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 also is a signal for the opportunist 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 (Tham-mavongsa 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,
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 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 (Sheik 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 (Caldenwood 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 10⁴-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, swimming was abolished at 1 mM (Fig. 2A). In addition, rhamnolipids are essential for P. aeruginosa swimming (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,
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 \( 2.6 \pm 0.1 \)-fold after 14 h (Fig. 3A). Elastase production was also decreased \( 2.1 \pm 0.2 \)-fold after 6 h (Fig. 3B). Similarly, with adenosine, EPS levels were decreased \( 7.5 \pm 0.6 \)-fold (Fig. 3C), PQS was reduced \( 5.5 \pm 0.2 \)-fold (Fig. 3D), and siderophore pyoverdine production reduced (Fig. 3E). Confirming this result, extracellular siderophore production was examined on
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

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.
Adenosine decreases virulence in P. aeruginosa

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 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 pchE gene fragment 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 by Fur, including siderophores, proteases, exotoxin A and haem/haemoglobin utilization, since adenosine does not bind Fur.

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 (31 genes), pyochelin biosynthesis (31 genes), 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 gene aprDEF.

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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  | pvdQ      | -18.4       | Hypothetical protein |
| PA2385  | pvdA      | -7.0        | Probable acylase |
| PA2386  | pvdN      | -26.0       | L-ornithine N5-oxygenase |
| PA2389  | pvdO      | -3.0        | Conserved hypothetical protein |
| PA2392  | pvdP      | -3.0        | Probable ATP-binding/permease fusion ABC transporter |
| PA2393  |           | -17.1       | Probable dipeptidase precursor |
| PA2394  |           | -7.5        | Probable aminotransferase |
| PA2395  |           | -5.7        | Hypothetical protein |
| PA2396  |           | -8.0        | Hypothetical protein |
| PA2397  |           | -6.1        | Pyoverdine biosynthesis protein PvdE |
| PA2398  |           | -8.6        | Ferrivopyoverdine receptor |
| PA2399  |           | -4.9        | Pyoverdine synthetase D |
| PA2400  |           | -7.0        | Probable non-ribosomal peptide synthetase |
| PA2401  |           | -7.0        | Probable non-ribosomal peptide synthetase |
| PA2402  |           | -4.6        | Probable non-ribosomal peptide synthetase |
| PA2403  |           | -7.5        | Probable ATP-binding component of ABC transporter |
| PA2404  |           | -2.8        | Probable permease of ABC transporter |
| PA2411  |           | -5.7        | Probable thioesterase |
| PA2412  |           | -16.0       | Conserved hypothetical protein |
| PA2413  |           | -3.5        | Probable class III aminotransferase |
| PA2424  |           | -22.6       | Probable non-ribosomal peptide synthetase |
| PA2425  |           | -4.3        | Probable thioesterase |
| PA2426  |           | -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  |           | -4.0        | Hypothetical protein |
| PA4225  | pchG      | -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 |
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.

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 (Diggie 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 1. cont.**

| Gene ID | Gene name | Fold change | Description |
|---------|-----------|-------------|-------------|
| PA3410  |           | –6.1        | Probable sigma-70 factor, ECF subfamily |
| PA472   |           | –2.8        | Probable sigma-70 factor, ECF subfamily |
| PA2468  | foxI      | –2.6        | Probable transcriptional regulator |
| PA2359  |           | –7.0        | |

Others

| Gene ID | Gene name | Fold change | Description |
|---------|-----------|-------------|-------------|
| 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  | pflR      | –3.7        | Two-component response regulator PflR |
| 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.
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).

