RNase E and RNase J are needed for S-adenosylmethionine homeostasis in *Sinorhizobium meliloti*

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Abstract

The ribonucleases (RNases) E and J play major roles in *E. coli* and *Bacillus subtilis*, respectively, and co-exist in *Sinorhizobium meliloti*. We analysed *S. meliloti* 2011 mutants with mini-Tn5 insertions in the corresponding genes *rne* and *rnj* and found many overlapping effects. We observed similar changes in mRNA levels, including lower mRNA levels of the motility and chemotaxis related genes *flaA, flgB* and *cheR* and higher levels of *ndeA* (important for glucan export). The acyl-homoserine lactone (AHL) levels were also higher during exponential growth in both RNase mutants, despite no increase in the expression of the *sinI* AHL synthase gene. Furthermore, several RNAs from both mutants migrated aberrantly in denaturing gels at 300 V but not under stronger denaturing conditions at 1300 V. The similarities between the two mutants could be explained by increased levels of the key methyl donor S-adenosylmethionine (SAM), since this may result in faster AHL synthesis leading to higher AHL accumulation as well as in uncontrolled methylation of macromolecules including RNA, which may strengthen RNA secondary structures. Indeed, we found that in both mutants the N⁶-methyladenosine content was increased almost threefold and the SAM level was increased at least sevenfold. Complementation by induced ectopic expression of the respective RNase restored the AHL and SAM levels in each of the mutants. In summary, our data show that both RNase E and RNase J are needed for SAM homeostasis in *S. meliloti*.

INTRODUCTION

Ribonucleases (RNases) are major players in posttranscriptional gene regulation and often have overlapping functions or are essential (reviewed in [1, 2]). Bacterial RNases have been analysed extensively in *E. coli* and *Bacillus subtilis*, but little is known about these enzymes in other bacteria, such as *Sinorhizobium meliloti*. *S. meliloti* is an alphaproteobacterium living in soil and capable of fixing molecular nitrogen in symbiosis with alfalfa and several other legume plants, thus performing ecologically safe nitrogen fertilization [3, 4]. It is a widely studied model organism but the mechanisms of its RNA-based gene regulation are just emerging [5].

One of the most important bacterial RNases is the endoribonuclease, RNase E, which is essential for growth in most bacteria analysed in this respect, including *S. meliloti* [6–8]. *E. coli* RNase E participates in rRNA [9, 10] and tRNA processing [11], is crucial for mRNA turnover [12–14], prefers 5'-monophosphorylated over 5'-triphosphorylated RNA [15], cleaves at single-stranded, AU-rich regions [12] and is localized at the membrane [16]. Its catalytic activity resides in a conserved N-terminal domain, while the C-terminal unstructured domain serves as a scaffold for the assembly of a protein complex called degradosome [17, 18]. The major components of the *E. coli* degradosome are the DEAD-box RNA helicase RhlB, the phosphorolytic 3’–5’ exoribonuclease polynucleotide phosphorylase

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Received 29 August 2016; Accepted 24 January 2017

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Keywords: RNase; RNase E; RNase J; S-adenosylmethionine; N⁶-methyladenosine; acyl-homoserine lactone; quorum sensing; RNA methylation; *Sinorhizobium*

Abbreviations: AHL, acyl-homoserine lactone; FC, fold change; m⁴A, N⁶-methyladenosine; OD₆₀₀, optical density at 600 nm; QS, quorum sensing; SAM, S-adenosylmethionine.

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Seven supplementary figures and three supplementary tables are available with the online Supplementary Material.
(PNPase) and enolase [19, 20]. Degradosomes of other bacteria, which are assembled around RNase E, have different compositions, but often an RNA helicase and a 3′–5′ exoribonuclease are present in the complex (reviewed in [8]). Recently, in an RNase E-enriched fraction of S. meliloti, we identified the DEAD-box RNA helicase RhlE but not an exoribonuclease candidate [21].

In contrast to E. coli, B. subtilis lacks RNase E but harbours the endoribonucleases RNase J1 (forming a dimer with its parologue RNase J2) and RNase Y, which directly control the abundance of most mRNAs and were first considered to be essential [22–27]. However, in a B. subtilis strain devoid of prophages, the RNase J1 and RNase Y genes were successfully destroyed and the corresponding knockout mutants had major defects and prolonged doubling times [28]. A special feature of RNase J1 is its 5′–3′ exoribonucleolytic activity in addition to an endoribonucleolytic activity with an RNase E-like specificity [29, 30].

The key bacterial RNases E, J and Y are often present in bacteria in different combinations [8, 24]. RNase J of several bacteria harbouring RNase E but not RNase Y, such as S. meliloti, Mycobacterium smegmatis, Rhodobacter sphaeroides and Streptomyces coelicolor, was analysed previously [31–34]. RNase J is also non-essential in these bacteria and is necessary for 5′-matura-bion of 16S rRNA like in Bacillales [35, 36]. Transcriptomic analyses revealed that in contrast to the major role of RNase J1 in B. subtilis and Staphylococcus aureus [27, 36], only one RNase J homologue in R. sphaeroides is required for clearance of a limited number of mRNA and rRNA degradation products [33]. Genomewide analyses of RNase J functions in other bacteria have not yet been performed.

