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Interkingdom adenosine signal reduces *Pseudomonas aeruginosa* pathogenicity

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Summary

Pseudomonas aeruginosa **is becoming recognized as an important pathogen in the gastrointestinal (GI) tract. Here we demonstrate that adenosine, derived from hydrolysis of ATP from the eucaryotic host, is a potent interkingdom signal in the GI tract for this pathogen. The addition of adenosine nearly abolished** ***P. aeruginosa*** **biofilm formation and abolished swarming by preventing production of rhamnolipids. Since the adenosine metabolite inosine did not affect biofilm formation and since a mutant unable to metabolize adenosine behaved like the wild-type strain, adenosine metabolism is not required to reduce pathogenicity. Adenosine also reduces production of the virulence factors pyocyanin, elastase, extracellular polysaccharide, siderophores and the** ***Pseudomonas*** **quinolone signal which led to reduced virulence with** ***Caenorhabditis elegans*****. To provide insights into how adenosine reduces the virulence of** ***P. aeruginosa*****, a whole-transcriptome analysis was conducted which revealed that adenosine addition represses genes similar to an iron-replete condition; however, adenosine did not directly bind Fur. Therefore, adenosine decreases** ***P. aeruginosa*** **pathogenicity as an interkingdom signal by causing genes related to iron acquisition to be repressed.**

Introduction

Bacteria communicate via chemical signals (Fuqua *et al.*, 1994; Chen *et al.*, 2002) and within the gastrointestinal (GI) tract, where 500–1000 different bacterial species interact (Xu and Gordon, 2003), bacterial signals influence the host. For example, indole from commensal *Escherichia coli* strengthens the epithelial cell barrier of the host (Bansal *et al.*, 2010), and the *Pseudomonas aeruginosa* quorum sensing molecule 3-oxododecanoyl homoserine lactone is a putative ligand for peroxisome proliferator-activated receptors for human lung epithelial cells (Jahoor *et al.*, 2008). Similarly, host signals in the GI tract influence bacterial behaviour; for example, noradrenaline is a signal for enterohaemorrhagic *E. coli* since it increases chemotaxis, motility, surface colonization and attachment of the bacterium (Bansal *et al.*, 2007). Noradrenaline is also a signal for the opportunistic pathogen *P. aeruginosa* since it stimulates its growth (Freestone *et al.*, 1999) and expression of its virulence determinant PA-I lectin (Alverdy *et al.*, 2000). Therefore, interkingdom signalling is important in the GI tract.

Within the GI tract, adenosine contributes to the secretion of electrolytes, the downregulation of inflammation and protection against ischemic injury (Roman and Fitz, 1999; Haskó and Cronstein, 2004). Adenosine is released in copious amounts into the lumen in a rabbit model of enteropathogenic *E. coli* (EPEC) infection (Crane and Shulgina, 2009), and it is generated by the breakdown of secreted ATP (Crane *et al.*, 2002). As with noradrenaline, adenosine is recognized by bacteria as it induces production of the PA-I lectin in *P. aeruginosa* which plays an important role in disruption of the barrier function of epithelial cells (Kohler *et al.*, 2005; Patel *et al.*, 2007). Adenosine also stimulates EPEC growth (Crane and Shulgina, 2009) and enables *Staphylococcus aureus* and *Bacillus anthracis* to escape phagocytic clearance (Thammavongsa *et al.*, 2009). These studies clearly demonstrate the relevance of adenosine in the context of GI tract infections.

Along with increasing adenosine concentrations, *Pseudomonas* sp. populations increase dramatically in patients with severe systemic inflammatory response syndrome (Shimizu *et al.*, 2006). Although the opportunistic pathogen *P. aeruginosa* is better known as a respiratory and wound pathogen, up to 12% of the normal population carry *P. aeruginosa* in the GI tract (Bodey *et al.*, 1983). Animal studies have shown that direct introduction of *P. aeruginosa* into the caecum of normal mice does not lead to death (Laughlin *et al.*, 2000); however,

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Pseudomonas sp. levels have been shown to increase by as much as 100-fold while beneficial bacteria are significantly decreased in patients with severe systemic inflammatory response syndrome (Shimizu *et al*., 2006). *Pseudomonas aeruginosa* has also been shown to lead to mortality rapidly when injected into the mouse stomach (Schook *et al*., 1976). In fact, the mere presence of *Pseudomonas* group in the GI tract of critically ill surgical patients has been associated with a nearly threefold increase in mortality (Marshall *et al*., 1993). Moreover, *P. aeruginosa* induces one of the most rapid and significant decreases in transepithelial electrical resistance compared with other bacteria (Kohler *et al*., 2005).

Since iron is essential for bacterial growth and found at extraordinarily low levels in the host (10^{-18} M) (Sritharan, 2006), the genes for its acquisition are highly regulated (Venturi *et al*., 1995; Ochsner *et al*., 2002; Cornelis *et al*., 2009). To this end, the ferric uptake regulator (Fur) regulates many iron-related genes including virulence genes in many bacteria (Sheikh and Taylor, 2009). For example, in *Vibrio cholera*, an important virulence factor, toxin-coregulated pilus, is positively regulated by Fur (Mey *et al*., 2005). Also, Shiga and Shiga-like toxins are induced by low iron concentrations in *E. coli* (Calderwood and Mekalanos, 1987). In *P. aeruginosa*, not only the siderophores pyoverdine and pyochelin but also several virulence factors (e.g. exotoxin A, elastase and haemagglutinin) are modulated by iron and Fur (Bjorn *et al*., 1979).

Here we demonstrate that adenosine has several diverse effects on *P. aeruginosa* which include reducing biofilm formation dramatically due to abolished swarming as well as reducing virulence factors and pathogenicity in an animal model. Through a whole-transcriptome approach, we determined that the mechanism for these effects is that adenosine causes virulence genes related to iron acquisition to be repressed.

Results

Our hypothesis was that adenosine has a significant effect on *P. aeruginosa* physiology. To test this, we assayed the effect of adenosine (10 mM) on biofilm formation and other virulence factors. This concentration of adenosine was chosen based on that (i) 5 mM adenosine is estimated to be present in the lumen of the intestine (Kimura *et al*., 2005), (ii) there is a 400% increase in *P. aeruginosa* PA-I lectin expression upon exposure to 10 mM adenosine (Patel *et al*., 2007), and (iii) 2.4 μM adenosine was found in the lumen of 10 individuals with Crohn's disease or ulcerative colitis (Egan *et al*., 1999), while the extracellular adenosine concentration can increase 10^9-fold in human intestinal epithelial cells under hypoxic conditions as a result of increased conversion of adenosine monophosphate to adenosine by elevated 5′-ectonucleasidase and reduced activity of adenosine deaminase and adenosine kinase (Patel *et al*., 2007).

Unmetabolized adenosine decreases biofilm formation

The addition of 10 mM adenosine decreased the specific growth rate of PA14 by 25% (1.01 ± 0.06 h^{-1} versus 1.35 ± 0.01 h^{-1} for no adenosine) in LB medium. More significantly, 10 mM adenosine nearly abolished static biofilm formation in 96-well plates (25-fold decrease) (Fig. 1A). Similarly, adenosine reduced biofilm formation by 2.5-fold in M9 glucose medium (Fig. 1A). We also investigated whether adenosine could induce biofilm dispersal. However, no significant effect was observed by the addition of adenosine.

