

Diribonuclease activity eliminates toxic diribonucleotide accumulation

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Running title: Diribonuclease activity is essential

ABSTRACT

RNA degradation is a central process in cells, which is vital for transcriptional regulation. The degradation process is initiated by endoribonucleolytic fragmentation of long RNA polymers, followed by exoribonucleolytic cleavage to release mononucleotides from the fragment ends. In this process, the degradation of short oligonucleotides, especially diribonucleotides, into mononucleotides requires specific enzymatic activity, which in Gram-negative bacteria is provided by oligoribonuclease (Orn). Orn is unique in that it is the only essential exoribonuclease in *Escherichia coli*. Recent studies have shown that Orn cleaves only diribonucleotides under physiological conditions, suggesting that the accumulation of dinucleotides poisons cells. Yet, related organisms, such as *Pseudomonas aeruginosa*, display a growth defect but are viable without Orn, contesting its essentiality. Here, we took advantage of *P. aeruginosa orn* mutants to elucidate the mechanisms of their survival. Genetic screening for suppressors that restored colony morphology identified *yciV*. Purified YciV exhibited diribonuclease activity. YciV is present in all γ -proteobacteria, suggesting that the enzyme from *P. aeruginosa* is distinct from its *E. coli* ortholog. Phylogenetic analysis revealed differences between the two orthologs that were mapped to the active site of the enzyme and correlated with differences in substrate profiles. The expression of *P. aeruginosa* YciV in *E. coli* eliminated the necessity of *orn*. Similarly, the deletion of *yciV* from *P. aeruginosa* caused *orn* to become essential. Together, these results show that diribonuclease activity is required in γ -proteobacteria and that diribonucleotides may be utilized to monitor the efficacy of RNA degradation. Because Orn is found in higher eukaryotes, these observations indicate a conserved mechanism for monitoring RNA degradation.

INTRODUCTION

Since the discovery of RNase A, numerous studies have sought to identify enzymes that complete the turnover of RNA polymers into mononucleotides. One set of early studies biochemically identified and named oligoribonuclease (Orn) because the enzyme degraded RNA oligoribonucleotides from two to five nucleotides (Datta and Niyogi, 1975; Niyogi and Datta, 1975; Stevens and Niyogi, 1967). The view that one enzyme processes all short RNA oligoribonucleotides has been accepted for prokaryotic and eukaryotic systems (Arraiano et al., 2010; Belasco, 2010; Condon, 2007; Deutscher, 2006; Hui et al., 2014). The gene encoding *orn* was subsequently discovered in *E. coli* and revealed to be essential (Ghosh and Deutscher, 1999), which is unique among all other exoribonucleases. Three mechanisms have been proposed to explain the essentiality of *orn*: (1) loss of *orn* leads to depletion of mononucleotides, (2) accumulation of oligoribonucleotides is toxic to cells, or (3) an unknown moonlighting activity of Orn is essential. Which of these mechanisms is the basis for essentiality remained unsolved.

A separate line of study reported that the bacterial second messenger cyclic-di-GMP (c-di-GMP) is degraded through a two-step process in which the cyclic nucleotide is linearized into a pGpG diribonucleotide by one set of enzyme(s), and is then hydrolyzed to two GMPs by another set of enzyme(s) (Ross et al., 1987). The enzyme responsible for linearizing c-di-GMP, an EAL domain-containing protein, was identified first (Chang et al., 2001). The enzyme required for hydrolyzing pGpG was identified later as Orn in two independent studies using *Pseudomonas aeruginosa* (Cohen et al., 2015; Orr et al., 2015), enabled by the fact that for unknown reasons a transposon insertion mutant of *orn* was viable in *P. aeruginosa* (Liberati et al., 2006). A study that tested all other known 3' to 5' exoribonucleases in *P. aeruginosa* showed that only Orn from this organism, or functionally equivalent nano-RNases from other bacterial species, could cleave pGpG (Orr et al., 2018). Taken together, these studies suggested that Orn cleaves dinucleotides.

However, we wondered how Orn differentiates between these diribonucleotides and oligoribonucleotide substrates of different lengths and avoids acting on longer RNA molecules, which would sequester the enzyme from the short substrates. We were surprised to discover that purified Orn has a more than 100-fold preference for diribonucleotides over longer substrates (Kim et al., 2019). For example, in ex vivo experiments, a 7 nucleotide-long RNA was degraded by *P. aeruginosa* lysates into mononucleotides, whereas the lysates of Δorn accumulated diribonucleotides (Kim et al., 2019). The molecular basis of the stark selectivity of Orn for diribonucleotides was revealed by the co-crystal structure of Orn and dinucleotides. The structures revealed that Orn has a highly restricted substrate-binding site that also serves as the catalytic

site. This substrate binding pocket consists of a surface patch that selects for the 5' phosphate, a leucine wedge to orient the dinucleotide substrate for cleavage, and a flexible loop that moves the catalytic histidine in place; together, these conserved features determine the high selectivity of Orn for diribonucleotides (Kim et al., 2019).

The discovery that Orn only cleaves diribonucleotide substrates led us to revisit the basis for essentiality of *orn* in γ -proteobacteria. When Orn was thought to cleave oligoribonucleotides of all lengths, the absence of Orn would have resulted in the sequestration of many nucleotides in these oligonucleotide fragments, and it could be assumed that cells would be depleted of monoribonucleotides. However, because Orn predominantly cleaves dinucleotides, the degradation of long RNA molecules (mRNA, rRNA) should release the majority of the molecules as mononucleotides by other exoribonucleases, and only a small amount will become dinucleotides. The reason why *orn* is essential in this model is unclear.

To improve our understanding of *orn*'s essentiality, we took advantage of the fact that *P. aeruginosa* Δorn is viable but exhibits a small-colony phenotype. In this study, we obtained suppressor mutants that restored the colony morphology to wild-type levels. These suppressor strains featured alterations of the *yciV* gene. The *yciV* gene encodes an enzyme with a Polymerase and Histidinol Phosphatase (PHP) domain and has been reported to cleave short oligonucleotides. Indeed, we found that purified YciV acts as a 5'-3' exoribonuclease that can act on short nucleic acid substrates. Yet, YciV is found in the genomes of many γ -proteobacteria, including *E. coli*. So, if YciV contributes to the survival of the *P. aeruginosa* Δorn strain, why doesn't it assist survival of an *E. coli* Δorn strain as well? Through a combination of biochemical studies, structural characterization, and bioinformatic analysis, we found that *P. aeruginosa* YciV has unique residues in its catalytic site that specifically correlate with increased cleavage of diribonucleotide substrates. Since the only suppressor of Δorn is *yciV*, these results suggest that diribonucleotides are biologically active molecules that have adverse effects if they accumulate in the cell. Additional proteomic studies show that cells have low levels of Orn and YciV and suggest that diribonucleotides are recognized in the cell as a mechanism to monitor the RNA degradation process.