The role of S. meliloti RNase J in rRNA maturation was studied using a mutant with a mini-Tn5 insertion destroying the only one rnj gene [31, 37]. The rRNA maturation defect of this RNase J mutant was complemented perfectly by constitutive ectopic rnj expression [31]. In contrast, an S. meliloti strain with a knockout mutation of the RNase E encoding gene rne is still not available. A previous analysis of an RNase E mutant with a mini-Tn5 insertion in the 3′-half of rne, which constitutively expresses rne from a plasmid, revealed rapid degradation of the autoinducer synthase mRNA sinl mRNA and diminished autoinducer (acylhomoserine lactone) amounts due to rne overexpression [7]. RNase E seems to be essential in S. meliloti and the RNase E mutant most probably expresses a truncated RNase E that has defects in degradosome formation [7]. The S. meliloti degradosome probably plays a role in the post-transcriptional regulation of sinl mRNA, since full-length RNase E is needed for degradation of this mRNA upon overexpression of the small RNA (sRNA) RcsR1 [21].

The autoinducer synthase Sinl is a part of the Sin quorum sensing (QS) system of S. meliloti, a bacterial communication system using long-chain acyl-homoserine lactones (AHLs) as signal molecules [38, 39]. For AHL synthesis, autoinducer synthases use acyl–acyl carrier proteins and S-adenosylmethionine (SAM), the methyl donor for the majority of methyltransferases [40, 41]. Expression of sinl critically depends on the transcription regulator SinR [38, 42]. Additionally, the SinR homologue ExpR senses AHLs and transcriptionally regulates sinR, sinl and other genes depending on population density, which is reflected by the AHL concentration [43, 44]. As mentioned above, RNase E and the sRNA RcsR1 are involved in sinl mRNA regulation [7, 21]. Furthermore, posttranscriptional regulation of sinl and expR by the RNA chaperone Hfq has also been described [45–47]. The role of RNase J in posttranscriptional regulation of QS in S. meliloti has not yet been studied.

The aim of this work was to compare the existing RNase E and RNase J mutants to the parental strain 2011 in order to learn more about the roles of these RNases in S. meliloti. Our analyses revealed striking overlapping effects in the RNase mutants including higher AHL and Nε-methyladenosine levels during exponential growth, strongly increased SAM levels, and aberrant electrophoretic behaviour of sRNAs pointing to RNA hypermodification. We found that both RNases are necessary to maintain balanced SAM levels in S. meliloti and suggest that deregulation of SAM metabolism leads to overlapping secondary effects in the two RNase mutants.

**METHODS**

**Strains, plasmids, cultivation and cloning**

We used the S. meliloti strain Rm2011 (referred to here as strain 2011; [48, 49]) and its RNase E and RNase J mutants 2011rne::Tn5 and 2011rnj::Tn5, which harbour mini-Tn5 insertions in the respective genes rne (SMc01336) and rnj (SMc01929) [7, 31, 37]. The mutant strains are referred to here as 2011rne and 2011rnj, respectively. Strain 2011 is closely related to strain 1021, the S. meliloti strain with the first sequenced genome and both strains are expR mutants [43, 50, 51]. Bacterial strains and plasmids are listed in Table S1 (available in the online Supplementary Material). S. meliloti was cultivated on tryptone yeast extract medium [52] with appropriate antibiotics (250 μg streptomycin ml⁻¹, 120 μg neomycin ml⁻¹, 20 μg gentamycin ml⁻¹ and 20 μg tetracycline ml⁻¹). Liquid cultures were grown semi-aerobically (50 ml culture in 100 ml Erlenmeyer flasks at 140 r.p.m. and 30 °C). E. coli was grown in LB broth. OD₆0₀ was measured with a Specord 50 (Analytik Jena). Diparental conjugation was used to transfer plasmids from E. coli S17-1 to S. meliloti as described by [53]. For cloning of gene rnj between the NdeI and XbaI restriction sites of plasmid pSRK-Gm, primers Rnj-NdeI-fwd (5′-CGCAGATATGAAAAAAGCAAGGCGGACTCAC-3′) and RnjStrep-XbaI-rev (5′-TGCTCTAGATCATTTCTCAGAACTCGGGTGGCGACAGAGGGCGCCCTTCTGTATAAACCCGTGCAC-3′) were used (restriction sites are underlined). Genes cloned in pRK415 are expressed constitutively [7, 54]. pWB
plasmids are pSRK-derivatives and allow for induced gene expression upon IPTG addition [55].

**AHL detection**

AHLs were extracted with 0.3 ml chloroform from 1 ml bacterial culture supernatant, evaporated, resuspended in 30 µl of acetone and detected using two different systems. First, *A. tumefaciens* NTL4 (pZLR4) expressing beta-galactosidase from an AHL/TraR dependent promoter [56] was used as described previously [7]. Briefly, identical volumes of the AHL extracts from strains to be compared were spotted onto agar with the *A. tumefaciens* reporter strain. Intensity of blue colour after incubation at 32 °C overnight corresponds to the amount of AHL. For quantitative analysis of the blue spots the program ImageJ (https://imagej.nih.gov/ij) was used. Spots of mutants and complemented strains on each plate were normalized to the spot of strain 2011, which was set to 100 %.

