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Oligoribonuclease is a central feature of c-di-GMP signalling in the opportunistic pathogen

*Pseudomonas aeruginosa*

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The second messenger cyclic diguanylate (c-di-GMP) controls diverse cellular processes among Bacteria. Diguanylate cyclases (DGCs) synthesize c-di-GMP while it is degraded by c-di-GMP-specific phosphodiesterases (PDEs). Nearly 80% of these PDEs are predicted to depend on the catalytic function of EAL domains, which hydrolyse a single phosphodiester group in c-di-GMP to produce 5′-phosphoguanylyl-(3′,5′)-guanosine (pGpG). However, to degrade pGpG and prevent its accumulation, bacterial cells require an additional nuclease, the identity of which remains unknown. Here we identify oligoribonuclease (Orn) - a 3′-5′ exonuclease highly conserved among Actinobacteria, Bet-, Delta- and Gammaproteobacteria - as an enzyme responsible for pGpG degradation. We found that a *Pseudomonas aeruginosa* Δorn mutant had high intracellular c-di-GMP levels, causing this strain to overexpress extracellular polymers and overproduce biofilm. Although recombinant Orn degraded small RNAs in vitro, this enzyme had a proclivity for degrading RNA oligomers comprised of 2 to 5 nucleotides ("nanoRNAs"), including pGpG. Corresponding with this activity, Δorn cells possessed highly elevated pGpG levels. We found that pGpG inhibited the activity of EAL-dependent PDEs in cell lysates. This inhibition could be alleviated by Orn. These data suggest that elevated levels of pGpG might exert product inhibition on PDEs in vivo, thereby increasing intracellular c-di-GMP. We propose that Orn provides homeostatic control of intracellular pGpG and that this activity may be fundamental to c-di-GMP signal transduction.

*Pseudomonas aeruginosa* | biofilm | cyclic diguanylate | EAL domain

Significance statement

Many bacteria possess enzymes that synthesize and degrade the intracellular second messenger cyclic diguanylate (c-di-GMP). Bacteria use this molecule to relay environmental signals into physiological responses that control motility, virulence and biofilm formation. There are two pathways for enzymatic c-di-GMP degradation. One of these pathways involves the production of an intermediate molecule called pGpG. While many enzymes responsible for c-di-GMP degradation have been characterized, microbiologists have long sought those responsible for pGpG degradation. Here we identify that oligoribonuclease (Orn) mediates pGpG degradation, and show that Orn is important for c-di-GMP signalling in the human pathogen *Pseudomonas aeruginosa*. This discovery reveals that "nanoRNAs," which have been considered housekeeping proteins crucial for mRNA turnover, also have a key role in c-di-GMP signalling.

Introduction

Enzymes that are predicted to “make and break” c-di-GMP have been identified in nearly every known bacterial phylum (1). These enzymes are found in many species in vast numbers and are associated with diverse cellular processes. In many bacteria, low levels of intracellular c-di-GMP upregulate motility and virulence factor expression, whereas high levels promote extracellular polysaccharide (EPS) production, biofilm development and cell cycle progression (1-4).

C-di-GMP is enzymatically synthesized by proteins harbouring GGDEF domains and degraded by EAL and HD-GYP domain-containing proteins (5). EAL domains catalyse the asymmetric hydrolysis of c-di-GMP to yield the linear dinucleotide 5′-phosphoguanylyl-(3′,5′)-guanosine (pGpG) (6). By contrast, HD-GYP domains degrade c-di-GMP to GMP (7-9). Degradation of c-di-GMP by an EAL-dependent pathway thus requires a second phosphodiesterase (PDE) to eliminate pGpG and recycle this dinucleotide into the cellular guanosine pool. Although the activity of this second enzyme was predicted to be an important part of c-di-GMP degradation more than 25 years ago (10), the identities of the proteins involved in degrading pGpG have remained enigmatic.

