



RNase III influences microaerobic symbiotic pathways and RNA regulation in *Sinorhizobium meliloti*

Sabina K. Guedes-García^{a,1}, Natalia I. García-Tomsig^{a,2,3}, Rute G. Matos^{b,4},
Margarida Saramago^{b,5}, Cecilia M. Arraiano^{b,6}, José I. Jiménez Zurdo^{a,7,*}

^a Structure, Dynamics and Function of Rhizobacterial Genomes (RhizoRNA Lab), Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas (CSIC), Granada, Spain

^b Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Oeiras, Portugal

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ABSTRACT

Bacterial ribonucleases (RNases) are central components of post-transcriptional networks underlying environmental adaptation. However, their contribution to the ecological specialization of bacteria with complex life-styles, such as nitrogen-fixing legume symbionts, remains poorly understood. Here, we investigated the role of the double-stranded RNase III ortholog (*SmRNase III*) in *Sinorhizobium meliloti*, the symbiotic partner of alfalfa (*Medicago sativa* L.). Loss of *SmRNase III* function affected the expression of nearly 30% of protein-coding genes and 12% of annotated non-coding RNAs (sRNAs). Remarkably, more than 70% of these changes occurred under the microaerobic conditions typical of symbiotic nodules. Many *SmRNase III*-dependent transcripts encode pathways supporting microaerobic metabolism and nitrogen fixation in endosymbiotic bacteroids. Analysis of sequencing read coverage identified putative consensus cleavage signatures enriched in mRNA 5' untranslated regions, suggesting preferential processing at these sites. Altered expression of sRNAs and/or their predicted mRNA targets further supports a role for *SmRNase III* in sRNA-mediated silencing. Consistently, *in vitro* assays showed that base-pairing between *nifK* (encoding the β -subunit of the nitrogenase MoFe protein) and the antisense RNA *asNifK* promotes *SmRNase III*-mediated cleavage. *In vivo* assays further supported that silencing of *nifK* and *dctA* (encoding a major dicarboxylate transporter) requires *SmRNase III*, with *dctA* regulation involving a base-pairing interaction between the *trans*-sRNA *AbcR1* and a predicted *SmRNase III* cleavage site within the mRNA. Our findings reveal a major impact of *SmRNase III* on shaping the symbiotic transcriptome of *S. meliloti* and provide a foundation for deeper investigation into RNase III-mediated regulation in rhizobia.

1. Introduction

Ribonucleases (RNases) catalyze the processing and degradation of transcripts to adjust their levels to cellular needs. Consequently, these enzymes are regarded as global post-transcriptional regulators of gene expression (Arraiano et al., 2010). Bacteria are equipped with large sets of RNases with a diversity of activity mechanisms and substrate

preference. Specifically, the RNase III family gathers structurally and functionally conserved endonucleolytic enzymes that cleave double-stranded RNA (dsRNA) in a strict metal-dependent manner. Eukaryotic RNase III orthologues, such as Rnt1, Drosha, and Dicer, play a central role in processing dsRNA structures to produce mature short interfering RNAs, including microRNAs (miRNAs) and small interfering RNAs (siRNAs) (Hammond, 2005). In contrast, in bacteria RNA

* Corresponding author.

E-mail address: jjz@eez.csic.es (J.I. Jiménez Zurdo).

¹ 0009-0007-4204-6145

² Present address: Instituto de Biomedicina y Biotecnología de Cantabria, Universidad de Cantabria-CSIC, Cantabria, Santander, Spain

³ 0000-0002-1161-8709

⁴ 0000-0002-9788-7562

⁵ 0000-0003-0458-1036

⁶ 0000-0003-1934-9301

⁷ 0000-0002-6378-2199

processing and decay are largely attributed to RNase E, an endoribonuclease that targets single-stranded RNA (ssRNA), including small regulatory RNAs (sRNAs). Meanwhile, the major role assigned to bacterial RNase III is the maturation of ribosomal RNA (rRNA) (Lejars et al., 2021). However, the comprehensive characterization of the prototypical member of the family, the *Escherichia coli* RNase III (EcRNase III), encoded by the conserved *rnc* gene, has uncovered a broader impact of this enzyme in RNA metabolism. For example, RNase III regulates its own synthesis by removing 5' RNA structures that preclude degradation of the *rnc* mRNA, and in a similar way it also regulates the degradation of *pnp* mRNA encoding the polynucleotide phosphorylase (PNPase) exoribonuclease (Bardwell et al., 1989; Matsunaga et al., 1996; Régnier and Grunberg-Manago, 1990; Meur and Portier, 1992; Jarrige, 2001). It stands out that the specific activity on dsRNA substrates (*in vitro* and *in vivo*) envisages a prevalent role of RNase III as effector of mRNA processing and silencing upon base pairing with sRNAs of either the antisense (asRNAs) or *trans*-acting classes. Indeed, asRNA-mediated regulation of plasmid copy number and toxin-antitoxin systems is known to involve RNase III in some bacteria (Vogel et al., 2004; Lasa et al., 2011; Viegas et al., 2010; Svensson and Sharma, 2021; Mediati et al., 2022; Lejars et al., 2022). However, the function of RNase III in global adaptive regulatory networks has been only investigated in the model *E. coli* and a few other pathogens such as *Bordetella pertussis*, *Bacillus subtilis* or *Staphylococcus aureus* (Durand et al., 2012; Ifill et al., 2021; Lejars and Hajnsdorf, 2023). RNase III is not essential but *rnc* knockout severely hampers growth and influences virulence traits such as motility, biofilm development or host cell invasion in these bacteria.

The root nodule nitrogen (N₂)-fixing symbiosis between soil-dwelling rhizobia and legume plants is another noteworthy example of a host-microbe interaction but with a beneficial outcome. Successful legume infection demands rhizobia to rapidly adapt to a variety of environments within the plant (Oldroyd et al., 2011). In particular, the root nodules hosting mature differentiated intracellular bacteroids provide the microaerobic conditions required by nitrogenase to reduce the inert atmospheric dinitrogen to usable ammonia (Dusha and Kondrosi, 1993; Mendoza, 1995; Patriarca et al., 2002; Yurgel and Kahn, 2008; Batista and Dixon, 2019). Therefore, symbiotic gene expression reprogramming driven by plant cues in rhizobia is expected to concern post-transcriptional regulation by both sRNAs and RNases. *Sinorhizobium meliloti*, the symbiont of alfalfa (*Medicago sativa* L.) and related legumes, is a model rhizobial species with a well-characterized non-coding transcriptome (Jiménez-Zurdo et al., 2013; Schlüter et al., 2013; Jiménez-Zurdo and Robledo, 2015; Robledo et al., 2020). We previously reported on the catalytic properties and impact on symbiosis of *S. meliloti* RNase III (*SmRNase III*) (Saramago et al., 2018). This enzyme consists of an N-terminal catalytic domain (NucD) followed by a dsRNA-binding domain (dsRBD), both highly conserved. *SmRNase III* cleaves efficiently dsRNA substrates *in vitro* in the presence of either Mg²⁺ or Mn²⁺ as metal co-factors. However, the optimal activity conditions for *E. coli* and *S. meliloti* RNase III such as pH and metal concentration differ significantly, suggesting a mechanistic and functional adaptation to the environments specific to the lifestyle of each bacterial species (Saramago et al., 2018; Srivastava and Srivastava, 1996; Sun, 2005). Accordingly, lack of *SmRNase III* not only delays *S. meliloti* free-living growth but also nodulation and the onset of symbiotic nitrogen fixation.

In this work, we used strand-specific RNA-Seq to assess *SmRNase III*-dependent alterations in the abundance of both protein-coding and non-protein coding transcripts upon *S. meliloti* growth under aerobic or microaerobic conditions. Our data unveiled a widespread influence of this RNase on the steady-state levels of mRNAs encoding proteins central to bacteroid metabolism and N₂ fixation. Furthermore, we observed an impact of RNase III on the sRNA-mediated post-transcriptional regulation of these genes, positioning this enzyme as a key player in the riboregulatory network governing symbiotic pathways.

2. Materials and methodology

2.1. Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study along with their sources and relevant characteristics are listed in Table S1. *S. meliloti* strains were grown at 30°C in tryptone-yeast complex TY medium (Berlinger, 1974). To mimic the nodule microaerobic environment, bacteria were first grown to logarithmic phase (OD₆₀₀ of 0.4) in TY broth and microaerobiosis was then imposed by flushing flasks for 10 min with the mixture 2% O₂/98% N₂. Bacteria were further cultured under this condition at 60 rpm for 4 h prior to RNA extraction. *E. coli* strains were cultured in Luria-Bertani (LB) medium at 37°C. If required, antibiotics were added to the media at the following concentrations (µg/ml): tetracycline (Tc) 200, (Km) 50 for *E. coli* and 180 for *S. meliloti*.

