

1 **A subset of exoribonucleases serve as degradative enzymes for**  
2 **pGpG in c-di-GMP signaling**

3  
4 **Running Title**

5 RNases degrade pGpG to complete c-di-GMP degradation

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24

25 **Abstract**

26       Cyclic-di-GMP (c-di-GMP) is a bacterial second messenger that regulates  
27 processes such as biofilm formation and virulence. During degradation, c-di-GMP  
28 is first linearized to pGpG and subsequently hydrolyzed to two GMPs by a  
29 previously unknown enzyme, which was recently identified in *Pseudomonas*  
30 *aeruginosa* as the 3' to 5' exoribonuclease Oligoribonuclease (Orn). Mutants of  
31 *orn* accumulated pGpG, which inhibited linearization of c-di-GMP. This product  
32 inhibition led to elevated c-di-GMP levels, resulting in increased aggregate and  
33 biofilm formation. Thus, the hydrolysis of pGpG is crucial to maintenance of c-di-  
34 GMP homeostasis. How species that utilize c-di-GMP signaling but lack an *orn*  
35 ortholog hydrolyze pGpG remains unknown. Because Orn is an exoribonuclease,  
36 we asked whether pGpG hydrolysis can be carried out by genes that encode  
37 protein domains found in exoribonucleases. From a screen of these genes from  
38 *Vibrio cholerae* and *Bacillus anthracis*, we found only enzymes known to cleave  
39 oligoribonucleotides (*orn* and *nrnA*), rescued the *P. aeruginosa*  $\Delta orn$  phenotypes  
40 to wild-type. Thus, we tested additional RNases with demonstrated activity  
41 against short oligoribonucleotides. These experiments show that only  
42 exoribonucleases previously reported to degrade short RNAs (*nrnA*, *nrnB*, *nrnC*  
43 and *orn*) can also hydrolyze pGpG. A *B. subtilis* *nrnA* and *nrnB* mutant had  
44 elevated c-di-GMP, suggesting that these two genes serve as the primary  
45 enzymes to degrade pGpG. These results indicate that the requirement for pGpG  
46 hydrolysis to complete c-di-GMP signaling is conserved across species. The final

47 steps of RNA turnover and c-di-GMP turnover appear to converge at a subset of  
48 RNases specific for short oligoribonucleotides.  
49

50 **Importance**

51       The bacterial cyclic-di-GMP (c-di-GMP) signaling molecule regulates  
52 complex processes such as biofilm formation. C-di-GMP is degraded in two-  
53 steps: linearization into pGpG and subsequently cleavage to two GMPs. The 3' to  
54 5' exonuclease oligoribonuclease (Orn) serves as the enzyme that degrades  
55 pGpG in *Pseudomonas aeruginosa*. Many phyla contain species that utilize c-di-  
56 GMP signaling but lack an Orn homolog and the protein that functions to degrade  
57 pGpG remains uncharacterized. Here, systematic screening of genes encoding  
58 proteins containing domains found in exoribonucleases revealed a subset of  
59 genes encoded within the genomes of *Bacillus anthracis* and *Vibrio cholerae* that  
60 degrade pGpG to GMP and are functionally analogous to Orn. Feedback  
61 inhibition by pGpG is a conserved process as strains lacking these genes  
62 accumulate c-di-GMP.

## 63 Introduction

64 Bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) was  
65 originally described in 1987 by the Benziman lab as an allosteric activator of  
66 cellulose synthase in *Acetobacter xylinum* (since renamed *Komagataeibacter*  
67 *xylinus*) (1). C-di-GMP is utilized by many bacterial species to govern behaviors  
68 such as biofilm formation, motility, virulence, development, and cell cycle  
69 progression, making c-di-GMP a crucial regulator of bacterial lifestyle transitions.  
70 In general, high levels of c-di-GMP promote a sessile, biofilm forming lifestyle  
71 while low levels of c-di-GMP promote a motile, planktonic lifestyle (see (2) for a  
72 comprehensive review of c-di-GMP signaling).

73 In their initial report, the Benziman lab demonstrated that c-di-GMP is  
74 synthesized from two GTP molecules by enzymes with diguanylate cyclase  
75 (DGC) activity. C-di-GMP is degraded to two GMP molecules via a two-step  
76 process. First, it is hydrolyzed into linear 5'-phosphoguanylyl-(3',5')-guanosine  
77 (pGpG) by enzymes the authors referred to as phosphodiesterase A. This  
78 linearization process can be inhibited by  $\text{Ca}^{2+}$  ions (1) while the subsequent  
79 hydrolysis of pGpG to two GMPs is not inhibited by  $\text{Ca}^{2+}$ , which the authors  
80 interpreted as evidence for a second, distinct enzyme which they termed  
81 phosphodiesterase B (1). Numerous follow-up experimental and bioinformatics  
82 studies revealed the motifs and domains for DGC activity (GGDEF domains) (3-  
83 5) and c-di-GMP linearization activity (EAL (4, 6, 7) and HD-GYP (8) domains),  
84 yet the identity of the enzyme responsible for pGpG cleavage remained  
85 unknown. While EAL domain and HD-GYP proteins have been shown to degrade

86 pGpG in vitro, their contribution to pGpG turnover in bacterial cells remains under  
87 investigation. Recently, two publications identified Orn as the primary PDE-B in  
88 *Pseudomonas aeruginosa* (9, 10). Using cell lysates, we showed that <sup>32</sup>P-labeled  
89 pGpG is turned over at a much lower rate in the PA14  $\Delta orn$  compared to wild  
90 type (9). The  $\Delta orn$  strain likely continued to express EAL and HD-GYP domain  
91 proteins, but their contribution towards pGpG turnover was less than 5% of Orn,  
92 indicating that Orn is the primary enzyme responsible for pGpG hydrolysis in  
93 vivo.

94       While c-di-GMP signaling is used across the bacterial domain, homologs  
95 of *orn* are restricted to Betaproteobacteria, Deltaproteobacteria  
96 Gammaproteobacteria, and Actinobacteria (9). For bacterial phyla that utilize c-  
97 di-GMP signaling but lack *orn* homologs, these organisms must encode another  
98 group of enzymes that fulfill the role of Orn in pGpG cleavage. Orn is a 3' to 5'  
99 exoribonuclease that is the major enzyme responsible for degrading short  
100 oligoribonucleotides in *Escherichia coli*. Orn was first isolated from *E. coli* in the  
101 1970's and shown to degrade short polyA oligos (5-mer and shorter) in vitro (11,  
102 12). The *orn* gene is essential in *E. coli*. To determine the function of Orn in vivo,  
103 a temperature-dependent mutant was generated by introducing a chromosomal  
104 interruption in the *orn* locus while supplying *orn* on a temperature-sensitive  
105 plasmid (13). Upon growth of this temperature-dependent *orn* mutant *E. coli* at  
106 the non-permissive conditions, the strain accumulated oligoribonucleotides that  
107 are 2-5 nucleotides long (13). In bacterial species that do not encode an *orn*  
108 homolog, other RNases were later identified to degrade oligoribonucleotides by

109 screening for genes that rescue growth of the *E. coli orn* mutant. Genes that  
110 rescued the *orn* mutant included NrnA and NrnB, which are widely found in  
111 Firmicutes (14, 15), and NrnC, which is widely found in Alphaproteobacteria (16).  
112 However, direct evidence of a role in degradation of short RNA in vivo was  
113 lacking. In addition, two RNases, YhaM and RNase J1, from *B. subtilis* also  
114 partially rescued the *E. coli* conditional *orn* deletion mutant (15). In vitro, the 3' to  
115 5' exoribonuclease YhaM (17) can degrade 5-mer oligo RNA, but was able to  
116 degrade oligo DNA at a faster rate, suggesting that DNA could be a preferred  
117 substrate (15). The 5' to 3' exoribonuclease RNase J1 (18) had low activity in  
118 vitro against 5-mer cytosine and adenine (15). These reports suggest that other  
119 RNases may degrade pGpG to terminate c-di-GMP signaling in species that lack  
120 *orn*. Currently these candidates have not been experimentally tested for  
121 hydrolysis of pGpG and their effects on c-di-GMP signaling. We thus used a  
122 similar complementation approach to assay the effect of RNases on pGpG  
123 turnover.

124 *P. aeruginosa*  $\Delta orn$  mutants are viable, but have increased levels of  
125 cytosolic c-di-GMP due to pGpG feedback inhibition, resulting in elevated c-di-  
126 GMP-regulated processes such as biofilm formation (19, 20). We hypothesized  
127 that genes encoding domains found in known RNA exonucleases could cleave  
128 pGpG in species that do not encode *orn* and should be able to restore the  
129 behavior of the *P. aeruginosa*  $\Delta orn$  strain to wild type. Thus, we identified genes  
130 that contained domains found in RNA exoribonucleases from *B. anthracis*, an  
131 organism that lacks *orn*, and *V. cholerae*, another species that encodes *orn* and

132 is well-known to utilize c-di-GMP signaling and thus may encode additional  
133 proteins for pGpG turnover. These genes were tested for their ability to degrade  
134 pGpG through complementation of the *P. aeruginosa*  $\Delta orn$  strain. Of the genes  
135 tested, only the known “nanoRNases” NrnA, NrnB, and NrnC could reduce  
136 aggregation of the *P. aeruginosa*  $\Delta orn$  strain to wild type levels. Cells that  
137 express NrnA, NrnB and NrnC reduced levels of pGpG and c-di-GMP found in  
138 the *P. aeruginosa*  $\Delta orn$  strain. Purified recombinant NrnA, NrnB and NrnC  
139 proteins were able to cleave pGpG in a manner similar to Orn. *Bacillus subtilis*  
140 lacking both *nmA* and *nmB* accumulated c-di-GMP. These results demonstrate  
141 that a specific subset of RNases act to hydrolyze pGpG, indicating that RNases  
142 serve as the final processing enzyme to terminate c-di-GMP signaling across  
143 bacteria.

