

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

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Submitted to Proceedings of the National Academy of Sciences of the United States of America

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, Beta-, 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 "nanoRNases," 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 "nanoRNases," which have been considered housekeeping proteins crucial for mRNA turnover, also have a key role in c-di-GMP signalling.

Reserved for Publication Footnotes

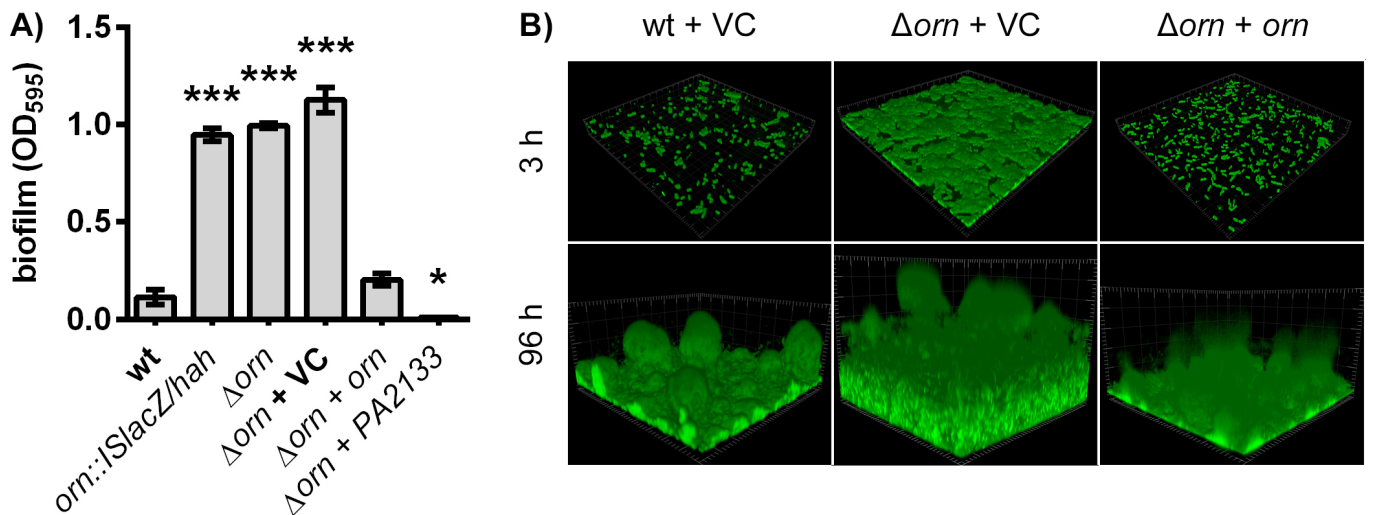


Fig. 1. Loss of *orn* increases *P. aeruginosa* surface attachment and biofilm development. (A) Biofilm formation in microtiter plates. Values represent the means and standard deviations of 3 biological replicates. * $P \leq 0.05$ and *** $P \leq 0.001$ versus wild type (wt) with Student's t-test. (B)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

