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

Dorit Cohen^a, Undine Mechold^b, Hadas Nevenzal^a, Yafit Yarmiyhu^a, Trevor E. Randall^c, Denice C. Bay^d, Jacquelyn D. Rich^e, Matthew R. Parsek^e, Volkhard Kaever^f, Joe J. Harrison^{c,1}, and Ehud Banin^{a,1}

^aCenter for Advanced Materials and Nanotechnology, The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan 5290002, Israel; ^bUnité de Biochimie des Interactions Macromoléculaires, Département de Biologie Structurale et Chimie, Institut Pasteur, 75724 Paris Cedex 15, France; ^cDepartment of Biological Sciences, University of Calgary, Calgary, AB, Canada T2N 1N4; ^dDepartment of Medical Microbiology, University of Manitoba, Winnipeg, MB, Canada R3E 0J9; ^eDepartment of Microbiology, University of Washington, Seattle, WA 98105; and ^fResearch Core Unit Metabolomics, Institute of Pharmacology, Hannover Medical School, Hannover 30625, Germany

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The second messenger cyclic diguanylate (c-di-GMP) controls diverse cellular processes among bacteria. Diguanylate cyclases synthesize c-di-GMP, whereas it is degraded by c-di-GMP-specific phosphodiesterases (PDEs). Nearly 80% of these PDEs are predicted to depend on the catalytic function of glutamate-alanine-leucine (EAL) domains, which hydrolyze 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 the primary enzyme responsible for pGpG degradation in *Pseudomonas aeruginosa* cells. We found that a *P. 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 two to five nucleotides (nanoRNAs), including pGpG. Corresponding with this activity, Δorn cells possessed highly elevated pGpG levels. We found that pGpG reduced the rate of c-di-GMP degradation in cell lysates and inhibited the activity of EAL-dependent PDEs (PA2133, PvrR, and purified recombinant RocR) from *P. aeruginosa*. This pGpG-dependent inhibition was alleviated by the addition of Orn. These data suggest that elevated levels of pGpG exert product inhibition on EAL-dependent PDEs, thereby increasing intracellular c-di-GMP in Δorn cells. Thus, we propose that Orn provides homeostatic control of intracellular pGpG under native physiological conditions and that this activity is fundamental to c-di-GMP signal transduction.

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

Enzymes that are predicted to “make and break” second messenger cyclic diguanylate (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 up-regulate motility and virulence factor expression, whereas high levels promote extracellular polysaccharide (EPS) production, biofilm development, and cell cycle progression (1, 2).

c-di-GMP is enzymatically synthesized by proteins harboring glycine-glycine-aspartate-glutamate-phenylalanine (GGDEF) domains and degraded by glutamate-alanine-leucine (EAL) and histidine-aspartate-glycine-tyrosine-proline (HD-GYP) domain-containing proteins (3). EAL domains catalyze the asymmetric hydrolysis of c-di-GMP to yield the linear dinucleotide 5'-phosphoguanylyl-(3',5')-guanosine (pGpG) (4). By contrast, HD-GYP domains degrade c-di-GMP to GMP (5–7). 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 y ago (8), the identities of the proteins involved in degrading pGpG have remained enigmatic.

RNAs that are two to five nucleotides in length have been termed nanoRNAs (9). These short oligonucleotides, including pGpG, are degraded by nanoribonucleases (nanoRNases) (9). One of the most likely candidates for pGpG degradation in vivo is oligoribonuclease (*orn*) (1). *Escherichia coli* Orn and its orthologs are required to complete the degradation of mRNA to mononucleotides. Although some bacteria possess multiple redundant nanoRNases (10), Orn is essential for the viability of other species, including *E. coli* (9, 11). 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 (12). Recently, a method was devised to trigger the posttranslational 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 causing changes in promoter-specific gene expression (13). Nevertheless, because Orn depletion did not

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 5'-phosphoguanylyl-(3',5')-guanosine (pGpG). Although 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 signaling in the human pathogen *Pseudomonas aeruginosa*. This discovery reveals that nanoribonucleases, which have been considered housekeeping proteins crucial for mRNA turnover, also have a key role in c-di-GMP signaling.