Because *P. aeruginosa* can metabolize adenosine to inosine via its adenosine deaminase (PA0148 protein) (Heurlier *et al*., 2006) (Fig. S1), we examined whether inosine could also regulate biofilm formation like adenosine. However, inosine up to 10 mM had little effect on *P. aeruginosa* PA14 biofilm formation (Fig. 1B). Additionally, the adenosine deaminase mutant was also used to test its response to adenosine. The results showed adenosine decreases adenosine deaminase mutant biofilm formation as well as wild-type PA14 (Fig. 1A). Therefore, adenosine dramatically reduces *P. aeruginosa* biofilm formation in a manner that does not depend on its metabolism.

To examine how adenosine affects biofilm architecture as well as to study the effect of adenosine under flow conditions, biofilm formation was tested using a flow cell chamber. Addition of adenosine almost abolished biofilm formation (Fig. 1C). These results were quantified using COMSTAT statistical analysis which showed a 68 ± 21-fold decrease in biomass, a 109 ± 24-fold decrease in thickness and a 127 ± 13-fold decrease in maximum substratum coverage upon addition of adenosine (Table S1).

Adenosine abolishes swarming by reducing rhamnolipid production

Since motility is usually directly related to infection and biofilm formation (Feldman *et al*., 1998; O'Toole and Kolter, 1998), we investigated the effect of adenosine on swimming and swarming motility. No distinct effect of adenosine was observed on swimming; however, swarming was abolished at 1 mM (Fig. 2A). In addition, rhamnolipids are essential for *P. aeruginosa* swarming (Caiazza *et al*., 2005), so we investigated the effect of adenosine on rhamnolipids production on semisolid surfaces and found the rhamnolipid zone was reduced significantly at 1 mM and abolished at 10 mM adenosine (Fig. 2B). As expected, based on its lack of impact on biofilm formation,

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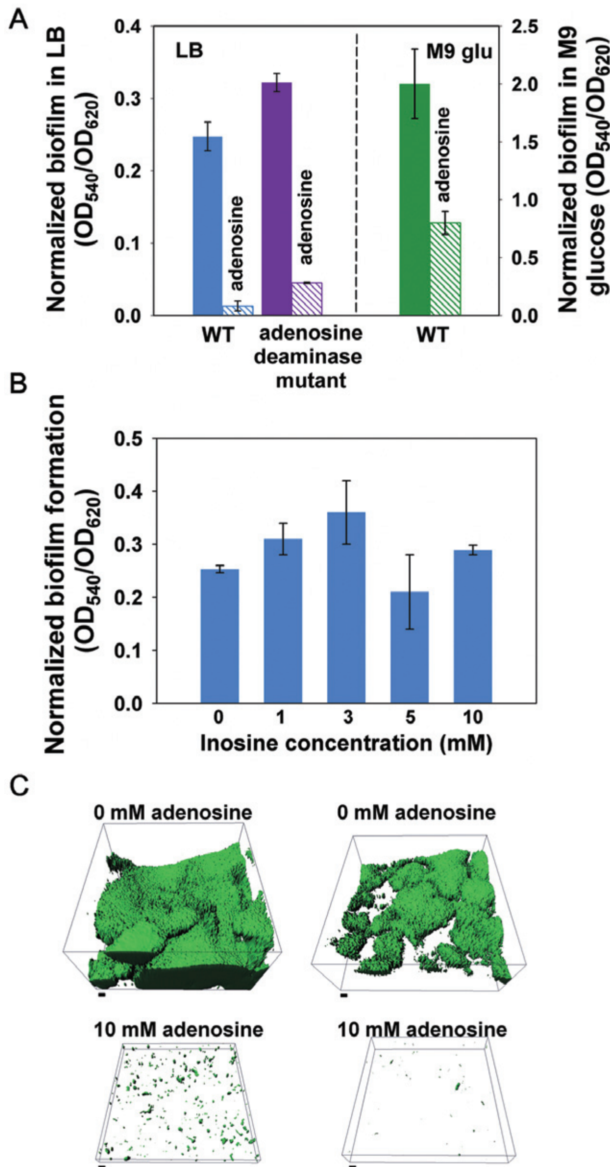

Fig. 1. Normalized biofilm formation with adenosine and inosine.
A and B. Total biofilm formation was assayed at 37°C after 24 h in 96-well plates without shaking with 10 mM adenosine in LB and M9 glucose media (A) and with 0, 1, 3, 5 and 10 mM inosine in LB medium (B). Six wells were used for each strain. To remove growth effects, biofilm formation was normalized by dividing total biofilm by the turbidity at 620 nm for each strain.
C. Biofilm formation at 37°C after 6 days in 5% LB with 0 and 10 mM adenosine in flow cells. Two representative IMARIS images of each condition are shown. Scale bars represent 10 μm. Data are from two independent experiments.

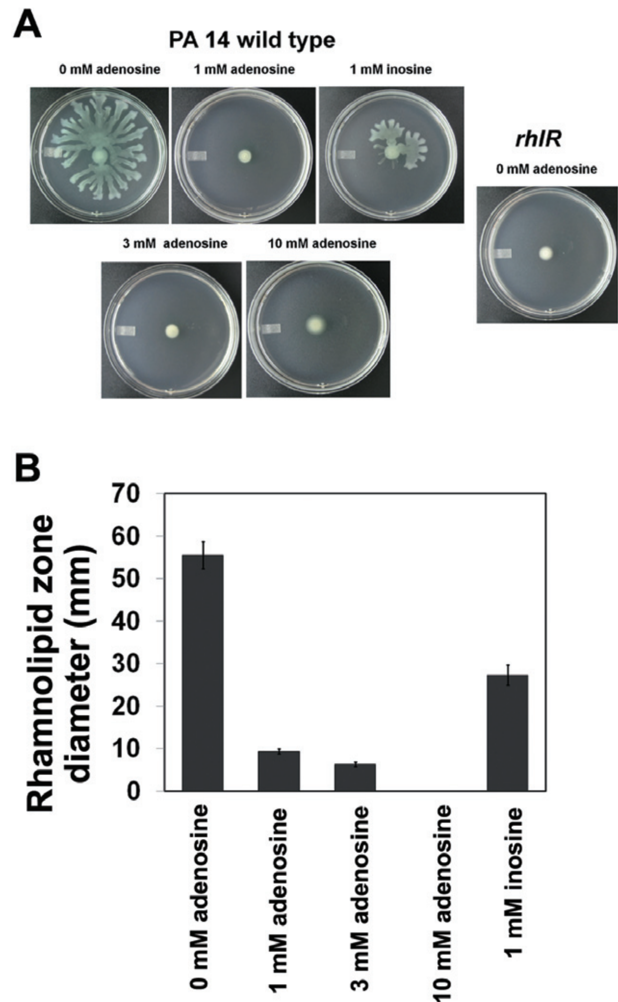

Fig. 2. Swarming motility and rhamnolipid production with adenosine.
A. PA14 was spotted onto BM2 agar plates containing 1, 3 and 10 mM adenosine and 1 mM inosine. The isogenic *rhlR* mutant was used as a negative control.
B. The rhamnolipid zone diameter was measured after 20 h at 37°C. Three swarming plates were used for each culture. Data are from two independent cultures.