Two independent experiments, each with three technical replicates, were evaluated. Second, *E. coli* Top10 (pSB403) expressing luxR,luxI::luxCDABE from *Vibrio fisheri* in the presence of AHLs [57] was grown to an OD_{600} of 0.4 in microwell plates. Luminescence was measured 4 h after AHL addition with a frequency of 4000 ms⁻¹. Two independent experiments were performed, each with four technical replicates.

**Fluorescence measurement and Western blot analysis for SinI'-EGFP detection**

SinI'-EGFP levels in *S. meliloti* were analysed as follows. Cultures grown to the indicated OD_{600} were harvested and washed in 0.9 % NaCl. First, 1 ml samples in microtite plates were examined with a fluorescence binocular microscope (exciting 480/40 nm; emission, 510 nm filter). Then the difference of fluorescence in the RNase mutants in comparison to strain 2011 was measured using a fluorescence reader (exciting wavelength of 485 nm and emission at 538 nm). In addition, SinI'-EGFP levels were determined by Western blot analysis. For this, 30 ml liquid culture was harvested at the indicated OD_{600} and resuspended in 500 µl buffer containing 50 mM Tris·HCl pH 7.5, 250 mM KCl, 2 mM EDTA, 1 mM beta-mercaptoethanol, 0.1 mM DTT, and 0.5 mM PMSF. Cells were lysed by sonication. After centrifugation at 13 000 g for 20 min at 4 °C, the protein concentration of the cleared lysate was determined by the Bradford method. Equal amounts of lysates were separated in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. EGFP was detected with anti-EGFP (Clontech) and anti-mouse-IgG peroxidase conjugate (BD Bioscience) antibodies. Signals were visualized using a chemiluminescence imager (Fusion SL4, Vilber) and quantified with the provided software (Fusion, Vilber).

**Isolation and analysis of nucleic acids**

For isolation of total DNA, we used the method of Masterson et al. [58]. Total RNA for microarray analysis and real-time quantitative reverse transcription-PCR (qRT-PCR) was purified with RNeasy (Qiagen). RNA for microarray analysis was isolated from cultures grown to an OD_{600} of 1.3 (late transition stage from exponential to stationary phase, Fig. S1). The ODs of the cultures used for qRT-PCR analysis are indicated. Oligonucleotides used for RT-PCR and PCR efficiencies of primer pairs determined using serial dilutions of RNA are listed in Table S2. Conditions for qRT-PCR were as previously described [7, 59]. Briefly, RNA was treated with DNase I to remove residual DNA and a one-step RT-PCR kit (Qiagen) and 4 ng µl⁻¹ of total RNA per reaction mixture were used. The steady-state mRNA level in a mutant strain was calculated relative to the level in the parental strain and relative to the internal standard rpoB [60]. Total RNA for separation in gels or for primer extension was isolated at an OD_{600} of 1.0 with TRIzol as described by Schlüter et al. [61]. 10 % polyacrylamide – 7 M urea gels were run for 2 h at 300 V. Alternatively, 6 % sequencing gels (Roth) were pre-warmed at 50 °C and run at 1300 V. The oligonucleotide probes for detection of EcpR1 (formerly Sra33), AbcR1 (formerly C16), 6S RNA, SRP RNA and 55 rRNA in Northern blot hybridization were described previously [5, 31, 62, 63]. For Northern blot analysis of tRNA-Asp, oligonucleotide 5'-GCACTCTAAACGACTGACCTAC-3' was used as probe. Primer extension analysis of rRNA was described previously [31].

**Quantification of N⁶-methyladenosine**

For determination of the N⁶-methyladenosine (m⁶A) content in total RNA, we used the colorimetric m⁶A Methylation Quantification Kit from Abcam. Total RNA was isolated with TRIzol from 8 ml liquid cultures (OD_{600} 0.5) of *S. meliloti* 2011, its RNase E and RNase J mutants and from *E. coli* strain DH5α. Each 200 ng sample of RNA was bound to strip-wells using a kit-provided binding solution. N⁶-methyladenosine was detected using specific m⁶A-capture and -detection antibodies and resulting signal was quantified by reading absorbency at a wavelength of 450 nm. A provided positive control was used to calculate the percentage of m⁶A in the total RNA. Samples were measured in duplicates in at least two independent experiments.