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¹To whom correspondence may be addressed. Email: jjharris@ucalgary.ca or ehud.banin@biu.ac.il.

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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 SDs of three biological replicates. * $P \leq 0.05$ and *** $P \leq 0.001$ vs. WT with Student *t* test. (B) Biofilm development in flow cells. Each square on the grid is $20 \times 20 \mu\text{m}$. VC, vector control.

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 signaling was not evident.

Here we report 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 previously unidentified opportunity to investigate the function of Orn in bacterial c-di-GMP signaling. 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 EAL-dependent PDEs produced in cell lysates or purified in vitro inhibits c-di-GMP degradation and that this inhibition can be alleviated through addition of purified Orn. Collectively, these data suggest that Orn has a key role in *P. aeruginosa* c-di-GMP signal transduction. Finally, we demonstrate that the *E. coli* ortholog of Orn alleviates pGpG accumulation and restores wild-type (WT) phenotypes to a *P. aeruginosa* Δ*orn* mutant, suggesting that orthologs 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) (14). Thus, to investigate the function of oligoribonuclease, we constructed an 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 transposon mutant library (14), produced similar results (Fig. 1A). To investigate these findings further, we grew PAO1 and the Δ*orn*

mutant expressing 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 WT copy of *orn* on a plasmid complemented all of these phenotypes (Fig. 1 and Fig. S1). Repeating the complementation analysis with an allele of *orn* bearing a site-directed mutation at a conserved catalytic residue (D11A, E13Q, or D162A) failed to reverse the phenotypes of the Δ*orn* strain (Fig. 1A and Fig. S1). We conclude from these collective data that the ribonuclease activity of 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 WT and Δ*orn* cells and quantified c-di-GMP using LC tandem MS (LC-MS/MS). We observed that cultures of Δ*orn* cells produced approximately twofold more c-di-GMP than the ancestral PAO1 strain (Fig. 2A). WT 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 plasmid 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 genes for synthesis of the biofilm matrix, including extracellular polysaccharides (*pel* and *psl*) and an adhesin (*cdrA*) (15). We observed that transcription of these genes was up-regulated 1.7- to 2.6-fold by loss of *orn* (Fig. S2A–C). Deletions in *pelF* and *pslD* abolished biofilm formation (Fig. S2D) and aggregation (Fig. S2E) by the Δ*orn* strain (SI Results). These data indicate that loss of *orn* transcriptionally up-regulates a hallmark c-di-GMP regulon and that this contributes to 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 signaling in the Δ*orn* strain led us to hypothesize that the loss of *orn* might radically alter intracellular pools of pGpG and GMP. To test this, we extracted nucleotide pools from WT and Δ*orn* strains and measured pGpG and GMP using LC-MS/MS. Loss of *orn* resulted in a fivefold decrease in cellular GMP (Fig. 2C). Although pGpG was not detected in WT cells, there was a massive increase in this dinucleotide in the Δ*orn* strain (Fig. 2B). In fact, these data suggest that Δ*orn* cells had intracellular pGpG concentrations that far exceeded those of GMP. We estimated that WT *P. aeruginosa* had an average intracellular concentration of 134 μM GMP, undetectable levels of pGpG, and 1.7 μM c-di-GMP, whereas the Δ*orn* strain had ~26 μM GMP, 88 μM pGpG, and 3.3 μM c-di-GMP (see SI Results for calculation). 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 y ago (16, 17). These studies indicated that Orn is a manganese (Mn²⁺)-dependent 3'→5' exonuclease that produces 5'-phosphorylated ribonucleotide monomers from polyribonucleotides (16). 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