| Gene ID | Gene name | Adenosine versus no adenosine | PA2384 mutant versus WT | High versus low iron | Descriptions |
|---------|-----------|-------------------------------|-------------------------|----------------------|--------------|
| **Pyoverdine synthesis and transport** | | | | | |
| PA2384  | pvdQ      | –18.4                         | –148                   |                      | Hypothetical protein |
| PA2385  | pvdA      | –17.2                         | –117                   | –203                 | L-ornithine N5-oxygenase |
| PA2386  | pvdM      | –26.0                         | –19.0                  | –216                 | Conserved hypothetical protein |
| PA2389  | pvdN      | –3.0                          | –3.6                   | –14                  | Probable ATP-binding/permease fusion ABC transporter |
| PA2390  | pvdO      | –3.0                          | –3.6                   | –14                  | Hypothetical protein |
| PA2392  | pvdP      | –3.3                          | –4.7                   | –14                  | Probable dipeptidase precursor |
| PA2393  | pvdR      | –17.2                         | –8.0                   | –38                  | Probable aminotransferase |
| PA2394  | pvdS      | –7.5                          | –3.8                   | –38                  | Hypothetical protein |
| PA2395  | pvdT      | –5.7                          | –2.9                   | –38                  | Probable non-ribosomal peptide synthetase |
| PA2396  | pvdU      | –8.0                          | –6.7                   | –45                  | Probable non-ribosomal peptide synthetase |
| PA2397  | pvdV      | –6.1                          | –7.6                   | –33                  | Pyoverdine biosynthesis protein PvdE |
| PA2398  | tprA      | –8.6                          | –2.6                   | –35                  | Ferripyoverdine receptor |
| PA2399  | pvdD      | –4.9                          | –4.2                   | –39                  | Pyoverdine synthetase D |
| PA2400  | pvdE      | –7.0                          | –11.9                  | –30                  | Probable haem-degrading factor |
| PA2401  | pvdF      | –7.0                          | –11.9                  | –30                  | Probable haem-degrading factor |
| PA2402  | pvdG      | –4.6                          | –5.1                   | –30                  | Probable haem-degrading factor |
| PA2403  | pvdH      | –5.3                          | –5.1                   | –15                  | Probable haem-degrading factor |
| PA2404  | pvdI      | –8.0                          | –15                   | –15                  | Probable haem-degrading factor |
| PA2405  | pvdJ      | –6.1                          | –15                   | –15                  | Probable haem-degrading factor |
| PA2406  | pvdK      | –4.0                          | –15                   | –15                  | Probable haem-degrading factor |
| PA2407  | pvdL      | –7.5                          | –15                   | –15                  | Probable haem-degrading factor |
| PA2408  | pvdM      | –7.5                          | –15                   | –15                  | Probable haem-degrading factor |
| PA2409  | pvdN      | –3.0                          | –15                   | –15                  | Probable haem-degrading factor |
| PA2410  | pvdO      | –5.7                          | –29.8                  | –126                 | Probable haem-degrading factor |
| PA2411  | pvdP      | –16.0                         | –14.2                  | –126                 | Conserved hypothetical protein |
| PA2412  | pvdQ      | –3.5                          | –10.6                  | –65                  | Probable class III aminotransferase |
| PA2413  | pvdR      | –22.6                         | –7.9                   | –34                  | Probable non-ribosomal peptide synthetase |
| PA2414  | pvdS      | –4.3                          | –8.1                   | –34                  | Probable non-ribosomal peptide synthetase |
| PA2415  | pvdT      | –27.9                         | –7.3                   | –177                 | Sigma factor PvdS |
| PA2416  | pvdU      | –6.5                          | –6.1                   | –55                  | Hypothetical protein |
| **Pyochelin synthesis and transport** | | | | | |
| PA4175  | piv       | –2.8                          | –7                    |                      | Probable endoprotease Arg-C precursor |
| PA4218  | tpiX      | –3.7                          | –47.8                 | –49                  | Probable transporter |
| PA4219  | tpiC      | –4.0                          | –17.7                 | –49                  | Hypothetical protein |
| PA4220  | tpiB      | –4.3                          | –48.8                 | –182                 | Hypothetical protein |
| PA4221  | tpiA      | –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                  | Probable transporter |
| PA4225  | pchF      | –4.9                          | –37.6                 | –55                  | Probable transporter |
| PA4226  | pchE      | –4.23                         | –85.0                 | –55                  | Dihydroxyphenylglycine synthetase |
| **Haem uptake and utilization** | | | | | |
| PA0672  | hemO      | –9.2                          | –9.6                   | –138                 | Hypothetical protein |
| PA0675  | hemN      | –5.3                          | –4.1                   | –138                 | Probable sigma-70 factor, ECF subfamily |
| PA0676  | hemP      | –3.0                          | –3.0                   | –138                 | Probable transmembrane sensor |
| PA3410  | phuT      | –6.1                          | –2.1                   | –140                 | Probable sigma-70 factor, ECF subfamily |
| PA4708  | phuS      | –3.5                          | –2.0                   | –138                 | Hypothetical protein |
| PA4709  | phuU      | –4.3                          | –3.1                   | –138                 | Probable haem-degrading factor |
| PA4710  | phuR      | –4.0                          | –3.5                   | –7                   | Probable outer membrane haem receptor |

