

## Special Issue Article

# The world of ribonucleases from pseudomonads: a short trip through the main features and singularities

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## Summary

The development of synthetic biology has brought an unprecedented increase in the number molecular tools applicable into a microbial chassis. The exploration of such tools into different bacteria revealed not only the challenges of context dependency of biological functions but also the complexity and diversity of regulatory layers in bacterial cells. Most of the standardized genetic tools and principles/functions have been mostly based on model microorganisms, namely *Escherichia coli*. In contrast, the non-model pseudomonads lack a deeper understanding of their regulatory layers and have limited molecular tools. They are resistant pathogens and promising alternative bacterial chassis, making them attractive targets for further studies. Ribonucleases (RNases) are key players in the post-transcriptional control of gene expression by degrading or processing the RNA molecules in the cell. These enzymes act according to the cellular requirements and can also be seen as the recyclers of ribonucleotides, allowing a continuous input of these cellular resources. This makes these post-transcriptional regulators perfect candidates to regulate microbial physiology. This review summarizes the current knowledge and unique properties of ribonucleases in the world of pseudomonads, taking into account genomic context analysis, biological function and strategies to use ribonucleases to improve biotechnological processes.

## Introduction

The establishment of microbes as viable means of producing valuable compounds has led to the development of methods to expand their natural biosynthetic capabilities. Throughout the years, synthetic biology has contributed to an increasing number of biological functions that can be engineered inside a bacterial cell. The designing process of such microbial chassis uses a combination of different chemical and genomic editing tools. However, with these advances came the realization that different bacterial hosts would respond differently to novel biological functions in accordance with their regulatory layers. Synthetic biologists have adopted some bacterial hosts, in most cases the widely studied *Escherichia coli* and *Bacillus subtilis*, for plugging-in and plugging-out genetic circuits for specific purposes. Exploration of non-model microorganisms has also been made in synthetic biology, but it has been hampered by the lack of understanding of their layers of control and the limited toolboxes available. The general assumptions that regulatory mechanisms and genome-editing tools from model microorganisms work in other hosts often lead to unreliable results and point to the problem of portability in synthetic biology. Organisms that present natural biotechnologically relevant traits are important systems to study and engineer. To fully unlock the potential of non-model bacteria, it is necessary to understand their fundamental biology, namely their regulatory rules for gene expression.

The non-model bacterial group of pseudomonads have been in the focus of attention due to their relevant features. They are ubiquitous, metabolically versatile Gram-negative bacteria (Goldberg, 2000), capable of adapting to different physicochemical and nutritional niches, to endure both endogenous and exogenous stresses, and they are even able to synthesize many bioactive compounds (including antibiotics) (Gurney and Thomas, 2011; Nikel *et al.*, 2014). All these traits are encoded by approximately 1500 genes, which are common to all *Pseudomonas* species that have been sequenced so far (Loper *et al.*, 2012; Nikel *et al.*, 2014). For instance, *Pseudomonas putida* is able to colonize soil, water and

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the plant rhizosphere (Timmis, 2002). The prototype species of the genus, *Pseudomonas aeruginosa*, is an opportunistic pathogen for plants, animals and humans, it is metabolically versatile, and it can be found in numerous aquatic and soil habitats (Campa *et al.*, 1993). Despite the considerable differences between the genomes of these two distinctive species, such as a distinct GC content and nucleotide substitutions at synonymous sites, the two genomes are strongly related in the organization and content of coding sequences (Weinel *et al.*, 2002).

Attaining a proper balance between RNA synthesis and degradation is essential for the modulation of cellular functions. Post-transcriptional control involves a series of steps after RNA transcription, which are important to control RNA lifetime and functionality. This is a highly regulated process orchestrated by the concerted action of ribonucleases (RNases). They can be classified as endoribonucleases if they cleave RNA molecules internally or as exoribonucleases if the cleavage of the RNA molecule is made from one of its extremities. Additional aspects can be considered such as the capability of these enzymes to cleave single and/or double stranded RNA, the nature of the products released, the specificity of the enzyme for substrates with a define shape and/or sequences, their ability to digest DNA besides RNA, and their processive or distributive action in cells (Arraiano *et al.*, 2013). Deactivation of single or multiple RNases can affect the longevity of the bulk intracellular mRNA, and this approach has been crucial in the understanding of mechanisms of mRNA decay (Arraiano *et al.*, 2010). They are involved in almost every aspect of RNA metabolism, including the control of non-coding RNAs (ncRNAs), native regulators that have inspired the design of many synthetic RNA devices for the engineering of bacterial metabolism (see Leistra *et al.*, 2019; and Xie *et al.*, 2021 for a review).

RNases constitute a regulatory layer in *Pseudomonas* that deserves further exploration. A high-throughput analysis of *P. aeruginosa* transcriptome revealed a great extent of processed transcripts and showed a wider level of complexity in RNA processing than it was previously anticipated (Gill *et al.*, 2018). For instance, the majority of the processing events occurred within defined sequence motifs indicative of distinct endonucleolytic activities. The most prevalent cleavages were associated with RNase E suggesting the instrumental role of this enzyme in the regulation of a number of RNAs in *Pseudomonas*. The understanding of the role of this and other ribonucleases will expand our knowledge regarding the role and significance of RNA processing in maintaining a dynamic, flexible and robust bacterial transcriptome.

In this mini-review, it was gathered the information regarding RNases that has been explored in the

pseudomonad's world, revealing the main contrasting features with the *E. coli* model. For a more exhaustive description of what is known about the role of these RNases, please consult previous reviews on the subject, for example (Arraiano *et al.*, 2010; B aria *et al.*, 2016; Mohanty and Kushner, 2016; Mohanty and Kushner, 2018; Bechhofer and Deutscher, 2019).

## Endoribonucleases

In  $\gamma$ -proteobacteria, there are two main endoribonucleases involved in RNA degradation: RNase III, specific for double-stranded RNA, and RNase E, which cleaves single-stranded RNA. Many others are known to be involved in the degradation process, and here, we review the main features described for those studied in pseudomonads.

### RNase E

RNase E is a large protein composed of an essential N-terminal domain required for catalytic activity, and a C-terminal region lacking enzymatic activity that is mostly unstructured. This region works as a scaffold since it has several microdomain sites to interact with other proteins, such as the RNA chaperone Hfq (Morita *et al.*, 2005; Ikeda *et al.*, 2011) or the components of the degradosome, a multienzyme RNA-degrading complex (see Ait-Bara *et al.*, 2015, for a review).

RNase E can act together with an exoribonuclease [polynucleotide phosphorylase (PNPase) or RNase R], an ATP-dependent RNA helicase (such as RhlB) and a glycolytic enzyme (enolase or aconitase). The specific composition of the degradosome can change depending on the microorganism and/or growth condition (Purusharth *et al.*, 2005; Barria *et al.*, 2013; Redko *et al.*, 2013). Even though the catalytic domain of RNase E is well conserved within the Proteobacteria, the noncatalytic region is poorly conserved, both in sequence and in length (Marcaida *et al.*, 2006). This may contribute to the functional plasticity in the composition of the complex (Ait-Bara *et al.*, 2015).

Inside the *Pseudomonadales* order, it was discovered that the cold-adapted Antarctic bacterium *Pseudomonas syringae* has a multiprotein complex containing RNase E, RNase R, and the DEAD-box helicase RhlE. Therefore, in this case, the hydrolytic exoribonuclease activity (RNase R) substitutes the phosphorolytic activity of PNPase (Purusharth *et al.*, 2005). This bacterium grows well at 4°C, a condition that favours the formation of secondary structures by the RNA molecules. In this environment, the high processivity of RNase R through RNA secondary structures might be more advantageous in the context of the degradosome complex, to conserve

energy (ATP) and work more efficiently on the turnover of the RNAs. Analysis by mass spectrometry of the degradosome composition in *P. aeruginosa* included RNase E, PNPase and the RNA helicase DeaD, revealing a similar composition to the *E. coli* complex (Van den Bossche *et al.*, 2016).