**Analysis of SAM levels**

To compare the SAM levels in *S. meliloti* strains, we used the Bridge-It S-Adenosyl Methionine (SAM) Fluorescence Assay Kit (Mediomics). Bacteria were grown to an OD_{600} of 0.5, pelletted and washed twice in cold water. A bacterial pellet corresponding to 1 ml bacterial suspension with an OD_{600} of 8.0 was resuspended in 40 µl SAM extraction buffer. Bacterial lysates in the SAM extraction buffer (samples) did not show fluorescence. The lysates were treated with the assay solution according to the manufacturer’s instructions and the fluorescence of the samples was measured (F) along with the fluorescence of the assay solution (F₀). Fluorescence intensity (F-F₀) was calculated and used to determine the SAM concentrations in the samples by comparing the obtained values to a SAM-standard curve prepared with dilutions of SAM of known concentrations.
Transcriptome profiling experiments
RNA was isolated from four independent bacterial cultures of *S. meliloti* 2011rne, *S. meliloti* 2011rnj and the parental strain 2011, as described above. cDNA synthesis, Cy3- and Cy5 labelling, hybridization employing the Sm14kOli microarray (ArrayExpress Accession No. A-MEXP-1760), image acquisition and data analysis were performed as described previously [64]. Spot detection, image segmentation and signal quantification were performed using ImaGene 8.0 software (Biodiscovery, Los Angeles, CA, USA). Spots were flagged as 'low signal spots' if \( R \leq 2.0 \) in both channels, where \( R = (\text{signal mean} - \text{background mean})/\text{background standard deviation} \). The log2 value of the ratio of intensities was calculated for each remaining spot using the formula \( M_i = \log_2 R_i/G_i \), where \( R_i = I_{ch1(i)} - B_{ch1(i)} \) and \( G_i = I_{ch2(i)} - B_{ch2(i)} \) where \( I_{ch1(i)} \) and \( I_{ch2(i)} \) are the intensities of spots in channel 1 and channel 2, respectively, and \( B_{ch1(i)} \) and \( B_{ch2(i)} \) are the background intensities of spots in channel 1 and channel 2 respectively. The mean intensity \( \bar{A}_i \) was calculated for each spot as follows: \( \bar{A}_i = \log_2 (R_i/G_i)^{0.5} \). A normalization method based on local regression that accounts for intensity and spatial dependence in dye biases was used [65]. Normalization and t-statistics were carried out using the EMMA2.8.2 microarray data analysis software developed at the Bioinformatics Resource Facility, Center for Biotechnology, Bielefeld University [66]. Genes were classified as differentially expressed with \( P < 0.05 \) and \( M > 1 \) or \( M < -1 \) (at least a twofold difference). Transcriptome data are available at ArrayExpress (Accession No. E-MTAB-5435).

RESULTS

Microarrays reveal overlapping effects in the RNase E and RNase J mutants
To address the roles of RNase E and RNase J in *S. meliloti*, the two RNase mutant strains 2011rne and 2011rnj with mini-Tn5 insertions in the genes *rne* and *rnj*, respectively, were compared to the parental strain 2011 by microarrays. The analysis was performed with total RNA isolated from cultures grown in TY medium to an OD600 of 1.3 (Fig. S1), a condition at which a previous study uncovered *sinI* as a preferred target of RNase E [7].

Our analysis revealed 134 differentially expressed genes, which belong to different functional groups (Tables 1 and S3). We found genes with elevated or decreased mRNA levels in one of the mutants or in both mutants. Although more than half of the affected genes (87 genes) showed increased mRNA levels, as expected for mutants impaired in RNA decay, the levels of 46 transcripts were decreased, indicating probable indirect effects. It is noteworthy that 10 genes related to motility and chemotaxis showed decreased mRNA levels and no up-regulated genes belonging to this category were detected. Furthermore, 25% of the affected genes were up- or down-regulated in both mutants, revealing overlapping effects (Tables 1 and S3). Using qRT-PCR we confirmed the overlapping effects for the symbiotically important gene *ndvA* encoding a cyclic beta-1,2-glucan ABC transporter (with higher mRNA levels in the mutants) and the flagella and chemotaxis genes *flaA, flgB* and *cheR*, which belong to different operons (with lower mRNA levels in the mutants; Table 2).

Motility and chemotaxis genes, and *ndvA* are under QS control depending on the AHL-sensing protein ExpR [67, 68]. Because *expR* is disrupted in strain 2011 and its RNase mutants [43, 44], this implies that the changes in the mRNA levels of *ndvA, flaA, flgB, cheR*, and other motility and chemotaxis genes (Table 1) are not based on AHL/ExpR regulation but rather at another level of regulation [68]. Since RNase E is important for QS [7], we decided to analyse the AHL levels in both RNase mutants.

**Both RNase mutants show increased AHL levels at low population density despite no increase in *sinI* expression**

Previously, no difference in the AHL levels of the RNase E mutant 2011rne and the parental strain 2011 were detected at an OD600 of 1.3, while AHLs were diminished at this OD in strain 2011rne (pRKrne) that overexpresses the complementary gene *rne* [7]. AHL accumulation in the RNase J mutant 2011rnj was not analysed. Here we compared the AHL levels in cultures of strain 2011, the two RNase mutants, and the complemented mutants containing pRKrne and pRKrnj, respectively. AHLs were extracted from supernatants of exponentially growing cultures (at an OD600 of 0.5) and during transition to the stationary phase (OD600 of 1.3; see Fig. S1).

At an OD600 of 0.5 we found increased AHL levels in both RNase mutants compared to strain 2011, while fewer AHLs were detected in strains 2011rne (pRKrne) and 2011rnj (pRKrnj) (Figs 1a and S2). The decreased AHL levels in the last two strains most probably resulted from overexpression of the respective complementary gene (Fig. S3; [7]). In contrast, at an OD600 of 1.3 no differences were found between strain 2011 and both RNase mutants, while the AHL levels in strains 2011rne (pRKrne) and 2011rnj (pRKrnj) were still decreased (Fig. 1a). The decrease in the AHL amount of strain 2011rne (pRKrne) is in line with previous results [7].

