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RNase J is required for processing of a small number of RNAs in *Rhodobacter sphaeroides*

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All bacteria contain multiple exoribonucleases to ensure a fast breakdown of different RNA molecules, either for maturation or for complete degradation to the level of mononucleotides. This efficient RNA degradation plays pivotal roles in the post-transcriptional gene regulation, in RNA processing and maturation as well as in RNA quality control mechanisms and global adaption to stress conditions. Besides different 3′-to-5′ exoribonucleases mostly with overlapping functions in vivo many bacteria additionally possess the 5′-to-3′ exoribonuclease, RNase J, to date the only known bacterial ribonuclease with this activity. An RNA-seq approach was applied to identify specific targets of RNase J in the α-proteobacterium *Rhodobacter sphaeroides*. Only few transcripts were strongly affected by the lack of RNase J implying that its function is mostly required for specific processing/degradation steps in this bacterium. The accumulation of diverse RNA fragments in the RNase J deletion mutant points to RNA features that apparently cannot be targeted by the conventional 3′-exoribonucleases in Gram-negative bacteria.

Introduction

Ribonucleases play crucial roles at different steps of the cellular metabolism in bacteria. On the level of post-transcriptional gene regulation the permanent but coordinated and fine tuned degradation of mRNAs offers a fast and direct impact on the availability of mRNA for the translation machinery. Ribosomal and tRNAs constitute more than 90% of total RNA within a cell and specific processing steps are required for their maturation from precursor molecules. They are considered as durable, but are also subjected to decay under certain circumstance e.g., quality control mechanisms ensure immediate decay of defective or disassembled rRNA molecules, thereby preventing the accumulation of non-functional ribosomes that might interfere with their intact counterparts. Under nutrient deprived growth or during entrance into stationary phase the decomposition of rRNAs from excess ribosomes is part of the resource scavenging cell metabolism.[1,2] Principally the course of degradation of mRNAs or the more stable rRNAs and tRNAs follows the same scheme. An endonucleolytic cleavage of the RNA is followed by exonucleolytic degradation of the generated fragments. In *Escherichia coli* and other Gram-negative bacteria the initiation of mRNA decay is primarily ascribed to the essential, single-strand specific endoribonuclease RNase E. Internal cleavage of an mRNA by RNase E is followed by a rapid degradation of resulting fragments by processive 3′-to-5′ exoribonucleases, namely RNase R, PNPase and RNase II.[3,4] The extreme stability of assembled rRNA is based on its inaccessibility for endo- or exoribonucleases due to protecting ribosomal proteins. Defective or disassembled molecules exhibit exposed entry sites that can be easily attacked by ribonucleases.[1] rRNA degradation under starvation conditions mainly affects free ribosome subunits, while intact 70S ribosomes are protected. It is assumed that initial endonucleolytic cleavages occur within rRNA regions that are located on the interface of both subunits. Once the translational activity in starved cells decreases, the number of non-translating, dissociated subunits with exposed cleavage sites increases and they are becoming targets for ribonucleolytic enzymes.[5] At present the enzymes responsible for the initial endoribonucleolytic cleavage in the ribosome decay pathways are unknown. But the aforementioned exoribonucleases involved in the mRNA decay also take part in the ribosome degradation pathways. Interestingly individual enzymes have different impact on ribosomes decay in the course of quality control or under starvation conditions. Quality control primarily requires RNase R and PNPase, while removal of RNA fragments during starvation is mainly accomplished by RNase R, RNase II and in addition RNase PH.[6] Deletion strains that lack one of these four exoribonucleases show normal growth due to a redundant functionality of these exoribonucleases with overlapping substrates. The redundancy can partially be explained by similar ribonucleolytic characteristics of most of these enzymes.[7-9] RNase R is capable of degrading structured RNAs by itself while PNPase is able to degrade RNAs with a moderate degree of secondary structure due to complex formation with RNA-helicase RhlB.[10,11] Just recently

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Table 1. List of RNAs accumulating in 2.4.1Δ*rnj*

RNA fragment	Gene number	Chromosomal position	Length	
			RNA-seq	Northern
nuoI_int	RSP_0107	1.820.677–844	167 - 81 nt	150 - 80 nt
0381_3′	RSP_0381	2.112.245–332	88 nt	~75 nt
0959_int	RSP_0959	2.715.898–983	85 nt	~75 nt
rnpA_5′	RSP_1060	2.818.046–104	59 nt	< 75 nt
dnaK_int	RSP_1173	2.940.067–315	249 nt	< 300 nt
ftsI_int	RSP_2098	697.538–611	74 nt	~85 nt
fbaB_3′	RSP_4045	1.126.140–201	60 nt	< 75 nt
16S_int	RSP_4294	1.151–299	147 nt	< 150 nt
23S_int	RSP_4295	4.219–315	95/84 nt	100/90 nt

Fragments with their respective length have been identified by RNA sequencing (RNA-seq) and were validated by northern blot (Northern). Chromosomal positions correspond to location of fragments on chromosome 1 (GenBank accession number: CP000143). Names of RNA fragments consist of the annotated gene and relative position of the fragment pertaining the open reading frame (ORF) of the corresponding gene. *_int*: within ORF, *_5′*: overlap with 5′-UTR and ORF, *_3′*: overlap with ORF and 3′-UTR.

it was shown that also RNase PH is responsible for degradation of fragments with extended stem loop motifs.[12] The processive ribonucleolytic activity of RNase II however is restricted to stretched, single stranded regions and stops around 7 nt before reaching a stem loop reviewed in ref.[13] Two further enzymes ensure the complete digestion of RNA fragments in a cell. Oligoribonuclease (Orn) degrades RNA fragments of 2–5 nt in length that are permanently released from the previously described RNases.[14] Intensively structured RNAs cannot be digested at once by processive exoribonucleases as they gradually arrest within helical regions and thereby loose contact to these substrates. The addition of poly(A) tails to the 3′-end of structured RNAs by the poly(A) polymerase (PAP) facilitates rebinding of an RNase and continuing digestion of the target RNA.[15]