RNase E is an essential in *E. coli* and other bacteria (Ait-Bara *et al.*, 2015; Table 1). There are also indications that it is essential for growth in *Pseudomonas* (Lee *et al.*, 2015; Poulsen *et al.*, 2019; Table 1). Analyses of genomic location have been useful in providing clues about functional annotations for many proteins. Our analysis revealed that the genomic context for this ribonuclease is similar between *P. aeruginosa* PAO1, *P. putida* KT2440, *Pseudomonas fluorescens* SBW25 and *Pseudomonas pv. tomato* D300 (Fig. 1A).

The catalytic activity of RNase E resides in its N-terminal half and it is required for bacterial growth. In *P. putida*, an RNase E<sup>N-term</sup> mutant, which does not contain the C-terminal region, was created to abolish degradosome component interaction. The growth profile of *P. putida* in the absence of degradosome formation was not significantly affected at the optimal growth temperature. However, in cold conditions (10°C) or at higher temperatures (40°C), the mutant presented a strong defect on growth. These results highlighted the need of a full-length RNase E by *P. putida* KT2440 in order to cope with the temperature stress conditions tested (Apura *et al.*, 2021).

The characterization of the *P. putida* RNase E<sup>N-term</sup> revealed the highly pleiotropic role of this enzyme. The analysis of its response to various oxidative agents revealed a higher sensitivity to the redox and ROS

damage, in comparison with the wild-type strain (Apura *et al.*, 2021). An impairment in motility was observed in *P. putida* cells harbouring the degradosome mutation, as previously observed in a *S. typhimurium* RNase E similar mutant (Viegas *et al.*, 2013). Analysis of the colony morphology of RNase E mutant also revealed a possible connection of this RNase in the regulation of the production of the extracellular polymeric substance (EPS) in *P. putida* on solid surfaces (Apura *et al.*, 2021).

To study the effects of RNase E depletion in *P. aeruginosa*, Sharp *et al.* created a conditional RNase E depletion mutant (Sharp *et al.*, 2019). In *P. aeruginosa*, the enzyme plays an important role in the expression of virulence factors through different mechanisms (Sharp *et al.*, 2019). This opportunistic pathogen employs a type III secretion system (T3SS) to inject effector proteins into host cells, and it was seen that RNase E plays a role in positively regulating the expression of T3SS genes (Sharp *et al.*, 2019). The fact that RNase E is required for the efficient production of ExsA transcription factor, a master regulator of T3SS gene expression, may be one of the possible mechanisms. On the other hand, this endoribonuclease negatively regulates the expression of a type VI secretion system (T6SS) gene and limits biofilm formation in this organism (Sharp *et al.*, 2019). Therefore, it seems that this enzyme can regulate the expression of virulence factors in *P. aeruginosa* through multiple ways.

In *P. aeruginosa* cells infected with the giant *Pseudomonas* phage phiKZ, the degradosome activity is inhibited. This happens because the phage encodes a protein, the 'degradosome interacting protein' or Dip, that directly binds to two RNA binding sites within the

**Table 1.** Essentiality of ribonuclease genes in *E. coli* and *Pseudomonas*.

Ribonuclease	Gene	Essentiality <sup>a</sup>		Mutants available in <i>Pseudomonas</i> <sup>b</sup>
		<i>E. coli</i>	<i>P. aeruginosa</i>	
RNase E	<i>me</i>	Yes	Yes	<i>P. putida</i> KT2440 RNase E <sup>N-term</sup> mutant only retains catalytic domain (Apura <i>et al.</i> , 2021) <i>P. aeruginosa</i> ClpXP-based RNase E depletion system (Sharp <i>et al.</i> , 2019)
YbeY	<i>ybeY</i>	No	Yes	<i>P. aeruginosa</i> mutants (PA14, PAO1 and PAK strains) (Xia <i>et al.</i> , 2020a)
RNase III	<i>mc</i>	No	Conditional	<i>P. putida</i> (KT2440) (Apura <i>et al.</i> , 2020)
RNase G	<i>mg</i>	No	No	<i>P. putida</i> KT2440 (Apura <i>et al.</i> , 2020)
MazF	<i>mazF</i>	No	No	<i>P. putida</i> PaW85 (isogenic to KT2440) (Rosendahl <i>et al.</i> , 2020)
PNPase	<i>pnp</i>	No	Yes	<i>P. putida</i> KT2440 (Apura <i>et al.</i> , 2020; Favaro and Deho, 2003), <i>P. putida</i> TMB (Favaro and Deho, 2003)
RNase PH	<i>rph</i>	No	No	—
RNase R	<i>mr</i>	No	No	<i>P. putida</i> KT2440 (Fonseca <i>et al.</i> , 2008; Apura <i>et al.</i> , 2020), <i>P. syringae</i> (Purusharth <i>et al.</i> , 2007)
Oligoribonuclease	<i>orn</i>	Yes	No	<i>P. aeruginosa</i> PA14 (Orr <i>et al.</i> , 2015; Chen <i>et al.</i> , 2016a) <i>P. aeruginosa</i> PAO1 (Goldman <i>et al.</i> , 2011; Cohen <i>et al.</i> , 2015)

Details on the essentiality of ribonuclease genes in *E. coli* and *P. aeruginosa*, as predicted from studies that created Tn-mutant libraries (third column). On the right column are indicated the ribonuclease mutants available in different pseudomonads, based on experimental data details discriminated on the bottom row.

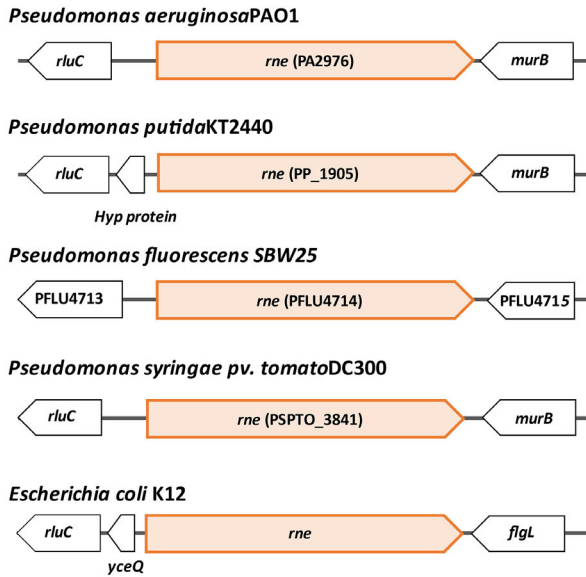
**a.** As predicted from Tn-mutant libraries in *P. aeruginosa* (Lee *et al.*, 2015; Poulsen *et al.*, 2019).

**b.** based on experimental evidence.

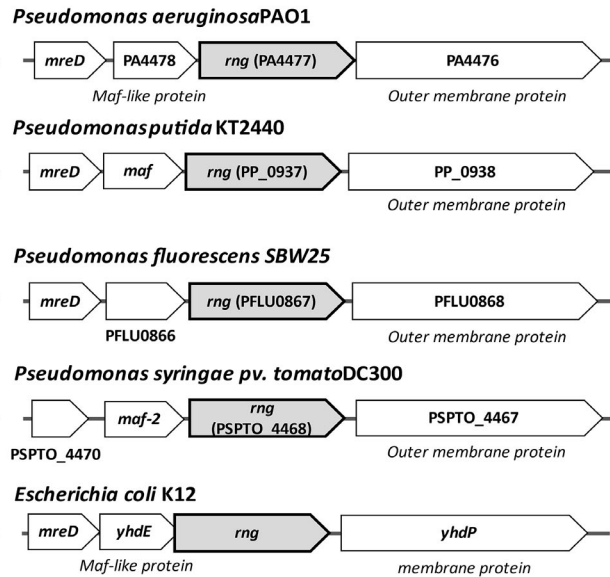
(A)