2.2. Oligonucleotides

Table S2 provides the sequences and experimental applications of the oligonucleotides used in this work.

2.3. Construction of mutant strains

Strain Sm2020Δ*rnc* was generated by allelic exchange with pK18Δ*rnc* in Sm2020 as described (Saramago et al., 2018). Plasmids for constitutive expression of *nifK* and for IPTG-inducible expression of asRNA275 (formerly SmelA031; Schlüter et al., 2010) were generated as follows. The *nifK* coding sequence was amplified by PCR from genomic DNA of strain Sm2011 with Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific) using the primers NifKFBamHI and NifKRNheI. The purified PCR product was digested with BamHI/NheI, and inserted into pR-eGFP, yielding pRnifK::eGFP. To generate pSKI-asRNA275, the DNA sequence of asRNA275 and the promoter sequence *sinR-P_{sinI}-TSS_{sinI}* were amplified by PCR with Sma_{asRNA275F}/Sma_{asRNA275XbaI} and *sinR-NdeIF*/TSS3_28pb_{b_{sinR}} primer pairs. These overlapping amplification fragments were jointly used as the template for a second PCR with primers *sinR-NdeIF* and Sma_{asRNA275XbaI}. The final PCR product was restricted with NdeI and XbaI, and then inserted into pSRKKm. Plasmid constructs were verified by Sanger sequencing. Both plasmids, pSKI-asRNA275 and pRnifK::eGFP, were mobilized to Sm2020 and Sm2020Δ*rnc* strains by biparental mating. Similarly, plasmid pSKIAbcR1, which drives IPTG-induced expression of the AbcR1 sRNA (García-Tomsig et al., 2022), was conjugated into the same strains to assess AbcR1- and *SmRNase III*-dependent endogenous *dctA* levels.

2.4. RNA isolation

Total RNA was extracted from bacterial cultures using acid phenol-chloroform as described (Del Val et al., 2007). RNA samples were further treated with Invitrogen TURBO™ DNase for 1 h at 37°C, and subsequently cleaned up with the RNeasy Mini Kit (Qiagen) following the manufacturer guidelines. To enhance retention of the small RNA fraction, samples were mixed with seven volumes of 100% EtOH prior to column loading.

2.5. RNA sequencing and data analysis

RNA samples derived from wild type and SmΔ*rnc* strains grown under aerobic and microaerobic conditions (three biological replicates per strain and growth condition) were used to generate strand-specific cDNA libraries that were sequenced on the Illumina NextSeq Mid 500 platform to render paired-end reads (2 × 75 pb). Data were processed using R software (<https://www.r-project.org/>). Demultiplexed sequencing reads were mapped to the *S. meliloti* Rm1021 reference genome with the Rbowtie2 package v2.2.3 using standard parameters

(Shlüter et al., 2013; Au et al., 2010). Optical duplicates were removed with SAMtools (Li et al., 2009). Uniquely mapped reads were assigned to protein coding genes or non-coding RNAs with the Rsubread package v3.12 (Liao et al., 2019). Differential gene expression analysis was conducted with the DESeq 2 package v1.34.0 (Love et al., 2014). Coverage and transcriptome-wide read distributions were visualized with the Integrative Genomic Viewer (IGV) v2.12.3 software (Robinson et al., 2011). Plots were generated using the ggplot2 package v3.3.6 (Wickham, 2009). Searches for conserved motifs were done with the MEME algorithm (<http://meme-suite.org/index.html>). Venn diagrams were generated with the Venny 2.0 tool (<https://bioinfogp.cnb.csic.es/tools/venny/index2.0.2.html>). IntaRNA (v 3.2.0; <http://rna.informatik.uni-freiburg.de/>) was used to predict sRNA-mRNA base-pairing interactions. RNA secondary structures were predicted using RNAfold from the ViennaRNA package (Lorenz et al., 2011).

2.6. RT-qPCR

cDNA was synthesized with the Takara Prime Script RT Master Mix using 1 µg of total RNA. RT-qPCR was performed on a QuantStudio 3 system (Thermo Fisher Scientific) using the Takara TB Green Premix ExTaqII with 0.5 µl of cDNA. Transcript abundance ratios were calculated as the average $\Delta\Delta$ -CT (where CT is the threshold cycle) of the results of three amplifications using three independent RNA extracts, where $\Delta\Delta$ CT represents the level of gene expression in the IPTG-induced strain relative to the untreated control strain. The seemingly constitutive gene *SMC01852*, encoding a phosphofructokinase, was used to normalize gene expression (Becker et al., 2004). Non-reverse transcriptase (-RT) control reactions on the RNA samples were performed simultaneously to confirm the absence of DNA contamination.

2.7. In vitro activity assays

The plasmid encoding His-tagged *SmRNase III* was transformed into *E. coli* BL21(DE3) recArnc105 strain (Amarasinghe et al., 2001). The expression and purification of the recombinant *SmRNase III* was performed as previously described (Saramago et al., 2018). DNA templates for *in vitro* transcription of *SmRNaseIII* substrates were generated by PCR using genomic DNA from *S. meliloti* Sm2011 strain (primer pairs on Table S2). The phage T7 RNA polymerase promoter sequence was incorporated into the forward primers. *In vitro* transcription was carried out using the TransGen Biotech T7 High Efficiency Transcription Kit by overnight incubation at 37°C. The DNA template was removed by incubation of the reactions 30 min at 37°C in the presence of 1 U DNase. RNA transcripts were purified by electrophoresis on an 8.3 M urea/6% polyacrylamide gel. Gel slices containing the RNA transcripts were eluted overnight at room temperature with elution buffer [0.5 M ammonium acetate pH 5.2, 1 mM EDTA, 2.5% (v/v) phenol pH 4.3]. The RNA was ethanol-precipitated, resuspended in RNase free water and quantified using the Invitrogen Qubit™ RNA HS Assay Kit. The RNA substrates *as275* and *nifK* mRNA were labeled at their 5' ends with [γ -³²ATP] using New England Biolabs T4 Polynucleotide Kinase in a standard reaction. Labeled substrates yields (cpm/µl) were determined by scintillation counting. The hybridization between labeled and non-labeled substrates to generate dsRNA was always performed in 30 mM Tris-HCl solution (pH 8) by incubation for 10 min at 80°C, followed by 45 min at 37°C. The molar ratio of unlabeled to labeled substrate molecules was 5:1.

SmRNaseIII activity assays were performed in a final volume of 50 µl containing the activity buffer (30 mM Tris-HCl pH 8, 160 mM NaCl, 10 mM MgCl₂ and 0.1 mM DTT) and the RNA substrate (concentrations indicated in the figure legends). As a control, mock-treated reactions were incubated until the end of the assay. The reactions were initiated by the addition of the enzyme, and further incubated at 30°C. At designated time points, 5-µl aliquots were withdrawn and mixed with formamide containing dye supplemented with 10 mM EDTA. Reaction

products were resolved in a 7 M urea/polyacrylamide gel (polyacrylamide concentrations are indicated in the respective figure legends) and visualized by digital imaging of the exposure plate (Fuji), on a Personal Molecular Imager FX scanner and analyzed with the Quantity One software (Bio-Rad). Each activity assay was performed at least in triplicate.

3. Results

3.1. *SmRNase III*-dependent alterations of the *S. meliloti* transcriptome

Lack of *SmRNase III* confers pleiotropic free-living and symbiotic phenotypes to *S. meliloti* (Saramago et al., 2018), suggesting a broad influence of this enzyme on the turnover of the cellular transcripts. We have therefore investigated the differences in the transcriptomic profiles between a wild-type strain (Sm2011) and its *rnc* deletion mutant derivative (*SmΔrnc*) when cultured under the aerobic and microaerobic (2% O₂) conditions prevailing during *S. meliloti* free-living growth and symbiotic N₂-fixation, respectively. Illumina-based sequencing of rRNA-depleted bacterial RNA delivered an average of 10 million reads per sample, which we mapped to the *S. meliloti* Rm1021 reference genome retrieved from the RhizoGATE database (Becker et al., 2009). This annotation incorporates the *trans*-sRNA (605) and asRNA (3720) loci identified experimentally by genome-wide determination of transcription start sites (Schlüter et al., 2010, 2013). After normalization of read counts, only loci with coverage exceeding 10 reads in at least two of the experimental conditions were retained for further analysis. Genes were considered differentially expressed between strains and/or growth conditions if they exhibited at least a 2-fold change in read counts (i.e., log₂FC ≥ 1) with a *P* value ≤ 0.05 across the three biological replicates (Dataset S1). These replicates showed a high degree of concordance in gene expression (*R* > 0.95), as revealed by multidimensional scaling (MDS) and by clustering of the top 250 differentially expressed-genes (Fig. S1A), thus confirming the reliability and robustness of our transcriptomic dataset. Besides, the absence of reads mapping to the *rnc* locus in the mutant strain (Fig. S1B), along with the upregulation of *bona fide* N₂-fixation genes in Sm2011 under microaerobic conditions (Fig. S1C) further validated the experimental setup. As independent experimental validation, RT-qPCR confirmed upregulation of *nifA* and *fixK1* in both the wild-type strain and the *SmΔrnc* mutant, specifically under microaerobic conditions (Fig. S1D).

