

Mapping the RNA-Seq Trash Bin: Unusual Transcripts in Prokaryotic Transcriptome Sequencing Data

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Abstract

Prokaryotic transcripts constitute almost always uninterrupted intervals when mapped back to the genome. Split reads, i.e., RNA-seq reads consisting of parts that only map to discontinuous loci, are thus disregarded in most analysis pipelines. There are, however, some well-known exceptions, in particular tRNA splicing and circularized small RNAs in Archaea as well as self-splicing introns. Here, we reanalyze a series of published RNA-seq data sets, screening them specifically for non-contiguously mapping reads. We recover most of the known cases together with several novel archaeal ncRNAs associated with circularized products. In Eubacteria, only a handful of interesting candidates were obtained beyond a few previously described group I and group II introns. Most of the atypically mapping reads do not appear to correspond to well-defined, specifically processed products. Whether this diffuse background is, at least in part, an incidental by-product of prokaryotic RNA processing or whether it consists entirely of technical artefacts of reverse transcription or amplification remains unknown.

Keywords: RNA-seq, self-splicing introns, split tRNAs, circular sRNAs

1. Introduction

Common wisdom has it that prokaryotic transcripts correspond to intervals on the genomic DNA. In archaea, several exceptions to this simple rule are well known. As in eukaryotes, some of their tRNAs have introns that are spliced out by dedicated splicing endonucleases (1; 2). In contrast to Eukarya, enzymatically spliced introns can also be found in mRNAs (3) and in rRNAs (4). In some archaeal species, furthermore, tRNAs are composed of pieces that independently transcribed from different genomic locations (5; 2; 6; 7; 8).

Archaeal non-coding RNAs often are processed to yield a circular form. Large ORF-containing introns derived from rRNAs form stable RNA species in (9). Circular forms of both 23S and 16S rRNAs appear as processing intermediates during rRNA maturation (10). Circularized RNAs are produced from tRNA introns in (5), and a circularized box C/D snoRNA from *Pyrococcus furiosus* (11) turned out to be typical for box C/D snoRNAs, see also (8) for an example in *Nanoarchaeum equitans*. A recent study based on RNase R treated RNA libraries systematically mapped circularized RNAs and showed that circularized RNAs are also abundant in *Sulfolobus solfataricus* and its relatives (12). In contrast to this rather complex situation in Archaea, Eubacterial transcriptomes are not known to harbour spliced transcripts with the exception of the hosts of self-splicing group I and group II introns (13; 14; 15).

It is well known, on the other hand, that reverse transcription can generate artefactual sequences that look

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like splicing products, i.e., by leaving out stable RNA secondary structure features (16; 17; 18). Most analyses of prokaryotic RNA-seq data thus completely neglect sequencing reads that do not map as a single, uninterrupted interval. Of course, this strategy also hides any true splicing or circularization products. The purpose of this contribution is to systematically explore the content of the “trash bin” of RNA-seq analysis, aiming at the identification of atypically processed RNAs.

2. Methods

Sequence Data. Publicly available RNA-seq data were downloaded from the short read archive for 4 Archea and 6 Eubacteria in the Electronic Supplement¹. All these RNA-seq data were produced with non-strand-specific protocols. With the exception of the read data for *Escherichia coli* and *Salmonella enterica* all reads are single ended with length between 30 to 100 nts. According to requirements the raw reads were quality trimmed with FASTX-Toolkit and adapter clipped with Cutadapt (19).

Annotation. Annotation sources are the GFF files for each analyzed species downloaded from the NCBI² and the Rfam³ ftp servers, respectively. From the NCBI files all genes are extracted and the corresponding annotated elements, i.e., CDS, tRNA, and rRNA, are used. All genes that did not code for one of these elements are grouped into the separate class, “other”. Since NCBI annotation files often miss non-coding RNAs (ncRNAs) and regulatory elements such as riboswitches, these were instead adopted from the Rfam gff files. A detailed summary of all used annotation items and their sources is provided in the Electronic Supplement.