Significance

Many bacteria possess enzymes that synthesize and degrade the intracellular second messenger cyclic diguanylate (c-di-GMP). Bacteria use this molecule to relay environmental signals into physiological responses that control motility, virulence and biofilm formation. There are two pathways for enzymatic c-di-GMP degradation. One of these pathways involves the production of an intermediate molecule called pGpG. While many enzymes responsible for c-di-GMP degradation have been characterized, microbiologists have long sought those responsible for pGpG degradation. Here we identify that oligoribonuclease (Orn) mediates pGpG degradation, and show that Orn is important for c-di-GMP signalling in the human pathogen *Pseudomonas aeruginosa*. This discovery reveals that "nanoRNAs," which have been considered housekeeping proteins crucial for mRNA turnover, also have a key role in c-di-GMP signalling.

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RNAs that are 2 to 5 nucleotides in length have been termed nanoRNAs (11). These short oligonucleotides, including pGpG, are degraded by "nanoRNases" (11). One of the most likely candidates for pGpG degradation in vivo is oligoribonuclease (orn) (1). *Escherichia coli* Orn and its orthologues are required to complete the degradation of mRNA to mononucleotides. Although some bacteria possess multiple redundant nanoRNases (12), Orn is essential for the viability of other species, including *E. coli* (11, 13, 14). Thus, the role of Orn in bacterial physiology has been investigated primarily by using systems for the conditional expression or depletion of this enzyme.

*Pseudomonas aeruginosa* is a formidable opportunistic pathogen that has served as a model organism for studying biofilm formation. This developmental process is regulated by c-di-GMP (15). Recently, a method was devised to trigger the post-translational degradation of Orn in *P. aeruginosa*. Work with this system demonstrated that nanoRNAs accumulate in vivo and that these oligonucleotides may prime RNA transcription, shifting transcriptional start sites and leading to global changes in promoter-specific gene expression (16). Nevertheless, because Orn depletion did not seem to cause expression changes in genes known to belong to a c-di-GMP regulon, an additional role for Orn in c-di-GMP signalling was not evident.

Here we report the observation that *P. aeruginosa* PAO1 with a complete and precisely engineered deletion of *orn* is viable, and surprisingly, exhibits elevated levels of c-di-GMP. This finding provided a new opportunity to investigate the function of Orn in bacterial c-di-GMP signal transduction. We present biochemical and genetic evidence that Orn degrades pGpG in vitro as well as in vivo. We also show that the addition of pGpG to cell lysates inhibits PDE activity, which can be alleviated through addition of purified Orn. Collectively, these data suggest that Orn has a key role in bacterial physiology.

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**Fig. 1.** Loss of *orn* increases *P. aeruginosa* surface attachment and biofilm development. (A) Biofilm formation in microtiter plates. Values represent the means and standard deviations of 3 biological replicates. *P* ≤ 0.05 and ***P* ≤ 0.001 versus wild type (wt) with Student’s t-test. (B) Biofilm development in flow cells. Each square on the grid is 20 × 20 μm. VC denotes the vector control.

**Fig. 2.** Loss of *orn* increases intracellular c-di-GMP and pGpG. Intracellular levels of guanosine nucleotides were measured by LC-MS/MS. (A) c-di-GMP. (B) pGpG. (C) GMP. Values represent the means and standard deviations of 3 biological replicates. *P* ≤ 0.05, **P* ≤ 0.005 and ***P* ≤ 0.001 versus wild type (wt) + vector control (VC) with Student’s t-test.
role in *P. aeruginosa* c-di-GMP signal transduction. Finally, we demonstrate that the *E. coli* orthologue of Orn alleviates pGpG accumulation and restores wild type phenotypes to a *P. aeruginosa* Δorn mutant, suggesting that orthologues of Orn may be a central feature of c-di-GMP signaling in other species of bacteria.