144

## 145 **Results**

### 146 A screen identifies exoribonucleases that rescue cell aggregation and biofilm 147 formation in *P. aeruginosa* PA14 $\Delta orn$

148 A bioinformatic approach was used to identify candidate exoribonucleases  
149 for screening to identify additional enzymes responsible for turning over pGpG.  
150 Previously reported exoribonucleases in *E. coli* and *B. subtilis* include  
151 Oligoribonuclease, RNase B, RNase BN, RNase D, RNase J, RNase PH, RNase  
152 R, RNase T, PNPase, YhaM and Nrn proteins (21-24). These proteins were used  
153 as a starting point for bioinformatic identification of putative exoribonucleases  
154 based on Pfam domains (see Table S1). The Pfam HMM model obtained from



155 the Pfam database version 31 (March 2017) was searched against the complete  
156 proteomes of *B. anthracis* str. Ames and *V. cholerae* serotype O1 using the  
157 Hmmer 3.1b2 hmmsearch command (25), and resulted in a list of fifty-one unique  
158 protein sequences with a significant E-value as reported by Hmmer (See Table  
159 S1) (26). Of these fifty-one, fifty genes (*polC* (BA3955) was not available) were  
160 obtained from the *B. anthracis* and *V. cholerae* (27) Gateway clone set libraries  
161 and introduced into a replicative plasmid in *P. aeruginosa*.

162 The PA14  $\Delta orn$  strain has elevated levels of pGpG and c-di-GMP resulting  
163 in increased autoaggregation (Fig. 1) (9). The ability of each of the 50 genes to  
164 cleave pGpG was tested by trans complementation of the PA14  $\Delta orn$  to reduce  
165 autoaggregation. Expression of the PA14 *orn* (*orn<sub>Pa</sub>*) complemented the PA14  
166  $\Delta orn$  mutant and prevented aggregate formation, whereas the vector control  
167 aggregated. Expression of genes encoding RNase B, RNase BN, RNase D,  
168 RNase J, RNase PH or PNPase domains in  $\Delta orn$  did not prevent aggregation  
169 indicating that they do not hydrolyze pGpG (Fig. 1). Of the genes encoding the  
170 RNase T domain, only VC0341 (*orn<sub>Vc</sub>*) from *V. cholerae* was able to reverse  
171 aggregation (Fig. 1). For genes encoding DHH or DHHA1 domains, only BA4852  
172 (*nrnA<sub>Ba</sub>*) from *B. anthracis* prevented aggregation.

173 In addition to aggregation, the  $\Delta orn$  strain forms more pellicle biofilm (9).  
174 The pellicle biofilm was assayed using a crystal violet microtiter plate biofilm  
175 assay (28). Complementation of a PA14  $\Delta orn$  mutant with *orn<sub>Pa</sub>* decreased  
176 biofilm two-fold compared to the empty vector ( $p < 0.05$ , Fig. 2A). Expression of  
177 BA4852 (*nrnA<sub>Ba</sub>*) and VC0341 (*orn<sub>Vc</sub>*) reduced the biofilm similar to expression of

178 *orn<sub>Pa</sub>* ( $p > 0.05$ ) while expression of other RNases tested had no effect in the  
179  $\Delta orn$  strain (Fig. 2A). Similar to the aggregation assay, only *VC0341* (*orn<sub>Vc</sub>*) and  
180 *BA4852* (*nrnA<sub>Ba</sub>*) were able to reduce the enhanced biofilm formation of PA14  
181  $\Delta orn$ . These results suggest that Orn and NrnA are able to degrade pGpG in *V.*  
182 *cholerae* and *B. anthracis*, respectively.

183 Both *BA4852* (*nrnA<sub>Ba</sub>*) and *VC0341* (*orn<sub>Vc</sub>*) are 3' to 5' exoribonucleases  
184 with known activity against short oligoribonucleotides. NrnA from *B. subtilis* was  
185 originally identified from a screen that rescued the growth of an *E. coli orn*  
186 conditional mutant (14). From similar screens, other RNases from *Bacillus*  
187 *subtilis* and *Caulobacter crescentus*, namely *nrnB*, *rnjA*, *yhaM* and *nrnC*, were  
188 also identified that could hydrolyze short oligoribonucleotides in vitro (14-16). We  
189 therefore asked whether these proteins could cleave pGpG by assaying for  
190 complementation of the PA14  $\Delta orn$  strain. *nrnA*, *nrnB*, *rnjA*, and *yhaM* were  
191 cloned from *B. subtilis* 168 and *nrnC* was cloned from *C. crescentus* CB15 and  
192 expressed in PA14  $\Delta orn$ . Expression of *B. subtilis nrnA* (*nrnA<sub>Bs</sub>*), *B. subtilis nrnB*  
193 (*nrnB<sub>Bs</sub>*), and *C. crescentus nrnC* (*nrnC<sub>Cc</sub>*) were able to prevent aggregation of  
194 the PA14 *orn* mutant while *yhaM* and *rnjA* were not (Fig. 2B). These strains were  
195 also assayed for pellicle biofilm formation. Complementation with *nrnA<sub>Bs</sub>*, *nrnB<sub>Bs</sub>*  
196 and *nrnC<sub>Cc</sub>* reduced  $A_{595}$  readings to  $0.15 \pm 0.02$ ,  $0.14 \pm 0.2$ , and  $0.15 \pm 0.02$ ,  
197 respectively, as compared to the vector control  $A_{595}$  readings  $0.47 \pm 0.05$  (Fig.  
198 2C). This reduction is similar to complementation with PA14 *orn*. Expression of *B.*  
199 *subtilis rnaseJ1* (*rnjA<sub>Bs</sub>*) and *B. subtilis yhaM* (*yhaM<sub>Bs</sub>*) did not prevent  
200 aggregation, but resulted in a modest reduction in biofilm with  $A_{595}$  readings of

201 0.31  $\pm$  0.02 and at 0.36  $\pm$  0.04, respectively (Fig. 2C). When combined with the  
202 aggregation data, expression of *rnjA<sub>BS</sub>* and *yhaM<sub>BS</sub>* do not efficiently complement  
203 the  $\Delta orn$  mutant. Since pGpG accumulation causes decreased c-di-GMP  
204 turnover via feedback inhibition of the phosphodiesterase responsible for  
205 linearizing c-di-GMP, these data suggest that the genes *nrnA*, *nrnB*, and *nrnC*  
206 could degrade pGpG in species that do not have *orn*.

207

#### 208 Orn, NrnA, NrnB and NrnC convert pGpG to GMP

209 The elevated c-di-GMP-related phenotypes seen in the PA14  $\Delta orn$  mutant  
210 strain was shown to be complemented by *orn<sub>Pa</sub>*, but not catalytically inactive  
211 alleles of *orn<sub>Pa</sub>* (9, 10). As previously reported (9), the rate of pGpG turnover in  
212 whole cell lysates was barely detectable after 20 mins incubation in the empty  
213 vector control, while plasmid-provided PA14 *orn* showed full conversion of pGpG  
214 to GMP by 20 mins, with a half life of ~6 mins (Fig. 3A). To determine the ability  
215 of each of the RNases to degrade pGpG, the lysates of PA14  $\Delta orn$  expressing  
216 each RNase from *B. anthracis* and *V. cholerae* were tested for their ability to  
217 hydrolyze <sup>32</sup>P-pGpG to <sup>32</sup>P-GMP. Of the strains expressing RNases from *B.*  
218 *anthracis*, only BA4852 (*nrnA<sub>Ba</sub>*) decreased the pGpG half life to 0.23 minutes  
219 (Fig. 3B). Of the strains expressing RNases from *V. cholerae*, VC0341 (*orn<sub>Vc</sub>*)  
220 reduced the pGpG half life to 0.25 minutes (Fig. 3C), while the expression of  
221 other RNases did not alter rates of pGpG degradation. When complemented with  
222 *nrnA<sub>BS</sub>*, *nrnB<sub>BS</sub>*, and *nrnC<sub>Cc</sub>*, the pGpG half life was decreased to 16.5 minutes 2.7  
223 minutes and 1.5 minutes, respectively (Fig. 3D). Complementation with the other

224 RNases had similar pGpG hydrolysis rates to the empty vector control. These  
225 results suggest that these genes act on pGpG turnover in a manner similar to *orn*  
226 in *P. aeruginosa*.

227 To support the enzymatic activity of NrnA, NrnB and NrnC against pGpG,  
228 purified recombinant NrnA<sub>BS</sub>, NrnB<sub>BS</sub>, and NrnC<sub>Cc</sub> proteins were tested for the  
229 ability degrade pGpG. As expected, all were able to convert pGpG to GMP.  
230 When using 10 nM of each enzyme, the pGpG turnover rates were determined to  
231 be  $517.4 \pm 7.846$  nM/min for Orn<sub>Vc</sub>,  $338.1 \pm 14.3$  nM/min for NrnA<sub>BS</sub>,  $271 \pm 26.31$   
232 nM/min for NrnB<sub>BS</sub> and  $150.6 \pm 14.49$  nM/min for NrnC<sub>Cc</sub> (Fig. 4).