RESULTS

A transposon mutagenesis screen of suppressor mutants for the restoration of colony size

To identify other genes involved in the small colony variant phenotype observed in the Δorn strain, a suppressor screen was carried out using transposon mutagenesis to identify mutants that would restore Δorn colony size to that of the wild type. A pBT20 plasmid containing a mini-mariner-based transposon was introduced into *P. aeruginosa* PA14 Δorn (Kulasekara et al., 2005). Screening approximately 40,000 colonies identified ~200 that fully or partially restored the Δorn colony size to that of the wild type. A second screen of these initial mutants yielded four insertions that reproducibly restored colony size. When the transposon from each strain was cloned and sequenced, they mapped to the same genomic region, including one insertion in *PA14_22770*, one insertion in *PA14_22780*, and two insertions in the intergenic region between *PA14_22800* and *PA14_22820* (**Fig. 1**). To determine the level of suppression, the suppressor strains were plated on LB agar plates to determine colony size. They were also grown overnight in liquid culture to assess aggregation, as it was previously shown that *P. aeruginosa* Δorn is prone to aggregation in liquid medium. All four suppressor strains restored the colony size of Δorn to the parental PA14 colony size. Similarly, the four transposon insertion mutations suppressed the aggregation phenotype. To assess whether the insertion of the transposons into the particular locations disrupted the expression of *PA14_22770*, *PA14_2280*, and *PA14_22800*, in-frame deletion mutations of each gene were generated in combination with Δorn . Surprisingly, in-frame deletion of these genes in the Δorn background failed to recapitulate the suppression of the small colony size and aggregation observed for transposon insertions (**Fig. 1, Sup 1**). These findings indicated that the genes disrupted by the transposon insertion were not responsible for the observed suppression.

Genomic fragment overlapping with genes disrupted by transposons restores colony size of Δorn to PA14 wild-type

We reasoned that a complementation approach could be used to identify the genes responsible for suppression of the Δorn growth phenotype. Genomic libraries were prepared for the Δorn transposon suppressor strains by fragmenting their genomic DNA with a limited *AluI* digest and cloning these fragments into a plasmid. The genomic libraries were then conjugated into Δorn and tested for colony size restoration. We screened approximately 20,000 colonies and identified hundreds of colonies with increased colony size (**Fig. 2, Sup 2**). A secondary screen was conducted to identify strains that grew like wild-type PA14 in liquid medium. This approach allowed the identification of suppressors in each genomic library (**Fig. 2 sup 2B**, red box). Sequencing of these suppressor plasmids revealed a common genomic region shared among all suppressed strains (**Fig. 2A**). The shared gDNA region containing the full

fragment (*yciI*, intergenic region, *yciB*, *PA14_22820*, and *PA14_22830*) was cloned and tested for suppression of Δorn . This common fragment suppresses the small colony phenotype of Δorn (**Fig. 2B**, FL). To determine which genes were required to mediate suppression, several subfragments were generated: F1 (intergenic region with *yciB*), F2 (intergenic region with *PA14_22820* and *PA14_22830*), and F3 (intergenic region). Only F2 restored normal colony size and growth to that of the wild type, whereas F1 and F3 failed to complement the Δorn phenotype (**Fig. 2B**), suggesting that *PA14_22820* is the gene responsible for complementation. We hypothesized that the restoration of colony size and growth might be due to the degradation of accumulated dinucleotides by Δorn complemented by FL and F2. To test this hypothesis, lysates were generated from *P. aeruginosa* strains, including Δorn complemented with the vector control, FL, F1, F2, and F3. Each lysate was mixed with 5'-³²P-radiolabeled pGG or pAAAAAGG and aliquots were taken at the indicated time intervals (**Fig. 2C**). FL and F2 in Δorn lysates were able to degrade pGpG into GMP, whereas F1 and F3 failed to complete the degradation of pGpG (**Fig. 2C**, top). Furthermore, 5'-³²P-AAAAAGG was degraded into mononucleotides by FL and F2 in Δorn lysates, whereas lysates of F1 and F3 in Δorn accumulated intermediate dinucleotide (**Fig. 2C**, bottom). Taken together, these results show that the expression of *PA14_22820* leads to the degradation of pGG and pAA dinucleotides in Δorn lysates and the restoration of Δorn growth.

The catalytic activity of PA14_22820 (yciV) is required for suppression of Δorn phenotype and degradation of dinucleotides

The F2 fragments consist of an intergenic region, full-length *PA14_22820*, and partial *PA14_22830*. We investigated whether *PA14_22820* alone was sufficient to complement the small colony morphology phenotype of Δorn . Complementation of Δorn with *PA14_22820* under an IPTG-inducible plasmid restored normal colony sizes in contrast to the vector control (**Fig. 3A**). The lysates in Δorn complemented with *yciV* were capable of cleaving pGG into GMP, similar to what was observed for the F2 activity (**Fig. 3B** and **3C**). *PA14_22820* encodes a YciV protein containing a PHP domain. To understand the features of YciV that enable the cleavage of diribonucleotides, YciV_{Pa} was aligned to the YciV sequence from *C. violaceum*, *E. coli*, *V. cholerae*, *S. enterica*, and *Y. pseudotuberculosis* (**Fig. 3 Supplement 1**). We identified multiple residues near the AMP-binding site (H38, D39, E63, H194, and H253) and phosphate-binding site (R101) in the *C. violaceum* structure (Ghodge and Raushel, 2015) and introduced alanine substitution mutations into the corresponding residue in YciV_{Pa}. To determine the activity of these mutant alleles, we tested their ability to increase the colony size of Δorn , an effect indicative of diribonuclease activity. While *yciV*^{WT} suppressed Δorn with an average colony size of 0.8 mm², the mutant alleles of *yciV* failed to restore colony size (**Fig. 3A**). When lysates from Δorn expressing these variants were tested for their ability to degrade pGG into GMP, none of the alanine-

substitution variants had activity comparable to that of wild-type *yciV* (**Fig. 3B** and **3C**). Taken together, YciV suppression of the colony size of Δorn strains requires cleavage of dinucleotides.

RNase AM_{Pa} is a 5' to 3' oligoribonuclease

YciV was renamed RNase AM as an enzyme that cleaves ribo- and deoxyribo-oligonucleotides from the 5' to 3' direction with enhanced activity toward 3',5'-pAp and less activity toward longer substrates in XXX (Ghodge and Raushel, 2015). To determine whether RNase AM_{Pa} alone can cleave dinucleotides, RNase AM_{Pa} protein was purified and tested for activity against 2 aminopurine (2AP)-containing oligonucleotide substrates. 2AP released from oligonucleotides exhibits enhanced fluorescence (Jean and Hall, 2001). Purified RNase AM_{Pa} was able to cleave pAp(2AP), but all variants with active-site alanine substitutions failed to cleave the same substrate, supporting our findings with radiolabeled substrates (**Fig. 3D**). To determine whether RNase AM_{Pa} also cleaves from the 5' end, we utilized two substrates that were four nucleotides long with a non-hydrolyzable phosphothioate linkage between the second and third nucleotides. For one substrate, the 2AP is first base at the 5' end and allows detection of 5' cleavage of the substrate. For the other substrate, 2AP is the last base at the 3' end and allows the detection of 3' cleavage of the substrate. Purified RNase AM_{Pa} was only able to release 2AP from the 5' end but not the 3' end (**Fig. 3E**). These results indicate that RNase AM_{Pa} cleaves short oligoribonucleotides of various lengths from the 5' end.

SCV and dinucleotide degradation activity are partially restored by RNase AM orthologs.

