Intra- and Intergenomic Variation of Ribosomal RNA Operons in Concurrent Alteromonas macleodii Strains

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Abstract Biodiversity estimates based on ribosomal operon sequence diversity rely on the premise that a sequence is characteristic of a single specific taxon or operational taxonomic unit (OTU). Here, we have studied the sequence diversity of 14 ribosomal RNA operons (rrn) contained in the genomes of two isolates (five operons in each genome) and four metagenomic fosmids, all from the same seawater sample. Complete sequencing of the isolate genomes and the fosmids establish that they represent strains of the same species, Alteromonas macleodii, with average nucleotide identity (ANI) values >97 %. Nonetheless, we observed high levels of intragenomic heterogeneity (i.e., variability between operons of a single genome) affecting multiple regions of the 16S and 23S rRNA genes as well as the internally transcribed spacer 1 (ITS-1) region. Furthermore, the ribosomal operons exhibited intergenomic heterogeneity (i.e., variability between operons located in separate genomes) in each of these regions, compounding the variability. Our data reveal the extensive heterogeneity observed in natural populations of A. macleodii at a single point in time and support the idea that distinct lineages of A. macleodii exist in the deep Mediterranean. These findings highlight the potential of rRNA fingerprinting methods to misrepresent species diversity while simultaneously failing to recognize the ecological significance of individual strains.

Introduction

The rRNA operon quickly gained prominence as the backbone of bacterial identification and phylogenetics with the advent of molecular genomics [1, 2]. The ubiquitous nature of the rRNA operon (i.e., found in all organisms) in conjunction with its functional necessity and remarkable degree of conservation made rRNA a logical target for determining the evolutionary relatedness across all organisms [3]. Furthermore, the size of the larger rRNA genes (16S and 23S), together with the numerous variable domains that they encompass, make these genes good molecular markers across various phylogenetic scales [1]. Evidence that the rRNA operon is fairly resistant to horizontal transfer [4] further cemented its place as the gold standard for bacterial identification and phylogenetic analysis. As a result, over the last 40 years, the concepts underlying microbial diversity and evolution have been greatly advanced with the vast majority of these studies involving rRNA sequencing [5].

Despite these advances, evidence has been accumulating over the past 15 years highlighting the limitations of rRNA sequencing for microbial diversity studies. The presence of multiple rRNA operons in numerous microorganisms, for example, limits species-level resolution of bacterial identity. Bacteria are known to house from one (e.g., Prochlorococcus marinus MIT 9312) to 15 (e.g., Photobacterium profundum SS9_15) rRNA operons [6]. Homogenization through recombination [7] can minimize the effects of multiple rRNA operons, in which case quantitative estimates of biodiversity could be determined using normalization measures. However, homogenization does not occur in all
bacteria [8, 9], and heterogeneity within the 16S rRNA gene is well documented for a number of organisms, including *Aeromonas* spp. [10], *Halocarcula* sp. [11], *Pseudomonas* spp. [12], *Fellionella* spp. [13], and *Vibrio* spp. [14–16]. In these cases, intragenomic heterogeneity in the 16S RNA results in poor species and subspecies classification as well as overestimation of microbial diversity [8, 9, 17]. Similar arguments have been made for the internally transcribed spacer 1 (ITS-1) region [18–20], highlighting both quantitative (i.e., taxonomic numbers) and qualitative (i.e., taxonomic identity) concerns. The potential quantitative bias resulting from multiple rRNA operons in individual genomes is often recognized, yet much less attention has been given to the problems linked to the identity of rRNA gene sequences.

Conversely, isolates sharing identical or nearly-identical 16S rRNA genes exhibit significant divergence in protein-coding sequences, suggesting that 16S rRNA analysis can simultaneously underestimate functional diversity and ecologically distinct and relevant strains of bacteria [21, 22]. Strains of *Escherichia coli*, for example, can vary in gene content by >1,000 protein-coding gene sequences, and these differences correlate with ecological and phenotypic differences between strains [23, 24]. Similarly, pathogenic strains representing diverse phenotypic characteristics of both *Vibrio cholerae* and *Bacillus cereus* show significant variation in gene content due to lateral gene transfers (LGT) [25] and the presence of large plasmids [26], respectively, despite being classified as an individual species based on rRNA sequence similarity. This phenomenon is also seen in nonpathogenic bacteria, such as *Alteromonas macleodii*, in which phenotypic ‘ecotypes’ have been identified among closely related strains [27].

