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 inde-

pendent 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 P2Y$_2$ 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 (*flgABCEFHIJKL*, *flhAB*, *fliFHKLMQ*, *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 Luria–Bertani medium (LB) (Fig. S1A, Table S1); for example, the *pyrF* mutant had a specific growth rate of $0.90 \pm 0.01 \, h^{-1}$ compared with $1.59 \pm 0.04 \, h^{-1}$ 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.

Purines do not affect biofilm formation

To examine whether purine nucleotide synthesis also regulates biofilm formation like the pyrimidine uracil, we tested biofilm formation with mutants lacking the purine nucleotide synthetic pathway. The *purH* mutant, deficient in inosine monophosphate synthesis (used for adenosine and guanosine), formed biofilms as well as wild-type PA14 (Fig. S2). Additionally, the *purE* mutant (lacks phosphoribosylaminoimidazole carboxylase for 5′-phosphoribosyl-4-carboxy-5-aminoimidazole synthesis) and the *PA14_01760* mutant (putative non-specific ribonucleoside hydrolase that may catalyse adenosine to adenine and guanosine to guanine) showed normal 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-
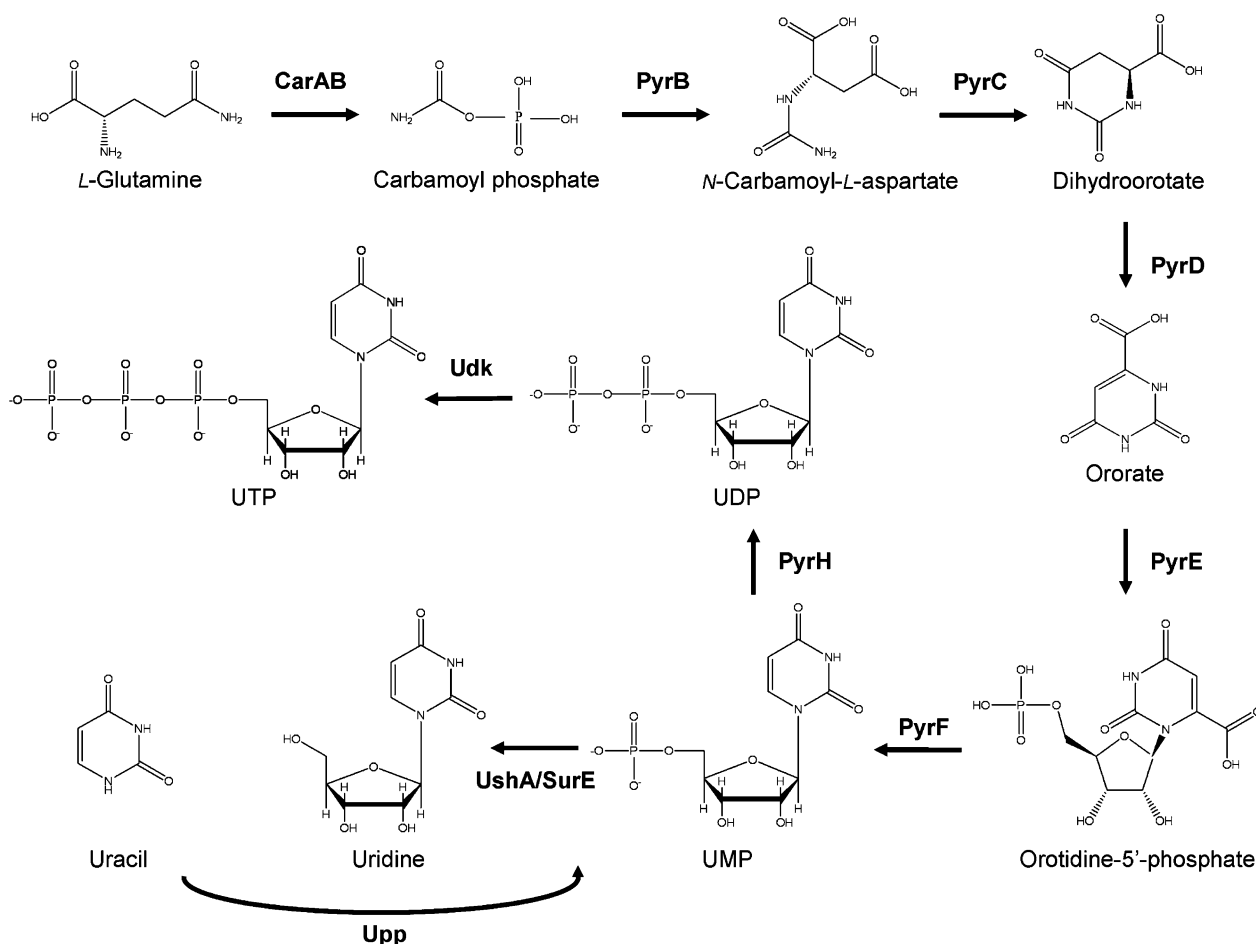
Fig. 1. UMP synthesis pathway in *P. aeruginosa* PA14 (Kanehisa and Goto, 2000). Abbreviations are CarA for the carbamoylphosphate synthetase large subunit, CarB for the carbamoylphosphate synthetase small subunit, PyrB for aspartate carbamoyltransferase, PyrC for dihydroorotase, PyrD for dihydroorotate dehydrogenase, PyrE for orotate phosphoribosyl-transferase, 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.

