Oligoribonuclease is the primary degradative enzyme for pGpG in Pseudomonas aeruginosa that is required for cyclic-di-GMP turnover

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The bacterial second messenger cyclic di-GMP (c-di-GMP) controls biofilm formation and other phenotypes relevant to pathogenesis. Cyclic-di-GMP is synthesized by diguanylate cyclases (DGCs). Phosphodiesterases (PDE-As) end signaling by linearizing c-di-GMP to 5′-phosphoguanosyl-(3′,5′)-guanosine (pGpG), which is then hydrolyzed to two GMP molecules by yet unidentified enzymes termed PDE-Bs. We show that pGpG inhibits a PDE-A from Pseudomonas aeruginosa. In a dual DGC and PDE-A reaction, excess pGpG extends the half-life of c-di-GMP, indicating that removal of pGpG is critical for c-di-GMP homeostasis. Thus, we sought to identify the PDE-B enzyme(s) responsible for pGpG degradation. A differential radial capillary action of ligand assay-based screen for pGpG binding proteins identified oligoribonuclease (Orn), an exoribonuclease that hydrolyzes two- to five-nucleotide-long RNAs. Purified Orn rapidly converts pGpG into GMP. To determine whether Orn is the primary enzyme responsible for degrading pGpG, we assayed cell lysates of WT and orn strains of P. aeruginosa PA14 for pGpG stability. The lysates from orn showed 25-fold decrease in pGpG hydrolysis. Complementation with WT, but not active site mutants, restored hydrolysis. Accumulation of pGpG in the orn strain could inhibit PDE-As, increasing c-di-GMP concentration. In support, we observed increased transcription from the c-di-GMP-regulated pel promoter. Additionally, the c-di-GMP–governed auto-aggregation and biofilm phenotypes were elevated in the orn strain in a pel-dependent manner. Finally, we directly detect elevated pGpG and c-di-GMP in the orn strain. Thus, we identified that Orn serves as the primary PDE-B enzyme that removes pGpG, which is necessary to complete the final step in the c-di-GMP degradation pathway.

In addition to regulation by extracellular signals, c-di-GMP homeostasis is subject to feedback inhibition. Crystal structures of the DGCs PleD from Caulobacter crescentus and WspR from Pseudomonas aeruginosa show that c-di-GMP binds to an RxxD domain I-site to inhibit c-di-GMP synthesis (17, 18). Additionally, the Xanthomonas campestris XCC4471 DGC lacking an RxxD motif can also be inhibited by excess c-di-GMP through c-di-GMP binding to and occluding the active site (19). The linearized c-di-GMP hydrolysis product, 5′-phosphoguanosyl-(3′,5′)-guanosine (pGpG), also plays an active role in cyclic dinucleotide turnover. Purified YfgF from Escherichia coli, a PDE-A, is inhibited by excess pGpG through an unknown mechanism (20). Here, we show that the EAL domain PDE-A RocR from the P. aeruginosa PA14 strain is also inhibited by excess pGpG via direct competition with c-di-GMP binding in the active site. Accordingly, the addition of excess pGpG results in an increased c-di-GMP half-life in vitro, suggesting that removal of pGpG is required for terminating c-di-GMP signaling.

To elucidate the mechanism of c-di-GMP signal termination, we sought to identify the PDE-B(s) responsible for cleaving pGpG. Previously, HD-GYP domain-containing proteins were found to inhibit HD-GYP domain-containing proteins were found to inhibit...
proposed to be the PDE-Bs involved in pGpG hydrolysis because they bind pGpG with higher affinity than c-di-GMP (21). How- ever, HD-GYP domain proteins are missing in some genomes that contain other c-di-GMP signaling machinery (1), suggesting that other enzymes must be responsible for PDE-B activity. To identify PDE-B(s), we used a screen based on the differential radial capillary action of ligand assay (DRaCALA) (22, 23) to probe an ORF library of Vibrion cholerae El Tor N16961 (24) for proteins that bind pGpG. This screen identified oligoribonuclease (Orn) as a protein that binds pGpG, but not c-di-GMP. Orn is an exoribonuclease that cleaves two- to five-nucleotide-long RNA molecules (25). We found that purified Orn from both V. cholerae and P. aeruginosa bound and cleaved pGpG. To determine whether Orn is the primary PDE-B in bacteria, we show that whole cell lysates of an orn transposon mutant (orn:tn) from the P. aeruginosa PA14 Non-Redundant Transposon Insertion Mutant Library (26) and an in-frame deletion mutant of orn (Δorn) were decreased in pGpG cleaving activity by 25-fold compared with the parental strain. Complementation with WT orn, but not active site point mutants, restored pGpG hydrolysis in cell lysates. Thus, loss of orn is expected to increase pGpG concentration in vivo and may inhibit PDE-A activity to also elevate c-di-GMP concentrations in the Δorn strain. In support of elevated c-di-GMP in the Δorn strain, we observed three times more activity from the c-di-GMP-responsive pel promoter FleQ (27). We also demonstrate that two c-di-GMP-governed phenotypes, biofilm formation (28) and aggregation (29), are enhanced in the Δorn strain, and are dependent on the c-di-GMP-regulated PEL exopolysaccharides (30). Using LC-MS/MS, we determined that higher levels of both pGpG and c-di-GMP in extracts of the Δorn strain compared with WT. Taken together, these results indicate that Orn is the primary PDE-B responsible for degrading pGpG in P. aeruginosa.