The α-proteobacterium *Rhodobacter sphaeroides* encodes protein homologs of all abovementioned exoribonucleases, but not RNase II and oligoribonuclease. Intensive studies of the *puf* operon in this organism showed that similar principles for mRNA degradation apply as in *E. coli*.[16] But in contrast to *E. coli*, *R. sphaeroides* additionally possesses RNase J1, the only prokaryotic exoribonuclease that degrades RNA in 5′-to-3′ direction, provided the target RNA bears a 5′-monophosphate.[17,18] In the Gram-positive *Bacillus subtilis*, the best studied organism regarding the functions of RNase J1, the deletion of the gene *rnjA*, encoding RNase J1, leads to a slow down in growth and major defects in cell morphology, sporulation and competence.[19] This is in accordance with the role of RNase J1 as one of the global regulators of mRNA degradation in *B. subtilis*.[20,21] On the one hand it degrades mRNA 3′-fragments generated by internal cleavages of RNase Y, the functional homolog of RNase E in *B. subtilis*.[22] On the other hand RNase J1 is able to directly attack mRNAs from their 5′-end probably after conversion of the 5′-triphosphate to a monophosphate group by the RNA-pyrophosphohydrolase RppH.[23] Besides these roles RNase J1 is also responsible for 5′-maturation of 16S rRNA.[24] Also in other bacteria rRNA processing depends on RNase J as shown for *Sinorhizobium meliloti* and *Mycobacterium smegmatis*.[25,26] In *R. sphaeroides* we previously showed that RNase J is responsible for the final 5′-processing of the 23S rRNA.[27] Here we present the identification of RNA fragments that strongly accumulate in an RNase J deletion strain, implying the existence of RNA related features that prevent the degradation by conventional 3′-to-5′ exoribonucleases.

Results

RNA-seq analysis of an RNase J deletion mutant 2.4.1Δrnj

We have recently shown that RNase J is responsible for the final 5′-maturation of all three 23S rRNA fragments in *R. sphaeroides*.[27] To identify further RNA targets of RNase J in *R. sphaeroides* we performed comparative RNA-seq analysis with total RNA isolated from the RNase J deletion mutant 2.4.1Δ*rnj* and the wild type strain 2.4.1 grown under micro-aerobic conditions in exponential phase (OD_{660} 0.4). Prior to cDNA preparation RNA samples were treated with tobacco acid pyrophosphatase (TAP) to subsequently capture both primary and processed RNA fragments by sequencing.[28] RNA-seq (sequencing on a GAIIx machine, Illumina) resulted in a total of 6.7 million reads for the wild type and 4.9 million reads for the mutant. Examination of data was performed with the Integrated Genome Browser, for a more exhaustive viewing of BAM files containing individual reads we used the Tablet software.[29,30] Comparison of RNA-seq data from wild type and 2.4.1Δ*rnj* revealed about 30 different abundant RNA fragments that were apparently exclusively enriched in the RNase J deletion mutant. We also observed some less abundant RNA fragments which accumulated in the mutant, but the total number of accumulated fragments was small (< than 2% of all genes) compared with the changes observed for an RNaseJ1 mutant in *B. subtilis*.[21] Because our cDNA library preparation did not include an RNA fragmentation step, the read coverage of the RNAs is mainly limited to their immediate 5′-end with a length of 107 nt. To ascertain the 3′-ends of fragments that apparently outreached length limit, we took advantage of available data from a rudimentary RNA-seq approach using the same RNA samples for sequencing on a 454 platform (Roche), generally generating read lengths longer than 200 nt. The accumulated fragments in 2.4.1Δ*rnj* ranged in size from 60 to 250 nt and were distributively found, located in 5′-or 3′-regions and at internal positions of annotated open reading frames. In few cases the accumulated fragments originated from internal regions of rRNAs (**Fig. 1** and **S1**).

Validation of RNase J specific targets by northern blot

Out of the roughly 30 strongly accumulated RNAs we chose 15 fragments showing the highest enrichment in the 2.4.1Δ*rnj*

RNA-seq data for validation by northern blot. To exclude that the accumulating RNAs in the mutant result from polar effects of the inserted kanamycin cassette on downstream genes we included two complemented mutant strains in our northern blot analysis. The strain Δ*rnj*RK::*rnj~His* harbors a plasmid-borne *rnj-His*$_6$ gene, while a second mutant strain Δ*rnj*RK::*rnj-DH80KA~His* expresses an inactive RNase J-DH80KA-His$_6$ variant harboring two point mutations within the catalytic center.[27,31] For about half of the RNAs we tested by northern blot their increased levels in 2.4.1Δ*rnj* could be confirmed (**Tab. 1**). For some fragments northern blot detection completely failed, probably due to their low abundance. In some cases RNA fragments of the expected size were detected in similar amounts in the mutant as well as in the wild type (data not shown). This corroborates the presence of technical artifacts in the RNA-seq data probably due to biased amplification during cDNA library preparation.