Our analysis evidenced that microaerobiosis imposed to Sm2011 log cultures drove differential expression of 796 out of the ~6200 protein-coding genes (mRNAs) predicted in the genome (~13%), roughly confirming previous microarray data from similar experiments (Becker et al., 2004) (Fig. 1A). Additionally, we also identified 97 microaerobiosis-dependent sRNAs, 37 and 60 of those annotated as *trans*-acting and asRNAs, respectively, all of them with an unknown function. *SmRNase III* knockout caused misregulation of 654 mRNAs (10.5% of the genome) in aerobic conditions, whereas upon microaerobic growth this number raised to 1706 (27%). Microaerobiosis similarly boosted differential expression of sRNAs in *SmΔrnc*, increasing their number from 96 (40 *trans*-sRNAs/56 asRNAs) identified in aerobically-growth bacteria to 300 (118 *trans*-sRNAs /182 asRNAs) (Dataset S1). It is also noteworthy that a substantial number of leader mRNAs, 302 in total, are differentially expressed in the absence of *SmRNase III* under both oxic and microoxic culture conditions (Fig. 1A). Volcano plots illustrating the scattering of differentially expressed mRNAs and sRNAs in *SmΔrnc* when compared to the wild-type strain showed that 524 of those were downregulated by the loss of *SmRNase III* activity, whilst 388 were upregulated during bacterial growth in aerobic conditions. Under microaerobiosis, lack of *SmRNase III* negatively affected accumulation of 1156 transcripts, whereas the levels of 1186 increased (Fig. 1B). Notably, in this condition the genes affected most significantly mapped to the *S. meliloti* symbiotic megaplasmid pSymA. Further comparative analysis of our transcriptomic datasets, illustrated

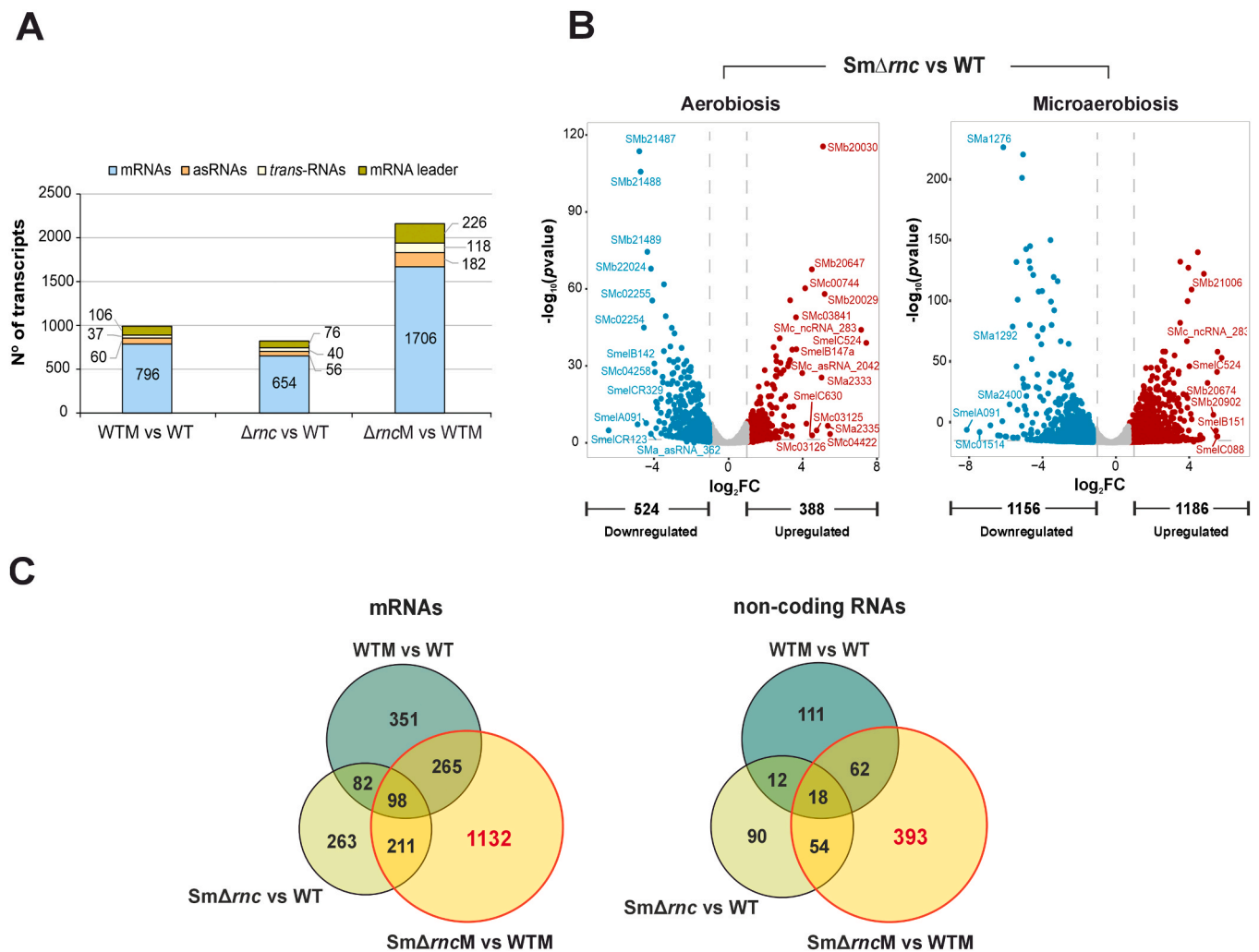


Fig. 1. Overview of the RNA-Seq data. A) Number of differentially expressed mRNAs, asRNAs, *trans*-sRNAs, and mRNA leaders identified in each transcriptomic comparison. M, microaerobic conditions; WT, wild-type strain; Δrnc , *SmRNase III* deletion mutant (*Sm Δrnc*). B) Volcano plots showing the $\log_2$ fold change (x-axis) versus the $-\log_{10}$ adjusted *P*-value (y-axis) for each gene in the *Sm Δrnc* vs WT comparison in aerobic and microaerobic conditions as indicated on top. Genes with statistically significant differential expression ($P < 0.05$) are highlighted in color: upregulated genes in red and downregulated genes in blue. Genes without significant differential expression are shown in gray. Horizontal and vertical dashed lines indicate the significance threshold ($P < 0.05$) and fold change cutoff ($\log_2 FC \geq 1$), respectively. C) Proportional Venn diagrams comparing differentially expressed mRNAs and sRNAs across transcriptomic comparisons. Numbers of misregulated transcripts in the mutant and specifically under microaerobic conditions are highlighted in red.

through proportional Venn diagrams, revealed that the absence of *SmRNase III* alone is sufficient to cause misregulation of 1606 mRNAs and 537 sRNAs across both tested growth conditions. This corresponds to approximately 25% of the protein-coding and 12% of the non-coding transcriptome of *S. meliloti*, indicating that *SmRNase III* exerts a broad regulatory influence, either directly or indirectly (Fig. 1C). Notably, over 70% of these transcripts (1132 mRNAs and 393 sRNAs) are specifically affected under microaerobic conditions. These findings underscore the pivotal role of *SmRNase III* in maintaining *S. meliloti* RNA homeostasis, particularly under the microoxic conditions characteristic of symbiotic root nodules.