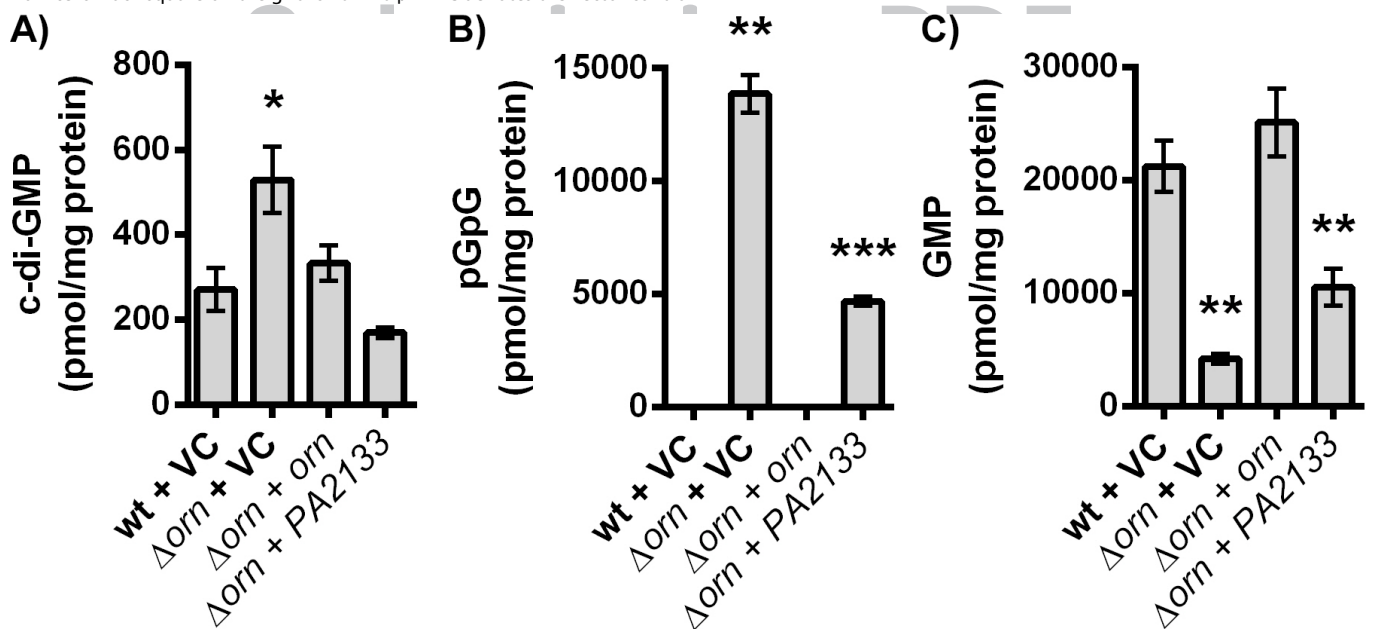


Fig. 2. Loss of *orn* increases intracellular c-di-GMP and pGpG. Intracellular levels of guanosine nucleotides were measured by LC-MS/MS. (A) c-di-GMP. (B) pGpG. (C) GMP. Values represent the means and standard deviations of 3 biological replicates. * $P \leq 0.05$, ** $P \leq 0.005$ and *** $P \leq 0.001$ versus wild type (wt) + vector control (VC) with Student's t-test.

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

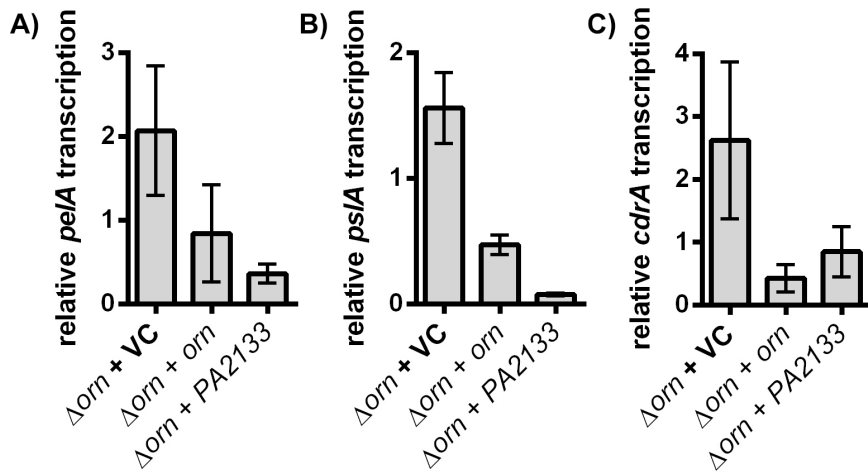


Fig. 3. The *P. aeruginosa* Δorn mutant overexpresses genes for extracellular polymers. RT-PCR was used to quantify relative transcript levels for (A) *peiA*, (B) *psIA*, and (C) *cdrA*. Values represent the means and standard deviations of 3 biological replicates each performed in 3 technical replicates, as normalized to the *ampR* gene in RNA from wild type + vector control (VC) samples. Relative expression levels were calculated using the comparative $2^{-\Delta\Delta CT}$ method (30).