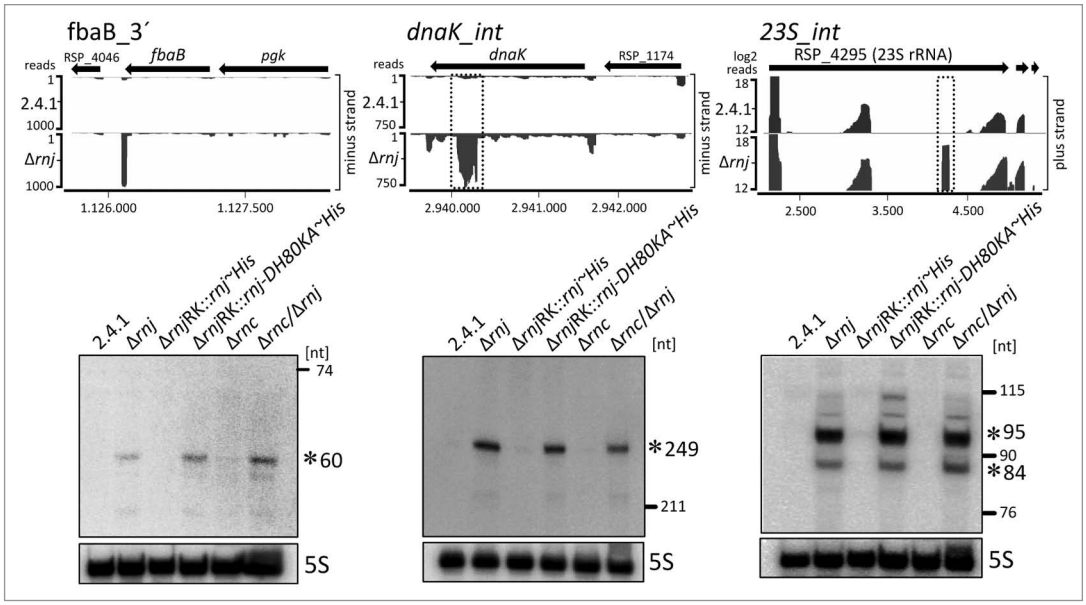

Figure 1. Identification of accumulating RNAs in an RNase J deletion mutant of *R. sphaeroides,* Δ*rnj*, by RNA-seq. **Top**: Examples of regions with accumulated reads in the RNA-seq data of Δ*rnj* compared with the wild type 2.4.1 data visualized by the Integrated Genome Browser. Left: 3′-terminus of the *fbaB* mRNA. Middle: internal fragment of *dnaK* mRNA. Right: internal fragment from the 3′-half of the 23S rRNA. Relevant regions are accented by pointed frames. **Bottom**: Northern blot validation of the Δ*rnj* specific RNAs. Fifteen micrograms of total RNA isolated from different *R. sphaeroides* strains (OD_{660} 0.6) were separated on 10% polyacrylamid gels containing 7.5M urea. Wild type (2.4.1), RNase J deletion mutant (Δ*rnj*), RNase J deletion mutant complemented with RNase J~His$_6$ (Δ*rnj*RK::*rnj~His*), RNase J deletion mutant complemented with point-mutated inactive RNase J~His$_6$ variant (Δ*rnj*RK::*rnj-DH80KA~His*), RNase III deletion mutant (Δ*rnc*), RNase J/RNase III double-mutant (Δ*rnj*/Δ*rnc*). After blotting RNAs were detected by 5′-radioactively labeled DNA-oligonucleotides. Sizes indicated with a bar on the right of the blots are derived from probing against RNAs with known length (coding piece of tmRNA, 211 nt; 5S rRNA, 115 nt;. tRNA-Ala, 76 nt). Sizes marked with an asterisk correspond to the RNA-seq obtained lengths of the respective RNA.

Accumulation of mRNA derived fragments

Northern blot validation is shown in **Figure 1 and 2** and summarized in **Table 1**. All northern blots described below confirmed the accumulation of specific RNA fragments in the RNase J deletion mutant 2.4.1Δ*rnj* and the complementation strain Δ*rnj*RK::*rnj-DH80KA~His* which expresses an inactive variant of RNase J. We confirmed the accumulation of *fbaB_3′*, a 60 nt long fragment matching the 3′-terminal region of fructose bisphosphate aldolase encoding mRNA (RSP_4045) (**Fig. 1**). The *dnaK_int* RNA is a roughly 250 nt long fragment located within the open reading frame of *dnaK* mRNA (RSP_1173), encoding the bacterial homolog of the heat-shock protein DnaK (**Fig. 1**). The *rnpA_5′* fragment has a length of approximately 60 nt according RNA-seq and corresponds to the immediate 5′-region of the *rnpA* open reading frame, encoding the protein component of RNase P (RSP_1060). The northern blot revealed two *rnpA_5′* variants differing roughly 5 nt in length from each other (**Fig. 2** and **S1**). The *ftsI_int* RNA originates from an internal region of the *ftsI* mRNA (RSP_2098) which encodes a glycosyltransferase involved in peptidoglycan synthesis. According to RNA-seq *ftsI_int* has a length of 74 nt whereby northern blot revealed a prominent approximately 85 nt long fragment and several less abundant fragments smaller than 74 nt (**Fig. 2** and **S1**). *0959_int* matches an internal part of the RSP_0959 mRNA, encoding an ATPase related to the exodeoxyribonuclease V involved in DNA recombination and repair. RNA-seq points to accumulation of an 85 nt long RNA fragment in 2.4.1Δ*rnj*. We detected an RNA with this expected size in wild type, the deletion mutants as well as both complemented strains. Nevertheless a shorter about 74 nt fragment was specifically detected only in 2.4.1Δ*rnj* and Δ*rnj*RK::*rnj-DH80KA~His* (**Fig. 2** and **S1**).

rRNA fragments in 2.4.1Δrnj show growth-stage dependent accumulation

We confirmed the accumulation for two rRNA derived fragments in 2.4.1Δ*rnj*, in the following named *16S_int* and *23S_int* RNA. The *16S_int* fragment is an 149 nt RNA spanning nt 1151 to 1300 of the 16S rRNA (orthologous genes: RSP_4294/4347/4352) (Fig. Two and S1). This region comprises the helices 41, 42 and 43 (*E. coli* numbering). Helix 41 in 16S rRNA of *E. coli* has a crucial role in specific inhibition of RNase T2, also designated as RNase I. This unspecific RNase is mainly located in the periplasmic space and involved in scavenging exogenous RNA. When RNase T2 enters the cytoplasm e.g., due to aged or depolarized inner membrane their potentially cytotoxic