3.2. Widespread effect of *SmRNase III* on genes involved in iron uptake, N_2 fixation and microaerobic metabolism

To identify the cellular processes affected by the absence of *SmRNase III* in *S. meliloti*, we performed a gene ontology (GO) analysis on the RNA-Seq datasets (Alexa et al., 2006) (Fig. 2). *SmRNase III*-dependent genes with predictable function, and identified under aerobic and microaerobic growth conditions were categorized into 72 and 82 established GO terms ($P < 0.05$), respectively (Dataset S1). A significant

number of genes with altered expression in the *Sm Δrnc* mutant are involved in biosynthetic and energy metabolism pathways. A notable subset of these genes is responsible for the uptake of iron complexed with the siderophore rhizobactin 1021, which is specifically secreted by the *S. meliloti* 1021/2011 strains (Persmark et al., 1993). The oxygen downshift promoted upregulation of the whole set of regulatory and structural genes involved in rhizobactin 1021 biosynthesis and transport in the wild-type strain. However, in the *Sm Δrnc* mutant this iron uptake pathway was markedly silenced under both aerobic and microaerobic growth conditions, suggesting that *SmRNase III* positively regulates these genes at the post-transcriptional level in an O_2 -independent manner.

SmRNase III also influenced the expression of numerous genes known to support bacteroid metabolism within root nodules, most notably in microaerobic cultures. These include some involved in N_2 fixation, microaerobic N metabolism or dicarboxylate transport. The levels of mRNAs encoding the master transcriptional regulators of symbiotic nitrogen fixation, *nifA* and *fixK1/2*, increased in the *Sm Δrnc* mutant, indicating negative post-transcriptional regulation by *SmRNase III*. Of note, we found a similar *SmRNase III*-dependent expression pattern for the two copies of the FixK-dependent gene cluster *fixNOQP*,

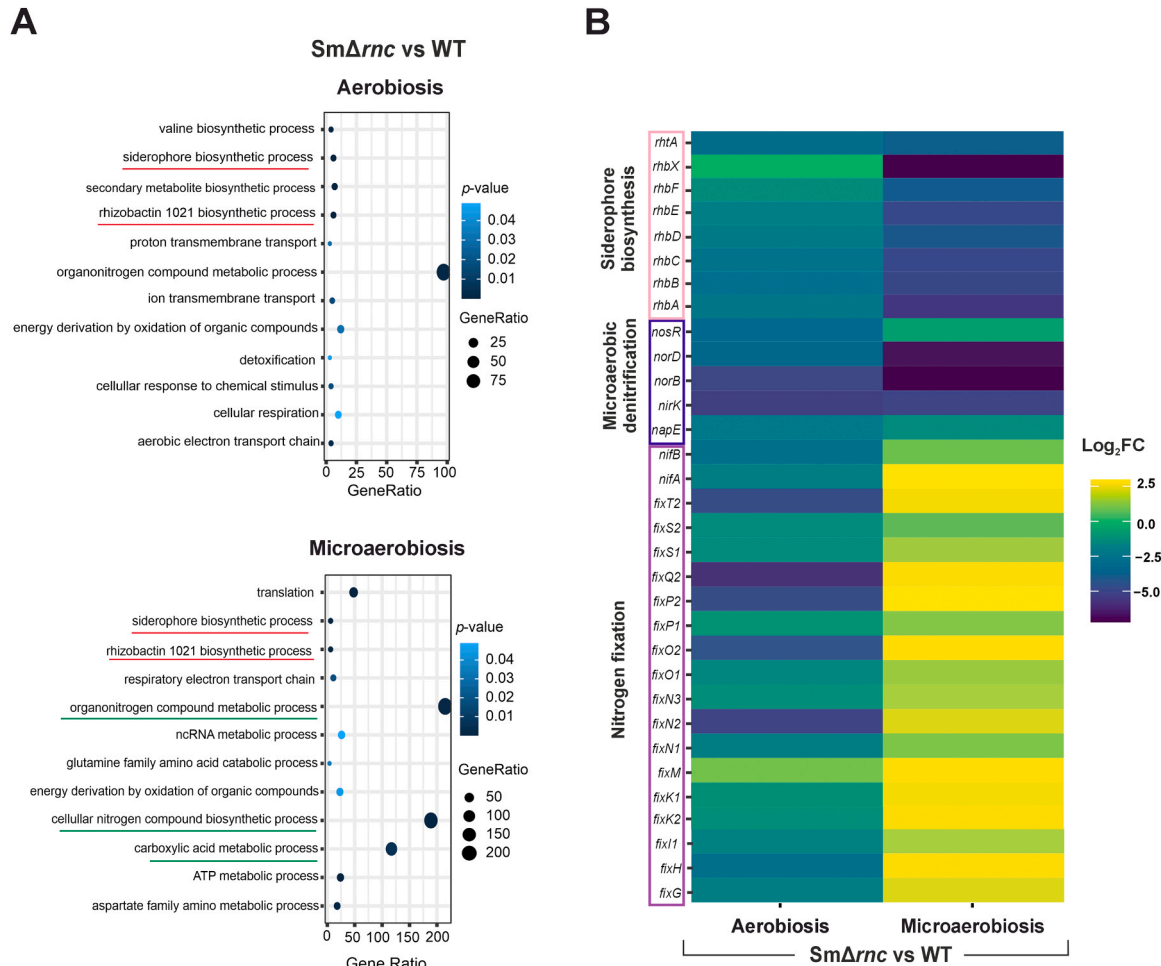


Fig. 2. Gene ontology (GO) analysis of *SmRNase III*-dependent mRNAs. A) Dot plot showing misregulated pathways in the *Sm Δ *rnc** mutant under aerobic and microaerobic conditions. Pathways highlighted in red are downregulated, while those in green are upregulated. Dot size reflects the number of genes associated with each GO term, and color intensity indicates statistical significance (*P*-value indicated to the right). B) Heatmap of differentially expressed genes involved in iron uptake, microaerobic denitrification, and N_2 fixation pathways. Color intensity represents relative expression levels, with yellow indicating higher expression and dark green lower expression in the *Sm Δ *rnc** mutant compared to the wild-type strain (WT).

required for the synthesis of the *cbb3* cytochrome oxidase (Salas et al., 2021). Denitrification was another noteworthy microaerobic metabolic pathway influenced by *SmRNase III*. Specifically, *napE*, *nirK*, and the gene cluster *norECBQD*, which encode periplasmic nitrate reductase, copper nitrite reductase and nitric oxide reductase, respectively, were all downregulated in *Sm Δ *rnc**, more significantly in microaerobiosis (Salas et al., 2021). Overall, these findings predict a major impact of *SmRNase III* in the post-transcriptional fine-tuning of bacteroid metabolism.

3.3. Inferring putative *SmRNase III* substrates from the RNA-Seq data

The misregulated mRNAs and sRNAs in *Sm Δ *rnc** are either direct *SmRNase III* substrates or indirectly influenced secondary targets. A detailed analysis of sequencing coverage across annotated genomic features may reveal transcript-processing signatures, thereby facilitating the identification of potential endogenous substrates for *SmRNase III*. In addition to its own mRNA (*rnc*), bacterial RNase III is well known for processing *pnp* mRNA, as well as rRNA precursors and their interspaced tRNAs (Court et al., 2013; Jarrige, 2001; Lejars et al., 2021; Meur and Portier, 1992). In our experiments, *pnp*, 23S rRNA, and some tRNAs

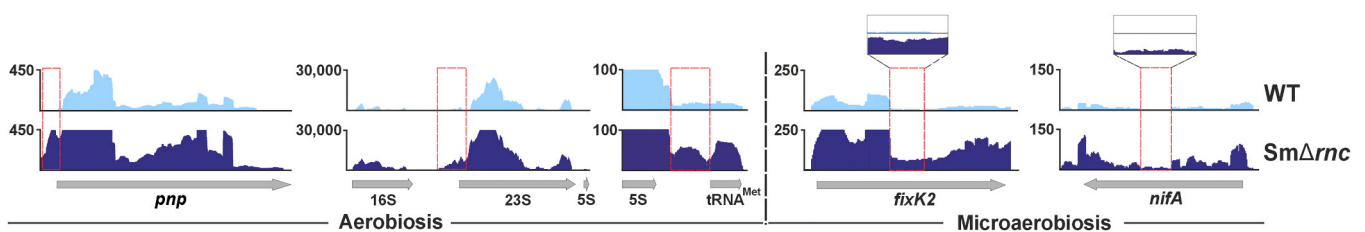


Fig. 3. Read coverage profiles of putative endogenous *SmRNase III* substrates in the wild-type strain (WT) and the *Sm Δ *rnc** mutant. Culture conditions are indicated below each panel. The height of the coverage tracks reflects the relative abundance of transcripts. Boxes with red dotted lines demarcate 5' regions putatively cleaved by *SmRNase III*, or sequence stretches showing a sharp drop in read density in the wild-type strain, consistent with potential *SmRNase III* cleavage sites (zoomed in for *fixK2* and *nifA* mRNAs).

likely co-transcribed with rRNA genes (e.g., tRNA^{Met} encoded by *SMC02623*) accumulated in *SmΔrnc* as 5'-extended isoforms of the respective wild-type mature RNA species (Fig. 3). These coverage patterns suggest that *SmRNase III* is involved in the maturation of *pnp*, rRNA and tRNA precursors primarily through cleavage at their 5' regions, as described in other bacterial species (Court et al., 2013; Lejars et al., 2021). A genome-wide search for elongated mRNA isoforms with significantly increased accumulation in their virtual 5'-untranslated regions (UTR; extending from nucleotide positions -50 to +100 relative to the translation start codon) in *SmΔrnc*, compared to the wild-type, identified a list of 141 and 585 *SmRNase III* possible target candidates in aerobiosis and microaerobiosis, respectively. This list also includes the *S. meliloti rnc* mRNA as the 5'-UTR of the *rnc* gene was not deleted in the mutant.