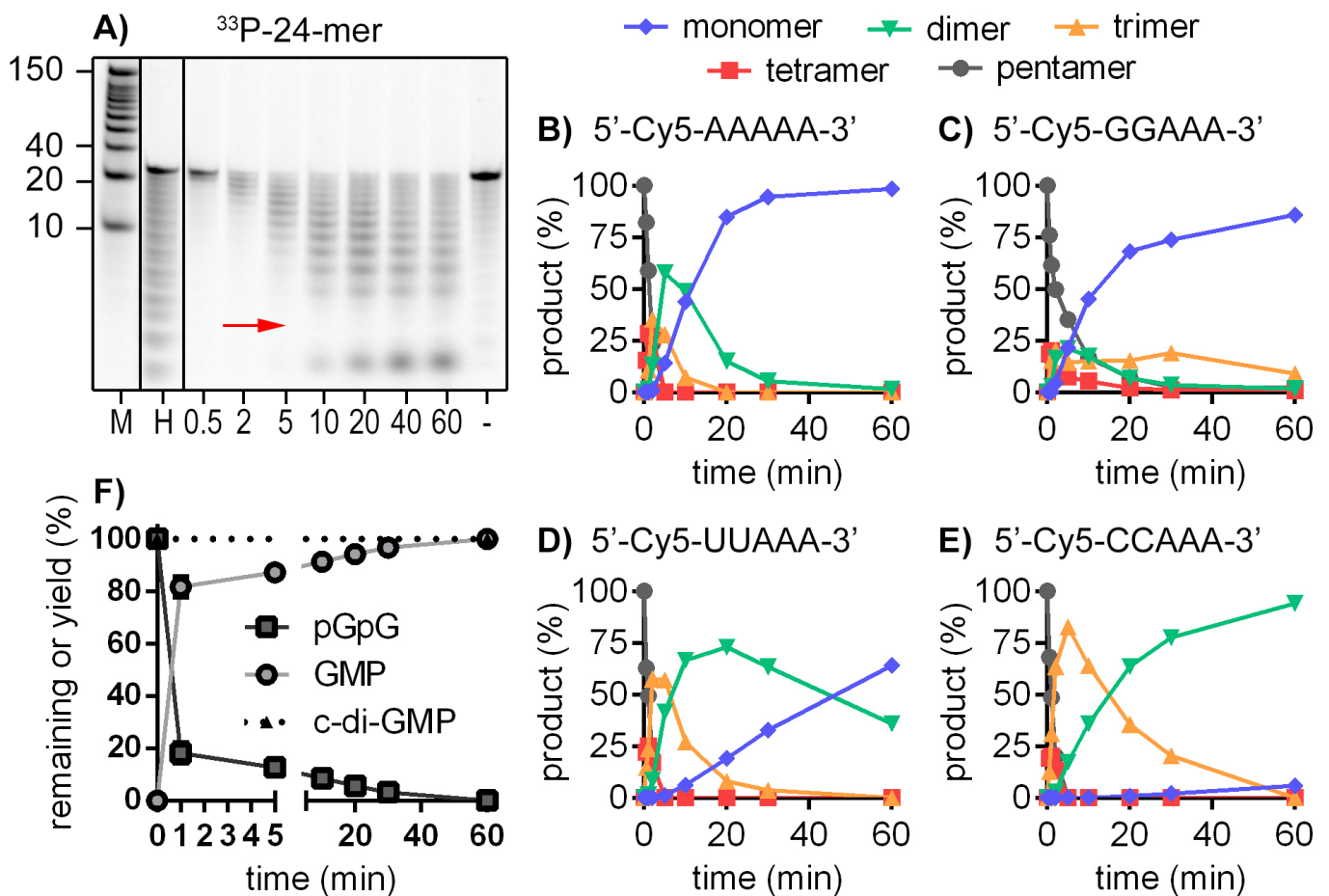


Fig. 4. Orn preferentially degrades nanoRNAs and purine dinucleotides *in vitro*. (A) Degradation of the 24-mer 5'-³³P-CACACACACACACACACACACA-3'. The red arrow indicates the preferential elimination of nanoRNAs. M denotes the MilleniumTM RNA marker, H denotes acid hydrolysis, and "-" denotes a control with no enzyme. The region corresponding (B) Degradation and semi-quantitation of 5'-Cy5-AAAAA-3', (C) 5'-Cy5-GGAAA-3', (D) 5'-Cy5-UUAAA-3', and (E) 5'-Cy5-CCAAA-3'. Each semi-quantitation is one representative of three technical replicates. (F) Degradation of pGpG and production of GMP by 100 ng Orn *in vitro* as assessed by LC-MS/MS. Each data point is the mean and standard deviation of 3 replicates and is expressed as the percentage yield of GMP, or the amount of pGpG or c-di-GMP remaining.