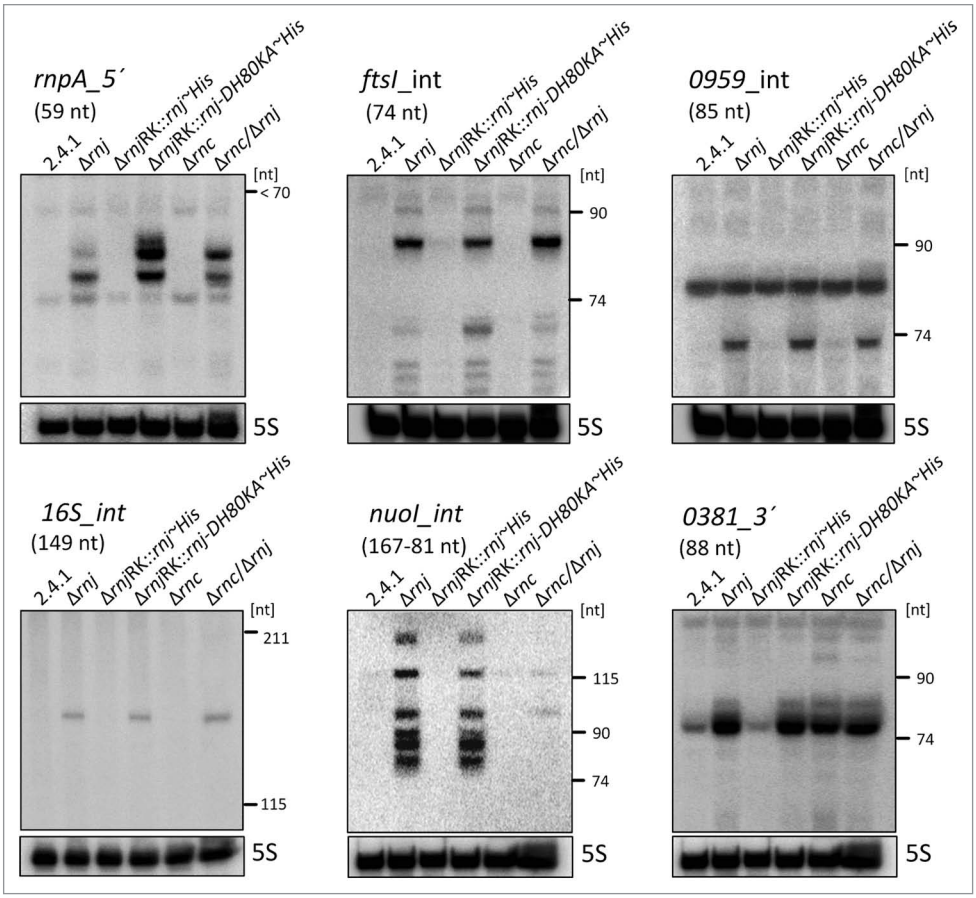

Figure 2. Northern blot validation of RNAs accumulating in Δ*rnj*, revealed by RNA-seq analysis. Total RNA (15 µg) isolated from different *R. sphaeroides* strains (OD_{660} 0.6) were separated on denaturing 10% polyacrylamid gels. Wild type (2.4.1), RNase J deletion mutant (Δ*rnj*), RNase J deletion mutant complemented with RNase J~His_6 (Δ*rnj*RK::*rnj~His*), RNase J deletion mutant complemented with point-mutated inactive RNase J~His_6 variant (Δ*rnj*RK::*rnj-DH80KA~His*), RNase III deletion mutant (Δ*rnc*), RNase J/RNase III double-mutant (Δ*rnj*/Δ*rnc*). RNAs were detected by 5′-radioactively labeled DNA-oligonucleotides. Numbers in parentheses indicate expected length according RNA-seq data. Membranes were probed repeatedly after stripping. 5S rRNA served as a loading control. Sizes indicated with bars on the right of the blots are derived from probing against RNAs with known length (5S rRNA, 115 nt; tRNA-Ser (RSP_4314), 90 nt; tRNA-Gly (RSP_4305), 74 nt).

activity is prevented through its specific binding to helix 41.[32] In the case of the *23S_int* fragment RNA-seq data pointed to two alternative 5′- and 3′-ends, between nt 2090 to 2186 of the 23S rRNA (orthologous genes: RSP_4295/4350/4355) resulting in two alternative fragments 95 and 84 nt in length, respectively (**Fig. 1**). The abundance of the *23S_int* fragments in 2.4.1Δ*rnj* is enormous, this is reflected by roughly 450.000 reads for these fragments during RNA-seq, equivalent to almost one-tenth of all reads in the 2.4.1Δ*rnj* RNA-seq data set. Only the 5.8S-like rRNA showed a higher read number (1.500.000) while other typically highly abundant RNAs such as tRNAs only reached read numbers between 5.000 to 60.000. In this respect it is also surprising that *23S_int* is virtually not detectable in wild type by RNA-seq or northern blot (**Fig. 1**). The *23S_int* corresponds to the RNA component of the L1 protuberance in the 50S ribosomal subunit and includes helices 76, 77 and 78 (*E. coli* numbering according Petrov et al.[33]). This very flexible region protrudes to the exterior of the large ribosomal subunit and is implicated in the release of deacylated tRNA from the E site.[34]

The high abundance of the rRNA fragments *23S_int* in 2.4.1Δ*rnj* was surprising to us as ribosome degradation during exponential growth is negligible and mainly occurs during slow down of growth preceding entry into stationary phase.[2] We asked whether there are growth phase dependent differences in the abundance of the rRNA derived fragments and determined their steady-state levels by northern blot in 2.4.1Δ*rnj* grown under standard micro-aerobic conditions at different growth stages (**Fig. 3**). The growth behavior of the RNase J deletion mutant 2.4.1Δ*rnj* showed no deviations to the growth of the wild type (**Fig. 3A**). Relative quantification of fragments at different growth stages was performed by normalizing against 5S rRNA. Afterwards the highest signal intensity was set to 100% and compared with the intensities at other growth phases (**Fig. 3C**/D). The highest amounts of *16S_int* and *23S_int* were detected at the transition to stationary phase (T_4, OD_{660} 1.3). The relative amount of *16S_int* continuously increase during growth to transition phase (T_4) compared with the early exponential phase (T_1). The relative amount of *23S_int* RNA in 2.4.1Δ*rnj* already reached a plateau at T_2 followed by only a marginal increase until T_4. The relative abundance of both fragments showed a strong decline at stationary phase (T_5). Tracking the abundance of RNase J in *R. sphaeroides* 2.4.1 wild type during micro-aerobic growth by western blot revealed that RNase J is constitutively expressed during growth from early exponential to stationary phase (**Fig. 3B**).

