Maturation of 23S rRNA includes removal of helix H1 in many bacteria

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\section*{ABSTRACT}

In most bacteria, the three ribosomal RNAs (rRNAs) are encoded together in each of several near-identical operons. As soon as the nascent precursor rRNA emerges from RNA polymerase, ribosome assembly begins. This process entails ribosomal binding, rRNA folding, rRNA modification, and rRNA processing. In the model organisms \textit{Escherichia coli} and \textit{Bacillus subtilis}, rRNA processing results in similar mature rRNAs, despite substantial differences in the cohort of RNases involved. A recent study of \textit{Flavobacterium johnsoniae}, a member of the phylum Bacteroidota (formerly Bacteroidetes), revealed that helix H1 of 23S rRNA is absent from ribosomes, apparently a consequence of rRNA maturation. In this work, we mined rRNA-seq data from 19 individual organisms and ocean metatranscriptomic samples to compare rRNA processing across diverse bacterial lineages. We found that mature ribosomes from multiple clades lack H1, and typically these ribosomes also lack an encoded H98. For all groups analysed, H1 is predicted to form in precursor rRNA as part of a longer leader-trailer helix. Hence, we infer that evolutionary loss of H98 sets the stage for H1 removal during 50S subunit maturation.

\section*{Introduction}

Biogenesis of the ribosome entails folding of ribosomal RNA (rRNA), binding of ribosomal (r) proteins, modification of rRNA, and processing of rRNA (reviewed in [1]). In \textit{Escherichia coli}, all three rRNA molecules (16S, 23S, 5S) are encoded (along with one or two tRNAs) in each of seven operons. Subunit assembly begins co-transcriptionally, as primary r proteins interact with the nascent pre-rRNA. This facilitates rRNA folding and subsequent r protein-binding events, allowing ribonucleoparticles of increasing size and complexity to form, until complete subunits are ultimately made. In the context of subunit formation, the long pre-rRNA transcript, which includes structures important for assembly [2–4], is processed by various RNases [5]. Sequences flanking the 16S rRNA form a > 40 basepair (bp) leader-trailer (LT) helix [6], while sequences flanking the 23S rRNA form a ∼ 30 bp LT helix [3]. RNase III, a double-stranded endonuclease, recognizes each LT helix and cleaves both strands, generating the pre-16S (17S) and pre-23S molecules. For 30S subunit maturation, subsequent cleavage events by RNases E and G remove the remaining leader strand of the 17S precursor, while YbeY and/or 3′-to-5′ exonucleases remove the remaining trailer strand [5]. For 50S subunit maturation, RNase T and an unknown nuclease further trim the precursor molecule to generate the mature 3′ and 5′ ends, respectively [5].

In most other bacteria, rRNA genes are similarly arranged in operons. Sequences flanking the 16S and 23S regions typically show complementarity [7], consistent with LT helices akin to those of \textit{E. coli}, and RNase III is present in nearly all bacteria [8]. While these observations suggest common aspects of rRNA processing across bacteria, there are also clear indications of variation. For example, intergenic regions of rRNA operons exhibit a high degree of sequence diversity [9–11]. Moreover, studies of \textit{Bacillus subtilis} have revealed a unique cast of RNases involved in rRNA maturation [5]. Following initial cleavage of pre-rRNA by RNase III, the double-stranded endonucleases Mini-III and RNase M5 generate the mature ends of 23S and 5S rRNAs, respectively [12,13]. For 30S subunit maturation, the 5′-to-3′ exonuclease RNase J1 removes the 5′ leader strand, while YqG (homologous to \textit{E. coli} YbeY) cuts off the remaining 3′ trailer. Notably, \textit{B. subtilis} completely lacks RNase E, G, and T, underscoring mechanistic differences in rRNA processing between \textit{E. coli} and \textit{B. subtilis} [5].