**Results**

**Loss of orn enhances biofilm formation by *P. aeruginosa* PAO1.** Transposon mutagenesis indicates that *P. aeruginosa* can withstand mutational inactivation of orn (*PA4951*) (17, 18). Thus, to investigate the function of oligoribonuclease, we constructed an
in-frame deletion of orn in *P. aeruginosa* PAO1. A prominent phenotype of Δorn mutant cells grown in shaken cultures was their tendency to clump and attach to the sides of the culture tubes (Fig. S1). This observation suggested that inactivation of orn may increase surface attachment and biofilm growth. To test this, we carried out microtiter dish biofilm formation assays. The Δorn mutant accumulated significantly increased biofilm biomass in microtiter plates relative to the ancestral PAO1 strain (Fig. 1A). We noted that the orn-G11::Tn5 transposon mutant, which was obtained from the *P. aeruginosa* PAO1 two-allele transposon mutant library (17), produced similar results (Fig. 1A). To investigate these findings further, we grew PAO1 and the Δorn mutant expressing green fluorescent protein (GFP) in continuous flow chambers. We saw that the Δorn mutant attached to the interior of flow cells more rapidly and produced mature biofilms with much greater thickness than the parental PAO1 strain (Fig. 1B). Introducing a wild type copy of orn on a plasmid complemented all of these phenotypes (Fig. 1 and Fig. S1). We conclude from these collective data that Orn suppresses *P. aeruginosa* aggregation, surface attachment and biofilm formation.

A Δorn mutant strain has elevated intracellular c-di-GMP and overexpresses extracellular polymers. Because Orn is predicted to participate in a two-step pathway for the degradation of c-di-GMP, we postulated that the increased biofilm growth of the Δorn mutant strain might result from elevated intracellular c-di-GMP concentrations. To test this hypothesis, we extracted nucleotide pools from wild type and Δorn cells and quantified c-di-GMP using liquid chromatography tandem mass-spectrometry (LC-MS/MS). We observed that cultures of Δorn cells produced ~2-fold more c-di-GMP than the ancestral PAO1 strain (Fig. 2A). Wild type levels of c-di-GMP in the Δorn strain were restored by genetic complementation (Fig. 2A). To further corroborate the involvement of c-di-GMP in regulating the biofilm associated traits of the Δorn mutant, a plasmid encoding an EAL-dependent phosphodiesterase (pGpG) and GMP. To test this, we began by engineering a Δorn mutant that had additional deletions in *psl* and *cdr* genes. To test this, we began by engineering a Δorn mutant that had additional deletions in *psl* and *cdr* genes. Since Δorn cells display elevated intracellular c-di-GMP, we hypothesized that loss of orn should activate the transcription of *pel*, *psl* and *cdr* genes. To test this, we began by engineering a Δorn mutant that had additional deletions in *pelF* and *psdD*, which are genes predicted to participate in the degradation of pGpG (20 µM) inhibited c-di-GMP degradation, and this was alleviated by adding 100 ng of purified Orn. (B) pGpG inhibited c-di-GMP degradation in a concentration-dependent fashion, and this inhibition was reduced when c-di-GMP concentrations were increased 10-fold. A representative biological replicate is shown and each data point represents the mean and standard error for 3 technical replicates.

In *P. aeruginosa*, c-di-GMP regulates the expression of the *pel*, *psl* and *cdr* operons (15, 19, 20), which encode genes for extracellular polysaccharides (Pel and Psl) and a biofilm matrix protein (CdrA). Since Δorn cells display elevated intracellular c-di-GMP, we hypothesized that the loss of orn should activate the transcription of *pel*, *psl* and *cdr* genes. To test this, we began by engineering a Δorn mutant that had additional deletions in *pelF* and *psdD*, which are genes predicted to participate in the degradation of pGpG (20 µM) inhibited c-di-GMP degradation, and this was alleviated by adding 100 ng of purified Orn. (B) pGpG inhibited c-di-GMP degradation in a concentration-dependent fashion, and this inhibition was reduced when c-di-GMP concentrations were increased 10-fold. A representative biological replicate is shown and each data point represents the mean and standard error for 3 technical replicates.