Effect of RNase III deletion on RNase J dependent RNA fragments

For most of the detected fragments in 2.4.1Δ*rnj* we assume that they are remnants from incomplete decay of the corresponding mRNAs and therefore are probably initially generated by RNase E cleavages. Additionally the detected internal fragments could also represent 3′-terminal truncated forms of RNase E degradation products caused by 3′-exoribonucleases that are paused during degradation. However participation of RNase E in generation of the *23S_int* RNA fragments is unlikely regarding their positions within the secondary structure of the corresponding

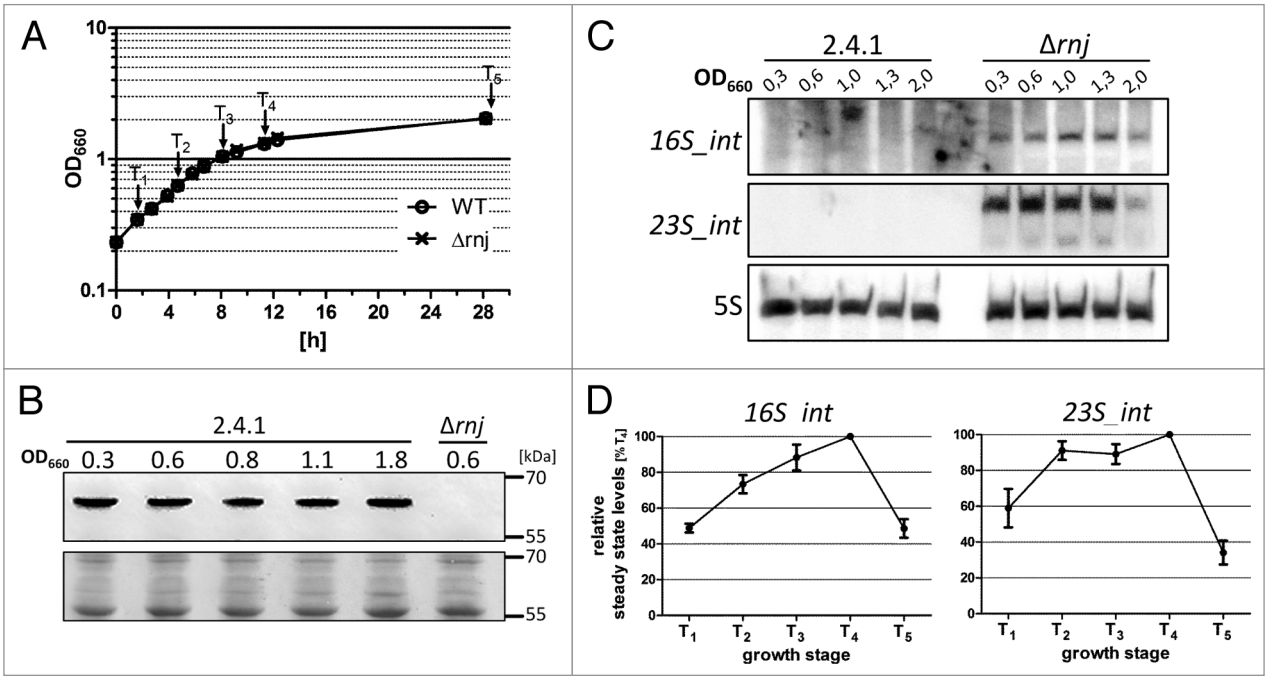

Figure 3. Steady-state levels of RNase J in 2.4.1 wild type and rRNA fragments accumulating in the RNase J deletion mutant 2.4.1Δ*rnj* during micro-aerobic growth. **(A)** Growth curve (optical density at 660 nm) from *R. sphaeroides* wild type (dot, WT) and RNase J deletion mutant (cross, Δ*rnj*) micro-aerobically grown in standard media. Results represent the mean of three independent experiments. Samples for RNA isolation were collected at time points indicated by arrows (T_1 to T_5). **(B)** Soluble protein fractions (20 µg) from different growth stages of wild type (2.4.1) and deletion mutant (Δ*rnj*) (slightly different to RNA sampling indicated in 3A) were separated by 10% SDS-PAGE and blotted onto nitrocellulose membrane. **Top**: Western blot. Membrane was incubated with anti RNase J antiserum. RNase J was detected using a horseradish peroxidase coupled secondary antibody and X-ray film developing. **Bottom**: Ponceau staining was used as a loading control. **(C)** Northern blots of total RNA (20 µg) isolated from cultures of *R. sphaeroides* wild type (2.4.1) and RNase J deletion mutant (Δ*rnj*) at different growth stages. The indicated OD_{660} corresponds to time points T_1-T_5 in the growth curve shown on the left. The membrane was consecutively probed and stripped with radioactively labeled DNA-oligonucleotides complementary to the RNAs named on the left. 5S rRNA served as a loading control. **(D)** Relative abundance of RNA fragments accumulating in Δ*rnj* during micro-aerobic growth. Quantification of fragments was normalized against 5S rRNA. Percentage quantities result from comparison to signal intensities at time point T_4 (OD_{660} 1.3) which were defined as 100%. Error bars indicate standard deviation within biological triplicates.

helices in the 23S rRNA of *R. sphaeroides* structure available from the Comparative RNA web (CRW) site, see ref.[35] Both alternative 5′- and 3′-ends of the *23S_int* fragments are located in a helical stem structure quite opposite to each other (**Fig. S2**). This suggests the generation of the *23S_int* RNA from cleavages by the double-strand specific endoribonuclease RNase III within the L1 protuberance RNA component that is subsequently degraded by RNase J. To prove this assumption northern blot analysis with total RNA of an RNase J/RNase III double deletion mutant, 2.4.1Δ*rnc*/Δ*rnj* and an RNase III mutant, 2.4.1Δ*rnc* was performed. Despite the presence of potential RNase III processing sites, the 2.4.1Δ*rnc*/Δ*rnj* strain accumulated *23S_int* to the same extent as 2.4.1Δ*rnj* (**Fig. 1**). An unchanged accumulation in 2.4.1Δ*rnc*/Δ*rnj* holds true for all of the RNA fragments mentioned before (Fig. One and 2). Interestingly, at least in two cases we observed a correlation between RNase J dependent RNA fragments and the presence of RNase III. One example is *nuoI_int*, corresponding to an internal part of the *nuoI* mRNA (RSP_0107), encoding a subunit of the NADH-quinone oxidoreductase. RNA-seq analysis revealed fragments of these mRNA accumulating in 2.4.1Δ*rnj* with lengths between 81 and 167 nt. Northern blot analysis of total RNA from 2.4.1Δ*rnj* and 2.4.1Δ*rnj*RK::*rnj-DH80KA-His* showed at least six alternative fragments within this range of length. These fragments were almost undetectable in the 2.4.1Δ*rnc*/Δ*rnj* strain (**Fig. 2**). A more precise inspection of the individual, length varying fragments revealed that the predominant part of fragments bear the same 3′-end but have differing 5′-ends. Interestingly, RNA-seq data revealed quite a few reads for a short RNA in antisense orientation to the 3′-region of *nuoI_int* (**Fig. S3**). These observations suggest that RNase III might generate fragments of *nuoI* mRNA that are then exclusively degraded by RNase J. The generation of the *0381_3′* fragment is mysterious and cannot be explained by the canonical degradation pathway. This 88 nt long RNA represents the 3′-terminus of the RSP_0381 mRNA encoding a protein related to PhaP, a polyhydroxyalkanoate-granule associated protein. This mRNA harbors a Rho-independent transcription terminator as predicted by TransTermHP.[36] *0381_3′* is detectable in wild type to low extent, and strongly accumulated in 2.4.1Δ*rnj* and 2.4.1Δ*rnc*/Δ*rnj*. But also in an RNase III single deletion mutant 2.4.1Δ*rnc*, *0381_3′* was highly abundant (**Fig. 2**). Interestingly, preliminary data of a dRNA-seq approach with *R. sphaeroides* suggests that *0381_3′* seems more likely to be a small RNA that is transcribed from the 3′-region of the RSP_0381 locus than it is a degradation intermediate. This assumption is based on the enrichment of