Recently, Jha \textit{et al.} mapped the 5′ and 3′ ends of the rRNAs in mature ribosomes of \textit{Flavobacterium johnsoniae}, a representative of the Bacteroidota (formerly Bacteroidetes) [14]. They found that, in 5 of 6 cases, the actual termini differ from those predicted in the annotated genome. Most notably,
nucleotides 1–8 and 2891–2902 of 23S rRNA, which include the two strands of helix H1, are missing in the mature ribosome. Covariation analysis indicates that H1 is a conserved feature of bacterial 23S rRNA [15], and complementary strands corresponding to H1 are encoded in the *F. johnsoniae* genome. Hence, it was inferred that, in *F. johnsoniae*, H1 forms in the precursor rRNA and is later removed during subunit maturation [14].

In this work, we mined RNA-seq data from 19 individual bacteria and SMART-seq data from the ocean microbiome to compare rRNA processing in diverse lineages. We found that helix H1 is absent from mature 50S subunits in more than half of the bacteria analysed, and loss of H1 generally coincides with the absence of helix H98 from 23S rRNA.

**Results**

High-throughput sequencing methods have been developed to globally identify the 5' ends (dRNA-seq/TSS-seq), 3' ends (Term-seq), or both ends (Rend-seq) of RNA molecules. Ribosomal RNA is highly abundant in growing bacteria, and the lion's share of rRNA in the cell is fully processed, contained in ribosomes. To identify the ends of mature 23S, 16S, and 5S rRNA in various lineages, we analysed Rend-seq data from 14 organisms, dRNA-seq/TSS-seq data (-TEX/-RppH) from 16 organisms, and Term-seq data from 2 organisms (Table 1). For each organism, 5' read end coverage was plotted with respect to the annotated 5' ends of the rRNA genes, and 3' read end coverage (when obtained) was plotted with respect to the annotated 3' ends of the RNA genes. In most plots, a single large peak was observed, corresponding to the mature end of the rRNA (Figure 1, Figures S1-S23). Assignments of the mapped ends, determined here and reported previously [14], are shown in Figure 2–4. Throughout this report, we use the universal numbering of rRNA nucleotides, based on structural conservation, with *E. coli* as the reference model [15].

One known caveat to Rend-seq is that 5' end mapping cannot always achieve nucleotide resolution [16]. This stems from the ability of reverse transcriptase to incorporate an additional adenosine after reaching the end of the RNA template. If the resulting cDNA happens to match the genome sequence, such reads will be included and can shift the coverage peak by one nucleotide. To help mitigate this, we also analysed independent dRNA-seq datasets, when possible.

**Mapping rRNA ends in individual organisms**

**23S rRNA**

The experimentally deduced ends of 23S rRNA matched those predicted from the genome in surprisingly few cases (3 of 20 for 5' end; 1 of 7 for 3' end) (Figure 2). Only for *E. coli* did the mapping data agree with the genome annotation for both ends. Genomes CP0009977.1 of *Vibrio natriegens* and CP000487.1 of *Campylobacter foetus* are wildly misannotated, based on comparisons with other strains of the same species, which helps explain the large discrepancies seen in these cases. Notably, the mapping data indicate that helix H1 of 23S rRNA is missing from mature ribosomes of several lineages. A9 represents the 5' nucleotide of *Bacteroides thetaiotamicron* 23S rRNA, just as observed in *F. johnsoniae*. Nucleotides 7 and 2890 represent the 5’ and 3’ ends of 23S rRNA in *Zymomonas mobilis* and *Caulobacter crescentus*, indicating equivalent removal of both strands of H1. A6 represents the 5’ end of 23S rRNA in both *Campylobacter foetus* and *Helicobacter pylori*, consistent with severe truncation (if not removal) of H1. In all organisms analysed, complementary strands corresponding to H1 are encoded in the genome (Figure 2, underscored regions). Yet, ribosomes of Alphaproteobacteria (*Z. mobilis*, *C. crescentus*) and Bacteroidia (*B. thetaiotamicron*, *F. johnsoniae*) lack H1, while ribosomes of Campylobactera (*C. foetus*, *H. pylori*) lack or mostly lack H1. We infer that, in these organisms, H1 forms during 50S biogenesis and is subsequently removed or processed.