Since the Rfam annotation did not feature well-known group I introns, we reasoned that either the Rfam seed alignment (RF00028) does not cover the diversity of bacterial group I introns, or the presence of open reading frames in these introns hampers infernal search. We therefore split the Rfam seed alignment as well as 14 alignments of group I subtypes (<http://www.rna.whu.edu.cn/gissd/>, (20)) into 27 overlapping blocks along the 5'→3' direction of the intron, constructed individual CMs, scanned the genomes for

these sub-CMs and reconstructed potential group I introns by chaining adjacent hits in the correct order (non-overlapping 5' and 3' sub-CMs, and ≤5 kb distance between sub-CMs). The resulting 8 candidates, of which all but 2 have been described in literature (21; 22; 23), are listed in the Supplement.

For group II introns, we downloaded 35 intron sequences listed in the group II intron database (<http://webapps2.ucalgary.ca/~groupii/> on Feb 4th 2013, (24)) for different strains of the species considered here. Of these, 9 could be located in our reference genomes by blastn.

Read Mapping. All reads were mapped with segemehl, version 0.1.4 (25; 26) with the split read option -S. Depending on the read length of RNA-seq data, the minimum fragment length Z and the minimum fragment score U we set to combinations from $-Z\ 20\ -U\ 18$ to $(-Z\ 14\ -U\ 12)$. These small values are motivated by the need to emphasize split reads. For all other parameters the default values were used. Reads that remained unmapped in the first pass were remapped with Remapper, a component of the segemehl suite.

Reads that were mapped with splits were assigned to one of three categories: “normal”, same strand, same chromosom and insert between 15 nt and 200 kb, and matched fragments colinear with the genomic DNA; “circular”, same strand, same chromosom and junction distance less than 200 kb with fragment order inverted relative to genomic DNA; “(strand)switched”, same chromosom, junction distance less than 200 kb and fragments located on opposite strand. Splice sites determined by the read mapping were clustered with haarz, a component of the segemehl suite, to determine median split positions. The results of the mapping procedure are summarized in Tab. 1.

Overlaps between mapped reads and annotation data were computed with the help of BEDTools (27).

Analysis of ribosomal RNA loci. To compare rRNA split read patterns across species we performed the following steps: (i) For each species operon structures have been defined based on the rRNA gene annotation. For bacteria 16S-23S-5S rRNA operons are used and 16S-23S rRNA operon in archaea. (ii) The sequences of the rRNA operons including 300 nt flanking sequence have been extracted from the corresponding genomes. In species with multiple copies of rRNA operons a clustalw alignment has been calculated and the consensus sequence extracted. Either the consensus sequence or the sequence of a unique encoded operon has been

¹Machine readable data files and additional information can be found at <http://www.bioinf.uni-leipzig.de/supplements/12-002>.

²<ftp://ftp.ncbi.nih.gov/genomes/Bacteria/>

³<ftp://ftp.sanger.ac.uk/pub/databases/Rfam/11.0/genome.gff3.tar.gz>

Table 1: Summary of mapped reads.

Species	input	mappable	unsplit	split	read class		
					normal	circular	switch
Eubacteria							
<i>Bacillus cereus</i>	15,498,220	15,264,233	15,250,993	13,240	3,853	1,631	6,324
<i>Escherichia coli</i>	52,515,346	44,429,568	44,115,280	314,288	8,573	20,544	39,587
<i>Salmonella enterica</i>	31,924,568	27,752,771	27,737,761	15,010	543	2,481	4,421
<i>Pseudomonas PA14</i>	78,141,620	65,573,260	65,300,316	272,944	12,271	8,706	12,372
<i>Helicobacter pylori</i> 26695	82,847,902	40,152,294	39,146,732	1,005,562	17,930	53,709	77,095
<i>Synechocystis PCC6803</i>	31,985,927	15,080,656	15,031,302	49,354	39,956	165	250
Archaea							
<i>Nanoarchaeum equitans</i>	17,253,447	11,173,688	11,096,897	35,034	7,393	12,860	1,932
<i>Ignicoccus hospitalis</i>	17,253,447	5,302,517	5,181,769	76,039	6,254	39,994	3,656
<i>Pyrococcus furiosus</i>	6,449,461	8,691,213	8,474,477	216,736	11,536	54,795	17,095
<i>Sulfolobus solfataricus</i>	17,356,356	11,965,214	11,921,178	44,036	3,681	6,893	11,623