1 mM inosine did not reduce swarming (Fig. 2A) and rhamnolipid production (Fig. 2B) to the extent seen with adenosine. Therefore, adenosine abolishes the swarming of *P. aeruginosa* by preventing rhamnolipid production. These significant effects are due to the specific action of adenosine.

Adenosine reduces pyocyanin, elastase, extracellular polysaccharide (EPS), Pseudomonas *quinolone signal (PQS) and siderophore production*

Since adenosine is produced in the GI tract where it comes in contact with *P. aeruginosa* (Patel *et al*., 2007), we investigated the effect of adenosine on several additional virulence phenotypes. The production of pyocyanin was decreased 2.6 ± 0.1-fold after 14 h (Fig. 3A). Elastase production was also decreased 2.1 ± 0.2-fold after 6 h (Fig. 3B). Similarly, with adenosine, EPS levels were decreased 7.5 ± 0.6-fold (Fig. 3C), PQS was reduced 5.5 ± 0.2-fold (Fig. 3D), and siderophore pyoverdine production reduced (Fig. 3E). Confirming this result, extracellular siderophore production was examined on

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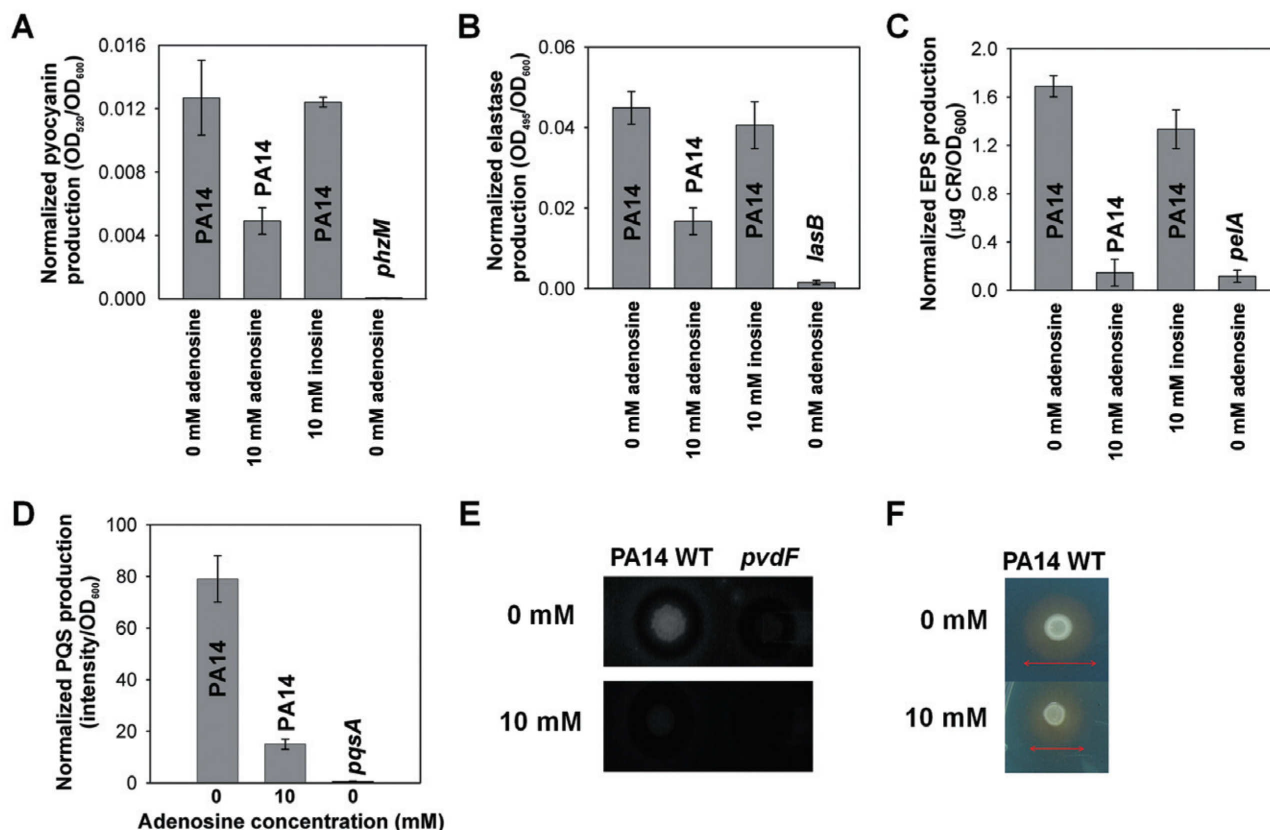

Fig. 3. Virulence factor production with adenosine. Changes in the levels of (A) pyocyanin, (B) elastase, (C) EPS, (D) PQS, (E) pyoverdine and (F) siderophore in *P. aeruginosa* PA14 in the presence of 10 mM adenosine. Data are from two independent experiments.

chrome azurol S (CAS) agar plates where adenosine decreased siderophore production by 1.5-fold after 16 h (Fig. 3F). Moreover, inosine up to 10 mM had little effect on the production of pyocyanin, elastase and EPS (Fig. 3A–C). Therefore, additional adenosine in LB medium decreased consistently pyocyanin production, elastase activity, EPS production, PQS production and pyoverdine production.

Adenosine reduces the pathogenicity of P. aeruginosa *to* Caenorhabditis elegans

To further characterize the effect of adenosine on the ability of PA14 to act as a pathogen, we investigated infection with *Caenorhabditis elegans* (Tan *et al*., 1999) as the animal model. The *C. elegans* slow-killing model involves an infection-like process and correlates with the accumulation of PA14 within worm intestines (Tan *et al*., 1999). In addition, many of the *P. aeruginosa* virulence factors are required for slow-killing (Tan *et al*., 1999).

L4 stage hermaphrodite worms were exposed to lawns of PA14 grown on NGM agar plates with or without adenosine for 10 days [the lifespan of adult *C. elegans* is approximately 10 days (Wolkow *et al*., 2000)]. In the first 3 days, the death of the worms was not due to adenosine; however, after 3 days, our results indicate that PA14 with adenosine is much less pathogenic to *C. elegans* (Fig. 4) since adenosine reduced PA14 killing by 3 ± 1-, 3.3 ± 0.7- and 1.9 ± 0.5-fold at 120 h, 144 h and 168 h respectively. Hence, with adenosine, the death rate with *P. aeruginosa* was reduced to that basically of normal worm death in the presence of the non-virulent control strain *E. coli* OP50. Note that adenosine had no effect on worm death with *E. coli* OP50 (results not shown); hence, adenosine did not affect *C. elegans*.