**16S rRNA**

The large number of discrepancies between the mapped and annotated ends of 23S rRNA prompted us to examine the other rRNAs as well. The mapped 5’ end of 16S rRNA matched the genome annotation in 6 of 19 cases, while the mapped 3’ end matched the genome annotation in 2 of 7 cases (Figure 3). The 5’ termini of 16S rRNA from *C. fritschii* and *F. thermus* could not be mapped, because the corresponding genomes (obtained via shotgun sequencing) are incomplete and lack data just upstream of the 16S genes. For most bacteria, one to six additional 5’ nucleotides are present, as compared to the reference *E. coli* molecule. On the 3’ end of 16S rRNA, 2–4 nucleotides beyond 1542 are often seen, with the terminal sequence UUUCU (nt 1540–1544) being most common. These additional 3’ nucleotides (1543–1544) can potentially pair with mRNA as part of an extended SD-ASD helix [14]; hence, even small differences in 3’ tail length may affect ribosome function.

**5S rRNA**

When the 5’ read ends of the *E. coli* Rend-seq data were mapped back to the genome, the peak of coverage was seen at position −1 (Figure 1(e)). Fairly high coverage was also seen at position 0, corresponding to U1 of 5S rRNA. These data might be explained by some natural length heterogeneity of this molecule. However, analogous data from a related organism, *V. natriegens*, showed a sharp peak corresponding to U1 (Figure S2, Figure 4), and independent dRNA-seq data from *E. coli* mapped U1 as the 5’ nucleotide (Figure S1). Hence, we suspect that the Rend-seq peak at −1 is most likely due to the RT-dependent artefact discussed above. The 3’ end of *E. coli* 5S rRNA, as determined by Rend-seq, precisely matched the genome annotation, as expected (Figure 1(f)).

For other bacteria analysed, the mapped 5’ end of 5S rRNA matched the genome annotation in 5 of 19 cases, while the mapped 3’ end matched the genome annotation in 2 of 6 cases (Figure 4). In the case of *Bordetella pertussis*, there were two peaks of read coverage – one corresponding to C1 and another larger peak corresponding to A10 (Figure S3). This raises the possibility that A10, which lies near a helical junction in 5S rRNA, is vulnerable to RNase cleavage in this organism.
Loss of H1 of 23S rRNA is largely concentrated in specific groups of the taxonomic tree

The apparent removal of H1 from ribosomes of several lineages prompted us to investigate the presence and absence of H1 in 23S rRNA more broadly across Bacteria. To this end, we exploited the fact that RNA sequenced using the SMART-seq approach is captured all the way to the 5′ end. In addition, the strand transfer of the polymerase at the 5′ end upon recognition of the Template Switching Oligo (TSO) during library preparation results in the true 5′ ends of RNAs being marked in such SMART-seq data by a non-encoded GGG that is complementary to the 5′-most locked cytosine bases of the TSO. We thus identified all reads from the ribosomal section of a massive ocean metatranscriptomics data set starting with a GGG, and aligned them against a database of 2940 representative bacterial 23S rRNA sequences. The position of each alignment provided information on the location of the 5′ end of the RNA while the full alignment identified the species of origin among our reference 23S rRNA sequences. Stringent quality filtering resulted in 498 species with assigned 5′ ends. A histogram of the locations of these 5′ ends relative to the true 5′ end of E. coli shows a clearly bimodal distribution (Figure S5(a)), one peak of which (around zero offset relative to E. coli) corresponds to species in which H1 is present, and the other peak of which (around an offset of 6 relative to E. coli) corresponds to species in which H1 is absent, with only 36 species among the 498 featuring offsets that are difficult to interpret as either a proper H1 or a proper 23S rRNA without H1 and thus classified as outliers. We conclude that even though this automatic approach of assigning 5′ ends to 23S rRNA en masse from the byproducts of a metatranscriptomics study is likely more noisy than manual inspection of individual data sets specifically constructed to reveal 5′ ends, it is able to provide reliable information on the presence and absence of H1 for hundreds of bacterial species.