Results

pGpG Inhibits RocR Phosphodiesterase Activity by Binding to the Active Site

The PDE-A YfgF from E. coli is inhibited by the addition of 100 μM of pGpG (20). We asked whether PDE-A inhibition by pGpG is a generalizable phenomenon. The RocR PDE-A from P. aeruginosa PA14 was used to determine inhibition by pGpG. In the absence of competitor (Fig. 1A), RocR rapidly converts 89% of the radiolabeled c-di-GMP to pGpG within 5 min as measured after separation by TLC. The addition of 100-fold excess of pGpG nearly completely inhibited c-di-GMP linearization (Fig. 1A). These results demonstrate that pGpG inhibits PDE-A activity. To determine whether pGpG is competing with c-di-GMP for active site binding in RocR, we performed experiments in which binding of radiolabeled c-di-GMP to purified RocR was assessed in the presence of different unlabeled nucleotide competitors. RocR binds c-di-GMP with a fraction bound of 0.54 ± 0.03 in the absence of competitor, whereas the addition of 50 μM unlabeled pGpG reduced fraction bound to 0.02 ± 0.005 (P < 0.001), indicating that pGpG competes for c-di-GMP binding (Fig. 1B, black bars). To see whether binding is mutually exclusive, RocR was incubated with radiolabeled pGpG in the presence of the same panel of unlabeled nucleotide competitors (Fig. 1B, white bars). The addition of 50 μM c-di-GMP reduced fraction bound from 0.48 ± 0.01 to 0.13 ± 0.03 (P < 0.001), indicating that c-di-GMP is also able to prevent pGpG binding. No other nucleotide had a significant effect on fraction of pGpG or c-di-GMP bound to RocR (Fig. 1B). Thus, RocR binding to pGpG and c-di-GMP is specific and mutually exclusive. Crystal structures of another EAL domain PDE-A from Vibrio cholerae were shown to have pGpG binds in the active site where c-di-GMP cleavage occurs (31). Because the binding is occurring at the same site, the relative affinity of RocR binding to pGpG and c-di-GMP is important to determine whether pGpG competition of c-di-GMP binding could occur in physiological conditions. The dissociation constant (Kd) of RocR binding to pGpG and c-di-GMP was found to be 3.6 ± 0.4 and 0.60 ± 0.07 μM respectively (Fig. 1C). The c-di-GMP concentration in WT P. aeruginosa has been reported to be up to 11 μM (32). Therefore, rapid turn-over of c-di-GMP can yield pGpG concentrations that exceed the Kd for RocR if pGpG is not also quickly removed from the cell.

Excess pGpG Extends c-di-GMP Half-Life in Vitro. Inhibition of RocR by pGpG should effectively increase the half-life of c-di-GMP. To test this in vitro, a coupled DGC and PDE-A reaction was performed to trace the synthesis and linearization of c-di-GMP in the presence of different intermediates in the c-di-GMP biosynthesis and degradation pathways. Specifically, the DGC WspR and PDE-A from P. aeruginosa PA14 were incubated with radiolabeled α-32P-GTP in the presence of (i) no competitor, (ii) GMP, (iii) c-di-GMP, or (iv) pGpG. The reactions were stopped at different times by the addition of the divalent metal chelator EDTA and heat inactivation. The reaction products were separated by TLC, and intensities of radiolabeled GTP, c-di-GMP, and pGpG were quantified (Fig. 2). In the absence of any competitor, the α-32P-GTP is converted to c-di-GMP, which is then linearized to pGpG. Adding 100-fold excess GMP shows no effect on enzymatic activity, as expected (Fig. 2A and B). Addition of excess c-di-GMP inhibited the ability of WspR to convert α-32P-GTP to c-di-GMP, which is consistent with inhibition through binding to

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Fig. 1. pGpG inhibits RocR phosphodiesterase activity by competing for c-di-GMP binding in the active site. (A) The rate of c-di-GMP hydrolysis by RocR in the absence of competitor (NC, circle) or 500 μM pGpG competitor (triangle). (B) The fraction bound of 32P-c-di-GMP (black) or 32P-pGpG (white) to RocR (10 μM) was quantified by DRaCALA in the presence of no competitor (NC) or 500 μM excess unlabeled nucleotide competitor. (C) The dissociation constants (Kd) for RocR binding to 32P-c-di-GMP (black) and 32P-pGpG (white) as determined by DRaCALA. All data shown represent the average and SD of triplicate independent experiments.
the I-site (Fig. 2C) (17, 18). Most importantly, the addition of excess pGpG reduced the turnover of c-di-GMP (Fig. 2D), supporting our hypothesis that elevated concentrations of pGpG can increase the half-life of c-di-GMP by reducing PDE-A activity.

Identification of pGpG Binding Proteins from V. cholerae ORFeome Library. Based on the above results, the PDE-B responsible for hydrolyzing pGpG is critical for completing the c-di-GMP degradation pathway. To identify this enzyme, we used a high-throughput DRaCALA-based screen to interrogate pGpG binding to a library of ORFs (22, 23). The V. cholerae El Tor N16961 complete genome ORF library (ORFeome) was used because 97% of the ORFs are represented (24), and V. cholerae uses c-di-GMP signaling extensively (33, 34). Each ORF was recombined into two destination vectors with an isopropyl-β-D-1-thiogalactopyranoside (IPTG)-inducible T7 promoter, one containing a 10x histidine N-terminal tag and a second containing a 10x histidinemaltose binding protein (MBP) N-terminal tag. These two libraries were individually introduced into an E. coli T7Iq expression strain and arrayed in 96-well plates. Following IPTG induction, whole cell lysates were generated and assayed for binding to pGpG using DRaCALA. The fraction bounds for each ORF tested are listed in Table S1. Positive hits were defined as 3 SDs above the mean fraction bound of the entire library. For validation, each positive hit was repicked from the expression library and reassayed for pGpG binding. The fraction of each ORF bound to pGpG is shown for both ORFeomes with validated hits in color (Fig. 3). A list of hits and their known or predicted functions is provided in Table S2.

