



**HAL**  
open science

## Oligoribonuclease is a central feature of cyclic diguanylate signaling in *Pseudomonas aeruginosa*.

Dorit Cohen, Undine Mechold, Hadas Nevenzal, Denice C Bay, Jacquelyn D Rich, Matthew R Parsek, Volkhard Kaefer, Joe J Harrison, Ehud Banin

### ► To cite this version:

Dorit Cohen, Undine Mechold, Hadas Nevenzal, Denice C Bay, Jacquelyn D Rich, et al.. Oligoribonuclease is a central feature of cyclic diguanylate signaling in *Pseudomonas aeruginosa*.. Proceedings of the National Academy of Sciences of the United States of America, 2015, 112 (36), pp.11359-64. 10.1073/pnas.1421450112 . pasteur-01427579

HAL Id: pasteur-01427579

<https://pasteur.hal.science/pasteur-01427579>

Submitted on 5 Jan 2017

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial - NoDerivatives 4.0 International License

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

Dorit Cohen<sup>a</sup>, Undine Mechold<sup>b</sup>, Hadas Nevenzal<sup>a</sup>, Denice C. Bay<sup>c</sup>, Jacquelyn D. Rich<sup>c</sup>, Matthew R. Parsek<sup>d</sup>, Volkhard Kaever<sup>e</sup>, Joe J. Harrison<sup>c,\*</sup>,<sup>1</sup> and Ehud Banin<sup>a,\*</sup>,<sup>2</sup>

<sup>a</sup> Center for Advanced Materials and Nanotechnology, The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan, 5290002, Israel <sup>b</sup> Institut Pasteur, CNRS URA3528, Unité de Biochimie des Interactions macromoléculaires, Département de Biologie Structurale et Chimie, 75724 Paris Cedex 15, France <sup>c</sup> Department of Biological Sciences, University of Calgary, 2500 University Drive NW, Calgary, Alberta, T2N 1N4, Canada <sup>d</sup> Department of Microbiology, University of Washington, 1705 NE Pacific Street, Seattle, WA, 98105, USA <sup>e</sup> Research Core Unit Metabolomics, Institute of Pharmacology, Hannover Medical School, Hannover, 30625, Germany \*Correspondence may be directed to either author <sup>1</sup> Telephone: 403-220-7627; Fax: 403-289-9311; E-mail: jjharris@ucalgary.ca <sup>2</sup> Telephone: 972-3-5317288; Fax: 972-3-7384511; E-mail: ehud.banin@biu.ac.il

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

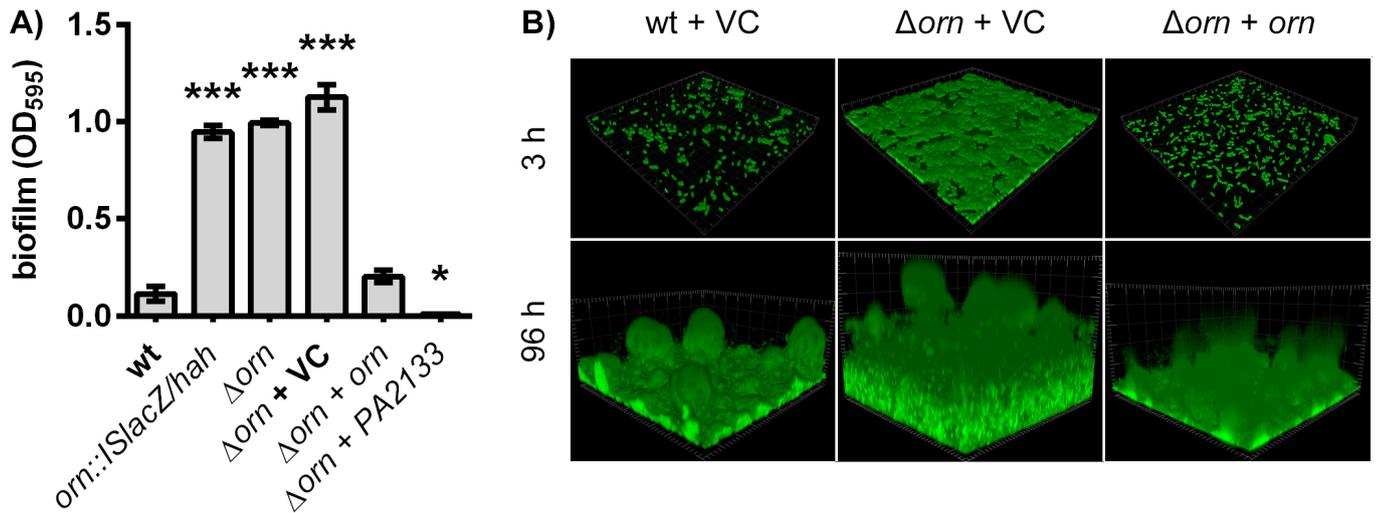
enzymes are found in many species in vast numbers and are associated with diverse cellular processes. In many bacteria, low levels of intracellular c-di-GMP upregulate motility and virulence factor expression, whereas high levels promote extracellular polysaccharide (EPS) production, biofilm development 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.