RNase III-mediated cleavage may also occur within either mRNA CDSs or sRNAs at sites that are particularly enriched in sequencing reads from *rnc* knock-out mutants compared with wild-type bacteria (Gordon et al., 2017). This was evident, for example, in the *fixK2* and *nifA* mRNAs, where wild-type sequencing data revealed complete absence of coverage over specific sequence stretches (Fig. 3). Further scanning of the *S. meliloti* genome in 20-nt windows to search for this coverage hallmark unveiled 572 mRNAs, notably including *fixK2* and *nifA*, and 72 sRNAs as *SmRNase III* target candidates. To investigate potential structural or sequence-based cleavage signatures we performed an analysis in which 100-nt sequences flanking the predicted cleavage sites were extracted and compared. Based on structural similarity determined by RNAfold predictions, the sequences were grouped into four distinct structural clusters. Structural conservation among cluster members was assessed through a dot-bracket heatmap (Fig. S2). This approach enables the inference of conserved structural control points that may be functionally relevant and potentially involved in RNA processing by *SmRNase III* as described (Gordon et al., 2017; Huang et al., 2020). Although RNase III primarily recognizes structural features, it also exhibits moderate preferences for specific local sequences at cleavage sites, such as AU-rich regions flanking the site and GC-rich sequences in the resulting 2-nucleotide overhangs, which may influence the efficiency and precision of RNA processing. (Zhang and Nicholson, 1997; Huang et al., 2020). To identify such potential consensus sequences, we analyzed each cluster individually. No conserved motifs were found in structural Clusters I, II, and III. However, Cluster IV consistently displayed the conserved motif 'UCGGCAU' across all included sequences, with an E-value of 1.4×10^{-3} (Fig. S2).

To evaluate whether *SmRNase III* preferentially cleaves 5'-UTR regions, we compared predicted cleavage sites with annotated virtual 5' UTRs (Dataset S2). A chi-squared test did reveal a significant preference for cleavage within 5'-UTRs, suggesting that *SmRNase III* modulates transcript stability or maturation at this level. Predicted *SmRNase III* substrates are enriched in mRNAs involved in amino acid catabolism, nucleotide metabolism, and gene regulation. Notably, pathways related to the degradation of glutamine family and arginine-derived amino acids, as well as cyclic nucleotide biosynthesis, were also significantly enriched, highlighting a potential role in N balance and metabolic signaling. Additionally, enrichment in transcriptional and RNA biosynthetic processes suggests that *SmRNase III* may regulate gene expression at both transcriptional and post-transcriptional levels. These findings support a model in which *SmRNase III*-mediated RNA processing is tightly integrated with cellular metabolic reprogramming and stress adaptation.

3.4. *SmRNase III* regulation of mRNA turnover induced by antisense transcription

Transcriptomic analysis of *SmΔrnc* unveiled a total of 56 and 182 asRNAs differentially expressed after aerobic and microaerobic bacterial growth, respectively, with 20 common to both conditions (Fig. 1A). Notably, among these transcripts are *SMc_asRNA_744*,

SMc_asRNA_1551, *SM_asRNA_1120*, *SMA_asRNA_380*, and *SMc_asRNA_936*, which are antisense to the protein-coding genes *metB*, *dgkA*, *dgkB*, *napC*, and *cyaE1*, respectively. These genes are involved in fundamental metabolic and regulatory pathways, including methionine biosynthesis, membrane lipid turnover, periplasmic nitrate reduction, and nucleotide signaling.

Specifically, 36 and 162 asRNAs were misregulated in the absence of *SmRNase III* under aerobic and microaerobic conditions, respectively. Notably, nearly 90% (142) of the microaerobically misregulated asRNAs were upregulated in the mutant strain, including those antisense to key N₂ fixation genes such as *nifK* (encoding a nitrogenase structural component), *nifN* (involved in Fe-Mo cofactor biosynthesis), and *fixX* (encoding a ferredoxin). The asRNA *SMc_asRNA_1268*, antisense to *nouN*, was also induced in the *SmRNase III* mutant in microaerobiosis. *NouN* is a component of the NADH-ubiquinone oxidoreductase complex, a central enzyme in aerobic respiration and energy production. Collectively, these findings suggest a prominent role for the concerted activity of asRNAs and *SmRNase III* in fine-tuning aerobic energy metabolism and microaerobic N₂ fixation in endosymbiotic bacteroids.

3.5. *SmRNase III* mediates *nifK* silencing driven by *asnifK1*

To further investigate the role of *SmRNase III* as an effector of mRNA silencing mediated by asRNAs, we performed a detailed analysis of the interaction between *nifK* mRNA and asRNA275. The *nifK* mRNA (*SMA0829*), which encodes the β -subunit (FeMo protein) of the *S. meliloti* nitrogenase complex, is not among the predicted *SmRNase III* putative substrates (Dataset S2). However, among the three sRNAs likely transcribed antisense to *nifK*, asRNA275 (also annotated as *SMA_asRNA_275* or *SmEla031*), though it is also not among the *SmRNase III* target candidates (Dataset S2), was prominently represented in our RNA-Seq dataset, showing upregulation in the *SmΔrnc* mutant under microaerobic conditions (Fig. 4A). Accordingly, we designated this transcript as *asnifK1*.

We initially performed *in vitro* cleavage assays with *asnifK1* and *nifK* transcripts using purified *SmRNase III* (1 μ M) and Mg²⁺ as a cofactor (Fig. 4B). The enzyme was first incubated with radiolabeled *asnifK1* and *nifK* in separate reactions. These experiments revealed modest cleavage of *asnifK1* by *SmRNase III*, as evidenced by slight, time-dependent degradation of the full-length transcript (269 nt), whereas the *nifK* substrate (298 nt) remained unaffected (Fig. 4B, panels a and b). As expected, shorter RNA species detected on gels, commonly observed in this type of assays due to incomplete synthesis caused by premature transcription termination and/or radiolysis events, were unaffected by *SmRNase III* activity. However, when radiolabeled *nifK* was incubated with unlabeled *asnifK1* to promote duplex formation, the resulting dsRNA substrate was completely degraded after 15 min of incubation with *SmRNase III*, leading to the accumulation of a small RNA species that, according to the labeling strategy, contains the *nifK* 5' end (Fig. 4B, panel c). To test whether prior processing of the asRNA affects duplex cleavage, unlabeled *asnifK1* was pre-incubated with *SmRNase III* before the addition of radiolabeled *nifK*. Under these conditions, *nifK* degradation and accumulation of the same 5'-end cleavage product were still detectable, albeit only after long incubation (60 min) and at much reduced levels (Fig. 4B, panel d), thus confirming that *asnifK1* is pre-processed, which likely compromises duplex formation. These assays support the prediction that *asnifK1* and *nifK* individually function as poor substrates for *SmRNase III* activity, whereas duplex formation markedly enhances *nifK* cleavage *in vitro*.