**Endoribonucleases**

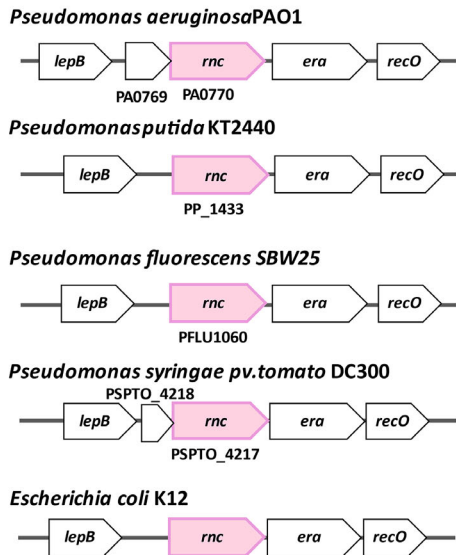
**RNase E**



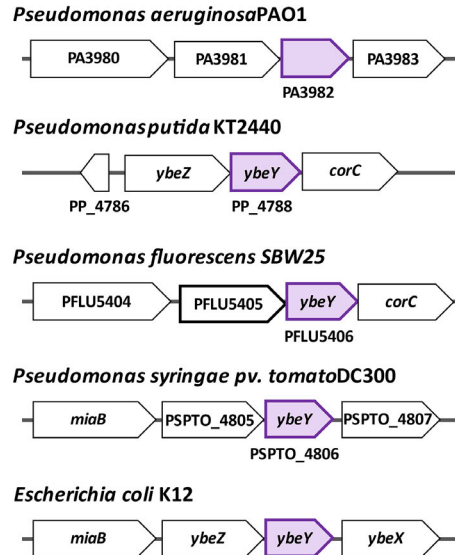
**RNase G**



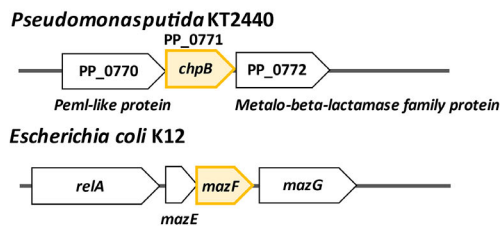
**RNase III**



**YbeY**



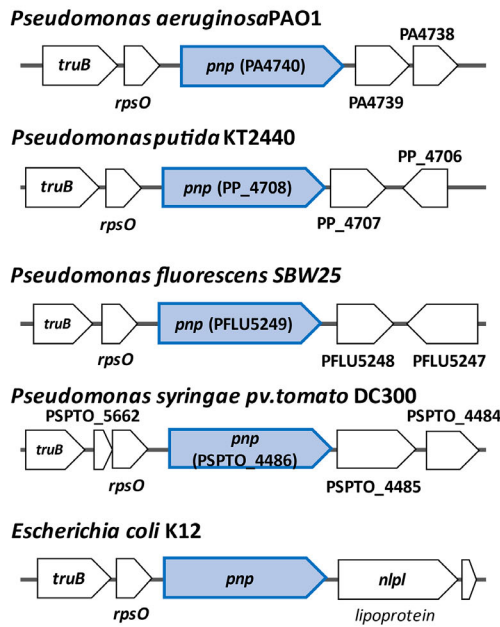
**mazF interferase**



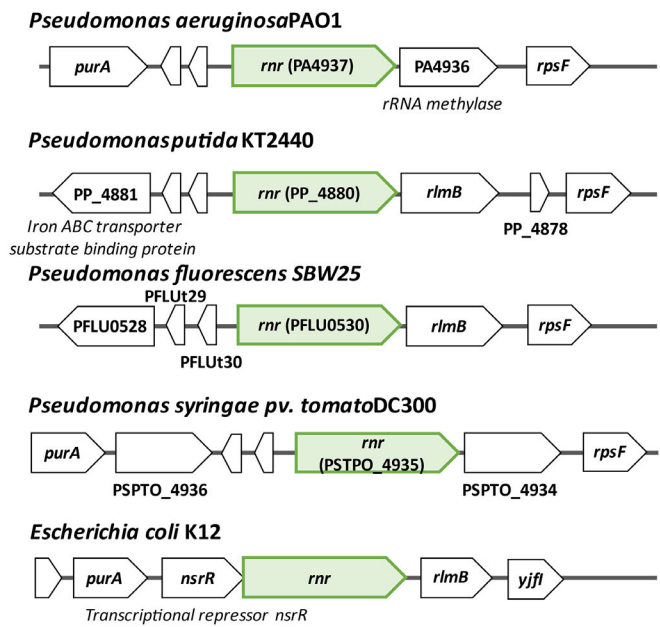
(B)

### Exoribonucleases

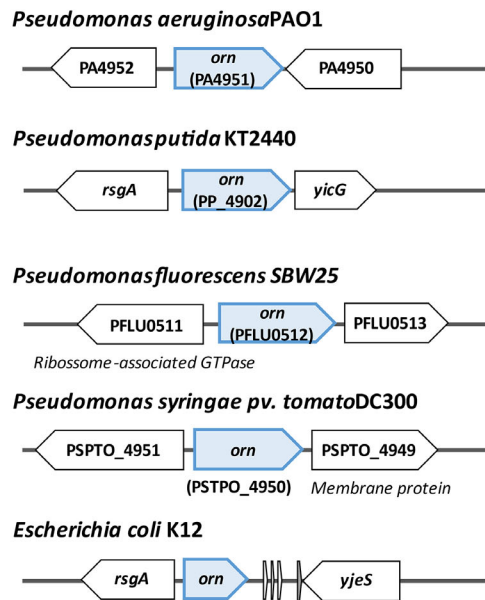
#### PNPase



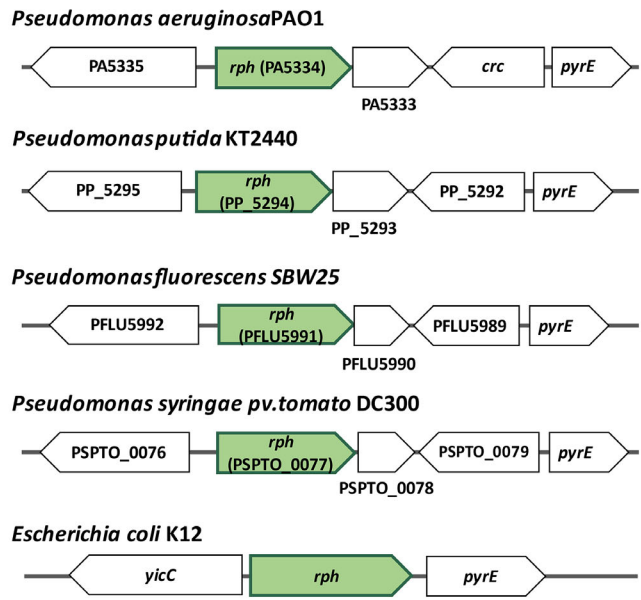
#### RNase R



#### Oligoribonuclease



#### RNase PH



**Fig. 1.** Comparison of the genomic context of the ribonuclease genes in different *Pseudomonas* strains (*P. aeruginosa* PAO1, *P. putida* KT2440, *P. fluorescens* SBW25, *P. syringae* pv. *tomato* DC300) and *Escherichia coli* K12. Ribonucleases are divided according to specificity of activity in endoribonucleases and exoribonucleases (panels A and B, respectively). Ribonuclease genes are colour-highlighted, and each arrow corresponds to a gene that is identified by its name and/or the corresponding *locus* tag. The genomic context of each *Pseudomonas*' RNase was retrieved from the *Pseudomonas* Genome database [<http://www.pseudomonas.com>; (Winsor *et al.*, 2016)] and/or Microbial Genome Annotation & Analysis Platform (MicroScope) (Vallenet *et al.*, 2020). *E. coli* genomic context was retrieved from xBASE (Chaudhuri *et al.*, 2008).

C-terminal region of the host RNase E, preventing substrate RNAs from being bound and degraded by the RNA degradosome during the virus infection cycle. Interestingly, the binding places of Dip are conserved in the *E. coli* RNase E and the interaction and functional inhibition was also confirmed (Van den Bossche *et al.*, 2016). In *E. coli*, two other phage proteins were previously reported to have independently evolved to influence the activity of the RNA degradosome of their host in order to support efficient phage infection (Marchand *et al.*, 2001; Qi *et al.*, 2015).