#### 233 234 HD-GYPs do not cleave pGpG in cells lacking *orn*

235 Previous studies have shown that HD-GYPs can cleave both c-di-GMP  
236 and pGpG in vitro (8). This has led to the suggestion that proteins containing the  
237 HD-GYP domain can act to both linearize c-di-GMP and cleave pGpG in vivo.  
238 However, deconvolution of the in vivo pGpG hydrolysis activity of HD-GYP from  
239 Orn was difficult due to essentiality of *orn* in other proteobacterial species. Using  
240 the viable *P. aeruginosa*  $\Delta orn$  strain, we asked whether HD-GYP proteins can  
241 cleave pGpG by expressing each of the nine genes from *V. cholerae* that contain  
242 a HD-GYP domain in an  $\Delta orn$  background. Lysates from these strains were  
243 tested for pGpG turnover by addition of  $^{32}\text{P}$ -pGpG. Similar to the vector control,  
244 expression of any of the HD-GYP genes failed to increase pGpG hydrolysis  
245 (Figure 5). Since expression of Orn<sub>Vc</sub> was able to restore pGpG hydrolysis, these  
246 results indicate that HD-GYP proteins do not cleave pGpG in vivo.

247 The intracellular concentration of pGpG and c-di-GMP in *P. aeruginosa* PA14  
248  $\Delta orn$  is reduced by complementation with *nrnA*, *nrnB*, *nrnC*, VC0341, and  
249 BA4852

250 To confirm that these changes in phenotype were due to reducing c-di-  
251 GMP in the complementation strains, nucleotides were extracted from wild type  
252 PA14 and the PA14  $\Delta orn$  strains containing empty vector or vector expressing  
253 wild type *orn*<sub>Pa</sub>, *nrnA*<sub>Bs</sub>, *nrnB*<sub>Bs</sub>, *nrnC*<sub>Cc</sub>, VC0341 (*orn*<sub>Vc</sub>), and BA4852 (*nrnA*<sub>Ba</sub>)  
254 and the levels of c-di-GMP and pGpG were detected by LC-MS/MS. The PA14  
255 strain with vector control had  $2.2 \pm 0.4 \mu\text{M}$  pGpG, while PA14  $\Delta orn$  strain with  
256 vector control had  $17.4 \pm 3.7 \mu\text{M}$  pGpG. Complementation of the PA14  $\Delta orn$   
257 strain with all genes tested reduced pGpG and c-di-GMP levels (Table 1).  
258 Together, these results demonstrate that a specific subset of RNases can cleave  
259 pGpG to terminate c-di-GMP signaling.

260  
261 *B. subtilis* 168  $\Delta nrnA \Delta nrnB$  double mutant and  $\Delta nrnA \Delta nrnB \Delta yhaM$  triple mutant  
262 have elevated levels of c-di-GMP

263 The ability of *nrnA*<sub>Bs</sub> and *nrnB*<sub>Bs</sub> from *B. subtilis* to complement *P.*  
264 *aeruginosa*  $\Delta orn$  suggests that these enzymes could be responsible for pGpG  
265 cleavage in *B. subtilis* in a manner that is analogous to Orn function in *P.*  
266 *aeruginosa*. Thus, we generated an unmarked  $\Delta nrnA \Delta nrnB$  double mutant in *B.*  
267 *subtilis* and assayed for c-di-GMP levels using a fluorescent riboswitch reporter  
268 of c-di-GMP levels. This riboswitch reporter construct consists of a constitutively  
269 active promoter followed by a c-di-GMP-specific riboswitch from *B. licheniformis*

found upstream of the *lch* operon (*lchAA* UTR) fused to *yfp*. When the riboswitch is bound to c-di-GMP, it forms a terminator prior to *yfp*, resulting in lower fluorescence levels; when the riboswitch is not bound to c-di-GMP, it folds differently permitting transcription elongation through the *yfp* gene, resulting in elevated fluorescence (Fig. 6A). As a control, we used a constitutively active promoter without the riboswitch before the *yfp* reporter ( $P_{\text{const}}\text{-}yfp$ , Fig. 6A). As expected, the control reporter showed no differences in fluorescence between the wild type and the  $\Delta nrnA \Delta nrnB$  double mutant, with the same histogram distribution of fluorescence intensity in both strains (Fig. 6B, 6D). Inserting the *lchAA* UTR containing the c-di-GMP-specific riboswitch between the promoter and *yfp* is expected to render *yfp* expression sensitive to c-di-GMP levels. The  $\Delta nrnA \Delta nrnB$  had very low fluorescence compared to wild type indicating that c-di-GMP is indeed higher in this strain (Fig. 6C, 6E). As previously reported in *P. aeruginosa* (9, 10), this could be due to pGpG accumulation that competitively inhibits linearization of c-di-GMP. Although YhaM could not rescue aggregation in our assay in *P. aeruginosa*, (Fig. 2B) it could partially reduce pellicle biofilm formation (Fig. 2C). These data, in conjunction with the report that expression of *yhaM* could partially rescue an *E. coli orn* mutant and purified YhaM could turn over RNAs (15), led us to also generate an unmarked  $\Delta yhaM$  mutant. The  $\Delta yhaM$  had similar YFP levels as the parental 168 strain (Fig. S1). Furthermore, the  $\Delta nrnA \Delta nrnB \Delta yhaM$  triple mutant had similar results (Fig. S2) as the  $\Delta nrnA \Delta nrnB$  double mutant. These results indicate that NrnA and NrnB are the enzymes primarily responsible for degradation of pGpG in *B. subtilis*.

293 To support the changes in c-di-GMP observed with the fluorescent  
294 riboswitch reporter construct, c-di-GMP and pGpG extracted from wild type *B.*  
295 *subtilis* and the  $\Delta nrnA \Delta nrnB$  strains were quantified by LC-MS/MS in which  
296 pGpG and c-di-GMP generated two daughter ions (Table 2). For the wild type,  
297 the concentration of pGpG was below the limit of detection. In contrast, the  
298  $\Delta nrnA \Delta nrnB$  double mutant strain exhibited 1.9  $\mu\text{M}$  of pGpG. For c-di-GMP, wild-  
299 type bacteria had 1  $\mu\text{M}$  while the  $\Delta nrnA \Delta nrnB$  double mutant strain had 3. This  
300 3-fold increase agrees with the effect on the fluorescent c-di-GMP reporter.  
301 These results demonstrate that NrnA and NrnB degrade pGpG in *B. subtilis* and  
302 suggest that product inhibition of c-di-GMP linearization by pGpG is a  
303 widespread phenomenon.

304 **Discussion**

305 A subset of RNases degrade pGpG

306       Only Orn, NrnA, NrnB and NrnC can degrade pGpG. These four genes  
307 have previously been referred to as “nanoRNases” to describe the enzymes that  
308 can cleave “extremely short oligonucleotides” that are shorter than microRNA  
309 (14). pGpG and other linearized dinucleotides from signaling cyclic dinucleotides  
310 are two-nucleotide-long RNA molecules and represent appropriate substrates for  
311 nanoRNases. Despite being functionally similar, these four proteins contain  
312 different domains and different catalytic sites. Orn belongs to the RNase T  
313 superfamily (PFAM PF00929), NrnA and NrnB belong to the NrnA family with two  
314 adjacent DHH and DHHA1 domains (PF01368 and PF02272) (14, 15) and NrnC  
315 belongs to the RNase D superfamily (PF01612) (16). Nonetheless, these specific  
316 proteins appear to be distinct from other members of their family since other  
317 RNases and proteins that share these domains do not appear to cleave pGpG.

318       Whether additional proteins that were not identified in this study can turn  
319 over pGpG remains an outstanding question. It is possible that the transgenic  
320 approach used in this screen could result in false negatives and yet-unidentified  
321 exoribonuclease families would not have been included in the candidate for  
322 screening. A more general question is what are the total number and identity of  
323 exoribonucleases in prokaryotes. The most well characterized exoribonucleases  
324 are in two model organisms: *E. coli* and *B. subtilis*. *E. coli* encodes Orn, PNPase,  
325 Rbn RNase II, Rnd, Rnr, Rph and Rnt (21). Of the RNases found in *E. coli*, *B.*  
326 *subtilis* encodes only PNPase, Rph and Rnr (22). In the past decade, a number



327 of additional exoribonucleases have been characterized in *B. subtilis* including  
328 RNase J, NrnA, NrnB and YhaM (14, 15). Thus, there likely are additional yet  
329 uncharacterized genes that degrade short oligonucleotides and thus can cleave  
330 pGpG and other linear dinucleotide intermediates of c-di-nucleotide turnover. The  
331 enzymes that complement PA14  $\Delta orn$  were previously identified through  
332 their ability to rescue lethality in a conditional *orn* mutant in *E. coli*. However,  
333 while YhaM and RNase J also rescued growth of the *E. coli orn* mutant, they did  
334 not complement the biofilm phenotypes observed in PA14  $\Delta orn$ . These  
335 differences indicate that complementation of Orn essentiality in *E. coli* is a  
336 distinct phenotype from complementation of the *orn* activity in *P. aeruginosa*.  
337 Future experiments using the PA14  $\Delta orn$  strain as a surrogate host can allow  
338 identification of genes encoding enzymes from targeted organisms or from  
339 complex microbiomes.

340  
341 Enzymes that cleave linear dinucleotides are required to reduce cellular  
342 concentration of cyclic dinucleotides.