Adenosine represses the iron regulon

To determine the mechanism by which adenosine affects *P. aeruginosa* physiology, we performed a whole-transcriptome analysis. Exposure to 10 mM adenosine for 7 h altered significantly the expression of 281 genes as compared with the untreated control (Table 1). Of these, 88 genes were induced, while 193 genes were repressed. As expected, induced genes included those for xanthine dehydrogenase (*xdhA* and *xdhB*, induced 13-fold and 9.8-fold respectively) that are involved in adenosine metabolism from hypoxanthine to uric acid (Fig. S1). In addition, two genes that are involved in the degradation of anthranilate (*antA* and *antB*), a precursor of PQS (Farrow and

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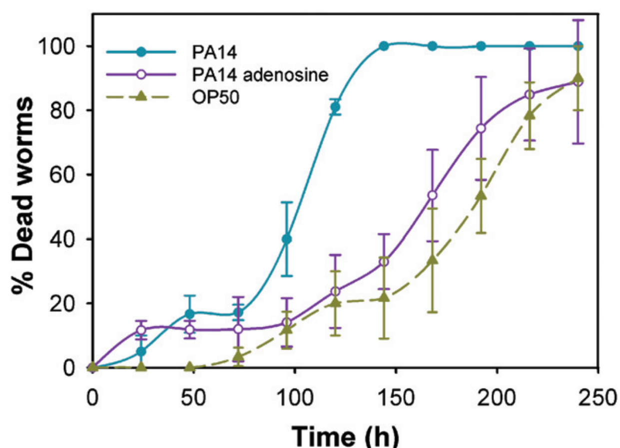

Fig. 4. Killing of *C. elegans* by *P. aeruginosa* in the present of adenosine on NGM agar plates. L4 stage hermaphrodite worms were exposed to wild-type *P. aeruginosa* PA14 grown on NGM medium with (blue closed circles) or without (purple open circles) 10 mM adenosine. *Escherichia coli* OP50 on the same medium plates was used as the negative control (yellow closed triangles). Data represent the mean ± standard deviation; $n = 3$ plates, 20 worms per plate.

Pesci, 2007), were induced 3.5- and 3.0-fold, suggesting that adenosine decreased PQS production by increasing degradation of its precursor.

In addition, 79 genes related to iron acquisition were repressed (Tables 1 and 2) including those involved in pyoverdine biosynthesis (31 genes), pyochelin biosynthesis (*pchEFG*), a siderophore receptor (*pirA*), a two-component response regulator (*pfeR*), the ferric enterobactin receptor precursor (*pfeA*), a ferric enterobactin transporter (*fepG*), an outer membrane receptor for iron transport (PA4514) and an outer membrane haem receptor (*phuR*). These iron-related genes regulated by adenosine compared well to the iron regulon identified by deleting PA2384 (a hypothetical protein involved in the positive regulation of iron uptake) (Zheng *et al.*, 2007) and identified by high iron concentrations (Ochsner *et al.*, 2002) (Table 2). Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) confirmed the main microarray results; i.e. repression of PA2384, *pvdF*, *pvdS* and *pchE* (Table S2).

Several other virulence-related genes were also significantly repressed by 10 mM adenosine (Table 1). These include exotoxin A regulation gene *toxR*, motility and attachment genes *ygcA* and PA2407, protein secretion genes *icmF1* and PA0687, and alkaline protease and secretion genes *aprDEF*.

Adenosine does not bind Fur

Since 79 genes were repressed by adenosine involved in iron acquisition including siderophores, proteases, exotoxin A and haem/haemoglobin utilization, since adenosine represses virulence and PQS, and since several bacteria regulate their iron acquisition systems and virulence via Fur (Sheikh and Taylor, 2009), we checked adenosine binding to Fur. To test this, we purified wild-type Fur (Fig. S3A and B) and used electrophoretic mobility shift assays (EMSA) with a 178 bp fragment of the *pvdS* promoter which contains a Fur box (Fig. S2A); *pvdS* encodes a positive regulatory factor for pyoverdine siderophore production and is repressed by Fur binding (Ochsner *et al.*, 1995). Our microarray results also showed *pvdS* was repressed 28-fold upon the addition of adenosine (Table 1). Although we confirmed that Fur binds the *pvdS* promoter, there was not a significant shift in the promoter fragment upon addition of 100 μM adenosine (Fig. S3C). Similar EMSA results were obtained with a 205 bp *pchR* promoter region and a 156 bp *fagA* promoter fragment of the (Fur-associated gene A) promoter which contain a Fur box (Fig. S2B); purified Fur binds the *fagA* promoter but there was not a large increase in binding upon addition of adenosine (Fig. S3D). *fagA* was repressed 20-fold upon the addition of adenosine in our transcriptome study (Table 2), and *fagA* is repressed 120-fold in the presence of high iron concentrations (Ochsner *et al.*, 2002). Furthermore, Fur binds this promoter (Hassett *et al.*, 1997); hence, this promoter is regulated by Fur under iron-replete conditions. The inability of adenosine to bind to dimeric Fur was also confirmed using isothermal titration calorimetry.

Discussion

Adenosine from the host behaves as an interkingdom signal which results in a dramatic reduction in biofilm formation in flow cells via a reduction of swarming/rhamnolipid production and EPS. The inhibition of biofilm formation with adenosine is important as biofilms cause persistent infections that are responsible for many human diseases related to bacteria (Costerton *et al.*, 1999); therefore, adenosine may have some utility as a non-toxic therapeutic in treating biofilm infections. In addition, several other virulence factors were decreased by adenosine including pyocyanin, pyoverdine, elastase, EPS and PQS. Corroborating this reduction in a wide range of virulence factors, adenosine reduced the ability of *P. aeruginosa* to kill *C. elegans* in a slow-killing assay. All these significant effects are due to the specific action of adenosine rather than its degradation product and structural analogue inosine. This is the first report of changes in these virulence factors (swarming, rhamnolipids, biofilm formation) and pathogenicity with adenosine.

Our results indicate adenosine represses at least 79 genes in *P. aeruginosa* that are related to iron acquisition (Table 2). It appears that through some unknown mechanism, adenosine represses the virulence genes controlled

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Table 1. Partial list of differentially expressed genes (greater than 2.4-fold) after 7 h in LB medium upon addition of 10 mM adenosine versus no adenosine for PA14.