Strain 2011 and its derivatives do not possess a functional *expR* gene [43, 44]. To test whether the above results are valid for strains expressing *expR*, plasmid pWBexpR was used, in which *expR* is under the control of a tightly regulated *lac* promoter. Growth of the pWBexpR-containing strains in the absence of IPTG led to a highly mucoid phenotype on plates (not shown), suggesting that leaky expression of *expR* is sufficient for functional QS [43]. This was confirmed by the similar AHL accumulation during the growth of strains 2011(pWBexpR) and Sm2B3001, the latter with a restored *expR* on the chromosome (data not shown; for AHL accumulation during growth of strains 2011 and Sm2B3001 see [21]). A comparison of the AHL levels of the pWBexpR-containing strains (2011, RNase mutants and mutants containing pRKrne or pRKrnj) revealed differences
### Table 1. Genes differentially expressed in the RNase E and RNase J mutants 2011rne and 2011rnj in comparison to the parental strain 2011 as revealed by microarray analysis (for details see Table S3)

<table>
<thead>
<tr>
<th>Functional category</th>
<th>Gene ID number</th>
<th>Gene name or description</th>
<th>Change in the mRNA level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Translation, ribosomal structure and biogenesis</td>
<td>SMc00364</td>
<td>rplT</td>
<td>up in rne</td>
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<td></td>
<td>SMc00475</td>
<td>adaS</td>
<td>up in rne</td>
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<tr>
<td></td>
<td>SMc00649</td>
<td>RNA methyltransferase</td>
<td>up in rne</td>
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<tr>
<td>Transcription</td>
<td>SMb21140</td>
<td>Transcriptional regulator</td>
<td>up in rne</td>
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<td></td>
<td>SMA1387</td>
<td>LysR family transcriptional regulator</td>
<td>up in rne</td>
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<tr>
<td></td>
<td>SMc03843</td>
<td>Transcriptional regulator</td>
<td>up in rnj</td>
</tr>
<tr>
<td></td>
<td>SMc00108</td>
<td>Acetyl transferase</td>
<td>up in rnj</td>
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<td></td>
<td>SMA2387</td>
<td>TetR family transcriptional regulator</td>
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<td></td>
<td>SMb20714</td>
<td>LacI family transcriptional regulator</td>
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<td>SMc00283</td>
<td>Transcriptional regulator</td>
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<td>Replication, recombination and repair</td>
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<td>helO, ATP-dependent helicase</td>
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<td>SMc02905</td>
<td>dnaX</td>
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<td>ABC-type multidrug transport system</td>
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<td>SMb21206</td>
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<td>flgB</td>
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<td>mcpX</td>
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<td>SMc03037</td>
<td>flaA</td>
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<td>flaB</td>
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<td>SMc04114</td>
<td>pilA1</td>
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<td></td>
<td>SMc00159</td>
<td>Signal peptide protein, similarity to TadG</td>
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<td>Posttranslational modification, protein turnover, chaperones</td>
<td>SMc02361</td>
<td>cyeH</td>
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<td></td>
<td>SMc03849</td>
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<td></td>
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<td>Similarity to sugar kinase</td>
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<td>down in \textit{rnj}</td>
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<td></td>
<td></td>
<td>4 genes</td>
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Table 2. Real-time RT-PCR (qRT-PCR) verification of the differential expression of genes, which was originally detected by microarrays

For qRT-PCR, the mRNA level in strain 2011 was set to 1, and fold changes (FCs) in the RNase E and RNase J mutants (2011rne and 2011rnj) were calculated. At least two independent experiments with technical duplicates were evaluated.

<table>
<thead>
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<th>Gene ID number</th>
<th>Gene name</th>
<th>Functional description</th>
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<th>qRT-PCR FC</th>
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<td>SMc03900</td>
<td>mbvA</td>
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<td>3.0±1.3, 4.9±2.1</td>
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<td>SMc03037</td>
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<td>flagellin A</td>
<td>−4.1, −4.3</td>
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<td>flgB</td>
<td>flagellar basal body rod</td>
<td>−2.0, −2.8</td>
<td>−2.7±0.9, −2.5±1.3</td>
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<tr>
<td>SMc03009</td>
<td>cheR</td>
<td>chemotaxis</td>
<td>−2.9, −2.4</td>
<td>−14±6.3, −4.4±3.8</td>
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</tbody>
</table>
like those observed in the absence of pWBexpR, with the exception of strain 2011rne (pRKrne) at an OD$_{600}$ of 1.3 (compare Fig 1b to Fig. 1a). Thus, the changes in AHL levels of the RNase mutants at OD$_{600}$ of 0.5 are independent of expR. Since these changes are in the focus of this work, all further experiments were performed with the expR-deficient strains allowing for analysis of sinI expression and AHL accumulation in a simpler genetic background.

Next we tested whether the differences in AHL levels at the two ODs can be attributed to differences in the levels of sinI mRNA. With the exception of the previously described down-regulation of this mRNA in strain 2011rne (pRKrne) at an OD$_{600}$ of 1.3 [7], no strong changes were observed, regardless of strain or condition (Fig 1c). Importantly, the increased AHL levels in the mutants at an OD$_{600}$ of 0.5 were not due to higher sinI mRNA abundance (compare Fig. 1a to Fig. 1c).