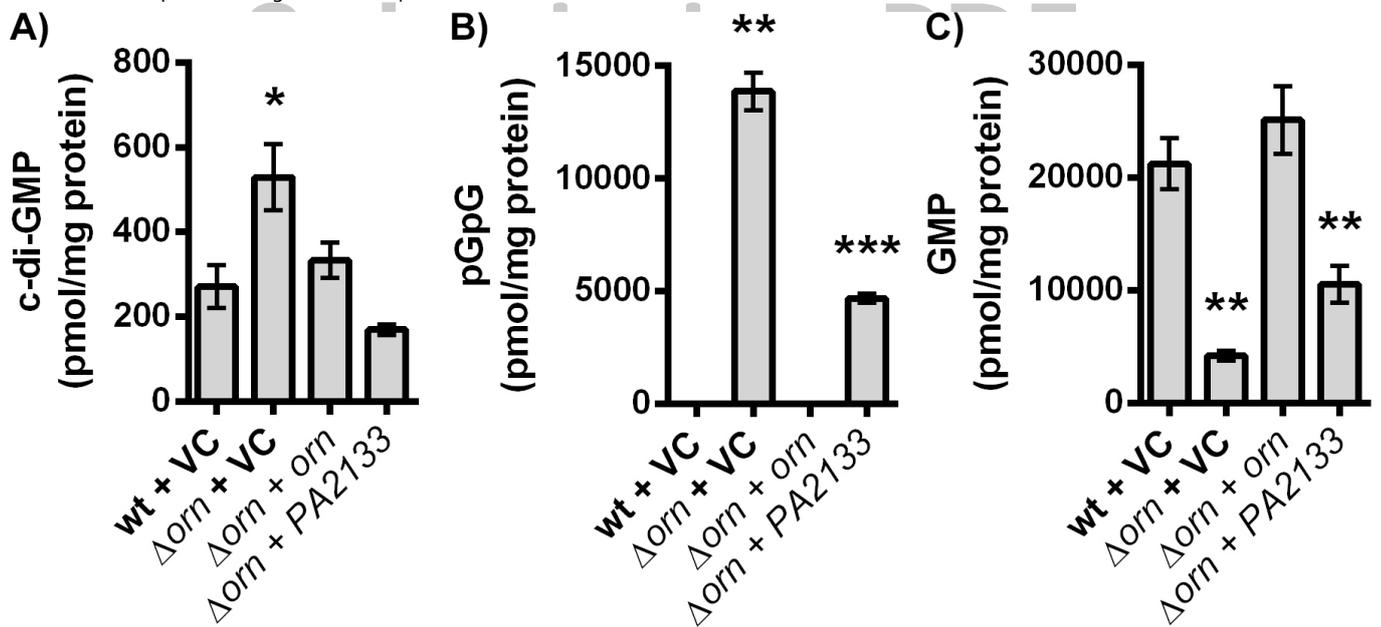
## Significance

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  $20 \times 20 \mu\text{m}$ . 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