**Fig. 5.** pGpG inhibits the activity of EAL-dependent PDEs in lysates of cells overexpressing PA2133. C-di-GMP was measured by LC-MS/MS. (A) The addition of pGpG (20 µM) inhibited c-di-GMP degradation, and this was alleviated by adding 100 ng of purified Orn. (B) pGpG inhibited c-di-GMP degradation in a concentration-dependent fashion, and this inhibition was reduced when c-di-GMP concentrations were increased 10-fold. A representative biological replicate is shown and each data point represents the mean and standard error for 3 technical replicates.
LC-MS/MS. Loss of orn resulted in a 5-fold decrease in cellular GMP (Fig. 2). Although pGpG could not be detected in wild type cells, there was a massive increase in this dinucleotide in the Δorn strain (Fig. 2). In fact, these data suggest that loss of orn resulted in intracellular pGpG concentrations that far exceeded those of GMP (Fig. 2). We estimated that wild type P. aeruginosa had an average intracellular concentration of 2.7 mM GMP and an undetectable levels of pGpG, whereas the Δorn strain had approximately 0.5 mM GMP and 1.8 mM pGpG (see SI Materials and Methods).

These data suggest that Orn has a key role in degrading pGpG in vitro. Orn efficiently degrades pGpG. The biochemical activity of Orn was first characterized for E. coli nearly 40 years ago (21, 22). These studies indicated that Orn is a manganese (Mn2+)-dependent 3′-5′ exonuclease that produces 5′-phosphorylated ribonucleotide monomers from polynucleotides (21). The rate at which E. coli Orn degraded RNA oligomers was inversely proportional to the chain length of the substrate, and moreover, the presence of guanine at the 5′ or 3′ end seemed to be strongly inhibitory on the enzyme activity (22). Provision of 5′-Cy5-CC-3′ suggested that Orn has relatively low activity against a GpG substrate (22). P. aeruginosa Orn has high identity (67%) with the E. coli protein. Because our data indicate that Orn has a crucial role in degrading pGpG, we sought to reassess the biochemical functionality of Orn. To do this we produced and purified recombinant P. aeruginosa Orn. Subsequently, the exonuclease activity of this enzyme was assessed by tracking the time-dependent degradation of 32P- or Cy5-labeled RNA oligomers. The products of these reactions were then electrophoresed in 40% polyacrylamide (PAA) gels, imaged, and quantified.

In the presence of Mn2+, Orn degraded a 32P-labeled RNA 24-mer (5′-CACACACACACACACACACACA-3′) (Fig. 4A). Similar to the work with E. coli, P. aeruginosa Orn showed a notable predilection for degrading nanorNAs. This was evident from the preferential elimination of RNA molecules ranging in size from 2 to 5 ribonucleotides during degradation of the 24-mer (Fig. 4A). Next, the preference of P. aeruginosa Orn for specific nanoRNAs was evaluated. Because dinucleotides and trinucleotides can be difficult to synthesize, and since guanosine homopolymers can form stable quadruplexes that may interfere with enzyme assays, the activity of Orn against RNA homo- and heteropolymers was investigated. To begin, we examined the kinetics of 5′-Cy5-A AAAA-3′, 5′-Cy5-U AAAA-3′, 5′-Cy5-GAAAA-3′, and 5′-Cy5-C CAAA-3′ degradation. Although Orn degraded all of these substrates, this enzyme displayed a preference for purine substrates and bias against pyrimidines (Fig. 4B–4E). 5′-Cy5-C CAAA-3′ was degraded the least efficiently of all these substrates (Fig. 4E). Corresponding with this observation, a 5′-Cy5-CCCCC-3′ homopentamer was degraded much less efficiently than the RNA homopentamer containing purines at the 3′ end (Fig. S3). The stepwise degradation of pentamers into intermediates also informed the relative promiscuity of Orn for degrading RNA homodimers. Orn degraded 5′-Cy5-AAA-3′ most rapidly, followed by 5′-Cy5-G G-3′ and 5′-Cy5-UU-3′. The degradation of 5′-Cy5-UU-3′ was inefficient and the production of 5′-Cy5-C-3′ monomers was barely detectable in these assays (Fig. 4E and Fig. S3).