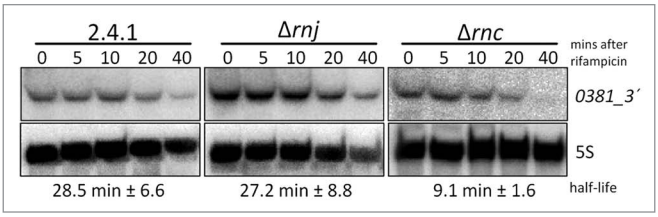

Figure 4. Stability of *0381_3′* RNA in different RNase deletion strains. For half-life determination of *0381_3′* RNA cultures were grown microaerobically to an OD_{660} of 0.6 (2.4.1 wild type, Δ*rnj* RNase J deletion, Δ*rnc* RNase III deletion, Δ*rnj*/Δ*rnc* RNase J/RNase III double deletion strain). Rifampicin was added to a final concentration of 0.2 mg/ml and samples for RNA isolation were taken at timepoints as indicated. Twenty micrograms of total RNA were separated on denaturing 10% polyacrylamide gels. After blotting onto a nylon membrane *0381_3′* RNA was detected by the 5′-radioactively labeled DNA-oligonucleotide NB_*0381_3′* (**Table S1**). Afterwards membrane was stripped and reprobed with a 5S probe. Calculated half-lifes with standard deviation of *0381_3′* RNA are given under the panels. Quantification based on biological triplicates was performed by using Quantity One software (Biorad). 5S rRNA was used for normalization.

RNA-seq reads for the *0381_3`* in RNA treated with terminator exonuclease (TEX) compared with read numbers with non-TEX treated RNA (**Fig. S4**).[28] Considering that *0381_int* might be an individually transcribed RNA we performed half-life experiments to test whether the accumulation of *0381_int* RNA in RNase deletion strains rather results from increased transcription rates than increased transcript stability. While the half-life of *0381_int* RNA is not affected in the RNase J deletion strain (27 min in 2.4.1Δ*rnj* compared with 28 min in the wild type), we surprisingly observed a strong decline of transcript stability to about 9 min, in the RNase III deletion strain, 2.4.1Δ*rnc* (**Fig. 4**).

Discussion

Our comparative RNA-seq approach between *R. sphaeroides* 2.4.1 wild type and an RNase J deletion strain 2.4.1Δ*rnj* identified a small number of RNA fragments that accumulated to high levels in absence of RNase J. This is in agreement with the unaffected growth behavior of the RNase J deletion strain compared with that of the wild type (**Fig. 3A**).[27] Apparently RNase J has only limited participation in the global mRNA degradation of *R. sphaeroides*. This is in contrast to *B. subtilis*, where under severe depletion conditions of RNase J1 roughly 30% of all mRNAs were affected.[21] Just recently an RNA-seq approach with the Crenarchaeon *Sulfolobus acidocaldarius* revealed global alterations of the transcriptome by deletion of aCPSF2 an archaeal homolog of RNase J.[37] The identification of RNA fragments exclusively accumulating in the RNase J deletion strain 2.4.1Δ*rnj* and virtually not detectable in the wild type leads to a number of questions regarding different aspects of RNA metabolism. According to the major model of RNA degradation in Gram-negative bacteria RNAs are cleaved endonucleolytically by RNase E and subsequently become degraded by processive 3′-exoribonucleases. What determines RNA fragments in *R. sphaeroides* to become specific targets for the 5′-exoribonuclease RNase J or why are they not degraded by the classical 3′-exoribonucleases RNase R or PNPase? A third 3′-exoribonuclease, RNase II, typically involved in the RNA decay of Gram-negative bacteria is not encoded in the *R. sphaeroides* chromosome. Structured 3′-termini that are inaccessible for processive 3′-exoribonucleases can explain an RNase J dependent degradation. PNPase and RNase R from *E. coli* are capable to bind and degrade structured regions providing 3′-terminal single stranded regions with a minimum of 7 nt or 11 nt respectively in length are present.[10,38] All accumulating RNA fragments described in our study of the RNase J deletion mutant exhibit single stranded 3′-ends of 3 - 5 nt as predicted by Mfold (**Fig. S5**).[39] This is most likely insufficient for effective binding and processing of RNAs by 3′ exoribonucleases. However single stranded 3′-ends of only 2 nt are adequate for adding poly(A) tails to mRNA fragments by poly(A) polymerase I (PAPI). These attached "toeholds" in turn facilitate rebinding of exoribonucleases and recurrent attack at otherwise inaccessible structured 3′-termini.[40,41] Interestingly, genomes of α-proteobacteria like *R. sphaeroides* do not encode poly(A) polymerase I and therefore appear to lack a poly(A) tail-assisted pathway for the 3′-to-5′ decay of structured RNA intermediates.[42] However *R. sphaeroides* harbors the bifunctional PNPase. Beside its phosphorolytic RNA cleavage activity this enzyme can also operate reversely, by synthesizing heteropolymeric 3′-terminal tails that serve a similar purpose as poly(A) tails.[43] In *Bacillus subtilis* depletion of RNase J1 leads to accumulation of 3′-fragments of many different RNAs and it is suggested that 5′-to-3′ degradation by RNase J1 is the primary pathway for the decay of transcription terminators in this Gram-positive bacterium.[44,45] Obviously in the Gram-negative *R. sphaeroides* the decay of at least some degradation intermediates also relies on the 5′-to-3′ orientated degradation by RNase J. Maybe these RNA fragments are omitted by the classical 3′-to-5′ orientated RNA decay machinery because adding of heteropolymeric tails at 3′-termini of RNA fragments by PNPase is hindered or not efficient enough to serve as "toehold" for 3′-exoribonucleases.