The V. cholerae ORFeome library includes known c-di-GMP binding proteins: 28 of 31 GGDEF domain proteins (33), all 5 PilZ domain proteins (35), and all 3 c-di-GMP-binding transcription factors (36–38). None of these proteins were identified in this screen, indicating that the assay is specific for pGpG-binding proteins. We expected to identify EAL domain and HD-GYP domain proteins, both of which have been shown to bind pGpG (21, 31). The V. cholerae ORFeome library includes 11 of 12 EAL domain proteins encoded in the genome, 9 of 10 dual GGDEF-EAL domain proteins, and all 9 HD-GYP domain proteins (33). Of these, four EAL domain-containing proteins, VC1592, VC1641, VC1652, and VC1710 (Fig. 3, blue dots); four GGDEF-EAL domain containing proteins, VC0653, VC0658, VC2750, and VCA0680 (Fig. 3, green dots); and two HD-GYP domain containing proteins, VCA0681 and VCA0931 (Fig. 3, purple dots), bound pGpG in the screen. Not all EAL or HD-GYP domain ORF lysates showed binding in this system, which could reflect either genuine inability to bind pGpG or low protein expression in E. coli, resulting in concentrations below the Kd.

Because pGpG is a two-nucleotide-long RNA, we expected ribonucleases (RNases) to bind as well. The ORFeome library included all 15 annotated RNases from the V. cholerae genome, of which 2 were found to bind pGpG: VC0210 and VC0341 (Fig. 3, red dots). Additionally, the screen identified five ORFs that were not previously known to interact with pGpG: VC0371, VC2147, VC2459, VC2708, and VCA0593 (Fig. 3, orange dots). Sequence homology predictions indicate that four of these five proteins are predicted to interact with nucleic acids or nucleotides, whereas VCA0593 has no predicted function (Table S2). VC0341 is a homolog of Orn, which is a known exoribonuclease that cleaves two- to five-nucleotide-long RNAs (25). Because VC0341 was shown to bind in both the His-tagged and the His-MBP–tagged ORFeomes, it was chosen for further study.

Orn Binds to pGpG Specifically and Can Cleave It into GMP. To fully validate the interaction between Orn and pGpG observed in cell lysates, Orn from P. aeruginosa PA14 and V. cholerae were His-MBP tagged, purified, and assayed for pGpG binding by DRaCALA. pGpG bound both P. aeruginosa and V. cholerae Orn with fraction bounds of 0.32 ± 0.02 and 0.27 ± 0.01 in the absence of competitor (NC) (Fig. 4A and C). Addition of excess unlabeled pGpG effectively competed radiolabeled pGpG binding, reducing the fraction bounds to 0.009 ± 0.008 and 0.03 ± 0.003 for P. aeruginosa and V. cholerae Orn (P < 0.05; Fig. 4A and C). However, Orn binding to pGpG was not affected by the addition of excess c-di-GMP or any other guanine-containing nucleotides tested, indicating that Orn binds pGpG specifically (Fig. 4A and C). Finally, Orn from P. aeruginosa and V. cholerae bound to pGpG with high affinity, with a Kd of 40 ± 2 and 25 ± 2 nM, respectively (Fig. 4B and D).

Having demonstrated specific and high-affinity binding, we then investigated the ability of Orn to hydrolyze pGpG. Purified P. aeruginosa His-MBP-Orn degraded pGpG (Fig. 4F) into GMP within 10 s at room temperature. Orn belongs to the DEDDh subfamily of 3′ to 5′ exoribonucleases that have a highly conserved active site motif (39). The locations of the DEDDh active site residues were shown in the solved crystal structure of Xanthomonas campestris Orn (40), and an alignment of P. aeruginosa PA14 Orn with X. campestris Orn revealed the active site residues D111, E13, D111, H157, and D162 (Fig. S1). Single alanine point mutants in the signature motif of another DEDDh RNase, RNase T of Xanthomonas campestris Orn (40), and an alignment of P. aeruginosa PA14 Orn with X. campestris Orn revealed the active site residues D111, E13, D111, H157, and D162 (Fig. S1). Single alanine point mutants in the signature motif of another DEDDh RNase, RNase T of E. coli, had significantly reduced catalytic activity (41). Therefore, we introduced single alanine substitutions into each residue of the DEDDh motif (D111A, E13A, D111A, H157A, and D162A) of P. aeruginosa Orn to assay for pGpG binding and degradation. All of the Orn variants bound pGpG (Fig. 4E) but failed to cleave pGpG (Fig. 4F). Taken together, these results demonstrate that
purified Orn binds pGpG with nanomolar affinity and requires an intact active site for pGpG hydrolysis activity.