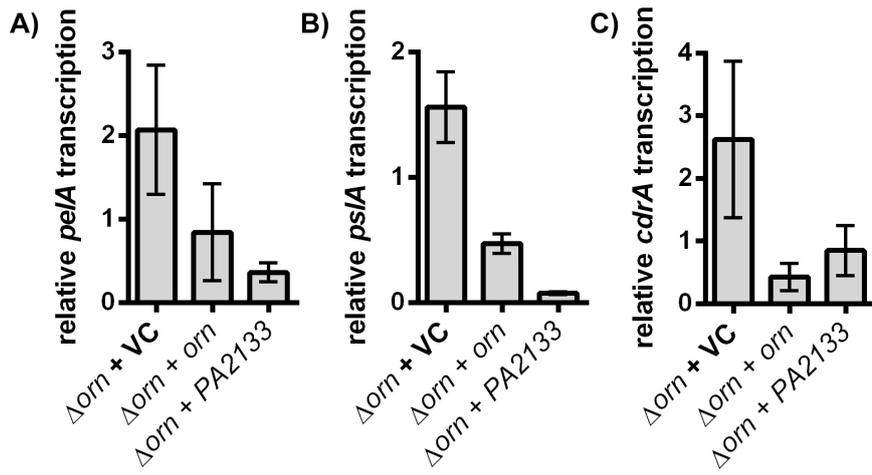


Fig. 3. The *P. aeruginosa*  $\Delta orn$  mutant overexpresses genes for extracellular polymers. RT-PCR was used to quantify relative transcript levels for (A) *peiA*, (B) *psIA*, and (C) *cdrA*. Values represent the means and standard deviations of 3 biological replicates each performed in 3 technical 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).