Previously, calcium-sensitivity has been used to distinguish between EAL-dependent PDEs and the putative enzymes for pGpG degradation. Specifically, EAL-dependent PDEs were found to be inhibited by 1 mM Ca2+, whereas the putative PDEs for pGpG degradation were insensitive to this metal cation (10). We noted that the ribonuclease activity of Orn was Ca2+-insensitive (Fig. S4).

Lastly, we directly assessed the ability of recombinant Orn to degrade pGpG. Under the tested conditions, LC-MS/MS indicated that 100 ng of clean, purified Orn degraded pGpG to GMP with an initial reaction rate of at least ~107 molecule min⁻¹ (Fig. 4F). Recombinant Orn exhibited no activity against c-di-GMP (Fig. 4F). Overall, we conclude from these data that Orn has a preference for purine rich RNA substrates and that this enzyme can readily degrade pGpG in vitro.

pGpG inhibits c-di-GMP degradation in lysates of cells lacking Orn. The build-up of an enzymatic end product frequently leads to negative feedback that inhibits the enzyme that produced it. This led us to hypothesize that high levels of pGpG may inhibit c-di-GMP-specific PDEs. This is a daunting problem to test in P. aeruginosa since there are many EAL domain-containing proteins. To initially test this hypothesis, a crude cell lysate was prepared from a P. aeruginosa Δorn strain that had been transformed with a plasmid encoding the phosphodiesterase PA2133. Subsequently, we added c-di-GMP to aliquots of the cell lysate and quantified its time-dependent degradation by LC-MS/MS. In tandem with these assays, c-di-GMP degradation was measured in aliquots of the same cell lysate except that 20 μM pGpG was added to the reactions. We observed that the addition of pGpG significantly inhibited the degradation of c-di-GMP (Fig. 5A). Adding purified recombinant Orn to cell lysates rapidly eliminated pGpG from the reaction mixture (Fig. S5B), and completely alleviated inhibition (Fig. 5A). Increasing concentrations of pGpG over a range of 2 to 20 μM increased inhibition (Fig. 5B), and moreover, increasing the amount of c-di-GMP 10-fold reduced the level of inhibition (Fig. 5B). These observations suggest that competitive inhibition may at least partially explain pGpG-dependent inhibition of PA2133. pGpG-dependent inhibition of c-di-GMP degradation was not uniquely characteristic of PA2133, and we also observed product inhibition in lysates of Δorn cells overexpressing the EAL-dependent PDE PvrR (Fig. S5A). In sum, these collective data suggest that the elimination of pGpG by Orn may be important for the normal catalytic functioning of EAL-domain-dependent PDEs.