used as reference operon. The only exceptions are *N. equitans* and *H. pylori*. In *N. equitans* the 16S and 23S rRNA are transcribed from separate loci, in *H. pylori* the 16S rRNA and an operon comprising the 23S and 5S rRNAs. The separate parts were concatenated with an intervening stretch of 160 Ns as reference sequence. (iii) For each species all rRNA gene overlapping reads have been remapped onto the reference operon. Hence all rRNA reads are projected to a single locus for each species. (iv) To compare split-read patterns between species, the species specific reference operons were aligned using `clustalw` (28) and the mapped read coordinates transferred onto the alignment.

3. Results

3.1. Archaea

Several types of “atypical” RNAs are well known in Archaea. The most prominent form among them circularized RNAs. As expected we observe circularized precursor forms for both the 16S and 23S RNAs, see e.g. (10). In addition, large numbers of additional circularized products are observed, Fig. 1. The rRNA loci also feature substantial numbers of apparently spliced reads.

Most snoRNAs in Archaea also form circularized forms. Somewhat surprisingly, these are readily detectable from RNA-seq data even without prior treatment of the libraries to enrich circularized products as in the recent work of (12). The association of circularized products with small ncRNAs allows us to detect a number of novel ncRNA species in each of the four Archaea, Tab. 2. The number of new candidates depends

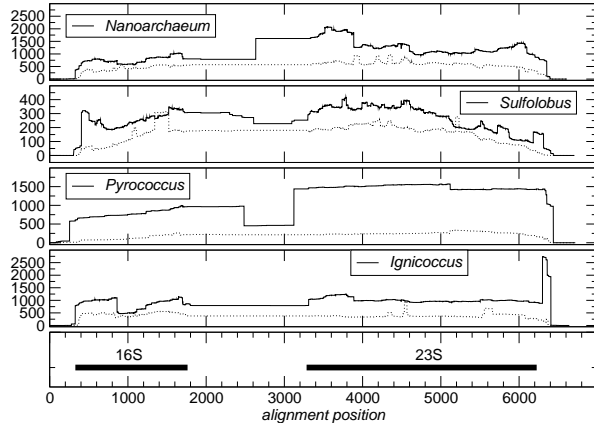


Figure 1: Density of circularized (thick line) and “spliced” reads (thin line) at the ribosomal rRNA loci. Coordinates refer to an multiple sequence alignment of the four Archaea species. For *N. equitans* the two separate RNAs genes are concatenated with 160 Ns linker.

strongly on the species, presumably in response to the quality of the available genome annotation.

Enzymatic splicing to tRNAs is a well-known phenomenon in Archaea. It is typically invisible in RNA-seq data, however, because tRNAs are normally multi-copy genes and tRNAs with introns typically have nearly identical paralogs without an intron. In this situation, mature tRNAs are mapped to the intro-less locus even the molecule in reality was produced by splicing for the locus with intron. In 20 cases the intron is visible as a circularized by-product of splicing; a detailed table can be found in the Electronic Supplement.

In *Sulfolobus* an enzymatically spliced intron inter-

Table 2: Novel ncRNAs in Archaea. Since the RNA-seq data are not strand specific, the reading direction remains undetermined in most cases. Promoter or terminator elements annotated in the UCSC Archaea Browser identify a likely reading direction. Read support was added up for alternative junctions within a few nucleotides. In the Note column, ‘mult.’ designates the presence of multiple products, and ~ denotes loci adjacent to annotated ncRNAs.