Orn Is the Primary Enzyme Responsible for pGpG Degradation. Our results led us to ask whether Orn is the primary PDE-B responsible for turnover of pGpG in *P. aeruginosa*. An orn transposon mutant (orn::tn) and a control strain cat::tn (chloramphenicol acetyltransferase, which is not expected to affect pGpG hydrolysis) from the PA14 Transposon Library (26) were grown to midlog, lysed, and assayed for the ability to degrade radiolabeled pGpG. Lysates of the control cat::tn strain rapidly degraded pGpG (Fig. 5A). In contrast, the orn::tn strain showed a 10-fold reduced rate in pGpG cleaving ability, indicating that Orn is the primary enzyme involved in degradation (Fig. 5A). The orn mutant extracts still converted a small fraction of pGpG into GMP, indicating that there are additional proteins with PDE-B activity. Because purified HD-GYP domain phosphodiesterases from *P. aeruginosa* were shown to bind pGpG with higher affinity than c-di-GMP (21), we tested also pGpG degradation in cell lysates of transposon mutants of the two HD-GYP genes from *P. aeruginosa*. Lysates from PA4108::tn and PA4781::tn showed no reduction in rates of pGpG cleavage compared with cat::tn (Fig. 5A), so their individual contribution to pGpG turnover in vivo is less than Orn. To further validate the defect of the orn::tn strain, an in-frame deletion of orn was generated in *P. aeruginosa* PA14, and the cell lysate was analyzed for pGpG degradation. The Δorn strain showed a 25-fold reduced pGpG cleavage rate compared with the parental strain, consistent with the pGpG hydrolysis defect seen in the orn::tn strain (Fig. 5A and B). Complementation with WT orn on an IPTG-inducible plasmid restored the ability to hydrolyze pGpG, whereas complementation with the catalytically inactive orn alleles did not (Fig. 5C). Addition of purified WT Orn from PA14 to the Δorn lysate restored hydrolysis activity, whereas addition of the purified catalytically inactive proteins (D11A, E13A, D111A, H157A, and D162A) had no effect (Fig. 5D). Taken together, these results demonstrate that Orn is the primary enzyme responsible for removing pGpG in *P. aeruginosa*.

Aggregation and Biofilm Are Increased in an Orn Mutant and Dependent on the PEL Polysaccharide. We assayed the Δorn strain for two phenotypes positively regulated by c-di-GMP: auto-aggregation (29) and biofilm formation (28). The auto-aggregation assay measures the ability of bacterial aggregates to settle. WT *P. aeruginosa* PA14 cultures remain in suspension, whereas the Δorn strain formed larger aggregates that settled to the bottom of the culture tube (Fig. 6A). Complementation of Δorn with active orn on a plasmid abolished aggregate formation, whereas complementation with catalytically inactive alleles did not (Fig. 6B). In a crystal violet microtiter plate assay for biofilm formation (42), cultures of WT *P. aeruginosa* PA14 formed less biofilm than Δorn, with A595 readings of 0.84 ± 0.37 and 1.98 ± 0.73, respectively (*P* < 0.05; Fig. 6C).

In PA14, extracellular matrix products such as PEL exopolysaccharides are required for biofilm formation (43). Cyclic-di-GMP elevates the production of the PEL exopolysaccharides (28) by increasing transcription of the pel operon (27) and activating biosynthesis (30). Therefore, we generated a ΔornΔpel double deletion mutant to ask whether PEL is required for the increased
The above data approximately threefold increase in concentration of Orn (μM) for WT (P < 0.05; Fig. 6D). Together, our results support the hypothesis that the loss of orn elevates c-di-GMP to promote c-di-GMP-regulated biofilms.

In additional support for the role of c-di-GMP in aggregation, overexpression of enzymes that either make or linearize c-di-GMP have opposing effects on aggregation in the Δorn strain. Elevating c-di-GMP via overexpression of the DGGs PA1107 and wspR increased aggregation. Conversely, decreasing c-di-GMP via expression of the PDE-As PA2133 and rocR was unable to completely reverse bacterial aggregation (Fig. S2). These results indicate that because the Δorn mutant lacks the ability to rapidly hydrolyze pGpG, it cannot complete degradation of c-di-GMP even when overexpressing EAL PDE-As.

Intracellular pGpG and c-di-GMP Are Elevated in the Oligoribonuclease Mutant. To confirm the presence of increased c-di-GMP and pGpG in the PA14 Δorn strain, both nucleotides were directly detected by LC-MS/MS. WT and Δorn cultures were grown midlog (OD₆₀₀ = 0.6) and late-log (OD₆₀₀ = 2.0), and the nucleotides were extracted for analysis. Both c-di-GMP and pGpG were detected in the PA14 WT and Δorn mutant strains. At midlog, the Δorn strain had 5.9-fold increased c-di-GMP compared with WT, with 2.86 ± 0.67 vs. 0.49 ± 0.05 μM, respectively (P < 0.001; Table 1). We also detect 6.6-fold more pGpG in the Δorn strain than WT in midlog (70.8 ± 1.6 vs. 10.8 ± 1.4 μM, P < 0.001; Table 1). Lower concentrations of c-di-GMP and pGpG were detected from both strains at late-log, although both nucleotides remained higher in the Δorn strain. At late-log, the Δorn strain had 8.1-fold increased c-di-GMP compared with WT (0.65 ± 0.20 vs. 0.08 ± 0.01 μM, respectively; P < 0.05) and 6.6-fold increased pGpG compared with WT (26.3 ± 3.6 vs. 4.0 ± 1.0 μM, respectively, P < 0.005; Table 1). The above data confirm that pGpG and c-di-GMP are both elevated in orn mutants at both growth phases. All together, our results show that in aggregation and biofilm formation we observe in the Δorn mutant. The ΔornΔpel double mutant remained in suspension like the WT and the Δpel single mutant cultures (Fig. 6A), indicating that PEL is required for the aggregation observed in the Δorn strain. The pel operon is also known to be required for in vitro pellicle biofilm formation in P. aeruginosa PA14 (43). As expected, the Δpel mutant was unable to form a pellicle biofilm in a microtiter plate assay, with a crystal violet A₅₉₅ reading of 0.13 ± 0.02 compared with 0.84 ± 0.37 for WT (P < 0.05; Fig. 6C). The ability to form biofilm is also lost in the ΔornΔpel double deletion strain, which had an A₅₉₅ reading of 0.21 ± 0.05 (P < 0.05 compared with WT; Fig. 6C). These data indicate that the increased aggregation and biofilm formation seen in the Δorn strain is PEL dependent.