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 genes for uracil catabolism (8- to 74-fold increases for PA0439-PA0444), and *lasI* was induced by twofold (the *pyrF* mutation repressed *lasAB*). Interestingly, 10 mM uracil repressed

genes related to iron acquisition (−2.1 to −2.3-fold for the two component system *pfeRS*, −13-fold for PA2426 *pvdS* which encodes sigma factor PvdS, −2.3-fold for the siderophore receptor PA0931, and −1.3 to −4.3-fold for pyochelin synthesis genes PA4224-PA4231) (Table S2). It is not clear in detail how uracil regulates expression of these genes, but uracil may enhance iron uptake, and then the increased iron concentrations repress these iron-related genes including virulence factors which are regulated by iron (Ochsner *et al.*, 2002).

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
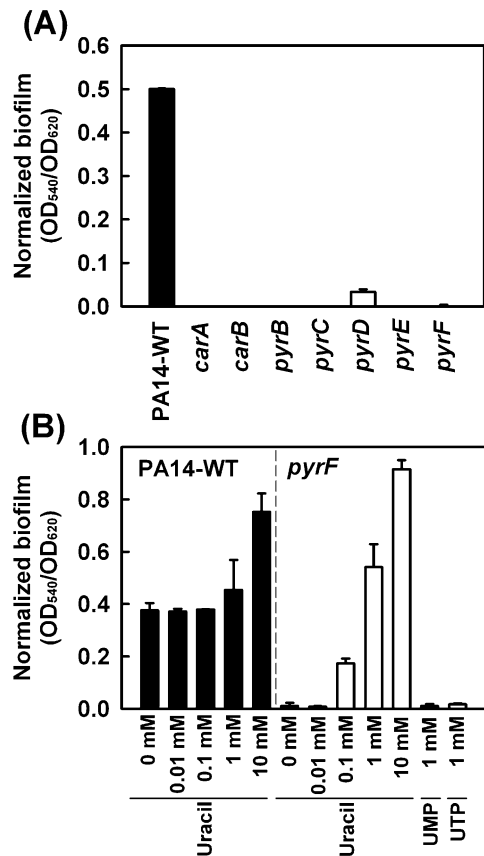
(A)

(B)

Fig. 2. Uracil controls biofilm formation.
A. Biofilm formation in the *P. aeruginosa* PA14 uracil synthesis mutants *carA*, *carB*, *pyrB*, *pyrC*, *pyrD*, *pyrE* and *pyrF*.
B. Biofilm formation of the *pyrF* mutant upon addition of uracil, UMP and UTP. Biofilm formation was examined in LB medium after 24 h. Six to 10 wells were used for each culture. Biofilm was normalized by cell growth, and data show the average of the two independent experiments ± SD.