Coordinates		Note	
<i>Pyrococcus furiosus</i>			
128135	128190	?	8
258945	259007	?	915
505270	505323	?	4
505760	505814	+	1
860511	860567	?	3
<i>Sulfolobus solfataricus</i>			
434665	434719	-	2
1275505	1275576	?	71 3 variants
<i>Nanoarchaeum equitans</i>			
432130	432227	+	159 5' of 16S
396865	396957	+	95 3' of 23S
339418	339570	?	53 mult.
248142	248285	?	1
<i>Ignicoccus hospitalis</i>			
28125	28202	?	883
54013	54076	-	9
62481	62544	?	3
62543	62607	?	7 ~ previous
69658	69725	?	411
74304	74365	?	4
507227	507289	?	112
576736	576811	?	828
598273	598363	?	41
599309	599358	?	2
617433	617521	+	2017 ~ Iho-sR86
720628	720706	?	18
734264	734345	+	20 3' of 23S
824008	824070	+	8 ~ Iho-sR109
1000660	1000778	+	6 ~ Iho-sR131
1000717	1000778	+	3 ~ Iho-sR131
1066825	1066891	?	500
1266699	1266795	?	461 mult.

rupts the coding sequence of the *cbf5* gene (29). This case is readily detectable in the form multiple splitreads of the normal type. A second well-supported candidate is located close to the annotated translation start site of the putative protein SSO1586. With a length of 144 nt it preserves the reading frame. Since the entire sequence of the putative protein is conserved it might encode a functional isoform.

An interesting case of trans-splicing are the split tRNAs reported in *Nanoarchaeum* (30; 6; 8). Some of

Table 3: Splice junction overlaps with crisper and rRNA.

Species	all	crisper	rRNA
Eubacteria			
<i>Bacillus cereus</i>	11,808	0	7,955
<i>Escherichia coli</i>	68,704	6	37,616
<i>Salmonella enterica</i>	7,445	0	6,050
<i>Pseudomonas PA14</i>	33,349	528	16,819
<i>Helicobacter pylori 26695</i>	148,734	0	114,388
<i>Synechocystis PCC6803</i>	40,371	7	786
Archaea			
<i>Nanoarchaeum equitans</i>	22,185	0	16,607
<i>Ignicoccus hospitalis</i>	49,904	0	12,615
<i>Pyrococcus furiosus</i>	83,426	23,544	23,502
<i>Sulfolobus solfataricus</i>	22,197	150	13,781

them are not directly observable as split reads, however. This is the case e.g. for tRNA-Lys and tRNA-Gln, which have nearly identical paralogs that attract the mature tRNA reads to the unspliced loci irrespective of their true origin. The tRNA-Met and tRNA-Glu are visible at least with a few reads, while tRNA-His is invisible. This is explained by that high conservation of tRNA genes and the fact that RNA-seq data used here comprise a mixture of *N. equitans* and *I. hospitalis*. The tRNA-His sequence in *I. hospitalis* thus captures the transspliced tRNA-His reads from *N. equitans*. No other transsplicing events supported by a larger number of reads was observed in the data sets analyzed here.

Given the high expression levels of rRNAs, it is not surprising that a large fraction (ranging from 25% in *Ignicoccus* to 75% in *Nanoarchaeum*) of split reads maps to the rRNA loci, see Tab. 3. The number of spliced reads nevertheless is systematically smaller than the number of reads crossing a circularization point, see Fig. 1 above.

Surprisingly, about a quarter of the split read data for *Pyrococcus* maps to the CISPR loci. It is tempting to speculate that inclusion of an organisms own sequences in CRISPRs is akin to an autoimmune reaction. Without further validation, however, we cannot rule out that artifacts in reverse transcription or amplification are responsible for these “trans-spliced” reads.