Orn is essential in many γ -proteobacteria, but not in *P. aeruginosa*, which has a small colony morphology. We asked if the different requirement for *orn* is due to the other γ -proteobacteria not encoding *rnm*. Notably, RNase AM orthologs are present in *E. coli*, *V. cholerae*, and *Y. pestis*, for all of which *orn* has been reported to be essential (Ghosh and Deutscher, 1999; Kamp et al., 2013; Palace et al., 2014). To determine whether the reason for *orn* essentiality is due to species-specific differences in the function or activity of these enzymes, RNase AM orthologs were expressed in *P. aeruginosa* Δorn and assessed for restoration of normal growth and colony morphology. Surprisingly, all RNase AM orthologs, including RNase AM_{Ec}, RNase AM_{Vc}, RNase AM_{St}, and RNase AM_{Yp}, were able to suppress small-colony morphology in a similar manner as RNase AM_{Pa} (**Fig. 4A**).

When the purified proteins were tested for the degradation of dinucleotides, only RNase AM_{Pa} was able to effectively cleave the pAp(2AP) dinucleotide, whereas the orthologs from other γ -proteobacteria had little activity (**Fig. 4B**). To understand the activity of

RNase AM in cells, we tested lysates from Δorn cells expressing each of the RNase AM orthologs for the degradation of ^{32}P -labeled GG and AAAAAGG. While the expression of RNase AM_{Pa} in Δorn allowed complete cleavage of pGpG into GMP within 5 min, other RNase AM orthologs showed slower degradation over time: RNase AM_{Ec} and RNase AM_{Vc} required 30 min to degrade pGpG, whereas RNase AM_{St} and RNase AM_{Ye} were unable to cleave all of the pGpG within 30 min (**Fig. 4C**). For the 7-mer substrate, *P. aeruginosa* Δorn has been shown to accumulate 5' dinucleotides, indicating that other 3' exoribonucleases can degrade RNA to dinucleotides (**Fig. 2**) (Kim et al., 2019). The expression of RNase AM_{Pa} allowed the completion of the degradation of the accumulated diribonucleotide to mononucleotide. When RNase AM from *E. coli*, *V. cholerae*, *S. enterica*, and *Y. pseudotuberculosis* were overexpressed, less than 50% of the dinucleotide is cleaved to mononucleotide. To better understand whether there were differences in substrate preference for these different orthologs of RNase AM, the lysates of Δorn expressing each of the *yciV* orthologs were tested for activity against all 16 dinucleotides. Previously, Orn has been reported to degrade all 16 dinucleotides (Kim et al., 2019). The lysates of Δorn expressing RNase AM_{Pa} were able to degrade >50% of dinucleotides, except AA, CC, UU, UA, and UC (**Fig. 4 sup 1**). In contrast, lysates of Δorn expressing RNase AM_{Ec}, RNase AM_{Vc}, RNase AM_{St}, and RNase AM_{Yp} proficiently processed a subset of dinucleotides, whereas the remaining dinucleotides were poor substrates. The dinucleotide substrates included AG and GU by RNase AM_{Ec}; AG, AU, GG, GC, and GU by RNase AM_{Vc}; GG and GC by RNase AM_{Se}; and GG by RNase AM_{Yp}. These results indicate that RNase AM_{Pa} has a narrower substrate spectrum than Orn. However, orthologs from other γ -proteobacteria have even lower activity and more restricted substrate profiles, distinguishing themselves from RNase AM_{Pa}.

Unique residues of RNase AM_{Pa} located near the active site of the enzyme

These results led us to investigate whether there are unique features of RNase AM_{Pa} that differ from orthologs found in other γ -proteobacteria. Phylogenetic analysis of RNase AM orthologs identified 1,621 RNase AM sequences between 200 and 400 amino acids. Several groups were identified when clustered by relatedness, including a group consisting of *Pseudomonadales* (yellow), *Enterobacterales* (purple), and *Burkholderiales* (green) (**Fig. 5A**). A comparison of the logo generated for sequences near the catalytic site for each group revealed key residues that were highly conserved, and two residues that distinguished the groups (**Fig. 5B**). In particular, RNase AM from *Pseudomonadales* has two key residues, P¹³⁵ and D¹⁶⁵, which differ from the corresponding residues in *Burkholderiales* and *Enterobacterales*.

Next, we sought structural insights into the substrate specificity of RNase AM orthologs. Although RNase AM_{Pa} has resisted crystallization to date, we were able to determine the

crystal structure of full-length RNase AM from *V. cholerae*, RNase AM_{Vc}, at a maximum resolution of 1.5 Å (**Fig. 5C**). The electron density resolved the entire sequence from residues 1 to 279, with the last 11 residues of the protein being disordered. RNase AM was incubated with excess pGpG prior to crystallization, yet no nucleotide density was discernible at the active site. However, the active site contained three divalent cations (Mg²⁺ or Mn²⁺), which are coordinated by strictly conserved residues, and two sulfate ions from the crystallization buffer (**Fig. 5C**, insets). One of the sulfate ions is coordinated by the metal ions instead of the protein, likely mimicking the scissile phosphodiester of a substrate. The presence of divalent cations, which are crucial for catalytic activity, and solvent molecules, which can compete for substrate binding, may explain the lack of bound substrates in the crystals. A comparison with known experimentally determined structures using a Foldseek search identified *E. coli* (PDB 7ug9, (Sharma et al., 2022)) and *C. violaceum* (PDB 2yb1, (Cummings et al., 2014)) RNase AM as close structural orthologs, despite only 43% and 40% sequence identity, respectively (van Kempen et al., 2024). A phosphoesterase from *Bifidobacterium adolescentis* also shows a high degree of structural homology, but its sequence identity is less than 30% (PDB 3e0F, (Han et al., 2011)). While the structure of RNase AM_{Ec} represents the closest ortholog of RNase AM_{Vc} with known structure and the two structures align with a root mean square deviation (rmsd) of 0.6 Å over 190 Cα positions, the insertion lobe that contains the residues distinguishing the different groups of RNase AM sequences was not resolved in the structure of the *E. coli* protein. The structures of RNase AM_{Cv} and *B. adolescentis* phosphoesterase resolve this region and align to RNase AM_{Vc} with rmsd values of 1.2 Å and 1.8 Å, respectively, but belong to distinct phylogenetic orders.

Since there are no reported crystal structures of RNase AM with bound substrates, we resorted to docking predictions of pGG against the crystal structure of RNase AM_{Vc} employing the DiffDock method (Corso et al., 2024; Corso et al., 2023). In addition to a moderate confidence score (-0.83 for the top-ranking model), the solution places the phosphate groups of the dinucleotide, where two sulfate ions are resolved in the crystal structure (**Fig. 5D**). Consequently, the scissile phosphodiester is in close proximity to the three metal ions at the active site of RNase AM. Furthermore, the 5' phosphate is in a position comparable to that of AMP in the crystal structure of RNase AM_{Cv} (Cummings et al., 2014). The residue F¹⁵⁴ of RNase AM_{Cv}, which is strictly conserved in the RNase AM family, forms a π -stacking interaction with the AMP base. Although the corresponding residue of RNase AM_{Vc} exhibits the same rotamer, the bases of the docked pGG do not overlap with AMP (**Fig. 5D**, inset). Instead, the bases appear splayed. This conformation may allow residue F¹⁵⁴ to function as a wedge when inserted between the two bases of the dinucleotide substrate. Such a role would be similar to the leucine wedge observed in Orn and NrnC, and could be relevant for the specific activity

of these enzymes against dinucleotides (Kim et al., 2019; Lormand et al., 2021). The 3' side of the docked pGG faces the periphery of the active-site groove, suggesting that there is no strict length preference for 5'-3' RNase activity.