As a primary assessment of *nifK* regulation by *asnifK1* *in vivo*, we employed a dual-plasmid assay in which the asRNA and its mRNA target were expressed independently from compatible plasmids, bypassing their endogenous transcriptional regulation. To confirm IPTG-induced overexpression of *asnifK1* from plasmid pSKI-*asnifK1*, we first performed RT-qPCR in the wild-type strain grown aerobically in TY medium (Fig. 4C, left). Under these conditions, abundance of *nifK* mRNA,

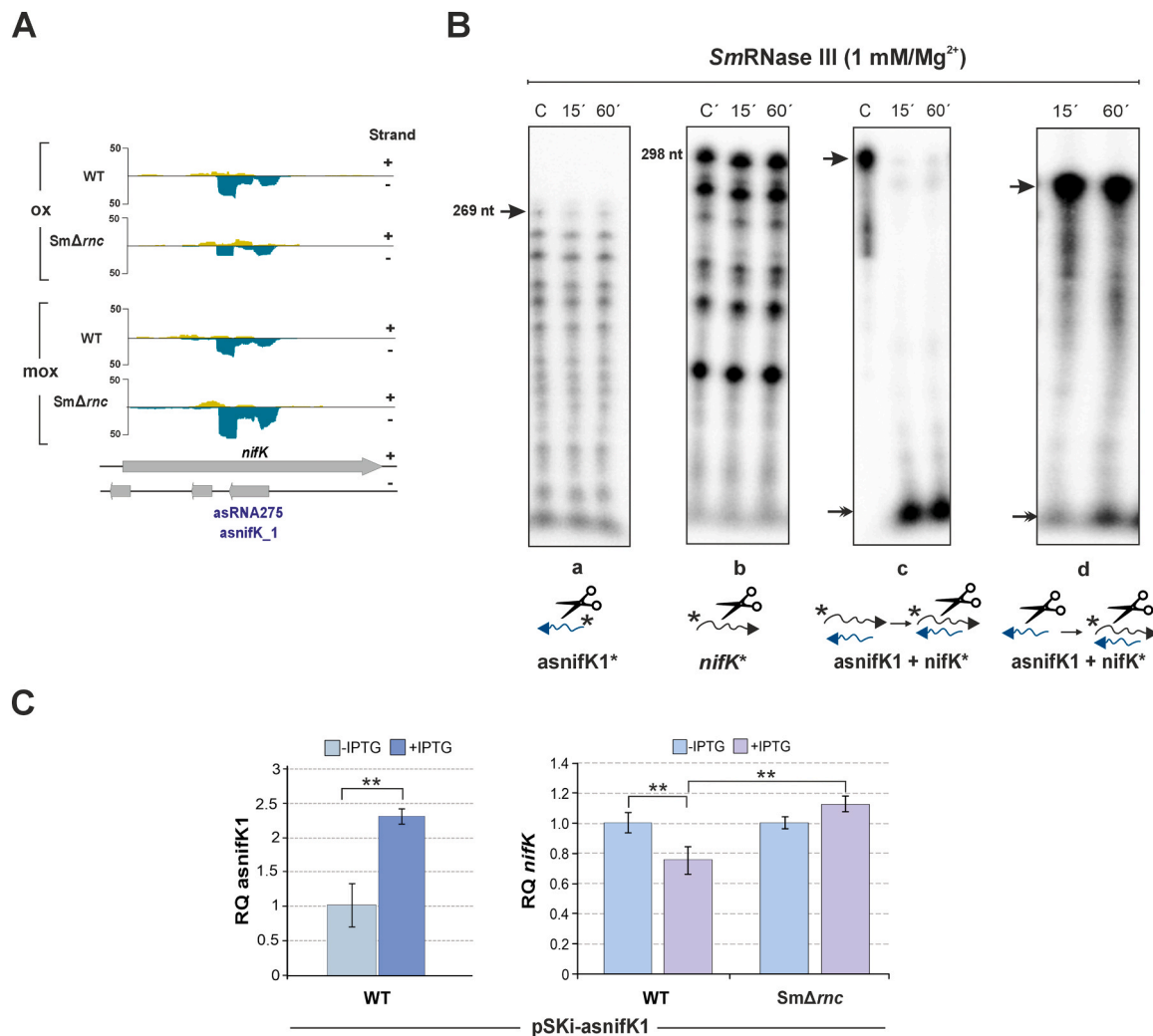


Fig. 4. *SmRNase III*-assisted *asnifK1*-mediated regulation of *nifK*. A) Read coverage of *nifK* and *asnifK1* in wild-type (WT) and *SmΔrnc* strains under aerobic and microaerobic conditions. B) *SmRNase III* activity on *asnifK1*, *nifK*, and its duplex. *In vitro* reactions were carried out at 30 °C for the indicated conditions and time points and analyzed on 7 M urea/6% polyacrylamide gels. "C" denotes control reactions without the enzyme. Single and double arrowheads indicate full-length RNA species (when assayed independently) and cleavage products, respectively. The order in which the enzyme and the RNA substrates were added is shown below each panel; *, radiolabeled RNA. C) RT-qPCR analysis of the impact of ectopic IPTG-induced *asnifK1* overexpression from plasmid pSKI-*asnifK1* on *nifK* mRNA abundance. Determinations were carried out in duplicate for RNA from three biological replicates (n = 6). Relative quantification (RQ) values of *asNifK* (left) and *nifK* (right) were normalized to *SMC01852* as a constitutive control. Asterisks indicate statistically significant differences according to ANOVA (P < 0.05).

constitutively expressed from a synthetic promoter (*Psyn*) on plasmid pR-*nifK*, was significantly reduced in wild-type cells overexpressing *asnifK1* following IPTG induction, but remained unchanged in the *SmΔrnc* mutant (Fig. 4C, right). These results provide genetic evidence, additional to that obtained from the *in vitro* assays, supporting the involvement of *SmRNase III* in *asnifK1*-dependent silencing of *nifK* mRNA *in vivo*.

3.6. *SmRNase III* assists regulation by *trans*-sRNAs

Bacterial RNase III also functions as a silencing enzyme for mRNAs that base-pair with *trans*-encoded sRNA regulators. In *S. meliloti*, the targetomes of the homologous *trans*-sRNAs AbcR1 and AbcR2, which function as central regulatory hubs within extensive metabolic networks, have been well characterized (García-Tomsig et al., 2022). Interestingly, 111 and 146 *SmRNase III*-dependent mRNAs are part of the AbcR1 and AbcR2 regulons, respectively (Dataset S3; Fig. S3A). Functional analysis of these mRNA sets revealed enrichment in pathways related to carbohydrate metabolism, environmental stress response, microaerobic adaptation, N₂ fixation, redox homeostasis, and

the transport of various nutrients (Dataset S3). Specifically, the mRNA set overlapping the *SmRNase III* and AbcR1 regulons includes *dctA*, which encodes a key dicarboxylate transporter. Previously, we reported that *dctA* co-purifies with an MS2-tagged AbcR1 in affinity-chromatography experiments, showing enrichment at its 3' region and thereby supporting the *in vivo* interaction between both transcripts (Fig. S3A) (García-Tomsig et al., 2022). The steady-state levels of AbcR1 remained unaffected by microaerobic conditions or deletion of the *rnc* gene, suggesting that *SmRNase III* does not participate in its turnover (Dataset S1; Fig. S3B). Conversely, *SmRNase III* influences *dctA* in a condition-dependent manner. Under aerobic conditions, *dctA* is upregulated in the *SmΔrnc* mutant, whereas its levels decrease in microaerobiosis (Fig. S3B). Consistent with these observations, *dctA*, but not AbcR1, was identified as a putative *SmRNase III* substrate in our predictions (Dataset S2). *In vitro* assays failed to reveal *SmRNase III*-mediated cleavage of AbcR1 and, strikingly, also of *dctA*, whether tested as individual substrates or as a duplex (Fig. S4). IntaRNA-based computational predictions suggest that the two previously characterized AbcR1 anti-Shine-Dalgarno regulatory motifs, aSDa (nt 22–32) and aSDb (nt 50–58), may function as seed-pairing regions that interact at different

sites within the *dctA* CDS, with a bias toward the 3' region of the mRNA (positions 1281–1287 and 850–866 relative to 50 nt upstream of the AUG start codon, respectively; Fig. 5A). We interpret the seemingly negative *in vitro* results as evidence that the short, imperfect base-pairing with AbcR1 is likely required for *dctA* cleavage but depends on the RNA chaperone Hfq, as strongly suggested by our previous identification of both AbcR1 and *dctA* as Hfq-binding RNAs in *S. meliloti* (Torres-Quesada et al., 2014).