*A. macleodii* is a ubiquitous copiotrophic bacterium [28, 29] that appears important in biogeochemical cycling of organic compounds in open waters [27, 30, 31]. In the ‘deep ecotype’ category, two strains of *A. macleodii* isolated from the same deep Mediterranean seawater sample have been isolated and fully sequenced: AltDE [27] and AltDE1 [32]. Furthermore, a metagenomic fosmid library was constructed using the same sample and over 200 fosmids were identified as *A. macleodii* [32] including four fosmids containing rRNA operons. In this study, we use the two fully sequenced genomes and the four fosmids to examine the intragenomic and intergenomic heterogeneity of the rRNA operons present in concurrent lineages of *A. macleodii*. Our findings reveal significant intragenomic heterogeneity throughout the entire rRNA operon as well as significant intergenomic heterogeneity among the concurrent lineages. These results underscore rRNA operon variability, which is amplified due to the presence of multiple closely related strains within a single physical niche.

**Methods and Materials**

**rRNA Sequences**

The complete genomes of AltDE, accession number CP001103 [27], and AltDE1, accession number NC_019393 [32] as well as the fosmid metagenomic library construction [32] have been described previously. Briefly, a 200 L sample of seawater was obtained from the deep (1000 m) Adriatic Sea in the Mediterranean for metagenomic and culture-based analyses. An aliquot of the sample was plated on marine agar, from which the isolates AltDE [27] and AltDE1 [32] were sequenced. The remaining sample was filtered, and the extracted DNA was used to prepare fosmid libraries for microbial community analysis [32]. The resulting fosmids (ca. 50,000) were screened for 16S rRNA genes using a 16S-ITS amplification strategy [33], and eight (out of 62 16S rRNA positive fosmids) showed a high similarity to the 16S rRNA of *A. macleodii*. The *A. macleodii* subset of 16S rRNA fosmids was completely sequenced and compared with the AltDE genome. From this comparison, four fosmids (NCBI BioProject PRJNA168986) were found to contain the entire rRNA operon and show homology to *A. macleodii*. rRNA sequences utilized in this study were evaluated using Geneious Pro 5.0.1 program.

**rRNA Alignment and Annotation**

The four fosmid rRNA operons were aligned to either AltDE or AltDE1 using Basic Local Alignment Search Tool (BLAST) followed by visual inspection. To identify variable regions in the rRNA operons, sequences were aligned and compared using MUSCLE version 3.6 [34] and ClustalW [35]. All 14 rRNA operons were annotated using the Integrative System for Genomic Analysis (ISGA) platform [36] for consistency.

**Data Analysis**

Predicted secondary structures were obtained using UNA-Fold software [37]. Average nucleotide identity (ANI) values were calculated using the BLASTn algorithm run with the following settings: X=150 (drop-off value for gapped alignment), q=−1 (penalty for nucleotide mismatch), and F=T (filter for low-complexity sequences[38]); the rest of the parameters were at default settings. The results were then filtered to get sequences with minimum id of 70 % and coverage of 50 %. Dissimilarity percentages between variants of a given hypervariable region were calculated by dividing the number of dissimilar nucleotides between the two most distinct variants by the total number of nucleotides.
In Silico Amplification Analysis

Phylogenetic analysis was performed using portions of the 16S rRNA genes discussed here as well as that of Alteromonas SN2 (accession number CP002339.1) and Pseudo
dna atlantica (accession number CP000388.1). Four sets of primers were used for this in silico 16S rRNA analysis.
The first set (S-D-Bact-0564-b-S-15/S-D-Bact-1061-b-A-17) resulted in the highest overall coverage and specificity
for Bacteria during a recent study evaluating universal primers, while the second set (S-D-Bact-0341-b-S-17/S-D-Bact-0515-b-A-19) provided the highest coverage of Bacteria
for Illumina sequences[39]. The third and fourth sets were described as the most (E341/E1406R) and least
(E969/E1492) accurate primers, respectively, for ocean samples in a recent study that examined 16S rRNA sequencing
from different environments [40]. Maximum likelihood trees were generated in MEGA 5.05 using 100 replicates
for bootstrapping [41]. Variable ITS-1 lengths were determined using the universal 16S primer 1392f (5′ GSACA
CACCGCCCGT 3′) and the bacterial 23S primer 125r (5′ GGTTCBCCCCATCRG 3′) often used in automated ribosomal
intergenic spacer analysis (ARISA) [42]. Taxonomic units based on in silico ARISA data are commonly
described as the number of distinct ITS peaks, i.e., ITS-1 length [43]. Here, we calculated operational taxonomic units
(OTUs) using the number of distinct ITS-1 lengths with an
additional criterian of a minimal of >5 nt difference from the
nearest neighbor to account for peak width.