Mapping the positions of the two residues that are conserved in *Pseudomonadales* RNase AM sequences but differ in all other groups, P¹³⁵ and D¹⁶⁵, onto the structure of RNase AM_{vc} illustrated their proximity to the active site of the enzyme (**Fig. 5C** and **5D**). In addition, we modeled the structure of RNase AM_{pa} by using AF2 and ColabFold (Jumper et al., 2021; Mirdita et al., 2022) (**Fig. 5E**). The high-confidence model predicted a short insertion in the active site-proximal lobe and a C-terminal helix in the *P. aeruginosa* model, both of which are missing from the corresponding structure of the *V. cholerae* homolog. The RNase AM_{pa} model superimposes well with the crystal structure of RNase AM_{vc}, aligning with an average rmsd of 1.1 Å. However, closer inspection of the superposition revealed higher rmsd values for residues in the aforementioned lobe than for those of the domain containing the catalytic core of the enzyme (**Fig. 5E**). The *Pseudomonadales* signature residues are part of this lobe. It is conceivable that the unique features of this group of RNase AM enzymes collectively or individually contribute to the shifted higher activity of RNase AM_{pa} towards dinucleotides.

Low levels of Orn and RNase AM in *P. aeruginosa* suggest that diribonuclease activity is limited

Since *P. aeruginosa* encodes both *orn* and *yciV*, it is unclear why the Δorn mutant has a phenotype when the *yciV* gene is present in the genome. To better understand this, proteomic analysis was performed on the PA14 vector control, Δorn vector control, Δorn pMMB-F2, Δorn pMMB-*orn*_{pa}, and Δorn pMMB-*yciV*_{pa}. Global protein expression profiles were investigated using euclidian-based clustering at the sample and individual protein levels (**Fig. 6A**). At the strain level, individual biological replicates grouped well into their individual strains. The Δorn and PA14 have distinctive proteomes clustered into two groups: proteins increasing in abundance and proteins decreasing in abundance upon *orn* knockout. Upon complementation with *orn*, the proteome was restored to wild-type levels. Conversely, complementation with either the F2 fragment or *yciV*_{pa}, while restoring colony size and dinucleotide cleavage, did not restore global proteome levels. PCA analysis of these samples showed that Δorn had a very different proteome from PA14 or Δorn complemented with *orn*_{pa} (**Fig. 6B**). In contrast, the expression of the F2 fragment or *yciV*_{pa}, while restoring colony size and cleavage of dinucleotides, yielded a distinct proteome from either PA14 or Δorn (**Fig. 6B**). Within the proteomic data, the protein level of Orn in PA14 was detectable compared to that in Δorn , but Orn appeared to be expressed at a very low level in the wild-type strain (**Fig. 6C**). Complementation with *orn* on a plasmid greatly increased Orn protein levels, which corresponds to the

increased diribonuclease activity reported previously (Kim et al., 2019). The expression of F2 and *yciV_{Pa}* in a Δorn genetic background did not affect Orn protein levels, which remained undetectable. Protein expression of RNase AM_{Pa} in wild-type PA14 was undetectable but increased in Δorn (**Fig. 6D**). Complementation with *orn* did not alter RNase AM_{Pa} as compared to Δorn with the vector control. In contrast, F2 and *yciV* expression significantly increased RNase AM_{Pa}. The increased protein levels of RNase AM_{Pa} in Δorn likely explain why Δorn has a phenotype but is viable, since the amount of RNase AM_{Pa} is not high enough to fully complement the activity of Orn, but sufficient for the cells to survive the accumulation of dinucleotides.

Dinuclease activity is the basis for *orn* essentiality in *E. coli* and *P. aeruginosa*

To reconcile the difference in genetic essentiality of *orn* in *E. coli* and *P. aeruginosa*, two sets of genetic experiments were performed to determine whether the *orn* gene can be deleted from the genome. In the first experiment, *E. coli* TW6 was transformed with an empty pMMB vector and tested for deletion of *orn* by lambda red recombinase (Datsenko and Wanner, 2000; Murphy et al., 2000; Yu et al., 2000). When TW6 pMMB was transformed with a PCR product consisting of the chloramphenicol resistance (Cm^R) gene flanked by 50 bp of sequences upstream and downstream of the *E. coli orn* gene, only three Cm^R colonies arose (**Fig. 7A**). When tested by PCR, all three Cm^R colonies had the wild-type *orn* gene, suggesting that the resistance marker was integrated elsewhere in the genome. When TW6 pMMB-*orn_{PA}* was transformed with the same *orn* deletion fragment, 49 Cm^R colonies appeared. PCR testing of 16 of these colonies chosen at random revealed that 13 of them had the *orn_{Ec}* gene deleted from the chromosome (**Fig. 7A**). When TW6 pMMB-F2 was transformed with the same *orn* deletion fragment, over 100 colonies appeared. PCR testing of 16 of these colonies chosen at random revealed that 12 of them had the *orn_{Ec}* gene deleted from the chromosome (**Fig. 7A**). These results suggest the essentiality of *orn* in *E. coli* is due to diribonuclease activity of Orn and this activity can be replaced by RNase AM_{Pa}. In the second experiment, we generated a co-integrant by selecting on gentamicin for the integration of plasmid with an *orn* deletion construct flanked by 1 kilobase upstream and downstream of *orn* and a gentamicin resistance (Orr et al 2015). When grown in the absence of selection, the integrated plasmid can be recombined out to yield either the wild-type or Δorn mutant. After counter-selection against the co-integrant, we assessed the frequency in which the Δorn arose after resolution of the co-integrant. PCR analysis of 90 colonies resolved from the PA14 co-integrant showed that 30% were Δorn and 70% returned to wild type. When 103 resolved colonies from a PA14 $\Delta yciV$ co-integrant was assessed, no Δorn was detected (**Fig. 7B**). Ninety resolved colonies from co-integrant in PA14 $\Delta yciV$ pMMB-*orn_{PA}* yielded 40% Δorn and 60% wild-type (**Fig. 7B**). Lastly, co-integrant resolved from PA14 $\Delta yciV$ with pMMB-F2 yielded 15% Δorn gene (**Fig. 7B**). Together, these data show that both *E. coli* and *P. aeruginosa* require

diribonuclease activity for viability, with *P. aeruginosa* genomes encoding a second enzyme supporting this activity. These results also indicate that the removal of diribonucleotides is an essential process.

DISCUSSION

Orn was initially identified as an oligoribonuclease (Datta and Niyogi, 1975; Niyogi and Datta, 1975; Stevens and Niyogi, 1967) that is essential in *E. coli* (Ghosh and Deutscher, 1999). The historical results suggest that the inability to cleave a large array of oligoribonucleotides would result in the depletion of mononucleotides and leave the cell unable to grow. More recently, studies have shown that Orn is the primary enzyme that degrades pGpG to GMP to complete the c-di-GMP signaling cycle (Cohen et al., 2015; Orr et al., 2015). Subsequent investigations into why Orn cleaves pGpG revealed that it selectively cleaves dinucleotides over longer oligonucleotides (Kim et al., 2019). The co-crystal structure revealed that diribonucleotides fit snugly into the catalytic site of Orn. Orn has a 5' phosphate cap and an activation loop that restrict the length of substrate that can enter (Kim et al., 2019). While these studies clearly revealed that Orn is a diribonuclease within a cell, they raised questions as to why Orn would be essential, as most of the degraded RNA would be released as mononucleotides by other 3' to 5' exoribonucleases and only a small fraction of nucleotides would be sequestered as diribonucleotides (**Fig. 8**). Hence, it is unlikely that depletion of the mononucleotide pool is the reason for the essentiality of *orn*.