Several biotechnological applications have been proposed based on this interaction of Dip with the RNase E, to inhibit RNA degradation without killing the bacterial cell (Dendooven *et al.*, 2017). For instance, the co-expression of Dip (or the addition of a small-molecule inhibitor) may be used as means to stabilize the mRNA of a recombinant protein in order to improve its expression in bacteria. Dip or Dip-based small-molecule inhibitors could also potentially be used to improve the performance of CRISPR-Cas and long antisense RNAs to knock down targeted gene expression. Preventing degradation by the bacterial RNase E would enable the use of longer CRISPR RNA precursors. A heterologous expression of Dip protects phage mRNA, increasing expression efficiency during the infection cycle. Therefore, applications in the field of phage therapy for the treatment of *P. aeruginosa* infections have also been envisaged (Brussow, 2012; Rose *et al.*, 2014).

### RNase G

RNase G, originally known as CafA protein, is a paralog of RNase E (Okada *et al.*, 1994), and it has a strong homology with its N-terminal sequence. However, contrary to RNase E, RNase G is not essential in *E. coli* (see Ait-Bara *et al.*, 2015, for a review) and *P. aeruginosa* (Table 1). In  $\gamma$ -proteobacteria, RNase E and RNase G are both conserved, suggesting that RNase G has an important conserved function that is distinct from the function of RNase E. RNase G cooperates with RNase E in 16S ribosomal RNA (rRNA) maturation (Li *et al.*, 1999; Wachi *et al.*, 1999) and in the degradation of several transcripts such as the mRNA encoding enolase (Kaga *et al.*, 2002; Lee *et al.*, 2002).

The enzyme is encoded by the *rng* gene (or *cafA*), and its genomic context is conserved between

*Pseudomonas* (Fig. 1A). A phenotypical characterization of a *rng* mutant, in *P. putida*, showed an absence of cold sensitivity and a higher robustness of growth than the wild type under heat stress (Apura *et al.*, 2021). The RNase G mutation has been previously linked to an increased expression of heat shock proteins (HSPs) in *E. coli* (Lee *et al.*, 2002) and *Stenotrophomonas maltophilia* (Bernardini *et al.*, 2015), and this might also be the case in *P. putida* KT2440, where these proteins are conserved, consequently explaining the increased temperature tolerance observed. Despite this characterization, the information regarding the concrete function of RNase G in *Pseudomonas* is still scarce.

### RNase III

RNase III is a widely conserved enzyme, found in almost all eubacteria, and has homologues in eukaryotic species. It is specific for double-stranded RNA, but substrate selection also requires other structural and sequence determinants, such as the helix length, the strength of base-pairing and correct positioning of specific nucleotide pairs (Pertzev and Nicholson, 2006; Arraiano *et al.*, 2010; Bernardini *et al.*, 2015).

RNase III has multiple roles in rRNA and mRNA processing (Regnier and Grunberg-Manago, 1989; Arraiano *et al.*, 2010; Lim *et al.*, 2012), in the maturation of phages (Nicholson, 1999), in CRISPR RNAs (Deltcheva *et al.*, 2011) and in the degradation of ncRNAs (Vogel *et al.*, 2004; Opdyke *et al.*, 2011; Viegas *et al.*, 2011). This endoribonuclease regulates its own expression through the cleavage of its messenger RNA (Bardwell *et al.*, 1989). Furthermore, it also controls the decay of the messenger RNA of the exoribonuclease PNPase (Portier *et al.*, 1987; Takata *et al.*, 1987).

The genomic organization of the RNase III encoding gene, forming the *mc-era-recO* operon (Fig. 1A), is conserved in several species (Powell *et al.*, 1999; Apura *et al.*, 2021). The downstream gene *era* encodes a GTP binding protein and is essential (Takiff *et al.*, 1989). Mutations in *rnc* genes with polar effects on *era* mRNA levels are non-viable. The enzyme was predicted to be condition-specific essential gene (only required under a subset of growth conditions) in *P. aeruginosa* (Lee *et al.*, 2015; Poulsen *et al.*, 2019); however, a RNase III<sup>-</sup> defective mutant in *P. putida* (PP-RNase III<sup>-</sup>) is available (Apura *et al.*, 2021; Table 1). In resemblance with the

mutant in *E. coli* and other bacteria (Studier, 1975; Takiff *et al.*, 1989; Babitzke *et al.*, 1993; Nicholson, 1999; Viegas *et al.*, 2007; Saramago *et al.*, 2018), the PP-RNase III mutant exhibits a slower growth rate (Zangrossi *et al.*, 2000; Apura *et al.*, 2021). This phenotype was attributed to a possible reduction in the kinetics of rRNA processing, due to the accumulation of the 30S rRNA, the larger precursor of 16S, 23S and 5S rRNAs forms (Apura *et al.*, 2021).

In a recent phenotypical and morphological study characterization, PP-RNase III mutant presented a reduction in motility. The mutation also seemed to impact on EPS production and increase the strain's sensitivity to redox and ROS damage (Apura *et al.*, 2021). Apura *et al.* also pinpointed the need of RNase III in the adaptation to cold and heat. In the absence of RNase III, there is a severe defect on the growth of *P. putida* KT2440 upon cold and heat temperature shifts.

### YbeY

YbeY is a highly conserved bacterial endoribonuclease, whose loss of function is either lethal or can result in pleiotropic changes (Vercruyssen *et al.*, 2014; Leskinen *et al.*, 2015). Its biochemical activity has been characterized only in few bacteria (Jacob *et al.*, 2013; Vercruyssen *et al.*, 2014), mostly in *E. coli* (Zhan *et al.*, 2005; Rasouly *et al.*, 2010) and *S. meliloti* (Saramago *et al.*, 2017). YbeY activity, originally defined as a metal-dependent hydrolase (Zhan *et al.*, 2005), has been associated with the maturation of 16S rRNA, ribosome quality control, regulation by small non-coding RNAs (ncRNAs) (Pandey *et al.*, 2011; Pandey *et al.*, 2014; Vercruyssen *et al.*, 2014) and stress responses (Davies *et al.*, 2010; Jacob *et al.*, 2013; Vercruyssen *et al.*, 2014; Saramago *et al.*, 2017). Unlike its *E. coli* orthologue, *S. meliloti* YbeY can cleave double-stranded RNAs (dsRNA), and there is evidence that YbeY can work as a gene silencing enzyme, through the dsRNA degradation of hybrids of ncRNAs with their targets (Saramago *et al.*, 2017).

Contrarily to what was inferred from studies using transposon mutant libraries (Vercruyssen *et al.*, 2014; Lee *et al.*, 2015), this enzyme is not essential in *P. aeruginosa* (Table 1). YbeY mutants were obtained in different *P. aeruginosa* strains (Xia *et al.*, 2020a). These mutants showed growth defects and an accumulation of 16S rRNA precursors, which is explained by the role of YbeY in 16S rRNA maturation, ribosome assembly and pathogenesis (Xia *et al.*, 2020a).

The genomic localization of *ybeY* in an operon with the coding gene of the PhoH-like protein, YbeZ, seems to be conserved between *Pseudomonas* and *E. coli* (Fig. 1A). YbeZ was described as containing an ATP binding and a nucleoside triphosphate hydrolase domain

(Xia *et al.*, 2020b). A functional connection between YbeY and YbeZ was confirmed in *P. aeruginosa* (Xia *et al.*, 2020a). YbeZ binds to YbeY and is involved in the maturation of 16S rRNA and the response to oxidative stress (Xia *et al.*, 2020a).

The majority of the studies of YbeY in pseudomonads have focused on the role of the enzyme on the regulation of virulence determinants in *P. aeruginosa* (PA3982 (PA14\_12310 in the PA14 genome)). This endoribonuclease demonstrated a critical role in the virulence of *P. aeruginosa* at different levels. In this pathogen, YbeY controls bacterial resistance to oxidative stresses through the ncRNA ReaL (Xia *et al.*, 2020a). This occurs because ReaL directly represses the translation of the stationary-phase sigma factor RpoS, a global regulator of multiple stress response genes and quorum sensing systems (Schuster *et al.*, 2004). YbeY is responsible for the degradation of ReaL, and therefore, it indirectly controls the expression of *rpoS* and oxidative stress response genes. Additionally, it was determined that *P. aeruginosa* YbeY regulates the expression of the type III and type VI secretion system genes (T3SS and T6SS), two major virulence factors of this bacterium. It also regulates biofilm formation through the regulation of the ncRNAs RsmZ/Y (Xia *et al.*, 2020b).