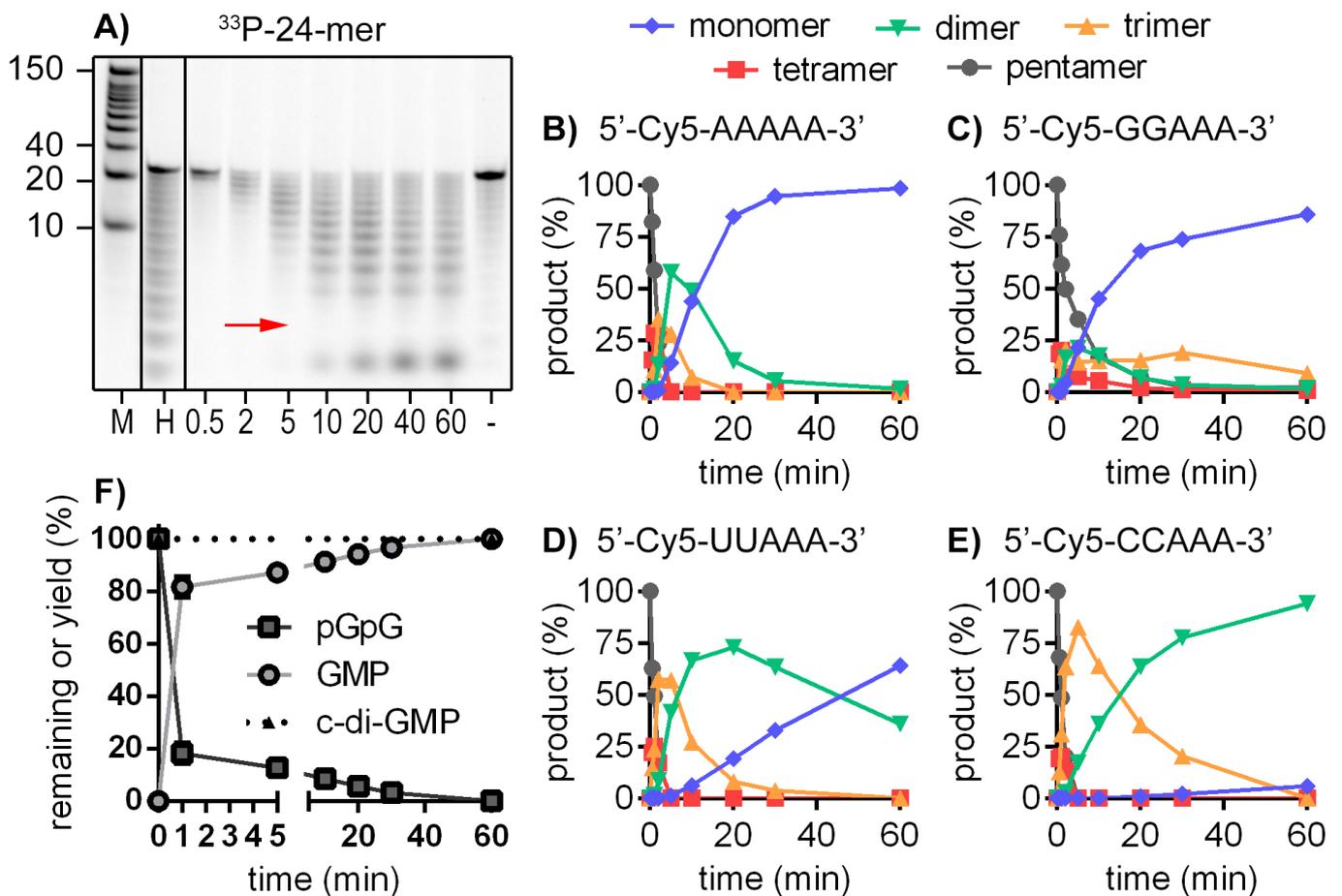


Fig. 4. Orn preferentially degrades nanoRNAs and purine dinucleotides *in vitro*. (A) Degradation of the 24-mer 5'- $^{33}P$ -CACACACACACACACACACACA-3'. The red arrow indicates the preferential elimination of nanoRNAs. M denotes the Millenium<sup>TM</sup> RNA marker, H denotes acid hydrolysis, and "-" denotes the 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*  $\Delta orn$  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

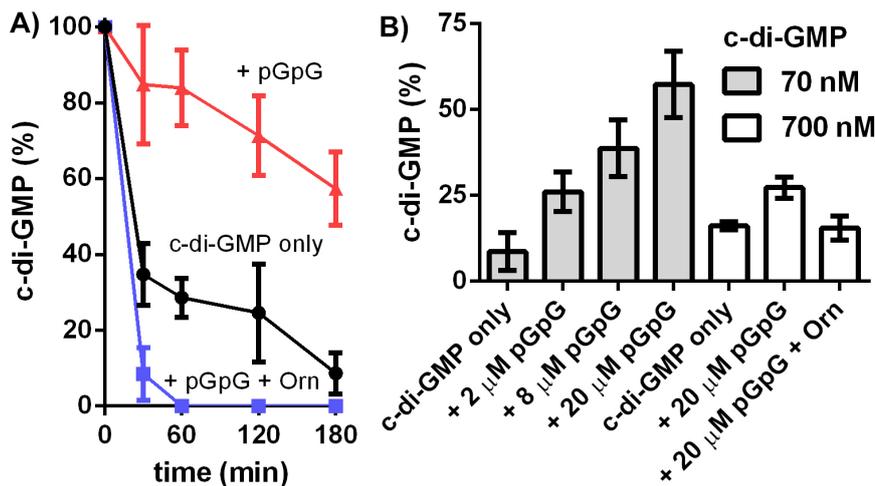


Fig. 5. pGpG inhibits the activity of EAL-dependent PDEs in lysates of cells overexpressing PA2133. C-di-GMP was measured by LC-MS/MS. (A) The addition of pGpG (20 μM) inhibited c-di-GMP degradation, and this was alleviated by adding 100 ng of purified Orn. (B) pGpG inhibited c-di-GMP degradation in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

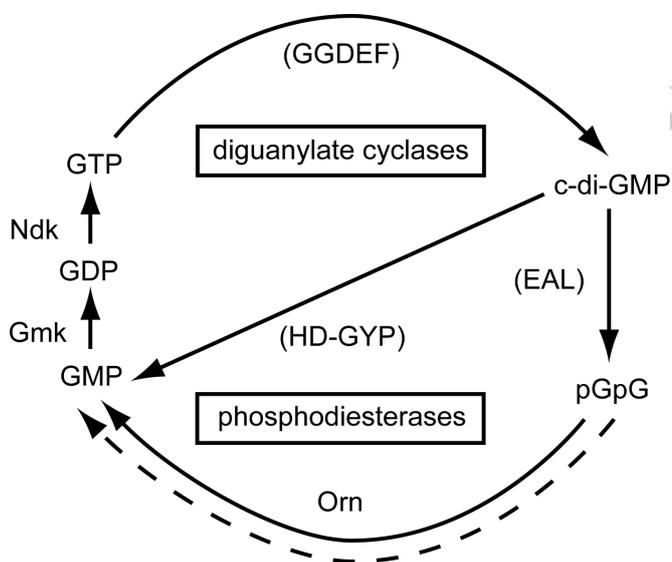


Fig. 6. Guanosine biochemistry fundamental to c-di-GMP signal transduction in *P. aeruginosa*. Solid lines indicate reactions mediated by the named types of enzymes. Gmk and Ndk denote guanylate kinase and nucleoside diphosphate kinase, respectively. The dashed line indicates a reaction that may be mediated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