To better understand the basis of Orn essentiality, we took advantage of *P. aeruginosa*, an organism in which *orn* mutants were viable, despite having a small-colony phenotype. Through genetic experiments, we identified RNase AM_{Pa} (previously YciV) as a gene product that can act as a diribonuclease and restore the degradation of diribonucleotides to the Δorn strain. To our surprise, this gene was not unique to *P. aeruginosa*, but was also present in other γ -proteobacteria in which *orn* was essential (Ghodge and Raushel, 2015). Phylogenetic analysis revealed that *P. aeruginosa* RNase AM is part of a clade with unique sequences in the catalytic site, which maps to the 5' base of the dinucleotide substrate. When assessed for activity, RNase AM_{Pa} was far more active than RNase AM from other γ -proteobacteria. The introduction of RNase AM_{Pa} into *E. coli* permitted the deletion of the *orn* gene from the chromosome. Similarly, removal of the *yciV* gene from *P. aeruginosa* makes the *orn* gene essential.

These results show that a minimal level of diribonuclease activity is required in γ -proteobacteria. The diribonuclease activity can be provided by Orn, which is an efficient 3' to 5' diribonuclease, or by RNase AM_{Pa}, which is a less efficient 5' to 3' oligoribonuclease. Based on the in vitro and ex vivo results presented here and those reported earlier, Orn is a far more efficient enzyme (Kim et al., 2019). RNase AM_{Pa} is more active than RNase AM from other γ -proteobacteria. Despite these differences in activity of RNase AM, overexpression of RNase AM from any γ -proteobacteria restored the normal colony size of Δorn , with higher expression levels likely compensating for the

differences in activity profiles. These results provide further support for the existence of a threshold concentration of diribonucleotides in the cell that causes a small colony morphology, but not sufficiently high to cause toxicity. In addition, RNase AM_{Pa} is able to suppress the essentiality of *orn* in both *P. aeruginosa* and *E. coli*. Because these two enzymes are different in protein sequence, domain structure, and activity (strict diribonuclease vs a 5' oligoribonuclease), the basis for essentiality must be a requirement for diribonuclease activity.

The essentiality of *orn* has been previously explained by three scenarios (Ghosh and Deutscher, 1999). First, the absence of *orn* leads to the accumulation of oligoribonucleotides and the consequent depletion of mononucleotides. Second, oligoribonucleotides inhibit enzymes involved in essential metabolic processes. Third, Orn has additional moonlighting functions that are essential. Our findings rule out the third scenario, as diribonuclease activity rather than the Orn protein itself is required for cell viability. The essentiality of diribonuclease activity can be attributed to either depletion of monoribonucleotides or accumulation of diribonucleotides. Because the majority of RNA polymers (rRNA, tRNA, and mRNA) are degraded directly into monoribonucleotides (**Fig. 8**) and growth on rich media should, in addition, enable de novo synthesis of nucleotides, depletion of mononucleotides is an unlikely mechanism for the essentiality of diribonucleases. This leaves the intriguing possibility that accumulated dinucleotides are biologically active molecules that negatively affect γ -proteobacteria. Data for RNase AM_{Pa} suggest that it has stronger substrate preference but less activity than Orn (**Fig. 5 supplement 1**), suggesting that only a subset of diribonucleotides is causing toxicity. Future work should identify the specific diribonucleotide(s) and the mechanisms that inhibit cell growth. Similar requirement for diribonuclease has been observed for NrnC (Lormand et al., 2021) in α -proteobacteria including *Caulobacter crescentus*, *Bartonella henselae*, and *Brucella abortus* (Christen et al., 2011; Liu et al., 2012; Sternon et al., 2018) suggesting that elevated dinucleotides levels are detected in many organism. Accumulation of diribonucleotides would thus serve as a signal to the cell that there is a defect in RNA degradation and recycling. In *P. aeruginosa*, the ability of the Δorn mutant to increase expression of RNase AM_{Pa} suggest that this may serve as a quality control mechanism to reduce diribonucleotide levels. Since orthologs of *orn* are present in eukaryotes from yeast to plants to humans (Zhang et al., 1998) and appears to be required for mitochondrial function (Bruni et al., 2013), this quality control mechanism for RNA degradation may be a broadly conserved process.

FIGURE LEGENDS

Figure 1. Transposon suppressor screen for restoration of Δorn colony size identified mutants with insertions in CpxR, YciI, and YciB. **A.** map of the transposon insertions. **B.** photograph of plates showing in colony size of transposon suppressor mutant compared to PA14 and PA14 Δorn **C.** photograph of tubes containing overnight culture of transposon mutant, PA14, and PA14 Δorn for visualization of bacterial aggregates.

Figure 2. complementation of suppressor in Δorn restores colony size and hydrolysis activity to wild-type level. **A.** The genomic region surrounding the transposon insertion sites. **B.** Photograph of colony size expressing indicated gene fragments in Δorn . Bacterial cultures were diluted and dripped on LB agar plate containing carbenicillin for 30 hours. Quantification of colony sizes are shown plot. **C.** Degradation of AAAAAGG or GG by Δorn complemented with suppressor fragments. ^{32}P -GG (top, 1 μ M total) and ^{32}P -AAAAAGG (bottom, 1 μ M total) by whole cell lysates of Δorn complemented with indicated gene fragments. Samples were stopped at the indicated time (min) and analyzed by 20 % denaturing PAGE.

Figure 3. Catalytic active-site mutation of yciV are required for diribonuclease activity.

A. Photograph of PA14 Δorn expressing yciV or alanine substitution alleles were grown on LB-agar plate containing 1 mM IPTG for 30 hours. Graph shows colonies size as measured by Fuji. **B.** Degradation of ^{32}P -pGpG (total 1 μ M) by whole cell lysates PA14 Δorn complemented with yciV or variants at indicated alanine substitutions was assessed. Samples are stopped at the indicated times (min) and analyzed by 20 % denaturing PAGE. All data shown represent the average of triplicate independent experiments. **C.** The graph shows the quantification of triplicate data by the amount of remaining pGpG in **B.** **D.** Cleavage of pAp(2AP) by purified YciV or YciV with indicated alanine substitution. **E.** Cleavage of (2AP)ApsGG and AApsG(2AP) by purified YciV shows that YciV is a 5' to 3' exonuclease.

Figure 4. RNase AM homologues from different γ -proteobacteria has reduced diribonuclease activity.

A. Photograph of PA14 Δorn expressing yciV from indicated γ -proteobacteria were grown on LB-agar plate containing 1 mM IPTG for 30 hours. Graph shows colonies size as measured by Fuji. **B.** Cleavage of pAp(2AP) by purified RNase AM orthologs from γ -proteobacteria. **C.** Degradation of ^{32}P -GG (top) or ^{32}P -AAAAAGG by whole cell lysates PA14 Δorn expressing yciV from indicated γ -proteobacteria was assessed. Samples are

stopped at the indicated times (min) and analyzed by 20 % denaturing PAGE. All data shown represent the average of triplicate independent experiments.