Another question arises with accumulating rRNA fragments in the RNase J deletion strain from *R. sphaeroides*. Since rRNAs generally are considered as very stable the extreme abundance of the 23S fragment *23S_int* in 2.4.1Δ*rnj* is quite surprising.[2] rRNA degradation basically takes place at the entry of growth into stationary phase or in the course of quality control.[1] We detected the highest levels for rRNA fragments *16S_int* and *23S_int* in 2.4.1Δ*rnj* at the transition stage (**Fig. 3D**). This suggests their generation in the process of adaptation to stationary growth conditions. However it is unclear why *23S_int* is already highly abundant during exponential phase of 2.4.1Δ*rnj* (**Fig. 3C**/D). In this regard *23S_int* might also represent a stable degradation intermediate generated in the course of quality control mechanisms for rRNAs. In bacteria these mechanisms are not well understood. So far it is assumed that defect or misassembled ribosomal subunits contain exposed RNA cleavage sites that are targeted by an unknown endoribonuclease followed by removal of the resulting fragments mainly by PNPase and RNase R.[46] In the course of 23S rRNA quality control in *E. coli* several closely spaced initial cleavages in the region of

helix 71 were identified. This helix 71 is normally located in the subunit interface of 70S ribosomes.[6] The *23S_int* RNA identified in our study is located in the helices 76, 77 and 78 of the large ribosomal subunit a region that is called L1 protuberance because it protrudes out of the 70S ribosome.[34] The ribosomes from *R. sphaeroides* as well as from other proteobacteria naturally contain fragmented 23S rRNA molecules. Initial fragmentation of 23S rRNA is performed by RNase III followed by 5′-and 3′-exoribonucleolytic processing of the resulting fragments.[47] We previously reported that the RNase J deletion mutant 2.4.1Δ*rnj* exclusively harbors premature 23S rRNA fragments with prolonged 5′- and 3′-ends.[27] RNA quality control mechanisms could take effect on a subpopulation of ribosomes from 2.4.1Δ*rnj* that are misassembled because of the incompletely processed 23S rRNA fragments. As the presence of *23S_int* in 2.4.1Δ*rnc*/Δ*rnj* excludes that RNase III is the preceding ribonuclease that cleaves in the double-stranded region of helix 76, one of the questions that have to be answered in future work is, which ribonuclease is responsible for generation of the *23S_int* fragment.

The accumulation of *nuoI_int* RNAs with varying lengths in 2.4.1Δ*rnj* but not in 2.4.1Δ*rnc*/Δ*rnj* points toward an RNase III dependent generation of these fragments and their subsequent degradation by RNase J. Double-stranded RNA regions that comprise RNase III processing sites can be intrinsic features of RNA transcripts as found in e.g., polycistronic ribosomal precursor RNAs and diverse mRNAs as *rpsO-pnp* or *bdm* mRNA.[48-50] Alternatively, helical RNase III cleavage sites can emerge from RNA-RNA interactions between mRNA and small antisense RNAs.[51] Our RNA-seq data imply the presence of a low abundant *cis*-encoded antisense RNA (asRNA) whose 3′-part overlaps with the *nuoI_int* region (**Fig. S3**). It is tempting to speculate that duplex formation between such an asRNA and the *nuoI* region generates RNase III processing sites that may result in *nuoI_int* RNA fragments detectable in 2.4.1Δ*rnj* but not in wild type 2.4.1 due to their immediate degradation by RNase J. It was however not possible to unambiguously prove the existence of this low abundant asRNA. The *nuo* operon encodes 14 subunits of the energy conserving NADH dehydrogenase also called complex I and a conserved antisense RNA associated with the *nuo* operon has also been described for different *Streptomyces* bacteria. But in *Streptomyces* bacteria the position of an asRNA is opposite of *nuoE* and *nuoF* and therefore differs to the anti-*nuoI* location identified in our study.[52]

In case of the *0381_3′* RNA accumulating in 2.4.1Δ*rnj*, 2.4.1Δ*rnj*/Δ*rnc* and 2.4.1Δ*rnc* we considered that this RNA is rather an individually transcribed small RNA than a 3′-terminal degradation intermediate of the RSP_0381 mRNA. Yet unpublished data of a dRNA-seq approach that allows distinction between primary and processed transcripts hint to a putative transcriptional start site at the 5′end of the *0381_3′* RNA (**Fig. S4**).[28] sRNAs from 3′-regions of mRNAs that are produced by overlapping sense transcription with a shared transcription terminator have already been described for *E. coli* and *Salmonella typhimurium*.[53,54] Half-life determination revealed that the strong accumulation of *0381_3′* in 2.4.1Δ*rnj* and 2.4.1Δ*rnc* is not the consequence of increased stability of this RNA supporting the view of increased production. We could not detect promoter activity for the sequence directly upstream of the *0381_3′* RNA in a reporter construct (data not shown), indicating that accumulation after TEX treatment is due to other features of this RNA 5′end than a triphosphate. Lacking promoter activity in our reporter construct does not exclude transcriptional initiation within the RSP_0381 coding region further upstream. A sequence-based prediction of promoters in *R. sphaeroides* is only possible for a subset of promoters for some alternative sigma factors. Promoters recognized by the house-keeping sigma factor have very low sequence similarity. At present the mechanisms of generation of the *0381_3′* RNA remain obscure and most likely involve indirect effect of RNase J and RNase III, which increase synthesis of part of *0381* mRNA.