E. coli orn complements the P. aeruginosa Δorn strain and degrades pGpG in vitro. Our data provide evidence that Orn has an important role in P. aeruginosa c-di-GMP signal transduction. Although orn is evolutionarily conserved among many bacteria, testing this role in other species is challenging because Orn may be essential for viability. This is especially pertinent to E. coli, another paradigm species for studying c-di-GMP signal transduction (2). Given this quandary and the previous report that E. coli Orn may not degrade diguanosine substrates efficiently in vitro (22), we turned to interspecies genetic complementation to look for a potential function of the E. coli orthologue of orn in c-di-GMP signalling. Here, we postulated that E. coli orn may complement the defects of a P. aeruginosa orn mutant, and vice versa. To test this hypothesis, we began with an E. coli strain in which the native promoter of orn was replaced with preC (11). This strain had a considerable growth defect in the absence of the inducer anhydrotetracycline (Atc). P. aeruginosa orn expressed ectopically from a plasmid restored the growth of this strain in the absence of Atc (Fig. S6A). Analogously, E. coli orn expressed from a plasmid abolished the hyper-biofilm formation phenotype of the P. aeruginosa Δorn strain and eliminated the accumulation of intracellular pGpG (Fig. S6B – S6E). Altogether, these data suggest that orthologues of Orn from other bacterial species, including E. coli, have the capacity to function in the two-step pathway for c-di-GMP degradation.

Discussion
C-di-GMP was originally identified as an allosteric activator of cellulose synthase in Gluconacetobacter xylinus (10). This pioneering work recognized that c-di-GMP is degraded in two steps. Deactivation of c-di-GMP in G. xylinus protein fractions occurred when a Ca2+-sensitive phosphodiesterase, termed PDE-A,
cleft c-di-GMP to yield the inactive open dimer, pGpG. Subsequently, a second Ca\(^{2+}\)-insensitive phosphodiesterase, termed PDE-B, split this product into two molecules of GMP. It was soon discovered that EAL-domain-dependent phosphodiesterases mediate PDE-A activity (23); however, the enzymes responsible for PDE-B activity have remained elusive.

Identifying the enzymes responsible for PDE-B activity has been complicated because different bacteria possess many different types of proteins that could have a role in PGP degradation. Biochemical investigations of several of these proteins have not provided evidence supporting their role in PGP degradation. For example, EAL-domain proteins may cleave pGpG; however, this activity occurs in vitro at a rate that is orders of magnitude slower than that of c-di-GMP degradation (6, 24). HD-GYP-domain proteins might also degrade pGpG; however, while there is some indication that HD-GYP-domains may degrade pGpG in vitro (9), the relevance of this activity has not been substantiated in vivo. Finally, nanoRNAses may degrade pGpG. It has been previously hypothesized that Orn mediates PDE-B activity (1); however, Orn was first studied in E. coli and investigations of this enzyme have been hampered because it is essential for E. coli cell viability (13).

Unlike E. coli, P. aeruginosa can withstand a loss-of-function mutation in orn. This discovery has enabled us to revisit the hypothesis that Orn mediates PDE-B activity. Here, we have provided multiple lines of evidence that Orn has a key role in not only PGP degradation, but also c-di-GMP signalling in vivo.

Thus oligoribonuclease has a role in guanosine metabolism that is thought to be fundamental to c-di-GMP signal transduction in P. aeruginosa (Fig. 6).

Investigations carried out in the 1970s examined the activity of E. coli Orn against a variety of dinucleotide substrates, and estimated that Orn had relatively low activity against GpG. Contrary to that work, our investigation suggests that P. aeruginosa Orn has a relatively high capability for degrading purine dimers such as pApA and pGpG (Fig. 4). The basis for this discrepancy in relative reaction rates is not clear. However, our direct measurements of pGpG in cells lacking Orn (Fig. 2 and Fig. S6), as well as the ability of E. coli Orn to complement the P. aeruginosa Δorn mutation (Fig. S6), suggest that Orn-dependent degradation of pGpG has a biologically relevant function, especially in the context of c-di-GMP signal transduction.

The mutational inactivation of orn is pleiotropic (16). We reasoned that overexpressing the c-di-GMP-specific PDE PA2133 could distinguish the subset of genes and phenotypes differentially regulated by c-di-GMP from other changes due to loss of orn. Overexpression of PA2133 eliminated intracellular c-di-GMP in the Δorn strain (Fig. 2). As expected, this also abolished bacterial hyper-production (Fig. 1) as well as overexpression of pelA, pelB and cdhA (Fig. 3).