343 The termination of cyclic di-nucleotide signaling requires cleavage of the  
344 linear dinucleotide intermediate. In the absence of Orn in *P. aeruginosa*, pGpG is  
345 not degraded and can competitively inhibit linearization of c-di-GMP (9, 10)  
346 (Figure 7). As a consequence, c-di-GMP accumulates, leading to prolonged  
347 signaling and enhanced c-di-GMP dependent phenotypes (9, 10). Data shown  
348 here for *B. subtilis* indicate that NrnA and NrnB degrade pGpG in this organism.  
349 YhaM is not likely to be important in pGpG turnover since the c-di-GMP

350 riboswitch reporter showed similar c-di-GMP levels in the parental 168 strain and  
351 the  $\Delta yhaM$  single mutant. As observed in *P. aeruginosa*, the loss of the primary  
352 enzymes responsible for pGpG hydrolysis in *B. subtilis* leads to accumulation of  
353 pGpG and c-di-GMP. These results suggest that feedback inhibition by pGpG on  
354 the enzymes that linearize c-di-GMP is a conserved property of c-di-GMP  
355 signaling. This feedback inhibition appears to also hold true for c-di-AMP  
356 signaling. C-di-AMP is linearized by enzymes that contain HD (29) and DHH-  
357 DHHA1 domain (30). Recent studies of PDE2 in *Staphylococcus aureus* revealed  
358 that this protein cleaves pApA in c-di-AMP signaling (31). Furthermore, in the  
359 absence of *pde2*, *S. aureus* cells accumulate both pApA and c-di-AMP (31)  
360 (Figure 7). Together, these studies suggest that feedback inhibition by the linear  
361 dinucleotide product of cyclic di-nucleotide turnover may be conserved. For  
362 cGAMP (32), linearization to pApG is mediated by three V-cGAP enzymes (33).  
363 How the pApG linear product of cGAMP is hydrolyzed to mononucleotide is  
364 currently unknown. Since cGAMP is produced in *V. cholerae*, we anticipate that  
365 Orn<sub>Vc</sub> can serve to degrade both pApG and pGpG dinucleotides. Future studies  
366 in additional organisms will reveal whether feedback inhibition of linearization  
367 enzyme by linear dinucleotides is a general property of the known bacterial cyclic  
368 dinucleotide signaling molecules: c-di-GMP, c-di-AMP and cGAMP (Figure 7).

369  
370 Proteins containing HD-GYP domain may not cleave pGpG in cells

371 Previous studies of HD-GYP proteins demonstrated that these proteins are able  
372 to degrade c-di-GMP and pGpG in vitro (2). In vivo studies in *V. cholerae*

373 revealed that expression of HD-GYP proteins reduced c-di-GMP levels (34).  
374 Furthermore, lysates of *E. coli* overexpressing of *V. cholerae* HD-GYP domain  
375 proteins was able to both degrade c-di-GMP into pGpG and subsequently to  
376 GMPs (35). However, the cleavage of pGpG to GMP cannot be specifically  
377 attributed to HD-GYP proteins due to the presence of Orn in the *E. coli* strain  
378 background. To clearly test pGpG hydrolysis activity of proteins containing a HD-  
379 GYP domain without Orn, we tested lysates of *P. aeruginosa*  $\Delta orn$  expressing  
380 each of the HD-GYP genes from *V. cholerae*. Since expression of these genes  
381 failed to increase pGpG cleavage, these results provide additional evidence that  
382 HD-GYP do not function as the main pGpG degrading enzymes in vivo (9, 10).

383

#### 384 NanoRNases degrade pGpG.

385 Unlike the linearization step of c-di-GMP, which relies on c-di-GMP-  
386 specific phosphodiesterases, our results suggest that the degradation of pGpG  
387 does not appear to require a pGpG-specific enzyme. Instead, the turnover of  
388 pGpG appears to be carried out by a subset of RNases. These RNases, dubbed  
389 “nanoRNases”, were identified in screens to find genes able to rescue growth in  
390 an *E. coli* conditional *orn* mutant and were shown to be able to turn over short  
391 oligoribonucleotides in vitro (14-16). However, although RNase J1 and YhaM  
392 were shown to partially rescue the *E. coli orn* growth defect (15), we did not  
393 observe that these enzymes were able to hydrolyze pGpG or rescue the *P.*  
394 *aeruginosa orn* biofilm and aggregation phenotypes, suggesting that not all  
395 enzymes possessing nanoRNase activity have pGpG degrading activity.

396 Nevertheless, the final steps of c-di-GMP and RNA turnover appear to intersect  
397 at RNases. Thus, the relative affinity for and rate of cleavage of  
398 oligoribonucleotides of different sequence and length may matter during periods  
399 in which bacteria need to rapidly remove c-di-GMP. Whether this overlap in  
400 source of oligoribonucleotides substrates for these RNases has consequences  
401 for cellular regulation or mRNA turnover is at present an open question.

402         The current experiments have focused on the identification of the  
403 enzymes responsible for cleaving pGpG. Since nanoRNases are hypothesized to  
404 cleave all short oligoribonucleotides regardless of sequence, we also expect  
405 them to have activity against the linearized form of the other two cyclic  
406 dinucleotide signaling molecules (pApA from c-di-AMP and pApG from cAG).  
407 Whether the linear cAG can also engage in product inhibition is currently  
408 unknown. However, the finding that all cyclic di-nucleotides share the final  
409 processing enzymes that are also responsible for degrading 2-mer RNAs would  
410 not be surprising.

411

412 **Materials and Methods**

413 *Strains and culture conditions*

414 The strains, plasmids, and primers used in this study are listed in Tables  
415 S2, S3, and S4, respectively. Bacteria were grown in LB or LB-agar  
416 supplemented with 50 µg/mL carbenicillin at 37 °C except when otherwise noted.  
417 Plasmids were induced with 1 mM IPTG. All *B. subtilis* strains in this study are  
418 derived from 168. To make  $\Delta yhaM$ ,  $\Delta nrnA \Delta nrnB$ , and  $\Delta nrnA \Delta nrnB \Delta yhaM$ ,  
419 strains harboring gene knockouts of locus tags BSU29250, BSU18200 and  
420 BSU09930 were obtained from the BKE collection. The erythromycin-resistance  
421 cassette inserted in each locus was then removed in each strain, and markerless  
422 deletions were created through transformation with pDR244 ((36), Bacillus  
423 Genetic Stock Center). A series of transformation protocols were performed with  
424 each BKE strain as well as pDR244 until the double and triple mutant strains  
425 were achieved. Removal of the erythromycin-resistance cassette was verified by  
426 Sanger sequencing. For construction of the fluorescent *yfp* reporters used in this  
427 study, integration at the *amyE* locus of 168 was performed with plasmids derived  
428 from pJG019 (GenBank: KX499653.1). To construct pRSL\_F4, the *lchAA* leader  
429 sequence (complete sequence is provided in the supplemental materials) was  
430 synthesized (GenScript) and inserted at the HindIII restriction site of the vector.  
431 pJG019 and pRSL\_F4 were transformed into 168,  $\Delta yhaM$ ,  $\Delta nrnA \Delta nrnB$ , and  
432  $\Delta nrnA \Delta nrnB \Delta yhaM$  by using cells induced for competence through growth in  
433 nitrogen limiting media (37).

434

435 *Cloning*

436 The *V. cholerae* O1 biovar El Tor str. N16961 and *B. anthracis* Gateway  
437 compatible ORFeome libraries were obtained from BEI Resources. The ORFs  
438 were moved into the desired expression vectors (see Table S2 for primers) using  
439 LR-clonase enzyme II (Invitrogen) and introduced into chemically competent *E.*  
440 *coli* strain T7lq (NEB) following the manufacturer's protocols. The *B. subtilis*  
441 *nrnA*, *nrnB*, *rnjA* and *yhaM* and the *C. crescentus* *nrnC* were cloned using the  
442 primers shown in Table S1.

443

444 *Protein purification*

445 His<sub>10</sub>-VC0341, His<sub>10</sub>-BA4852, His<sub>10</sub>-Nr<sub>n</sub>A, His<sub>10</sub>-Nr<sub>n</sub>B, His<sub>10</sub>-Nr<sub>n</sub>C, and  
446 His<sub>10</sub>-YhaM were purified from *E. coli* T7lq strains containing expression  
447 plasmids (Table S2) as described previously (38). Briefly, strains were grown in  
448 LB with appropriate antibiotic at 37°C overnight, subcultured in fresh media and  
449 grown to OD<sub>600</sub> ~ 1.0 when protein production was induced with the addition of 1  
450 mM IPTG. Induced bacteria were pelleted and resuspended in 10 mM Tris, pH 8,  
451 100 mM NaCl, and 25 mM imidazole and frozen at -80°C until purification.  
452 Proteins were purified over a Ni-NTA column followed by desalting on a  
453 Sephadex G-25 column into reaction buffer. Proteins were flash frozen in liquid  
454 nitrogen for storage at -80°C until use.

455

456 *Synthesis of radiolabeled dinucleotides*

457  $^{32}\text{P}$ -pGpG was generated by the linearization of  $^{32}\text{P}$ -c-di-GMP with RocR  
458 from *P. aeruginosa*. For this reaction,  $^{32}\text{P}$ -c-di-GMP (0.167  $\mu\text{M}$  final) was  
459 incubated with RocR (20  $\mu\text{M}$  final) in 10 mM Tris, pH 8, 100 mM NaCl and 5 mM  
460  $\text{MgCl}_2$  at room temperature for 1 h and the reaction was stopped by heat  
461 inactivation at 98 °C for 10 min, then passed over a 3 kDa molecular weight  
462 cutoff column to remove the protein.  $^{32}\text{P}$ -c-di-GMP was enzymatically  
463 synthesized as previously described (39). Purity was checked by TLC.