When projecting the presence and absence of H1 onto the GTDB taxonomic tree (Figure S24), we find that the vast majority (278 out of 284) of all species within Gammaproteobacteria, for which we can make a determination about H1, contain H1. Interestingly, the Gammaproteobacteria include two species, Actinobacillus equuli and Actinobacillus suis, within the same genus (red frame in Figure S24) with differing behaviour concerning H1 (each of which is strongly supported by the alignment data, see Figure S25). On the other hand, none of the 39 species within Bacteroidia or 109 species within Alphaproteobacteria contain H1. Members of Saccharimonadida and Fusobacteria also lack H1, whereas the one member of Clostridia (C. difficile) contains H1. Because these latter groups contain few representatives, little can be inferred about the consistency of H1 retention in these clades. Only within the Actinomycetia is there a lot of variability, with 11 out of 18 species that do not contain H1 and 7 that do. Notably, five organisms analysed by the automatic approach were also analysed manually (B. pertussis, E. coli, F. johnsoniae, N. meningitidis, and Z. mobilis), and H1 calls were consistent in all cases. We conclude that presence and absence of H1 largely follows the groups of the bacterial tree but that there are a few interesting outliers deserving future investigation. We also note that the pattern of presence and absence of H1 indicates that removal of H1 may have independently arisen multiple times in evolution.

Loss of H1 of 23S rRNA correlates with the absence of H98

One difference between the E. coli and F. johnsoniae 23S rRNA is that the latter naturally lacks H98, a poorly conserved helix (Figure 6). In the E. coli ribosome, H98 lies across nucleotides 10–12 and 2889–2894, and A9 and G2890 correspond to the terminal nucleotides of 23S rRNA in the F. johnsoniae ribosome. This raises the possibility that H98 might prevent H1 removal by protecting these rRNA regions from RNase cleavage. To investigate this idea, we scored for the presence of H98 in all the organisms analysed. For the manually investigated organisms, we found that, with few

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**Table 1. Bacteria analysed in this study.**

| Organism (Abbreviation) | Phylum, Class | Method(s) | H1 | H98 | Reference |
|-------------------------|---------------|-----------|----|-----|-----------|
| *Escherichia coli* (Eco) | Proteobacteria, Gammaproteobacteria | Rend-seq, dRNA-seq | YES | YES | [16][43] |
| *Vibrio natrievigen* (Vna) | Proteobacteria, Gammaproteobacteria | Rend-seq | YES | YES | [16] |
| *Bordetella pertussi* (Bpe) | Proteobacteria, Gammaproteobacteria | dRNA-seq | YES | YES | [44] |
| *Neisseria meningiti* (Nme) | Proteobacteria, Gammaproteobacteria | dRNA-seq | YES | YES | [45] |
| *Novosphingobium aromativorcan* (Nar) | Proteobacteria, Alphaproteobacteria | TSS-seq | YES | NO | [46] |
| *Zymomonas mobilis* (Zmo) | Proteobacteria, Alphaproteobacteria | TSS-seq | NO | NO | [47] |
| *Caulobacter crescentu* (Cct) | Proteobacteria, Alphaproteobacteria | Rend-seq, dRNA-seq | NO | NO | [16,40] |
| *Campylobacter foetu* (Cfe) | Campylobacterota, Gammaproteobacteria | dRNA-seq | NO | NO | [49] |
| *Helicobacter pylori* (Hyg) | Campylobacterota, Campylobacteria | dRNA-seq | NO | NO | [50] |
| *Chlorobaculum tepidum* (Cte) | Bacteroidia, Bacteroido | dRNA-seq | YES | NO | [51] |
| *Bacteroides thetaiotaomicron* (Bth) | Bacteroidia, Bacteroido | dRNA-seq | NO | NO | [41] |
| *Flavobacterium johnsoni* (Fjo) | Bacteroidia, Bacteroido | RNA-seq* | NO | NO | [41] |
| *Synechococcus elongat* (Sel) | Cyanobacteria, Cyanobacteria | dRNA-seq | YES | YES | [34] |
| *Chlorogloeopsis fritschi* (Cft) | Cyanobacteria, Cyanobacteria | dRNA-seq | YES | YES | [34] |
| *Fischerella thermals* (Fth) | Cyanobacteria, Cyanobacteria | dRNA-seq | YES | YES | [34] |
| *Streptomyces lividans* (Sil) | Actinobacteriota, Actinomycetia | dRNA-seq, Term-seq | YES | YES | [55] |
| *Mycobacterium tuberculosis* (Mtu) | Actinobacteriota, Actinomycetia | dRNA-seq | YES | YES | [56] |
| *Bacillus subtilis* (Bsu) | Firmicutes, Bacilli | Rend-seq | YES | YES | [16] |
| *Streptococcus pyogenes* (Spy) | Firmicutes, Bacilli | TSS-seq | YES | YES | [57] |
| *Clostridioides difficile* (Cd) | Firmicutes, A. Clostridia | TSS-seq | YES | YES | [54] |