Gene ID	Gene name	Fold change	Description
Nucleotide biosynthesis and metabolism			
PA1523	*xdhB*	9.8	Xanthine dehydrogenase
PA1524	*xdhA*	13.0	Xanthine dehydrogenase
Pyoverdine synthesis and transport			
PA2384		–18.4	Hypothetical protein
PA2385	*pvdQ*	–7.0	Probable acylase
PA2386	*pvdA*	–26.0	L-ornithine N5-oxygenase
PA2389		–3.0	Conserved hypothetical protein
PA2390		–3.0	Probable ATP-binding/permease fusion ABC transporter
PA2392	*pvdP*	–3.2	Hypothetical protein
PA2393		–17.1	Probable dipeptidase precursor
PA2394	*pvdN*	–7.5	Probable aminotransferase
PA2395	*pvdO*	–5.7	Hypothetical protein
PA2396	*pvdF*	–8.0	Hypothetical protein
PA2397	*pvdE*	–6.1	Pyoverdine biosynthesis protein PvdE
PA2398	*fpvA*	–8.6	Ferripyoverdine receptor
PA2399	*pvdD*	–4.9	Pyoverdine synthetase D
PA2400	*pvdJ*	–7.0	Probable non-ribosomal peptide synthetase
PA2401		–7.0	Probable non-ribosomal peptide synthetase
PA2402		–4.6	Probable non-ribosomal peptide synthetase
PA2408		–7.5	Probable ATP-binding component of ABC transporter
PA2409		–2.8	Probable permease of ABC transporter
PA2411		–5.7	Probable thioesterase
PA2412		–16.0	Conserved hypothetical protein
PA2413	*pvdH*	–3.5	Probable class III aminotransferase
PA2424	*pvdL*	–22.6	Probable non-ribosomal peptide synthetase
PA2425	*pvdG*	–4.3	Probable thioesterase
PA2426	*pvdS*	–27.9	Sigma factor PvdS
Pyochelin synthesis and transport			
PA4218		–3.7	Probable transporter
PA4219		–4.0	Hypothetical protein
PA4220		–4.3	Hypothetical protein
PA4221	*fptA*	–2.6	Fe(III)-pyochelin receptor precursor
PA4222		–3.5	Probable ATP-binding component of ABC transporter
PA4223		–4.6	Probable ATP-binding component of ABC transporter
PA4224	*pchG*	–4.0	Hypothetical protein
PA4225	*pchF*	–4.9	Pyochelin synthetase
PA4226	*pchE*	–4.3	Dihydroaeruginoic acid synthetase
Attachment and motility			
PA0993	*ygcA*	–5.3	Probable pili assembly chaperone
PA2407		–4.0	Probable adhesion protein
Iron transport or receptor genes			
PA4880		8.6	Probable bacterioferritin
PA0931	*pirA*	–3.5	Siderophore receptor protein
PA1302		–2.8	Probable haem utilization protein precursor
PA2688	*pfeA*	–12.1	Ferric enterobactin receptor precursor PfeA
PA4161	*fepG*	–5.3	Ferric enterobactin transport protein FepG
PA4514		–4.3	Probable outer membrane receptor for iron transport
PA4709	*phuS*	–4.3	Probable haem-degrading factor
PA4710	*phuR*	–4.0	Probable outer membrane haem receptor
Virulence-related genes			
PA0077	*icmF1*	–7.0	Protein secretion/export apparatus
PA0687		–4.6	Probable type II secretion system protein
PA1246	*aprD*	–4.3	Alkaline protease secretion protein AprD
PA1247	*aprE*	–4.6	Alkaline protease secretion protein AprE
PA1248	*aprF*	–5.7	Alkaline protease secretion protein AprF
Transcriptional regulators			
PA0547		4.9	Probable transcriptional regulator
PA3221	*casA*	4.0	CsaA protein
PA0675		–5.3	Probable sigma-70 factor, ECF subfamily
PA0707	*toxR*	–3.2	Transcriptional regulator ToxR
PA1300		–13.0	Probable sigma-70 factor, ECF subfamily
PA1912		–3.2	Probable sigma-70 factor, ECF subfamily
PA2312		–2.6	Probable transcriptional regulator

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Table 1. *cont.*

Gene ID	Gene name	Fold change	Description
PA3410		−6.1	Probable sigma-70 factor, ECF subfamily
PA0472		−2.8	Probable sigma-70 factor, ECF subfamily
PA2468	*foxI*	−2.6	Probable sigma-70 factor, ECF subfamily
PA2359		−7.0	Probable transcriptional regulator
Others			
PA2466	*foxA*	−19.7	Probable TonB-dependent receptor
PA2512	*antA*	3.5	Anthranilate dioxygenase large subunit
PA2513	*antB*	3.0	Anthranilate dioxygenase small subunit
PA2686	*pfeR*	−3.7	Two-component response regulator PfeR
PA4468	*sodM*	−16.0	Superoxide dismutase
PA4168	*fpvB*	−3.5	Probable TonB-dependent receptor

by Fur. The global transcriptional regulatory protein, Fur, controls iron homeostasis in most bacteria (Andrews *et al*., 2003) (at least in 170 different genera based on a protein cut-off of 57% identity), so our results here in regard to adenosine may be applicable to many strains.

Our results showing adenosine decreases the level of the *P. aeruginosa* virulence factors biofilm formation, swarming, rhamnolipids, pyocyanin, pyoverdine, elastase, EPS, and PQS and showing reduced *C. elegans* killing are in contrast to the increase in the levels of the *P. aeruginosa* virulence factor PA-I lectin/adhesion upon exposure to adenosine (Patel *et al*., 2007). Moreover, these authors also observed that the adenosine metabolite inosine was approximately 10-fold more potent than adenosine in increasing PA-I expression whereas our results clearly show that inosine has little effect on the phenotypes tested. Since the mammalian transcription factor hypoxia-inducible factor (HIF)-1alpha has been linked to increased PA-I expression (Patel *et al*., 2007), it is possible that adenosine specifically upregulates PA-I through a HIF-1alpha-dependent mechanism. Furthermore, we found adenosine decreases the specific growth rate of PA14 by 25% in LB medium, whereas adenosine stimulates EPEC growth in several types of media (Crane and Shulgina, 2009). Also, the effect of adenosine on the expression of several EPEC-secreted proteins, such as virulence factors EspA and EspB, is biphasic, with stimulation at lower adenosine concentrations and an inhibition at higher concentrations (Crane and Shulgina, 2009). In addition, Thammavongsa and colleagues (2009) showed Gram-positive *S. aureus* and *B. anthracis* avoid the host immune response in a manner dependent on adenosine which was converted from adenosine monophosphate by adenosine synthase A, a cell wall-anchored enzyme. However, our data indicate *P. aeruginosa* with adenosine has reduced pathogenicity in the animal model. Although speculative, this reduced pathogenicity in the GI tract may allow the bacterium to leave the GI tract. Hence, the effects of adenosine on growth and virulence differ depending on the bacterium and medium.

The regulation of swarming is complex and includes quorum sensing (Daniels *et al*., 2004), iron (Hegde *et al*., 2009) and rhamnolipids (Caiazza *et al*., 2005). Adenosine appears to reduce swarming by reducing rhamnolipid production (Fig. 2B). Also, hypothetical protein PvdQ is essential for normal swarming behaviour (Overhage *et al*., 2008), and *pvdQ* was repressed sevenfold in our microarray result.

PQS is a virulence factor, iron chelator and a quorum sensing signal produced by *P. aeruginosa*, which also regulates the production of elastase, pyocyanin, rhamnolipids and biofilm development (Diggle *et al*., 2006). The decrease in the production of PQS with 10 mM adenosine may be due to the increased degradation of anthranilate, a precursor of PQS as evidenced by increased expression of genes that encode an enzyme for anthranilate degradation, *antA* and *antB*. Hence, adenosine is a quorum-quenching compound in that it reduces a quorum sensing compound, PQS, without causing severe toxicity, so cells have less chance of developing resistance to it (Rasko and Sperandio, 2010).