464

#### 465 *Cell lysate and enzymatic activity assays*

466 The activity of whole cell lysates and purified proteins against  $^{32}\text{P}$ -pGpG  
467 was assayed as previously described (9). Briefly, 0.1  $\mu\text{M}$  of purified in reaction  
468 buffer (50 mM Tris, pH 8, 100 mM NaCl, and 5 mM  $\text{MgCl}_2$  for NrnA, NrnB, NrnC,  
469 and YhaM; 50 mM Tris, pH 8, 100 mM NaCl, and 5 mM  $\text{MnCl}_2$  for Orn) was  
470 incubated with 0.1 mM of pGpG spiked with 4 pM  $^{32}\text{P}$ -pGpG tracer. For cell  
471 lysates, PA14  $\Delta orn$  carrying the indicated complementation vectors were grown  
472 overnight, subcultured 1:100 into fresh LB supplemented with carbenicillin, and  
473 induced with 100 mM IPTG and grown at 37°C or 30°C as indicated to  $\text{OD}_{600} \sim$   
474 0.4 with shaking. The cultures were pelleted and resuspended in 1/10<sup>th</sup> volume of  
475 reaction buffer, adjusted to the same  $\text{OD}_{600}$ , and supplemented with 10  $\mu\text{g}/\text{mL}$   
476 DNase, 250  $\mu\text{g}/\text{mL}$  lysozyme, 10 mM PMSF, and lysed by sonication. At  
477 indicated times, aliquots were removed and the reaction stopped by adding an  
478 equal volume of 0.2 M EDTA, pH 8, and heated at 98 °C for 10 min.

479

480 *Thin-layer chromatography*

481        Performed as previously described (9). Briefly, 0.5  $\mu$ L of each sample was  
482 spotted on polyethyleneimine-cellulose TLC plates (EMD Chemicals), dried, and  
483 developed in mobile phase consisting of 1:1.5 (vol:vol) saturated  $\text{NH}_4\text{SO}_4$  and 1.5  
484 M  $\text{KH}_2\text{PO}_4$ , pH 3.60. The TLC plate was dried and imaged using Fujifilm FLA-  
485 7000 phosphorimager (GE) and the intensity of the radiolabel was quantified  
486 using Fujifilm Multi Gauge software v3.0.

487

488 *Microtiter plate crystal violet biofilm assay*

489        Briefly, overnight cultures were diluted 1:100 in LB and grown as static  
490 cultures in a 96-well polystyrene plate (Greiner) at 30 °C inside a humidified  
491 chamber for 24 h. The cultures were washed of planktonic cells and stained with  
492 crystal violet as previously described (28). The  $A_{595}$  was measured on a  
493 SpectraMax M5 spectrophotometer (Molecular Devices).

494

495 *Aggregation assay*

496        Cultures of *P. aeruginosa* strains were grown in 10 mL LB with appropriate  
497 antibiotic and IPTG induction for 24 h at 37 °C with shaking. Culture tubes were  
498 allowed to settling at room temperature for 30 mins and photographed.

499

500 *Fluorescence microscopy & quantification*

501        *B. subtilis* 168 WT,  $\Delta nrmA$   $\Delta nrmB$ , and  $\Delta nrmA$   $\Delta nrmB$   $\Delta yhaM$ -derived  
502 reporter strains were grown at 37°C on LB plates supplemented with 1.5% Bacto



503 agar and 5 ug/mL chloramphenicol, when appropriate. Single colonies were used  
504 to inoculate liquid MSgg medium (40) and grown at 37°C shaking overnight. The  
505 following day, cultures of each strain were inoculated 1:50 on fresh medium and  
506 grown at 37°C shaking until reaching an optical density at 600 nm ( $OD_{600}$ ) of 1.0.  
507 Aliquots of these cultures were placed on 1.5% low-melting agarose MSgg pads  
508 and allowed to dry for 10 minutes. Agarose pads were inverted onto a glass  
509 bottom dish (Willco Wells). Cells were imaged at room temperature using a Zeiss  
510 Axio-Observer Z1 inverted fluorescence microscope, equipped with a Rolera EM-  
511 C<sub>2</sub> electron-multiplying charge-coupled (EMCC) camera, and an environmental  
512 chamber. Fluorescence intensity per cell was quantified using Outfi analysis  
513 software (41). Images were analyzed and adjusted with FIJI software (42).

#### 514 515 *Quantification of Intracellular c-di-GMP and pGpG in P. aeruginosa*

516 Extraction, quantification and CFU determination were performed as  
517 previously described (9) using previously published MS and UPLC parameters  
518 (43, 44). Briefly, *P. aeruginosa* strains were grown overnight in LB at 37 °C with  
519 shaking, subcultured 1:100 in LB, and grown at 37 °C with shaking. Cells were  
520 pelleted, resuspended in 100 µL ice-cold 40:40:20 (vol:vol:vol) MeOH,  
521 acetonitrile, and water with 0.1 N formic acid, incubated 30 min at –20 °C for  
522 lysis, and neutralized after a 30-min incubation with 4 µL 15% (wt/vol)  $NH_4NCO_3$ .  
523 Cellular debris was pelleted, and the supernatant was removed for desiccation by  
524 a Savant SpeedVac Concentrator (Thermo Scientific). Desiccated samples were  
525 suspended in 100 µL ultra-pure water, and insoluble material was pelleted. The

soluble supernatant was filtered through a Titan syringe filter (PVDF, 0.45  $\mu$ m, 4 mm) before quantification of c-di-GMP and pGpG using LC-MS/MS on a Quattro Premier XE mass spectrometer (Waters) coupled with an Acquity Ultra Performance LC system (Waters). Cyclic-di-GMP was detected in 10- $\mu$ L injections of filtered extracts. For the detection of pGpG, filtered extracts were diluted 1:100 in ultra-pure water, and 10- $\mu$ L injections of the diluted extracts were then analyzed. The intracellular concentrations of c-di-GMP and pGpG were determined by calculating the total number of colony-forming units in each sample and multiplying this value by the intracellular volume of a single bacterium. The total c-di-GMP and pGpG extracted in each sample were then divided by the total intracellular volume of the cells in the sample to provide the intracellular concentration of each analyte.

#### *Metabolite extraction and quantification of c-di-GMP and pGpG in B. subtilis*

Three independent replicates of *B. subtilis* 168 WT and  $\Delta nrmA \Delta nrmB$  were grown overnight in liquid MSgg medium (40) shaking at 37°C. The following day cultures of each strain were inoculated 1:50 and grown shaking at 37°C until reaching an optical density at 600 nm (OD<sub>600</sub>) of 1.0. Metabolite extraction was described previously (45). 5 mL cultures were passed through 0.2  $\mu$ m nylon filters (EMD Millipore). Metabolism was quenched and metabolites were extracted by inverting the filters into petri dishes that contained 1.5 mL pre-chilled extraction solvent composed of 40:40:20 acetonitrile/methanol/water. Dishes were placed on dry ice for 15 minutes before the wash was collected in

549 microcentrifuge tubes and allowed to spin at max speed for 5 minutes at 4°C.  
550 The supernatant was then transferred to new microcentrifuge tubes and placed in  
551 a vacuum centrifuge until metabolite extracts were dry. Detection of c-di-GMP by  
552 LC-MS/MS was described previously (46). Briefly, bacterial extract was  
553 resuspended in Solvent A (10 mM tributylamine in water, pH 5.0) and centrifuged  
554 twice to remove insoluble particles. Metabolites were then separated on a  
555 Synergi Fusion-RP column (4 µm particle size, 80 Å pore size, 150 mm x 2 mm,  
556 Phenomenex) using a Shimadzu high performance liquid chromatography  
557 machine and simultaneously analyzed by a triple quadrupole mass spectrometer  
558 (3200 QTRAP, ABSCIEX). The total run time was 20 min at a binary flow rate of  
559 0.5 ml min<sup>-1</sup>, with 10 mM tributylamine in water (pH 5.0) as Solvent A and 100%  
560 methanol as Solvent B. The following gradient was performed: 0.01 min, 0% B, 4  
561 min, 0% B, 11 min, 50% B, 13 min, 100% B, 15 min, 100% B, 16 min, 0% B, 20  
562 min, 0% B. C-di-GMP and pGpG were detected by multiple reaction monitoring  
563 (MRM) under negative mode using the ion pairs 689/79 and 689/344 (c-di-GMP)  
564 and 707/79 and 707/150 (pGpG). C-di-GMP and pGpG were quantified using the  
565 Analyst<sup>®</sup> software (version 1.6.2) by calculating the total peak area and  
566 normalized by total ion current (TIC). Authentic c-di-GMP and pGpG standards  
567 were injected and analyzed alongside samples.  
568

569 **Figure legends**

570 **Figure 1. A subset of genes with RNase domains reduce aggregation by**

571 **PA14  $\Delta orn$ .** Photograph of overnight cultures of PA14  $\Delta orn$  with empty vector

572 (EV), complementation vectors expressing the indicated genes. Genes are

573 grouped by RNase domain. Strains were growing with shaking and induction

574 overnight, allowed to sediment for 30 minutes by gravity, and photographed.

575 Daggers indicate strains grown and induced at 30°C while the remaining were

576 grown and induced at 37°C. Red boxes indicate genes that prevented

577 autoaggregation.

578

579 **Figure 2. *nrnA*, *nrnB*, and *nrnC* can reduce biofilm formation and**

580 **aggregation by PA14  $\Delta orn$ .**

581 (A) Quantification of the crystal violet assay for pellicle biofilm formation of the

582 PA14  $\Delta orn$  strain with either empty vector or complementation by indicated

583 genes carried on a pMMB-based a single-copy IPTG inducible plasmid after 24 h

584 of static growth. (B) Photographs of the aggregation assay and (C) quantification

585 of the crystal violet assay for pellicle biofilm formation of PA14  $\Delta orn$

586 complemented with the indicated genes carried on a pMMB-based plasmid.