Another measure of elevated c-di-GMP levels is through the activity of the transcription factor FleQ, which leads to increased transcription from the pel promoter when bound to c-di-GMP (27). The pel promoter fused to lacZ (P₈₇₋₉₇lacZ) was integrated into the chromosome of both P. aeruginosa PA14 WT and Δorn strains. These strains were grown to midlog and assayed for β-galactosidase activity. Approximately threefold increase in β-galactosidase activity was observed in the Δorn strain compared with WT, with 563 ± 230 Miller units compared with 185 ± 64 Miller units, respectively (P < 0.05; Fig. 6D). Together, our results support the hypothesis that the loss of orn elevates c-di-GMP to promote c-di-GMP-regulated biofilms.
**Discussion**

**Oligoribonucleases as PDE-8s.** Early characterization of c-di-GMP from the Benziman laboratory identified that c-di-GMP is linearized by PDE-As into pGpG, which is then degraded into two GMPS through the action of a presumed PDE-B (44). Through nearly three decades of c-di-GMP research that followed, the identity of the PDE-B remained unknown. We demonstrate here that the 3′-5′ exoribonuclease Orn is the primary PDE-B in *P. aeruginosa*. The linearized product of c-di-GMP hydrolysis by PDE-As, pGpG, is a two-nucleotide-long RNA that matches the known substrates of Orn. We present several lines of evidence indicating that Orn is the primary PDE-B for c-di-GMP in *P. aeruginosa*: (i) Orn binds pGpG and not c-di-GMP, (ii) Orn rapidly degrades pGpG in vitro, (iii) lysates of *P. aeruginosa* orn mutants are greatly reduced for PDE-B activity, (iv) DEDDH catalytic residues used to degrade oligoribonucleotides are required for pGpG cleavage, and (v) loss of orn results in enhanced intracellular pGpG and c-di-GMP. This additional role for Orn is important because we demonstrate that the loss of Orn results in increased c-di-GMP and c-di-GMP governed phenotypes, likely due to accumulated pGpG inhibiting PDE-As as shown in our model (Fig. 7A).

Orn degrades short oligoRNAs regardless of sequence (45), raising the interesting possibility that it could degrade all linearized bacterial cyclic dinucleotides. In addition to c-di-GMP, two other cyclic dinucleotide signaling molecules have thus far been identified in bacteria. Cyclic di-AMP (c-di-AMP) is synthesized by diadenylate cyclases, which are widely distributed among bacterial species (46), and a hybrid cAMP-GMP is synthesized by DncV in *V. cholerae* (47) and an unknown cyclase in *Geobacter sulfurreducens* (48). Cyclic-di-AMP is linearized by HD domain and DHH-DHHA-1 domain phosphodiesterases (49, 50), and cAMP-GMP is linearized by HD-GYP proteins in *V. cholerae* (51). The linearized cyclic di-nucleotides could be hydrolyzed by Orn. However, not all species that use cyclic dinucleotides posses an Orn homolog (Fig. 7B). In a search of the clusters of orthologous group (COG) database, only Actinobacteria, Fibrobacteres, Betaproteobacteria, Deltaproteobacteria, Gammaproteobacteria, and a few unclassified species are predicted to encode Orn (52) (Fig. 7B). *Bacillus subtilis* has both c-di-GMP (53) and c-di-AMP signaling systems (54, 55) but lacks orn. Nevertheless, *B. subtilis* is capable of degrading two- to five-nucleotide-long RNAs using the potential Orn functional homologs NrnA, NnrB, yhaM, and RNase J1. All of these enzymes have oligoribonuclease activity and were able to partially complement an *E. coli orn* conditional mutant (56, 57). NnrA homologs are found in many bacteria that lack Orn (58). Additionally, another RNase with oligoribonuclease activity, NrnC, was recently identified in *Bartonella birtlesii* and is widely found in Alphaproteobacteria (59). Nearly all bacteria that encode diguanylate and diadenylate cyclases encode at least one RNase capable of degrading short oligoRNAs. Based on this genomic distribution, we predict these RNases are likely the primary PDE- Bs for hydrolyzing linearized cyclic dinucleotides into mononucleotides.

Our observation that residual PDE-B activity is present in mutants lacking orn indicates that other proteins can cleave pGpG. Proposed PDE-B candidates include the HD-GYP domain proteins in *P. aeruginosa* PA14 did not show a defect in pGpG removal in our system, in contrast to the large defect observed for the orn mutant. Despite being shown to be

Addition to its known role degrading oligoribonucleotides (25), Orn is the major PDE-B that hydrolyzes pGpG in *P. aeruginosa*.

**Table 1. Quantification of c-di-GMP and pGpG in PA14 and PA14 Δorn**

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<th>PA14 (μM)</th>
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<td>c-di-GMP</td>
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Data shown are mean ± SD of three independent samples.