Figure 5. RNase AM structure and key sequences.

A. Phylogenetic tree and key sequence conservation of RNase AM proteins. Unrooted phylogenetic tree of RNase AM sequences with sequences of interest highlighted by stars on branch tips. Only one star is shown to represent both RNase AM from *Salmonella typhimurium* (RNase AM_{St}) and *Escherichia coli* (RNase AM_{Ec}), which are closely related and overlap on the tree. The branch tips are colored by taxonomic order for the organism for each RNase AM sequence with the color key shown to the right. The major clades are labeled by the most representative taxonomic order. Only the top 10 most populated taxonomic orders and top 18 taxonomic families are shown for clarity, all others are gray. **B.** Sequence logo for major clades in the RNase AM phylogeny. Each logo shows information entropy of important sequence motifs. The residue numbering for *Pseudomonas aeruginosa* on top of sequences. Stars highlight conservation in the *Pseudomonadales* clade that is not conserved in *Enterobacterales* or *Burkholderiales*. **C. Crystal structure of RNase AM_{Vc}.** The cartoon shows the structure of *V. cholerae* RNase AM bound to divalent metal ions and two sulfate molecules (shown here as spheres) at the active site. Close-up views highlight key residues that interact with the ligands. The conserved sequence features shown in (B) are labeled and colored in blue or purple for the RNase AM family and *Pseudomonadales* group, respectively. **D. Predicted dinucleotide-binding site and pose.** Computational docking of pGG to the crystal structure of RNase AM_{Vc} resulted in a top-ranked solution in which the dinucleotide was placed at the active site of the enzyme. Ligands from the crystallization are shown as transparent spheres. The inset shows a close-up view of the docking solution and crystallographic ligands, in addition to AMP from the crystal structure of RNase AM_{Cv} (PDB 2yb1). The side chain of residue F¹⁵⁴ in RNase AM_{Vc} is shown as sticks. **E. Structural model of RNase AM_{Pa}.** The AF2 model of *P. aeruginosa* RNase AM was aligned with the crystal structure of RNase AM_{Vc}. The model is shown as a cartoon putty, with the putty thickness and color gradient indicating the rmsd between corresponding residues. An insertion and a predicted C-terminal helix in RNase AM_{Pa} are shown and labeled in light green. The positions of *Pseudomonadales*-specific sequence changes are highlighted as black spheres representing their corresponding C α positions.

Figure 6. Proteomic analysis of *P. aeruginosa* Δ orn and complemented strains.

A. Normalized protein abundances were of each sample (x-axis) and each protein (y-axis) were clustered by euclidean distance with a complete linkage method. The clustering resulted in grouping of samples relative to strain under two discriminate sub trees. **B.** A principal component analysis of the normalized protein abundances data. **C.** A bar graph representing the calculated intensity of Orn_{Pa} in each strain. **D.** A bar graph representing the calculated intensity of RNase AM_{Pa} in each strain.

Figure 7. Diribonuclease activity is required in γ -proteobacteria.

A. PCR product to delete *orn_{Ec}* is introduced into *E. coli* via lambda red recombination yielding the indicated number of Cm^R colonies. PCR testing determine the number of colonies that *orn* is substituted with the chloramphenicol acetyl transferase gene. **B.** *P. aeruginosa* harboring a co-integrant for the Δorn deletion was resolved by growth in the absence of selection followed by counterselection on sucrose. Colonies were assessed by PCR to determine the number of colonies that reverted to wild-type or deleted the *orn* gene.

Figure 8. Model for flux of ribonucleotides.

RNA distribution in growing *P. aeruginosa* cells. Amount of nucleotides (nts) is based on an estimate of ~10,000 ribosomes in a cell. Each ribosome has ~4,400 nts for a total of $\sim 4.4 \times 10^7$ nts. Each of the mononucleotides (adenine, guanine, cytosine, and uracil) includes, mono-, di-, and tri-phosphate version and estimated at ~1 mM for each of the four bases. In this estimate, there would be $\sim 4 \times 10^6$ nts in the cell as mononucleotides. For diribonucleotides, a prior estimate of pGpG shows that there is ~10 μ M in the cell. Assuming all of the linear dinucleotides are present at similar concentrations, then there would ~160 μ M or $\sim 1.6 \times 10^5$ nts per cell. In Δorn mutants, the concentration of pGpG increases to ~70 μ M or $\sim 1.12 \times 10^6$ nts per cell.

Figure 1 Supplemented 1. Colony size and aggregation assay of suppressor mutant in Δorn . **A.** PA14 Δorn with indicated gene deletion LB-plate showing small colonies compared to PA14 wild-type **B.** Auto-aggregation assay of overnight cultures of PA14, Δorn , $\Delta orn \Delta yciL$, $\Delta orn \Delta fleQ$, $\Delta orn \Delta cpxR$, and $\Delta orn \Delta 22770$ mutants.

Figure 2 Supplemented 1. Whole-genome complementation screen identifies the restoration of Δorn growth defect. PA14 Δorn was transformed using donor gDNA from PA14 Δorn , PA14_27420::tn, PA14 *yciL*::tn, and PA14 *fleQ*::tn. Genomic DNA libraries from transposon suppressor strains are enzymatically digested using AluI restriction enzyme. The shared region is indicated in Figure 2A (grey). **A.** Photographs of LB agar plates containing *fleQ*::tn library showing different colony sizes compared to pMMB. **B.** Growth phenotype of overnight culture using LB-agar plate colonies of *fleQ*::tn library.

Figure 4 Supplemented 1. Protein sequence alignment of RNase AM from *P. aeruginosa*, *E. coli*, *Salmonella enterica*, *Yersinia pseudotuberculosis*, *Vibrio cholerae*, and *Chromobacterium violaceum*.

Figure 5 Supplemented 1. Cleavage of diribonucleotides by RNase AM from *P. aeruginosa*, *E. coli*, *Vibrio cholerae*, *Salmonella enterica*, *Yersinia pseudotuberculosis*, and Orn from *P. aeruginosa*. Each of the diribonucleotide is labeled at the 5' end with γ -³²P-ATP and assayed for cleavage in extracts of *P. aeruginosa* Δorn expressing the indicated *yciV* ortholog for 30 minutes. The reaction is stop, separated on 20% denaturing PAGE, and analyzed for cleavage compared to uncleaved controls. These reactions were conducted duplicated.

Figure 7 Supplemented 1. Data for PCR analysis of *E. coli* and *P. aeruginosa* *orn* mutants. PCR primers in supplemental Table 1 is used to perform colony PCR on *E. coli* Cm^R colonies or *P. aeruginosa* sucrose resistant colonies. PCR products were separated on 1% TAE agarose gel and visualized by ethidium bromide staining. The expected product size for the endogenous *orn* gene in *E. coli* is ~0.6 kb and the expected size with the *cat* gene replacement is ~1.2 kb. The expected product size for the wild-type allele of *orn* is ~1.7 kb and for the *orn* deletion is ~1.1 kb.

MATERIALS AND METHODS

Strains, plasmid, and growth condition.