Fig. 3. Orn preferentially degrades purine dinucleotides in vitro. (A) Degradation of exogenously added pGpG (20 μ M) after 60 min in cell lysates as measured by LC-MS/MS. A representative independent experiment is shown, and each datum point represents the mean and SE for three technical replicates. (B) Degradation of pGpG and production of GMP by 100 ng Orn in vitro as assessed by LC-MS/MS. Each datum point is the mean and SD of three replicates and is expressed as the percentage yield of GMP, or the amount of pGpG or c-di-GMP remaining. (C–F) Degradation and semiquantitation of Cy5-pentamers. Each semiquantitation is one representative of three technical replicates.

specifically in the context of the Δorn strain. However, a corollary from these collective data is that Orn exonuclease activity is essential for the elimination of pGpG in WT cells and that this homeostatic function is crucial for the normal catalytic functioning of EAL domain-dependent PDEs.

Orn Is Highly Conserved Among Bacteria but Is Not Likely the Only NanoRNase Important for c-di-GMP Signal Transduction. Orn is essential for the viability of *E. coli*, another paradigm species for studying c-di-GMP signal transduction (2). Therefore, we used interspecies genetic complementation to test whether *E. coli* and *P. aeruginosa* Orn proteins are functional orthologs (SI Results). We observed that *P. aeruginosa* Orn could rescue the growth defect of an *E. coli* conditional orn mutant (Fig. S5A) and that *E. coli* Orn was able to reverse the phenotypes of the *P. aeruginosa* Δorn mutant (Fig. S5B–E). These data suggest that *E. coli* Orn has the capacity to function in the two-step pathway for c-di-GMP degradation.

It is thus tempting to speculate that orthologs of Orn may function in c-di-GMP signal transduction in other organisms, and therefore, a tBLASTn survey of Orn was performed for 3,109 complete genomes in the National Center for Biotechnology Information (NCBI) database (as of October 31, 2013). This search identified proteins from 568 additional bacterial species with >50% identity to *P. aeruginosa* PAO1 Orn (Dataset S1). A Bayesian analysis of Orn sequences placed these orthologs into four phyla (Fig. S6A). This primarily includes Proteobacteria (especially Beta-

Delta- and Gammaproteobacteria) and Actinobacteria, but also single species representing Gemmatimonadetes and Fibrobacteres.

A cofrequency analysis indicated that the number of genome-sequenced bacterial species possessing both Orn and EAL domain proteins (23%) was slightly greater than the number of those possessing Orn but no EAL domain proteins (13%) (Fig. S6B). At first glance this seems to suggest coselection between Orn and EAL domain proteins. However, Orn may have a crucial role in completing the degradation of RNA (11), and thus it is no surprise that some bacterial species that have Orn do not possess EAL domain proteins. What is more intriguing, however, is that many bacterial species that possess EAL-dependent PDEs do not have an apparent ortholog of Orn (Fig. S6B). Indeed, 15 additional well-sampled bacterial phyla that are thought to possess EAL domain proteins (1) do not have Orn. These phyla include members of Alphaproteobacteria such as *Gluconacetobacter xylinus*, which is the species in which c-di-GMP was discovered (8). Moreover, a large number of all genome-sequenced bacterial species (10%) are predicted to possess EAL domain proteins yet have neither Orn nor HD-GYP domain proteins (Fig. S6B), the latter of which have been postulated to possess functional redundancy to Orn (Discussion). This finding 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.

Fig. 4. pGpG inhibits c-di-GMP degradation in cell lysates and inhibits activity of EAL-dependent PDEs in vitro. C-di-GMP was measured by LC-MS/MS unless otherwise noted in this legend. (A) Degradation of c-di-GMP (70 nM) after 60 min in cell lysates with and without added pGpG (20 μ M). (B) Degradation kinetics of c-di-GMP (70 nM) in lysates of Δorn cells overexpressing PA2133 with and without the addition of pGpG (20 μ M). Inhibition was alleviated by adding 100 ng of purified Orn. (C) C-di-GMP and pGpG concentration-dependent inhibition of c-di-GMP degradation in lysates of Δorn cells overexpressing PA2133 (measured at 60 min). Where indicated, 100 ng of purified Orn was added. (D) Degradation of MANT-c-di-GMP (400 nM) by purified RocR (1 μ M) in vitro, with and without the addition of pGpG (400 μ M) or purified Orn (1 μ M). In all panels, a representative independent experiment is shown, and each datum point represents the mean and SD for three technical replicates.

c-di-GMP was originally identified as an allosteric activator of cellulose synthase in *G. xylinus* (8). This 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 domains mediate PDE-A activity (20); however, the enzymes responsible for PDE-B activity have remained elusive.