*P value was determined by Student unpaired two-tailed t test assuming equal variance.
essential in *E. coli* (60), *orn* mutants in *P. aeruginosa* are viable (26, 61), suggesting some functional redundancy. However, *P. aeruginosa* PA14 has no homologs of NrnA (56), NrnB (57), yhaM (expected value >1 in a protein BLAST search), RNase J1 (62), or NrcC (59). Other RNases may possess oligoribonuclease activity that partially compensates for the loss of *orn*. Our DraCALA screen identified one other RNase, VC0419 (predicted based on homology to be RNase G), but purified VC0419 did not hydrolyze pGpG. Purified N-terminal His- and His-MBP-tagged RNase G from *E. coli* have been shown to be active in vitro against longer RNA substrates (≥10 nt) (63–65). RNase G has a 5′ monophosphosphate sensor pocket that binds 5′ monophosphorylated RNAs and is distinct from the active site (66, 67). It is possible that pGpG, which has a 5′ monophosphate, is bound by the 5′ sensor of VC0419, but cannot be accessed by the active site due to its short length. Other PDE-Bs responsible for residual pGpG cleavage may have been missed in our ORFeome binding screen. The screen is limited by the exogenous expression of targets in *E. coli*. For example, RNase E (VC2030) is structurally similar to RNase G and possesses a 5′ sensor pocket (67) and thus would be expected to bind pGpG, but SDS/PAGE analysis of the RNase E whole cell lysates from the ORFeome libraries showed the protein was poorly expressed. Another limitation of assaying whole cell lysates is that pGpG formation and aggregation.

**Intracellular Concentration of pGpG and c-di-GMP.** We were able to detect pGpG in both PA14 and the Δ*orn* mutant by LC-MS/MS. As expected, the level of pGpG in the Δ*orn* mutant was compared with WT. The levels of pGpG detected in the Δ*orn* strain at both mid- and late-log are well above the *Kₐ* of RocR binding to pGpG. As a consequence, pGpG can compete for c-di-GMP binding to inhibit RocR activity, leading to the observed increase in c-di-GMP concentration. There are two potential sources of pGpG in the cell: linearization of c-di-GMP and normal RNA processing and turnover (69). Future studies will determine the relative contribution of each source. The detection of pGpG in the PA14 indicates a potential physiological role for pGpG in *P. aeruginosa* during normal growth conditions. Other positive hits from the ORFeome screen represent potential cellular receptors for other pGpG-regulated processes.

A previous study reported ~11 μM of c-di-GMP in *P. aeruginosa* PAO1 grown to midlog in rich media (32). We detect ~0.5 μM c-di-GMP for PA14 grown at midlog phase. The difference in c-di-GMP levels is likely due to differences between strains and estimations of cell size. The concentration of c-di-GMP for PA14 we detect is below the reported dissociation constants for *P. aeruginosa* c-di-GMP receptors, which are in the low micromolar range [Alg44 *Kₐ* = 5.6 μM (70), PelD *Kₐ* = 1 μM (30), and FleQ *Kₐ* = 15–25 μM (27)]. This level of c-di-GMP is consistent with PA14 cells behaving as motile, planktonic cells at midlog phase, which is indicative of reduced c-di-GMP signaling.

**Cyclic-di-GMP Phenotypes in Δorn Mutants.** The Δ*orn* strain formed more PEL-dependent biofilm and aggregates and had higher levels of pGpG and c-di-GMP. The connection between pGpG and elevated c-di-GMP is through pGpG inhibition of the PDE-As. The pGpG inhibition of PDE-A activity has also been observed for Ygf from *E. coli* (20). Ygf and RocR EAL domains are linked to different sensor domains and have different oligomer states (20, 71, 72), yet are both inhibited by pGpG. From our DraCALA-based ORFeome screen, several other EAL and HD-GYP proteins also bind pGpG, suggesting that product inhibition by pGpG may be a general phenomenon. As a consequence, pGpG generated from c-di-GMP hydrolysis will act to extend c-di-GMP signaling unless a PDE-B is active to reduce the pGpG pool (Fig. 7A). As expected, deletion of the primary PDE-B

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**Fig. 7.** The distribution of RNases, GGDEF domain proteins, and diadenylate cyclase domain proteins. (A) A model showing the synthesis and degradation of c-di-GMP with the inclusion of pGpG-mediated feedback inhibition of EAL-domain PDE-As and Orn as a PDE-B. (B) A simplified phylogenetic tree of prokaryotic and archaeal species from the COG database and a table showing the number of species from each class that encodes the following proteins: Orn, NrnA, NrnB, GGDEF domains, and diadenylate cyclase (DisA) domains. The number of species were determined by a COG database search: Orn = COG1949; NrnA = COG0618; NrnB = COG2404; GGDEF = COG2199; COG3706; DisA = COG1623, COG1624.
(om) in *P. aeruginosa* resulted in the accumulation of c-di-GMP and increased biofilm formation and auto-aggregation. These observations will likely extend to other bacteria that use Orn homologs to degrade pGpG.