The pleiotropic function of YbeY in *P. aeruginosa*, altogether with the multiple roles discovered in other bacteria such as in the regulation of bacterial resistance to heat shock (Rasouly *et al.*, 2010), oxidative stresses (Vercruyssen *et al.*, 2014) and regulation of multiple ncRNAs (Pandey *et al.*, 2011; Pandey *et al.*, 2014; Vercruyssen *et al.*, 2014; Leskinen *et al.*, 2015; Saramago *et al.*, 2017), can contribute to unlock the biotechnological applications of *Pseudomonas* strains. For instance, ncRNAs are native regulators that offer a wide suite of tools for engineering metabolic pathways and can be used as a regulator switch to turn on/off genes with biotechnological or agricultural interest (reviewed in Leistra *et al.*, 2019; Apura *et al.*, 2020; Xie *et al.*, 2021).

### MazF interferase

Most bacteria and a large number of archaea encode toxin genes on their genomes, which can be cotranscribed with their cognate antitoxins in an operon (toxin antitoxin or TA operons), to form a stable complex that is not expressed in the cell under normal growth conditions (Engelberg-Kulka *et al.*, 2004; Buts *et al.*, 2005; Gerdes *et al.*, 2005). MazF interferase is a toxin that has sequence-specific endoribonuclease activity (Aizenman *et al.*, 1996; Inouye, 2006). In *E. coli*, it cleaves mRNAs at ACA sequences to inhibit protein synthesis. MazF is a part of a TA system along with its cognate antitoxin MazE (Inouye, 2006).

The induction of MazF leads to a complete inhibition of growth, but cells are fully metabolically active to produce energy, amino acids and nucleic acids and to synthesize RNA and protein. The primary role of these toxins seems to be the freezing of cells in a quasi-dormant state so that cells are protected from environmental stresses in their habitats or from bactericidal chemicals and treatments, yet maintaining a fully metabolically active state. When the life-threatening stress is gone, MazE antitoxin is produced to detoxify MazF and cells reactivate growth (see (Inouye, 2006) for a review).

Besides MazF widely studied role in bacterial pathogenicity, persistence in multi-drug resistance, bio-film formation and bacterial evolution, TA systems have also revolutionized the field of biotechnology. Synthetic circuits can exploit shifts in cellular physiological state due to MazF activity, diverting intracellular resources via programmable mRNA decay (Venturelli *et al.*, 2017). Using this approach, it is possible to enhance target functions that compete directly with biomass synthesis, exploiting microbes as 'cell factories' to synthesize chemicals or biomolecules of interest.

*E. coli* cells engineered to overproduce MazF, can produce only a protein of interest when the ACA sequences in the mRNA for this protein are changed to non-MazF cleavable sequences (see Suzuki *et al.*, 2005; Inouye, 2006, for a review). Then, MazF downregulates transcripts that compete with the protected synthetic circuit for limiting resources, yielding an increase in protected gene expression (Venturelli *et al.*, 2017). This system called 'Single Protein Production' (SPP) allows the alteration of the ACA sequences of the proteins of interest to non-MazF cleavable sequences without changing their amino acid sequences. The small size of MazF (111 amino acids) and availability of its cognate antitoxin confers advantage for the implementation of this system. And in fact, a number of *E. coli*, yeast and human proteins have been successfully expressed at high yield in the absence of cellular protein production (Suzuki *et al.*, 2005; Inouye, 2006; Venturelli *et al.*, 2017).

*P. putida* is widely used for production of chemical compounds, and it is also a xenobiotic decomposer in environmental engineering (Poblete-Castro *et al.*, 2012). This bacterium encodes an homologue of this non-essential RNA interferase, the MazFpp (Fig. 1A – *chpB* – PP\_0771 and Table 1), that specifically recognizes the unique triplet UAC (Miyamoto *et al.*, 2016). A possible resource of this bacterium, to improve its resistance against specific stresses, could be the use of specific RNAs that elude the MazFpp. The quasi-dormant state of the cells, achieved through the overexpression of the MazFpp enzyme, would allow the resources of the cells

to be directed for the expression of RNAs of interest. As such, further characterization of the toxin enzyme that regulates its growth would be beneficial to understand its industrial potential.

### Exoribonucleases

Exoribonucleases are the enzymes that degrade the RNA from one of its extremities. Most of the information on exoribonucleases in bacteria comes from studies in *E. coli* and *B. subtilis*, and although the general characteristics are true for other bacteria, the exploration of different bacterial systems has unveiled novel information. For instance, the  $\gamma$ -division of Proteobacteria often contains homologues of the hydrolytic RNase II and RNase R. However, pseudomonads are an exception in this subdivision and only encode RNase R (Condon and Putzer, 2002).

### PNPase

PNPase, encoded by *pnp* gene, is widely conserved among bacteria, and homologous genes are found in several eukaryotes (Hayes *et al.*, 1996; Leszczyniecka *et al.*, 2002; Piwowarski *et al.*, 2003; Inouye, 2006). This enzyme has a processive, sequence-independent, phospholytic activity, being capable of synthesizing and degrading single-stranded RNA, depending on the availability of inorganic phosphate. It stalls in the presence of structured RNAs, requiring the presence of a poly(A) tail in the 3'-end of the RNA, or the association with other proteins [e.g. a helicase or the degradosome complex (Liou *et al.*, 2002; Lin and Lin-Chao, 2005)]. PNPase requires a minimal 3' overhang of 7–10 unpaired ribonucleotides to bind to its substrates (Py *et al.*, 1996; Cheng and Deutscher, 2005). It is mainly involved in the processive degradation of RNA, but PNPase can also add heteropolymeric tails to its substrates (Mohanty and Kushner, 2000; Slomovic *et al.*, 2008).

A conserved genomic linkage was observed between the gene encoding this exoribonuclease and the upstream *rpoS* gene, which codifies the RNA polymerase sigma factor RpoS (Favaro and Deho, 2003; see Fig. 1B). PNPase exoribonuclease was predicted to be one of the essential genes in the core genome of *P. aeruginosa* (Lee *et al.*, 2015; Poulsen *et al.*, 2019), although obtaining of mutants with transposon insertions in the last 10% of the gene was reported (Veracruz *et al.*, 2014). *P. putida* PNPase mutants were constructed (Apura *et al.*, 2021) with no significant defects on growth rate (Table 1).

In *P. putida*, PNPase was first identified in 2003 (Favaro and Deho, 2003). Contrary to what was observed in *E. coli*, the *P. putida pnp* mutant was able to



grow at low temperatures (Favaro and Deho, 2003; Apura *et al.*, 2021). This mutant did not exhibit growth arrest under cold acclimation, and it presented different expression patterns in terms of mRNA and protein levels when compared to a similar mutant in *E. coli* (Favaro and Deho, 2003). Also, the swimming halo formed by the PNPase mutant in KT2440 was only slightly impaired, not having the same impact as in the *E. coli pnp* mutant (Pobre and Arraiano, 2015; Apura *et al.*, 2021). These observations highlight that the patterns of *pnp* regulation can be different between bacteria and features from model microorganisms should not be considered as universal.

In *P. aeruginosa*, PNPase was shown to be important for the regulation of determinant virulence factors, such as the type III secretion system (T3SS), type VI secretion system and pyocin synthesis genes (Li *et al.*, 2013; Chen *et al.*, 2016b; Fan *et al.*, 2019). It was also shown that PNPase degrades ncRNA P27, responsible for the direct translational control of the quorum sensing signal synthase RhII (Chen *et al.*, 2019). In this bacterium, this exoribonuclease interacts with RNase E and RNA helicase DeaD to form an RNA degradosome, which plays an important role in RNA processing (Van den Bossche *et al.*, 2016).