Another key observation is that pGpG inhibits the degradation of c-di-GMP in lysates of cells overexpressing EAL-dependent PDEs (Fig. 5 and Fig. S5). Product inhibition is a type of negative feedback where the product of an enzyme-catalysed reaction binds to the enzyme and inhibits its activity. This type of inhibition may occur through more than one mechanism. For instance, pGpG may competitively bind the active site of EAL-dependent PDEs, preventing c-di-GMP from binding the enzyme. Alternatively, pGpG may bind at another site, creating allosteric inhibition. The concentration dependence of product inhibition, as well as the alleviation of inhibition by increasing c-di-GMP concentration (Fig. S5B), suggest that pGpG-dependent inhibition of EAL-domain PDEs may be at least partially competitive in nature.

A question that arises from this work is whether or not pGpG-dependent inhibition of EAL-dependent PDEs may contribute to elevated intracellular c-di-GMP in the Δorn strain. Because Orn rapidly eliminates pGpG and because c-di-GMP degradation is difficult to detect in lysates of wild type cells, we utilized Δorn cells overexpressing PA2133 or PvrR. We observed that under these conditions, c-di-GMP degradation was inhibited by 2- to 20-μM pGpG in vitro. Our direct measurements suggest that Δorn cells may have as much as 1.8 mM intracellular pGpG, which is 90- to 900-times greater than those concentrations causing product inhibition in cell lysates. However, these data require careful interpretation. The optimized in vitro assay utilized 70 to 700 nM c-di-GMP; whereas we estimated that intracellular c-di-GMP may be as high as 70 μM in the Δorn strain (see SI Materials and Methods). Thus, c-di-GMP concentrations are 95- to 950-times less than those expected in vivo. We would estimate, therefore, that the stoichiometric ratios of pGpG and c-di-GMP would be approximately similar in both in vitro assays and in Δorn cells. Thus, we propose that pGpG-dependent inhibition of EAL domain PDEs may be biologically relevant, increasing intracellular c-di-GMP in the Δorn strain. We acknowledge that not all PDE containing enzymes in the cell may be subject to feedback inhibition by pGpG. However, if it happens for even a few, this may tip the balance of DGC and PDE activity to favour the build-up of c-di-GMP in the Δorn strain.

Using LC-MS/MS, pGpG could not be detected in lysates of wild type cells, and therefore, it is not clear whether pGpG-dependent feedback control of EAL-domain PDEs is operational under normal circumstances. Rather, Orn seems to be responsible for naturally low levels of pGpG in wild type P. aeruginosa cells.

Thus our data suggest that Orn provides homeostatic control of intracellular pGpG and that this may enable the responsiveness of c-di-GMP signalling cascades by preventing feedback inhibition of enzymes crucial for c-di-GMP degradation.

Genetic complementation of the P. aeruginosa Δorn strain indicates that E. coli Orn may degrade pGpG in vivo. Thus it is tempting to speculate that orthologues of Orn may function in c-di-GMP signal transduction in other organisms. A tBLASTn survey of 3109 completely sequenced genomes in the NCBI database (as of October 31, 2013) identified protein sequences from 568 additional bacterial species with >50% identity to P. aeruginosa PA01 Orn (see Supplementary Dataset). A Bayesian analysis of Orn sequences placed these orthologues into four phyla (Fig. S7). This primarily includes Proteobacteria (especially β-, δ- and γ-Proteobacteria) and Actinobacteria, but also single species representing Gemmatimonadetes (Gemmatimonas aurantiaca) and Fibrobacteres (Fibrobacter succinogenes). Orn may have a crucial role in completing the degradation of RNA (13), and thus it may be no surprise that some bacterial proteins may be orthologues of Orn.