Since mRNA and protein levels must not correlate [69], we considered the possibility that the RNase mutants may have higher SinI protein levels during exponential growth. So far,
antibodies against SinI are not available and therefore we analysed a translational sinI'-egfp fusion. The fusion gene is under the control of the sinI promoter on pLK64 and the resulting mRNA contains the sinI 5'-UTR and the first nine sinI codons [7, 42]. However, at an OD_{600} of 0.5 the fluorescence in the two RNase mutants was lower than in strain 2011, although the sinI'-egfp mRNA level was not changed (Fig. 1d). The lower SinI'-EGFP protein level in the mutants was also confirmed by Western blot analysis with EGFP-specific antibodies (Fig. 1d), indicating lower sinI expression in the RNase mutants. In summary, the AHL levels were elevated during exponential growth of both RNase mutants despite no increase in the expression of the AHL synthase gene sinI.

The similarities between the two RNase mutants prompted us to test whether RNase J is necessary for diminishing the AHL levels upon overexpression of the sRNA RcsR1 as previously described for RNase E [21]. The AHL levels were decreased in the rcsR1-overexpressing RNase J mutant and in rcsR1-overexpressing strain 2011, but not in the rcsR1-overexpressing RNase E mutant (Fig. 2). This result was independent of the growth phase (Fig. 2). We conclude that in contrast to RNase E, RNase J is not involved in the RcsR1 pathway of sinI mRNA degradation. It is noteworthy that this experiment also confirmed the increased AHL levels in both RNase mutants at an OD_{600} of 0.5.

Small RNAs with aberrant electrophoretic behaviour in both RNase mutants

Maturation of rRNA is affected in the RNase J mutant [31]. To test whether this is also the case in the RNase E mutant, the RNA profiles of strain 2011 and the RNase mutants were compared by separation of total RNA in a denaturing polyacrylamide gel (Fig. 3a). We found that, in contrast to the RNase J mutant, the 5.8S-like 23S rRNA fragment of the RNase E mutant has wild-type length (Fig. 3a). Additional primer extension analyses revealed that the 5-ends of all rRNA species are exactly the same in strain 2011 and the RNase E mutant (Fig. S4). Thus, the insertion of mini-Tn5 in the C-terminal part of the rne gene does not affect rRNA maturation in S. meliloti.

Careful inspection of the tRNA profiles in Fig. 3a, however, revealed similarities between the mutants, which distinguish them from strain 2011 (marked with arrows on the right-hand side). Indeed, Northern blot analysis of tRNA-Asp revealed faster migration in denaturing polyacrylamide gel run at 300 V for the RNase mutants than for strain 2011 (Fig. S5). Thus we asked whether the electrophoretic behaviour of small RNAs (sRNAs) from the RNase mutants is affected and performed Northern blot analysis of four previously analysed sRNAs (EcpR1, AbcR1, 6S RNA and SRP RNA; see Methods). We observed similar aberrant migration of the sRNAs from the two RNase mutants in denaturing polyacrylamide gels run at 300 V (Fig. 3b).

The migration behaviour was partly (for EcpR1) or fully (for the other analysed sRNAs) restored in the complemented strains 2011rne (pRKrne) and 2011rnj (pRKrnj) (Fig. 3b). Electrophoresis under stronger denaturing conditions (sequencing gel at 1300 V and 50 °C) revealed that the sRNAs of strain 2011 and the RNase mutants have identical lengths (Figs. 3c and 3d). Together the results in Fig. 3(b, c) suggest the existence of strong RNA secondary structures in the mutants, which can be caused by RNA modifications such as RNA methylation [70].

\[ \text{m}^6\text{A} \text{ content is increased in the RNase mutants} \]

The above data do not explain the higher AHL accumulation in cultures of both RNase mutants during exponential growth, but document striking similarities between them. Since SAM is used for both AHL synthesis and RNA methylation [41], we considered that higher SAM levels in the mutants may lead to faster AHL synthesis and uncontrolled RNA modification resulting in higher AHL levels and aberrant gel migration of sRNAs, respectively.

To provide evidence for generally increased RNA methylation in the RNase mutants, we determined the \( \text{m}^6\text{A} \) content in total RNA of the parental strain 2011 and the two RNase mutants. As a control we used RNA from E. coli, for which we determined 0.3 % \( \text{m}^6\text{A} \) (Fig. 3d), a value very similar to the recently determined \( \text{m}^6\text{A} \)-content of E. coli mRNA [71]. The presence of \( \text{m}^6\text{A} \) in S. meliloti was not analysed previously. We found 0.13 % \( \text{m}^6\text{A} \) in S. meliloti 2011 RNA, and approximately 0.3 % \( \text{m}^6\text{A} \) in the two RNase mutants (Fig. 3d). The significantly higher \( \text{m}^6\text{A} \) level in the two RNase mutants supports the proposed hypermethylation of their RNA.
Both RNases are needed for SAM homeostasis in S. meliloti

Next we compared the SAM levels in the parental strain, the RNase mutants and the complemented strains 2011rne (pRKrne) and 2011rnj (pRKrnj) using a fluorescence assay kit (see Methods). We found that the SAM levels were increased at least sevenfold in the RNase mutants and were almost restored to wild-type levels in the complemented strains (Figs 4 and S7). The restored SAM levels in the complemented strains were paralleled by successful complementation of RNA gel migration behaviour for most tested sRNAs (Fig. 3b), but not by restored AHL levels (Fig. 1a). The last observation could be explained by constitutive expression of the complementing genes rne and rnj from pRKrne and pKRrnj, respectively, which results in overexpression (Fig. S3; [7]). To test whether regulated ectopic production of the RNases in the mutants will restore the AHL levels along with the SAM levels, we used the pSRK-derived plasmids pWBrne and pWBrnj allowing IPTG-induced expression of rne and rnj, respectively.