Identifying the enzymes responsible for PDE-B activity has been complicated because bacteria possess many 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 (21). This mechanism may explain the reduction of pGpG in *P. aeruginosa* Δorn cells transformed with a plasmid that highly overexpresses PA2133 (Fig. 2). However, overexpression of PA2133 was still insufficient to restore native levels of pGpG to Δorn cells (Fig. 2B). HD-GYP domain proteins might also degrade pGpG; however, whereas HD-GYP domains may degrade pGpG *in vitro* (7), the relevance of this activity has not been substantiated *in vivo*. Finally, nano-RNAses 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* viability (11).

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 signaling in vivo. Although there may be other PDEs that degrade pGpG, it is clear from this work that Orn has a role in guanosine homeostasis that is fundamental to c-di-GMP signal transduction in *P. aeruginosa* (Fig. 5).

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 (17). 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. 3 C and D). 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. S5), as well as the ability of *E. coli* Orn to complement the *P. aeruginosa* Δorn mutation (Fig. S5), suggest that Orn-dependent degradation of pGpG has a biologically relevant function, especially in the context of c-di-GMP signaling.

The mutational inactivation of *orn* is pleiotropic (13). Among other phenotypes, this results in the accumulation of nanoRNAs—including pGpG—that may differentially prime transcription (13). 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 hyperproduction (Fig. 1), aggregation (Fig. S1), and overexpression of *pelA*, *pslA*, and *cdrA* (Fig. S2).

A key observation is that pGpG inhibits the degradation of c-di-GMP in lysates of Δorn cells overexpressing EAL-dependent PDEs, as well as purified RocR *in vitro* (Fig. 4). A question that arises from this work is whether or not this product inhibition may lead to elevated c-di-GMP *in vivo*. Data in this report do not provide compelling evidence that this mechanism would be operable under normal circumstances. For instance, pGpG could

Fig. 5. Guanosine biochemistry fundamental to c-di-GMP signal transduction in *P. aeruginosa*. Solid lines indicate reactions and enzymes in which physiological relevance has been substantiated *in vivo*. The dashed lines indicate a reaction that may be mediated by other unknown cellular enzymes or through the activity of the named enzyme, which has been inferred by sequence or structural similarity. Gmk and Ndk denote guanylate kinase and nucleoside diphosphate kinase, respectively.

not be detected in nucleotide extracts from WT *P. aeruginosa* by LC-MS/MS (Fig. 2). Also, although it is possible that Orn activity may be differentially regulated under certain conditions, there is little evidence for differential Orn expression in the Gene Expression Omnibus database (22). Last, we investigated *orn* gene expression by RT-PCR in planktonic cultures and biofilms. There was no more than a 1.7-fold change in *orn* transcription measured between any two conditions tested (Fig. S7).

However, by contrast we propose that pGpG-dependent inhibition of PDEs is a likely scenario for the Δom strain. In contrast to WT cells, intracellular pGpG in Δom cells was greater than $>80 \mu\text{M}$, exceeding levels of GMP (Fig. 2 and [SI Results](#)). LC-MS/MS measurements indicated that the molar ratio of pGpG to c-di-GMP was nearly 30:1 in Δom cells (Fig. 2). Similar stoichiometric ratios of pGpG to c-di-GMP (between 25:1 and 1,000:1) inhibited EAL domain-dependent PDEs in cell lysates (even when those proteins were over-expressed; Fig. 4B) and inhibited the activity of purified RocR in vitro (Fig. 4D and [Fig. S4B](#)). Thus, pGpG-dependent inhibition of EAL domain PDEs may be relevant specifically in the context of the Δom 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 favor the buildup of c-di-GMP in the Δom mutant.

