impact biofilm formation using a crystal violet biofilm screen. We identified 137 mutants with over threefold enhanced biofilm formation and identified 88 mutants with over 10-fold reduced biofilm formation. The mutants with decreased biofilm formation include 20 flagella and related proteins (figABCDEHJL, flhAB, fliFHKLQM, motAB), and two type IV fimbriae/pili biogenesis proteins (pilFX) that are well characterized for affecting biofilm formation (Klausen et al., 2003). Among those with decreased biofilm formation, all seven mutants (carA, carB, pyrB, pyrC, pyrD, pyrE and pyrF) of the UMP synthetic pathway (Fig. 1) formed dramatically reduced biofilms (Fig. 2A). These mutants grew more slowly in LB medium (Fig. S1A, Table S1); for example, the pyrF mutant had a specific growth rate of 0.90 ± 0.01 h⁻¹ compared with 1.59 ± 0.04 h⁻¹ for the wild-type strain. All of the six other mutants (carA, carB, pyrB, pyrC, pyrD and pyrE) showed similar growth (turbidity in 96-well plates) and biofilm formation compared with the pyrF mutant (Table S1).

The mutants for the biosynthesis of other pyrimidines and purines were not identified by screening for altered biofilm formation. Hence, the effects were not due to growth defects nor were they related to general metabolic defects in nucleic acid biosynthesis. This suggested that the products of this metabolic pathway regulate biofilm formation, so we investigated whether UMP, UTP or uracil may serve as an internal signal for biofilm formation by using a pyrF mutant (PyrF catalyses the last step of UMP synthesis and encodes orotidine-5′-phosphate decarboxylase). Addition of uracil (0.1–1 mM), but not UMP and UTP, increased biofilm formation in the pyrF mutant (Fig. 2B); note that LB medium contains approximately 0.2 mM uracil, and complementation by uracil was expected as P. aeruginosa has a pyrimidine salvage pathway to utilize uracil but not UMP or UTP. Neither UMP nor UTP affected growth of the wild-type strain and the pyrF mutant at 1 mM; hence, both nucleotides are probably not transported into cells. As higher concentrations of uracil (10 mM) enhanced biofilm formation of wild-type PA14 (Fig. 2B), uracil, but not UMP and UTP, serves as a consistent positive regulator of biofilm formation in P. aeruginosa; note these experiments do not rule out the possibility that UMP or UTP may mediate the biofilm effect intracellularly.

Uracil influences quorum sensing in P. aeruginosa

To examine whether uracil influences virulence, biofilm formation and biofilm formation. Thus, the purine nucleotides, adenine and guanine, do not affect biofilm formation whereas all of the car and pyr mutants related to uracil synthesis showed decreased biofilm formation.

UMP synthesis induces QS genes

To determine which genes are regulated by uracil in biofilms, we analysed the whole transcriptome for the pyrF mutant versus wild-type P. aeruginosa for cells grown on glass wool (Table 1). Remarkably, all three known QS systems were repressed upon inhibition of UMP synthesis (overall 298 genes were repressed and 147 were induced). The pyrF mutation repressed the transcriptional factor for the Rhl QS system, rhlR, ~4.9-fold as well as repressed rhlA (~21-fold) and rhlB (~20-fold). The deficiency in UMP synthesis also repressed lasAB of the Las QS system ~16- to ~39-fold and repressed pqsH (encodes a FAD-dependent monooxygenase for the last step of PQS synthesis) ~2.6-fold. In addition, many virulence factors were repressed upon inhibition of UMP syn-
thesis as seen by repression of the genes for phenazine synthesis, chemotaxis, alkaline proteases, type II secretion and type IV pilus formation (Table 1).

To corroborate these microarray results and to show they are directly related to uracil, we analysed the changes in differential gene expression of the whole transcriptome upon addition of uracil to the pyrF cells in biofilms. As expected, uracil addition induced expression of nearly all of the genes repressed by the pyrF mutation (Table 1); therefore, the changes in the QS pathways are caused by uracil, as extracellular uracil restored transcription of 252 out of 298 genes repressed by the pyrF mutation.

To explore the effect of uracil further, we also added 10 mM uracil to the wild-type strain in LB medium and studied the whole transcriptome response of biofilm cells. As expected, uracil treatment induced the expression of nearly all of the genes repressed by the pyrF mutation (Table 1); therefore, the changes in the QS pathways are caused by uracil, as extracellular uracil restored transcription of 252 out of 298 genes repressed by the pyrF mutation.

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UMP synthesis increases QS phenotypes

The DNA microarray results suggested uracil influences genes related to QS for all three known QS systems in P. aeruginosa. As QS systems are regulated by cell growth, we investigated the growth of the pyrF mutant in defined M9 glucose medium with exogenous uracil. The pyrF mutant grew well in minimal medium with 0.1, 1 or 10 mM uracil.