To determine the IPTG concentration needed to complement the rRNA processing defect of the RNase J mutant,
Fig. 4. SAM levels are strongly increased in both RNase mutants. SAM levels in S. meliloti 2011, the RNase mutants 2011rne (rne) and 2011rnj (rnj), and the complemented mutants containing pRKrne and pRKrnj, respectively, were analysed with a fluorescence assay. Shown are mean values and standard deviations from two independent biological experiments, each performed in triplicate. The mean values were used for determination of the SAM concentrations (in µM) in the samples, with the help of a standard curve corresponding to known SAM concentrations (Fig. S7). Each sample corresponded to 1 ml culture of an OD$_{600}$ of 8.0 (see Methods).

Strain 2011rne (rne) 2011rnj (rnj) (pRKrne) (pRKrnj) µM SAM in sample 0.9 6.8 6.6 1.2 1.3

Discussion

In this work we compared S. meliloti RNase E and RNase J mutants to the isogenic strain 2011 in order to learn more about the function of these RNases. Our microarray analysis revealed 67 genes, the expression of which (directly or indirectly) is influenced by RNase J. Thus, the function of RNase J in S. meliloti is broader than in R. sphaeroides, where only several RNA degradation products accumulated in an RNase J null mutant [33]. Further, our analysis revealed a relatively low number of genes with changed mRNA levels in the RNase E mutants when compared to studies with E. coli. For example, 60% of all E. coli genes were affected in the absence of RNase E [14], while only 100 genes were found to be affected in the S. meliloti RNase E mutant in our study. However, as mentioned in the Introduction, the used S. meliloti RNase E mutant probably expresses a functional catalytic N-terminal part of RNase E while lacking the C-terminal part of the protein [7]. Thus, the 100 genes with changed expression in the S. meliloti RNase E mutant directly or indirectly depend on full-length RNase E and probably on degradosome formation. In comparison, 185 transcripts showed changed abundance in four different isogenic degradosome mutants of E. coli [72]. Furthermore, previous studies with E. coli were performed with cells in the exponential growth phase [14, 72], while our analysis was performed at late transition to the stationary phase (Fig. S1), when fewer genes are expected to be active than in exponentially growing bacteria [73]. Thus, the relatively low number of transcripts affected in the S. meliloti RNase E mutant may also be due to the applied growth conditions.

Strain 2011rne (rne) 2011rnj (rnj) (pWRnre) (pWRnre) (pWRrnj) (pWRrnj) µM SAM in sample 0.9 6.8 6.6 1.2 1.3

and then to a decrease (at 1 mM IPTG; see Fig. 6a), while a continuous decrease in AHL levels was observed with increased rne overexpression (Fig. 6b). In agreement with previous results, no AHLs were detected when 1 mM IPTG was applied [7].

Altogether, Figs 5 and 6 show that complementing the RNase mutants with respect to the SAM levels leads to normalization of the AHL levels. Furthermore, these data show that balanced expression of both RNases is needed for SAM homeostasis in S. meliloti.

Discussion

In this work we compared S. meliloti RNase E and RNase J mutants to the isogenic strain 2011 in order to learn more about the function of these RNases. Our microarray analysis revealed 67 genes, the expression of which (directly or indirectly) is influenced by RNase J. Thus, the function of RNase J in S. meliloti is broader than in R. sphaeroides, where only several RNA degradation products accumulated in an RNase J null mutant [33]. Further, our analysis revealed a relatively low number of genes with changed mRNA levels in the RNase E mutants when compared to studies with E. coli. For example, 60% of all E. coli genes were affected in the absence of RNase E [14], while only 100 genes were found to be affected in the S. meliloti RNase E mutant in our study. However, as mentioned in the Introduction, the used S. meliloti RNase E mutant probably expresses a functional catalytic N-terminal part of RNase E while lacking the C-terminal part of the protein [7]. Thus, the 100 genes with changed expression in the S. meliloti RNase E mutant directly or indirectly depend on full-length RNase E and probably on degradosome formation. In comparison, 185 transcripts showed changed abundance in four different isogenic degradosome mutants of E. coli [72]. Furthermore, previous studies with E. coli were performed with cells in the exponential growth phase [14, 72], while our analysis was performed at late transition to the stationary phase (Fig. S1), when fewer genes are expected to be active than in exponentially growing bacteria [73]. Thus, the relatively low number of transcripts affected in the S. meliloti RNase E mutant may also be due to the applied growth conditions.