**Other iron-regulated genes**

| Gene ID | Gene name | Fold change |
|---------|-----------|-------------|
| PA0472  | toxR      | –28         |
| PA0707  | pirA      | –3.5        |
| PA1134  | aprD      | –3.3        |
| PA1245  | aprE      | –4.6        |
| PA1246  | aprF      | –5.7        |
| PA1300  | –13.0     | –8.2        |
| PA1301  | –4.6      | –46         |

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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 2. cont. |
| --- |
| **Gene ID** | **Gene name** | **Fold change** | **Descriptions** |
| PA1302 | cyoD | −2.8 | Probable haem utilisation protein precursor |
| PA1320 | cyoD | −3.7 | Cytochrome o ubiquinol oxidase subunit IV |
| PA2033 | −7.0 | −4.9 | Hypothetical protein |
| PA2034 | −4.9 | −95 | Hypothetical protein |
| PA2452 | −4.9 | −52 | Hypothetical protein |
| PA2460 | −2.8 | −10.1 | Hypothetical protein |
| PA2466 | foxA | −19.7 | Probable TonB-dependent receptor |
| PA2467 | foxR | −3.3 | −30 | Probable transmembrane sensor |
| PA2468 | foxl | −2.6 | −30 | Probable sigma-70 factor, ECF subfamily |
| PA2466 | 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 | fxB | −3.5 | Probable TonB-dependent receptor |
| PA4467 | −9.9 | −33.9 | Hypothetical protein |
| PA4468 | sodM | −16.0 | −16.7 | Superoxide dismutase |
| PA4469 | −16.0 | −119 | Hypothetical protein |
| PA4470 | fumC | −18.4 | −9.9 | Fumarate hydratase |
| PA4471 | fagA | −19.7 | −20.3 | −119 | Hypothetical protein |
| PA4514 | −4.3 | −119 | Probable outer membrane receptor for iron transport |
| PA4570 | −7.5 | −10.0 | −403 | Hypothetical protein |
| PA4895 | −2.6 | −20 | Probable transmembrane sensor |

| 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 Δ::ftrB, Δ::furB, Δ::pqsA | Liberati et al. (2006) |
| PA14_16250 (PA3724, lasB) | PA14_16250 Δ::ftrB, Δ::furB, Δ::pqsA | Liberati et al. (2006) |
| PA14_09490 (PA4209, pheA) | PA14_09490 Δ::ftrB, Δ::furB, Δ::pqsA | Liberati et al. (2006) |
| PA14_33700 (PA2396, pvdF) | PA14_33700 Δ::ftrB, Δ::furB, Δ::pqsA | Liberati et al. (2006) |
| PA14_19120 (PA3477, rhlR) | PA14_19120 Δ::ftrB, Δ::furB, Δ::pqsA | Liberati et al. (2006) |
| PA14_24480 (PA3064, pelA) | PA14_24480 Δ::ftrB, Δ::furB, Δ::pqsA | Liberati et al. (2006) |
| PA14_51430 (PA0996, pqsA) | PA14_51430 Δ::ftrB, Δ::furB, Δ::pqsA | Liberati et al. (2006) |
| _E. coli_ | | |
| BL21(DE3) | F' ompT hsdS (r-c m-c) gal dcm l (DE3) Δ (pRUV5-3) 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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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 (Martinez-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...
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 dl-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 76 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 C. elegans; lane 3: whole cell lysate from E. coli; lane 4: whole cell lysate from P. aeruginosa.

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

C. Binding of native Fur to the pvdS promoter. Lanes 1–8: labelled pvdS promoter; lanes 2 and 3: addition of 7.8 μM Fur and 11.7 μM respectively; lanes 4: 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; lane 4: addition of 100-fold excess of unlabelled pchR promoter fragment. Each experiment was performed at least twice.

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