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 pyo-

cyanin 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 phosphoribosyltansferase (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.

Table 1. Uracil regulates QS and virulence factors.

PA14 #	PAO1 #	Gene	*pyrF* versus WT	PyrF + uracil versus WT	PyrF + uracil versus *pyrF*	Description
				Fold changes		
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	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 monooxygenase
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	PA4209	phzM	−3.5	1.1	3.5	Phenazine-specific methyltransferase
PA14_09480	PA4210	phzA1	−10.6	−1.1	8.6	Phenazine biosynthesis protein
PA14_09470	PA4211	phzB1	−18.4	1	18.4	Phenazine biosynthesis protein
PA14_09400	PA4217	phzS	−7.5	1.2	9.8	Flavin-containing monooxygenase
Pyochelin synthesis						
PA14_09290	PA4224	pchG	−5.7	−1.9	2.8	Pyochelin biosynthesis protein PchG
PA14_09280	PA4225	pchF	−7	−2.5	3.5	Pyochelin synthetase
PA14_09270	PA4226	pchE	−5.7	−1.7	3	Dihydroaeruginoic acid synthetase
PA14_09260	PA4227	pchR	−2.1	1.1	2.5	Transcriptional regulator PchR
PA14_09240	PA4228	pchD	−5.3	−1.3	4	Pyochelin biosynthesis protein PchD
PA14_09230	PA4229	pchC	−5.7	−1.7	3.5	Pyochelin biosynthesis protein PchC
PA14_09220	PA4230	pchB	−5.7	−1.3	4	Salicylate biosynthesis protein PchB
PA14_09210	PA4231	pchA	−7.5	−1.5	4.6	Salicylate biosynthesis isochorismate synthase
Pyoverdine synthesis						
PA14_33260	PA2426	pvdS	−11.3	−4.6	1.9	Sigma factor PvdS
Chemotaxis						
PA14_02200	PA0175		−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		−2.8	1.1	3.2	Probable purine-binding chemotaxis protein
PA14_02250	PA0178		−3.7	−1.1	3.5	Probable 2-component sensor
PA14_02260	PA0179		−3.7	1.1	4.3	Probable 2-component response regulator
PA14_02270	PA0180		−3.7	−1.1	3.7	Probable chemotaxis transducer
PA14_28050	PA2788		−14.9	−1.1	12.1	Probable chemotaxis transducer
Multidrug efflux transporter						
PA14_09540	PA4205	mexG	−32	−1.2	26	Hypothetical protein
PA14_09530	PA4206	mexH	−24.3	1.1	22.6	Efflux membrane fusion protein precursor
PA14_09520	PA4207	mexI	−14.9	−1.1	13	Efflux transporter
PA14_09500	PA4208	opmD	−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	aprI	−2.6	0.5	3.5	Alkaline proteinase inhibitor AprI
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	Flp pilus assembly protein TadD
PA14_55860	PA4300	tadC	−2.8	1.1	3	Flp pilus assembly protein TadC
PA14_55880	PA4301	tadB	−2	−1.1	2	Flp pilus assembly protein TadB
PA14_55890	PA4302	tadA	−4.6	1.3	6.5	Flp pilus assembly protein, ATPase CpaF
PA14_55900	PA4303	tadZ	−6.5	1	4.3	Flp pilus assembly protein, ATPase CpaE
PA14_55920	PA4304	rcpA	−7	−1.2	7	Flp pilus assembly protein, secretin CpaC
PA14_55930	PA4305	rcpC	−5.7	1.5	9.8	Flp pilus assembly protein CpaB
PA14_55940	PA4306	flp	−29.9	1.6	59.7	Flp pilus assembly protein, pilin Flp