in-frame deletion of *orn* in *P. aeruginosa* PAO1. A prominent phenotype of  $\Delta orn$  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  $\Delta orn$  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  $\Delta orn$  mutant expressing green fluorescent protein (GFP) in continuous flow chambers. We saw that the  $\Delta orn$  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  $\Delta orn$  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  $\Delta orn$  mutant strain might result from elevated intracellular c-di-GMP concentrations. To test this hypothesis, we extracted nucleotide pools from wild type and  $\Delta orn$  cells and quantified c-di-GMP using liquid chromatography tandem mass-spectrometry (LC-MS/MS). We observed that cultures of  $\Delta orn$  cells produced ~2-fold more c-di-GMP than the ancestral PAO1 strain (Fig. 2A). Wild type levels of c-di-GMP in the  $\Delta orn$  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  $\Delta orn$  mutant, a plasmid encoding an EAL-dependent PDE (*PA2133*) was transformed into this strain. This eliminated c-di-GMP-related phenotypes of the  $\Delta orn$  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  $\Delta orn$  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  $\Delta orn$  mutant that had additional deletions in *pelF* and *pslD*. A  $\Delta orn \Delta pelF \Delta pslD$  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  $\Delta orn$  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  $\Delta orn$  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  $\Delta orn$  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  $\Delta orn$  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  $\Delta orn$  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  $\Delta orn$  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-CAAA-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-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 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*  $\Delta orn$  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  $\mu M$  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  $\mu M$  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  $\Delta orn$  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*  $\Delta orn$  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*  $\Delta orn$  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,

681 cleaved c-di-GMP to yield the inactive open dimer, pGpG. Sub-  
682 sequently, a second Ca<sup>2+</sup>-insensitive phosphodiesterase, termed  
683 PDE-B, split this product into two molecules of GMP. It  
684 was soon discovered that EAL-domain-dependent phosphodi-  
685 esterases mediate PDE-A activity (23); however, the enzymes  
686 responsible for PDE-B activity have remained elusive.

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

703 Unlike *E. coli*, *P. aeruginosa* can withstand a loss-of-function  
704 mutation in *orn*. This discovery has enabled us to revisit the  
705 hypothesis that Orn mediates PDE-B activity. Here, we have  
706 provided multiple lines of evidence that Orn has a key role in  
707 not only pGpG degradation, but also c-di-GMP signalling *in vivo*.  
708 Thus oligoribonuclease has a role in guanosine metabolism that  
709 is thought to be fundamental to c-di-GMP signal transduction in  
710 *P. aeruginosa* (Fig. 6).

711 Investigations carried out in the 1970s examined the activity  
712 of *E. coli* Orn against a variety of dinucleotide substrates, and  
713 estimated that Orn had relatively low activity against GpG. Con-  
714 trary to that work, our investigation suggests that *P. aeruginosa*  
715 Orn has a relatively high capability for degrading purine dimers  
716 such as pApA and pGpG (Fig. 4). The basis for this discrepancy  
717 in relative reaction rates is not clear. However, our direct mea-  
718 surements of pGpG in cells lacking Orn (Fig. 2 and Fig. S6), as  
719 well as the ability of *E. coli* Orn to complement the *P. aeruginosa*  
720  $\Delta orn$  mutation (Fig. S6), suggest that Orn-dependent degradation  
721 of pGpG has a biologically relevant function, especially in the  
722 context of c-di-GMP signal transduction.

723 The mutational inactivation of *orn* is pleiotropic (16). We  
724 reasoned that overexpressing the c-di-GMP-specific PDE PA2133  
725 could distinguish the subset of genes and phenotypes differen-  
726 tially regulated by c-di-GMP from other changes due to loss of  
727 *orn*. Overexpression of PA2133 eliminated intracellular c-di-GMP  
728 in the  $\Delta orn$  strain (Fig. 2). As expected, this also abolished biofilm  
729 hyper-production (Fig. 1) as well as overexpression of *pelA*, *pslA*  
730 and *cdrA* (Fig. 3).

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

745 A question that arises from this work is whether or not pGpG-  
746 dependent inhibition of EAL-dependent PDEs may contribute to  
747 elevated intracellular c-di-GMP in the  $\Delta orn$  strain. Because Orn  
748