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

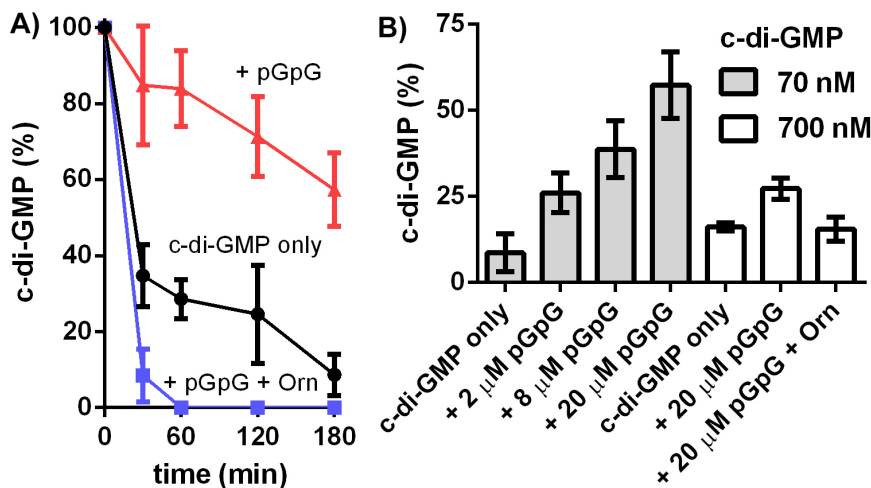


Fig. 5. pGpG inhibits the activity of EAL-dependent PDEs in lysates of cells overexpressing PA2133. C-di-GMP was measured by LC-MS/MS. (A) The addition of pGpG (20 μ M) inhibited c-di-GMP degradation, and this was alleviated by adding 100 ng of purified Orn. (B) pGpG inhibited c-di-GMP degradation in 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.

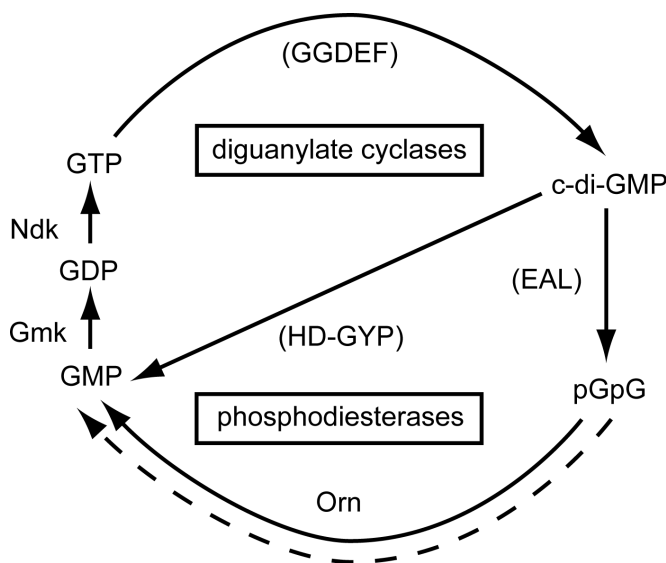


Fig. 6. Guanosine biochemistry fundamental to c-di-GMP signal transduction in *P. aeruginosa*. Solid lines indicate reactions mediated by the named types of enzymes. Gmk and Ndk denote guanylate kinase and nucleoside diphosphate kinase, respectively. The dashed line indicates a reaction that may be mediated by another unknown cellular enzyme.