587 Values shown are the average and SD of three independent experiments. \*

588 indicates  $p < 0.05$  Students' unpaired two-tailed *t*-test.

589

590 **Figure 3. A subset of RNases can rescue PA14  $\Delta orn$  pGpG hydrolysis**

591 **defect.** The rate of pGpG cleavage by whole cell lysates of *P. aeruginosa* PA14

592  $\Delta orn$  complemented with the indicated genes carried on a pMMB-based plasmid.

593 (A) The 3' to 5' exoribonucleases from *B. anthracis*, (B) the 3' to 5'

594 exoribonucleases from *V. cholerae*, and (C) *nrnA<sub>BS</sub>*, *nrnB<sub>BS</sub>*, *rnjA<sub>BS</sub>*, *yhaM<sub>BS</sub>* and

595 *nrnC<sub>Cc</sub>*. \* indicates  $p < 0.05$  Students' unpaired two-tailed *t*-test.

596

597 **Figure 4. Hydrolysis of pGpG by purified RNases.** The rate of  $^{32}\text{P}$ -pGpG

598 hydrolysis by 10 nM purified Orn<sub>Vc</sub>, NrnA<sub>BS</sub>, NrnB<sub>BS</sub>, and NrnC<sub>Cc</sub> incubated with 1

599  $\mu\text{M}$  pGpG supplemented with  $^{32}\text{P}$ -pGpG tracer over a period of 30 min. Aliquots

600 were removed for analysis and the reaction was stopped by addition of EDTA at

601 the indicated time points. Radiolabeled nucleotides were separated by TLC and

602 the fraction of  $^{32}\text{P}$ -pGpG remaining over total radiolabel was quantified. Values

603 shown are the average and SD of three independent experiments. \* indicates  $p <$

604 0.05 Students' unpaired two-tailed *t*-test.

605

606 **Figure 5. Proteins containing HD-GYP domain do not cleave pGpG in cells**

607 **lacking *orn*.** Lysates from PA14  $\Delta orn$  expressing individual genes encoding an

608 HD-GYP domain from *V. cholerae* were tested for pGpG hydrolysis by monitoring

609 conversion of  $^{32}\text{P}$ -pGpG to GMP. Values shown are the average and SD of three

610 independent experiments. \* indicates  $p < 0.05$  Students' unpaired two-tailed *t*-

611 test.

612

613 **Figure 6. Cyclic di-GMP fluorescence riboswitch detection of c-di-GMP**

614 **levels in *B. subtilis* 168.** Representative images of fluorescence of the

615 constitutively expressed YFP reporter  $P_{\text{const}}\text{-yfp}$  (A) or the c-di-GMP riboswitch  
616 reporter construct  $P_{\text{const}}\text{-lchAA UTR-yfp}$  (B) in either *B. subtilis* 168 wild type (WT)  
617 or the double deletion mutant  $\Delta nrmA \Delta nrmB$ . Histograms of the quantification of  
618 average fluorescence intensity of *B. subtilis* 168 wild type and  $\Delta nrmA \Delta nrmB$  with  
619  $P_{\text{const}}\text{-yfp}$  (C) or  $P_{\text{const}}\text{-lchAA UTR-yfp}$  cells (D) ( $n \sim 300$ ).

620

621 **Figure 7. Model for degradation of cyclic dinucleotides.** Cartoon of the two-  
622 step degradation process of (A) c-di-GMP, (B) c-di-AMP, and (C) cGAMP. Step 1  
623 is cyclic dinucleotides linearization (indicated by the gray boxes). Step 2 is pNpN  
624 hydrolysis (indicated by the green boxes). In scenarios in which the linear  
625 dinucleotide accumulates, there is feedback inhibition on the enzymes that  
626 linearize cyclic dinucleotides. Dashed lines indicate potential inhibition and ?  
627 mark indicate the presence of additional categories of enzymes that hydrolyze  
628 dinucleotides.

629

630 **Table 1. Intracellular concentration of pGpG following complementation of**  
631 **PA14  $\Delta orn$  strains**

Strain	$\mu\text{M pGpG}^*$	$\mu\text{M c-di-GMP}^*$
Wild type pMMB	$2.2 \pm 0.4$	$0.016 \pm 0.008$
$\Delta orn$ pMMB	$17.4 \pm 3.7$	$0.58 \pm 0.10$
$\Delta orn$ pMMB- <i>orn</i> <sub>Pa</sub>	$5.2 \pm 1.4$	$0.028 \pm 0.013$
$\Delta orn$ pMMB- <i>orn</i> <sub>Vc</sub>	$6.5 \pm 1.8$	$0.017 \pm 0.011$
$\Delta orn$ pMMB- <i>nrmA</i> <sub>Bs</sub>	$3.6 \pm 1.4$	$0.022 \pm 0.016$

$\Delta orn$ pMMB- <i>nrnA</i> <sub>BS</sub>	3.3 ± 1.2	0.024 ± 0.015
$\Delta orn$ pMMB- <i>nrnB</i> <sub>BS</sub>	2.5 ± 1.1	0.023 ± 0.011
$\Delta orn$ pMMB- <i>nrnC</i> <sub>Cc</sub>	2.4 ± 1.1	0.020 ± 0.001

633 \* average and standard deviation of 3 experiments, calculated assuming the  
 634 volume of a single bacterium equals  $4.3 \times 10^{-1}$  fL (9).  
 635

636 **Table 2. Intracellular concentration of pGpG and c-di-GMP in *B. subtilis* and**  
 637  **$\Delta nrnA \Delta nrnB$  strains**

			$\Delta nrnA \Delta nrnB$	Fold Change
WT 168 (□M)			(□M)	(ΔWT)
pGpG	daughter ion 1	ND	1.8 ± 0.6	NA
	daughter ion 2	ND	1.8 ± 0.4	NA
c-di-GMP	daughter ion 1	0.8 ± 0.2	2.4 ± 0.5	3.0
	daughter ion 2	0.9 ± 0.1	3.0 ± 0.9	3.3