Strains and plasmids used are listed in Table 1 and 2. *P. aeruginosa* and *E. coli* were grown in lysogeny broth (LB) at 37 °C. Plasmids are maintained using antibiotics at 15 µg/mL gentamicin, 50 µg/mL carbenicillin, and 50 µg/mL kanamycin, where appropriate. In-frame deletion mutants of *P. aeruginosa* were generated by conjugation of a non-replicative plasmid containing 1 kilobase upstream and downstream of the gene to be deleted. *P. aeruginosa* co-integrants were selected using 75 µg/mL gentamicin and 25 µg/mL irgasan. The plasmid was counter-selected by plating on 6% sucrose. In-frame deletion mutants were screened by PCR.

Transposon mutagenesis screen

The pBT20 mariner transposon from *E. coli* SM10 λpir was introduced into *P. aeruginosa* PA14 Δ*orn* by conjugation (Kulasekara et al., 2005). The bacteria were mixed and 50 µL were spotted onto a pre-dried and prewarmed LB agar plate. The spots were allowed to fully absorb and dry for ~ 10 mins and incubated for an additional 90 min at 37 °C. Each spot was swabbed and resuspended in 1 mL LB. Two hundred microliters of each resuspension were plated onto an LB agar plate containing 75 µg/mL gentamicin and 25 µg/mL irgasan, yielding ~ 100 - 150 colonies per plate. A total of 250 plates were generated, yielding ~40,000 colonies. After overnight incubation at 37 °C, colonies were visually screened for increased colony size. Colonies of interest were picked, restreaked for single colonies for validation of colony size changes, and saved for sequencing.

Sequencing of transposon mutants

Genomic DNA was isolated using ArchivePure DNA Cell/Tissue Kit (5 PRIME) according to manufacturer's instructions. Purified genomic DNA was digested using the blunt cutting restriction enzymes *Stu*I and *Msc*I according to manufacturer's instructions (New England Biolabs). The digested genomic DNA was cloned into *Stu*I linearized pVL-blunt and plated on an LB agar plate containing 50 µg/mL kanamycin and 15 µg/mL gentamicin. Resulting colonies were isolated and miniprep using Wizard Plus SV Miniprep DNA Purification Kit (Promega) and sequenced using the transposon specific (and/or plasmid specific) primers.

Whole genome complementation

The *P. aeruginosa* PA14 Δ*orn*, PA14 Δ*orn* *ycil::tn*, PA14 Δ*orn* *fleQ::tn*, and PA14 Δ*orn* *PA14_27420::tn* strains were grown on LB agar plate containing 15 µg/ml gentamicin, then inoculated into LB supplemented with 15 µg/ml gentamicin at 37 °C with shaking overnight. Genomic DNA was extracted from cultures using Wizard gDNA preparation kit (Promega). Extracted gDNA was digested for 1 hour using serial diluted amounts of *Alu*I to generate blunt fragments. The digested DNA was separated on 1% TAE agarose gel. DNA fragments with sizes between ~4-9 kb were gel purified, and cloned into pMMB vector that was digested with *Sma*I and treated with calf intestinal phosphatase (CIP). This yielded ~ 20,000 colonies. The collected transformants were subsequently mated with PA14 *P. aeruginosa* Δ*orn* strain and plated on LB supplemented with 25 µg/

ml irgasan and 150 µg/ml carbenicillin resulting in ~100,000 colonies. Colonies with increased colony size were retested in liquid culture (LB with 50 µg/mL carbenicillin) to identify cells that grew similar to the parental PA14 strain. Plasmids from suppressor strains were minipreped and sequence to determine the genomic fragments responsible for suppression the Δorn mutation.

Preparation of whole cell lysates

Overnight cultures of *P. aeruginosa* PA14 Δorn , or complemented strains were subcultured into fresh media with 50 µg/ml carbenicillin and 1 mM IPTG, grown at 37 °C with shaking. All bacteria samples were collected by centrifuge and resuspended in 1/10 volume of 100 mM NaCl, and 10 mM Tris, pH 8, also supplemented with 25 µg/ml lysozyme, 10 µg/ml DNase, and 1mM PMSF and stored at –80 °C.

Assay of enzyme activity

The activity of whole cell lysates against ^{32}P -labeled substrates was measured by the appearance of ^{32}P -labeled substrates on 20% Urea PAGE containing 1× TBE buffer. The reactions including phosphorylated RNA (1 µM) and trace amount of radiolabeled substrate were performed at room temperature in reaction buffer (10 mM Tris, pH 8, 100 mM NaCl, 5 mM MgCl_2 and 10 µM MnCl_2). At indicated time, the reaction was stopped by transferring 2 µl of the reaction to tubes containing 5 µl of 0.2 M EDTA and heated at 98 °C for 5 min. Samples were separated on 20% urea PAGE containing 1× TBE buffer and 4 M urea. The gel was exposure to phosphorimager screen and imaged using Fujifilm FLA-7000 phosphorimager (GE). The intensity of the radiolabeled nucleotides was quantified using Fujifilm Multi Gauge software v3.0.

Site-directed mutagenesis

yciV site-directed mutagenesis were generated by using primers in Supplemental Table 3 with the Q5 Site-Directed Mutagenesis Kit (New England Biolabs).

Labeling of RNAs

5' unphosphorylated RNAs were purchased from TriLink Biotechnologies or Sigma. The single-strand oligoribonucleotides (GG or AAAAAGG) were used for 5'-labeled ^{32}P with T4 polynucleotide kinase (New England Biolabs) and [γ - ^{32}P]-ATP. Reactions were incubated at 37°C for 1 hour followed by heat inactivation of T4 PNK at 90°C for 10 minutes.

Aggregation assay

Indicated strain of *P. aeruginosa* are grown in LB agar plates supplemented with 15 µg/mL gentamicin. Three independent colonies were inoculated into glass tubes containing 2.5 mL of LB supplemented with 10 µg/ml gentamicin. The cultures were incubated in a fly-wheel in 37 °C for 16 hours. Culture tubes were photographed.

Colony morphology

Strains were grown overnight in LB media supplemented with 50 µg/ml carbenicillin. Strains were normalized to OD₆₀₀ 0.5, serial dilution to achieve 5 CFU/µl, then 10 µl dripped in parallel on LB agar plate containing 50 µg/ml carbenicillin and 1 mM IPTG.

Strains were grown at 37 °C for 30 hours then imaged. Colonies sizes were measured by Fiji. For all strains, the control strains were always included on the same plate.

2-Aminopurine RNase assays

To investigate the rate of dinucleotide processing and to determine the polarity of RNase AM protein homologs, we utilized an assay based on the differential fluorescence output of the nucleotide analog 2-aminopurine (2-AP) as described previously (Myers et al., 2023; Weiss et al., 2022; Zhou et al., 2017). This assay exploits the different fluorescent properties of 2-AP as this nucleobase exhibits reduced fluorescence output when base stacked with other nucleobases but shows an increase in fluorescence output when liberated from an RNA polymer by a phosphodiesterase cleavage event. The RNA substrate pAp(2AP) and the tetranucleotide substrates containing internal, potentially non-hydrolyzable phosphorothioate (*ps*) linkages pApApsGp(2AP) and p(2AP)pApsGpG were all purchased as purified HPLC purified single stranded RNAs from GE Healthcare Dharmacon. These reactions were conducted at room temperature in a volume of 75 µL in a black Corning 384 well plate in the presence of 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM MgCl₂, 10 µM MnCl₂ 5 µM of the indicated 2-AP containing RNA substrate, and the respective protein concentration. 2-AP release was monitored using a Spectramax M5 plate reader with the excitation wavelength set to 310 nm and the emission wavelength at 375 nm.