in-frame deletion of *orn* in *P. aeruginosa* PAO1. A prominent phenotype of Δ orn 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 Δ orn mutant accumulated significantly increased biofilm biomass in microtiter plate wells relative to the ancestral PAO1 strain (Fig. 1A). We noted that the *orn*-G11::ISlacZ/hah 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 Δ orn mutant expressing green fluorescent protein (GFP) in continuous flow chambers. We saw that the Δ orn 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 Δ orn 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 Δ orn mutant strain might result from elevated intracellular c-di-GMP concentrations. To test this hypothesis, we extracted nucleotide pools from wild type and Δ orn cells and quantified c-di-GMP using liquid chromatography tandem mass-spectrometry (LC-MS/MS). We observed that cultures of Δ orn cells produced ~2-fold more c-di-GMP than the ancestral PAO1 strain (Fig. 2A). Wild type levels of c-di-GMP in the Δ orn 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 Δ orn mutant, a plasmid encoding an EAL-dependent PDE (PA2133) was transformed into this strain. This eliminated c-di-GMP-related phenotypes of the Δ orn mutant strain (Fig. 1A and Fig. S1), and reduced intracellular c-di-GMP (Fig. 2A). These data indicate that inactivation of *orn* increases intracellular c-di-GMP and that this is associated with increased biofilm formation.

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 Δ orn 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 Δ orn mutant that had additional deletions in *pelF* and *pslD*. A Δ orn Δ pelF Δ pslD triple mutant neither clumped in shaken culture tubes (Fig. S1) nor formed biofilms in microtiter dishes (Fig. S2). Levels of *pel*, *psl* and *cdr* transcripts were also quantified using reverse-transcriptase polymerase chain reaction (RT-PCR). This revealed 2.1-, 1.7- and 2.6-fold increased expression for *pelA*, *pslA* and *cdrA* in the Δ orn background (Fig. 3). This transcriptional upregulation could be reversed by genetic complementation or by overexpressing PA2133 from a plasmid (Fig. 3). We conclude from these data that loss of *orn* transcriptionally upregulates a hallmark c-di-GMP regulon and that this contributes to the increased production of extracellular polymers in the Δ orn strain.

Inactivation of *orn* increases intracellular pGpG. Orn is expected to function in cellular nucleotide recycling. The putative biochemical function of Orn and the disruption of c-di-GMP signalling in the Δ orn strain led us to hypothesize that the inactivation of *orn* might dramatically alter intracellular pools of pGpG and GMP. To test this, we extracted nucleotide pools from wild type and Δ orn strains and measured pGpG and GMP using

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 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 vivo*.

Orn efficiently degrades pGpG *in vitro*. 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 (Mn^{2+})-dependent 3'→5' exonuclease that produces 5'-phosphorylated ribonucleotide monomers from polyribonucleotides (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 to enzyme activity (22). Previous work also 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 ^{33}P - 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 Mn^{2+} , Orn degraded a ^{33}P -labelled 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 heteropentamers was investigated. To begin, we examined the kinetics of 5'-Cy5-AAAAA-3', 5'-Cy5-UUAAA-3', 5'-Cy5-GGAAA-3', and 5'-Cy5-CCAAA-3' degradation. Although Orn degraded all of these pentamers, this enzyme displayed a preference for purine substrates and bias against pyrimidines (Fig. 4B–4E). 5'-Cy5-CCAAA-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 heteropentamer containing purines at the 3' end (Fig. S3). The stepwise degradation of pentamers into intermediates also informed the relative proclivity of Orn for degrading RNA homodimers. Orn degraded 5'-Cy5-AA-3' most rapidly, followed by 5'-Cy5-GG-3' and 5'-Cy5-UU-3'. The degradation of 5'-Cy5-CC-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 Ca^{2+} , whereas the putative PDEs for pGpG degradation were insensitive to this metal cation (10). We noted that the ribonuclease activity of Orn was Ca^{2+} -insensitive (Fig. S4).