Experimental procedures

Bacterial strains and growth conditions

All strains and plasmids used in this study are listed in Table 3, and the primers are shown in Table 4. *Pseudomonas aeruginosa* PA14 wild type and its isogenic mutants were obtained from the Harvard Medical School (Liberati *et al*., 2006). *Pseudomonas aeruginosa* and *E. coli* were grown in Luria–Bertani (LB) medium (Sambrook *et al*., 1989) at 37°C except where indicated. Gentamicin (15 μg ml^{-1}), kanamycin (50 μg ml^{-1}) and carbenicillin (100 μg ml^{-1}) were used to maintain plasmids. Adenosine was used at 10 mM unless noted.

Biofilm assays

Static biofilm formation was examined in 96-well polystyrene plates (Lee *et al*., 2009) after 24 h. Biofilm formation in flow cells was examined as described previously (Ueda and

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Table 2. Iron-related genes repressed by 10 mM adenosine and compared with those repressed in the absence of PA2384 (hypothetical protein involved in the positive regulation of iron uptake) (Zheng *et al*., 2007) and repressed by at high iron concentrations (Ochsner *et al*., 2002).

		Fold change			
Gene ID	Gene name	Adenosine versus no adenosine	PA2384 mutant versus WT	High versus low iron	Descriptions
Pyoverdine synthesis and transport					
PA2384		–18.4		–148	Hypothetical protein
PA2385	*pvdQ*	–7.0		–11	Probable acylase
PA2386	*pvdA*	–26.0	–19.0	–216	L-ornithine N5-oxygenase
PA2389		–3.0	–3.6	–14	Conserved hypothetical protein
PA2390		–3.0		–14	Probable ATP-binding/permease fusion ABC transporter
PA2392	*pvdP*	–3.3	–4.7	–14	Hypothetical protein
PA2393	*pvdM*	–17.2	–8.0	–38	Probable dipeptidase precursor
PA2394	*pvdN*	–7.5	–3.8	–38	Probable aminotransferase
PA2395	*pvdO*	–5.7	–2.9	–38	Hypothetical protein
PA2396	*pvdF*	–8.0	–6.7	–45	Hypothetical protein
PA2397	*pvdE*	–6.1	–7.6	–33	Pyoverdine biosynthesis protein PvdE
PA2398	*fpvA*	–8.6	–2.6	–35	Ferripyoverdine receptor
PA2399	*pvdD*	–4.9	–4.2	–19	Pyoverdine synthetase D
PA2400	*pvdJ*	–7.0	–11.9	–30	Probable non-ribosomal peptide synthetase
PA2401		–7.0	–11.9	–30	Probable non-ribosomal peptide synthetase
PA2402		–4.6	–5.1	–30	Probable non-ribosomal peptide synthetase
PA2403		–5.3		–15	Hypothetical protein
PA2404		–8.0		–15	Hypothetical protein
PA2405		–5.3		–15	Hypothetical protein
PA2406		–6.1		–15	Hypothetical protein
PA2407		–4.0		–15	Probable adhesion protein
PA2408		–7.5		–15	Probable ATP-binding component of ABC transporter
PA2409		–2.8		–15	Probable permease of ABC transporter
PA2410		–3.0		–15	Hypothetical protein
PA2411		–5.7	–29.8	–126	Probable thioesterase
PA2412		–16.0	–14.2	–126	Conserved hypothetical protein
PA2413	*pvdH*	–3.5	–10.6	–65	Probable class III aminotransferase
PA2424	*pvdL*	–22.6	–7.9	–34	Probable non-ribosomal peptide synthetase
PA2425	*pvdG*	–4.3	–8.1	–34	Probable thioesterase
PA2426	*pvdS*	–27.9	–7.3	–177	Sigma factor PvdS
PA2427	*pvdY*	–6.5		–61	Hypothetical protein
Pyochelin synthesis and transport					
PA4175	*piv*	–2.8		–7	Probable endoproteinase Arg-C precursor
PA4218	*fptX*	–3.7	–47.8	–49	Probable transporter
PA4219	*fptC*	–4.0	–17.7	–49	Hypothetical protein
PA4220	*fptB*	–4.3	–48.8	–182	Hypothetical protein
PA4221	*fptA*	–2.6	–22.6	–182	Fe(III)-pyochelin receptor precursor
PA4222	*pchI*	–3.45	–26.8	–55	Probable ATP-binding component of ABC transporter
PA4223	*pchH*	–4.6	–9.0	–55	Probable ATP-binding component of ABC transporter
PA4224	*pchG*	–4.0	–12.4	–55	Hypothetical protein
PA4225	*pchF*	–4.9	–37.6	–55	Pyochelin synthetase
PA4226	*pchE*	–4.23	–85.0	–55	Dihydroaeruginoic acid synthetase
Haem uptake and utilization					
PA0672	*hemO*	–9.2	–9.6	–138	Hypothetical protein
PA0675		–5.3	–4.1		Probable sigma-70 factor, ECF subfamily
PA0676		–3.0			Probable transmembrane sensor
PA3410		–6.1	–2.1	–40	Probable sigma-70 factor, ECF subfamily
PA4708	*phuT*	–3.5	–2.0	–12	Hypothetical protein
PA4709	*phuS*	–4.3	–3.1	–12	Probable haem-degrading factor
PA4710	*phuR*	–4.0	–3.5	–7	Probable outer membrane haem receptor
Other iron-regulated genes					
PA0472		–2.8		–46	Probable sigma-70 factor, ECF subfamily
PA0707	*toxR*	–3.3		–8	Transcriptional regulator ToxR
PA0931	*pirA*	–3.5			Siderophore receptor protein
PA1134		–3.3		–7	Hypothetical protein
PA1245		–7.5		–12	Hypothetical protein
PA1246	*aprD*	–4.3		–12	Alkaline protease secretion protein AprD
PA1247	*aprE*	–4.6		–12	Alkaline protease secretion protein AprE
PA1248	*aprF*	–5.7		–12	Alkaline protease secretion protein AprF
PA1300		–13.0	–8.2	–46	Probable sigma-70 factor, ECF subfamily
PA1301		–4.6		–46	Probable transmembrane sensor