749 rapidly eliminates pGpG and because c-di-GMP degradation is  
750 difficult to detect in lysates of wild type cells, we utilized  $\Delta orn$   
751 cells overexpressing PA2133 or PvrR. We observed that under  
752 these conditions, c-di-GMP degradation was inhibited by 2- to 20  
753  $\mu\text{M}$  pGpG *in vitro*. Our direct measurements suggest that  $\Delta orn$   
754 cells may have as much as 1.8 mM intracellular pGpG, which  
755 is 90- to 900-times greater than those concentrations causing  
756 product inhibition in cell lysates. However, these data require  
757 careful interpretation. The optimized *in vitro* assay utilized 70  
758 to 700 nM c-di-GMP, whereas we estimated that intracellular c-  
759 di-GMP may be as high as 70  $\mu\text{M}$  in the  $\Delta orn$  strain (see *SI*  
760 *Materials and Methods*). Thus, c-di-GMP concentrations are 95-  
761 to 950-times less *in vitro* than those expected *in vivo*. We  
762 estimate, therefore, that the stoichiometric ratios of pGpG and c-  
763 di-GMP would be approximately similar in both *in vitro* assays and  
764 in  $\Delta orn$  cells. Thus, we propose that pGpG-dependent inhibition  
765 of EAL domain PDEs may be biologically relevant, increasing  
766 intracellular c-di-GMP in the  $\Delta orn$  strain. We acknowledge that  
767 not all PDE containing enzymes in the cell may be subject to  
768 feedback inhibition by pGpG. However, if it happens for even a  
769 few, this may tip the balance of DGC and PDE activity to favour  
770 the build-up of c-di-GMP in the  $\Delta orn$  strain.

771 Using LC-MS/MS, pGpG could not be detected in lysates  
772 of wild type cells, and therefore, it is not clear whether pGpG-  
773 dependent feedback control of EAL-domain PDEs is operational  
774 under normal circumstances. Rather, Orn seems to be responsible  
775 for naturally low levels of pGpG in wild type *P. aeruginosa* cells.  
776 Thus our data suggest that Orn provides homeostatic control of  
777 intracellular pGpG and that this may enable the responsiveness  
778 of c-di-GMP signaling cascades by preventing feedback inhibition  
779 of enzymes crucial for c-di-GMP degradation.

780 Genetic complementation of the *P. aeruginosa*  $\Delta orn$  strain  
781 indicates that *E. coli* Orn may degrade pGpG *in vivo*. Thus it is  
782 tempting to speculate that orthologues of Orn may function in c-  
783 di-GMP signal transduction in other organisms. A tBLASTn sur-  
784 vey of 3109 completely sequenced genomes in the NCBI database  
785 (as of October 31, 2013) identified protein sequences from 568  
786 additional bacterial species with >50% identity to *P. aeruginosa*  
787 PAO1 Orn (see Supplementary Dataset). A Bayesian analysis of  
788 Orn sequences placed these orthologues into four phyla (Fig. S7).  
789 This primarily includes Proteobacteria (especially  $\beta$ -,  $\delta$ - and  $\gamma$ -  
790 Proteobacteria) and Actinobacteria, but also single species rep-  
791 resenting Gemmatimonadetes (*Gemmatimonas aurantiaca*) and  
792 Fibrobacteres (*Fibrobacter succinogenes*). Orn may have a crucial  
793 role in completing the degradation of RNA (13), and thus it is  
794 no surprise that some bacterial species on this list do not possess  
795 EAL-domain proteins. What is more intriguing though is that  
796 many bacterial species that possess EAL-dependent PDEs do not  
797 have an apparent orthologue of Orn. Indeed, fifteen additional  
798 well-sampled bacterial phyla that are thought to possess EAL-  
799 domain proteins (1) do not have Orn. This includes members of  
800 Alphaproteobacteria such as *G. xylinus*, which is the species in  
801 which c-di-GMP was discovered. This raises the intriguing possi-  
802 bility that there may be additional, phylogenetically distinct types  
803 of nanoRNAses that participate in c-di-GMP signal transduction  
804 in the place of oligoribonuclease.

## 805 Materials and Methods

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

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

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

820 **Nucleotide extractions and quantification by LC-MS/MS.** Nucleotide  
821 pools were extracted from cultures grown for ~5 h at 37°C in BM2 using ice-  
822 cold acetonitrile/methanol/water (40/40/20% v/v) according to established  
823 procedures (26). Protein concentrations were determined from the cell  
824 pellets of 1 ml aliquots from these cultures using a bicinchoninic acid (BCA)  
825 Protein Assay kit (Pierce). GMP, pGpG and c-di-GMP concentrations were  
826 determined by LC-MS/MS using a cXMP or [<sup>13</sup>C<sup>15</sup>N]c-di-GMP internal standard  
827 as previously described (26, 27).