We therefore used the *in vivo* pSKI overexpression system to assess endogenous AbcR1-dependent *dctA* regulation in both $\Delta abcR1$ and $\Delta abcR1/\Delta rnc$ backgrounds (Fig. 5B). Total RNA was extracted from aerobic log-phase cultures of the transconjugants, both before and 16 h after IPTG induction of wild-type AbcR1 and its regulatory variants AbcR1a and AbcR1b from the corresponding pSKI derivatives. These variants carry point mutations in aSDa or aSDb, respectively, which preserve the predicted secondary structure of AbcR1 while disrupting base pairing with the target mRNA (Fig. 5A) (García-Tomsig et al., 2022). RT-qPCR revealed that AbcR1 silences *dctA* in the presence of *SmrNase III*, whereas regulation is lost in the Δrnc background, in which *dctA* levels increase, confirming the RNA-Seq data. Silencing persists

with AbcR1b (mutated in aSDb) but is abolished with AbcR1a (mutated in aSDa), indicating aSDa-mediated regulation. Notably, the aSDa interaction site in *dctA*, but not that of aSDb, corresponds to one of several *SmrNase III* cleavage sites predicted within this mRNA (genomic position 1626,701 +/-20 nt; Dataset S2). Strikingly, we also observed stronger *dctA* silencing induced by AbcR1b in the wild-type background, likely due to the emergence of a novel interaction site with *dctA* in this mutant variant (Fig. S5; left). Interaction at this site may promote secondary-structure rearrangements or duplex formation with *dctA* in addition to that driven by aSDa, leading to more efficient cleavage by *SmrNase III*. Similarly, a new base-pairing site emerges with the AbcR1a mutant (Fig. S5, right), although this does not restore *SmrNase III*-dependent silencing. All in all, these results support that silencing of *dctA* by *SmrNase III* *in vivo* requires Hfq-dependent base pairing to AbcR1, primarily through the aSDa motif.

4. Discussion

Bacterial fitness in fluctuating environments depends largely on RNA homeostasis, which is maintained by the concerted action of RNases and

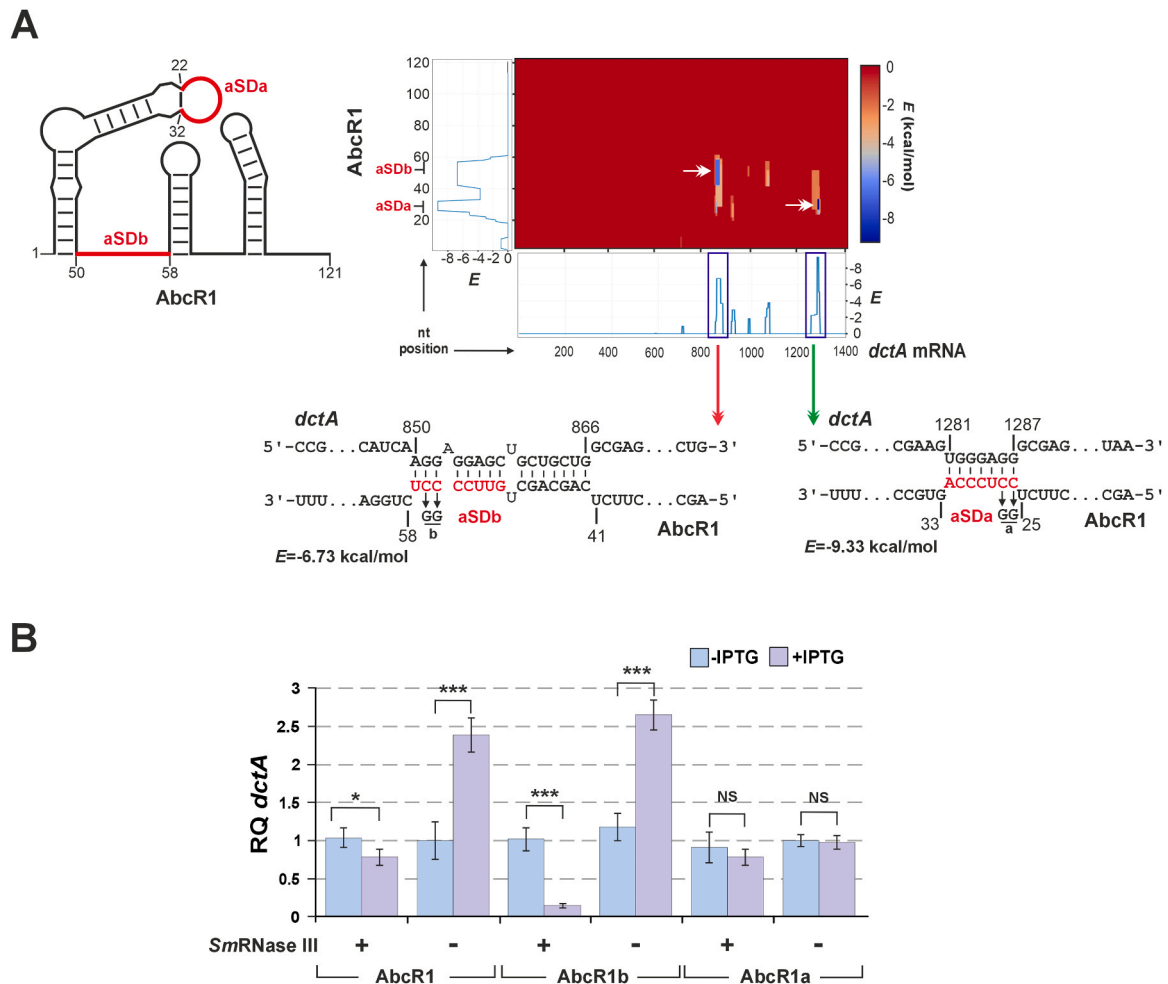


Fig. 5. *SmrNase III*-assisted *trans* regulation of *dctA* by AbcR1. A) Base-pairing between AbcR1 and *dctA*. The schematic (left) shows the predicted secondary structure of AbcR1, with the regulatory motifs aSDa and aSDb highlighted in red. The plot (right) displays the position-wise minimal-energy profiles for AbcR1 and *dctA*, together with a heatmap of predicted interactions. Axes indicate nucleotide positions along each RNA. White arrows mark the most probable wild-type regulatory interactions between aSDa/aSDb and *dctA*, which are illustrated below along with the minimum hybridization energy (*E*). Red and green arrows denote unproductive and productive base pairing, respectively. Nucleotide positions are given relative to 50 nt upstream of the *dctA* start codon and to the transcription start site of AbcR1. Nucleotides in red correspond to the targeting motifs, and point mutations within each motif are indicated. B) RT-qPCR analysis of the impact of AbcR1 on *dctA* mRNA abundance. AbcR1, AbcR1a, and AbcR1b were IPTG-induced in aerobic log cultures of $\Delta abcR1$ (*SmrNase III* +) and $\Delta abcR1/\Delta rnc$ (*SmrNase III* -) strains. Determinations were carried out in duplicate in RNA from three biological replicates (*n* = 6). Relative quantification (RQ) values of *dctA* were normalized to *Smc01852* as a constitutive control. Asterisks indicate statistically significant differences according to ANOVA (**p* < 0.05; ***p* < 0.01; ****p* < 0.001); NS indicates non-significant differences.

regulatory sRNAs. RNase III is central to this process because it cleaves dsRNA, shaping mRNA turnover and sRNA-guided regulation. Symbiotic N₂-fixing rhizobia must adapt from soil to the microaerobic environment of legume nodules. We show that the *S. meliloti* RNase III ortholog, *SmRNase III*, broadly influences steady-state mRNA and sRNA levels under nodule-like conditions. Our data further indicate that proper expression of major symbiotic metabolic pathways depends on the coordinated activity of *SmRNase III* and both antisense and *trans*-acting sRNAs.

4.1. *SmRNase III* is pivotal in shaping the microaerobic *S. meliloti* transcriptome

Root nodules that host endosymbiotic bacteroids create a complex environment that drives differential transcription of more than 20% of *S. meliloti* protein-coding genes (Becker et al., 2004). Consequently, strains carrying mutations in genes that pleiotropically affect cell physiology, such as *rnc*, or those involved in core symbiotic functions, typically induce empty or unevenly colonized, non-functional nodules. These defective nodules are unsuitable as biological material for elucidating the molecular mechanisms underlying bacterial-dependent symbiotic phenotypes. A commonly used alternative for investigating symbiotic pathways involves mimicking specific nodule conditions in free-living cultures, where viability of the mutants is uncompromised. In this context, imposing an oxygen downshift on rhizobial cultures, although it does not fully recreate the bacteroid transcriptome, is sufficient to induce expression of a subset of genes involved in bacteroid respiration and symbiotic N₂ fixation (Becker et al., 2004). Our transcriptomic analyses suggest that *SmRNase III* plays a central role in globally coordinating *S. meliloti* metabolism, influencing both coding and non-coding RNA populations in line with its known pleiotropic effects in free-living and symbiotic contexts (Saramago et al., 2018). In particular, *SmRNase III* is essential for maintaining the proper balance of the major microaerobic pathways active within nodule bacteroids, including the pSymA-encoded modules for iron acquisition, denitrification, and N₂ fixation. The collective misregulation of these pathways in the absence of *SmRNase III* indicates that this endoribonuclease acts as a key integrator that harmonizes iron homeostasis, N metabolism, and nitrogenase activity, thereby supporting *S. meliloti* fitness in the rhizosphere and ensuring efficient symbiotic performance within nodules. Altogether, the breadth and oxygen-dependent nature of the *SmRNase III* regulon suggest that this enzyme has evolved to function optimally in the nodule environment, buffering transcript levels to sustain both core cellular processes and genuine symbiotic functions.