638

639

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641

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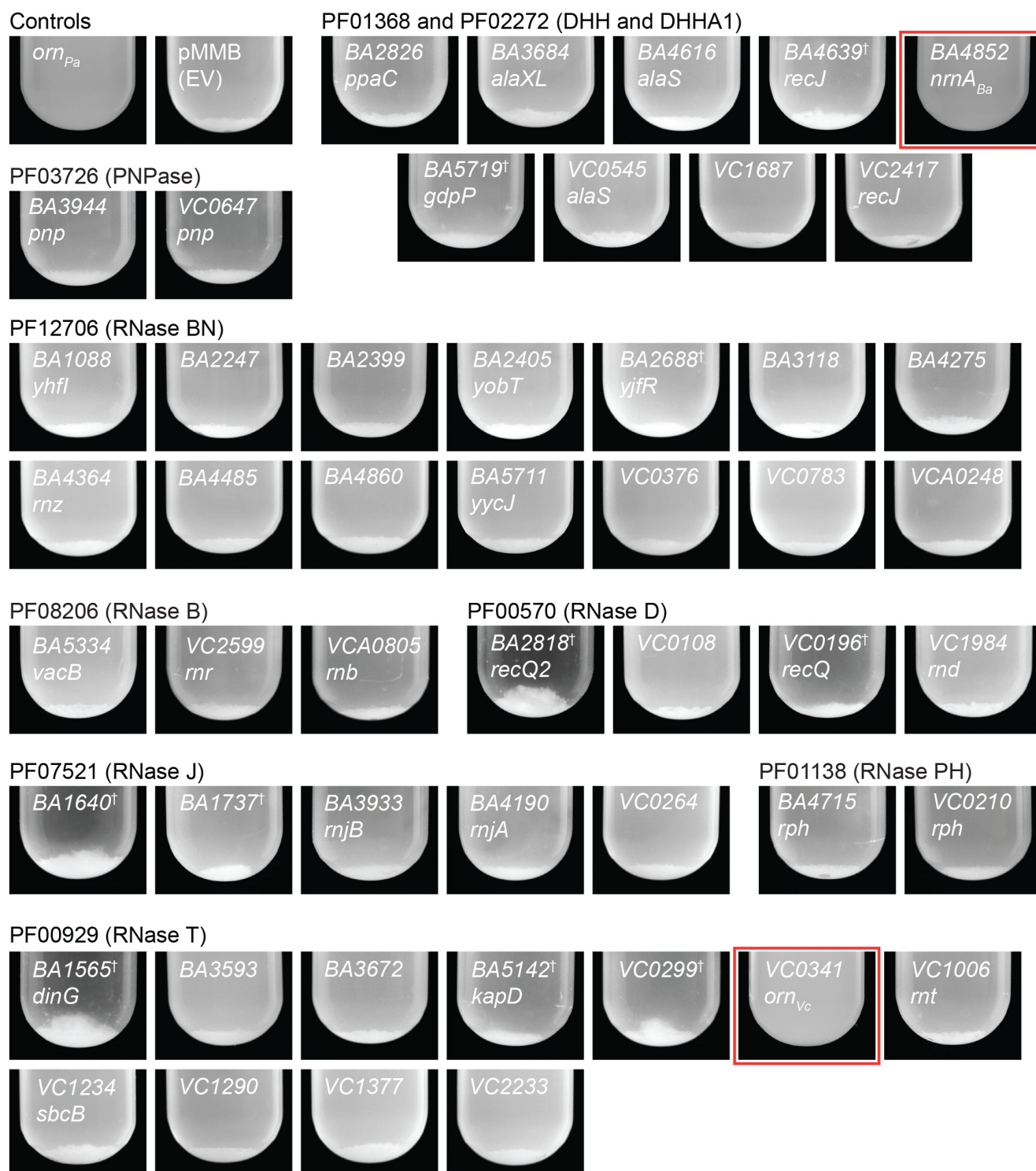
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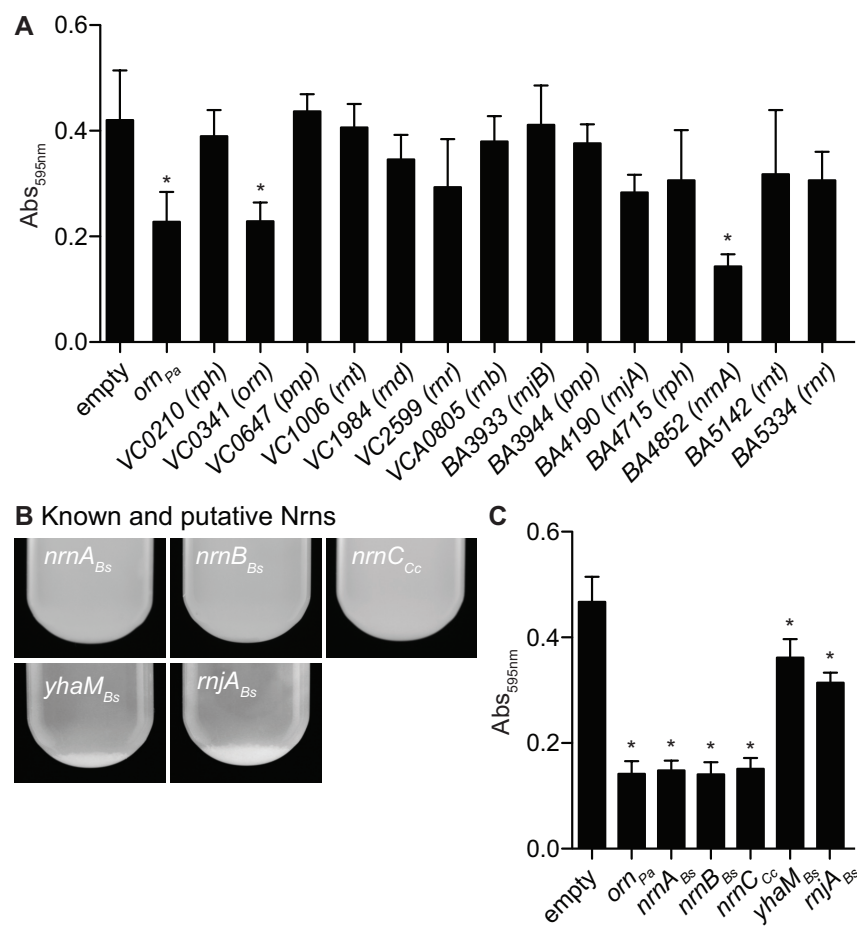
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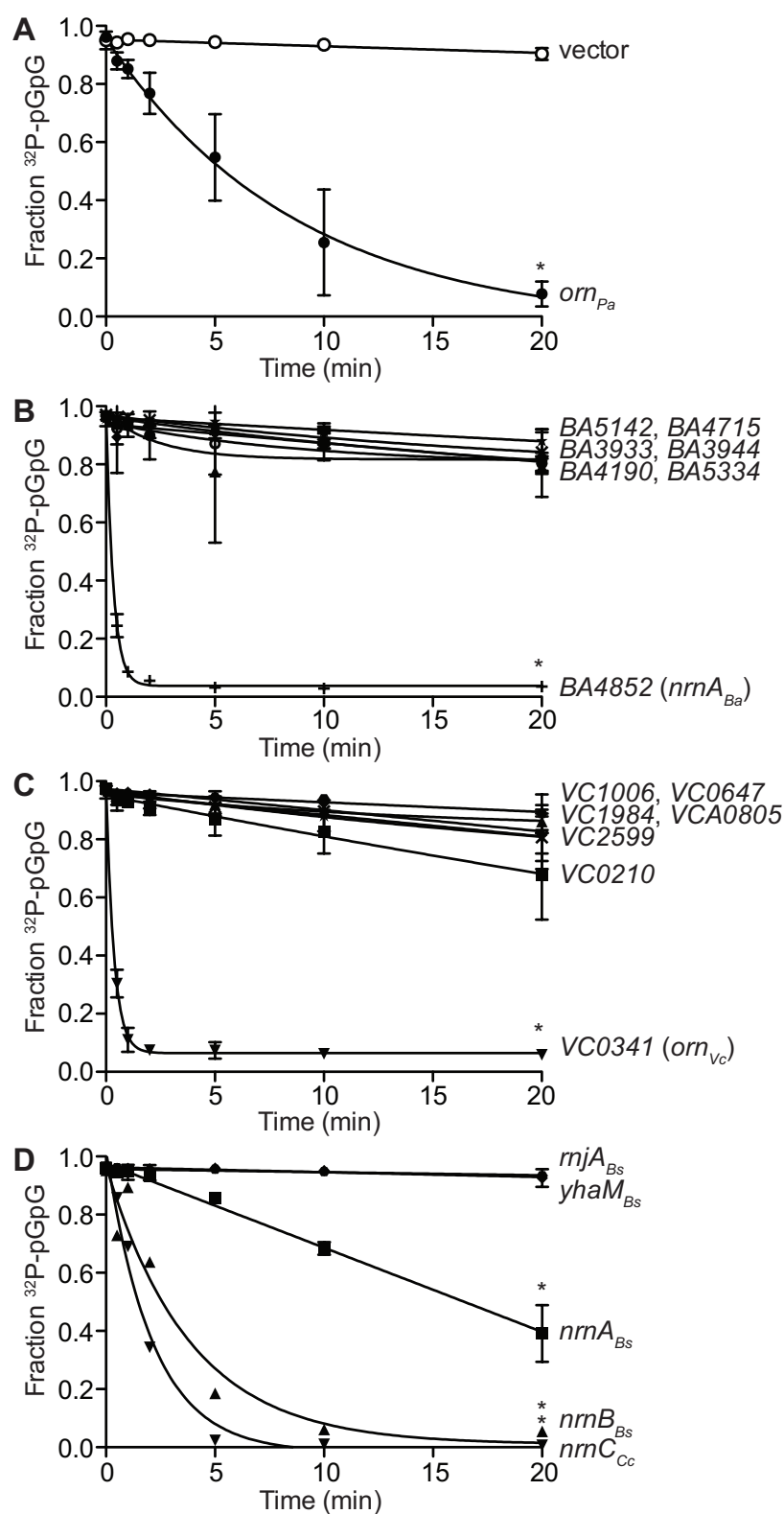