Phylogenetic analysis

All representative genomes from Genome Taxonomy Database (GTDB)(release r207) (Parks et al., 2022) were downloaded and protein sequences for each genome were predicted using prokka (version 1.14.6) (Seemann, 2014). Proteins predicted to belong to COG0613 by egg-nog-mapper (version 2.1.3) (Cantalapiedra et al., 2021) and SSF89550 based on interproscan(version 5.60-92.0) (Jones et al., 2014) were retrieved. These sequences were clustered to 70% identity with PSI-CD-hit (version 4.8.1) to remove redundancy (Huang et al., 2010). YciV sequences of interest were added into the dataset to assure their inclusion with NCBI gene identifiers ABJ12426.1 (*Pseudomonas aeruginosa*), NP_460680.1 (*Salmonella typhimurium*), NP_230822.1 (*Vibrio cholerae*), NP_415782.1 (*Escherichia coli*), YP_002347182.1 (*Yersinia pestis*) and WP_011135245.1 (*Chromobacterium violaceum*). The dataset was filtered to remove sequences below 200 amino acids and above 400 amino acids. The remaining 1,621 sequences were aligned with Clustal Omega (version 1.2.4) and default options (Sievers et al., 2011). A phylogenetic tree of the YciV genes was inferred with IQ-tree version 2.1.2 (Minh et al., 2019) with 10,000 ultrafast bootstrap replicates (UFBoot) (Hoang et al., 2018) using ModelFinder to determine the best model of evolution (Kalyaanamoorthy et al., 2017). Trees were visualized in iTol (Letunic and Bork, 2021). To generate the sequence logo, first, columns in the alignment with only gaps were removed with trimAl (Capella-Gutierrez et al., 2009). Logomaker (Tareen and Kinney, 2020) was implemented to generate sequence logos with information entropy from the sequence alignment.

Cloning, protein expression and purification

The open reading frame of RNase AM_{vc} (VC1177) from *Vibrio cholerae* O1 El Tor (residues 1–290) was codon optimized for expression in *E. coli* and synthesized by GeneArt (Life Technologies). The DNA fragment was inserted by In-Fusion seamless cloning (Takara) between BamHI and NotI sites of a modified pET28a vector (Novagen) yielding N-terminally His₆-tagged small ubiquitin-like modifier (SUMO) fusion proteins cleavable by recombinant Ulp-1 protease. Plasmids were verified by Sanger sequencing (Microsynth AG).

RNase AM_{vc} was overexpressed in *E. coli* BL21 T7 Express cells (New England Biolabs) from fresh transformants. Protein expression was induced with 0.5 mM IPTG for 16 hours at 16 °C after reaching an OD₆₀₀=1.0 in Terrific broth (TB) supplemented with 50 µg/mL kanamycin initially grown at 37 °C in a shaking incubator. Cells were harvested by centrifugation, resuspended in a minimal volume of Ni-NTA binding buffer (25 mM Tris-Cl pH 8.5, 500 mM NaCl, 20 mM imidazole, 10 % glycerol) and flash frozen in liquid nitrogen. After thawing, cells were lysed on ice by sonication, and insoluble debris was pelleted by centrifugation for 45 minutes at 40,000g and 4 °C. All further purification steps were carried out at 4 °C. Soluble supernatant was incubated with Ni-NTA resin (Qiagen) pre-equilibrated with Ni-NTA binding buffer for 1 hour on ice with gentle agitation. RNase AM_{vc}-bound resin was washed with three rounds of 10 column volumes (CV) Ni-NTA binding buffer by gravity flow, followed by elution with three rounds of 2 CV Ni-NTA elution buffer (25 mM Tris-HCl pH 8.5, 500 mM NaCl, 400 mM imidazole, 10 % glycerol). Eluates were buffer exchanged into gel filtration buffer (25 mM HEPES-NaOH pH 7.5, 500 mM NaCl, 10 % glycerol) with a HiPrep 26/10 desalting column (Cytiva) followed by overnight cleavage of the His₆-SUMO moiety by Ulp1-His₆. RNase AM_{vc} was separated from His-tagged Ulp-1 and SUMO by flowing-through a HisTrap Ni-NTA column (Cytiva). EDTA was added to the flow through containing RNase AM_{vc} at a final concentration of 10 mM, followed by concentration of RNase AM_{vc} with an Amicon Ultra 10K concentrator (Merck Millipore). Concentrated RNase AM_{vc} was injected onto a HiLoad 16/600 Superdex 200 gel filtration column (Cytiva) equilibrated in gel filtration buffer, and pure RNase AM_{vc} fractions were pooled, concentrated to >30 mg/mL, frozen in liquid nitrogen and stored at -80 °C.

Crystallization, data collection, and structure refinement

RNase AM_{vc} crystals were prepared in gel filtration buffer in the presence of pGG (Jena BioScience) by mixing RNase AM:pGpG in a 1:2 ratio followed by incubation at 19 °C for 30 minutes. Protein concentrations used in crystallization ranged from 5-20 mg/mL. Crystals were grown by hanging-drop vapor diffusion by mixing equal volumes (0.8 µL) of sample with reservoir solution. RNase AM_{vc} crystals grew at 19 °C over a reservoir solution of 0.1 M Bis-Tris (pH 5.0), 0.2 M ammonium sulfate and 22.5 % PEG 3350. Crystals were soaked in cryo-protectant composed of reservoir solution supplemented with 25% glycerol and frozen in liquid nitrogen. Data were collected by synchrotron radiation on frozen crystals at beamline P11 at Deutsches Elektronen-Synchrotron DESY, Hamburg, Germany. Diffraction data sets were processed using XDS (Kabsch, 2010), and initial structures were solved by molecular replacement using the software package Phenix (Liebschner et al., 2019) with the coordinates of CV1693 (PDB: 2yb1, (Cummings et al., 2014)) as the search model. Manual model building and refinement

were carried out with Coot (Emsley et al., 2010) and Phenix. Illustrations were prepared in PyMol (The PyMOL Molecular Graphics System, Version 3.0 Schrödinger, Inc). All data collection and refinement statistics are summarized in Supplementary Table S1.

Computational structural biology approaches

We used AlphaFold2 and ColabFold to predict the structure of monomeric RNase AM_{Pa} using default parameters (Jumper et al., 2021; Mirdita et al., 2022). Five models were generated and ranked. The top-ranked model was relaxed by molecular dynamics within ColabFold. To dock pGG to the crystal structure of RNase AM_{Vc}, we employed the DiffDock-L method using the DiffDock web interface (<https://huggingface.co/spaces/reginabarzilaygroup/DiffDock-Web>) (Corso et al., 2024; Corso et al., 2023). The crystallographic model of the protein from the final refinement step and the SMILES string for pGG were used as the inputs. Structural homology searches were carried out using Foldseek with the crystal structure of RNase AM_{Vc} as input and the PDB100 database as the search space (van Kempen et al., 2024). Structures were visualized in PyMOL, and rmsd values were calculated and illustrated using the PyMOL script ColorByRMSD and a custom color gradient (<https://raw.githubusercontent.com/PyMol-Scripts/PyMol-script-repo/master/colorbyrmsd.py>) (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC.).

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