Fig. 1. UMP synthesis pathway in P. aeruginosa PA14 (Kanehisa and Goto, 2000). Abbreviations are CarA for the carbamoylphosphate synthetase large subunit, CarB for the carbamoylphosphate synthetase small subunit, PyrB for aspartate carbamoyltransferase, PyrC for dihydroorotase, PyrD for dihydroorotate dehydrogenase, PyrE for orotate phosphoribosyltransferase, PyrF for orotidine-5′-phosphate decarboxylase, PyrH for uridylate kinase, Udk for uridylate diphosphate kinase, UshA for bifunctional UDP-sugar hydrolase and 5′-nucleotidase, SurE for stationary-phase survival protein and Upp for uracil phosphoribosyltransferase.
uracil supplement, but not with 0.01 mM uracil (Fig. S1B). The specific growth rate was 0.51 ± 0.05 h⁻¹ for wild-type PA14, and 0.49 ± 0.01, 0.47 ± 0.02 and 0.45 ± 0.04 h⁻¹ for the pyrF mutant with 0.1, 1 and 10 mM uracil respectively. Therefore, 0.1 mM uracil in M9 glucose medium restores normal growth, and higher concentrations of uracil are not utilized as a carbon, nitrogen or energy source. Hence, we tested the QS assays with uracil concentrations greater than 0.1 mM.

As LasB (elastase) is regulated by LasR (Wagner et al., 2004), we assayed elastase activity as an indicator of the Las QS system and found elastase activity increased 1.9- to 3.9-fold in the pyrF mutant compared with the wild-type strain upon adding uracil (Fig. 3A). We also examined three RhlR-regulated phenotypes, pyocyanin production (Wagner et al., 2004), rhamnolipid production (Wagner et al., 2004) and swarming motility (Déziel et al., 2003). As expected, poor pyocyanin production was found in the phzM mutant (PhzM is one of the key enzymes for pyocyanin production), and addition of uracil to the pyrF mutant (1 and 10 mM) enhanced pyocyanin production by 2.2- and 3.8-fold respectively. Rhamnolipid production also increased by 1.3-fold with additional uracil (Fig. 3A). Swarming motility was abolished in the pyrF mutant with 0.1 mM uracil but 1 mM uracil addition restored it (Fig. 3A). We also quantified PQS production because pqsH was repressed in the pyrF mutant (Table 1) and found PQS production by the pyrF mutant doubled as uracil concentrations increased from 0.1 to 1.0 mM uracil (Fig. 3A). Therefore, additional uracil in minimal medium increased consistently elastase activity, pyocyanin production, rhamnolipid production (slightly), swarming and PQS production in the pyrF mutant.

We also examined the effect of uracil addition to wild-type PA14 in M9 glucose medium (Fig. 3A). Elastase activity increased by 1.9-fold with 1 mM uracil, and pyocyanin increased by 1.8-fold with 10 mM uracil. However, rhamnolipid and PQS synthesis were not significantly changed with uracil addition, and swarming decreased with 10 mM uracil.

In LB medium, elastase activity, pyocyanin production, rhamnolipid production, swarming and PQS production were all abolished in the pyrF mutant and were all restored by uracil addition to the pyrF mutant (Fig. 3B). Therefore, results in both media show conclusively that UMP synthesis and extracellular uracil influence all five QS phenotypes and that the changes in QS phenotypes are not related to growth effects.

Upp is required to affect QS

Pseudomonas aeruginosa PA14 possesses uracil phosphoribosyltransferase (Upp) that participates in the uracil salvage pathway (Andersen et al., 1992). Upp catalyses uracil to UMP, hence the upp mutant is not able to utilize uracil for salvage and UMP synthesis. We hypothesized that if exogenous uracil influences QS phenotypes in the upp mutant, uracil itself impacts the regulation of QS or uracil may be utilized as a carbon/nitrogen source. If exogenous uracil does not influence QS phenotypes in the upp mutant, then an unidentified nucleotide derived from uracil may influence QS. We tested our hypothesis by performing QS assays with the upp mutant in the presence of uracil (Fig. 3C). Although exogenous uracil increased elastase activity by 1.5-fold; this increase is not significant in comparison with the results of the pyrF mutant (Fig. 3A). Exogenous uracil (0.1–10 mM) also did not increase the other QS phenotypes (Fig. 3C). Note that exogenous uracil did not affect the growth of the upp mutant in M9 glucose medium (Fig. S1C); hence, again, uracil did not serve as a carbon, nitrogen or energy source. These results suggest uracil utilization by Upp is necessary to influence the QS phenotypes.