828 **RNA isolation, cDNA synthesis and RT-PCR.** Approximately 1.0 × 10<sup>9</sup> cells  
829 grown in BM2 were harvested at an OD<sub>600</sub> = 0.5 and mixed with RNAprotect  
830 Bacteria Reagent (Qiagen) and stored at -80 °C. Isolation of total RNA and  
831 an assessment of its quantity and quality were carried out as previously  
832 described by Chugani *et al.* (28), and this is detailed in *SI Materials and*  
833 *Methods*. Three biological replicates were processed for each strain. First  
834 strand synthesis was carried out using the qScript cDNA Supermix kit (Quanta  
835 Biosciences) following the manufacturer's protocols. Quantitative PCR mea-  
836 surements were made with a CFX96 Touch™ Real-Time PCR Detection System  
837 (BioRad) using SsoAdvanced™ Universal SYBR® Green Supermix (BioRad).

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

- 840 1. Romling U, Galperin MY, & Gomelsky M (2013) Cyclic di-GMP: the first 25 years of a  
841 universal bacterial second messenger. *Microbiol. Mol. Biol. Rev.* 77(1):1-52.
- 842 2. Hengge R (2009) Principles of c-di-GMP signalling in bacteria. *Nat. Rev. Microbiol.* 7:263-273.
- 843 3. Tamayo R, Pratt JT, & Camilli A (2007) Roles of cyclic diguanylate in the regulation of  
844 bacterial pathogenesis. *Annu. Rev. Microbiol.* 61:131-148.
- 845 4. Mills E, Pultz IS, Kulasekara HD, & Miller SI (2011) The bacterial second messenger c-di-  
846 GMP: mechanisms of signalling. *Cell. Microbiol.* 13(8):1122-1129.
- 847 5. Schirmer T & Jenal U (2009) Structural and mechanistic determinants of c-di-GMP sig-  
848 nalling. *Nat. Rev. Microbiol.* 7(10):724-735.
- 849 6. Christen M, Christen B, Folcher M, Schauerte A, & Jenal U (2005) Identification and  
850 characterization of a cyclic di-GMP-specific phosphodiesterase and its allosteric control by  
851 GTP. *J. Biol. Chem.* 280(35):30829-30837.
- 852 7. Ryan RP, *et al.* (2006) Cell-cell signaling in *Xanthomonas campestris* involves an HD-GYP  
853 domain protein that functions in cyclic di-GMP turnover. *Proc. Natl. Acad. Sci. U. S. A.*  
854 103(17):6712-6717.
- 855 8. Bellini D, *et al.* (2013) Crystal structure of an HD-GYP domain cyclic-di-GMP phosphodi-  
856 esterase reveals an enzyme with a novel trinuclear catalytic iron center. *Mol. Microbiol.*
- 857 9. Stelitano V, *et al.* (2013) C-di-GMP hydrolysis by *Pseudomonas aeruginosa* HD-GYP phos-  
858 phodiesterases: analysis of the reaction mechanism and novel roles for pGpG. *PLoS One*  
859 8(9):e74920.
- 860 10. Ross P, *et al.* (1987) Regulation of cellulose synthesis in *Acetobacter xylinum* by cyclic  
861 diguanylic acid. *Nature* 325(6101):279-281.
- 862 11. Mechold U, Fang G, Ngo S, Ogryzko V, & Danchin A (2007) YtqI from *Bacillus subtilis* has  
863 both oligoribonuclease and pAp-phosphatase activity. *Nucleic Acids Res.* 35(13):4552-4561.
- 864 12. Fang M, *et al.* (2009) Degradation of nanoRNA is performed by multiple redundant RNases  
865 in *Bacillus subtilis*. *Nucleic Acids Res.* 37(15):5114-5125.
- 866 13. Ghosh S & Deutscher MP (1999) Oligoribonuclease is an essential component of the mRNA  
867 decay pathway. *Proc. Natl. Acad. Sci. U. S. A.* 96(8):4372-4377.
- 868 14. Vvedenskaya IO, *et al.* (2012) Growth phase-dependent control of transcription start site  
869 selection and gene expression by nanoRNAs. *Genes Dev.* 26(13):1498-1507.
- 870 15. Hickman JW, Tifrea DF, & Harwood CS (2005) A chemosensory system that regulates biofilm  
871 formation through modulation of cyclic diguanylate levels. *Proc. Natl. Acad. Sci. U. S. A.*  
872 102(40):14422-14427.
- 873 16. Goldman SR, *et al.* (2011) NanoRNAs prime transcription initiation *in vivo*. *Mol. Cell*  
874 42(6):817-825.
- 875 17. Jacobs MA, *et al.* (2003) Comprehensive transposon mutant library of *Pseudomonas aerugi-*

885 **RNA degradation assays.** <sup>33</sup>P- and Cy5-labeled RNA oligomers were  
886 prepared as described in *SI Materials and Methods*. Exonuclease assays were  
887 carried out using established protocols (11) and this is detailed in *SI Materials*  
888 *and Methods*.