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Table 2. *cont.*

Gene ID	Gene name	Fold change: Adenosine versus no adenosine	PA2384 mutant versus WT	High versus low iron	Descriptions
PA1302		−2.8			Probable haem utilization protein precursor
PA1320	*cyoD*	−3.7		−4	Cytochrome o ubiquinol oxidase subunit IV
PA2033		−7.0	−3.9	−95	Hypothetical protein
PA2034		−4.9		−95	Hypothetical protein
PA2452		−4.9	−10.1	−52	Hypothetical protein
PA2460		−2.8			Hypothetical protein
PA2466	*foxA*	−19.7			Probable TonB-dependent receptor
PA2467	*foxR*	−3.3		−30	Probable transmembrane sensor
PA2468	*foxI*	−2.6		−30	Probable sigma-70 factor, ECF subfamily
PA2686	*pfeR*	−3.7			Two-component response regulator PfeR
PA2688	*pfeA*	−12.1			Ferric enterobactin receptor precursor PfeA
PA4161	*fepG*	−5.3			Ferric enterobactin transport protein FepG
PA4168	*fpvB*	−3.5			Probable TonB-dependent receptor
PA4467		−9.9	−33.9	−119	Hypothetical protein
PA4468	*sodM*	−16.0	−16.7	−119	Superoxide dismutase
PA4469		−16.0	−17.2	−119	Hypothetical protein
PA4470	*fumC*	−18.4	−9.9	−119	Fumarate hydratase
PA4471	*fagA*	−19.7	−20.3	−119	Hypothetical protein
PA4514		−4.3			Probable outer membrane receptor for iron transport
PA4570		−7.5	−10.0	−403	Hypothetical protein
PA4895		−2.6		−20	Probable transmembrane sensor

Wood, 2010). Simulated three-dimensional images were obtained using IMARIS software (BITplane, Zurich, Switzerland), and biofilm parameters were determined using COMSTAT (Heydorn *et al*., 2000). Two independent cultures were used for each of these experiments.

Total RNA isolation and microarray analysis

The *P. aeruginosa* genome array (Affymetrix, P/N 510596) was used to investigate the impact of adenosine on gene expression of wild-type PA14. Cells were harvested after incubating for 7 h, and RNA was extracted as described (Ren *et al*., 2004) with RNA*later* buffer (Applied Biosystems, Foster City, CA) to stabilize the RNA. cDNA synthesis, fragmentation, hybridizations and data analysis were as described previously (González Barrios *et al*., 2006). The microarray raw data are deposited at the Gene Expression Omnibus (GSE29665) of the National Center for Biotechnology Information.

qRT-PCR

qRT-PCR was performed with total RNA isolated from two independent cultures using the StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA). The housekeeping gene *rplU* (Kuchma *et al*., 2007) was used to normalize the gene expression data.

Swarming and rhamnolipid assays

BM2 swarming agar plates were used (Overhage *et al*., 2008), and the swarming motility pattern was observed after

Table 3. Strains and plasmids used in this study.

Strain	Genotype or description	Reference
P. aeruginosa		
PA14	Wild-type strain	Liberati *et al*. (2006)
PA14_01830 (PA0148)	PA14_01830 Ω *Mar2xT7*, Gm^R	Liberati *et al*. (2006)
PA14_16250 (PA3724, *lasB*)	PA14_16250 Ω *Mar2xT7*, Gm^R	Liberati *et al*. (2006)
PA14_09490 (PA4209, *phzM*)	PA14_09490 Ω *Mar2xT7*, Gm^R	Liberati *et al*. (2006)
PA14_33700 (PA2396, *pvdF*)	PA14_33700 Ω *Mar2xT7*, Gm^R	Liberati *et al*. (2006)
PA14_19120 (PA3477, *rhlR*)	PA14_19120 Ω *Mar2xT7*, Gm^R	Liberati *et al*. (2006)
PA14_24480 (PA3064, *pelA*)	PA14_24480 Ω *Mar2xT7*, Gm^R	Liberati *et al*. (2006)
PA14_51430 (PA0996, *pqsA*)	PA14_51430 Ω *Mar2xT7*, Gm^R	Liberati *et al*. (2006)
E. coli		
BL21(DE3)	F^- *ompT hsdS*$_B$($r_B^- m_B^-$) *gal dcm* λ(DE3) Ω p*lac*UV5::T7 polymerase	Novagen
OP50	*E. coli* B strain (uracil auxotroph)	Brenner (1974)
Plasmids		
pET28b	Km^R, PT7 expression vector	Novagen
pET28b-Fur-cHis	Km^R, PT7::*fur-cHis*	This study

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Table 4. Primers used for PCR amplification, qRT-PCR and sequence verification in this study.

Primer name	Nucleotide sequence (5′ to 3′)
Primers for qRT-PCR	
rplU-F	AGGTTACCGCTGAAGTGGTTT
rplU-R	CCGGTGATCTTGATTTCAGTG
PA2384-RT-F	CTGGCCCGGCTGAAAGTGAT
PA2384-RT-R	CACCAGGGACAGCGGCGTAC
pvdF-RT-F	CGAACTGTTGCCGGACCTGAAG
pvdF-RT-R	CGGTTTCCTTGACGACCTTGC
pvdS-RT-F	GCCGGAAACCTCGCACATCAAC
pvdS-RT-R	CGTGCAGGCGGTACATCTCG
pchE-RT-F	CGCCTGGGCATGGCCGACTTCTATC
pchE-RT-R	ACGCCCTCCTCCAGTTGGGTTTCC
Primers for PCR	
Fur-F-XbaI	CTAGTCTAGAAAGAAGGAGATATACCATGGTTGAAAATAGCGAACTTCG
Fur-R-HindIII	CCCAAGCTTCTAGTGGTGGTGGTGGTGGTGCTTCTTCTTGCGCACGTAGAGCACC
T7 promoter	TAATACGACTCACTATAGGG
T7 terminator	GCTAGTTATTGCTCAGCGG
PpvdS(EMSA)-F	ACATTGGCGCGGCCATCCTTCTG
PpvdS(EMSA)-R	GTGGGGTAAGACCCACACATGGCGC
PfagA(EMSA)-F	GTTTTCGTTTCCGCCTGTTCG
PfagA(EMSA)-R	CAGCCAGTACCAGTGATCC
PpchR(EMSA)-F	TCGAGATAGCGGCGGACGAAGG
PpchR(EMSA)-R	GCCCCTGCAGCGAATGAAAAAGC

Restriction enzyme sites underlined.

20 h. Three plates were tested for each culture, and two independent cultures were used. The production of rhamnolipids was also measured on the semisolid surfaces of the BM2 swarm agar plates. The diameter of the transparent zone surrounding the motility halo of PA14 control plates was determined by adding a drop of 0.5% methylene blue (Fisher Scientific, Fair Lawn, NJ, USA) (Caiazza *et al*., 2005). The *rhlR* mutant (Liberati *et al*., 2006) was used as the negative control for swarming and rhamnolipid.

Virulence factor assays

PA14 was grown for 7–14 h, and pyocyanin was extracted from the supernatants with chloroform, re-extracted with HCl and assayed spectrophotometrically (Essar *et al*., 1990). The PA14 *phzM* mutant (Liberati *et al*., 2006) was used as the negative control.

Elastase activity was determined (Ohman *et al*., 1980) after 6 h using elastin-Congo Red (MP Biomedicals, 101637). The PA14 *lasB* mutant (Liberati *et al*., 2006) was used as the negative control.

EPS production was quantified via Congo red staining (Lee *et al*., 2007). PA14 *pelA* (Liberati *et al*., 2006) was used as the negative control.

PQS was extracted and assayed using thin-layer chromatography (Attila *et al*., 2008). Synthetic PQS (Syntech Solution, San Diego, CA, USA) was used as a standard, and the PA14 *pqsA* mutant (Liberati *et al*., 2006) was used as the negative control. PQS levels were determined and photographed using a Versa Doc 3000 imaging system (Bio-Rad, Hercules, CA, USA).