1H1 is either absent or severely truncated.
2Effectively Rend-seq, due to limited base hydrolysis of RNA.
exceptions, loss of H1 occurs in those organisms which also lack H98 (Table 1). The Bacteroidota lack H98, and ribosomes of F. johnsoniae and B. thetaiotamicron lack H1. The Campylobacterota lack H98, and ribosomes of H. pylori and C. foetus lack (or exhibit a severely truncated) H1. The Alphaproteobacteria lack H98, and ribosomes of Z. mobilis and C. crescentus lack H1, as do ribosomes from R. palustris [17]. One apparent exception to this trend entails N. aromaticivorans, another Alphaproteobacteria, whose ribosomes lack H98 and contain H1. Ribosomes of C. tepidum, a member of the Chlorobia, similarly retain H1 despite the absence of H98. For other groups of bacteria, including the Firmicutes, Actinobacteriota, Cyanobacteria, and Gammaproteobacteria, 23S RNA contains H98. In 12 of 12 cases analysed, ribosomes of these organisms retain H1. Overall, the co-occurrence of these elements, deemed significant by Fisher’s exact test ($p = 0.0003$), suggests that retention of H1 in the mature ribosome generally depends on the presence of H98. This pattern remains true for the automatically annotated organisms, with 442/457 containing both or lacking both helices (Figure S24). In 11 species, H98 is present without H1 (all in Actinomycetia or Gammaproteobacteria); and in 4 species, H1 is present without H98 (all in Gammaproteobacteria). Formally, this corresponds to a Fisher’s exact $p$-value of $1.3 \cdot 10^{-103}$ although this exaggerates the significance given that many species are closely related and thus cannot be
treated as independent. Nevertheless, it is clear that the removal of H1 and the absence of H98 go hand in hand.

**Discussion**

In this study, we mined RNA-seq datasets to identify the ends of rRNA in many bacteria. We found that helix H1 of 23S rRNA is absent from mature ribosomes in about half the organisms analysed. Removal of H1 correlates strongly with the absence of H98, suggesting that retention of H1 generally depends on the presence of H98. Helix H98 is positioned across nucleotides 10–12 and 2890–2896 and hence may protect these strands from cellular nucleases, enabling retention of H1. Loss of H98 by chromosomal deletion may have little consequence on ribosome function [18,19] but allow for H1 excision during subunit maturation. This could explain the multiple independent losses of H98 during evolution and the parallel removals of H1.
Structures of ribosomes from various bacteria, including representatives of Firmicutes (B. subtilis, S. aureus, E. faecalis), Deinococcus (T. thermophilus, D. radiodurans), Actinobacteria (M. smegmatis), and Gammaproteobacteria (E. coli, P. aeruginosa, A. baumannii), have now been solved [20–28]. All of these ribosomes contain H1 and H98, which adopt similar conformations regardless of the source organism.