889 **PDE activity assays.** PDE activity assays for whole cell lysates were  
890 performed according to the method of Kulasekara *et al.* (29) and this is  
891 detailed in *SI Materials and Methods*.

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

898 **Phylogenetic analyses.** Bacterial species surveys to identify *orn* ortho-  
899 logues and the construction of gene phylogenies is described in *SI Materials*  
900 *and Methods*.

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

- 910 *nosa*. *Proc. Natl. Acad. Sci. U. S. A.* 100(24):14339-14344.
- 911 18. Held K, Ramage E, Jacobs M, Gallagher L, & Manoil C (2012) Sequence-verified two-allele  
912 transposon mutant library for *Pseudomonas aeruginosa* PAO1. *J. Bacteriol.* 194(23):6387-6389.
- 913 19. Baraquet C, Murakami K, Parsek MR, & Harwood CS (2012) The FleQ protein from  
914 *Pseudomonas aeruginosa* functions as both a repressor and an activator to control gene  
915 expression from the *pel* operon promoter in response to c-di-GMP. *Nucleic Acids Res.*  
916 40(15):7207-7218.
- 917 20. Starkey M, *et al.* (2009) *Pseudomonas aeruginosa* rugose small colony variants have adapta-  
918 tions that likely promote persistence in the cystic fibrosis lung. *J. Bacteriol.* 191(11):3492-3503.
- 919 21. Niyogi SK & Datta AK (1975) A novel oligoribonuclease of *Escherichia coli*. I. Isolation and  
920 properties. *J. Biol. Chem.* 250(18):7307-7312.
- 921 22. Datta AK & Niyogi K (1975) A novel oligoribonuclease of *Escherichia coli*. II. Mechanism of  
922 action. *J. Biol. Chem.* 250(18):7313-7319.
- 923 23. Tal R, *et al.* (1998) Three *cdg* operons control cellular turnover of cyclic di-GMP in *Aceto-*  
924 *bacter xylinum*: genetic organization and occurrence of conserved domains in isoenzymes. *J.*  
925 *Bacteriol.* 180(17):4416-4425.
- 926 24. Schmidt AJ, Ryjenkov DA, & Gomelsky M (2005) The ubiquitous protein domain EAL  
927 is a cyclic diguanylate-specific phosphodiesterase: enzymatically active and inactive EAL  
928 domains. *J. Bacteriol.* 187(14):4774-4781.
- 929 25. O'Toole GA & Kolter R (1998) Initiation of biofilm formation in *Pseudomonas fluorescens*  
930 WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Mol.*  
931 *Microbiol.* 28(3):449-461.
- 932 26. Spangler C, Bohm A, Jenal U, Seifert R, & Kaever V (2010) A liquid chromatography-  
933 coupled tandem mass spectrometry method for quantitation of cyclic di-guanosine  
934 monophosphate. *J. Microbiol. Methods* 81(3):226-231.
- 935 27. Spangler C, Kaever V, & Seifert R (2011) Interaction of the diguanylate cyclase YdeH of  
936 *Escherichia coli* with 2',3'-substituted purine and pyrimidine nucleotides. *J. Pharmacol. Exp.*  
937 *Ther.* 336(1):234-241.
- 938 28. Chugani S, *et al.* (2012) Strain-dependent diversity in the *Pseudomonas aeruginosa* quorum-  
939 sensing regulon. *Proc. Natl. Acad. Sci. U. S. A.* 109(41):E2823-2831.
- 940 29. Kulasekara H, *et al.* (2006) Analysis of *Pseudomonas aeruginosa* diguanylate cyclases and  
941 phosphodiesterases reveals a role for bis-(3'-5')-cyclic-GMP in virulence. *Proc. Natl. Acad.*  
942 *Sci. U. S. A.* 103(8):2839-2844.
- 943 30. Livak KJ & Schmittgen TD (2001) Analysis of relative gene expression data using real-time  
944 quantitative PCR and the 2<sup>-ΔΔCT</sup> Method. *Methods* 25(4):402-408.