Table 1. *cont.*

PA14 #	PAO1 #	Gene	Fold changes			Description
			pyrF versus WT	*PyrF* + uracil versus WT	*PyrF* + uracil versus *pyrF*	
Others						
PA14_53250	PA0852	*cpbD*	−14.9	1.1	16	Chitin-binding protein CbpD precursor
PA14_39780	PA1914		−16	1.9	36.8	Conserved hypothetical protein
PA14_37770	PA2067		−16	−1.1	12.1	Probable hydrolase
PA14_37745	PA2069		−42.2	−1.1	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	*lecA*	−3	1.7	4.9	PA-I galactophilic lectin
PA14_26020	PA2939		−22.6	−1.1	22.6	Probable aminopeptidase
PA14_24650	PA3049	*rmf*	−22.6	1.6	39.4	Ribosome modulation factor
PA14_20610	PA3361	*lecB*	−2.1	1.2	3	Fucose-binding lectin PA-IIL
PA14_18120	PA3570	*mmsA*	−16	−1.4	11.3	Methylmalonate-semialdehyde dehydrogenase
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		−29.9	1.1	29.9	Cytochrome *c* oxidase subunit (cbb3-type)
PA14_10490	PA4134		−16	−1.3	13	Hypothetical protein
Induced genes upon deleting *pyrF*						
–	AF241171		4.9	1.1	−6.1	No significant similarity
PA14_00570	PA0045		4.6	1.1	−3.7	Hypothetical protein
PA14_00580	PA0046		4.6	1.1	−4.3	Hypothetical protein
PA14_03830	PA0293	*aguB*	10.6	−1.1	−9.8	*N*-carbamoylputrescine amidohydrolase
PA14_06420	PA0492		9.8	−2.1	−24.3	Conserved hypothetical protein
PA14_06430	PA0493		7	−1.6	−10.6	Probable biotin-requiring enzyme
PA14_06480	PA0496		6.5	−1.6	−8.6	Conserved hypothetical protein
PA14_54520	PA0755	*opdH*	12.1	1.4	−8.6	*Cis*-aconitate porin OpdH
PA14_54170	PA0782	*putA*	6.5	−1.1	−8.6	Proline dehydrogenase PutA
PA14_50770	PA1051		4.9	−1.2	−6.5	Probable transporter
PA14_46080	PA1420		5.3	1.7	−4	Hypothetical protein
PA14_46070	PA1421	*speB2*	9.8	−1.1	−9.8	Agmatinase
PA14_38170	PA2038		4.9	−1.3	−5.7	Hypothetical protein
PA14_35460	PA2252		7	1.2	−6.1	Probable Na/alanine/glycine symporter
PA14_27370	PA2840		4.9	−1.1	−3.7	Probable ATP-dependent RNA helicase
PA14_26910	PA2875		5.7	7	1.3	Conserved hypothetical protein
PA14_19470	PA3452	*mqoA*	5.3	1	−4.6	Malate:quinone oxidoreductase
PA14_17960	PA3582	*glpK*	6.1	−1.1	−5.7	Glycerol kinase
PA14_17930	PA3584	*glpD*	5.7	−1.1	−8	Glycerol-3-phosphate dehydrogenase
PA14_16010	PA3741		6.1	−1.1	−7.5	Hypothetical protein
PA14_13660	PA3885		5.7	1.5	−1.9	Hypothetical protein
PA14_11150	PA4077		7.5	4.9	−1.3	Probable transcriptional regulator
PA14_09660	PA4198		16	1.1	−13.9	Probable AMP-binding enzyme
PA14_67190	PA5088		7.5	2	−3	Hypothetical protein
PA14_67860	PA5139		4.9	−2.3	−10.6	Hypothetical protein
PA14_71890	PA5445		4.9	−1.1	−7.5	Probable coenzyme A transferase
PA14_72180	PA5469		7	−1.6	−10.6	Conserved hypothetical protein
PA14_72650	PA5506		6.5	1.9	−4	Hypothetical protein
PA14_72700	PA5509		6.1	1.1	−5.3	Hypothetical protein
PA14_72960	PA5530		4.9	1.1	−4.6	Probable MFS dicarboxylate transporter