Our study revealed just a significant role for 5′-to-3′ exoribonuclease RNase J in the turnover of a limited number of transcripts in *R. sphaeroides*. The structural characteristics of the RNA fragments accumulating in an RNase J deletion strain suggest that RNase J is responsible for the decay of degradation intermediates that cannot serve as substrates for the 3′-to-5′ exoribonucleases.

Material and Methods

All strains, plasmids and oligos used in this study are listed in **Table S1** and **S2** of the supplementary data.

Cultivation of strains

R. sphaeroides 2.4.1 strains were grown in malate minimal medium under micro-aerobic conditions (dissolved oxygen: »25 µM) at 32 °C.[55]

Construction of R. sphaeroides 2.4.1Δrnc and 2.4.1Δrnj/Δrnc mutant

R. sphaeroides strain 2.4.1Δ*rnc* was generated by homologous recombination of the suicide plasmid pPHU281::Δ*rnc::*Km^r. Briefly, 5′ and 3′ parts of the *rnc* open reading frame (RSP_1675) together with respective upstream and downstream sequences were PCR amplified using oligos 1675_KO_up_f, 1675_KO_up_r, 1675_KO_dwn_f and 1675_KO_dwn_r. These fragments were inserted simultaneously into EcoRI and HindIII sites of the suicide vector pPHU281 generating pPHU281::Δ*rnc_up_dwn*. A 1.3 kbp BamHI fragment containing a kanamycin resistance cassette from pUC4K was inserted into the BamHI sites of pPHU2.4.1Δ*rnc_up_dwn* to generate pPHU2.4.1Δ*rnc*::Km^r.[56] Alternatively a 2.6 kbp BamHI fragment containing a gentamycin cassette from pWKR209-CII was inserted to generate pPHU2.4.1Δ*rnc*::Gm^r.[57] The plasmid pPHU2.4.1Δ*rnc*::Km^r was transferred into *E. coli* S-17-1 and subsequently transferred to *R. sphaeroides* 2.4.1 by diparental conjugation to generate the RNase III deletion strain 2.4.1Δ*rnc*. The double-deletion mutant 2.4.1Δ*rnj*/Δ*rnc* was generated equally by transferring pPHU2.4.1Δ*rnc*::Gm^r into 2.4.1Δ*rnj* resulting in the kanamycin and gentamycin resistant strain 2.4.1Δ*rnj*/Δ*rnc*.[27] Successful deletion of *rnc* was verified by antibiotic resistance and PCR.

Construction of complementation strain 2.4.1ΔrnjRK::rnj-DH80KA~His$_6$

Using the pQE70::*rnj~His$_6$ E. coli* expression vector as template we performed overlap extension PCR to construct an *rnj* gene expressing a catalytic inactive RNase J variant. In this course Asp80 and His81 of RNase J were replaced by Lys80 and Ala81 resulting in the catalytic inactive RNase J-DH80KA variant.[31] Oligos used are listed in **Table S1**. The PCR fragment was inserted into SphI and BglII sites of pQE70 generating pQE70::*rnj-DH80KA*. Subsequently the gene *rnj-DH80KA~His$_6$* was amplified by PCR using oligos RSP_2534his6_415_f, RSP_2534his6_415_r. Cleavage of the PCR fragment with KpnI and EcoRI, followed by cloning with the same restriction sites into plasmid pRK415 resulted in plasmid pRK2.4.1*rnj-DH80KA~His$_6$*. This plasmid was subsequently transformed into *E. coli* S17–1 and conjugated with strain 2.4.1Δ*rnj* to obtain the complemented strain 2.4.1Δ*rnj*RK*rnj-DH80KA~His*.

RNA isolation and northern blot

RNA was prepared from cells growing at different growth stage using peqGold TriFast™ isolation system (PeqLab; #30–2020). 15–20 µg of total RNA samples were separated on 10% (v/v) polyacrylamid gels containing 7 M urea and 1 x TBE. Gel runs were performed at 300 Volt for approximately 3 h. RNA was transferred to Roti®-Nylon plus 0,45 µm (Roth; #K058.1) by semi-dry electroblotting in 1 x TBE (250 mA, 3 h) followed by crosslinking with UV-light. Northern blot analysis with radioactively labeled DNA-oligo was performed as described elsewhere.[58]

Half-life determination of 0381_3′

Cultures (400 ml) were grown micro-aerobically in 500 ml flasks to an OD_{660} of 0.6. After addition of 1.6 ml rifampicin (50 mg/ml solved in methanol, final concentration 200 µg/ml) samples (15 ml) were harvested on ice at indicated time-points. RNA isolation and northern blot were performed as described above. Quantification based on biological triplicates was performed by using Quantity One software (Biorad). 5S rRNA was used for normalization.

RNA-seq

Cultures from *R. sphaeroides* 2.4.1 wild type and 2.4.1Δ*rnj* were grown in biological triplicates under micro-aerobic conditions to a final OD_{660} of 0.4. From these cultures total RNA was isolated using the hot phenol method followed by DNase I (Invitrogen, #18047019) treatment. Equal amounts of triplicate RNA samples were pooled and used for cDNA library preparation at Vertis Biotechnology AG (Germany). cDNA library preparation and RNA sequencing (RNA-seq) was performed as previously described.[59] Shortly, RNA was poly(A)-tailed by poly(A) polymerase and treated with tobacco acid pyrophosphatase to remove 5′-PPP residues. After that, adaptor ligation at the 5′-end of the RNA was followed by first strand cDNA synthesis using an oligo(dT)-adaptor primer and the M-MLV reverse transcriptase. The resulting cDNAs were PCR amplified using primers designed for TruSeq sequencing (Illumina) and a high fidelity polymerase. Sequencing was performed on a Illumina Genome Analyzer IIx machine. Mapping of the obtained sequencing reads to the *R. sphaeroides* 2.4.1 genome (TaxID: 272943) was done by using the *segemehl* software.[60] The coverage graphs representing the number of reads per nucleotide were calculated as described in Dugar et al.[61] and visualized using the Integrated Genome Browser.[29] The raw, de-multiplexed reads as well as coverage files have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus under the accession number GSE54750 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE54750).[62]

Western blot

RNase J was detected in 20 µg soluble protein fraction of *R. sphaeroides* using polyclonal antiserum against His-tagged RNase J as described previously in Rische and Klug.[27]

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