### RNase R

RNase R, encoded by the *mnr* gene (previously *vacB*), is a 3'-5' hydrolytic exoribonuclease that belongs to the RNB family of enzymes, present in all domains of life (Cheng and Deutscher, 2002; Vincent and Deutscher, 2006). It is a processive and sequence-independent enzyme, with a wide impact on RNA metabolism (Cairrão *et al.*, 2003; Purusharth *et al.*, 2005; Andrade *et al.*, 2006; Domingues *et al.*, 2015). It is able to degrade highly structured RNA molecules and RNA duplex strands, provided there is a single-stranded 3' overhang (Hossain *et al.*, 2016). RNase R also has RNA helicase activity that is independent of its ribonuclease activity (Awano *et al.*, 2010). This ribonuclease is critical in RNA quality control, namely in the degradation of defective tRNAs (Vincent and Deutscher, 2006; Awano *et al.*, 2010) and rRNA (Cheng and Deutscher, 2003; Malecki *et al.*, 2014). This enzyme is a general stress-induced protein, whose levels are increased under several conditions, namely under cold-shock and under stationary-phase growth conditions (Cairrão *et al.*, 2003; Andrade *et al.*, 2006; Barria *et al.*, 2013).

Regarding the genomic context of RNase R, the gene immediately downstream of *mnr* seems to be conserved (see Fig. 1B), at least in function, since it encodes a rRNA methylase. The same does not happen with the upstream genomic region, albeit being more similar

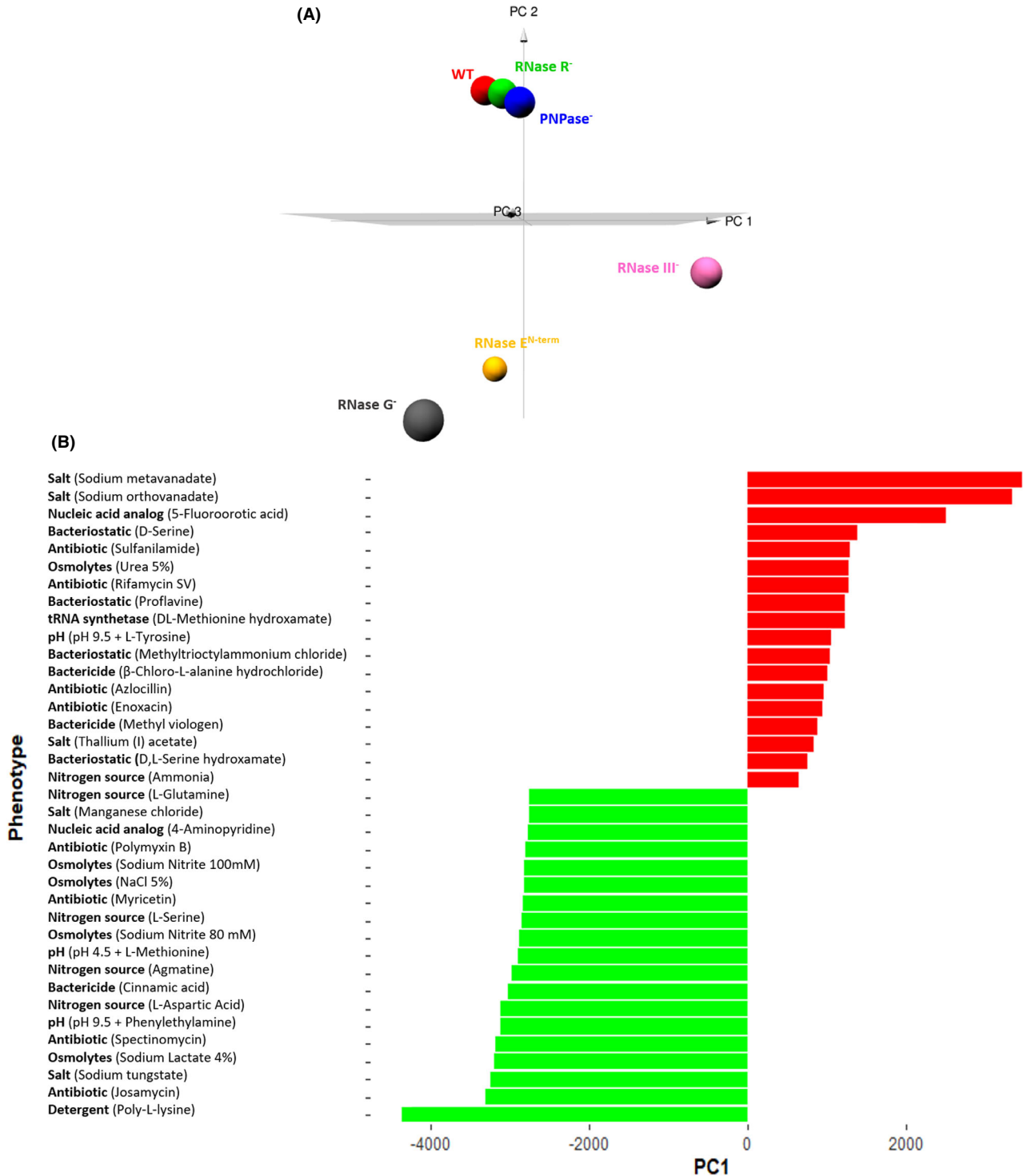
between *Pseudomonas* species than with *E. coli* (see Fig. 1B).

In *P. putida*, the *mnr* gene was inactivated (Table 1) and the mutant strain presented a reduction in the growth rate at 10°C (Reva *et al.*, 2006; Fonseca *et al.*, 2008; Apura *et al.*, 2021). These results go in agreement with the proposed role of RNase R in degrading the highly structured RNA, since secondary structures are more stable at low temperatures. From the analysis of transcriptome profiles of *P. putida* strains KT2440 (wild type) and KTRNR1 (a *mnr* mutant derivative of KT2440) using a genome-wide oligonucleotide-based DNA microarray, several transcripts were affected by the absence of this enzyme, being the majority related to the flagellar apparatus or the biosynthesis of cofactors (Fonseca *et al.*, 2008).