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Table 1. Uracil regulates QS and virulence factors.

| Gene      | PA14 # | Fold changes | Description                           |
|-----------|--------|--------------|---------------------------------------|
|          |        | pyrF versus WT | PyrF + uracil versus WT | PyrF + uracil versus pyrF |          |
| **Repressed genes upon deleting pyrF** |
|           |        |              |                                      |                          |          |
| **Quorum sensing** |
| PA14_19120 | PA3477 | rhlR        | –4.9 | 1.1 | 4.9 | Transcriptional regulator RhlR |
| PA14_19110 | PA3478 | rhlB        | –21.1 | –1.2 | 17.1 | Rhamnosyltransferase chain B |
| PA14_19100 | PA3479 | rhlA        | –19.7 | –1.4 | 16.0 | Rhamnosyltransferase chain A |
| PA14_45950 | PA1431 | rsaL        | –2.1 | 1.4 | 2.6 | Regulatory protein RsAL |
| PA14_40290 | PA1871 | lasA        | –39.4 | 1 | 32 | LasA protease precursor |
| PA14_16250 | PA3724 | lasB        | –16 | 1.1 | 19.7 | Elastase LasB |
| PA14_30630 | PA2587 | pqsH        | –2.6 | –1.1 | 2.5 | FAD-dependent monoxygenase |
| **Phenazine synthesis** |
| PA14_09460 | PA1901 | phzC2       | –17.1 | –1.1 | 13.9 | Phenazine biosynthesis protein PhzC |
| PA14_09450 | PA1902 | phzD2       | –18.4 | 1.3 | 19.7 | Phenazine biosynthesis protein PhzD |
| PA14_09440 | PA1903 | phzE2       | –14.9 | –1.1 | 14.9 | Phenazine biosynthesis protein PhzE |
| PA14_09420 | PA1904 | phzF2       | –17.1 | 1 | 17.1 | Phenazine biosynthesis protein |
| PA14_09410 | PA1905 | phzG2       | –12.1 | 1.2 | 13.9 | Pyridoxamine 5'-phosphate oxidase |
| PA14_09490 | PA2090 | phzH        | –3.5 | 1.1 | 3.5 | Phenazine-specific methyltransferase |
| PA14_09480 | PA210 | phzA1       | –10.6 | –1.1 | 8.6 | Phenazine biosynthesis protein |
| PA14_09470 | PA211 | phzB1       | –18.4 | 1 | 18.4 | Phenazine biosynthesis protein |
| PA14_09400 | PA217 | phzS        | –7.5 | 1.2 | 9.8 | Flavin-containing monooxygenase |
| **Pyochelin synthesis** |
| PA14_33260 | PA2426 | pvdS        | –11.3 | –4.6 | 1.9 | Sigma factor PvdS |
| **Chemotaxis** |
| PA14_02200 | PA0175 | aprD        | –2.8 | 1.2 | 3.5 | Probable chemotaxis methyltransferase |
| PA14_02220 | PA0176 | aer2        | –3.5 | 1.3 | 4.9 | Aerotaxis transducer Aer2 |
| PA14_02230 | PA0177 | aprE        | –2.8 | 1.1 | 3.2 | Probable purine-binding chemotaxis protein |
| PA14_02250 | PA0178 | aprF        | –3.7 | –1.1 | 3.5 | Probable 2-component sensor |
| PA14_02260 | PA0179 | aprG        | –3.7 | 1.1 | 4.3 | Probable 2-component response regulator |
| PA14_02270 | PA0180 | aprH        | –3.7 | –1.1 | 3.7 | Probable chemotaxis transducer |
| PA14_28050 | PA2788 | aprI        | –14.9 | –1.1 | 12.1 | Probable chemotaxis transducer |
| **Multidrug efflux transporter** |
| PA14_09540 | PA205 | mexG        | –32 | –1.2 | 26 | Hypothetical protein |
| PA14_09530 | PA206 | mexH        | –24.3 | 1.1 | 22.6 | Efflux membrane fusion protein precursor |
| PA14_09520 | PA207 | mexI        | –14.9 | –1.1 | 13 | Efflux transporter |
| PA14_09500 | PA208 | oprD        | –16 | –1.1 | 13.9 | Probable outer membrane efflux protein |
| **Virulence factors** |
| PA14_48115 | PA1246 | aprD        | –6.1 | –0.2 | 4.9 | Alkaline protease secretion protein AprD |
| PA14_48100 | PA1247 | aprE        | –4.9 | 0.2 | 5.7 | Alkaline protease secretion protein AprE |
| PA14_48090 | PA1248 | aprF        | –4 | 0.3 | 4.3 | Alkaline protease secretion protein AprF |
| PA14_48060 | PA1249 | aprA        | –10.6 | 0.6 | 36.8 | Alkaline metalloproteinase precursor |
| PA14_48040 | PA1250 | aprL        | –2.6 | 0.5 | 3.5 | Alkaline protease inhibitor AprL |
| PA14_36330 | PA2193 | hcnA        | –5.7 | –1.1 | 4.9 | Hydrogen cyanide synthase HcnA |
| PA14_36320 | PA2194 | hcnB        | –5.7 | –1.1 | 5.3 | Hydrogen cyanide synthase HcnB |
| PA14_36310 | PA2195 | hcnC        | –5.7 | 1.2 | 7 | Hydrogen cyanide synthase HcnC |
| PA14_34870 | PA2300 | chiC        | –32 | –1.2 | 34.3 | Chitinase |
| **Type II secretion system/Type IV pilus** |
| PA14_55850 | PA4299 | tadD        | –2.6 | 1.4 | 4.3 | Fli pilus assembly protein TadD |
| PA14_55860 | PA4300 | tadC        | –2.8 | 1.1 | 3 | Fli pilus assembly protein TadC |
| PA14_55880 | PA4301 | tadB        | –2 | –1.1 | 2 | Fli pilus assembly protein TadB |
| PA14_55890 | PA4302 | tadA        | –4.6 | 1.3 | 6.5 | Fli pilus assembly protein, ATPase CpaF |
| PA14_55900 | PA4303 | tadZ        | –6.5 | 1 | 4.3 | Fli pilus assembly protein, ATPase CpaE |
| PA14_55920 | PA4304 | rcpA        | –7 | –1.2 | 7 | Fli pilus assembly protein, secretin CpaC |
| PA14_55930 | PA4305 | rcpC        | –5.7 | 1.5 | 9.8 | Fli pilus assembly protein CpaB |
| PA14_55940 | PA4306 | flp         | –29.9 | 1.6 | 59.7 | Fli pilus assembly protein, pilin FliP |