3.2. Eubacteria

In contrast to Archaea, split reads are expected to be rare in Eubacteria. In fact, the only well-understood source are self-splicing introns. In the 6 genomes considered here, 8 group I and 9 group II introns could be

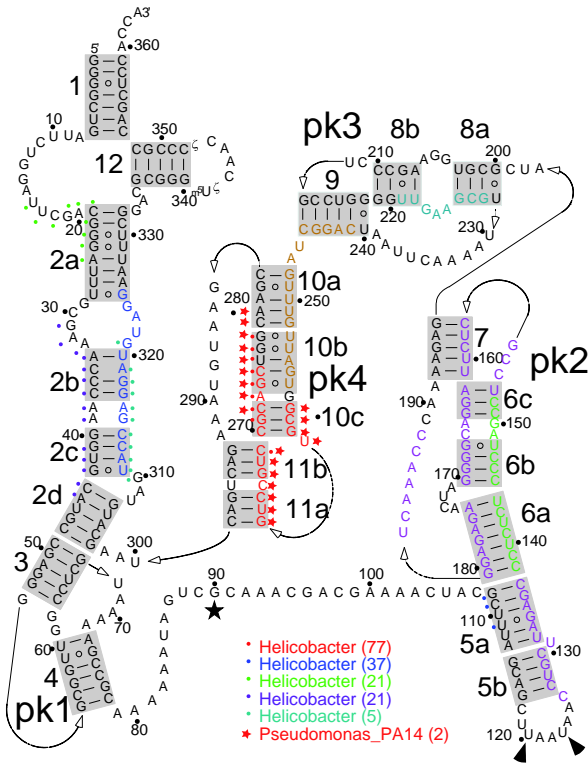


Figure 2: Mapping of “introns” observed in multiple reads from *Helicobacter* and *Pseudomonas* to the tmRNA structure (*E. coli* tmRNA model from (32)) shows that the excisions are concentrated in the pseudoknotted regions.

tentatively annotated computationally. Not all of them are visible in the RNA-seq data in the form of split-reads. Only a group I intron in the initiator-tRNA of *Synechocystis* (21) and a group I intron in the *recA* gene of *B. cereus* (31; 23) are well represented in our data. All of the detectable group II introns are located in *B. cereus* (22; 23). Only the B.c.I3 intron located within the DNA polymerase III subunit α is supported by many split reads. The two plasmid-borne introns designated B.c.I4 and B.c.I5 are visible as a single split read each. More details on the self-splicing introns can be found in the Electronic Supplement.

Surprisingly, our mapping data also show a large number of split and circularized reads that cannot be explained by known splicing mechanisms. As in Archaea, a large fraction of the split reads again maps to the rRNA operons, Tab. 3. With the exception of *Synechocystis*, rRNA accounts for the dominating part of the unusual RNAs. We have not been able to isolate candidates for well-defined stable processing products, however.

Beyond the self-splicing introns and the rRNA loci

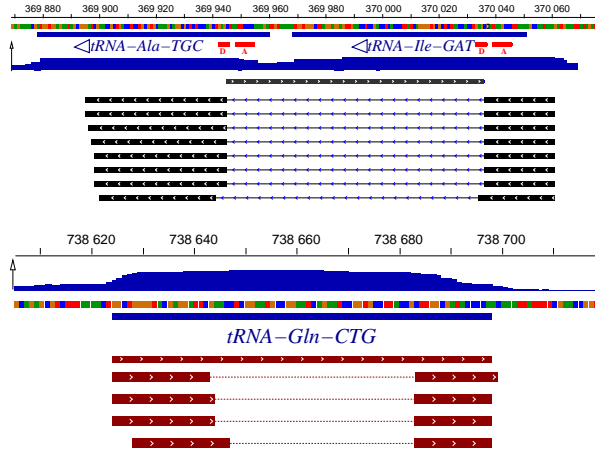


Figure 3: Unusual eubacterial reads associated with tRNAs. **Above:** Spliced fusion of two adjacent tRNAs in *H. pylori*. Red marks indicate the 5’-side of the acceptor stem and D-stem, resp. The apparent intron extends roughly from the end of the acceptor stem of tRNA-Ile to the begin of the D-stem of tRNA-Ala. The coverage suggests that the two adjacent tRNAs are produced from a single primary transcript. **Below:** A circularized tRNA in *Salmonella*.

only a moderate number of “splice sites” is supported by multiple, non-identical reads. Among the most peculiar examples are tmRNAs with missing subsequences, Fig. 2, which appear in several species. Although the excisions appear to be concentrated in the highly structured, pseudoknotted regions, only some of them are easily explained as “RTfact” resulting from the RT reading through the base of stem and thus omitting the entire structural domain enclosed by the stem.