The observed aberrant migration of sRNAs in gels is consistent with the existence of strong secondary structures in the sRNAs of the mutants, which still persist in denaturing gels at 300 V, but are resolved under the stronger denaturing conditions in sequencing gels (Fig. 3b, c). Residual secondary structures in denaturing gels were reported previously for chemically modified tRNAs [70]. Nucleotide modifications such as methylation are important for stable secondary and tertiary structures and specific functions of rRNAs and tRNAs [74]. Recently, methylated mRNAs and sRNAs were detected both in Eukarya and in Bacteria, and it was proposed that this may represent an additional level of post-transcriptional gene regulation [71, 75]. In contrast to the nucleotide modification at defined tRNA and rRNA

Conclusions

The observed aberrant migration of sRNAs in gels is consistent with the existence of strong secondary structures in the sRNAs of the mutants, which still persist in denaturing gels at 300 V, but are resolved under the stronger denaturing conditions in sequencing gels (Fig. 3b, c). Residual secondary structures in denaturing gels were reported previously for chemically modified tRNAs [70]. Nucleotide modifications such as methylation are important for stable secondary and tertiary structures and specific functions of rRNAs and tRNAs [74]. Recently, methylated mRNAs and sRNAs were detected both in Eukarya and in Bacteria, and it was proposed that this may represent an additional level of post-transcriptional gene regulation [71, 75]. In contrast to the nucleotide modification at defined tRNA and rRNA
positions, only a small portion of the sRNA and mRNA molecules are modified [71]. We speculate that the aberrant electrophoretic behaviour of sRNAs from the mutants is because of uncontrolled methylation due to increased cellular SAM concentrations. The observed increase in the m^6^A content in both RNase mutants compared to the parental strain is in line with this explanation. It is not clear whether sRNAs are naturally methylated in *S. meliloti*.

Fig. 5. Complementation of the RNase J mutant by regulated ectopic *rnj* expression using pWBrnj. Cultures of strain 2011, the RNase J mutant 2011rnj (*rnj*) and the complemented mutant containing pWBrnj were treated with different IPTG concentrations. Analysed were (a) maturation of the 5.8S-like rRNA detected by ethidium bromide staining of urea-polyacrylamide gel, (b) SAM levels measured by fluorescence assay and (c) AHLs detected by *lacZ* expression in *A. tumefaciens* NTL4 reporter strain. Strains and IPTG concentrations are indicated. In (b) mean values and standard deviations from three independent biological experiments are shown. Panel (c) shows results from a representative plate.

Fig. 6. Complementation of the RNase E mutant by regulated ectopic *rne* expression using pWBrne. Cultures of strain 2011, the RNase E mutant 2011rne (*rne*) and the complemented mutant containing pWBrne were treated with different IPTG concentrations. Analysed were (a) SAM levels measured by fluorescence assay and (b) AHLs detected by *lacZ* expression in *A. tumefaciens* NTL4 reporter strain. In (a) mean values and standard deviations from three independent biological experiments are shown. Panel (b) shows results from a representative plate. Strains and IPTG concentrations are indicated. C, negative control with acetone without AHLs.
Indeed, the strongly increased SAM amounts could explain the overlapping effects in the two RNase mutants. First, higher SAM concentration should lead to faster AHL synthesis, which may result in higher AHL accumulation even if the \textit{sinI} expression is lower in the mutants (as suggested by Fig. 1d). According to Figs 5 and 6 complementations of SAM and AHL levels correlate, supporting the idea of increased AHL accumulation in the mutants during exponential growth due to higher SAM concentrations. Second, higher SAM concentrations may lead to deregulated or aberrant methylation of DNA and proteins in addition to RNA. This may result in changes in gene expression, which, on this view, would be expected to be similar in the two mutants. This is in line with our data from the microarray analysis that 25% of the genes were similarly affected in both RNase mutants (Tables 1 and S3). Thus, it is possible that the similar changes in the \textit{flaA}, \textit{figB}, \textit{cheR} and \textit{ndaV} mRNA levels are due to such pleiotropic effects and are not related to QS, because the AHL-sensing protein \textit{ExpR} is not produced in strain 2011 and the two RNase mutants [43, 44, 67].

The reason for similarly increased SAM levels in the RNase mutants remains to be elucidated. The two RNases do not directly influence each other’s expression (Fig. S2) and RNase J was not among the proteins, which were recently co-purified with RNase E from \textit{S. meliloti} [21], suggesting that they also do not act in a protein complex. Another argument against a protein complex formed by the two RNases is provided by Fig. 1(d), which shows that RNase E (and more specifically its C-terminal part which is supposed to organize a degradosome), but not RNase J, is needed for RcSR1-dependent decrease in AHL levels. Overlapping effects among mutants lacking the important bacterial RNases E and III, which do not interact with each other, were previously described in \textit{E. coli} [14]. Most probably, \textit{S. meliloti} RNase E and RNase J have one or more common direct substrates with key role(s) in SAM homeostasis, which remain to be identified.

Taken together, our data revealed that while RNase E has a specific function in the RcSR1-dependent pathway of \textit{sinI} mRNA degradation and RNase J is specifically needed for 5'-maturation of mRNA, the two RNases together are necessary to maintain SAM homeostasis.

References

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Edited by: J. Stülke and T. Msadek

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