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Partial list of differentially expressed genes in biofilm cells in LB medium after 7 h for the pyrF mutant versus wild-type PA14 (WT), for the pyrF mutant with 1 mM uracil versus WT, and for the pyrF mutant without uracil.

### UMP synthesis increases virulence

As genes related to seven virulence factors (lasA, lasB, rhlRAB, phzABCDEFGMS, chiC, aprADEFI and tadZABCDG-rcpAC-flp) were repressed in the pyrF mutant, we compared the pathogenicity of wild-type PA14 and the pyrF mutant using our barley germination assay (Attila et al., 2008); previously, we had used this assay to corroborate virulence factors we identified in the poplar tree rhizosphere. The pyrF mutation increased barley germination by 1.8 ± 0.7-fold compared with wild-type PA14; hence, it reduced virulence. Furthermore, addition of 1 mM uracil to the pyrF mutant restored pathogenicity to wild-type levels, and addition of 1 mM uracil made the

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**Table 1. cont.**

| PA14 #   | PAO1 # | Gene   | pyrF versus WT | PyrF + uracil versus WT | PyrF + uracil versus pyrF | Description                      |
|----------|--------|--------|----------------|--------------------------|---------------------------|----------------------------------|
| PA14_53250 PA0852  | cbpD   | –14.9  | 1.1            | 16                       | Chitin-binding protein CbpD precursor |
| PA14_39780 PA1914  | –16    | 1.9    | 36.8           | 12.1                     | Probable hydrolase            |
| PA14_37770 PA2067  | –16    | –1.1   | 12.1           | 32                       | Probable carbamoyl transferase |
| PA14_35160 PA2274  | –13.9  | –1.2   | 13             | Hypothetical protein     |
| PA14_33870 PA2381  | –22.6  | 1.1    | 24.3           | Hypothetical protein     |
| PA14_31350 PA2566  | –17.1  | 1.5    | 21.1           | Conserved hypothetical protein |
| PA14_31290 PA2570  | 3      | 1.7    | 4.9            | PA-I galactophilic lectin |
| PA14_26020 PA2939  | –22.6  | –1.1   | 22.6           | Probable aminopeptidase  |
| PA14_24650 PA3049  | rnf    | –22.6  | 1.6            | 39.4                     | Ribosome modulation factor     |
| PA14_18120 PA3570  | lecA   | –3     | 1.7            | 4.9                      | Fucose-binding lectin PA-IIL   |
| PA14_11140 PA4078  | –17.1  | 1.1    | 16             | Probable non-ribosomal peptide synthetase |
| PA14_10560 PA4129  | –29.9  | –1.1   | 13.9           | Hypothetical protein     |
| PA14_10550 PA4130  | –26    | 1      | 26             | Probable sulfite or nitrite reductase |
| PA14_10540 PA4131  | –18.4  | 1      | 17.1           | Probable iron-sulfur protein |
| PA14_10500 PA4133  | –28.9  | 1.1    | 29.9           | Cytochrome c oxidase subunit (cbb3-type) |
| PA14_10490 PA4134  | –16    | –1.3   | 13             | Hypothetical protein     |

**Induced genes upon deleting pyrF**

| Description |
|-------------|
| No significant similarity |
| Hypothetical protein |
| Hypothetical protein |
| N-carbamoylputrescine amidohydrolase |
| Conserved hypothetical protein |
| Conserved hypothetical protein |
| Hypothetical protein |
| Conserved hypothetical protein |
| Proline dehydrogenase PutA |
| Hypothetical protein |
| Hypothetical protein |
| Malate:quinone oxidoreductase |
| Glycerol kinase |
| Glycerol-3-phosphate dehydrogenase |
| Hypothetical protein |
| Hypothetical protein |
| Hypothetical protein |
| Hypothetical protein |
| Probable transcriptional regulator |
| Probable AMP-binding enzyme |
| Hypothetical protein |
| Hypothetical protein |
| Probable coenzyme A transferase |
| Conserved hypothetical protein |
| Hypothetical protein |
| Hypothetical protein |
| Probable MFS dicarboxylate transporter |

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Fig. 3. Uracil and 5-fluorouracil control QS phenotypes.
A. Effect of uracil addition (0.1–10 mM) on wild-type *P. aeruginosa* PA14 and the *pyrF* mutant and of addition of 10 μM 5-fluorouracil to the wild-type strain in M9 glucose medium for LasB elastase activity, pyocyanin production, rhamnolipid production, swarming motility and PQS production. Negative controls were lasB (for elastase), phzM (for pyocyanin), rhlR (rhamnolipid and swarming) and pqsA (PQS). Data show the average of two independent experiments ± SD. Wild-type values were 0.03 ± 0.01 elastase absorbance at 495 nm per cell growth, 0.011 ± 0.002 pyocyanin absorbance at 520 nm per cell growth, 53 ± 2 μg rhamnolipid ml⁻¹, and 4.6 ± 0.4 cm for swarming. PQS production was quantified and compared with that of the wild-type strain and a purified standard.