4.2. *SmRNase III* substrates inferred from the transcriptomics datasets

Our experimental setup was conceived to assess the global impact of *SmRNase III* on *S. meliloti* transcriptome, rather than to precisely identify the endogenous substrates of the enzyme. In the mutant strain, upregulated and downregulated transcripts occur in similar numbers compared to the wild type. Although the differential accumulation of many of these RNA species likely reflects indirect regulatory effects, both negative and positive outcomes can be explained by *SmRNase III*-mediated cleavage at defined sites within its RNA targets, ultimately leading either to RNA degradation or to the production of stable processed transcripts. It has been proposed that sequence stretches over-represented in *rnc* knockout mutants relative to the wild-type strain likely correspond to RNase III cleavage sites (Gordon et al., 2017). A similar analysis of sequencing read coverage across all annotated genomic features in the *S. meliloti* genome identified putative cleavage signatures in 28% and 12% of *SmRNase III*-dependent mRNAs and sRNAs, respectively, predominantly within experimentally defined or predicted 5' UTRs. A preference for 5'-UTR cleavage has already been reported for *EcRNase III*, with the *rnc* (*EcRNase III*) and *pnp* (PNPase) transcripts serving as well-characterized examples (Bardwell et al.,

1989; Meur and Portier., 1992), both of which are also predicted *SmRNase III* targets in *S. meliloti*. RNase III-mediated removal of these regions promotes RNase E-dependent maturation or message decay. Interestingly, our predictions suggest that 5'-UTRs involved in a more complex post-transcriptional regulatory mechanisms are also substrates of *SmRNase III*. These include the leader region of thiamine biosynthesis mRNAs (*thi*), which acts as a thiamine-responsive transcriptional attenuator riboswitch in *Rhizobium etli* and *E. coli* (Miranda-Ríos et al., 2001; Rodionov et al., 2002; Guedich et al., 2016). Our pattern-based inference analysis thus provides a valuable resource for interpreting RNase-dependent transcriptional changes assessed by conventional RNA-Seq, while laying a foundation for deeper investigation into the mechanisms and regulatory roles of RNase III activity in rhizobia.

4.3. *SmRNase III* assists riboregulation of symbiotic microaerobic pathways

Several studies have shown that RNase III initiates cleavage of RNA duplexes formed by antisense interactions between sRNA regulators and their targets (Svensson and Sharma, 2021; Šetinová et al., 2018). Due to frequent misannotation, non-coding RNAs are often overlooked in standard RNA-Seq analyses. The accurate annotation of the *S. meliloti* genome therefore enables a more complete interpretation of *SmRNase III*-dependent regulation. In our dataset, asRNAs and *trans*-sRNAs, alongside mRNA leaders, were prominently represented among *SmRNase III*-dependent species, especially under microaerobic conditions. Most of these sRNAs remain functionally uncharacterized, and our results now place them within a symbiotic regulatory framework, providing a foundation for future reverse-genetics studies.

Massive accumulation of discrete antisense asRNAs in RNase III knockout mutants has been recognized as a hallmark of the pivotal role of this enzyme in mRNA silencing induced by antisense transcription (Lasa et al., 2011). In *S. meliloti*, antisense transcription skews towards pSymA-borne symbiotic genes that are highly expressed in the microaerobic environment of the nodule (Robledo et al., 2015). Of note, we found that the absence of *SmRNase III* significantly alters the abundance of a substantial number of asRNAs, particularly upon the oxygen downshift, with more than 90% of these transcripts being upregulated in the mutant. This subset is enriched in asRNAs that target mRNAs encoding both core and accessory components of the nitrogenase enzyme complex, including NifK, NifN, and FixX, which are significantly upregulated in microaerobic cultures of the *rnc* deletion mutant. Expression of several of these asRNAs, including asNifK1, which is transcribed antisense to the *nifK* mRNA, had been reliably detected by Northern blot probing of RNA extracted from nodule tissues (Robledo et al., 2015). Notably, our data revealed that the *nifK*-asNifK antisense interaction promotes *SmRNase III*-dependent *nifK* decay both *in vitro* and *in vivo*. Widespread antisense transcription of N₂ fixation genes within root nodules may counterbalance the strong microaerobic transcriptional output, helping to maintain the precise stoichiometry of the nitrogenase complex. In this context, our findings underscore a key role for *SmRNase III* in regulating the biogenesis and assembly of this enzymatic machinery essential for symbiosis.

RNase III has also been associated with post-transcriptional regulation by *trans*-acting sRNAs (Viegas et al., 2010). Nearly 20% of the *trans*-sRNAs predicted to be expressed by *S. meliloti* are *SmRNase III*-dependent under microaerobic conditions, suggesting that the enzyme may influence their regulatory activity. However, none of these *trans*-sRNAs has undergone functional characterization, and information regarding their potential mRNA targets is currently lacking. Nonetheless, we found that several mRNAs within the well-characterized metabolic target sets of the homologous, likely *SmRNase III*-independent, *trans*-sRNAs AbcR1 and AbcR2 also overlap with the *SmRNase III* regulon. Among these is *dctA*, which encodes a major C₄-dicarboxylate transporter for bacteroids (Yurgel and Kahn, 2004), is upregulated in the absence of *SmRNase III* specifically under aerobic conditions, and

appears in our list of predicted substrates of the enzyme. Our data confirmed that *in vivo* regulation of *dctA* by AbcR1 requires *SmrNase* III and revealed an uncommon regulatory mechanism involving base pairing deep within the *dctA* coding sequence, mediated by the first (aSDa) of the two known AbcR1 regulatory motifs, which function independently in target regulation (García-Tomsig et al., 2022). Strikingly, both the wild-type AbcR1 transcript and its mutant variants, AbcR1a and AbcR1b, can engage in multiple antisense interactions within *dctA*, some of which even enhance *SmrNase* III-dependent *dctA* decay. However, only the configuration that preserves the wild-type aSDa base-pairing to a *dctA* nucleotide stretch predicted to be cleaved by *SmrNase* III results in effective silencing. These findings thus hint at *SmrNase* III as a novel determinant of target specificity for AbcR1. AbcR1/2 are expressed in free-living *S. meliloti* bacteria and during early stages of host infection to fine-tune metabolic activity but, unlike *dctA*, are absent in the N₂ fixation zone of mature alfalfa nodules (Torres-Quesada et al., 2013). This seemingly opposite accumulation profiles supports a model in which *SmrNase* III-dependent silencing of *dctA* occurs endogenously prior to bacteroid differentiation, thereby fine-tuning *S. meliloti* metabolism during rhizosphere colonization and nodule infection, while enabling dicarboxylate uptake within nodules to meet the high-energy demands of N₂ fixation.

5. Conclusion

Our findings support that *SmrNase* III is a major regulator of RNA homeostasis in *S. meliloti*, especially under the microaerobic conditions found in root nodules. By controlling extensive coding and non-coding regulons, *SmrNase* III ensures proper expression of core symbiotic pathways. We identified an oxygen-dependent *SmrNase* III regulon with enriched cleavage signatures in 5'-UTRs, consistent with conserved RNase III targeting mechanisms in bacteria. *SmrNase* III also modulates antisense and *trans*-acting sRNAs, influencing both nitrogenase transcript turnover and AbcR1-dependent target regulation. Together, these results position *SmrNase* III as a pivotal regulator of metabolic and symbiotic functions in the shift of *S. meliloti* from a free-living to a symbiotic lifestyle.

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CRediT authorship contribution statement

Sabina K. Guedes-García: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Natalia I. García-Tomsig:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Rute G. Matos:** Writing – review & editing, Validation, Supervision, Resources. **Margarida Saramago:** Writing – review & editing, Validation, Supervision, Resources. **Cecilia M. Arraiano:** Writing – review & editing, Validation, Supervision, Resources. **Jiménez-Zurdo José I.:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project

administration, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare no competing interests.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2026.128526.

Data availability

Raw sequence data that support the findings of this study have been deposited in the Sequence Read Archive (SRA) with the accession code PRJNA1387402.

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