Results

Evaluation of the AltDE and AltDE1 genomes revealed five
rRNA operons in each organism, all with the expected (5′ 16S-23S-5S 3′) structure described above. In addition, four
metagenomic fosmids sequenced from the same environmental sample contained rRNA operons that were closely
related (>97 % sequence similarity) to either AltDE or
AltDE1. Two fosmids (AD1000-231-F10 and AD1000-
207-B8) corresponded to operon two (Op2) of both AltDE and AltDE1, while the remaining two fosmids (AD1000-
249-G9 and AD1000-112-F00) corresponded to operon
three (Op3) of AltDE1. In total, 14 rRNA operons were
compared and showed both conserved synteny (i.e., chromosomal positioning) and high overall sequence similarity
in the 16S (99.7–98.6 %; Table S1) and 23S (100–97.9 %,
with the exception of one fosmid exhibiting 94.9 %; Table
S2) rRNA genes. ANI values between the two genomes
(99 %) and between the fosmids and either of the genomes
(AD1000-112-F00, AD1000-207-B8, and AD1000-231-
F10 >99 % and AD1000-249-G9 >97 %) were also very
high. These data show that AltDE and AltDE1 as well as the
four fosmids represent closely related lineages of A. macleo-
dii coexisting in a relatively small (200 L seawater) environ-
mental sample.

Despite the overall sequence similarity between the A. macleodii lineages compared here, there were localized
variations in the rRNA genes. AltDE and AltDE1 contain
multiple variants in the 16S (Table 1) and 23S rRNA genes (Table 2), although AltDE1 displays a greater number of
variants (five compared to AltDE’s two) in rRNA genes.
None of the 16S or 23S rRNA genes from homologous
operons are identical between AltDE and AltDE1. The same
holds true for the fosmids, except in one case in which
AD1000-112-F00 contains an identical 23S rRNA gene to
the homologous operon (Op3) of AltDE1. For example, the
four 16S rRNA gene sequences associated with Op2 (Fig. 1b) each display a unique set of variants (Table 1), as
do the four 23S rRNA sequences (Fig. 2b, Table 2).
In contrast, there are identical 16S and 23S rRNA genes be-
tween nonhomologous operons of AltDE and AltDE1.

We observed the majority of 16S rRNA heterogeneity in
four hypervariable regions (H6, H14, H29, and P37) that
displayed a range of dissimilarities from 7 to 54 % (Fig. 1c).
The H6, also commonly referred to as the V1, region
revealed the greatest variability with three variants display-
ing 27–55 % dissimilarity. Intragenomic heterogeneity (also
to refered to as intercistronic heterogeneity) was observed
with AltDE1 exhibiting two H6 variants (H6-II, Op1, 3, and

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Table 1  Comparison of the 16S rRNA gene hypervariable
regions found in association with each operon in the two A.
macleodii genomes (AltDE and AltDE1) and the four A.
macleodii fosmids (AD1000-231-F10, AD1000-207-B8,
AD1000-112-F00, and AD1000-249-G9)

<table>
<thead>
<tr>
<th>Location</th>
<th>Associated 16S rRNA variants</th>
</tr>
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<tbody>
<tr>
<td>AltDE1 rRNA operon 1</td>
<td>H6-III H14-I H29-II P37-I</td>
</tr>
<tr>
<td>AltDE rRNA operons 2 and 4</td>
<td>H6-III H14-I H29-II P37-I</td>
</tr>
<tr>
<td>AltDE1 rRNA operon 2</td>
<td>H6-III H14-I H29-II P37-I</td>
</tr>
<tr>
<td>AltDE rRNA operons 1, 3, and 5</td>
<td>H6-I H14-II H29-I P37-I</td>
</tr>
<tr>
<td>AltDE1 rRNA operon 3</td>
<td>H6-I H14-II H29-I P37-I</td>
</tr>
<tr>
<td>AltDE1 rRNA operon 4</td>
<td>H6-II H14-I H29-II P37-II</td>
</tr>
<tr>
<td>AltDE1 rRNA operon 5</td>
<td>H6-II H14-I H29-II P37-II</td>
</tr>
<tr>
<td>AD1000-231-F10, AD1000-207-B8</td>
<td>H6-II H14-I H29-II P37-II</td>
</tr>
<tr>
<td>AD1000-112-F00, AD1000-249-G9</td>
<td>H6-I H14-II H29-I P37-II</td>
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5; H6-III, Op2 and 4), while AltDE1 exhibited three variants (H6-I, Op3; H6-II, Op2 and 4; H6-III, Op1 and 5). Heterogeneity was also observed among the fosmids, which contained representatives of all three H6 variants. AltDE exhibited more stability in the H14, H29 (also known as V5), and P37 regions, containing only a single variant of each region (H14-I, H29-II, and P37-I) in all five 16S rRNA genes. AltDE1, in contrast, exhibited intercistronic heterogeneity in each of these regions, displaying two variants at H14 (H14-I, Op1, 2, 4, and 5; H14-II, operon 3), H29 (H29-I, Op3-5; H29-II, Op1 and 2), and P37 (P37-I, Op1-3 and 5; P37-II, Op4). Representative sequences of both H14 and H29 variants were present in the four fosmids, while all four contained the same P37-II variant.