B. Effect of the *pyrF* mutation in LB medium and of adding 1 mM uracil to the *pyrF* mutant on LasB elastase activity, pyocyanin production, rhamnolipid production, swarming motility and PQS production. Data show the average of two independent experiments ± SD. Wild-type values were 0.12 ± 0.01 elastase absorbance at 495 nm per cell growth, 0.017 ± 0.001 pyocyanin absorbance at 520 nm per cell growth, 25 ± 5 μg rhamnolipid ml⁻¹ and 0.9 ± 0.4 cm for swarming. PQS production was quantified and compared with that of the wild-type strain and a purified standard.

C. Effect of uracil addition (0.1–10 mM) on elastase activity, pyocyanin production, rhamnolipid production, swarming motility and PQS production with the *P. aeruginosa upp* mutant in M9 glucose medium. Data show the average of two independent experiments ± SD. Wild-type values were 0.069 ± 0.008 elastase absorbance at 495 nm per cell growth, 0.022 ± 0.005 pyocyanin absorbance at 520 nm per cell growth, 56 ± 1 μg rhamnolipid ml⁻¹ and 4.4 ± 1.1 cm for swarming. PQS production was quantified and compared with that of the wild-type strain and a purified standard.
wild-type strain 2.0 ± 1.6-fold more virulent. These results show uracil synthesis and extracellular uracil influence virulence.

As extracellular uracil was clearly transported into cells as evidenced by the change in QS phenotypes, virulence and whole-genome transcription upon uracil addition, we checked to see if uracil was exported by *P. aeruginosa* PA14 cells and thereby was perhaps functioning as an extracellular signal. Using minimal medium to reduce the complexity of extracellular components and to avoid uracil in LB medium, we found that over a 24 h period, extracellular uracil increased 13-fold to 3.8 mM (similar results were obtained with *P. aeruginosa* PAO1); however, the small concentration implies uracil works intracellularly (not as a QS signal), and these small amounts may be due to cell lysis.

### 5-FU inhibits biofilm formation and QS phenotypes

Inhibition of biofilm formation is important as biofilms cause persistent infections that are responsible for many human diseases related to bacteria; therefore, discovering novel biofilm inhibitors is valuable. As shown in Fig. 2B, uracil regulates biofilm formation for both the *pyrF* mutant and wild-type PA14; hence, we screened uracil analogues for biofilm inhibition. Among the six uracil structural analogues tested, we identified 5-FU as an effective biofilm inhibitor (Fig. 4A). In LB medium, 5-FU (25 μM) decreased PA14 biofilm formation threefold with a 20% reduction in the specific growth rate (Fig. 4B). In M9 glucose medium, we found that 10 μM 5-FU inhibited biofilm formation by 56% (Fig. 4C) without affecting growth (in planktonic cultures, final turbidity at 620 nm was 0.132 ± 0.005 without 5-FU and 0.133 ± 0.001 with 5-FU). At higher concentrations, 25 μM 5-FU, biofilm formation was inhibited by 61%, but growth was also inhibited (final turbidity at 620 nm was 0.107 ± 0.001).

Additionally, we found 5-FU inhibits QS-regulated virulence factors for wild-type *P. aeruginosa* PA14 (Fig. 3A). In M9 glucose medium, all five QS phenotypes were nearly abolished: 5-FU (10 μM) repressed significantly elastase activity (86%) and the RhlR-regulated phenotypes of pyocyanin production (100%), rhamnolipid production (87%) and swarming (100%) as well as abolished PQS production. In addition, 5-FU decreased wild-type PA14 pathogenicity for barley (1.8 ± 1.2-fold more germination at 25 μM 5-FU). Hence, 5-FU is an effective biofilm inhibitor that works by inhibiting QS phenotypes.