Pyoverdine production was assayed by inspection under UV light (Martínez-Granero *et al*., 2005). Briefly, a single colony of PA14 was grown on LB agar plate with or without 10 mM adenosine for 16 h, then pyoverdine was observed by exposing the plates to UV light. The PA14 *pvdF* mutant (Liberati *et al*., 2006) was used as the negative control.

Siderophore production (pyoverdine and pyochelin) was assayed using CAS (Schwyn and Neilands, 1987). Briefly, overnight PA14 cells were washed and diluted with MM9 salts to a turbidity of 0.05. Diluted culture (1 μl) was spotted on CAS agar plates with or without 10 mM adenosine. After incubating for 16 h, the diameter of the orange halo on the blue agar plate was measured. Three CAS plates were used for each culture. The PA14 *pchE* mutant (Liberati *et al*., 2006) was used as the negative control for siderophore production. Two independent cultures were used in all of the virulence factor assays.

C. elegans *slow-killing assay*

To investigate the effect of adenosine on PA14, the *C. elegans* slow-killing assay was performed (Tan *et al*., 1999) using the wild-type Bristol N2 strain (*Caenorhabditis* Genetics Center). An overnight LB culture was used to inoculate bacterial cultures on NGM agar plates with 10 mM adenosine. Each plate was seeded with 20 early to mid-L4 stage hermaphrodite worms, and three replicates were used for each independent culture. The live worms were transferred onto fresh bacterial plates daily so that the bacterial lawn did not get thick; hence, there was good contact of the bacteria with adenosine on the agar plates. *Escherichia coli* OP50 was used as a negative control.

Fur purification

Primers Fur-F-XbaI and Fur-R-HindIII were designed to incorporate a XbaI restriction site at the 5′ end, a HindIII site at the 3′ end and a 6× His-tag at the C-terminus of the gene during the amplification of *fur* from *P. aeruginosa* PA14. The

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454 bp PCR-amplified fragment was cloned into the XbaI/HindIII site of expression vector pET28b(+) (Novagen, Madison, WI) to generate pET28b-Fur-cHis. The *fur* gene in pET28b-Fur-cHis is under the control of a T7 promoter. The pET28b-Fur-cHis plasmid was confirmed by DNA sequencing with the T7 promoter and T7 terminator primers.

Wild-type Fur was produced overnight in *E. coli* BL21 (DE3) with 1 mM IPTG at room temperature. The Fur protein was purified using a Ni-NTA resin (Qiagen, Valencia, CA) as described in the manufacturer's protocol. The Fur protein was dialysed against buffer (25 mM Tris-HCl, pH 7.6) at 4°C overnight. SDS-PAGE confirmed Fur was produced and was pure.

EMSA

EMSA was performed as described previously (Zhang *et al*., 2008). We choose three promoter regions (Fig. S2) containing the canonical Fur boxes for Fur binding and which had previously been shown to bind Fur: the *pvdS* promoter region (178 bp), the *fagA* promoter region (156 bp) and the *pchR* promoter region (205 bp). For the binding reaction, Fur (3.9 μM to 11.7 μM) was incubated at room temperature for 2 h with biotin-labelled target promoter (10 nM) and the non-specific competitor DNA (poly dI-dC, 1 μg) in 20 μl of 10 mM Tris (pH 7.5), 50 mM KCl, 1 mM DTT, 5% glycerol and 0.1 mg ml^{-1} BSA. Each experiment was performed at least twice.

Isothermal titration calorimetry (ITC)

Size exclusion chromatography (Superdex 76 26/60) was used to isolate dimeric Fur from monomeric Fur. ITC experiments were performed at 25°C using a VP-ITC microcalorimeter (Microcal). Dimeric FUR was equilibrated in protein buffer (100 mM Tris pH 8.0, 100 mM KCl and 1 mM TCEP) using size exclusion chromatography (Superdex 75 26/60; GE Healthcare) immediately prior to the ITC experiment. Adenosine was also solubilized in the protein buffer. To determine if adenosine binds FUR, 1 mM adenosine was titrated into 10 μM FUR. As a control, 1 mM adenosine was titrated into protein buffer alone. For each experiment, adenosine (10 μl per injection) was injected into the sample cell over a period of 20 s with a 250 s interval between titrations to allow for complete equilibration and baseline recovery. Twenty-eight injections were delivered during each experiment and the solution in the sample cell was stirred at 307 r.p.m. to ensure rapid mixing.

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Supporting information

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

Table S1. Biofilm COMSTAT flow cell measurements of *P. aeruginosa* PA14 in 5% LB with and without 10 mM adenosine after 6 days.

Table S2. qRT-PCR to confirm the main microarray results for repressed genes.

Fig. S1. Metabolites and precursors of adenosine. *Enzymes are from *P. aeruginosa*.

Fig. S2. Promoters used in this study.

A. Nucleotide sequence upstream of *pvdS* containing a Fur binding site (boxed). Primers used to amplify the promoter fragment are underlined, and the start codon ATG is shown in bold.

B. Nucleotide sequence upstream of *fagA*. Fur binding sites are boxed, primers used to amplify the promoter fragment are underlined and the start codon ATG is shown in bold.

C. Nucleotide sequence upstream of *pchR*. Fur binding sites are boxed, primers used to amplify the promoter fragment are underlined and start codon ATG is shown in bold.

Fig. S3. Fur binding to Fur box containing promoters with adenosine.

A. Induction of Fur expression: lane 1: protein marker; lane 2: whole cell lysate from *E. coli* BL21(DE3)/pET28b; and lane 3: whole cell lysate from *E. coli* BL21(DE3)/pET28b-Fur-cHis after IPTG induction.

B. Purification of native Fur: lane 1: protein marker; and lane 2: purified Fur-cHis.

C. Binding of native Fur to the *pvdS* promoter. Lanes 1–8: labelled *pvdS* promoter; lanes 2 and 3: addition of 7.8 μM and 11.7 μM Fur respectively; lanes 4 and 5: same as lanes 2 and 3 but with the addition of 100 μM adenosine; lane 6: addition of 100-fold excess of unlabelled *pvdS* promoter fragment with 11.7 μM Fur and 100 μM adenosine; lane 7: addition of 11.7 μM Fur and 2 mM EDTA; and lane 8: addition of 11.7 μM Fur, 2 mM EDTA and 100 μM adenosine.

D. Binding of wild-type Fur to the *fagA* promoter. Lanes 1–4: labelled *fagA* promoter; lanes 2–4: addition of 3.9 μM native Fur; lanes 3 and 4: addition of 100 μM adenosine; lanes 4: addition of 100-fold excess of unlabelled *fagA* promoter fragment.

E. Binding of native Fur to the *pchR* promoter. Lanes 1–4: labelled *pchR* promoter; lanes 2–4: addition of 7.8 μM native Fur; lanes 3 and 4: addition of 100 μM adenosine; and lane 4: addition of 100-fold excess of unlabelled *pchR* promoter fragment. Each experiment was performed at least twice.

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