In silico 16S rRNA gene amplification to assess whether the variation observed here disrupted appropriate classification of these lineages revealed inconsistent findings. Using primers that were recently shown to give the highest Bacterial coverage with Illumina sequences [39], we observed an underestimation of *Alteromonas* sp. (Fig. 3a). *Alteromonas* SN2, a different species than *A. macleodii* with an ANI of 74 % compared to AltDE [44], grouped together with all 14 operons examined here in a 99 % OTU. In contrast, primers from the same study that provided the overall highest

Table 2

<table>
<thead>
<tr>
<th>Location</th>
<th>Associated 23S rRNA variants</th>
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<tr>
<td>AltDE1 rRNA operon 1</td>
<td>H14-IV</td>
</tr>
<tr>
<td>AltDE1 rRNA operon 2</td>
<td>H14-I</td>
</tr>
<tr>
<td>AltDE1 rRNA operon 3, AD1000-112-F00</td>
<td>H14-I</td>
</tr>
<tr>
<td>AltDE1 rRNA operon 4</td>
<td>H14-II</td>
</tr>
<tr>
<td>AltDE1 rRNA operon 5</td>
<td>H14-I</td>
</tr>
<tr>
<td>AltDE rRNA operon 1</td>
<td>H14-V</td>
</tr>
<tr>
<td>AltDE rRNA operons 2 and 4</td>
<td>H14-V</td>
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<tr>
<td>AltDE rRNA operon 3</td>
<td>H14-IV</td>
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<tr>
<td>AltDE rRNA operon 5</td>
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<td>AD1000-249-G9</td>
<td>H14-VI</td>
</tr>
<tr>
<td>AD1000-207-B8</td>
<td>H14-III</td>
</tr>
<tr>
<td>AD1000-231-F10</td>
<td>H14-I</td>
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</table>

Fig. 1 Comparison of the four 16S rRNA gene hypervariable regions found in association with the two *A. macleodii* genomes and the four *A. macleodii* fosmids. a The secondary structure of the 16S rRNA gene with each hypervariable region discussed here marked. b Alignment of the four metagenomic fosmids, containing rRNA operons, to that of AltDE and AltDE1. c Four hypervariable regions were identified in the 16S rRNA gene, containing two to three variants each. The nucleotide numbers listed for each hypervariable region correspond to the AltDE1 16S rRNA nucleotides for Op1. Variable nucleotides in each region are denoted with red nucleotide letters
coverage and specificity for Bacteria distinguished the two species (Fig. 3b). Depending on which operons amplified the most, however, this distinction may or may not result in two distinct OTUs at 97 %. Similarly, two sets of primers evaluated specifically for their effectiveness within ocean samples [40] revealed one pair that was unable to distinguish between Alteromonas SN2 and A. macleodii at the 97 % cutoff (Fig. S1a) and one pair that could (Fig. S1b). In none of these cases were there clear clustering patterns between AltDE, AltDE1, and the fosmids. These data show that carefully optimized 16S rRNA gene amplification could possibly distinguish these closely related lineages as a single species, while they also highlight the inefficacy of 16S amplification to resolve ecologically relevant groupings within a species.