### Discussion

By carefully cataloguing the genes related to biofilm formation, we discovered here that uracil influences all three known QS pathways of the pathogen *P. aeruginosa*; hence, uracil is important for QS because in order to influence all three QS pathways it must be upstream of all three regulatory circuits. The lines of evidence showing the influence of uracil on QS are that: (i) disruption of UMP synthesis via the *pyrF* mutation represses transcription of hundreds of QS genes (Table 1), (ii) addition of extracel-
ular uracil to the pyrF mutant restores transcription of these QS genes to wild-type levels (Table 1), (iii) addition of uracil to the wild-type strain increases some QS phenotypes (Fig. 3A), (iv) the pyrF mutation abolishes at least five QS phenotypes including those regulated by LasR (elastase), RhlR (pyocyanin, rhamnolipid and swarming) and PQS (PQS production), and the addition of uracil restores these phenotypes (Fig. 3), (v) the disruption of UMP synthesis by seven independent mutations abolishes biofilm formation (Fig. 2A), and the addition of extracellular uracil restores biofilm formation (Fig. 2B), (vi) mutations in purine synthesis do not affect biofilm formation, and (vii) the pyrF mutation reduces virulence as shown by barley germination, and addition of uracil accentuates virulence.

Due to its importance, it is expected that others should have seen uracil-related genes in whole-transcriptome QS and biofilm studies. As expected, altered carAB and pyrBCDEF expression was observed in P. aeruginosa under various conditions; for example, pyrQ (a homologue of pyrC) was one of the most induced genes (45-fold) in cystic fibrosis sputum medium versus synthetic medium (Palmer et al., 2007), and this gene was induced 40-fold in sputum compared with minimal glucose medium (Palmer et al., 2005). Similarly, carAB and pyrF are induced fourfold in more pathogenic P. aeruginosa strains (Chugani and Greenberg, 2007). These results corroborate our barley germination results with the pyrF mutant by confirming that synthesis of uracil is important for pathogenesis and show that uracil-related genes are in the literature.

Metabolites are often identified as signalling molecules; for example, polyamines regulate swarming and biofilm formation, and rhamnolipids regulate swarming and biofilm structure (Monds and O'Toole, 2007). Indole from tryptophan is a cell signal for Escherichia coli, and it regulates biofilm formation in a different manner for E. coli and pseudomonads (Lee et al., 2007a). In this research, we show uracil enhances biofilm formation and QS-regulated virulence factors (elastase activity, pyocyanin production and rhamnolipid production) (Figs 2 and 3) without growth inhibition (Fig. S1B). These findings suggest that uracil may serve as a regulator for these phenotypes.

Iron availability is mediated by pyoverdine which regulates swarming for Pseudomonas putida (Matilla et al., 2007). In the pyrF mutant, pvdS, which encodes a sigma factor for iron acquisition, was highly repressed; hence, one possible mechanism is that swarming of the pyrF mutant is regulated by iron availability via uracil control of pvdS expression. Further evidence of this link between iron and uracil was seen upon adding uracil to wild-type biofilm cells as many iron-related genes were differentially expressed (Table S2).

The QS-regulated transcriptome was examined previously with the mutant P. aeruginosa PAO-JP2, which lacks both lasI and rhlI (Wagner et al., 2003). Addition of autoinducers induced a variety of the virulence factors, such as alkaline proteases (aprAD), phenazine biosynthesis (phzACDEFGMS), chitinase (cphD), LasAB, RhlRAB, MexGHI-OpmD and type II secretion (tadACD-rcpAC-flp). We found most of these genes are repressed in the pyrF mutant, and that uracil restores gene expression of these loci. This supports that uracil regulates many of the virulence factors via QS.

As uracil is a component for mRNA, one possible mechanism for global gene expression regulation by uracil is its influence on the transcription of AU-rich genes. Therefore, we examined the GC content of the top 10 induced and repressed genes in the pyrF versus wild-type microarray data (Table 1). The average GC content is 64.9% and 66.9% for the top 10 induced and repressed genes respectively. These values are not significantly different from the GC content of whole PAO1 genome, 66.6% (Stover et al., 2000). Therefore, uracil regulates gene expression independently of their GC content.

The impact of uracil on QS may be general in that all cells have uracil, and we have found that the main set of genes (e.g. carAB, pyrBCDFIL and uraA) induced in E. coli by the species-independent signal autoinducer-2 (Camilli and Bassler, 2006) are related to UMP biosynthesis and uracil transport (Lee et al., 2008). In addition, we have also found the cell signal indole (Lee et al., 2007b) represses this same set of genes in E. coli more than any others (e.g. carAB, pyrBCDFIL and uraA) (Lee et al., 2008). Hence, both known E. coli signals function through uracil synthesis and uracil-, autoinducer-2- and indole-based signalling are intertwined.

It is logical that procaryotic cells should use uracil in that they make use of three other RNA-based intracellular signals, cAMP, cGMP and c-diGMP. Our results show uracil addition to the upp mutant is not able to enhance QS phenotypes (Fig. 3C), although uracil addition to the pyrF mutant enhanced all five QS phenotypes (Fig. 3A and B). This suggests that the QS phenotypes are regulated in P. aeruginosa when uracil is utilized for pyrimidine nucleotide metabolism. In eucaryotes, UTP is an extracellular signal for exciting sympathetic neurons, for muscle cell proliferation and for endothelial cell adhesion (Lazarowski and Boucher, 2001); UTP release is stimulated in both neural and non-neural cells by mechanical stress (Lazarowski and Boucher, 2001).