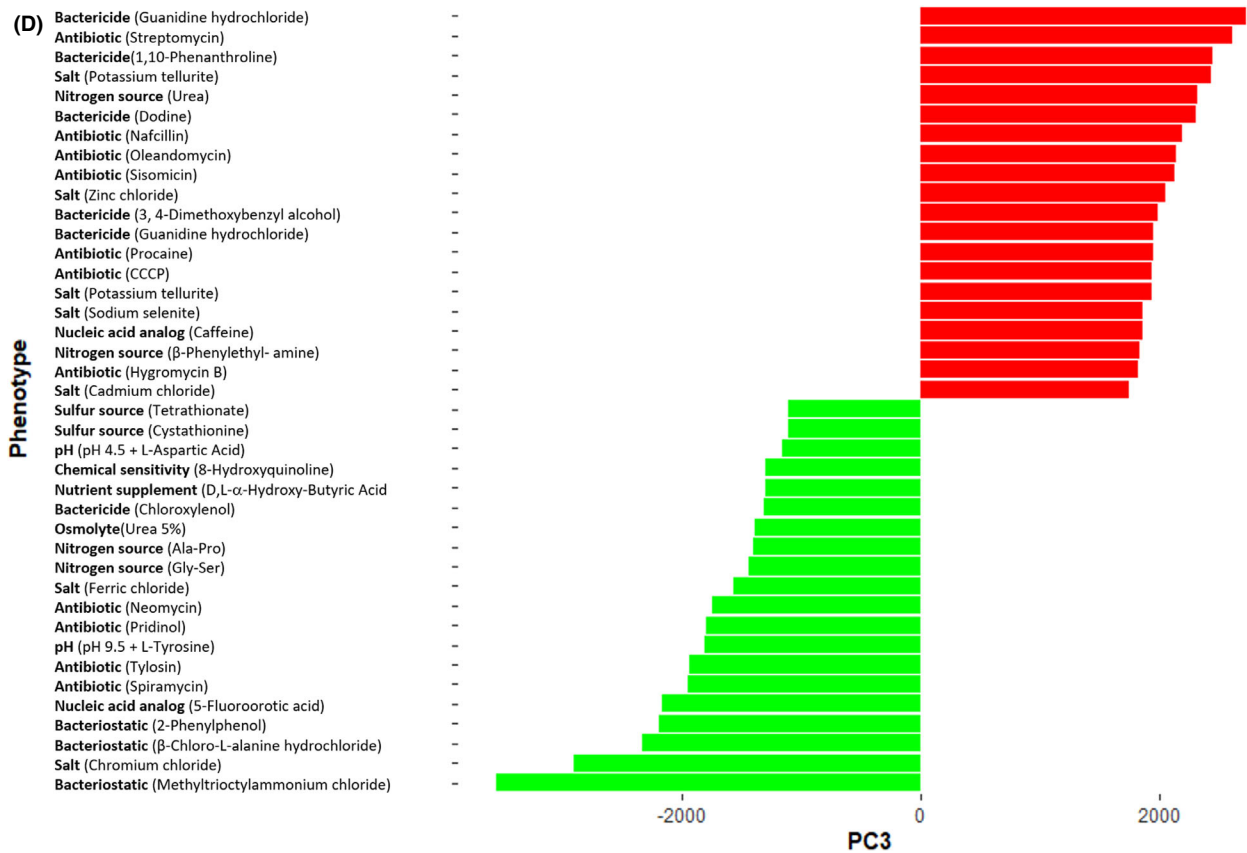
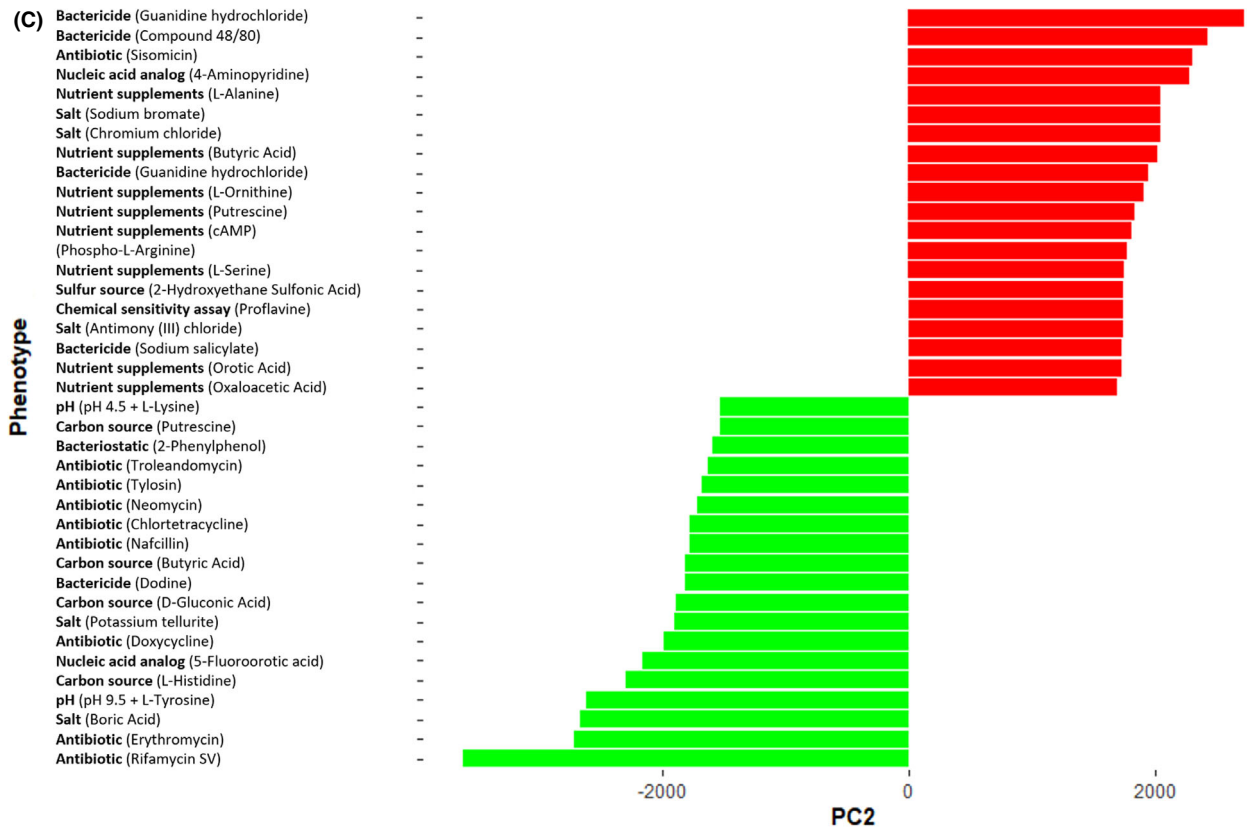
Due to the altered expression of flagellar genes, the swimming ability of the *mnr* mutant was evaluated and it revealed an increase in the size of the halo of 46% when compared to the wild-type strain (Fonseca *et al.*, 2008). This increase, however, was not observed in a different study, in which the mutant strain was slightly impaired in mobility when directly compared to the wild-type strain (Apura *et al.*, 2021). These differences were attributed to the fact that, in both studies, the media composition and the temperature used were different.

It was also shown that the absence of RNase R led to an increase in the mRNA levels of some RNA helicases, such as the RhIB (PP\_1295, or *rhIB*) and RhIE (PP\_4980, or *rhIE*), and ribonucleases, such as RNase E (PP\_1905 or *rne*), PNPase (PP\_4708 or *pnp*) and RNase D (PP\_4591 or *rnd*). It was also demonstrated that the growth rate of the *mnr* mutant strain at 10°C decreases, which is indicative of the importance of this ribonuclease at low temperatures (Fonseca *et al.*, 2008).

In *P. syringae*, RNase R was unexpectedly found to associate with RNase E and RhIE in the RNA degradosome complex (Purusharth *et al.*, 2005) (for further details of the RNA-degrading complex, go to the RNase E section). The identification of RNase R, instead of PNPase, in this complex further underlines the importance of studying the fundamental interactions between RNases to fully understand bacterial RNA metabolism. The mutant of this exoribonuclease (Table 1) was severely cold-sensitive. The *mnr* mutant presented a defective processing on the 16S rRNA and 5S rRNA, and it is possibly involved in the processing of 3' ends of both of these rRNAs. Similar to *E. coli*, the *P. syringae mnr* mutant also accumulated intermediates of the regulatory RNA, transfer messenger RNA (tmRNA) (Purusharth *et al.*, 2007). The genomic characterization of *mnr* gene revealed that this gene is present in a bicistronic operon and cotranscribed with the *trmH* gene (encoding a putative tRNA/rRNA methyl transferase) (Sulthana *et al.*,



**Fig. 2.** Distribution of *P. putida* strains with different RNase mutations based on their phenotypes. The area under the growth curve of the different phenotypes tested in BILOG analysis performed by Apura *et al.* (2021) for the *P. putida* KT2440 (WT) and 5 RNase single mutant strains: RNase G<sup>-</sup>, RNase E<sup>N-term</sup>, RNase III<sup>-</sup>, RNase R<sup>-</sup> and PNPase<sup>-</sup> were used as variables for a principal component analysis (PCA). The analysis was performed in R with the package FactoMineR, the variables were scaled using UV and centred. (A) PCA score graph, with three principal components. First component (PC1) is responsible for 64.7% of variation; PC2 responsible for 18.6% of variance and PC3 responsible for 8.2% of variance. Different strains are colour-highlighted: WT (red), RNase G<sup>-</sup> (grey), RNase E<sup>N-term</sup> (yellow), RNase III<sup>-</sup> (pink), RNase R<sup>-</sup> (green) and PNPase<sup>-</sup> (blue). (B) Top 20 positive and negative phenotypes responsible for the PC1. (C) Top 20 positive and negative phenotypes responsible for the PC2. (D) Top 20 positive and negative phenotypes responsible for the PC3. Phenotypes increased in the PC are in red, while phenotypes decreased in the PC are in green (in panels B, C and D).



2011). This exoribonuclease was characterized as a psychrophilic enzyme with an optimum activity at 22°C, presenting maximum activity towards poly(A) and poly(U) substrates and generating tetramer nucleotides as end-product (Sulthana *et al.*, 2011).