The 23S rRNA gene comparisons produced similar results to that observed in the 16S rRNA, in which AltDE, AltDE1, and the four fosmids exhibited considerable heterogeneity (Fig. 2). Most variability was observed in four hypervariable regions (H14, H15, H19, and H37) and two intervening sequences (IVS-I and IVS-II). The hypervariable regions showed up to 20 % dissimilarity between variants (Fig. 2c), with three to six variants observed for each region (Table 2). AltDE exhibited the least amount of variation housing nine variants in total (out of a total of 16 observed), whereas AltDE1 and the collective fosmids represented 12 variants each. The greatest 23S rRNA variability was observed in the IVS-1, which displayed four variants with up to 30 % dissimilarity (Fig. 4). AltDE again exhibited greater stability with Op1–4 containing the same variant and Op5 revealing only a single nucleotide difference. In contrast, AltDE1 harbored only one variant (Op1) similar to AltDE’s Op1–4, while the other two variants showed 30 % (Op2–4) or 25 % (Op5) dissimilarity. The fosmids collectively represented all three variants. Only two variants were observed in the IVS-2 region for AltDE and

Fig. 2 Comparison of the four 23S rRNA gene hypervariable regions found in association with the two A. macleodii genomes and the four A. macleodii fosmids. a The secondary structure of the 23S rRNA gene with each hypervariable region discussed here marked. b Alignment of the four metagenomic fosmids, containing rRNA operons, to that of AltDE and AltDE1. c Four hypervariable regions were identified in the 23S rRNA gene, containing three to six variants each. The nucleotide numbers listed for each hypervariable region correspond to the AltDE1 16S rRNA nucleotides for Op1. Variable nucleotides in each region are denoted with red nucleotide letters.
AltDE1, and the dissimilarity was much less at 2% (Fig.4). One fosmid, however, exhibited an IVS-2 variant with an 87 nucleotide deletion (Fig.4), suggesting that much greater variability in the IVS-2 region is possible among related strains.

Even more dramatic variations were observed in the ITS-1 region compared to that of the 16S and 23S rRNA genes. The *A. macleodii* lineages harbored ITS-1 regions ranging in size from 374 to 780 bp. The ITS-1 sequences displayed two general patterns, with four operons (AltDE Op1 and 2, AltDE1 Op1 and 5) exhibiting no tRNA (ca. 400 bp) and the remaining operons containing two tRNA (tRNA-Ile and tRNA-Ala) sequences (ca. 800 bp) (Table S3). To examine the ITS-1 region often used for ARISA analysis [42, 45], we determined the length between the 16S (1392f primer) and 23S genes (125r primer) for each of the 14 *A. macleodii* operons in silico. This analysis resulted in lengths ranging from 640 to 1,343 nucleotides with a total of 11 OTU identifications using distinct fragment lengths (Table S3). These results highlight the overestimation of diversity that results from the heterogeneity within a closely related population of *A. macleodii* strains across the entire rRNA gene.

Within the rRNA operons containing two tRNA (*n* = 10), two additional hypervariable regions (VR1 and VR2) were observed, each displaying three variants (Fig. 5). VR1 was located between the two tRNAs (Fig. 5a), while VR2 was identified downstream from the tRNA-Ile (Fig. 5b). In both cases, the three variants ranged in size from 15 to 117 bp (Fig. 5). In contrast to what was observed in the 16S and 23S rRNA genes, AltDE was more variable than AltDE1, containing all six variants compared to four (two VR1 and two VR2) variants in AltDE1. The fosmids harbored three VR1 and two VR2 variants.

**Discussion**

Since the 1970s, the rRNA operon has been the foundation of microbial phylogenetics due to its essential function, conserved sequence, structure and size, and limited horizontal transfer. In recent years, with the introduction of high throughput sequencing, a new dimension in microbial biodiversity analysis has emerged. rRNA profiling methods, such as 16S-based pyrosequencing [46–48] and ITS-based ARISA [49–52], have become common techniques to characterize microbial community structures in ecological samples. Because the majority of the rRNA genes remain...
unsequenced, diversity is measured as the number of OTUs in a given sample based on sequence similarity [53]. In this study, we compared the rRNA operons from two *A. macleodi* strains and four *A. macleodi* metagenomic fosmids, all of which are known to comprise a single *A. macleodi* OTU. The AltDE and AltDE1 genomes showed more than 99 % ANI over the core genome and the majority of fosmids had more than 99 % similarity to either of the strains over the shared genes outside the rRNA operon [32]. Our analysis of these 14 rRNA operons, however, clearly shows that intragenomic and intergenomic heterogeneity exist in areas of the rRNA operon that can affect consistent and appropriate analysis of these closely related strains.