As we discovered that uracil influences QS in P. aeruginosa, we hypothesized that well-known human anticancer drugs may be used to control P. aeruginosa pathogenicity. This realization is important in that this class of drugs is already screened for human toxicity and may be used rapidly in trials for diseases such as cystic fibrosis. Spe-
cifically, we showed 5-FU inhibits biofilm formation of *P. aeruginosa* (Fig. 4), and is non-toxic to *P. aeruginosa*, and this compound is already approved for treatment of human colon cancer (Wiebke et al., 2003); so it is relatively non-toxic to humans. Our results corroborate a previous report (Hussain et al., 1992) that showed 5-FU inhibits the biofilm formation of *Staphylococcus epidermidis* and suggest those original-surprising results may be related to uracil.

Therefore, for *P. aeruginosa*, 5-FU is one of the few known biofilm-inhibiting compounds that is non-toxic such as brominated furanones (Ren et al., 2001), ursoic acid (Ren et al., 2005) and indole derivatives (Lee et al., 2007b). Furthermore, 5-FU is one of the few known anti-virulence compounds (Cegelski et al., 2008); antivirulence compounds are an important way to fight infectious diseases because unlike antimicrobials, antivirulence compounds like 5-FU do not affect growth and so there is less chance of developing resistance (Hentzer et al., 2002).

### Experimental procedures

#### Bacterial strains

*Pseudomonas aeruginosa* PA14 and the isogenic transposon-insertion mutants were obtained from Dr Frederick Ausubel (Liberati et al., 2006) and are listed in Table S3. Strains were routinely pre-cultured in LB medium (Sambrook et al., 1989) or M9 minimal medium (Ausubel et al., 1998) with 0.2% glucose as a carbon source for PA14 or LB with 15 μg ml⁻¹ gentamicin for the isogenic mutants. All experiments were conducted at 37°C. Cell growth was measured using turbidity at 620 nm for the biofilm assay with 96-well plates or 600 nm for all other experiments. Conformation of the transposon insertion for the *pyrF* mutant was performed as described previously (Ueda and Wood, 2008). Gene-specific primers were designed as PA14_26890-VF (5′-GGGTGAAAGTCGGCAAGGAACCTCTT-3′) and PA14_26890-VR (5′-GGAGAATCTTCATCGACCGCCTTCAG-3′) to amplify wild-type *pyrF* gene. Using chromosomal DNA from PA14 wild type, 899 bp of the *pyrF* gene was amplified; this band was not amplified for the *pyrF* mutant. A DNA fragment corresponding to the end of the MAR2XT7 transposon and upstream flanking *pyrF* was amplified using the transposon-specific primer GB3a (5′-TACAGTTTACGAACGGATCCGGCAAGGC-3′) and chromosomal primer PA14_26890-VR, and using transposon-specific primer R1 (5′-ATCGACCCGACTCCCGCCAC-3′) and downstream chromosomal primer PA14_26890-VR with chromosomal DNA from the *pyrF* mutant; these two bands were not amplified for the PA14 wild type.

#### Biofilm assay

Comprehensive screening for altered biofilm mutants was carried out with the 5850 clones of the PA14 non-redundant mutant library (Liberati et al., 2006). The initial screen of biofilm formation was examined in LB medium using 96-well polystyrene plates and crystal violet staining (Lee et al., 2007b) using one well for each strain. Mutants with biofilm formation altered over threefold (inhibited or stimulated) were re-screened with 10 replicate wells and two independent cultures. Biofilm formation was normalized by planktonic cell growth to take into account changes in growth and is shown as normalized biofilm (OD₅₄₀/OD₆₂₀).

#### Biofilm inhibition with uracil analogues

Uracil analogues 5-aminouracil, 6-azauracil, 5-bromouracil, 5-FU (Fisher Scientific, Hanover Park, IL), 5-((trans-2-bromovinyl))-uracil (Sigma-Aldrich, St Louis, MO) and 5-nitouracil (MP Biomedical, Solon, OH) were tested as biofilm inhibitors in LB medium at 200 μM (10 to 200 μM for 5-FU). 5-FU was also tested for biofilm inhibition with M9 glucose medium at 10 μM and 25 μM. *P. aeruginosa* PA14 wild type was grown in LB medium or M9 glucose medium overnight, overnight cultures were diluted to a turbidity at 600 nm of 0.05 with LB medium or M9 glucose medium containing an uracil analogue, and biofilm formation in 96-well polystyrene plates was examined after 24 h. Ten wells were used for each condition, and two independent cultures were tested.