Lastly, we directly assessed the ability of recombinant Orn to degrade pGpG. Under the tested conditions, LC-MS/MS in-

dicated that 100 ng of clean, purified Orn degraded pGpG to GMP with an initial reaction rate of at least $\sim 10^{-7}$ mole 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 nanoRNA 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 vivo*.** 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 *ptetO* (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 Ca^{2+} -sensitive phosphodiesterase, termed PDE-A,

cleaved 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 pGpG degradation. Biochemical investigations of several of these proteins have not provided evidence supporting their role in pGpG 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 pGpG 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 biofilm hyper-production (Fig. 1) as well as overexpression of *pelA*, *pslA* and *cdrA* (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 to 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. 5B), 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 *in vitro* 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 signaling 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* PAO1 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 is no surprise that some bacterial species on this list do not possess EAL-domain proteins. What is more intriguing though is that many bacterial species that possess EAL-dependent PDEs do not have an apparent orthologue of Orn. Indeed, fifteen additional well-sampled bacterial phyla that are thought to possess EAL-domain proteins (1) do not have Orn. This includes members of Alphaproteobacteria such as *G. xylinus*, which is the species in which c-di-GMP was discovered. This raises the intriguing possibility that there may be additional, phylogenetically distinct types of nanoRNAses that participate in c-di-GMP signal transduction in the place of oligoribonuclease.

Materials and Methods

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids are described in Table S1. Primers are listed in Table S2. Plasmid and strain construction are described in *SI Materials and Methods*. *P. aeruginosa* was grown at 37 °C in lysogeny broth (LB), tryptic soy broth (TSB), Vogel-Bonner minimal medium (VBMM) or basal medium 2 (BM2), and *E. coli* was grown in LB (see *SI Materials and Methods* for media composition). Semi-solid medium was prepared by adding 1.0% noble agar to VBMM, and 1.5% agar to BM2 or LB. Antibiotics were added to media as required for selection as described in *SI Materials and Methods*.

Static and flow cell biofilm experiments. Crystal violet quantification of biofilms grown in BM2 in static microtiter plates was carried out using established protocols (25). Flow cell biofilms were grown in 1% TSB and then

imaged using a Leica TCS SPE confocal microscope as described in *SI Materials and Methods*. Z-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 acetonitrile/methanol/water (40/40/20% v/v) 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). GMP, pGpG and c-di-GMP concentrations were determined by LC-MS/MS using a cXMP or [¹³C¹⁵N]c-di-GMP internal standard as previously described (26, 27).

RNA isolation, cDNA synthesis and RT-PCR. Approximately 1.0 × 10⁹ cells grown in BM2 were harvested at an OD₆₀₀ = 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 Supermix (BioRad).

Protein production. Recombinant Orn was produced by using 0.2% L-arabinose to induce the expression of Orn-His×6 (from pDC12) in *E. coli* BL21(DE3). Cells were harvested and Orn-His×6 was purified using established protocols for high-performance liquid chromatography (HPLC) using a HisTrap™ HP Ni-affinity column (GE Healthcare) (see *SI Materials and Methods*).

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RNA degradation assays. ³³P- 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 according to the method of Kulasekara *et al.* (29) and this is detailed in *SI Materials and Methods*.

Interspecies genetic complementation. *E. coli* UM341 (Table S1), in which *ptetO* drives the expression of chromosomal *orn*, was transformed with plasmids expressing either the *E. coli* or *P. aeruginosa* orthologues of *orn*, or the appropriate vector control. Genetic complementation was evaluated by assessing bacterial growth in the presence or absence of anhydrotetracycline (Atc).

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

ACKNOWLEDGEMENTS. The authors thank J. A. Lemire and Y. Irie for constructive feedback on the manuscript, and Stacy Muise and Annette Garbe for technical assistance. EB was supported by a grant from the Israel Science Foundation (ISF) #1124/12. DC was supported by a FEMS Research Fellowship. JJH was supported by a Canada Research Chair (Tier II) from the Canadian Institutes of Health Research and a Discovery Grant from the Natural Sciences and Engineering Research Council (NSERC) #435631. MRP was supported by a grant from the National Institute for Allergy and Infectious Disease (NIAID) # 2R01AI077628-05A1..

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