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 with 1 mM uracil versus 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
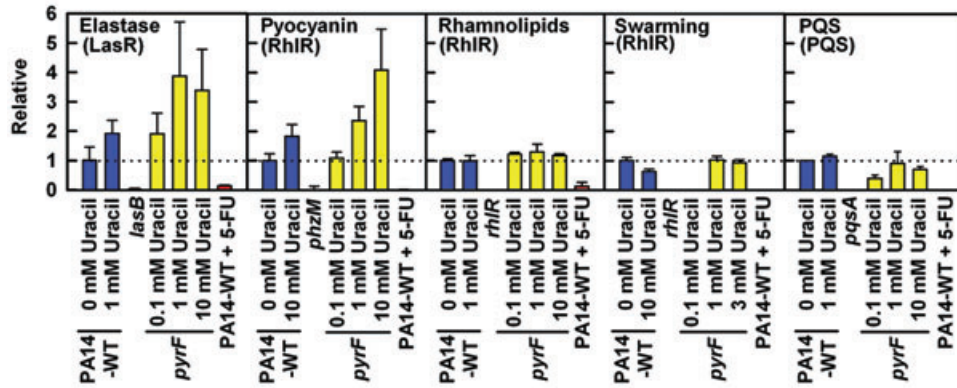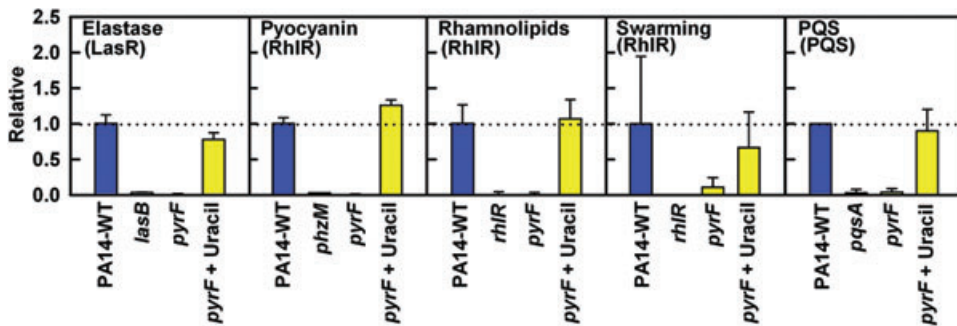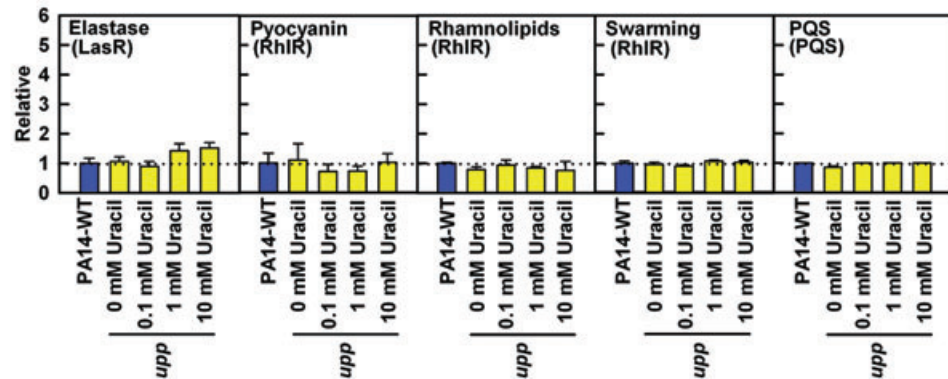
Fig. 3. Uracil and 5-fluorouracil control QS phenotypes.
A. Effect of uracil addition (0.1–10 mM) on wild-type *P. aeruginosa* PA14 and the *pyrF* mutant and of addition of 10 μM 5-fluorouracil to the wild-type strain in M9 glucose medium for LasB elastase activity, pyocyanin production, rhamnolipid production, swarming motility and PQS production. Negative controls were *lasB* (for elastase), *phzM* (for pyocyanin), *rhlR* (rhamonlipid 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.
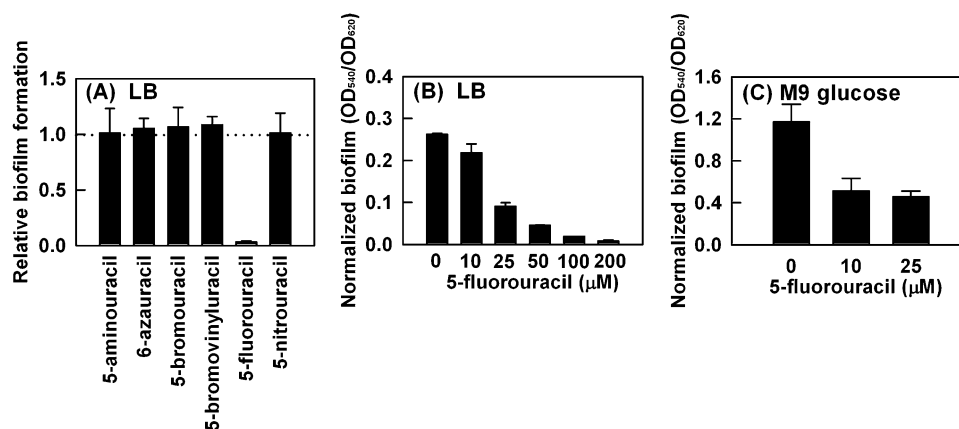
Fig. 4. 5-Fluorouracil inhibits biofilm formation.
A. Biofilm formation of wild-type *P. aeruginosa* PA14 upon addition of the uracil analogues 5-aminouracil, 6-azauracil, 5-bromouracil, 5-bromovinyluracil, 5-fluorouracil and 5-nitrouracil in LB medium. Each analogue was tested at 200 µM. Biofilm formation (OD$_{540}$) was normalized by cell growth (OD$_{620}$), and relative biofilm formation was calculated as the ratio of uracil analogue treatment to no treatment.
B. Biofilm formation of wild-type *P. aeruginosa* upon addition of 5-fluorouracil in LB medium. Biofilm formation was examined after 24 h, and 10 wells were used for each culture. Biofilm formation (OD$_{540}$) was normalized by cell growth (OD$_{620}$), and data show the average of the two independent experiments ± SD.
C. Biofilm formation of wild-type *P. aeruginosa* upon addition of 5-fluorouracil in M9 glucose medium. Biofilm formation was examined after 24 h, and 10 wells were used for each culture. Biofilm formation (OD$_{540}$) was normalized by cell growth (OD$_{620}$), and data show the average of the two independent experiments ± SD.