#### QS assays

LasB elastase activity was measured with cells grown to a turbidity at 600 nm of 2 as described previously based on spectrophotometric determination of the amount of elastin-Congo red reaction (Ohman et al., 1980). Elastin-Congo red was purchased from Sigma-Aldrich, and the lasB mutant was used as a negative control. Pyocyanin production was measured as described previously based on spectrophotometric determination after extraction with chloroform and 0.2 N HCl (Essar et al., 1990); the phzM mutant was used as a negative control. Rhamnolipids were quantified spectrophotometrically after diethylether extraction and sulfur acid/orcinol addition (Wilhelm et al., 2007); hms was used as a standard (Fisher Scientific), and the rhlR mutant was used as a negative control. Swarming motility (translocation on top of agar plates) was examined with BM-2 plates (62 mM potassium phosphate, 2 mM MgSO₄, 10 μM FeSO₄, 0.1% casamino acid, 0.4% glucose, 0.5% Bacto agar) (Overhage et al., 2008) with cells grown to a turbidity of 1 at 600 nm (Morohoshi et al., 2007) after 24 h; five plates were tested for each experiment and two independent cultures were used, and the rhlR mutant was used as a negative control. PQS was extracted and quantified by thin-layer chromatography (Gallagher et al., 2002) with the modifications of glass silica gel F₂₅₄ pre-coated plates (10 × 20 cm, VWR, West Chester, PA) and a 95:5 mixture of dichloromethane : methanol. The plate image was quantified by a Bio-Rad VersaDoc 3000 imaging system (Bio-Rad, Hercules, CA), and PQS (500 ng) was used as a positive control (Syntech Solution, San Diego, CA).

#### Barley virulence assay

Pathogenicity of *P. aeruginosa* was tested by a barley germination assay (Attila et al., 2008). Barley seeds (cultivar Belford, Stover Seed Company, Los Angeles, CA) were surface-sterilized in 1% sodium hypochlorite for 30 min. After
washing the seeds with sterile water 10 times, seeds were germinated in the absence or presence of bacteria. Germi-
nated seeds were counted after 3 days.

Whole-transcriptome analysis

The P. aeruginosa genome array (Affymetrix, P/N 510596) was used to investigate differential gene expression in bio-
films for PA14 versus the pyrF mutant, in biofilms for the pyrF mutant versus the pyrF mutant with 1 mM uracil and in bio-
films for 10 mM uracil added to PA14 versus no uracil addition as described previously (Attila et al., 2008). Biofilm cells were harvested from 10 g of glass wool after incubation for 7 h in LB with shaking at 250 r.p.m., and RNA was extracted with a RNAex Mini Kit (Qiagen, Valencia, CA). Global scaling was applied so the average signal intensity was 500. The probe array images were inspected for any image artefact. Background values, noise values and scaling factors of both arrays were examined and were comparable. The intensities of polyadenosine RNA controls were used to monitor the labelling process. For each binary microarray comparison of differential genes expression, if the gene with the larger trans-
scription rate did not have a consistent transcription rate based on the 13 probe pairs (P-value less than 0.05), these genes were discarded. A gene was considered differentially expressed when the P-value for comparing two chips was lower than 0.05 (to assure that the change in gene expres-
sion was statistically significant and that false positives arise less than 5%) and when the expression ratio was higher than the standard deviation for the whole microarrays (2.74 for the pyrF mutant versus wild-type PA14, 1.73 for the pyrF mutant with uracil versus wild-type PA14, 3.15 for the pyrF mutant versus the pyrF mutant with uracil and 1.94 for wild-type PA14 with uracil versus wild-type PA14) (Ren et al., 2004). The microarray raw data are deposited at the Gene Expression Omnibus (GSE9592) of the National Center for Biotechnol-
yogy Information.

Extracellular uracil

Uracil was quantified as a function of cell density in M9 minimal medium supplemented with 0.2% glucose and 0.2% sodium succinate using a reverse-phase high-pressure liquid chromato-
graph (Waters 515 with photodiode array detector, Milford, MA) with a Nova-Pak C18 column (Waters, 150 × 3.9 mm, 4 μm) and gradient elution with 100 mM ammonium acetate (pH 5.0) and acetonitrile as the mobile phases at a flow rate of 1 ml min⁻¹ (100.0 at 0 min, 94.6 at 8 min and 100:0 at 10 min). Under these conditions, the retention time for uracil was 1.75 min, and the absorbance maximum was 259 nm (uracil standard was used to verify peaks by co-elution).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Growth of the pyrF mutant in LB medium and in M9 glucose medium supplemented with uracil. Wild-type P. aeruginosa PA14 and the pyrF mutant were grown in (A)
LB medium supplemented with 0.01 to 10 mM uracil and in (B) M9 glucose medium supplemented with 0.01 to 10 mM uracil. (C) Growth of wild-type *P. aeruginosa* PA14 and the *upp* mutant in M9 glucose medium supplemented with 0.1 to 10 mM uracil. Data show the average of two independent experiments ± s.d.

**Fig. S2.** Biofilm formation of mutants deficient in adenine and guanine synthesis. Biofilm formation was examined in LB medium after 24 h, and 10 wells were used for each culture. Biofilm formation was normalized by cell growth, and data show the average of the two independent experiments ± s.d. **Table S1.** Biofilm formation and growth of the PA14 uracil-synthesis mutants, *carA*, *carB*, *pyrB*, *pyrC*, *pyrD*, *pyrE* and *pyrF*. Biofilm formation was examined in LB after 24 h, and growth was obtained from turbidity of planktonic cells in the biofilm assay.

**Table S2.** List of the repressed genes related to iron acquisition in biofilm cells of PA14 wild type in LB medium after 7 h in the presence of 10 mM uracil. **Table S3.** Strains used in this study.

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