### Oligoribonuclease

Oligoribonuclease (Orn), encoded by the gene *orn*, is a highly conserved 3'-5' exoribonuclease responsible for the degradation of short oligoribonucleotides of 2–5 nucleotides that result from the degradation process of other RNases (Stevens and Niyogi, 1967; Niyogi and Datta, 1975). It belongs to the DEDD family of exoribonucleases (Zuo and Deutscher, 2001) and produces mononucleotides, requiring the presence of divalent cations ( $Mn^{2+}$ ) (Niyogi and Datta, 1975). In order to degrade oligoribonucleotides, this enzyme needs a free 3'-OH end and is not sensitive to the 5'-phosphorylation state of the RNA (Datta and Niyogi, 1975). In *E. coli*, it is the only exoribonuclease necessary for cell viability (Ghosh and Deutscher, 1999; see Table 1). In the RNA metabolism, this ribonuclease acts at the final part of the degradation process.

Regarding the genomic context of *orn* gene, it seems to have conserved the upstream gene *rsgA*, which codifies to the small ribosomal subunit biogenesis GTPase RsgA, between the *Pseudomonas* species and *E. coli* (see Fig. 1B).

In *P. aeruginosa*, the absence of this enzyme does not compromise the viability of the cells (Jacobs *et al.*, 2003; see Table 1). Depletion of this exoribonuclease causes accumulation of oligoribonucleotides, which might act as primers for transcription initiation in many promoters, bringing global repercussions to gene expression (Goldman *et al.*, 2011). A phenotype observed in Orn mutant cells was their tendency to clump and attach to the sides of the culture tubes, when grown in shaken cultures. This ultimately contributed to the discovery of the role of Orn in the suppression of *P. aeruginosa* cell aggregation, surface attachment and biofilm formation (Cohen *et al.*, 2015). Additionally, Orn plays an essential role in cyclic-di-GMP (c-di-GMP) turnover, an important secondary messenger, whose levels interfere with the motile or sessile lifestyle of this bacterial pathogen (Romling *et al.*, 2013; Cohen *et al.*, 2015; Orr *et al.*, 2015). It was also discovered that Orn might play an important role in the regulation of T3SS, which contributes to the *in vitro* and *in vivo* virulence of *P. aeruginosa* (Chen *et al.*, 2016b).

### RNase PH

RNase PH, encoded by the *rph* gene, is an exoribonuclease belonging to the PDX family of exoribonucleases,

as PNPase. It is involved in tRNA metabolism, specifically in the processing of tRNA precursors (Deutscher *et al.*, 1988; Kelly *et al.*, 1992). RNase PH can act as a phosphorolytic RNase by removing nucleotides following the CCA terminus of tRNA and also as a nucleotidyl-transferase by adding nucleotides to the ends of RNA molecules (Jensen *et al.*, 1992; Kelly and Deutscher, 1992). In addition to the tRNA precursor, RNase PH can also cleave off the 3' end of other small RNAs, including M1, 6S and 4.5S RNA (Li *et al.*, 1998). Although the sequences of these RNAs are significantly different, their structures are homologous to that of tRNA, and thus, it is likely that RNase PH recognizes and digests the substrate in a structure-specific manner rather than a sequence-specific manner. In *E. coli*, deletion of *rph* has no effect on the growth or the viability of the cells (see Table 1). However, the combination of this deletion with genetic mutations causing depletion of RNase T or PNPase affects growth. This suggests that RNase PH has overlapping functions *in vivo* with both RNase T and PNPase (Kelly *et al.*, 1992).

The genomic context of RNase PH seems to be conserved in *Pseudomonas* species in terms of number and genomic orientation of the genes (Fig. 1B). Nevertheless, it needs to be further explored regarding the resemblance of the final product of neighbouring genes.

Previous studies report that the *E. coli* RNase PH can form several different oligomers; however, it was unclear whether the formation of a hexameric ring was necessary for the biological function of the enzyme. This was first elucidated in *P. aeruginosa*, in which the crystal structure of RNase PH was obtained and revealed that the hexameric ring formation is essential for the catalytic activity on the 3' end of tRNA and the binding with precursor tRNA (Choi *et al.*, 2004).

### Phenotypic impact of ribonucleases in the biotechnologically relevant *P. putida*

In a recent study of the phenotypical differences of *P. putida* strains with single mutations on different RNases (RNase E, RNase III, RNase G, RNase R and PNPase), it was shown that those differences are dependent of the RNase mutation (Apura *et al.*, 2021). In a principal component analysis (PCA) using the phenotypical characteristics of each strain, is possible to observe that the loss of RNase III function is the one that causes more phenotypical alterations on *P. putida*, being separated from wild type in the first component (PC1, responsible for 64.7% of variation) (Fig. 2A). There is an increase in resistance to salts, as vanadium salts; to acids, as 5-fluoroorotic acid; or to antibacterial drugs, as sulphanilamide; on the other hand, RNase III<sup>-</sup> loss some phenotypes such as the utilization of some

amino acids as nitrogen source, L-serine and L-aspartate; and resistance to antibiotics, as josamycin, spectinomycin or polymyxin B; or to poly-L-lysine (Fig. 2 panel B, top 20 positive and negative phenotypes responsible for the PC1). By the PCA scores, on Fig. 2 panel A, it is also possible to observe that RNase E<sup>N-term</sup> and RNase G<sup>-</sup> are separated from the wild type in the second component (PC2, responsible for 18.6% of variance) and, in this case, *P. putida* gains resistance to compounds, such as guanidine hydrochloride, sisomicin or 4-aminopyridine; and the ability to utilize supplements, such as alanine, butyric acid or ornithine. On the other hand, they are more sensitivity to some antibiotics, as erythromycin, neomycin and rifamycin SV; and lose ability to use some compounds as carbon sources, such as L-histidine and D-gluconic acid (Fig. 2 panel C, top 20 positive and negative phenotypes responsible for the PC2). The separation between those two strains occurs in the third component (PC3, responsible for 8.2% of variance), being the principal alterations in the phenotypes related to resistance to chemicals (Fig. 2 panel C, top 20 positive and negative phenotypes responsible for the PC3). In comparison, the suppression of RNase R and PNPase activities are the ones that lead to less significant phenotypical difference relative to the wild type.

### Concluding remarks

The appearance of alternative bacterial hosts for synthetic biology applications have exacerbated the realization that there is no metabolism applicable to all microorganisms. Resource utilization influences the predictability, function and evolutionary stability of engineered pathways and, at the same time, constrains the desired parameter space for synthetic circuit design. Redesigning cell's metabolism requires a deeper understanding of the very core metabolic wiring in bacteria (Aslan *et al.*, 2017). Cells have at their disposal a limited resource quota, which is balanced between cellular processes and synthetic circuit functions. Despite the large number of mechanisms to control target gene expression, which includes engineered promoters (Mutalik *et al.*, 2013), tunable protein degradation (Cameron and Collins, 2014) or CRISPR interference (Jiang *et al.*, 2013; Qi *et al.*, 2013; Liu *et al.*, 2017), limited technologies exist to globally redistribute resources and reprogramme cellular state.

RNases are here presented as an important regulatory layer that deserves further investigation to construct efficient biofactories in synthetic biology. RNases constitute an endogenous regulatory network of the chassis and play a key role in the quick adaptation of bacteria to different internal and external challenges. Post-transcriptional processes provide a source of regulation

that saves time and energy as the mRNA translation and stability are regulated by RNases and ncRNAs. By binding to target mRNAs, ncRNAs affect ribosome accessibility or RNase-mediated cleavage. Meanwhile, the processing and stabilities of ncRNAs are under the control of RNases. Therefore, identification of the target ncRNAs and mRNAs of RNases is essential for the elucidation of the RNase-mediated regulatory pathways.

The examples described highlight the importance of understanding fundamental biology, but also show how much is still needed to explore. The study of RNases will certainly be important in order to further increase pseudomonads biotechnological potential and to design ways to fight pathogenicity.

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### Conflict of interest

None declared.

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