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 µM (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, and 200 µM 5-FU inhibited biofilm formation 33-fold with a 50% 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-

lular 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 rhamnolipids production) (Figs 2 and 3) without growth inhibition (Fig. S1B). These findings support 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 auto-inducers induced a variety of the virulence factors, such as alkaline proteases (*aprADI*), phenazine biosynthesis (*phzACDEFGMS*), chitinase (*cpbD*), 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*, *pyrBCDIL* 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), ursolic acid (Ren *et al.*, 2005) and indole derivatives (Lee *et al.*, 2007b). Furthermore, 5-FU is one of the few known antivirulence 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′-GGGTGAAGGTCGGCAAGGAACTCTT-3′) and PA14_26890-VR (5′-GGAGAATCTCATCGACCGCCTTCAG-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′-TACAGTTTACGAACCGAACA GGC-3′) and chromosomal primer PA14_26890-VF and using transposon-specific primer R1 (5′-ATCGACCCAAGTA CCGCCAC-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 (OD$_{540}$/OD$_{620}$).

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-nitrouracil (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); rhamnose 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 MgSO$_4$, 10 μM FeSO$_4$, 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 F$_{254}$ 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. Germinated 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 biofilms for PA14 versus the *pyrF* mutant, in biofilms for the *pyrF* mutant versus the *pyrF* mutant with 1 mM uracil and in biofilms 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 RNeasy 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 transcription 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 expression 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 Biotechnology 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 chromatograph (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).

Acknowledgements

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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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