Orn may also be involved in other phenotypes regulated by c-di-GMP. Cystic fibrosis patients are chronically infected with mucoid strains of *P. aeruginosa*, leading to poor patient prognosis (73). The mucoid phenotype is due to high production of the alginate polysaccharide, which is synthesized by components of the *alg operon* (74). Cyclic-di-GMP regulates production posttranscriptionally by binding to the PAZ domain of AlgS4 to activate alginate production (70). Overexpression of the diguanylate cyclase PA1107 significantly enhanced alginate production in *P. aeruginosa* (70). In addition, transcription of the operon is regulated by several proteins, including the activator AlbG (75). A mucoid cystic fibrosis isolate FRD of *P. aeruginosa* becomes nonmucoid on deletion of *algB*, although the strain is still able to produce low levels of alginate (75). A genetic screen for suppressor mutants that restored alginate production of the *algB* mutant identified six transposon insertion mutants. Two of these insertions were in the *om* gene (61). A possible explanation based on our model (Fig. 7A) is that the loss of *om* resulted in pGpG accumulation, inhibition of PDE-A, and thus elevated c-di-GMP to allow for increased Alg44 activation and enhanced mucoidy. These observations indicate that the PDE-B must be present and highly active during c-di-GMP signaling termination to prevent pGpG-mediated PDE-A inhibition.

**Materials and Methods**

*V. cholerae* ORFeome Library pGpG Binding Screen. Gateway destination vectors were generated from pET-19-derived expression vectors pVL791 (N-terminal His tag, carbenicillin resistance) and pVL847 (N-terminal His, gentamycin resistance). The Gateway destination cassette was amplified from pRFA and cloned in frame with the N-terminal tags to produce the gateway adapted vectors pVL791 G4 and pVL847 G4. The *V. cholerae* O1 biovar El Tor strain N16961 PD0N221 library was obtained from BEI Resources. The library was grown in 1.5 mL lysogeny broth (LB) in 2-mL, 96-well plates (Greiner) with kanamycin (50 mg/mL) selection, and the plasmids were isolated using the 96-well MultiScreen HTS Kit (Millipore).

The ORFs were moved into the expression vector using LR-clonase enzyme II (Invitrogen) and introduced into chemically competent *E. coli* strain TL7q (NEB) following the manufacturer’s protocols. Recombinants were selected on LB agar plates containing either carbenicillin (50 mg/mL) or gentamicin (15 mg/mL). Multiple colonies from individual transformations were inoculated in LB M9-rich media in 96-well plate format and grown overnight with shaking at 30 °C with the appropriate antibiotic and then resuspended in 20% (vol/vol) glycerol and frozen at −80 °C.

*E. coli* TL7q containing the *V. cholerae* ORFs were inoculated from frozen stocks in LB M9 rich media with the appropriate antibiotic in 96-well plate format, grown overnight with shaking at 30 °C, subcultured 1:50 into 1.5-mL fresh LB M9 media with antibiotic, and grown for 4 h at 30 °C with shaking; 1 mM IPTG was added to induce protein expression and cultures were grown for an additional 4 h. The induced culture was pelleted, and cells were resuspended in 1/10th volume of binding buffer (10 mM Tris, pH 8.0, 100 mM NaCl, 5 mM CaCl2), as well as 10 μg/mL DNase, 250 μg/mL lysozyme, and 10 mM PMSF. Cells were lysed by three freeze/thaw cycles at −80 °C/G21 °C. ORFeome whole cell lysates were stored at −80 °C until analysis by DRaCALA.

Binding of radiolabeled ligand to the ORFeome whole cell lysates was determined by DRaCALA as previously described (22, 23). Briefly, 16 pmol 32P-pGpG was added to the ORFeome whole cell lysate 96-well plates using a Multiflo Microplate Dispenser (BioTek) and then applied to nitrocellulose sheets (GE Healthcare) using a 96-well pin tool (V&P Scientific). The nitrocellulose was air dried and imaged using a Fujifilm FLA-7000 phosphorimager (GE). The intensity of the radiolabeled nucleotides was quantified using Fujifilm Multi Gauge software v3.0.

**Strains and Culture Conditions.** The primes, plasmids, and strains used in this study are listed in Tables S3–S5, respectively. The in-frame deletion of *orn* was generated in *P. aeruginosa* PA14 using a Flp-FRT recombination system where the 1-kb region upstream and downstream were PCR amplified and restriction digested to introduce these fragments into a pEX-Gn-based plasmid for making in-frame deletions as previously described (76). Deletions were verified by PCR. The pel promoter-lacZ fusion on a pCTX plasmid was incorporated into the genomic att site as previously described (77).

The alanine point mutations were generated by QuickChange using the primers listed in Table S3. Silent mutations resulting in addition or removal of a restriction site were introduced to facilitate mutagenesis: these were: the addition of an AfeI site in D11A and H157A, addition of an MscI site in D111A, and removal of EcoRV in D162A. The orn alleles from *P. aeruginosa* PA14 were cloned into pVL847 for purification and pMMB for complementation. The pMMB and pVL847 plasmids were maintained with gentamicin (15 mg/mL) and induced with 1 mM IPTG. Transposon mutants from the PA14 Non-Redundant Transposon Insertion Mutant Library (26) were maintained with gentamicin (15 mg/mL).

**Protein Expression and Purification.** His-MBP-RecR, His-MBP-Orn from *V. cholerae*, and His-MBP-Orn and His-MBP-Orn variants from *P. aeruginosa* were purified as previously described (23). Briefly, *E. coli* TL7q strains or *E. coli* BL21(DE3) containing expression plasmids were cultured overnight, subcultured in fresh media, and grown to OD600 = 1.0 when expression was induced with 1 mM IPTG. Induced bacteria were pelleted and resuspended in 10 mL T25 M (100 mM NaCl, 25 mM imidazole) and frozen at −80 °C until purification. Proteins were purified over a Ni-NTA column followed by anion exchange on a Q-Sepharose column. Purified proteins were dialyzed twice against 10 mM Tris, pH 8, 100 mM NaCl, and 25% (vol/vol) glycerol, aliquoted, and frozen at −80 °C until use.