Cleavage of tRNAs as a response to stress, first discovered as response to phage infection in *E. coli* (33; 34), is a general phenomenon in all domains of life, see e.g. (35; 36; 37). At least in some cases, tRNA cleavage seems to have evolved into an internal regulation mechanism (38). Fragments of tRNAs, furthermore, may act as regulatory ncRNAs in both Eukarya (39; 40) and Archaea (41). Healing of the cleaved tRNAs is likewise a frequently observed phenomenon, see e.g. (42; 43). The ligases involved in tRNA splicing in Eukarya (44) and Archaea (45) utilize the 2’,3’-cyclic phosphates generated by endonucleolytic cleavage. Members of the same protein family have also been found in Eubacteria, see (46) for a recent review of RNA ligases. The *E. coli* ligase RtcB, a component of the RNA repair operon reassembles tRNAs cleaved in the anticodon loop (43). It has been shown to be capable of catalyze tRNAs splicing in yeast (47). It is not unreasonable to assume, therefore, that unexpected tRNA-derived RNAs, including “trans-splicing” products ap-

pear as by-products of the the tRNA cleavage/repair pathways, and hence are present in the cell. In *Helicobacter*, for example, we find what looks like a spliced common precursor of two adjacent tRNAs, see Figure 3. In *Salmonella*, the matured tRNA-Gln-CTG is associated with circularized reads.

4. Discussion

The preparation of RNA-seq libraries contains a reverse transcription step that may account for many of the observed non-canonical splicing events. Such RT artifacts have investigated in detail e.g. in (16; 17; 18). While we cannot rule out in most cases that the observed reads are such “RTfacts”, there are plausible alternative mechanism that could produce atypical transcript structures.

On other hand, the data contain a large number of true positive examples for both Archaea and Eubacteria in which splicing or circularization has been demonstrated in independent experiments. Hence clearly not all of the observed split reads are technical artefacts. In some cases, the molecular mechanisms the lead to the “spliced” RNAs is well known. This is the case for the self-splicing introns and for the processing of tRNAs (48) and rRNAs (10) in Archaea. The splicing endonuclease processing in Archaea has a broad range of target and is known to be involved also the transsplicing of tRNAs from independently encoded fragments as well as in the splicing of mRNAs. Homologous enzymes are present also in diverse eubacterial species, where they form a tRNA cleavage/repair pathway (briefly reviewed in the previous section). Thus there appears to be an ancient RNA repair system present all domains of life, which could account for many or even most of the spliced and circularized RNAs observed here.

In *E. coli*, the stress-induced toxin MazF cleaves certain single-stranded mRNA at or closely upstream of the start codon and removes a 43 nt fragment comprises the anti-Shine-Dalgarno from the 3' terminus of the 16S rRNA (49). Ribosomes with the truncated 16S rRNA specifically translate leaderless mRNAs, presumably as a stress response (50). The abundance of leaderless transcripts also in other proteobacteria (51; 52) might similar mechanisms are more wide-spread. In conjunction with a variety of RNA ligases (46), they might account for at least a part of the atypical sequences observed here.

Apparent splice junctions that are supported by multiple read counts, thus, are at least good candidates atypically processed RNAs that deserve further attention. In

Archaea, the combination of atypical reads and a local, (nearly) isolated peak of coverage provides at least a very strong indication of for processed ncRNAs. In all four Archaea considered here, additional candidates, Tab. 2, could be identified.

On the other hand, several well-described cases of atypical transcripts, such as the trans-spliced tRNAs in *Nanoarchaeum* were observed only in a very small number of reads. This can be explained only in part by the presence of unspliced paralogs that attract the processed reads to the contiguous locus in the mapping procedure because an unspliced alignment is always preferred over a spliced one. Low expression, or support by only a small number of splice junctions, thus does not necessarily imply that an atypical transcript is a technical artefact or, even if present in the cell, devoid of biological function.

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