To our knowledge, the only solved structure of a bacterial ribosome that lacks H1 and H98 is that of F. johnsoniae [14], a representative of Bacteroidia. In this structure, the 3’ terminal nucleotide (G2890) is tucked behind nucleotides 2790–2791, components of a small loop that replaces H98. As for the 5’ end of 23S RNA, the path of nucleotides 9–12 diverges (by nearly 180°) from that seen in H98/H1-containing ribosomes. This enables contacts between C9 (the 5’ terminal nucleotide) and uL22, an interaction which may protect the RNA from exonucleases. Whether other ribosomes that lack H1/H98 exhibit similar or analogous interactions remains to be determined.

The absence of H1 and H98 from mature ribosomes of numerous bacteria indicates that these elements are unnecessary for ribosome function. Previous studies have shown that base substitutions in either strand of H1 results in severely diminished levels of active 50S subunits in the cell [3]. Compensatory mutations in the opposite strand restore active subunits to normal levels, implying the importance of H1 secondary structure in 50S biogenesis and/or stability. Given that the sequences flanking 23S RNA exhibit complementarity in diverse bacteria [7] (Figure 2), we infer that H1 contributes to 50S biogenesis in the context of the larger LT structure. In most lineages that contain H98, processing of the LT structure leaves a remnant, H1, behind. On the other hand, in most lineages that lack H98, the entire LT structure, including H1, is removed.

Finally, our work exemplifies precise mapping of RNA 5’ ends from metatranscriptomic datasets. By taking advantage of the GGG tag generated in SMART-seq library construction, we are able to determine the true 5’ ends of the 23S RNA for hundreds of species in parallel. While the ability of the strand-switching polymerase in the SMART-seq protocol to identify 5’ ends is well recognized in general [29,30], the approach taken here is to our knowledge unique in that it does not involve a specialized library preparation protocol but can be applied to standard SMART-seq data. Its main caveats are that, since 5’ ends are not strongly enriched, it requires a high abundance RNA (which 23S clearly is naturally) and an approximate knowledge of the correct location of the 5’ end (in our case provided by the highly conserved 24–60 nt region of 23S rRNA). These conditions are not unique to 23S rRNA but hold for other ribosomal and high abundance messenger and noncoding RNAs as well, so the method should be applicable more broadly. Another caveat that we observe is that the method sometimes detects spurious 5’ ends associated with genomically encoded runs of Gs, presumably due to internal priming by the template switching oligo. For example, a run of three Gs close to the true 5’ end of the cyano bacterial 23S rRNA causes all Cyanobacteria to fail our quality control criteria, explaining their absence from our results in spite of their abundance in ocean communities. A detailed
Figure 5. Bacterial ribosomes contain or lack 23S rRNA helices H1 and H98. (a) Shown is a histogram of the offsets of 23S rRNA 5′ ends relative to the E. coli 23S rRNA 5′ end. The bimodal nature of the histogram allows classification of species into those containing H1 (offset between −4 and 4, brown range), those lacking H1 (offset between 5 and 12, grey range), and a small number, for which H1 status remains unknown. (b) Shown is a histogram of the distances between two conserved sequence motifs surrounding H98 in 23S rRNA. The bimodal nature of the histogram allows classification of species into those containing H98 (distance between 45 and 55, brown range), those lacking H98 (distance between 30 and 40, grey range), and a small number, for which H98 status remains unknown.

Figure 6. Comparison of 23S rRNA structure in ribosomes containing or lacking H1. (a) Shown is a superimposition of E. coli (cyan) and F. johnsoniae (tan) 23S rRNA in the vicinity of the 5′ and 3′ termini (red nucleotides, as indicated). Helices H2, H25, H94, and H99 (as indicated) are common features, whereas H1 and H98 are missing in the F. johnsoniae ribosome. Ribosomal proteins have been computationally omitted for clarity. This image was generated in PyMOL using PDB files 2QAM and 7JIL. (b) Comparison of secondary structure elements (E. coli, cyan, left; F. johnsoniae, tan, right) in the relevant portion of domain VI of 23S rRNA, with helices and terminal nucleotides indicated.

exploration of the capabilities of the method will be left for a future publication.