The corollary of these results is that Orn is responsible for low levels of pGpG in WT *P. aeruginosa*. These data suggest that homeostatic control of pGpG by Orn enables the responsiveness of c-di-GMP signaling networks by preventing feedback inhibition of PDEs under normal physiological conditions. Thus, we conclude that Orn is the primary PDE in *P. aeruginosa* that catalyses a crucial final step in c-di-GMP degradation. This finding reveals the possibility that Orn and other nanoRNAses, which have long been considered housekeeping enzymes essential for mRNA degradation (11), may participate in other RNA-based signal transduction processes in many species.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions. Bacterial strains, plasmids, and primers are described in [Tables S1](#) and [S2](#). Microbiological media, growth conditions, antibiotic selection, and molecular methods for plasmid and strain construction are described in [SI Materials and Methods](#).

Static and Flow Cell Biofilm Experiments. Crystal violet quantification of biofilms grown in basal medium 2 (BM2) in static microtiter plates was carried out using established protocols (23). Flow cell biofilms were grown in 1% tryptic soy broth (TSB) and then imaged using a Leica TCS SPE confocal microscope as described in [SI Materials and Methods](#). Z-stacks of 2D confocal images were rendered in three dimensions using Imaris (Bitplane). Last, biofilms were cultivated using a silicone tube model (24) in BM2 at 37 °C for gene expression measurements ([SI Materials and Methods](#)).

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% (vol/vol/vol)] according to established procedures (25). 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 [$^{13}\text{C}^{15}\text{N}$]c-di-GMP internal standard as previously described (25).

RNA Isolation, cDNA Synthesis, and RT-PCR. Approximately 1.0×10^9 cells grown in BM2 were harvested at an $\text{OD}_{600} = 0.5$, mixed with RNAprotect Bacteria Reagent (Qiagen), and stored at -80°C . Isolation of total RNA and assessment of its quantity and quality were performed as previously described (26) (*SI Materials and Methods*). First-strand synthesis was carried out using the qScript cDNA Supermix kit (Quanta Biosciences). Quantitative PCR (27) measurements were made with a CFX96 Touch Real-Time PCR Detection System (BioRad) using SsoAdvanced Universal SYBR Green Supermix (BioRad).

Protein Production. Recombinant Orn and RocR were produced by using 0.2% L-arabinose and 0.1 mM isopropyl- β -D-1-thiogalactopyranoside to induce the expression of Orn-His $\times$ 6 (from pDC15) and RocR-His $\times$ 6 (from pET26b::rocR) (18) in *E. coli* BL21(DE3) and Nico21(DE3), respectively. Cells were harvested and Orn-His $\times$ 6 or RocR-His $\times$ 6 were purified using established protocols for HPLC with a HisTrap HP column (GE Healthcare) (*SI Materials and Methods*).

RNA Degradation Assays. Exonuclease assays were carried out using ^{33}P - and Cy5-labeled RNA oligomers (9) electrophoresed and imaged in 40% polyacrylamide gels as described in *SI Materials and Methods*.

PDE Activity Assays. Established procedures for PDE assays using whole cell lysates (28) and MANT-c-di-GMP (19) are detailed in *SI Materials and Methods*.

Interspecies Genetic Complementation. *E. coli* UM341 (9), in which *ptetO* drives *orn* expression, was transformed with plasmids expressing either *E. coli* or *P. aeruginosa orn*, or the appropriate vector control. Genetic complementation was evaluated by assessing bacterial growth with or without of anhydrotetracycline (Atc).

Phylogenetic Analyses. Bioinformatics tools (29–33) used to identify *orn* orthologs and domain cofrequencies and to construct gene phylogenies are described in *SI Materials and Methods*.

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