We investigated orthologues of Orn in a variety of bacteria and found that Orn is found in many, but not all, Gram-negative and Gram-positive bacteria. For example, Orn orthologues are absent in Alphaproteobacteria (such as G. xylinus) and Actinobacteria, but also single species representing Gemmatimonadetes and Fibrobacteres. Given the potential for Orn to degrade RNA, our data suggest that Orn orthologues may have a role in RNA degradation in other bacteria. The optimized in vitro assay utilized 70 to 700 nM c-di-GMP; whereas we estimated that intracellular c-di-GMP may be as high as 70 μM in the Δorn strain (see SI Materials and Methods). Thus, c-di-GMP concentrations are 95- to 950-times less than those expected in vivo. We would estimate, therefore, that the stoichiometric ratios of pGpG and c-di-GMP would be approximately similar in both in vitro assays and in Δorn cells. Thus, we propose that pGpG-dependent inhibition of EAL domain PDEs may be biologically relevant, increasing intracellular c-di-GMP in the Δorn strain. We acknowledge that not all PDE containing enzymes in the cell may be subject to feedback inhibition by pGpG. However, if it happens for even a few, this may tip the balance of DGC and PDE activity to favour the build-up of c-di-GMP in the Δorn strain.

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imaged using a Leica TCS SPE confocal microscope as described in SI Materials and Methods. 2-stacks of two-dimensional confocal images were rendered in three-dimensions using Imaris (Bitplane Inc.).

Nucleotide extractions and quantification by LC-MS/MS. Nucleotide pools were extracted from cultures grown for ~5 h at 37°C in BM2 using ice-cold acetone (100%) and ethanol (100%) according to established procedures (26). Protein concentrations were determined from the cell pellets of 1 ml aliquots from these cultures using a bicinchoninic acid (BCA) Protein Assay Kit (Pierce). GTP, GMP, NPI, and NPII concentrations were determined by LC-MS/MS using a XeMR or [32P]c-di-GMP internal standard as previously described (26, 27).

RNA isolation, cDNA synthesis and RT-PCR. Approximately 1 × 10^7 cells grown in BM2 were harvested at an OD600 of 0.5 and mixed with RNAprotect Bacteria Reagent (Qiagen) and stored at -80°C. Isolation of total RNA and an assessment of its quantity and quality were carried out as previously described by Chugani et al. (28), and this is detailed in SI Materials and Methods. Three biological replicates were processed for each strain. First strand synthesis was carried out using the qScript cDNA Supermix kit (Quanta Biosciences) following the manufacturer’s protocols. Quantitative PCR measurements were made with a CFX96 Touch™ Real-Time PCR Detection System (BioRad) using SsoAdvanced™ Universal SYBR Green BioRad (BioRad).

Protein production. Recombinant Orn was produced by using 0.2% L-arabinose to induce the expression of Orn-His (from pDC12) in E. coli BL21(DE3). Cells were harvested and Orn-His6 was purified using established protocols for high-performance liquid chromatography (HPLC) using a HiTrap™ HP Ni-affinity column (GE Healthcare) (see SI Materials and Methods).

RNA degradation assays. 32P- and Cy5-labeled RNA oligomers were prepared as described in SI Materials and Methods. Exonuclease assays were carried out using established protocols (11) and this is detailed in SI Materials and Methods.

PDE activity assays. PDE activity assays for whole cell lysates were performed as described in SI Materials and Methods by Kulasekara et al. (29) and this is detailed in SI Materials and Methods.

Interspecies genetic complementation. E. coli UM341 (Table S1), in which porn expression of orn deletions, was transformed with plasmids expressing either the E. coli or P. aeruginosa origins of orn, or the appropriate vector control. Genetic complementation was evaluated using the expression of orn in the presence or absence of anhydrotetracycline (Apx).

Phylogenetic analyses. Bacterial species surveys to identify orn orthologues and the construction of gene phylogenies is described in SI Materials and Methods.

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17. Jacob MA, et al. (2003) Comprehensive transposon mutant library of Pseudomonas aerugi-

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