MATERIALS and METHODS

Mapping of rRNA ends

The mature ends of each rRNA molecule were identified from publicly available data. Because processed 5′ ends were sought, control dRNA-seq/TSS-seq datasets (e.g., minus terminator 5′-phosphate-dependent exonuclease, -TEX; or minus RNA 5′ pyrophosphohydrolase, -RppH) were analysed. For dRNA-seq, TSS-seq, and Term-seq data, raw reads were downloaded from SRA and converted to fastq using the SRA toolkit [31]. 5′ adapters were identified by minion [32] and trimmed using skewer [33] where necessary. Reads were aligned using Bowtie2 [34], converted to bam format using samtools [35], and read coverage was obtained using the bedtools genomcov command [36]. For rend-seq data, WIG format coverage files were used when available, and raw data analysed as above otherwise.

5′ and/or 3′ read coverage were each plotted with respect to the corresponding genome-annotated gene ends using in-house python scripts. For each 5′ and/or 3′ coverage plot, we observed a large peak corresponding to the mature 5′ and/or 3′ ends, due to the end enrichment protocols used.

Sequence alignments and helix calling

Figure 2–4 were generated from multiple alignments of the entire RNA made in clustalw2 [37], with gaps removed manually. Helix H1 was called as missing if three or fewer predicted base pairs remained, and H98 was determined to be missing by inspection of the multiple alignment. The genomes of Vibrio natriegens (CP009977.1) and Campylobacter foetus (CP000487.1) were compared to strains of the same species (NZ_CP016351.1 and CP059443.1 respectively), which confirmed misannotations of rRNA in the former cases.
Computational approach to bulk mapping of 23S 5' ends

Query sequence acquisition

Metatranscriptomic reads were obtained for 551 samples of a study published by the TARA Ocean’s consortium [38]. These libraries had been prepared with the SMARTer Stranded RNA-Seq Kit (Clontech) following Ribo-Zero depletion and only the reads designated by SortMeRNA as ribosomal in origin were provided by the authors of the TARA Ocean’s study.

Query sequence selection

The forward ends of the paired end samples were selected. Since the last 3 bps of the Template Switch Oligo, GGG, were present at the beginning of reads starting at the 5' end of rRNAs, reads were down selected to only those beginning with GGG and the GGG was stripped from these reads with CutAdapt v3.4 (-g ^GGG -discard-untrimmed) [39].

An alignment of the 23S RNA sequences from the species of Table 1 was used to define a conserved motif (GNGGATGCCTTGGCNNNNNNAGNCGANGAAGGACGTG) at positions 24–60. This motif was used to only retain reads containing this motif (thus predicted to originate from the vicinity of the 5' end of 23S rRNA) using CutAdapt v3.4 (-a GNGGATGCCTTGGCNNNNNNAGNCGANGAAGGACGTG -e 0.25 -action=none -discard-untrimmed -o 37). Following both CutAdapt steps, 103,968,502 reads remained from all samples.

23S rRNA sequence acquisition

A list of all organisms with NCBI type data and designated as species representative was obtained from the Genome Taxonomy Database (GTDB) [40]. Their annotated genomes were downloaded and the 23S rRNA sequence was extracted based on the annotation. Finally, 23S rRNA sequences were added for the five species (S. pyogenes, C. tepidum, C. difficile, B. thetaiotaomicron, and S. lividans) of Table 1, which were not already included. 23S rRNA subject sequences, with their accompanying NCBI accession and genome positions, were collected and are available in Table S1.