**Whole Cell Lysate Generation.** Overnight cultures of *P. aeruginosa* PA14 parental, mutant, or complemented strains were subcultured 1:50 into fresh LB media with appropriate antibiotic and IPTG conditions, grown to OD600 = 0.4 at 37 °C with shaking, resuspended in 1/10th volume of reaction buffer (10 mM Tris, pH 8, 100 mM NaCl, 5 mM MgCl2), 10 μg/mL DNase, 250 μg/mL of lysozyme, and 10 mM PMSF, and lysed by sonication.

**DRaCALA Measurement of Ligand Binding, Nucleotide Competition, and Dissociation Constant.** The use of DRaCALA to probe protein-ligand interactions has been previously described (23). To assay binding, pure protein in binding buffer (10 mM Tris, pH 8.0, 100 mM NaCl, 5 mM CaCl2) was mixed with radiolabeled ligand (4 pmol 32P-c-di-GMP or 32P-pGpG), applied to nitrocellulose sheets, dried, imaged, and the fraction bound quantified (23). For competition assays, excess of unlabeled nucleotides were added to radiolabeled ligand and purified protein before analysis by DRaCALA (23). To measure Kd, twofold serial dilutions of purified His-MBP-Orn from either *P. aeruginosa* or *V. cholerae* were made in binding buffer (10 mM Tris, pH 8, 100 mM NaCl, 5 mM CaCl2) and mixed with radiolabeled ligand and the fraction bound, and Kd was calculated as previously described (23).

**Cell Lysate and Protein Activity Assays.** The activity of whole cell lysates and purified proteins against 32P-labeled substrates was measured by monitoring the appearance of 32P-labeled products on TLC. The reactions were carried out at 37 °C in reaction buffer (10 mM Tris, pH 8, 100 mM NaCl, and 5 mM MgCl2). At appropriate times, aliquots were removed and the reaction stopped by adding an equal volume of 0.2 M EDTA, pH 8, and heated at 98 °C for 10 min. Samples were spotted on polyethyleneimine-cellulose TLC plates (EMD Chemicals), dried, and developed in mobile phase consisting of 1:1.5 (vol/vol) saturated NH4SO4 and 1.5 M K,HPO4, pH 3.60. The TLC plate was dried and imaged using Fujifilm FLA-7000 phosphorimager (GE). The intensity of the radiolabeled nucleotides was quantified using Fujifilm Multi Gauge software v3.0.

**Aggregation Assay.** Cultures of *P. aeruginosa* strains were grown in 10 mL LB with appropriate antibiotic and IPTG conditions for 24 h at 37 °C with shaking. Cultures were imaged after 30 min of aggregate settling at room temperature.

**Microtiter Plate Biofilm Assay.** *P. aeruginosa* PA14 WT and mutant strains were grown overnight in LB and grown as static cultures in a 96-well polystyrene plate (Greiner) at 30 °C inside a humidified chamber for 24 h. The cultures were washed of planktonic cells and stained with crystal violet as previously described (42). The A560 was measured on a SpectraMax M5 spectrophotometer (Molecular Devices).
β-Galactosidase Reporter Assay. P. aeruginosa strains containing the reporter were grown overnight in LB at 37 °C with shaking. Overnight cultures were subcultured in LB and grown at 37 °C with shaking to midlog (OD600 = 0.5). The β-galactosidase activity was measured according to previously published methods (78).

Quantification of Intracellular c-di-GMP and pGpG. P. aeruginosa PA14 WT and Δ orn strains were grown overnight in LB at 37 °C with shaking, subcultured 1:100 in 20 mL LB, and grown at 37 °C with shaking to midlog (OD600 = 0.6) and later-log (OD600 = 2); 15 mL was pelleted by centrifugation, resuspended with 100 μL ice-cold extraction buffer, 40:40:20 (vol:vol:vol) MeOH, acetonitrile, and water with 0.1 N formic acid, incubated 30 min at −20 °C for lysis, and neutralized after a 30-min incubation with 4 μL 15% (wt/vol) NH4CNO2. Cellular debris was pelleted, and the supernatant was removed for desiccation by a Savant SpeedVAC Concentrator (Thermo Scientific). Desiccated samples were suspended in 100 μL ultra-pure water, and insoluble material was pelleted by precipitation at 180(17):4161-4164 a table-top microcentrifuge at room temperature for 5 min. The resulting supernatant was filtered through a Titan syringe filter (PVDF, 0.45 μm, 4 mm) before quantification of c-di-GMP and pGpG. Quantification of c-di-GMP and pGpG in cellular extracts was performed using LC-MS/MS on a Quattro Premier XE mass spectrometer (Waters) coupled with an Acquity Ultra Performance LC system (Waters). Cyclic diguanylate-specific phosphodiesterase: Enzymatically active and inactive EAL domain isoenzymes. We appreciate assistance from Chen Zhang and the Michigan State University Mass Spectrometry Facility. M.W.O. was supported in part by a NIH/NIAID Training Grant in Host-Pathogen Interactions (T32-AI096521). V.T.L. was funded by National Institutes of Health (NIH) NIAID R21AI006063. C.M.W. was funded by National Science Foundation MCB1253684. H.O.S. was funded by National Science Foundation CHE0746446.