The limitations of rRNA analysis have been explored, for the most part, in isolates and often with host-associated pathogens, in particular [14, 15, 54]. Much less work has been done with free-living marine bacteria, which is of interest due to genotypic differences between free-living and host-associated bacteria [55]. Even less is known regarding mixed populations found in environmental samples, where free-living organisms undergo LGT with one another at a higher frequency than their endosymbiotic relatives [56]. Since LGT appears to play a role in 16S rRNA heterogeneity [12], one would expect to see high levels of 16S rRNA heterogeneity in environmental samples, where there is a heterogeneous mixture of closely-related, free-living microorganisms, such as that examined in this study. Our results support this assertion, revealing both intra- and intergenomic heterogeneity in three major regions of the rRNA operon. Furthermore, our data suggests that the level of heterogeneity is variable depending on the strain, with AltDE1 displaying considerably greater variability than AltDE in both the 16S and 23S genes.

A handful of studies have highlighted the potential for overestimating microbial diversity due to intragenomic heterogeneity of 16S rRNA genes [9, 10, 12, 14, 15, 57], while others show primer biases can cause the underestimation of certain bacterial groups [39, 40, 58]. Many studies highlighting flaws associated with rRNA profiling methods have also suggested strategies to strengthen these techniques, including the application of corrective statistical measures [59], stringent sequence trimming and minimum clustering thresholds of 97 % [60], normalization using genome size and 16S rRNA operon numbers [6], and the removal of portions of the 16S gene from intragenus diversity studies [12]. Despite these efforts, others have proposed the use of alternative markers, such as beta subunit of DNA polymerase (rpoB) [61, 62] to avoid these problems. Using optimized universal primers from recently published papers evaluating the efficacy of 16S rRNA classifications, we still obtained inconsistent results at the species level. This work supports a certain level of skepticism when evaluating 16S rRNA studies of microbial diversity, especially when classified at the species level, and highlights the need for an
alternative molecular marker to distinguish between closely-related strains.

Even more so than the 16S rRNA gene, the ITS-1 region of the rRNA operon is known to be quite variable and can contain one or two tRNA genes (either tRNA-Glu, tRNA-Ala, or both tRNA-Ala and tRNA-Ile) and ranges in size from 200 to 1,200 bp [63, 64]. This variation between tRNA genes has been used for differentiating species [65] and for the identification of Gram-positive bacteria [66]. The analysis of 490 species of microorganisms, however, revealed that nearly half (45 %) of them produced more than one ARISA fragment with 9 % producing more than four fragments [59]. In this study, in silico ARISA analysis resulted in an 11× overestimation of bacterial diversity in the case of A. macleodii, which shows that diversity studies based on ARISA may be very misleading.

What is also quite remarkable in our study is the extent to which intergenic (in addition to intragenomic) variability further complicated the rRNA heterogeneity throughout the rRNA operon when considering the total population of A. macleodii strains in their natural environment. Thus, even the normalization of data to the known number of rRNA operons in a given strain would not necessarily account for the potential heterogeneity present in a diverse natural ecosystem. Previously, it was suggested that intragenomic heterogeneity was more severe than that seen between closely related strains (i.e., intergenomic) in Veillonella spp. [57], although it should be noted that the strains compared originated from diverse clinical samples and test strains. Vibrio parahaemolyticus strains, in contrast, displayed substantial intergenomic heterogeneity between numerous genotypically distinct environmental and clinical isolates [15]. This argues that the heterogeneity issues discussed here may be specific to certain genera. Nonetheless, in aquatic environments where Alteromonas spp. and Vibrio spp. are common, the issue of rRNA heterogeneity (both intra- and intergenomic) needs to be carefully considered.

Understanding the selective force that maintains rRNA heterogeneity should be an area of focus in future studies. The number of rRNA operons [67] and rRNA heterogeneity [11, 16, 68, 69] reflects differences in ecological capabilities. For example, there is differential expression of heterogenous rRNA operons during different developmental stages [69] and in response to varying temperatures [11]. Within this context, our findings are notable due to the increased potential for ecologically relevant variation represented in a specific...
physical niche by A. macleodii lineages previously undistinguished from one another. It should be noted that the variability observed in this study did not alter the general structure of rRNA, indicating preserved overall functionality in all of the rRNA genes examined. Thus, it seems more likely that the differences observed contribute to more refined responses and subtle ecological differences.

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