**Figure 1.** A subset of genes with RNase domains reduce aggregation by PA14  $\Delta orn$ .

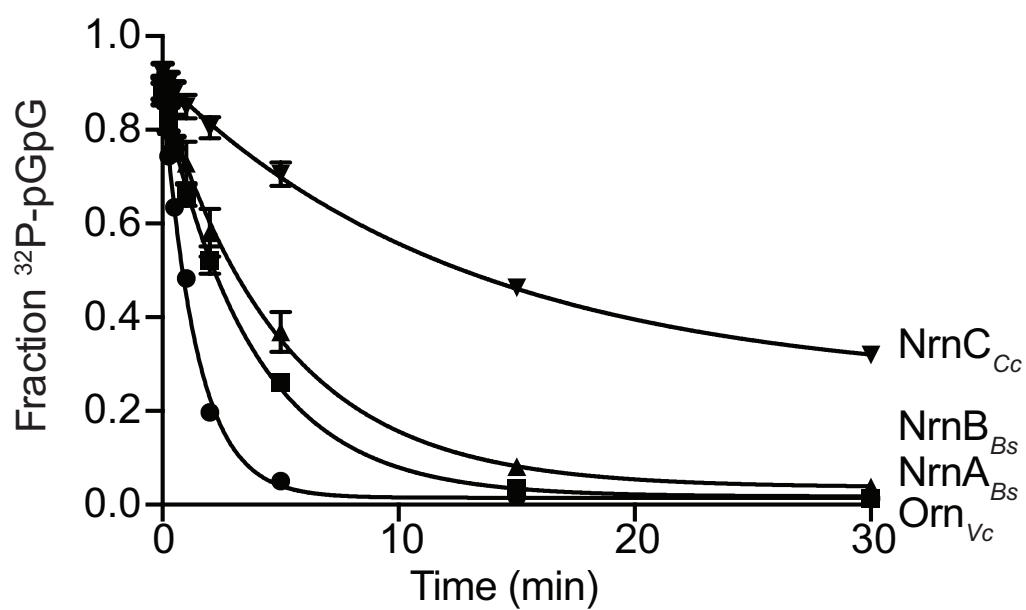


**Figure 2.** *nrmA*, *nrmB* and *nrmC* can reduce biofilm formation and aggregation by PA14  $\Delta orn$ .



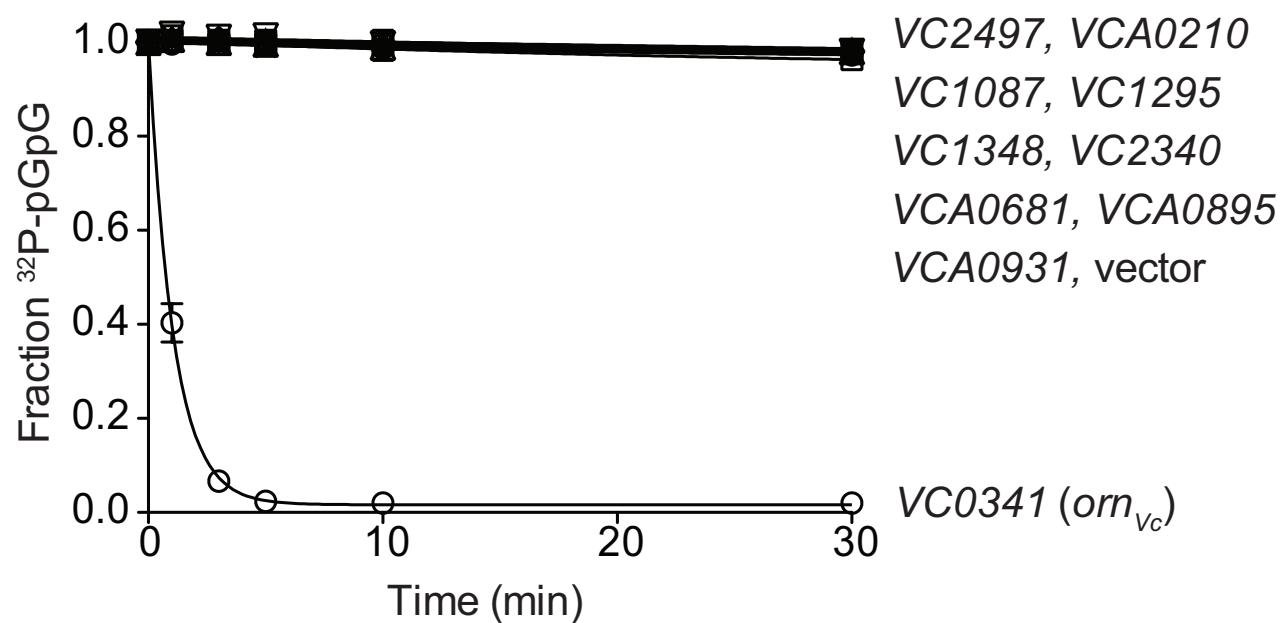


**Figure 3.** A subset of RNases can rescue PA14  $\Delta orn$  pGpG hydrolysis defect.

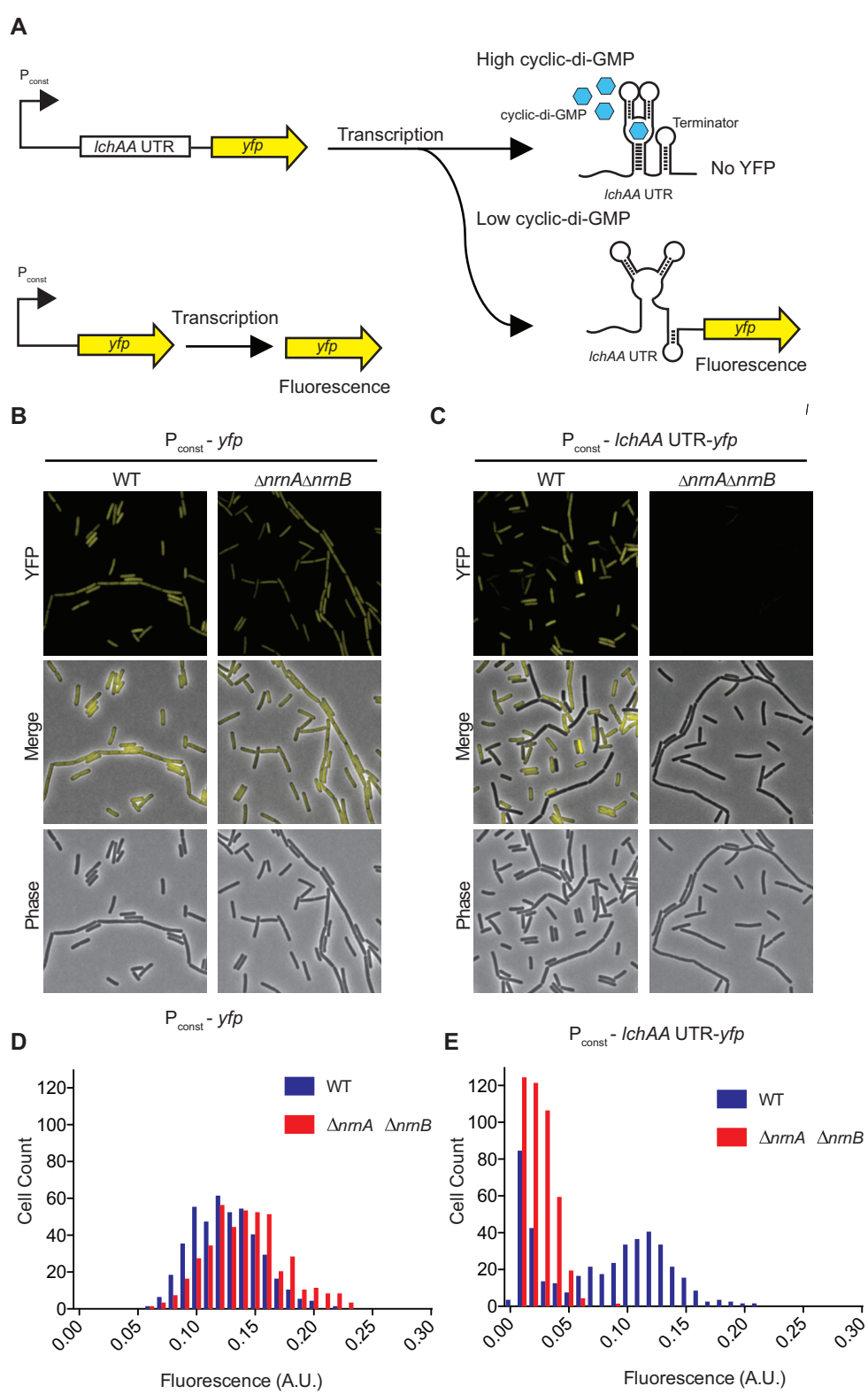


**Figure 4.** Hydrolysis of pGpG by purified RNases.

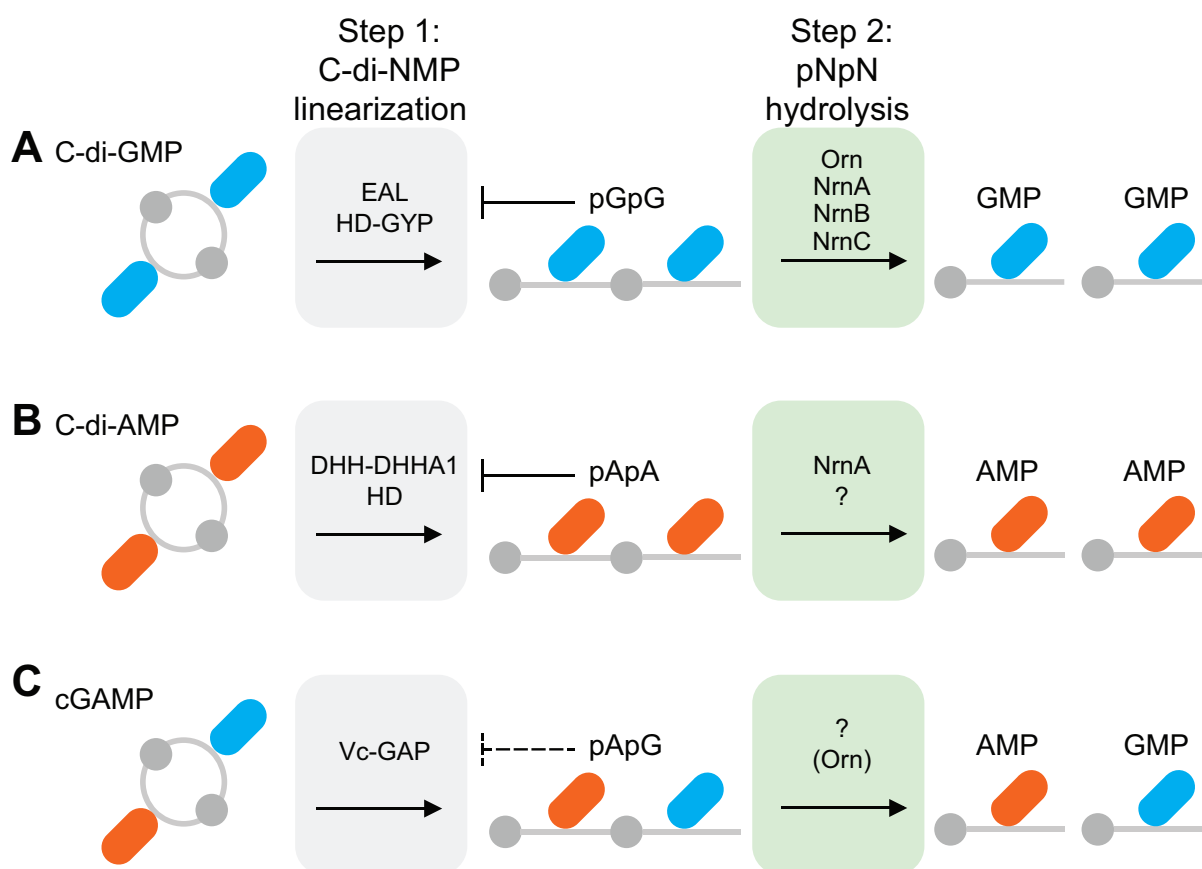




**Figure 5.** Proteins containing HD-GYP domain do not cleave pGpG in cells lacking *orn*.



**Figure 6.** Cyclic di-GMP fluorescence riboswitch detection of c-di-GMP levels in *B. subtilis* 168.



**Figure 7.** Model for degradation of cyclic dinucleotides.