Normalization of 23S rRNA annotations

The annotated 5' end of 23S rRNA sequences varied significantly between different NCBI assembled genomes, and even genomes sharing the same species presented significantly different (and often errant) annotated 23S 5’ ends. Thus, a 5' end normalization offset was generated for each collected 23S rRNA subject sequence by comparing the number of nucleotides upstream of the above-mentioned conserved motif (GNGGATGCCTTGGCNNNNNNAGNCGANGAAGGACGTG). E. coli was used as a template to normalize Δ for all 23S rRNA subject sequences, since the location of H1 on E. coli is well known. For each collected 23S rRNA subject sequence, i, the offset Δi was calculated as:

\[ Δ_i = \text{NumberntsBeforeMotif}_{E.\ coli} - \text{NumberntsBeforeMotif}_{i} \]

Query mapping and normalized 23S 5' end shift

Metatranscriptomic reads were aligned to the 23S rRNA subject sequences using LAMBDA2 [41]. Alignments were considered valid if their percent identity was above 95%, their query alignment start and/or subject alignment start was 1 (i.e. the alignment occurs at the beginning of the query and/or subject sequence), and the query alignment end equalled the query sequence length (i.e. the 3’ end of the query is completely aligned to the subject). The predicted normalized 5’ end shift for the read, SNS, was calculated as

\[ SNS = \text{QueryAlignmentStart} - \text{SubjectAlignmentStart} + Δ_i. \]

1,398,885 reads had at least one valid alignment.

Species normalized 5' shift prediction

All SNS values were grouped based on the species of the subject sequence. For each unique species, a histogram of the distribution of SNS values was generated. SNS values outside the range from −15 to 15 were discarded as implausible indicators of true 5' ends. To determine if (i) there was enough data, and (ii) the data had high agreement, two quality control criteria were imposed: (1) at least 20 of the remaining reads must map to the species, and (2) one 3 nucleotide sliding window from x-axis values −15 to 15 must contain at least 70% of the reads mapping to the species. Histograms passing both screening protocols were subsequently evaluated for their mode SNS value, which became the predicted normalized 5’ end shift value for the species, which is defined as λ.

Identification of helix H1

The predicted values from all species passing both screening protocols were collected. Based on a histogram of these values (Figure 5(a)) species whose λ value was between −4 and 4 were deemed to contain H1, species whose λ value was between 5 and 12 were deemed to lack H1. No call was made for species with λ values outside the −4 to 12 range. Values and H1 statuses of all species passing the quality control criteria are given in Table S1.

Identification of helix H98

H98 is present near the 3’ end of 23S rRNA. From the multiple sequence alignment of 23S rRNA of the 20 species of Table 1, two conserved motifs around the H98 position, one upstream (AGANNANNNNTTGTAGGNNNNNNNTG), and one downstream(GATAANNGCTGAAAGCATCTAAGNNNGAA NC) were identified. CutAdapt v3.4 (-a ’AGANNANNNNTTGTAGGNNNNNNNTGmin_overlap=28’ -g ’GATAANNGCTGAAAGCATCTAAGNNNGAANmin_overlap=31’ -discard-untrimmed -n 2 -e 0.25) was used to extract the portion of the subject sequence between both motifs, the length of which was defined as L.

A histogram of all L values revealed two groups of species: those containing H98 (the right group, i.e. those sequences with more nucleotides between the motifs, implying the presence of H98) and those without H98 (the left group) (Figure 5(b)). Based on this histogram, species whose L value was between 45 and 55 were deemed to contain H98, species whose L value was between 30 and 40 were deemed to lack H98, and the H98 status of all other species remained unknown. The number of nucleotides between the motifs
and the H98 status of all taxonomies passing the quality control criteria are given in Table S1.

**Visualization of taxonomic tree**

The GTDB taxonomic classification for each species passing the quality control criteria and having known H1 and H98 status was retrieved and converted to Newick format. The H1 and H98 status information for each of these taxonomies was converted into an iTOL annotation file using a custom script and the tree and its annotation visualized in iTOL [42].

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No potential conflict of interest was reported by the author(s).

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